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ORIGINAL ARTICLE

Basic Study

Rat model of anal sphincter injury and two approaches for stem cell administration

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Abstract

AIM

To establish a rat model of anal sphincter injury and test different systems to provide stem cells to injured area.

METHODS

Adipose-derived stem cells (ASCs) were isolated from BDIX rats and were transfected with green fluorescent protein (GFP) for cell tracking. Biosutures (sutures covered with ASCs) were prepared with 1.5 x 10^6 GFP-ASCs, and solutions of 10^6 GFP-ASCs in normal saline were prepared for injection. Anorectal normal anatomy was studied on Wistar and BDIX female rats. Then, we designed an anal sphincter injury model consisting of a 1-cm extra-mucosal miotomy beginning at the anal verge in the anterior middle line. The sphincter lesion was confirmed with conventional histology (hematoxylin and eosin) and immunofluorescence with 4', 6-diamidino-2-phenylindole (commonly known as DAPI), GFP and α-actin. Functional effect was assessed with basal anal manometry, prior to and after injury. After sphincter damage, 36 BDIX rats were randomized to three groups for: (1) Cell injection without repair; (2) biosuture repair; and (3) conventional suture repair and cell injection. Functional and safety studies were conducted on all the animals. Rats were sacrificed after 1, 4 or 7 d. Then, histological and immunofluorescence studies were performed on the surgical area.

RESULTS

With the described protocol, biosutures had been covered with at least 820000-860000 ASCs, with 100% viability. Our studies demonstrated that some ASCs remained adhered after suture passage through the muscle. Morphological assessment showed that the rat anal anatomy is comparable with human anatomy; two sphincters are present, but the external sphincter is poorly developed. Anal sphincter pressure data showed spontaneous, consistent, rhythmic anal contractions, taking the form of "plateaus" with multiple twitches (peaks) in each pressure wave. These basal contractions were very heterogeneous; their frequency was 0.91-4.17 per min (mean 1.6980, SD 0.57698), their mean duration was 26.67 s and mean number of peaks was 12.53. Our morphological assessment revealed that with the aforementioned surgical procedure, both sphincters were completely sectioned. In manometry, the described activity disappeared and was replaced by a gentle oscillation of basal line, without a recognizable pattern. Surprisingly, these findings appeared irrespective of injury repair or not. ASCs survived in this potentially septic area for 7 d, at least. We were able to identify them in 84% of animals, mainly in the muscular section area or in the tissue between the muscular endings. ASCs formed a kind of "conglomerate" in rats treated with injections, while in the biosuture group, they wrapped the suture. ASCs were also able to migrate to the damaged zone. No relevant adverse events or mortality could be related to the stem cells in our study. We also did not find unexpected tissue growths.

CONCLUSION

The proposed procedure produces a consistent sphincter lesion. Biosutures and injections are suitable for cell delivery. ASCs survive and are completely safe in this clinical setting.

Key words: Fecal incontinence; Experimental rat model; Anal sphincter; Cell implantation; Cell therapy; Stem cells; Mesenchymal stem cell

Core tip: Fecal incontinence is very frequent and associated with severe consequences for patients. Surgical treatment outcomes are not as good as they should be, mainly in the long term. Stem cells could improve these results, as demonstrated in other clinical settings. We report a simple rat model for experimental anal sphincter injury (a surgical section), and characterize it from morphological (conventional histology and immunofluorescence) and functional (anal manometry) points of view. Then, we describe two approaches for adipose-derived stem cell administration to the injured area (injection and biosutures) and demonstrate stem cell survival during at least 7 d, as well as their safety.

Trébol J, Georgiev-Hristov T, Vega-Clemente L, García-Gómez I, Carabias-Orgaz A, García-Arranz M, García-Olmo D. Rat model of anal sphincter injury and two approaches for stem cell administration. *World J Stem Cells* 2018; 10(1): 1-14 Available from: URL: http://www.wjgnet.com/1948-0210/full/v10/i1/1.htm DOI: http://dx.doi.org/10.4252/wjsc.v10.i1.1

INTRODUCTION

Fecal incontinence is a very prevalent nonfatal illness associated with considerable embarrassment, very relevant psychosocial repercussions (disability, anxiety, depression, social isolation and job loss; it is the second cause of institutionalization, *etc*.) and poor quality of life. It is estimated to affect 11%-15% of adults[1] as well as 2.2% of the general population and 47% of institutionalized people[2], but its real prevalence is probably much higher[3].

This condition also has a very important economic

impact, consisting of direct (diagnostic test, treatments, *etc.*) and indirect (labor production, secondary treatments, *etc.*) costs. In a Seattle study, annual healthcare costs augmented significantly in multivariate analysis, up to $2897 of 2005 (pads, barriers or institutionalization costs were not included)[4]. In a Dutch study, global costs grew €2169 yearly for each patient[5].

Fecal incontinence is a multifactorial disease. The most frequent morphological alteration is a sphincter lesion, found in almost 60% of patients, most of them obstetric (30%-40%). Sphincter lesions during delivery ranges from 11%[6] to 26.9%[7] and cause incontinence in 76.8%-82.8% of patients[7].

Although in the last years sacral neuromodulation has been growing exponentially, surgery remains the treatment of choice for the most severe or refractory cases, mainly if sphincter lesions are present. Sphincter repair is the most frequently performed and successful technique for traumatic lesions. Sphincter repair has good results in the short term, being excellent to good in 66%, moderate in 22% and poor in 12% of patients[8]. But this outcome does not persist in the long term[9] for reasons not well understood.

Stem cell (SC) therapy has demonstrated promising results in a wide variety of clinical settings. Application of adipose-derived stem cells (ASCs), one type of mesenchymal stem/stromal cells (MSCs), is a novel approach for enhancing regeneration or repair of damaged tissues[10-12]. ASCs have been tried in environments particularly unfavorable for healing, such as experimental colitis[13], sepsis[14], anal fistula[15] and Crohn´s[16], with promising results. ASCs have important proliferation and differentiation capabilities but also immunoregulatory and antiinflammatory properties[17]. ASCs are isolated easily from subcutaneous fat, in a process that yields 100 times more SCs than bone marrow aspirates[18].

Our research group has been working with ASCs since 2002 in the clinical and experimental setting and has been a pioneer on their use in digestive fistulizing diseases[15] and other conditions. We have conducted three clinical trials (phases Ⅰ, Ⅱ and Ⅲ)[19-21] with autologous ASCs and one with allogeneic (phase Ⅰ-Ⅱa)[22], and participated in two with allogeneic[23,24]. All these trials included more than 400 treated patients. Sutures covered with ASCs (named "biosutures") were designed and tested on colonic[25-26] and tracheal experimental anastomosis[27]. All these studies, and many others, have proven ASCs to be safe and possibly effective.

Our aim was to test ASCs for fecal incontinence in a murine model prior to study of their use in humans. The first step was to design an easy and reproducible model for sphincter injury, and the second to test two different ASCs administration systems.

MATERIALS AND METHODS

Animals employed

We used 50 adult female BDIX rats (selected for cell therapy because they are syngenic to mimic an autologous application), weighing 170–260 g and aged 16-24 wk (provided by Charles River Laboratories International, Inc., Wilmington, MA, United States), and 10 adult female Wistar rats (bred at the animal facility in the University Hospital La Paz, Madrid, Spain). Two days before surgery, animals were transferred to individual cages. They had free access to water and a standard diet (Panlab S.L.) and were housed in a restricted access room, with controlled temperature (23 ℃) and a 12-h light/12-h dark cycle.

Ten Wistar and fourteen BDIX rats were employed for anatomy studies, surgical damage model creation, obtaining ASCs, functional assays, and anesthetic dosage adjustments. Thirty-six BDIX rats were used for cell therapy and functional studies.

The present study was performed in accordance with the European Union guidelines for reducing pain and discomfort to experimental animals (86/609/CEE). Animals surpassed all sanitary controls. The study protocol was approved by the Ethical Committee for Animal Welfare of University Hospital La Paz, Madrid, Spain.

Anesthetic and surgical materials

For anesthesia induction, we used isoflurane (Forane®; Abbott Laboratories, Abbott Park, IL, United States) in oxygen (3 lpm O_2 and 5 lpm Forane® for induction and 1.5–2 lpm for maintenance). For functional studies, an intraperitoneal mixture of ketamine (Ketalar®; Pfizer, New York, NY, United States) and xylazine 2% (Xilagesic®; Calier, Buenos Aires, Argentina) was selected following Wang *et al*[28] and Zutshi *et al*[29]. We applied lower doses than those used in the mentioned studies: 50 mg/kg ketamine and 5 mg/kg xylazine for surgery with functional registry, and 30 mg/kg plus 3 mg/kg for functional study alone.

For surgery, standard microsurgical equipment, a 10 × to 40 × magnifying lens and 0.5 mL syringes with 30 G (0.3 mm) and 8 mm needles (BD Micro-Fine™; Becton Dickinson, Franklin Lakes, NJ, United States) for injecting ASCs were used. For suturing, 6/0 polyglactin 910 sutures with cylindrical needle (½ circumference and 17 mm length) were used (Vycril®; Ethicon, Johnson and Johnson, Somerville, NJ, United States).

Functional studies material

We performed basal anal manometry, modifying the model pioneered by Vinograd *et al*[30] and used by Wang *et al*[28] and Zutshi *et al*[29]. A 0.4 mL latex balloon (Kent Scientific Corp., Torrington, CT, United States) was connected through a rigid tube to two 3-way stopcocks; one of them had a free opening to set 0 values, and the other one was connected through one side to an infusion pump (that filled the system with normal saline solution) and to a Millar SPR-524 pressure transducer (Millar Inc., Houston, TX, United States) through the other side. The transducer was then connected to an amplifier (ML224) and this to a digital pulse detecting

system (Powerlab 4/30) with real time recording software (Chart v5.5); these three elements were from ADInstruments, Sidney, Australia.

Isolation, culture and marking of ASCs

ASCs were obtained from the subcutaneous fat tissue according to a previously described protocol in humans by Zuk *et al*[31], based on their adherence to culture, with some minor modifications. Stromal vascular fraction (SVF) was obtained and digested with type I collagenase (0.075%; Gibco®Thermo Fisher Scientific, Waltham, MA, United States). The digested tissue was centrifuged and the pellet was resuspended in 0.16 M NH_4Cl for erythrocyte lysis. Then, the cells were separated by filtering the product through a 70-μm nylon mesh, plated in culture dishes, and cultured at 37 ℃ in a humid atmosphere (90%–95%) with 5% carbon dioxide in the Gibco™ Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, United States) containing 10% phosphate buffered saline (PBS), 2 mmol/L glutamine, and 1% penicillin–streptomycin. Nonadherent cells were removed and subcultured 24 h after seeding; these were the ASCs.

In order to identify ASCs in animal tissue samples, we marked them. In the third subculture at 60% to 70% confluence, ASCs were infected with enhanced green fluorescent protein (eGFP) transducing lentivirus (CNIC, Madrid, Spain). Sorting of selected eGFP-ASCs (99.6% positive) was applied after another 2 to 3 passages.

ASCs characterization

Cell cultures were analyzed by four-color flow cytometry using a FACS Calibur (Becton Dickinson Biosciences–BDB, San José, CA, United States) after staining with fluorochrome (Alexa Fluor 647; Thermo Fisher Scientific) conjugated with two positive markers: anti-CD90 (BDB) and anti-CD29 (Millipore, Burlington, MA, United States) and two negative markers: anti-CD45 (BDB) and anti-CD11b (BDB). With these four markers and the confirmed plastic adherent capability, our cells fulfill at least two out of three minimal internationally-decided defining criteria[32,33]. Differentiation capability was proven in prior experiments with the same isolation protocol.

Biosuture preparation

Biosutures were obtained by culturing 30 cm 6/0 polyglactin 910 sutures with 1.5×10^6 eGFP-ASCs on ultra-low attachment plates (P6 ULA, Costar®; Corning, Corning, NY, United States) over 72 h, according to the published protocol with minor changes[25]. Then, the sutures were washed with saline and used. Cell viability was evaluated by trypan blue. Cell adherence to suture and needle was previously confirmed by fluorescence and electron microscopy[25,27], and cell density significantly decreased after two stitches in muscle tissue[27]. Thus, we decided to use each biosuture for only two stitches.

We performed some studies to calculate the real cell dose adhered to sutures and studied with immuno-fluorescence for several biosutures after their use, looking for cells, and then counted adhered cells in the last centimeter of the remaining suture.

Cell injection products

eGFP-ASCs (10^6 cells) diluted in 50 μL normal saline were applied in each case.

Surgical damage model

After detailed study of the anorectal morphology on three Wistar and three BDIX rats, we designed a simple procedure, as follows (Figure 1).

Firstly, the animal was placed over a heating blanket, in a supine position. Perineal antisepsis was performed. The anal canal was emptied and a probe (Abbocath® 14G or similar) was inserted through it.

An arciform 10 mm to 15 mm anterior perianal incision (3 mm from verge) was performed. A 6/0 stitch at the inferior border to the tail was knotted. Adipose tissue was dissected sideways (with scissors or gauze) to expose approximately 20 mm of anorectal conduit until visualization of a thin darker line (external sphincter) or up to the anal verge.

Then, the muscular layer was held with clamps and a small incision was made until herniation of the submucosa or the probe was visualized by transparency. Submucosal dissection was continued longitudinally and the muscular layer was sectioned in a longitudinal fashion up to the anal verge and near the stitch caudally and proximally until completing 10 mm. If a mucosal perforation occurred, an interrupted suture was performed, leaving knots on the parietal side.

Sphincter repair and cell therapy

Immediate repair was performed with three to four interrupted 6/0 stitches. In the biosuture group, one suture was used for two stitches and on the second one, a back and forth motion was used three to four times, trying to deposit more ASCs. For ASC injections, two injections were given on each side of the muscle incision (Figure 2).

Finally, the skin incision was closed with interrupted stitches, burying the knots in the depth (to keep the animals from biting the suture). We washed the wound with iodine solution and optionally applied an aerosol plastic dressing (Nobecutan®; Inibsa, Barcelona, Spain). Analgesia (petidine) was injected subcutaneously immediately afterwards and on the following day.

Measurement of anorectal pressure; data management

The system was purged to avoid air bubbles, and the latex balloon was filled to 20-90 mmHg (to remain turgescent). The rectal ampulla was emptied, and the balloon was inserted until the silk knot that secures it to the tube reached the verge. The registry took between 30-60 min to reach a stable and regular activity. The

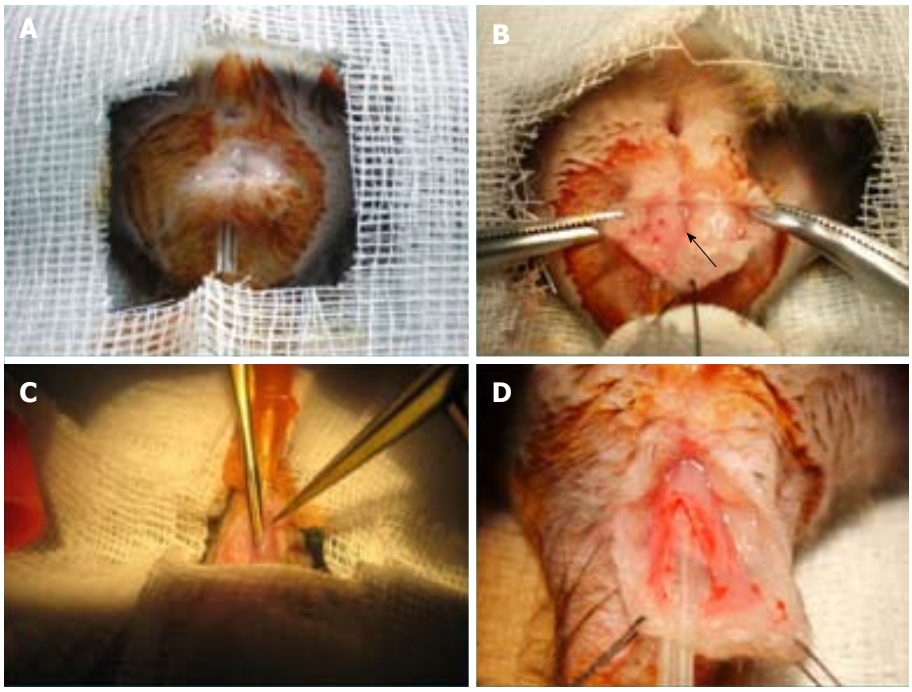

Figure 1 Sphincter anal injury model. A: An arciform 10-mm to 15-mm anterior perianal incision (3 mm to 5 mm from the anal verge) was performed; B: Adipose tissue was dissected sideways to expose 20 mm of conduit until visualization of the thin, darker line of the external sphincter was achieved (arrow); C: An incision was made in the muscular layer until submucosa herniation, then submucosal dissection progressed in a longitudinal fashion, downward (until the anal verge or near the marking stitch) and upward (until 10 mm had been completed); D: Final aspect of sphincter section.

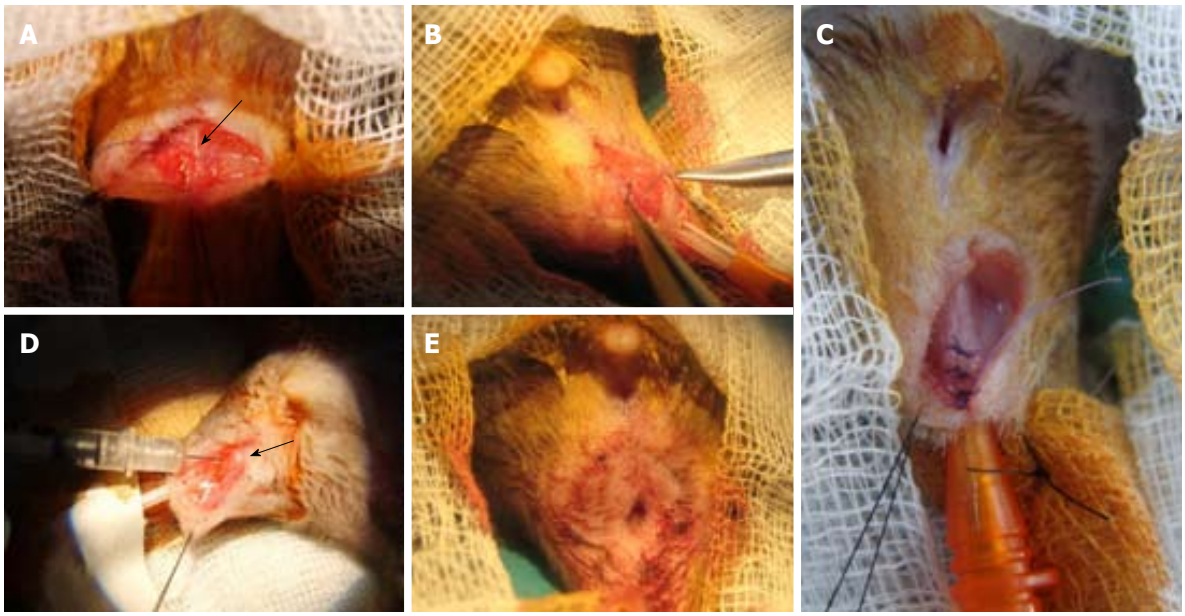

Figure 2 Sphincter repair and cell therapies. A–C: If immediate sphincter repair was programmed, it was performed with 3 to 4 stitches of 6/0 interrupted suture; A: If biosuture was used, sometimes a bubble formed on the outer aspect of the muscle (arrow); D: If an injection was given, two injections on each side of the muscle incision were done; generally, bubble formation was observed; E: Finally, the incision was closed with 6/0 interrupted sutures, burying the knots in the depth. More details about surgical and cell therapy techniques are provided in the text.

system was configured to detect 10 pulses per second, registered as mmHg, and at least 30 min of stable activity was registered.

Based on our experience and previously published papers, we picked the 12 min most stable registry selections as highly representative to study such. The area-under-the-contraction waveforms *vs* time curve (AUC), related to the selection baseline and not to 0 to correct for basal value (related to balloon inflation) variability, was obtained from these tracings. Total number of contractions (defined as a rise of at least 10 mmHg) and their frequency were determined for each selection. Since AUC could not reveal minor changes in every contraction, we selected the six best contractions

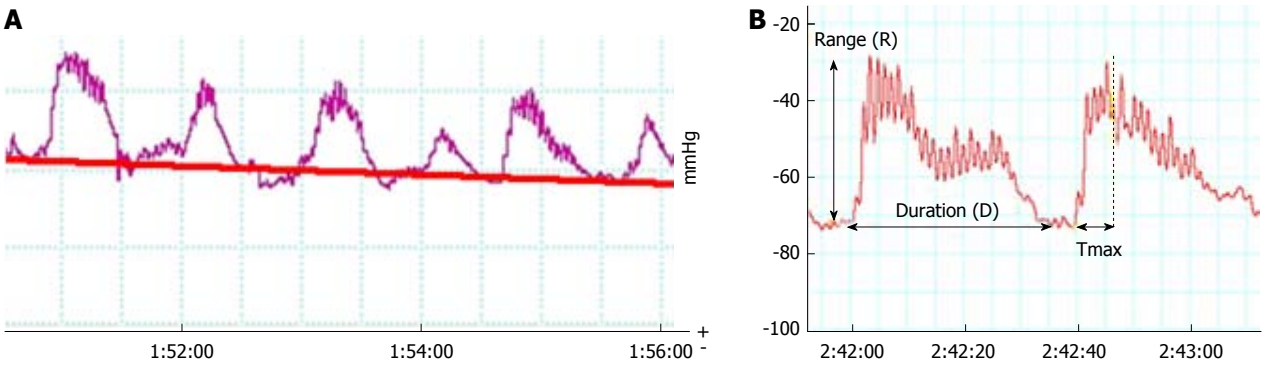

Figure 3 Basal anal manometry. A and B: Anal pressure data demonstrate spontaneous rhythmic contractions in the form of "plateaus", with multiple peaks. At least 30 min were registered and a segment of 12 min with the highest stability was selected. For these tracings selections, we calculated the area-under-the-contraction waveforms *vs* time curve related to the registry baseline. In A, it is the area between the purple and the red lines. To detect minor changes, we selected the six best contractions; for each one of these we determined (B): total duration (D), time to peak (Tmax), pressure range (range, R), and number of peaks. A mean value of these variables was calculated in each registry.

in the investigator´s viewpoint. For them, we calculated the mean values in each registry of the following: total duration (D), time to peak (Tmax), difference between maximal and minimal pressure value (range, R), and number of peaks (NP) (Figure 3).

Morphological assays

Animals were sacrificed at days 1, 4 or 7. Anorectal conduits were extracted, fixed in 4% formaldehyde, and embedded in paraffin.

Histopathology: Slices (5-μm thick) were stained with hematoxylin and eosin and examined under a Leica DM LS2 microscope.

Immunofluorescence: Incubation of slices with primary antiGFP (SC-8334; Santa Cruz Biotechnology, Dallas, TX, United States) and antiα-actin (MAB-1501; Millipore) antibodies was carried out overnight and then for 1 h with the secondary, antirabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR, United States) and antimouse Alexa Fluor 544 (Molecular Probes). Sections were mounted with antifade reagent, with 4', 6-diamidino-2-phenylindole (DAPI) (Prolong Gold; Molecular Probes) and viewed under a fluorescent microscope (Leica DMI6000B). To reduce the effect of spontaneous natural fluorescence, we used primary antibody omitted sections as negative controls and detected GFP presence and not GFP fluorescence (sometimes too faint).

Safety studies

Animals were examined for any local or systemic abnormality. If death occurred, an autopsy and an exam of the operated region were performed. If adverse events appeared, they were recorded and exhaustively analyzed. If any abnormal tissue was observed, a biopsy was done.

Experimental design

We divided 36 BDIX rats into three groups, with three subgroups of 4 animals based on time to sacrifice (1, 4 or 7 d): (1) group 1, no repair and ASCs injection; (2) group 2, repair with biosutures; (3) group 3, repair with conventional sutures and ASCs injection.

Statistical analysis

We used Kolmogórov-Smirnov test to assess if normal distribution criteria were met. In descriptive analyses, means, standard deviation and range were applied. To compare groups, we used ANOVA or Kruskal-Wallis test. *P*-value significance was 0.05. All statistics were performed using SPPS version 15.0 (IBM Corp., Armonk, NY, United States).

RESULTS

ASCs characterization

Flow cytometry confirmed eGFP expression > 95% until the 15th passage and mesenchymal phenotype by the wide expression (> 95%) of CD90 and CD29 and the absence (< 5%) of CD45 and CD11b.

Biosuture quality

Sometimes, we found some clots in the supernatant that contained grouped ASCs. Similar structures appeared occasionally when using biosutures on the outer side of the muscular layer and less frequently with injections.

Cellular distribution over the suture was heterogeneous (Figure 4).

Studies of cell count and viability showed the following: (1) in culture medium (seven determinations): 100000 to 280000 (mean 171428.57 with standard deviation (SD) 64627.14, viability with blue trypan was 98%; (2) adhered cells (detached with trypsin, three sutures): 820000 to 860000 (mean 840.000 with SD 20,000), viability was 100%.

We studied seven biosutures under microscopic immunofluorescence, prior to and after surgical use, and counted the remaining cells in their distal centimeter (Figure 4). (1) Some ASCs persistently adhered after

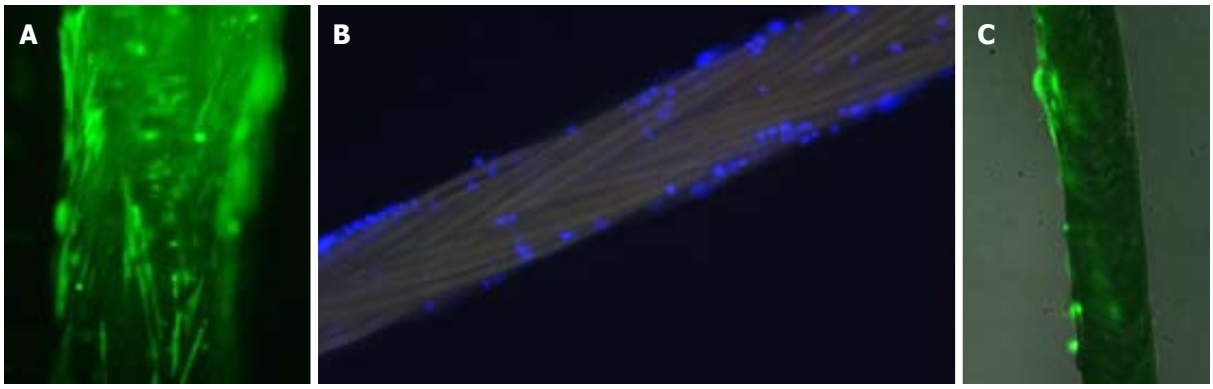

Figure 4 Biosuture samples. With the described protocol, cellular distribution over the suture was heterogeneous (A and B) and sutures were not fully covered; instead, there were "lumps" with clustered ASCs in the supernatant. After surgical use (C), some ASCs remained adhered and partially covering the suture, mainly in the proximal two-thirds of the suture. ASC: Adipose-derived stem cell.

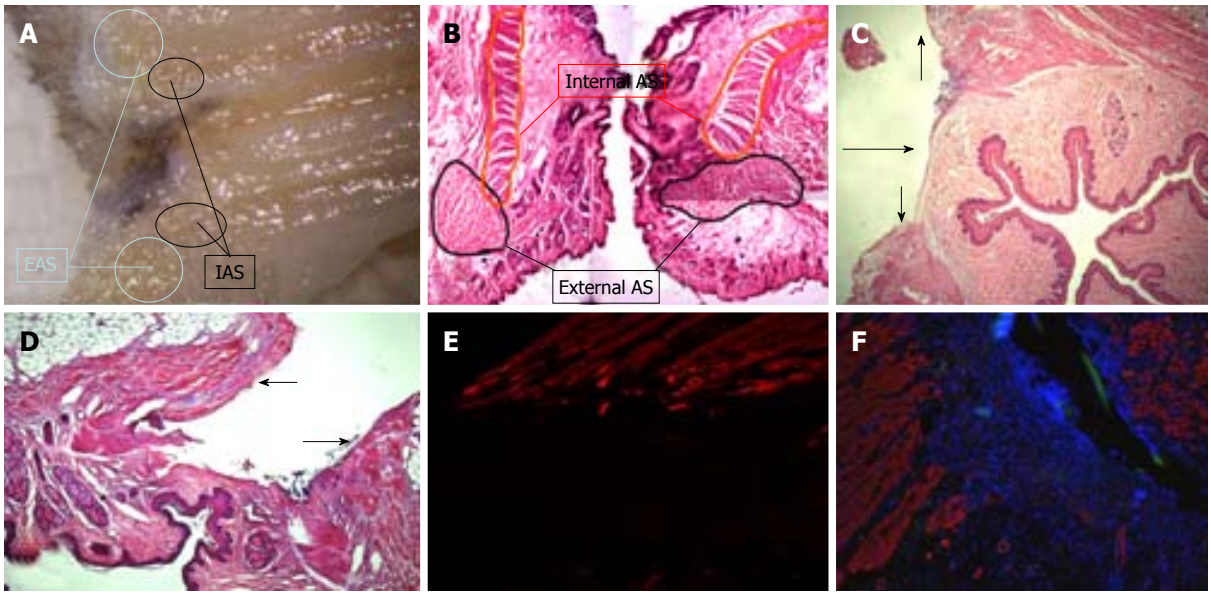

Figure 5 Anatomy and injury model study. Sometimes it is difficult to identify the external anal sphincter (see Figure 2), but it is present and included in the section as can be seen in A: A sagittal section; or in B: Coronal hematoxylin and eosin reconstruction (10 ×). The described model implies section of internal anal sphincter (C: Hematoxylin and eosin 10 ×) and external anal sphincter (D: Hematoxylin and eosin 10 ×). This was also evidenced on immunofluorescence staining at 10 × (E belongs to a non-repaired rat and F to a biosuture group rat; suture strains can be seen with green autofluorescence). EAS: External anal sphincter; IAS: Internal anal sphincter; AS: Anal sphincter.

its use, mainly in the proximal two-thirds of the suture. (2) cell count of the last centimeter ranged from zero to two ASCs per millimeter, so the majority of ASCs on the distal end were left on the animal tissue.

These results confirmed that the maximal dosage of ASCs that we could supply was 820000 to 860000, which was less than published data with the same charge of ASCs (80% of total, 1.2×10^6)[27]. Maybe, it could be a bit higher because with 98% to 100% cell viability some ASCs could divide.

Sphincter damage model: Morphologic and functional results

From a surgery viewpoint, it is frequently difficult to identify both sphincters-the external one because it is poorly developed (it has a slightly darker color), and the internal one because there is no clear continuity solution with rectal musculature (Figure 5). To identify anatomy, we made multiple coronal and sagittal sections and a 10 × reconstruction of hematoxylin and eosin imprints. It was very similar to that of humans, with the anal canal being 3 to 5 mm long and surrounded by two sphincters. The internal one consisted of smooth muscle, was an enlargement of the circular enteric musculature, and ended about 1 mm proximal to the anal verge. Immediately external to it, there are some longitudinal smooth fibers, and external to them the striated muscle bundles of the external sphincter, which ends close to the anal verge and has two to three different parts. A transition zone marks the change from rectum to anus, and there are some anal glands closer to it.

With the designed procedure (1 cm section), both

Table 1 Functional studies (anal manometry)

	Number of contractions	Frequency, *n* /min	Duration in s	Time to max in s	Peaks number, *n*	Range of mmHg	AUC
			Preoperative				
Number	36	36	36	36	36	36	36
Mean	19.489	1.6980	26.674752	8.882553	12.5323	25.9462	4884.9013
Standard deviation	7.1593	0.57698	6.6690300	2.9265056	3.52514	16.68935	3181.86004
Minimum	9.0	0.91	13.0000	4.2667	7.33	6.27	1227.46
Maximum	50.0	4.17	41.2800	15.6333	21.67	111.93	15790.69
			Postoperative				
Number	36	36	4	4	4	4	32
Mean	0.535	0.0446	26.425000	9.056250	7.5000	12.1950	418.2139
Standard deviation	1.5016	0.12513	5.8105507	4.2522727	2.48328	2.09572	361.06246
Minimum	0	0.00	20.4000	6.0750	5.00	10.39	12.91
Maximum	6.0	0.50	31.6000	15.1500	10.50	15.21	1610.80

A descriptive analysis is presented. In the first two columns, total number of contractions (defined as a rise higher than 10 mmHg) and their frequency are presented (studied within the 12-min selection). The following four columns focus on contraction characteristics: contraction duration, time to reach maximum from the beginning, number of twitches in each contraction, and range (maximum minus minimum). These values were calculated by selecting the six best contractions on each registry. Finally, the area under the pressure *vs* time curve (-AUC- calculated with the 12-min selection) values are presented. All of these variables are presented in basal (preoperative) and postoperative conditions.

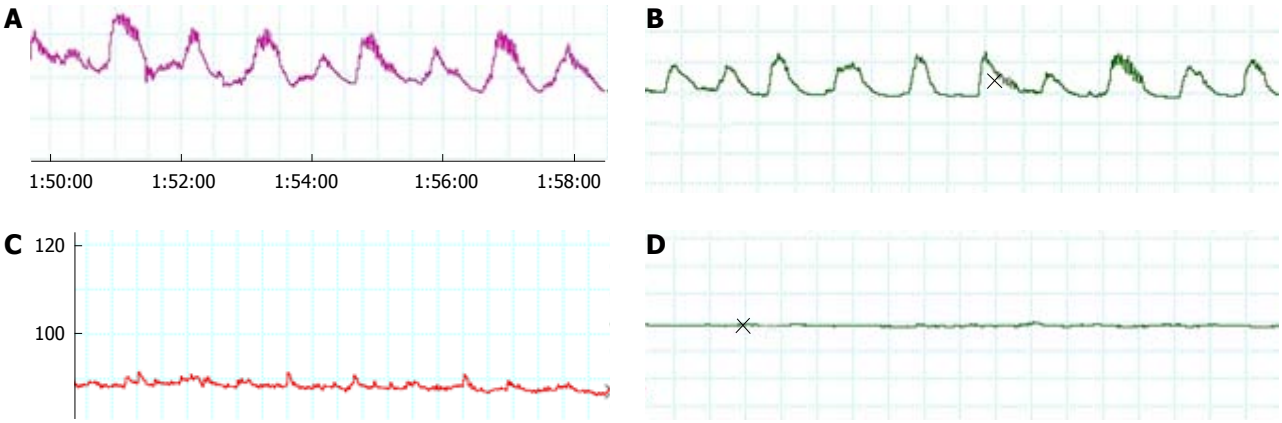

Figure 6 Functional studies (anal manometry). A and B: Once the time for registry stabilization (30 to 60 min) was surpassed, spontaneous, rhythmic contractions in the form of "plateaus" with multiple fasciculations (peaks) appeared; C and D: Postoperatively, findings were consistent with a total sphincter lesion, the activity disappeared and was substituted by a gentle oscillation of basal line, without a recognizable pattern and irrespective of the applied treatment. To visualize the values of registries analyzed parameters see Table 1.

sphincters were always sectioned, as confirmed by morphological studies (Figure 5).

Regarding functional assays, spontaneous, rhythmic contractions in the form of "plateaus" with multiple fasciculation-producing peaks appeared (Figure 6). These basal contractions were very heterogeneous, as we can see in Table 1, with a frequency of 0.91 to 4.17 per min (mean 1.6980 with SD 0.57698), a mean duration of 26.67 seconds, and a mean number of peaks of 12.53. Mean registered amplitude was 25.95 mmHg and the Tmax was 8.88 seconds with SD 2.93. Probably the most important component of this activity was the internal sphincter, but both could contribute. Postoperatively, findings were consistent with total sphincter lesion, irrespective of the applied treatment; the described activity disappeared and was substituted by a gentle oscillation of the basal line without a recognizable pattern (Figure 6). Only in 4 out of 36 animals did some small contractions appear (Table 1). There were no differences between groups at the immediate postoperative measurements (P = 0.329–0.73 on ANOVA). AUC decreased to less than one-tenth in the lesion group, without significant differences between groups (P = 0.134).

Cell tracking on animal samples

We analyzed the presence of GFP+ cells in 19 rats (Figure 7), finding them in 16 (84.21%) and in 100% of the 7 sacrificed at 7 d.

Regarding GFP+ cells' location, the majority of animals had many simultaneously. The most frequent were muscular section endings and the tissue between them (10/16 animals), then submucosa (9/16 animals), and surrounding sutures (6/16 animals; 100% of the 5 animals with biosutures). We could not find them at the mucosa. Concerning spatial disposition, the most frequent was in small groups (10/16) or making "conglomerates" (8/16; 5/6 of group 1 and 3/5 of group 3). Three animals presented with perivascular disposition.

We could not identify cells simultaneously expressing

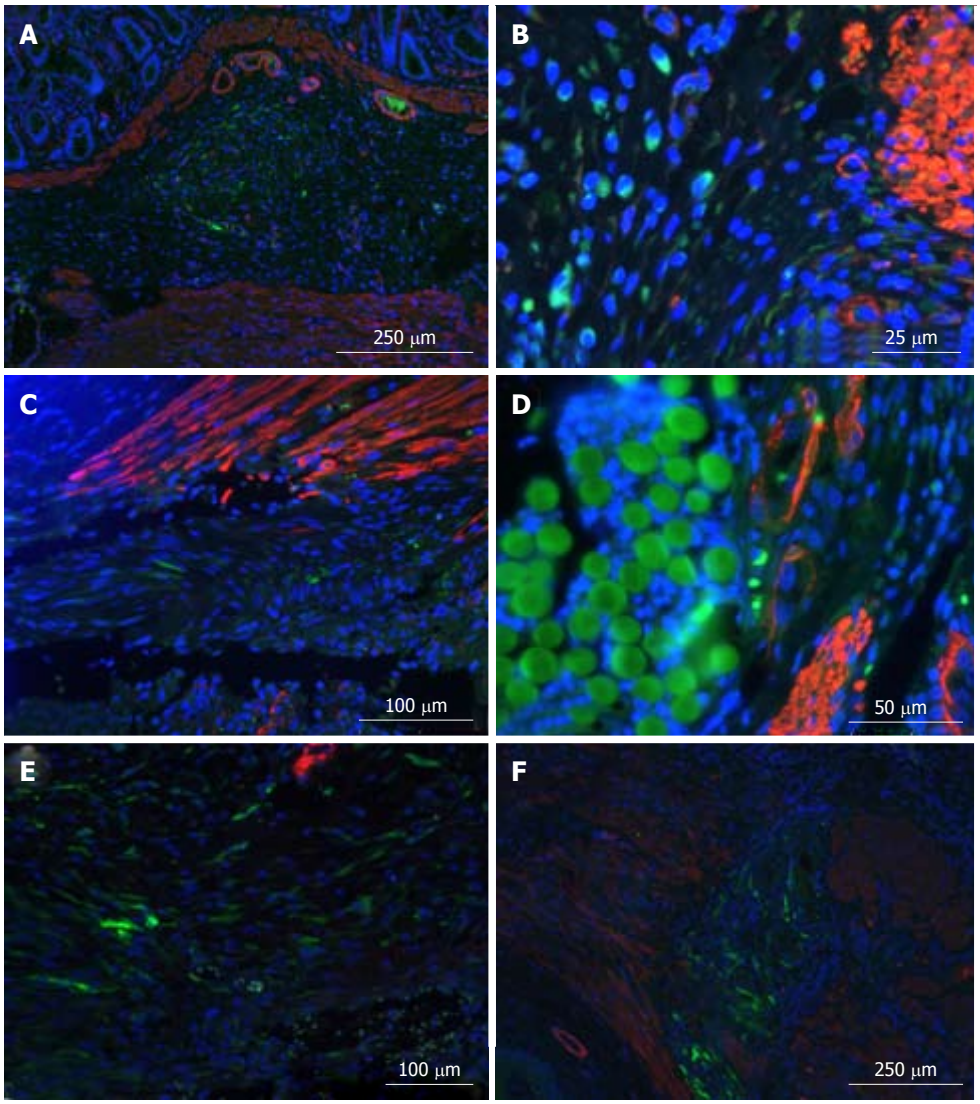

Figure 7 Immunolocalization of ASCs at surgical site. Cell nuclei were stained with DAPI (blue), ASCs with eGFP (green), and muscle with α-actin (red). On injected animals, ASCs tended to form "conglomerates" in the submucosa and were able to survive at least 7 d (A: G1 group rat sacrificed at 7 d). ASCs appeared in injury foci, even though they were deposited at some distance (*e.g.*, in "conglomerates"): In muscle section margins (B: G2 sacrificed at 7 d and C: G1 sacrificed at 1 d). In the biosuture group, ACSs also surround the suture (D: G2 sacrificed at 4 d). ASCs also appeared in the tissue that filled the space between muscle section margins (E: G3 group rat sacrificed at 4 d and F: G1 group rat sacrificed at 7 d). ASC: Adipose-derived stem cell; eGFP: Enhanced green fluorescent protein.

GFP and α-actin.

Safety

Five deaths took place (13.88%), without relevant findings on postmortem studies and unrelated to the treatment received; eighty percent were probably related to anesthetic drugs.

We found 17 adverse events, affecting 13 rats (incidence 36.11%). None of them were considered severe. In order of frequency, they were: fecal impaction; hypotonic anus and anal laceration (3 cases of each); perianal laceration, perianal phlegmon, and cutaneous suture dehiscence (2 cases of each) and 1 case of anal stenosis. We considered 2 cases attributable to received treatment (muscular suture), 3 cases doubtfully related, and 12 cases non-related. None seemed attributable to ASCs.

No significant differences were found between groups in the incidence of mortality or adverse events. So, SC therapy on this model was completely safe.

DISCUSSION

The anatomy of rat anal sphincters is similar to that of humans. The most remarkable differences are that the external sphincter is poorly developed and the internal sphincter is difficult to distinguish from the rectal muscular layer. Using the 1-cm section from the anal verge, we can include both, even though they are not well identified.

Related to function, several publications reflect that the external sphincter's contribution to basal tone in rats seems higher than in humans[29,30]. We decided to use the anesthetic ketamine/xylazine mixture,

because it was the one we found to provide more stable registries. This mixture was also used by Zutshi and colleagues[29,34-36], but other authors have employed ketamine with better results than ours[37].

Our results were similar to other publications with regard to detected activity. However, numeric values were in some cases very dissimilar, probably due to the different rat species employed (BDIX *vs* Sprague-Dawley) or slight methodological variations. Our frequency was similar to that of Vinograd (0.91–4.17 per min compared with 1–3[30]) or Zutshi *et al*[29], also duration (26.67 with SD 6.67 compared to 35.10 ± 2.53[29] or 26.23 ± 1.68[36]) and Tmax (8.88 second, SD 2.93, compared to 10[29] or 12.24 ± 0.078[36]). But our mean NP was higher (12.53 SD 3.53 compared to 8.9 ± 0.5[29] or to 7.22 ± 0.45[36]) and our range was also much wider (25.95, SD 16.69 compared to 10.01 ± 0.66[29] or to 12.0 ± 0.1[37]).

In our experiments, practically all spontaneous activity disappeared after surgery (irrespective of the reparation or SC presence). Nevertheless, Zutshi *et al*[29] and others[34] found some pressure waves, despite muscle disruption. The only possible explanation for this phenomenon is the difference in the injury, as they practice "a precise 3-mm to 4-mm incision" for selective sphincter section that might not be complete.

Compared with other published works using SCs for fecal incontinence in rats, our lesion is one of the most extensive. Lorenzi *et al*[38] described a left lateral selective sphincterotomy without specifying the length used later by Mazzanti *et al*[39]. Kang *et al*[40] performed cryoinjury without specifying the damaged volume; later, Bisson *et al*[41] published that cryoinjury must be at least 90º to be significant. Zutshi´s model[29] was also employed in other publications[34]. White *et al*[42] and Pathi *et al*[43] performed a total section of 7 mm, followed by rectal mucosa repair. Going further, Lane *et al*[37] performed a more aggressive injury ("proctoepisiotomy") without describing technical details and extension; Salcedo *et al*[36] applied an even more aggressive procedure involving an excision of 25% of both sphincters, but they did not perform an immediate postoperative control so we could not compare our functional results with theirs.

Aiming to minimize the effects of the poorly developed sphincters, some authors have tested bigger animals (rabbits[44-46] and dogs[47,48]), but there are only five published papers compared to ten for rats.

We can discuss clinical relevance of our injury model. Human obstetric trauma is more complex than a simple section during episiotomy or a perineum tear, it combines muscle injury, regional hypoxia, denervation, *etc*. Other factors could be added later in life, such as ageing, hormonal changes, surgeries, *etc*.

On the other hand, in the clinical setting, immediate repair offers better results, but the most frequent scenario is a repair indicated years later. This could modify some confounding factors that we have observed with immediate repair (mucosal tears, fecal contamination, *etc*.) that could compromise ASCs survivorship or effects.

In an effort to reproduce these complex effects, simulated childbirth injury models have been designed, mainly for urinary incontinence. It was first described by Resplande *et al*[49], who inserted an inflated 10F Foley catheter inside the vagina over 3 to 4 h to simulate labor; an episiotomy and extraction of inflated Foley was performed. Later, Healy *et al*[50] published a model for fecal incontinence using two intrapelvic, retrouterine balloons (6 Fr urinary catheters) placed through a 3-cm laparotomy over 1 h. We tested the model of Resplande, but we decided not to use it anymore because we observed high variability in injuries. We think that our model results in a more homogeneous injury. More studies on simulated childbirth models may be needed and also consideration of a delayed repair, as some authors have performed[51].

Regarding SC vehicles, we have employed biosutures and injections, finding GFP+ cells during at least 7 d with both. Biosutures can be used in a similar fashion to conventional sutures. The pioneer model with biosutures and colonic anastomoses studied in the short-term found fewer adherences on days 4 and 7 (preserving anastomotic resistance[25]), which was significantly greater on day 4 in a poor adhesion environment[26]. Later, tracheal anastomoses were studied for 60 d and a swift inflammatory response was found: on postoperative days 1 and 4, acute inflammation was substituted by chronic inflammation[27]. Yao *et al*[52] used embryonic SCs, added molecules to improve cell adherence (poly-L-lysine and fibronectin)[53], and applied them to rat tendons. Horváthy *et al*[54] employed bone marrow-MSCs and observed better cell adherence if the suture was covered with albumin and the SC surviving in implanted tissues during 5 wk. They recommended a 48 h culture as the best for clinical usage (over 168 h, 910 polyglactin loses resistance)[55]. No evidence exists about the best dose. However, we found that some cells tend to form "clusters" over the suture, in the culture medium and also remaining ASCs adhered to the sutures after their usage. These findings make us wonder about the suitability of this vehicle, so more studies on suture preparation are needed. Delivered cell dose could be more controlled by injection, but similar clusters can be observed sometimes, losing ASCs.

Now the questions that arise are how to get better SC delivery, survival, and function in tissues. An interesting approach is molecule addition to sutures, such as vascular endothelial growth factor (VEGF) by Bigalke *et al*[56]. Cytokines could also be of high relevance, mostly in models with delayed repair because they could already be normalized; some examples are stromal-derived factor-1 (SDF-1) and monocyte chemotactic protein-3 (MCP-3)[57]. As an example, Sun *et al*[58] performed a 50% excision of the anal sphincter, and they compared SDF-1 alone or combined with MSCs 3 wk later with interesting results.

Published animal investigations in this field have

employed muscle progenitors in 10 papers and BM-MSCs in 5 papers. All except one confirm the safety. Concerning results, generally good morphological and functional responses have been observed with questions about cell survival and only one long-term study[46]. In human research, there is one study unrelated to fecal incontinence that finds improvement with ASCs[20], three incontinence studies involving 38 patients with promising results[59-62], and seven ongoing clinical trials.

There are many questions remaining about the mechanism of action of ASCs, which is not totally understood nowadays. Their multi-lineage differentiation potential coupled with their immune-privilege and their ability to stimulate resident progenitor cells through paracrine secretion, as well as their angiogenic potential, are important. There is growing evidence about their immunomodulation capability. It is thought to be largely based on inhibition of T cell and B cell proliferation and dendritic cell maturation[63] and in the secretion of cytokines[64]. For example, Nemeth *et al*[65] observed MSCs' sepsis attenuation by macrophages reprogramming to increase IL-10, a cytokine that decreases neutrophil migration to tissues. There is much interest in identifying secretome and immunosuppressive properties of ASCs. Our research team has added some contributions. Georgiev-Hristov *et al*[27] showed an early switch from acute to chronic inflammation in the presence of ASCs after tracheal anastomosis. And, Riera del Moral *et al*[66] observed less acute and chronic inflammation and increasing fibrosis of aneurysm sacs in pigs.

Finally, our present results show that SC therapy on this model is safe, as no serious adverse reactions or neoplastic processes were observed. In this field, there is a worrisome paper in which 2/24 rats receiving 5 × 10^6 myogenic SCs developed local abnormal foci of growth that were benign[67].

Our proposed surgical procedure produces a consistent lesion of both sphincters and could be a model for posttraumatic fecal incontinence. Biosutures and injections are both suitable for cell delivery. Biosutures do not change the surgical technique or suture manageability. ASCs are able to survive in the complex area of anal sphincters, at least 7 d, and are safe in this clinical setting.

ARTICLE HIGHLIGHTS

Research background

Fecal incontinence is a very prevalent (11% to 15% of adults), nonfatal illness associated with devastating consequences mainly in the psychosocial sphere and quality of life. Although in the last years sacral neuromodulation has been improving the poor results of available treatments, surgery remains the choice for the most severe or refractory cases, mainly if sphincter lesions are present (the most frequently observed anatomic alteration). Sphincter repair is the most successful technique used for traumatic fecal incontinence, but its results are not very satisfactory, mainly in the long term. Stem cells (SCs) and adipose-derived stem cells (ASCs) have demonstrated promising results in a wide variety of clinical settings, including particularly unfavorable environments for wound healing, such as anal fistulas and Crohn´s disease.

Research motivation

To test if SC therapy could improve postoperative healing mechanisms in patients with fecal incontinence. If this hypothesis is correct, surgical outcomes could be improved and more patients would benefit from surgery in the short and long term.

Research objectives

The first objective was obtaining an in-depth knowledge of rat anal region anatomy so as to design an easy and reproducible model for fecal incontinence or sphincter injury. The second objective was to establish a method for studying rat anal sphincter function, defining the best anesthetic method and physiological test to be used with low morbidity. Finally, the main objective was to study the feasibility and safety of ASC administration to rat anal sphincters *via* different methods. All of the previously mentioned objectives were accomplished. This furnishes future investigations with the proposed animal model for study of potential SC efficacy; and, if the expected results are obtained, they will support trying this therapy on humans.

Research methods

Rat anal region normal anatomy was studied on BDIX and Wistar female rats. Once anatomy was well known, the authors studied a system capable of detecting the low-pressure waves supposed to be created in this area, and the capability of this system to detect sphincter lesions. Simultaneously, the authors needed to select a model for fecal incontinence. Since simulated childbirth injury models are complex, associate morbidity in animals and generate highly variable injuries, the authors decided to create a simple model of sphincter injury. The authors tested different injury models and finally selected an anterior extra-mucosal longitudinal myotomy of 1 cm. Researchers studied its morphology and physiology to verify if this procedure injured both sphincters in a constant way. Going further, to decide the best system to detect sphincter pressures, the authors tested different anesthetic drugs (inhaled isoflurane, intraperitoneal ketamine, intraperitoneal ketamine plus xylazine) and different systems to detect pressure, including an endorectal balloon that was retired slowly (similar to human manometry), a normal saline infusion through a mini-laparotomy in the rectum (until anus became opened by rectal increasing pressure), and basal anal manometry with a stationary endoanal balloon. Preliminary studies led the authors to select intraperitoneal ketamine plus xylazine (provided the most stable functional registries) and basal anal manometry with a stationary endoanal balloon as the best options. ASCs were obtained from subcutaneous fat from two BDIX rat males and later were marked with eGFP. Later, preparations of 10^6 ASCs in 50 μL of normal saline or biosutures of polyglactin 910 6/0 suture covered with 1.5 × 10^6 ASCs were prepared. Finally, those preparations were applied to 36 BDIX virgin female adult rats that underwent functional studies prior to and after the surgery. Then, safety and cell tracking studies were performed during a follow-up period lasting 7 d. Animals were distributed among three groups: biosuture repair; cell injection without repair; and conventional suture repair and cell injection. Moreover, some quality studies were performed with biosutures, trying to establish the real cell dose administered during their use.

Research results

A dose of 820000 to 860000 ASCs adhered to the suture, but not all of them remained on the animal tissue. The described lesion produced a constant injury to both anal sphincters. Rat anal sphincter spontaneous function is composed of heterogeneous, spontaneous, consistent, and rhythmic contractions in the form of "plateaus", with multiple fasciculations that disappeared consistently with the described surgical damage. ASCs are able to survive in this potentially septic area for at least 7 d. We were able to identify them in 84% of the studied animals, mainly in the muscular section area or in the tissue that appeared between both muscular endings. ASCs form a kind of "conglomerate" in rats treated with injections, while they wrapped around the suture in the biosuture group. ASCs were also able to migrate to the damaged zone, and the most frequent disposition was formation of small groupings. No relevant adverse events or mortality could be related to the SCs in our study. We also did not find unexpected tissue growths. So, this cell therapy was deemed as safe, at least in the short term. As unresolved issues, safety must be studied in the long term, ASC survival must be confirmed in longer follow-ups, systems to improve SC function and survival could be tested, and biosuture cell dosing must be studied

thoroughly.

Research conclusions

The authors propose an easy, reproducible and safe method for rat sphincter injury that could represent a model for posttraumatic fecal incontinence. ASC administration through cell injections or biosutures is feasible and safe. Both systems are suitable for cell delivery, and biosutures do not change the surgical technique or suture manageability. Applied ASCs are able to survive in this complex area. By this approach, ASCs could offer a benefit for postoperative healing in fecal incontinence.

Research perspectives

The model for rat anal sphincter injury can be used in future experiments for testing potential SC efficacy and obtaining long-term results. Anesthetic and functional study methodologies may need some minor changes. Areas prone to be studied deeply are: obtaining knowledge about the potential mechanism of action of ASCs; improving SC delivery, survival and function in the receiving animals (cytokine or molecule addition, *etc.*); and, supplying SCs through minimally invasive methods. This study allows continuing studies on animal models prior to human use, and, if the expected results are obtained, support trying this therapy on humans (only in empirical and highly controlled settings).

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REFERENCES

1 **Macmillan AK**, Merrie AE, Marshall RJ, Parry BR. The prevalence of fecal incontinence in community-dwelling adults: a systematic review of the literature. *Dis Colon Rectum* 2004; **47**: 1341-1349 [PMID: 15484348 DOI: 10.1007/s10350-004-0593-0]
2 **Nelson R**, Norton N, Cautley E, Furner S. Community-based prevalence of anal incontinence. *JAMA* 1995; **274**: 559-561 [PMID: 7629985 DOI: doi:10.1001/jama.1995.03530070057030]
3 **Johanson JF**, Lafferty J. Epidemiology of fecal incontinence: the silent affliction. *Am J Gastroenterol* 1996; **91**: 33-36 [PMID: 8561140]
4 **Dunivan GC**, Heymen S, Palsson OS, von Korff M, Turner MJ, Melville JL, Whitehead WE. Fecal incontinence in primary care: prevalence, diagnosis, and health care utilization. *Am J Obstet Gynecol* 2010; **202**: 493.e1-493.e6 [PMID: 20223447 DOI: 10.1016/j.ajog.2010.01.018]
5 **Deutekom M**, Dobben AC, Dijkgraaf MG, Terra MP, Stoker J, Bossuyt PM. Costs of outpatients with fecal incontinence. *Scand J Gastroenterol* 2005; **40**: 552-558 [PMID: 16036507 DOI: 10.1080/00365520510012172]
6 **Dudding TC**, Vaizey CJ, Kamm MA. Obstetric anal sphincter injury: incidence, risk factors, and management. *Ann Surg* 2008; **247**: 224-237 [PMID: 18216527 DOI: 10.1097/SLA.0b013e318142cdf4]
7 **Oberwalder M**, Connor J, Wexner SD. Meta-analysis to determine the incidence of obstetric anal sphincter damage. *Br J Surg* 2003; **90**: 1333-1337 [PMID: 14598410 DOI: 10.1002/bjs.4369]
8 **Madoff RD**. Surgical treatment options for fecal incontinence. *Gastroenterology* 2004; **126**: S48-S54 [PMID: 14978638 DOI: 10.1053/j.gastro.2003.10.015]
9 **Halverson AL**, Hull TL. Long-term outcome of overlapping anal sphincter repair. *Dis Colon Rectum* 2002; **45**: 345-348 [PMID: 12068192 DOI: 10.1007/s10350-004-6180-6]
10 **Mizuno H**, Tobita M, Uysal AC. Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem Cells* 2012; **30**: 804-810 [PMID: 22415904 DOI: 10.1002/stem.1076]
11 **Trebol Lopez J**, Georgiev Hristov T, García-Arranz M, García-Olmo D. Stem cell therapy for digestive tract diseases: current state and future perspectives. *Stem Cells Dev* 2011; **20**: 1113-1129 [PMID: 21187000 DOI: 10.1089/scd.2010.0277]
12 **Ma T**, Sun J, Zhao Z, Lei W, Chen Y, Wang X, Yang J, Shen Z. A brief review: adipose-derived stem cells and their therapeutic potential in cardiovascular diseases. *Stem Cell Res Ther* 2017; **8**: 124 [PMID: 28583198 DOI: 10.1186/s13287-017-0585-3]
13 **González MA**, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009; **136**: 978-989 [PMID: 19135996 DOI: 10.1053/j.gastro.2008.11.041]
14 **Gonzalez-Rey E**, Anderson P, González MA, Rico L, Büscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009; **58**: 929-939 [PMID: 19136511 DOI: 10.1136/gut.2008.168534]
15 **García-Olmo D**, García-Arranz M, García LG, Cuellar ES, Blanco IF, Prianes LA, Montes JA, Pinto FL, Marcos DH, García-Sancho L. Autologous stem cell transplantation for treatment of rectovaginal fistula in perianal Crohn's disease: a new cell-based therapy. *Int J Colorectal Dis* 2003; **18**: 451-454 [PMID: 12756590 DOI: 10.1007/s00384-003-0490-3]
16 **Oyama Y**, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. *Gastroenterology* 2005; **128**: 552-563 [PMID: 15765390 DOI: S0016508504021560]
17 **Uccelli A**, Moretta L, Pistoia V. Immunoregulatory function of mesenchymal stem cells. *Eur J Immunol* 2006; **36**: 2566-2573 [PMID: 17013987 DOI: 10.1002/eji.200636416]
18 **Aust L**, Devlin B, Foster SJ, Halvorsen YD, Hicok K, du Laney T, Sen A, Willingmyre GD, Gimble JM. Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy* 2004; **6**: 7-14 [PMID: 14985162 DOI: 10.1080/14653240310004539]
19 **García-Olmo D**, García-Arranz M, Herreros D, Pascual I, Peiro C, Rodríguez-Montes JA. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. *Dis Colon Rectum* 2005; **48**: 1416-1423 [PMID: 15933795 DOI: 10.1007/s10350-005-0052-6]
20 **Garcia-Olmo D**, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. *Dis Colon Rectum* 2009; **52**: 79-86 [PMID: 19273960 DOI: 10.1007/DCR.0b013e3181973487]
21 **Herreros MD**, Garcia-Arranz M, Guadalajara H, De-La-Quintana P, Garcia-Olmo D; FATT Collaborative Group. Autologous expanded adipose-derived stem cells for the treatment of complex cryptoglandular perianal fistulas: a phase III randomized clinical trial (FATT 1: fistula Advanced Therapy Trial 1) and long-term evaluation. *Dis Colon Rectum* 2012; **55**: 762-772 [PMID: 22706128 DOI: 10.1097/DCR.0b013e318255364a]
22 **García-Arranz M**, Herreros MD, González-Gómez C, de la Quintana P, Guadalajara H, Georgiev-Hristov T, Trébol J, Garcia-Olmo D. Treatment of Crohn's-Related Rectovaginal Fistula With Allogeneic Expanded-Adipose Derived Stem Cells: A Phase I-IIa Clinical Trial. *Stem Cells Transl Med* 2016; **5**: 1441-1446 [PMID: 27412883 DOI: 10.5966/sctm.2015-0356]
23 **de la Portilla F**, Alba F, García-Olmo D, Herrerías JM, González FX, Galindo A. Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: results from a multicenter phase I/IIa clinical trial. *Int J Colorectal Dis* 2013; **28**: 313-323 [PMID: 23053677 DOI: 10.1007/s00384-012-1581-9]
24 **Panés J**, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi

L, Grimaud JC, de la Portilla F, Goldin E, Richard MP, Leselbaum A, Danese S; ADMIRE CD Study Group Collaborators. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet* 2016; **388**: 1281-1290 [PMID: 27477896 DOI: 10.1016/S0140-6736(16)31203-X]

25 **Pascual I**, de Miguel GF, Gómez-Pinedo UA, de Miguel F, Arranz MG, García-Olmo D. Adipose-derived mesenchymal stem cells in biosutures do not improve healing of experimental colonic anastomoses. *Br J Surg* 2008; **95**: 1180-1184 [PMID: 18690635 DOI: 10.1002/bjs.6242]

26 **Pascual I**, Fernández de Miguel G, García Arranz M, García-Olmo D. Biosutures improve healing of experimental weak colonic anastomoses. *Int J Colorectal Dis* 2010; **25**: 1447-1451 [PMID: 20544210 DOI: 10.1007/s00384-010-0952-3]

27 **Georgiev-Hristov T**, García-Arranz M, García-Gómez I, García-Cabezas MA, Trébol J, Vega-Clemente L, Díaz-Agero P, García-Olmo D. Sutures enriched with adipose-derived stem cells decrease the local acute inflammation after tracheal anastomosis in a murine model. *Eur J Cardiothorac Surg* 2012; **42**: e40-e47 [PMID: 22689184 DOI: 10.1093/ejcts/ezs357]

28 **Wang EQ**, Soda DM, Fung HL. Nitroglycerin-induced relaxation of anorectal smooth muscle: evidence for apparent lack of tolerance development in the anaesthetized rat. *Br J Pharmacol* 2001; **134**: 418-424 [PMID: 11564661 DOI: 10.1038/sj.bjp.0704239]

29 **Zutshi M**, Salcedo LB, Zaszczurynski PJ, Hull TL, Butler RS, Damaser MS. Effects of sphincterotomy and pudendal nerve transection on the anal sphincter in a rat model. *Dis Colon Rectum* 2009; **52**: 1321-1329 [PMID: 19571711 DOI: 10.1007/DCR.0b013e31819f746d]

30 **Vinograd I**, Hanani M, Hadary A, Merguerian P, Nissan S. Animal model for the study of internal anal sphincter activity. *Eur Surg Res* 1985; **17**: 259-263 [PMID: 2864255]

31 **Zuk PA**, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001; **7**: 211-228 [PMID: 11304456 DOI: 10.1089/107632701300062859]

32 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]

33 **Bourin P**, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013; **15**: 641-648 [PMID: 23570660 DOI: 10.1016/j.jcyt.2013.02.006]

34 **Salcedo L**, Damaser M, Butler R, Jiang HH, Hull T, Zutshi M. Long-term effects on pressure and electromyography in a rat model of anal sphincter injury. *Dis Colon Rectum* 2010; **53**: 1209-1217 [PMID: 20628287 DOI: 10.1007/DCR.0b013e3181de7fe0]

35 **Salcedo L**, Mayorga M, Damaser M, Balog B, Butler R, Penn M, Zutshi M. Mesenchymal stem cells can improve anal pressures after anal sphincter injury. *Stem Cell Res* 2013; **10**: 95-102 [PMID: 23147650 DOI: 10.1016/j.scr.2012.10.002]

36 **Salcedo L**, Penn M, Damaser M, Balog B, Zutshi M. Functional outcome after anal sphincter injury and treatment with mesenchymal stem cells. *Stem Cells Transl Med* 2014; **3**: 760-767 [PMID: 24797828 DOI: 10.5966/sctm.2013-0157]

37 **Lane FL**, Jacobs SA, Craig JB, Nistor G, Markle D, Noblett KL, Osann K, Keirstead H. In vivo recovery of the injured anal sphincter after repair and injection of myogenic stem cells: an experimental model. *Dis Colon Rectum* 2013; **56**: 1290-1297 [PMID: 24105005 DOI: 10.1097/DCR.0b013e3182a4adfb]

38 **Lorenzi B**, Pessina F, Lorenzoni P, Urbani S, Vernillo R, Sgaragli G, Gerli R, Mazzanti B, Bosi A, Saccardi R, Lorenzi M. Treatment of experimental injury of anal sphincters with primary surgical repair and injection of bone marrow-derived mesenchymal stem cells. *Dis Colon Rectum* 2008; **51**: 411-420 [PMID: 18224375 DOI: 10.1007/s10350-007-9153-8]

39 **Mazzanti B**, Lorenzi B, Borghini A, Boieri M, Ballerini L, Saccardi R, Weber E, Pessina F. Local injection of bone marrow progenitor cells for the treatment of anal sphincter injury: in-vitro expanded versus minimally-manipulated cells. *Stem Cell Res Ther* 2016; **7**: 85 [PMID: 27328811 DOI: 10.1186/s13287-016-0344-x]

40 **Kang SB**, Lee HN, Lee JY, Park JS, Lee HS, Lee JY. Sphincter contractility after muscle-derived stem cells autograft into the cryoinjured anal sphincters of rats. *Dis Colon Rectum* 2008; **51**: 1367-1373 [PMID: 18536965 DOI: 10.1007/s10350-008-9360-y]

41 **Bisson A**, Fréret M, Drouot L, Jean L, Le Corre S, Gourcerol G, Doucet C, Michot F, Boyer O, Lamacz M. Restoration of anal sphincter function after myoblast cell therapy in incontinent rats. *Cell Transplant* 2015; **24**: 277-286 [PMID: 24143883 DOI: 10.3727/096368913X674053]

42 **White AB**, Keller PW, Acevedo JF, Word RA, Wai CY. Effect of myogenic stem cells on contractile properties of the repaired and unrepaired transected external anal sphincter in an animal model. *Obstet Gynecol* 2010; **115**: 815-823 [PMID: 20308844 DOI: 10.1097/AOG.0b013e3181d56cc5]

43 **Pathi SD**, Acevedo JF, Keller PW, Kishore AH, Miller RT, Wai CY, Word RA. Recovery of the injured external anal sphincter after injection of local or intravenous mesenchymal stem cells. *Obstet Gynecol* 2012; **119**: 134-144 [PMID: 22183221 DOI: 10.1097/AOG.0b013e3182397009]

44 **Aghaee-Afshar M**, Rezazadehkermani M, Asadi A, Malekpour-Afshar R, Shahesmaeili A, Nematollahi-mahani SN. Potential of human umbilical cord matrix and rabbit bone marrow-derived mesenchymal stem cells in repair of surgically incised rabbit external anal sphincter. *Dis Colon Rectum* 2009; **52**: 1753-1761 [PMID: 19966609 DOI: 10.1007/DCR.0b013e3181b55112]

45 **Kajbafzadeh AM**, Elmi A, Talab SS, Esfahani SA, Tourchi A. Functional external anal sphincter reconstruction for treatment of anal incontinence using muscle progenitor cell auto grafting. *Dis Colon Rectum* 2010; **53**: 1415-1421 [PMID: 20847624 DOI: 10.1007/DCR.0b013e3181e53088]

46 **Kajbafzadeh AM**, Kajbafzadeh M, Sabetkish S, Sabetkish N, Tavangar SM. Tissue-Engineered External Anal Sphincter Using Autologous Myogenic Satellite Cells and Extracellular Matrix: Functional and Histological Studies. *Ann Biomed Eng* 2016; **44**: 1773-1784 [PMID: 26424474 DOI: 10.1007/s10439-015-1468-3]

47 **Oh HK**, Lee HS, Lee JH, Oh SH, Lim JY, Ahn S, Hwang JY, Kang SB. Functional and histological evidence for the targeted therapy using biocompatible polycaprolactone beads and autologous myoblasts in a dog model of fecal incontinence. *Dis Colon Rectum* 2015; **58**: 517-525 [PMID: 25850839 DOI: 10.1097/DCR.0000000000000346]

48 **Oh HK**, Lee HS, Lee JH, Oh SH, Lim JY, Ahn S, Kang SB. Coadministration of basic fibroblast growth factor-loaded polycaprolactone beads and autologous myoblasts in a dog model of fecal incontinence. *Int J Colorectal Dis* 2015; **30**: 549-557 [PMID: 25592048 DOI: 10.1007/s00384-015-2121-1]

49 **Resplande J**, Gholami SS, Graziottin TM, Rogers R, Lin CS, Leng W, Lue TF. Long-term effect of ovariectomy and simulated birth trauma on the lower urinary tract of female rats. *J Urol* 2002; **168**: 323-330 [PMID: 12050564 DOI: 10.1016/S0022-5347(05)64915-4]

50 **Healy CF**, O'Herlihy C, O'Brien C, O'Connell PR, Jones JF. Experimental models of neuropathic fecal incontinence: an animal model of childbirth injury to the pudendal nerve and external anal sphincter. *Dis Colon Rectum* 2008; **51**: 1619-1626; discussion 1626 [PMID: 18779998 DOI: 10.1007/s10350-008-9283-7]

51 **Sun L**, Yeh J, Xie Z, Kuang M, Damaser MS, Zutshi M. Electrical Stimulation Followed by Mesenchymal Stem Cells Improves Anal Sphincter Anatomy and Function in a Rat Model at a Time Remote From Injury. *Dis Colon Rectum* 2016; **59**: 434-442 [PMID:

27050606 DOI: 10.1097/DCR.0000000000000548]
52 **Yao J**, Korotkova T, Riboh J, Chong A, Chang J, Smith RL. Bioactive sutures for tendon repair: assessment of a method of delivering pluripotential embryonic cells. *J Hand Surg Am* 2008; **33**: 1558-1564 [PMID: 18984338 DOI: 10.1016/j.jhsa.2008.06.010]
53 **Yao J**, Korotkova T, Smith RL. Viability and proliferation of pluripotential cells delivered to tendon repair sites using bioactive sutures--an in vitro study. *J Hand Surg Am* 2011; **36**: 252-258 [PMID: 21186083 DOI: 10.1016/j.jhsa.2010.10.004]
54 **Horváthy DB**, Vácz G, Cselenyák A, Weszl M, Kiss L, Lacza Z. Albumin-coated bioactive suture for cell transplantation. *Surg Innov* 2013; **20**: 249-255 [PMID: 22717700 DOI: 10.1177/1553350612451353]
55 **Horváthy D,** Vácz G, Szabó T, Renner K, Vajda K, Sándor B, Lacza Z. Absorption and tensility of bioactive sutures prepared for cell transplantation. *Materials* 2013; **6:** 544-550 [DOI: 10.3390/ma6020544]
56 **Bigalke C**, Luderer F, Wulf K, Storm T, Löbler M, Arbeiter D, Rau BM, Nizze H, Vollmar B, Schmitz KP, Klar E, Sternberg K. VEGF-releasing suture material for enhancement of vascularization: development, in vitro and in vivo study. *Acta Biomater* 2014; **10**: 5081-5089 [PMID: 25204522 DOI: 10.1016/j.actbio.2014.09.002]
57 **Salcedo L**, Sopko N, Jiang HH, Damaser M, Penn M, Zutshi M. Chemokine upregulation in response to anal sphincter and pudendal nerve injury: potential signals for stem cell homing. *Int J Colorectal Dis* 2011; **26**: 1577-1581 [PMID: 21706136 DOI: 10.1007/s00384-011-1269-6]
58 **Sun L**, Xie Z, Kuang M, Penn M, Damaser MS, Zutshi M. Regenerating the Anal Sphincter: Cytokines, Stem Cells, or Both? *Dis Colon Rectum* 2017; **60**: 416-425 [PMID: 28267010 DOI: 10.1097/DCR.0000000000000783]
59 **Frudinger A**, Kölle D, Schwaiger W, Pfeifer J, Paede J, Halligan S. Muscle-derived cell injection to treat anal incontinence due to obstetric trauma: pilot study with 1 year follow-up. *Gut* 2010; **59**: 55-61 [PMID: 19875391 DOI: 10.1136/gut.2009.181347]
60 **Frudinger A**, Pfeifer J, Paede J, Kolovetsiou-Kreiner V, Marksteiner R, Halligan S. Autologous skeletal-muscle-derived cell injection for anal incontinence due to obstetric trauma: a 5-year follow-up of an initial study of 10 patients. *Colorectal Dis* 2015; **17**: 794-801 [PMID: 25773013 DOI: 10.1111/codi.12947]
61 **Romaniszyn M**, Rozwadowska N, Malcher A, Kolanowski T, Walega P, Kurpisz M. Implantation of autologous muscle-derived stem cells in treatment of fecal incontinence: results of an experimental pilot study. *Tech Coloproctol* 2015; **19**: 685-696 [PMID: 26266767 DOI: 10.1007/s10151-015-1351-0]
62 **Sarveazad A**, Newstead GL, Mirzaei R, Joghataei MT, Bakhtiari M, Babahajian A, Mahjoubi B. A new method for treating fecal incontinence by implanting stem cells derived from human adipose tissue: preliminary findings of a randomized double-blind clinical trial. *Stem Cell Res Ther* 2017; **8**: 40 [PMID: 28222801 DOI: 10.1186/s13287-017-0489-2]
63 **Nauta AJ**, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 2007; **110**: 3499-3506 [PMID: 17664353 DOI: blood-2007-02-069716]
64 **Chen L**, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* 2008; **3**: e1886 [PMID: 18382669 DOI: 10.1371/journal.pone.0001886]
65 **Németh K**, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009; **15**: 42-49 [PMID: 19098906 DOI: 10.1038/nm.1905]
66 **Riera del Moral L**, Largo C, Ramirez JR, Vega Clemente L, Fernández Heredero A, Riera de Cubas L, Garcia-Olmo D, Garcia-Arranz M. Potential of mesenchymal stem cell in stabilization of abdominal aortic aneurysm sac. *J Surg Res* 2015; **195**: 325-333 [PMID: 25592273 DOI: 10.1016/j.jss.2014.12.020]
67 **Jacobs SA**, Lane FL, Pham QA, Nistor G, Robles R, Chua C, Boubion B, Osann K, Keirstead H. Safety assessment of myogenic stem cell transplantation and resulting tumor formation. *Female Pelvic Med Reconstr Surg* 2013; **19**: 362-368 [PMID: 24165451 DOI: 10.1097/SPV.0000000000000035]

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MINIREVIEWS

Spatiotemporal switching signals for cancer stem cell activation in pediatric origins of adulthood cancer: Towards a watch-and-wait lifetime strategy for cancer treatment

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Abstract

Pediatric origin of cancer stem cell hypothesis holds great promise and potential in adult cancer treatment, however; the road to innovation is full of obstacles as there are plenty of questions left unanswered. First, the key question is to characterize the nature of such stem cells (concept). Second, the quantitative imaging of pediatric stem cells should be implemented (technology). Conceptually, pediatric stem cell origins of adult cancer are based on the notion that plasticity in early life developmental programming evolves local environments to cancer. Technologically, such imaging in children is lacking as all imaging is designed for adult patients. We postulate that the need for quantitative imaging to measure space-time changes of plasticity in early life developmental programming in children may trigger research and development of the imaging technology. Such quantitative imaging of pediatric origin of adulthood cancer will help develop a spatiotemporal monitoring system to determine cancer initiation and progression. Clinical validation of such speculative hypothesis-that cancer originates in a pediatric environment-will help implement a wait-and-watch strategy for cancer treatment.

Key words: Pediatric origins of adult cancer; Imaging of single cells

Core tip: How does "spatiotemporal tracking of cancer stem cells" should be achieved in an organism for

pediatric origins of adult cancer? Improving the resolution of current imaging technologies down to the single cell level is essential. However, how single cells could be tracked label-free throughout the lifetime of a human body will be challenging. Such technologies, if developed, can potentially provide an evidence base for cancer prevention and treatment.

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INTRODUCTION

In the United States, cancer affected more than 1.4 million individuals in 2007, with a treatment cost over $206 billion, about 33% of the aggregate medical services of $686 billion (the National Cancer Institute)[1]. Much progress has been made in defining the genetic mutations, known as the hallmarks of cancer[2] and in revealing the biological principles of metastasis[3], but this has not been effectively translated into significant benefits to patients. "Cancer genomic research has come to a crossroads with the realization that intratumoral spatial and temporal heterogeneity is a confounding factor"[4]. The discovery of this intratumoral spatiotemporal heterogeneity drives our understanding of epigenetics, which in turn defines the role of the environment[5], including chemical factors, cellular[6], and physical factors like tissue elasticity[7]. Managing tumor microenvironment may offer a holistic regimen for cancer patients with better ratio of benefit over risks[8]. These imply current cancer treatment do not address the root of cancer initiation and progression. New strategies are desperately needed.

MUTATION-TARGETED THERAPY FAILS IN CLINIC

As many mutated gene targeting drugs fail clinically, including mutation-targeted kinase inhibitors (bosutinib, ibrutinib, and cabozantinib) for glioblastoma (GBM)[9], multi-anti-HER2 targeted drugs(trastuzumab, lapatinib and/or T-DM1) for breast cancer[10], HSP70[11] and drugs through the regulation of mutant *p53* and *TAp63* in *p53*-mutated pancreatic cancer cells[12], we realize that not only the genetic mutations, but also the epigenetic changes (the role of the environment), shape cancer initiation and progression, in some cases, which may likely initiate in fetus development. Data from fetal exposome indicates that "utero exposures link to childhood cancer risk, and advances in epigenomics help understanding the effects of biological phenomena, environmental stressors, environmental and lifestyle factors on eliciting changes in the epigenome, leading to cancer initiation and progression"[13]. These data showing mutated gene targeting drugs alone fail in clinic demands a new concept for cancer initiation and progression, thereby improving treatment paradigm. Combining targeted and nontargeted therapy potentially leads to a paradigm shift from current targeted treatment of cancer.

PEDIATRIC AND ADOLESCENT PATIENTS RESPOND TO CANCER TREATMENT DIFFERENTLY FROM ADULT PATIENTS

Pediatric and adolescent patients have been speculated to respond to cancer treatment differently from those adult patients; however, lack of clinical trials on this population of patients led to inconclusive data sets thus far. For example, clinical trials "designed to determine the maximum tolerated dose of chemotherapies" (https://clinicaltrials.gov/ct2/show/NCT00993044?cond=pediatric+origins+of+cancer&rank=1, accessed August 31, 2017) by Children's Hospital Los Angeles conducted in 2009 "a phase Ⅰ study of vincristine, escalating doses of irinotecan, temozolomide and bevacizumab (Vit-b) in pediatric and adolescent patients with recurrent or refractory solid tumors of non-hematopoietic origin" (ClinicalTrials.gov Identifier: NCT00993044) without conclusion as it recruited on 12 patients. The primary objective of this study is "to evaluate the efficacy of moxetumomab pasudotox in pediatric participants with relapsed or refractory B-cell acute lymphoblastic leukemia (ALL) or B-cell lymphoblastic lymphoma" (The study was terminated prior to a planned interim analysis based on "lack of required efficacy in the first 32 participants enrolled", sponsor: MedImmune LLC) (ClinicalTrials.gov Identifier: NCT02227108, first received: August 21, 2014). Pediatricians demand for children-specific clinical trials to gain better efficacies.

HYPOTHESIS OF PEDIATRIC ORIGINS OF ADULT CANCER

As environment-derived epigenetic changes affect stem cell initiating development, most likely it occurs in early embryonic and fetus development. Dr. David Barker first observed that "low birth weight (LBW) is associated with chronic diseases"[14], such as coronary artery disease (CAD)[15], Type Ⅱ diabetes mellitus (T2DM), cancer (breast), osteoporosis and various psychiatric conditions, which led him to conceptualize "fetal origins of adult disease" (FOAD)[16], a.k.a., "pediatric origin of adulthood diseases" (POAD), or "developmental and environmental origins of adult disease" (DEOAD). POAD is based on the notion that, plasticity in early life developmental programming evolves local environments

to increase survival and reproduction[17]. The period for developmental plasticity extends from preconception to early childhood, which involves epigenetic modifications in response to environmental changes, and exerts the effects during life history phase transitions[18]. However, little is known about life history phase transitions responding to specific environmental cues.

One way of deciphering life history phase transitions for POAD would be to map out all the cells and their descendants throughout a lifetime using quantitative imaging technologies. The term stem cell originated in the context of embryological development by German biologist Ernst Haeckel in 1868 to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved[19]. Such "a prototypical cancer stem cell is a distinct cell in the embryo, responsible for giving rise to cancer found later in adulthood." In 1953-Leroy Stevens discovered "teratomas that contained mixtures of differentiated and undifferentiated cells, including hair, bone, intestinal and blood tissue." This implies the embryonic origin of tumor. Capable of tracing origin of cancer to cancer stem cells (cancer initiating cells), researchers can develop an innovative approach to treat the root of cancer. POAD in cancer takes on a new concept as cancers are being redefined as "common chronic and aging disorders" instead of "invading aliens," implying human beings may need to co-exist with cancer[20]. Indeed, cancer genomics reveals that genetic mutations exist in a wide range of human tumors as shown with different techniques[21], including detection of CX43 mutations in leukemia with microfluidic device[22]; thus, we need to reassess current mutation-target therapy, in particular current pharmaceutical strategies, focusing on multiple mutation targets, largely limited to small molecule blockade of gain-of-function mutations in accessible subcellular localizations, which to date, have not yet proven to be very effective. There is still "much to be learned about optimizing tumor responses, managing side effects, and minimizing the significant stochastic risk of drug resistance that is still too high"[23]. Mounting literature on tissue microenvironments and cell differentiation, which likely signal cancer initiation and progression, argues against a role for acquired cancer gene mutations as a critical event in tumorigenesis, such as "genetic mutations associated with metastatic clear cell renal cell carcinoma"[24,25]. This has caused "a waning of excitement regarding the direction of molecular oncology because of the large number of candidate cancer genes combined with detection of genetic heterogeneity within tumor subclones"[26]. The influence of host tissue microenvironment and cell differentiation and the role of acquired somatic mutations in tumorigenesis are not mutually exclusive, but being intertwined, thereby demanding integrated management of cancer to avoid activating dormant tumor subclones so as to maintain tumor dormancy[20]. Spatiotemporal monitoring of fetal subclonal programming engenders potential applications in diagnosis, preventive and curative measures for adult diseases. Such strategies include the development of novel preventive measures that are predicated on diet (tissue remodeling, metabolism changes)[27], life style (diet)[28], behavior (exercise)[29], stress, and medical care. Quantitative imaging of spatiotemporal biomarker expression would help define certain therapeutic windows-time-to-treatment-for pediatric origins of adulthood cancer, allowing clinicians perhaps to adopt a watch-and-wait strategy for prevention-based strategy for some predicted cancers.

EVIDENCE TO SUPPORT THE HYPOTHESIS

Stem cell origin of cancer emerges as a leading force in cancer diagnostics and treatment; however, little is known about the pediatric origin of adult cancer (POAC). Here, we will focus on POAC.

Stem cell origin of cancer consists of two conceptual schools of mechanism of tumorigenesis: "reserve-born-with-preexisted stem cells" and "bone fide locally-produced stem cells"-both involve with stem cell developmental biology. The "reserve-born-with-pre-existed" concept involves "a cancer stem cell originating in the early development, which seeds in waiting for the right soil (spatial) and the temporal (switching signal) that contribute towards cancer." The "bone fide locally-produced stem cell" are derived from undergoing genetic modifications leading to dedifferentiation, a process triggered by spatiotemporal signaling molecules such as persistent inflammation. Neither of these two conceptually defined stem cells can be identified *in vivo* with current technologies (we cannot detect a single cell *in vivo*); thus, the identity of these stem cells remains controversial. Lineage tracing shows that "Lgr5-expressing chief cells recruited to function as stem cells to affect epithelial renewal following injury by activating Wnt signaling, thus acting for maintaining the homeostatic stem cell pool, while Lgr5+ chief cells act as a major cell-of-origin of gastric cancer in a non-variegated Lgr5-2A-CreERT2 mouse model"[30]. Clearly, the hypothesis of "cancer is to embryology as mutation is to genetics" postulates cancer as embryological phenomenon as reactivated in an entirely inappropriate context[31], thereby indicating a new approach to cancer-searching for such "inappropriate context" in stem cell development.

It has been puzzled to observe that some tissue types give rise to human cancers more often than other tissue types. It is interesting to find that "the lifetime risk of cancers of diverse types is strongly correlated (0.81) with the total number of divisions of the normal self-renewing cells (stem cells) maintaining that tissue's homeostasis"[32]. The tissue's homeostasis is regulated by environmental factors or inherited predispositions. Such "correlation between the incidence of cancers and the number of stem-cell divisions in the corresponding

normal tissues"[33] shed new light on how cancer initiates and progression.

The tissue-specific cancer risk (environmental factors) can regulate the lifetime number of tissue-specific stem-cell divisions[34], suggesting that "intrinsic risk factors contribute only modestly (less than 10%-30% of lifetime risk) to cancer development, based on that the rates of endogenous mutation accumulation by intrinsic processes are not sufficient to account for the observed cancer risks." "Concomitant activation of the Wnt pathway and suppression of Mapk signaling by two small molecule inhibitors (2i) in the presence of leukaemia inhibitory factor (LIF) (hereafter termed 2i/L) induces a naive state in mouse embryonic stem (ES) cells, indicating the epigenetic and genomic integrity is required for developmental potential of embryonic stem cells"[35]. All above data shows that cancer risk is heavily influenced by extrinsic factors. These signal transduction management is supported by the female ES cells that display 2i/L-ES-cell-like transcriptional signatures while preserving gamete-derived DNA methylation and autonomous developmental potential[36].

Increased lines of evidence show "inappropriate context" (*i.e.*, environment) in stem cell development may contribute to cancer development through their interactions with abnormal environmental elements such as inflammation. We pointed out that the crosstalk for tumorigenesis may have a critical stage characterized as a "therapeutic window", which can be identified by association of molecular, biochemical and biological events in the converge developmental stages of different types of stem cells [*e.g.*, normal stem cells (NSC), CSC and embryonic stem cells][37]. Such convergence of NSC and CSC demands spatiotemporal confinement of boundary for breaching to malignancy in response to stress (tissue injury/wound healing). Stress-responsive transcription factor levels rise to reach excess, thereby causing stem cell lineage commanders to cancer[38]. It is challenging to distinguish NSC and CSC as both share common features.

POAC DEMANDS FOR DEVELOPMENT OF INNOVATIVE DETECTION TECHNOLOGIES

Safety is the most important for POAC detection technologies, as pediatric development plays a critical role in adult life, including cognitive capacity, physical and physiological functions. In practice, the neglect of pediatric origin of adult diseases desperately calls for innovative concepts and technologies to be developed. The Chinese proverb state that from the health of three-year-old body, you can predict the health care needed for an 80-year-old - from seven-year-old to see a lifetime health situation-which makes sense based on the pediatric (fetal, childhood) origin of adulthood diseases. As the cost of human genome sequencing reaches $1000[39] or even $100, physicians can know human genome so well that genomics will play a vital role in the future – thereby mapping out each step molecular profiles in a lifetime. Quantitative imaging is needed to define "therapeutic windows"[40] with predictive values for single cells based on life style measurement and biomarker profiles, with suitable criteria robust enough to determine therapeutic intervention. A biological global positioning system (bGPS)[41] could be considered for tracking spatiotemporal cancer stem cell behaviors throughout the body. Quantitative imaging is expected to improve to the point where it is sufficiently sensitive to detect subclonal growth and progression on the single-cell level[20]. Quantitative imaging may include genetic-tagged labeling and non-genetic-tagged labeling, presumably for a lifetime and at the single cell level[41]. Ideal imaging would be label-free, not invasive or minimally invasive. Such technologies might include "Raman spectroscopy for spontaneous and coherent Raman scattering microscopic imaging in the context of single cells, laser tweezers, tissue sections, biopsies and condensing Raman spectrum for a single-cell phenotype analysis"[42,43], as currently used for defining nasopharyngeal carcinoma[44]. Raman profiling for the single-cell analysis requires establish Raman spectra of individual cells by using filtering methodologies for pre-processing of Raman spectra signature, allowing to distinguish and feature as Raman-based biomarkers for single-cells with capture of spatial and temporal changes.

Big data based on supercomputing, such as the team led by University of Washington's David Baker in collaboration with researchers at the United States Department of Energy Joint Genome Institute (DOE JGI), can lead to an integrated comprehensive approach to cancer. Part of this integration is a lifetime imaging system that can define the convergence of normal stem cell and cancer stem cell developmental stages to determine appropriate therapies and assess their effects[37], for example, in monitoring maintenance immunotherapy[45]. Spatiotemporal monitoring of single cells will be high demand in the future because cancer originates from a single cell. The hypothesis of origin of a functional single cell has gained attention through time, including the Nobel Prize committee. For example, the 2014 Nobel Prize in Physiology or Medicine was awarded to John O'Keefe, May-Britt Moser and Edvard Ⅰ. Moser for their discovery that neurons in the brain are firing in response to the positioning of the body in a known space, which is referred to as the biological positioning system. This finding implies on a single-cell origin of a biological function or a single cell origin of an organ. Currently, "cancer stem cell" and the cell of origin for a tumor are not necessarily the same, as these terms have not been used carefully throughout the literature. Publications frequently flip back and forth between normal stem cells and cancer stem cells, and it is often unclear whether they refer to a normal or transformed stem cell when referring to "stem cell". It is still in debate whether cancer exists as cancer stem cells

or cancer is through de-differentiation of an adult cell, as in colon cancer[46]. Tracking down the cancer-initiating cell (CIC) subset of human colon cancers helped identify "nicotinamide phosphoribosil transferase (NAMPT) as a novel therapeutic target in colon cancer progression and relapse"[47,48], suggesting a possible solution to the puzzle. All these imply "the single-cell origin of cancer in colon cancer, which is supported for clonal origins of synchronous multifocal tumors in the hepatobiliary and pancreatic system"[49] and in the same subclone of cells of colorectal cancer[50]. Another report shows the patterns of glioma cell of origin, as somatic Nf1 loss in CD133+ neural progenitor/stem cells during late embryogenesis results in optic gliomas at three months of age, demonstrating that the cell of origin dictates the time to tumorigenesis[7], which can expose a break time or a "therapeutic window" of cancer progression[40].

Thus, the long-term advantage of imaging the single-cell and monitoring the origin of cancer for staging cancer initiation and progression as well as utility of promising advances in immunotherapy remains to be seen in clinical trials on patients with malignancy. This FOAD concept, historically, was started with poor nutrition, and the "fetus adapts to survive but the ramifications of the FOAD extend beyond low birth weight (LBW) to responses to stressors later in life, resulting in various diseases[51]". In 2017, a population-based cohort study of families in Suihua, China, shows that "prenatal exposure to famine led to the development of hyperglycemia and type 2 diabetes in adulthood across consecutive generations[52]". The cohort study consisted of 1034 families - 2068 parents [parental generation (F1)] and 1183 offspring [offspring generation (F2)] – both F1 and F2 were affected by the Chinese Famine of 1959-1961. The found that, "Prenatal exposure to famine was associated with elevated risks of hyperglycemia (multivariable-adjusted OR: 1.93; 95%CI: 1.51, 2.48) and T2D (OR: 1.75; 95%CI: 1.20, 2.54) in adulthood in F1. Furthermore, compared with the offspring of nonexposed parents, the F2 with exposed parents- especially both exposed parents-had increased hyperglycemia risk (OR: 2.02; 95%CI: 1.12, 3.66) in adulthood." However, neither did they predict nor they could track the disease progression, so they could not come up with prevention and treatment ahead of incidences of disease. They did one-time point blood testing, not sufficient to trace the disease. By improving understanding of FOAD or/and POAD, therefore, healthcare experts can prescribe preventive measures and treatment for those at higher risk, by using precise quantitative spatiotemporal imaging. Such imaging will guide how one could manipulate stem cell developmental programs for therapeutic use through time and space of single-cell in stem cell development.

Another challenge is how many single cells (*i.e.*, the critical mass) should be analyzed for the clinical manifestation, in which treatment must be applied. How do we know a dormant subclone of cancer switches to a dominating subclone? How many single cells in such a dormant subclone or a dominating subclone can manifest in its clinical phenotype? A recent study shows that integrating 14226 single-cell RNA sequencing (scRNA-seq) profiles from 16 patient samples with bulk RNA-sequence profiles from 165 patient samples[53] into the body physiology[54,55], can reveal a comprehensive strategy of cancer prevention and treatment. Such comprehensive strategy is based on the tissue organization field theory (TOFT) on tumor initiation and development[56]. Studying the POAC process through single cell imaging may likely help map out TOFT-evolved changes within an organism during development. In fact, the tissue organization field is reorganized through time and environmental factors such as dietary and lifestyle, a concept that has been speculated and yet to be elucidated. For example, "evaluation of sociodemographic and health data collected from 2310922 (2.3 million) 16-19-year-old Jewish Israeli adolescents (mean age 17.3 ± 0.4, 59.5% male) shows that adolescent risk factors (*e.g.,* Body mass index) for developing acute myeloid leukemia (AML) corelate that higher BMI in adolescence with the higher AML incidence in adulthood in this multiethnic population"[57]. The possible mechanism for this impact may be through a "hotspot for pre-neoplastic metaplasia and malignancy" in a transitional zone (TOFT) between diverse types of cells[58]. Multiple models and lineage trace imaging of single cells may therefore lead to show how this transitional zone serves as a source of malignancy for the transitional progenitor. Thus, controlling such transition may prevent cancer. Transitional zones can be assessed with "ultrasound shear wave elastography (US-SWE) in the normal prostate, which can be used to correlate with multiparametric magnetic resonance imaging (mpMRI) tissue characteristics, specifically quantitatively defining the peripheral zone (PZ) and the transitional zone (TZ) for prostate cancer"[59]. In the transitional zone, interactions between different cell types are essential for multiple biological processes, demanding concomitant multiple-single cell tracing techniques to be developed. A new report shows that "the labelling of 'kiss-and-run' interactions between immune cells 'Labelling Immune Partnerships by SorTagging Intercellular Contacts' (LIPSTIC)", which captured the two-phase "interactions between dendritic cells and CD4+ T cells during T-cell priming *in vivo*"[60]. Phase #1, "an early, cognate stage, during which CD40–CD40L interactions occur specifically between T cells and antigen-loaded dendritic cells;" and phase #2, "non-cognate stage during which these interactions no longer require prior engagement of the T-cell receptor," as shown *in vivo* in mouse models. Such a direct measurement of dynamic cell–cell interactions is expected to use in clinical settings to observe pathological processes. For example, "integration of diffusion-weighted-magnetic resonance imaging with dynamic contrast-enhanced-magnetic resonance imaging for imaging biomarkers of response to treatment, can add predictive value of pathologic response to neoadjuvant therapy in breast cancer"[61]. Whether such imaging technologies can be adopt to

other cancer types remains to be elucidated.

CONCLUSION

We attempt to address the nature of cells responsible for POAC, however; given the limitation of literature, many questions remain to be addressed, such as how to identify and target stem cells for POAC in infants or in fetus to trace the development at the single cell level? For example, will it be based on the cell surface marker, cell density, or certain transcriptional or translational features such as genetic mutations? Should the latter a case be, how one can distinguish such a cell from others without destroying tissue? Without such knowledge, it will be impossible to follow the development of a single stem cell even when the technical hurdle to image and monitor cells at the single cell level is resolved. We can predict that comprehensive artificial intelligence of medicine will lead to how "spatiotemporal tracking of cancer stem cells" should be achieved in an organism for pediatric origin of cancer. Supercomputing atlas of collecting big databases of cancer characteristics will offer a spatiotemporal tracking of all single cells in an organism throughout the lifetime of a human, thereby demonstrating a pediatric onset of adult cancer. Such advanced technologies, if developed, can potentially provide an evidence base for prevention and watch-and-wait treatment of cancer.

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REFERENCES

1 **Li SC**, Loudon WG. A novel and generalizable organotypic slice platform to evaluate stem cell potential for targeting pediatric brain tumors. *Cancer Cell Int* 2008; **8**: 9 [PMID: 18498656 DOI: 10.1186/1475-2867-8-9]

2 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

3 **Lambert AW**, Pattabiraman DR, Weinberg RA. Emerging Biological Principles of Metastasis. *Cell* 2017; **168**: 670-691 [PMID: 28187288 DOI: 10.1016/j.cell.2016.11.037]

4 **Li SC**, Tachiki LM, Kabeer MH, Dethlefs BA, Anthony MJ, Loudon WG. Cancer genomic research at the crossroads: realizing the changing genetic landscape as intratumoral spatial and temporal heterogeneity becomes a confounding factor. *Cancer Cell Int* 2014; **14**: 115 [PMID: 25411563 DOI: 10.1186/s12935-014-0115-7]

5 **Li SC**, Kabeer MH, Vu LT, Keschrumrus V, Yin HZ, Dethlefs BA, Zhong JF, Weiss JH, Loudon WG. Training stem cells for treatment of malignant brain tumors. *World J Stem Cells* 2014; **6**: 432-440 [PMID: 25258664 DOI: 10.4252/wjsc.v6.i4.432]

6 **Zhang C**, Yang SJ, Wen Q, Zhong JF, Chen XL, Stucky A, Press MF, Zhang X. Human-derived normal mesenchymal stem/stromal cells in anticancer therapies. *J Cancer* 2017; **8**: 85-96 [PMID: 28123601 DOI: 10.7150/jca.16792]

7 **Solga AC**, Toonen JA, Pan Y, Cimino PJ, Ma Y, Castillon GA, Gianino SM, Ellisman MH, Lee DY, Gutmann DH. The cell of origin dictates the temporal course of neurofibromatosis-1 (Nf1) low-grade glioma formation. *Oncotarget* 2017; **8**: 47206-47215 [PMID: 28525381 DOI: 10.18632/oncotarget.17589]

8 **Li SC**, Vu LT, Luo JJ, Zhong JF, Li Z, Dethlefs BA, Loudon WG, Kabeer MH. Tissue Elasticity Bridges Cancer Stem Cells to the Tumor Microenvironment Through microRNAs: Implications for a "Watch-and-Wait" Approach to Cancer. *Curr Stem Cell Res Ther* 2017; **12**: 455-470 [PMID: 28270089 DOI: 10.2174/1574888X12666170307105941]

9 **Barrette AM**, Bouhaddou M, Birtwistle MR. Integrating Transcriptomic Data with Mechanistic Systems Pharmacology Models for Virtual Drug Combination Trials. *ACS Chem Neurosci* 2018; **9**: 118-129 [PMID: 28950062 DOI: 10.1021/acschemneuro.7b00197]

10 **Li B**, Tao W, Shao-Hua Z, Ze-Rui Q, Fu-Quan J, Fan L, Ze-Fei J. Remarkable response with pembrolizumab plus albumin-bound paclitaxel in 2 cases of HER2-positive metastatic breast cancer who have failed to multi-anti-HER2 targeted therapy. *Cancer Biol Ther* 2018; **15**: 1-4 [PMID: 29333945 DOI: 10.1080/15384047.2017.1414761]

11 **Calderwood SK**. Heat shock proteins and cancer: intracellular chaperones or extracellular signalling ligands? *Philos Trans R Soc Lond B Biol Sci* 2018; **373**: [PMID: 29203709 DOI: 10.1098/rstb.2016.0524]

12 **Ogata T**, Nakamura M, Sang M, Yoda H, Hiraoka K, Yin D, Sang M, Shimozato O, Ozaki T. Depletion of runt-related transcription factor 2 (RUNX2) enhances SAHA sensitivity of p53-mutated pancreatic cancer cells through the regulation of mutant p53 and TAp63. *PLoS One* 2017; **12**: e0179884 [PMID: 28671946 DOI: 10.1371/journal.pone.0179884]

13 **Ghantous A**, Hernandez-Vargas H, Byrnes G, Dwyer T, Herceg Z. Characterising the epigenome as a key component of the fetal exposome in evaluating in utero exposures and childhood cancer risk. *Mutagenesis* 2015; **30**: 733-742 [PMID: 25724893 DOI: 10.1093/mutage/gev010]

14 **Barker DJ**. The fetal and infant origins of adult disease. *BMJ* 1990; **301**: 1111 [PMID: 2252919]

15 **Alsaied T**, Omar K, James JF, Hinton RB, Crombleholme TM, Habli M. Fetal origins of adult cardiac disease: a novel approach to prevent fetal growth restriction induced cardiac dysfunction using insulin like growth factor. *Pediatr Res* 2017; **81**: 919-925 [PMID: 28099426 DOI: 10.1038/pr.2017.18]

16 **Calkins K**, Devaskar SU. Fetal origins of adult disease. *Curr Probl Pediatr Adolesc Health Care* 2011; **41**: 158-176 [PMID: 21684471 DOI: 10.1016/j.cppeds.2011.01.001]

17 **Singhal A**, Lucas A. Early origins of cardiovascular disease: is there a unifying hypothesis? *Lancet* 2004; **363**: 1642-1645 [PMID: 15145640 DOI: 10.1016/S0140-6736(04)16210-7]

18 **Hochberg Z**, Feil R, Constancia M, Fraga M, Junien C, Carel JC, Boileau P, Le Bouc Y, Deal CL, Lillycrop K, Scharfmann R, Sheppard A, Skinner M, Szyf M, Waterland RA, Waxman DJ, Whitelaw E, Ong K, Albertsson-Wikland K. Child health, developmental plasticity, and epigenetic programming. *Endocr Rev* 2011; **32**: 159-224 [PMID: 20971919 DOI: 10.1210/er.2009-0039]

19 **Ramalho-Santos M**, Willenbring H. On the origin of the term "stem cell". *Cell Stem Cell* 2007; **1**: 35-38 [PMID: 18371332 DOI: 10.1016/j.stem.2007.05.013]

20 **Li SC**, Lee KL, Luo J. Control dominating subclones for managing cancer progression and posttreatment recurrence by subclonal switchboard signal: implication for new therapies. *Stem Cells Dev* 2012; **21**: 503-506 [PMID: 21933025 DOI: 10.1089/scd.2011.0267]

21 **Liu Y**, Wen Q, Chen XL, Yang SJ, Gao L, Gao L, Zhang C, Li JL, Xiang XX, Wan K, Chen XH, Zhang X, Zhong JF. All-trans retinoic acid arrests cell cycle in leukemic bone marrow stromal cells by increasing intercellular communication through connexin 43-mediated gap junction. *J Hematol Oncol* 2015; **8**: 110 [PMID: 26446715 DOI: 10.1186/s13045-015-0212-7]

22 **Yang S**, Wen Q, Liu Y, Zhang C, Wang M, Chen G, Gong Y, Zhong J, Chen X, Stucky A, Zhong JF, Zhang X. Increased expression of CX43 on stromal cells promotes leukemia apoptosis. *Oncotarget* 2015; **6**: 44323-44331 [PMID: 26517241 DOI: 10.18632/

oncotarget.6249]
23 **Kaye FJ**, Ivey AM, Drane WE, Mendenhall WM, Allan RW. Response. *J Natl Cancer Inst* 2016; **109**: pii: djw191 [PMID: 27671685 DOI: 10.1093/jnci/djw191]
24 **Li Z**, Hao P, Wu Q, Li F, Zhao J, Wu K, Qu C, Chen Y, Li M, Chen X, Stucky A, Zhong J, Li L, Zhong JF. Genetic mutations associated with metastatic clear cell renal cell carcinoma. *Oncotarget* 2016; **7**: 16172-16179 [PMID: 26908440 DOI: 10.18632/oncotarget.7473]
25 **Baker SG**. A cancer theory kerfuffle can lead to new lines of research. *J Natl Cancer Inst* 2014; **107**: pii: dju405 [PMID: 25528755 DOI: 10.1093/jnci/dju405]
26 **Kaye FJ**. Re: a cancer theory kerfuffle can lead to new lines of research. *J Natl Cancer Inst* 2015; **107**: pii: djv060 [PMID: 25766401 DOI: 10.1093/jnci/djv060]
27 **Dias Rodrigues V**, Barroso de Pinho N, Abdelhay E, Viola JP, Correia MI, Brum Martucci R. Nutrition and Immune-Modulatory Intervention in Surgical Patients With Gastric Cancer. *Nutr Clin Pract* 2017; **32**: 122-129 [PMID: 27329862 DOI: 10.1177/0884533616653807]
28 **Uster A**, Ruehlin M, Mey S, Gisi D, Knols R, Imoberdorf R, Pless M, Ballmer PE. Effects of nutrition and physical exercise intervention in palliative cancer patients: A randomized controlled trial. *Clin Nutr* 2017: pii: S0261-5614(17)30201-7 [PMID: 28651827 DOI: 10.1016/j.clnu.2017.05.027]
29 **Wurz A**, Brunet J. The Effects of Physical Activity on Health and Quality of Life in Adolescent Cancer Survivors: A Systematic Review. *JMIR Cancer* 2016; **2**: e6 [PMID: 28410184 DOI: 10.2196/cancer.5431]
30 **Leushacke M**, Tan SH, Wong A, Swathi Y, Hajamohideen A, Tan LT, Goh J, Wong E, Denil SLIJ, Murakami K, Barker N. Lgr5-expressing chief cells drive epithelial regeneration and cancer in the oxyntic stomach. *Nat Cell Biol* 2017; **19**: 774-786 [PMID: 28581476 DOI: 10.1038/ncb3541]
31 **Cofre J**, Abdelhay E. Cancer Is to Embryology as Mutation Is to Genetics: Hypothesis of the Cancer as Embryological Phenomenon. *ScientificWorldJournal* 2017; **2017**: 3578090 [PMID: 28553657 DOI: 10.1155/2017/3578090]
32 **Tomasetti C**, Vogelstein B. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* 2015; **347**: 78-81 [PMID: 25554788 DOI: 10.1126/science.1260825]
33 **Tomasetti C**, Vogelstein B. On the slope of the regression between stem cell divisions and cancer risk, and the lack of correlation between stem cell divisions and environmental factors-associated cancer risk. *PLoS One* 2017; **12**: e0175535 [PMID: 28520721 DOI: 10.1371/journal.pone.0175535]
34 **Wu S**, Powers S, Zhu W, Hannun YA. Substantial contribution of extrinsic risk factors to cancer development. *Nature* 2016; **529**: 43-47 [PMID: 26675728 DOI: 10.1038/nature16166]
35 **Choi J**, Huebner AJ, Clement K, Walsh RM, Savol A, Lin K, Gu H, Di Stefano B, Brumbaugh J, Kim SY, Sharif J, Rose CM, Mohammad A, Odajima J, Charron J, Shioda T, Gnirke A, Gygi S, Koseki H, Sadreyev RI, Xiao A, Meissner A, Hochedlinger K. Prolonged Mek1/2 suppression impairs the developmental potential of embryonic stem cells. *Nature* 2017; **548**: 219-223 [PMID: 28746311 DOI: 10.1038/nature23274]
36 **Yagi M**, Kishigami S, Tanaka A, Semi K, Mizutani E, Wakayama S, Wakayama T, Yamamoto T, Yamada Y. Derivation of ground-state female ES cells maintaining gamete-derived DNA methylation. *Nature* 2017; **548**: 224-227 [PMID: 28746308 DOI: 10.1038/nature23286]
37 **Li SC**, Lee KL, Luo J, Zhong JF, Loudon WG. Convergence of normal stem cell and cancer stem cell developmental stage: Implication for differential therapies. *World J Stem Cells* 2011; **3**: 83-88 [PMID: 22007273 DOI: 10.4252/wjsc.v3.i9.83]
38 **Ge Y**, Gomez NC, Adam RC, Nikolova M, Yang H, Verma A, Lu CP, Polak L, Yuan S, Elemento O, Fuchs E. Stem Cell Lineage Infidelity Drives Wound Repair and Cancer. *Cell* 2017; **169**: 636-650.e14 [PMID: 28434617 DOI: 10.1016/j.cell.2017.03.042]
39 **Davies K.** The $1000 genome: The revolution in DNA sequencing and the new era of personalized medicine. New York: Free Press, 2010
40 **Li SC**, Han YP, Dethlefs BA, Loudon WG. Therapeutic window, a critical developmental stage for stem cell therapies. *Curr Stem Cell Res Ther* 2010; **5**: 297-293 [PMID: 20528752]
41 **Li SC**, Tachiki LM, Luo J, Dethlefs BA, Chen Z, Loudon WG. A biological global positioning system: considerations for tracking stem cell behaviors in the whole body. *Stem Cell Rev* 2010; **6**: 317-333 [PMID: 20237964 DOI: 10.1007/s12015-010-9130-9]
42 **Sun S**, Wang X, Gao X, Ren L, Su X, Bu D, Ning K. Condensing Raman spectrum for single-cell phenotype analysis. *BMC Bioinformatics* 2015; **16** Suppl 18: S15 [PMID: 26681607 DOI: 10.1186/1471-2105-16-S18-S15]
43 **Krafft C**, Schie IW, Meyer T, Schmitt M, Popp J. Developments in spontaneous and coherent Raman scattering microscopic imaging for biomedical applications. *Chem Soc Rev* 2016; **45**: 1819-1849 [PMID: 26497570 DOI: 10.1039/c5cs00564g]
44 **Liu M**, Lin J, Qiu S, Wu W, Liu G, Li Y, Gong H, Chen R, Chen G. Label-Free Classification of a Nasopharyngeal Carcinoma Tissue Test at Different Stages Based on Raman Spectroscopy. *J AOAC Int* 2017; **100**: 429-433 [PMID: 28118141 DOI: 10.5740/jaoacint.16-0191]
45 **Li SC**, Kabeer MH. Designer immunotherapy specific for cancer. *J Cell Sci Ther* 2013; **4**: e116 [DOI: 10.4172/2157-7013.1000e4116]
46 **Yamada M**, Sakurai T, Komeda Y, Nagai T, Kamata K, Minaga K, Yamao K, Takenaka M, Hagiwara S, Matsui S, Watanabe T, Nishida N, Kashida H, Kudo M. Clinical Significance of Bmi1 Expression in Inflammatory Bowel Disease. *Oncology* 2017; **93** Suppl 1: 20-26 [PMID: 29258116 DOI: 10.1159/000481225]
47 **Lucena-Cacace A**, Otero-Albiol D, Jiménez-García MP, Muñoz-Galvan S, Carnero A. NAMPT Is a Potent Oncogene in Colon Cancer Progression that Modulates Cancer Stem Cell Properties and Resistance to Therapy through Sirt1 and PARP. *Clin Cancer Res* 2017: Epub ahead of print [PMID: 29203587 DOI: 10.1158/1078-0432.CCR-17-2575]
48 **Lucena-Cacace A**, Otero-Albiol D, Jiménez-García MP, Peinado-Serrano J, Carnero A. NAMPT overexpression induces cancer stemness and defines a novel tumor signature for glioma prognosis. *Oncotarget* 2017; **8**: 99514-99530 [PMID: 29245920 DOI: 10.18632/oncotarget.20577]
49 **Jiang W**, Ding Y, Shen Y, Fan L, Zhou L, Li Z, Zheng Y, Zhao P, Liu L, Tong Z, Fang W, Wang W. Identifying the clonal origin of synchronous multifocal tumors in the hepatobiliary and pancreatic system using multi-omic platforms. *Oncotarget* 2017; **8**: 5016-5025 [PMID: 28008139 DOI: 10.18632/oncotarget.14018]
50 **Wu H**, Zhang XY, Hu Z, Hou Q, Zhang H, Li Y, Li S, Yue J, Jiang Z, Weissman SM, Pan X, Ju BG, Wu S. Evolution and heterogeneity of non-hereditary colorectal cancer revealed by single-cell exome sequencing. *Oncogene* 2017; **36**: 2857-2867 [PMID: 27941887 DOI: 10.1038/onc.2016.438]
51 **Katzmarzyk PT**, Barlow S, Bouchard C, Catalano PM, Hsia DS, Inge TH, Lovelady C, Raynor H, Redman LM, Staiano AE, Spruijt-Metz D, Symonds ME, Vickers M, Wilfley D, Yanovski JA. An evolving scientific basis for the prevention and treatment of pediatric obesity. *Int J Obes* (Lond) 2014; **38**: 887-905 [PMID: 24662696 DOI: 10.1038/ijo.2014.49]
52 **Li J**, Liu S, Li S, Feng R, Na L, Chu X, Wu X, Niu Y, Sun Z, Han T, Deng H, Meng X, Xu H, Zhang Z, Qu Q, Zhang Q, Li Y, Sun C. Prenatal exposure to famine and the development of hyperglycemia and type 2 diabetes in adulthood across consecutive generations: a population-based cohort study of families in Suihua, China. *Am J Clin Nutr* 2017; **105**: 221-227 [PMID: 27927634 DOI: 10.3945/ajcn.116.138792]
53 **Venteicher AS**, Tirosh I, Hebert C, Yizhak K, Neftel C, Filbin MG, Hovestadt V, Escalante LE, Shaw ML, Rodman C, Gillespie SM, Dionne D, Luo CC, Ravichandran H, Mylvaganam R, Mount C, Onozato ML, Nahed BV, Wakimoto H, Curry WT, Iafrate AJ, Rivera MN, Frosch MP, Golub TR, Brastianos PK, Getz G, Patel AP, Monje M, Cahill DP, Rozenblatt-Rosen O, Louis DN, Bernstein BE, Regev A, Suvà ML. Decoupling genetics, lineages,

and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science* 2017; **355**: [PMID: 28360267 DOI: 10.1126/science.aai8478]

54 **Sonnenschein C**, Soto AM. An Integrative Approach Toward Biology, Organisms, and Cancer. *Methods Mol Biol* 2018; **1702**: 15-26 [PMID: 29119499 DOI: 10.1007/978-1-4939-7456-6_2]

55 **Soto AM**, Longo G, Miquel PA, Montevil M, Mossio M, Perret N, Pocheville A, Sonnenschein C. Toward a theory of organisms: Three founding principles in search of a useful integration. *Prog Biophys Mol Biol* 2016; **122**: 77-82 [PMID: 27498204 DOI: 10.1016/j.pbiomolbio.2016.07.006]

56 **Sonnenschein C**, Soto AM. Carcinogenesis explained within the context of a theory of organisms. *Prog Biophys Mol Biol* 2016; **122**: 70-76 [PMID: 27498170 DOI: 10.1016/j.pbiomolbio.2016.07.004]

57 **Shamriz O**, Leiba M, Levine H, Derazne E, Keinan-Boker L, Kark JD. Higher body mass index in 16-19 year-old Jewish Adolescents of North African, Middle Eastern and European Origins is a Predictor of Acute Myeloid Leukemia: a cohort of 2.3 million Israelis. *Cancer Causes Control* 2017; **28**: 331-339 [PMID: 28258513 DOI: 10.1007/s10552-017-0863-5]

58 **Jiang M**, Li H, Zhang Y, Yang Y, Lu R, Liu K, Lin S, Lan X, Wang H, Wu H, Zhu J, Zhou Z, Xu J, Lee DK, Zhang L, Lee YC, Yuan J, Abrams JA, Wang TC, Sepulveda AR, Wu Q, Chen H, Sun X, She J, Chen X, Que J. Transitional basal cells at the squamous-columnar junction generate Barrett's oesophagus. *Nature* 2017; **550**: 529-533 [PMID: 29019984 DOI: 10.1038/nature24269]

59 **Harvey H**, Morgan V, Fromageau J, O'Shea T, Bamber J, deSouza NM. Ultrasound Shear Wave Elastography of the Normal Prostate: Interobserver Reproducibility and Comparison with Functional Magnetic Resonance Tissue Characteristics. *Ultrason Imaging* 2018; Epub ahead of print [PMID: 29353529 DOI: 10.1177/0161734618754487]

60 **Pasqual G**, Chudnovskiy A, Tas JMJ, Agudelo M, Schweitzer LD, Cui A, Hacohen N, Victora GD. Monitoring T cell-dendritic cell interactions in vivo by intercellular enzymatic labelling. *Nature* 2018; **553**: 496-500 [PMID: 29342141 DOI: 10.1038/nature25442]

61 **Kang H**, Hainline A, Arlinghaus LR, Elderidge S, Li X, Abramson VG, Chakravarthy AB, Abramson RG, Bingham B, Fakhoury K, Yankeelov TE. Combining multiparametric MRI with receptor information to optimize prediction of pathologic response to neoadjuvant therapy in breast cancer: preliminary results. *J Med Imaging* (Bellingham) 2018; **5**: 011015 [PMID: 29322067 DOI: 10.1117/1.JMI.5.1.011015]

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REVIEW

Physiologically based microenvironment for *in vitro* neural differentiation of adipose-derived stem cells

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Abstract

The limited capacity of nervous system to promote a spontaneous regeneration and the high rate of neurodegenerative diseases appearance are keys factors that stimulate researches both for defining the molecular mechanisms of pathophysiology and for evaluating putative strategies to induce neural tissue regeneration. In this latter aspect, the application of stem cells seems to be a promising approach, even if the control of their differentiation and the maintaining of a safe state of proliferation should be troubled. Here, we focus on adipose tissue-derived stem cells and we seek out the recent advances on the promotion of their neural differentiation, performing a critical integration of the basic biology and physiology of adipose tissue-derived stem cells with the functional modifications that the biophysical, biomechanical and biochemical microenvironment induces to cell phenotype. The pre-

clinical studies showed that the neural differentiation by cell stimulation with growth factors benefits from the integration with biomaterials and biophysical interaction like microgravity. All these elements have been reported as furnisher of microenvironments with desirable biological, physical and mechanical properties. A critical review of current knowledge is here proposed, underscoring that a real advance toward a stable, safe and controllable adipose stem cells clinical application will derive from a synergic multidisciplinary approach that involves material engineer, basic cell biology, cell and tissue physiology.

Key words: Adipose stem cells; Biomaterials; Neurodegeneration; Neural differentiation; Physiological microenvironment

Core tip: Adipose-derived stem cells are easily accessible from liposuction, obtained in large quantity and cultured for several months with low levels of senescence. Moreover, they could be induced toward a neural phenotype *in vitro*. The preclinical studies show that microenvironment has a predominant role. Our objective is to consolidate the current literature to better delineate the functional response of adipose-derived stem cells to biochemical, biophysical or dimensional stimuli. Specifically, chemicals - like drugs and growth factors-biomaterials and microgravity are here discussed as both single and co-applied parameters for inducing a neural lineage.

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INTRODUCTION

The majority of neurological diseases are characterized by primary or secondary neurodegeneration with the concomitance of different degree of inflammation[1,2]. Parkinson disease[3,4], multiple sclerosis[5], traumatic injury[6] or lysosomal storage disease with neurological symptoms like Krabbe disease[7] represent conditions in which the neural cells disappearance turn into decline of patient quality of life. Therapeutic approaches are mostly symptomatic and not restorative.

Due to the skills to immunomodulation, to support the brain parenchyma and to transdifferentiate, stem cells (SC) are under evaluation in preclinical tests for the promotion of neural regeneration.

Among the different sources of SC, the adipose stem cells (ASCs) are becoming more and more popular and attract the researchers' interest because they are easily accessible from subcutaneous liposuction, obtained in large quantity[8], cultured for several months *in vitro* with low levels of senescence[9,10] and applicable without ethical and political issues[11]. Moreover, ASCs have been shown to possess self-renewal property and multipotential differentiation toward adipocytes[12], chondrocytes[13,14], osteoblasts[15], myocytes[16], neurocytes[17], and other cell types[18], including neurons[19] and neural cells[20]. All these hallmarks give to ASCs potential application in regenerative medicine and clinical studies[21,22]. As regard the transdifferentiating potential of ASCs into neural cells, the transduction properties need to be further characterized. Longtime, the stimulation of ASCs by growth factors-enriched media has been the most applied procedure to induce a specific cell lineage[23], but recently it has been enlightened that the conventional two-dimensional systems do not mimic the cellular connections and the space distribution that occur *in vivo*[24,25], especially if compared with the structural complexity of nervous system. A reliable solution to this question resides in three-dimensional (3D) biomaterial scaffolds that show a great potential as engineered neural tissue for cell-based therapy[26,27].

This review integrates the basic physiology of ASC with the functional modifications of cell phenotype furnished by enrichment of microenvironment with appropriate biophysical, biomechanical and biochemical stimuli. In particular, the effects of chemicals-like drugs and growth factors-biomaterial and microgravity are discussed as both single and co-applied parameters for inducing ASCs toward the neural lineage.

NEURAL CELLS FROM ASCs, WHY IS IT DESIRABLE?

SCs are defined as unspecialized cells capable of self-renewing and of giving rise to a wide range of mature cell types[28]. During their proliferation, SCs do not follow the classical asymmetric cell division that generates a SC and a differentiated daughter at each division. Their "potential" resides in generating more SC and differentiated daughters[29]. Two types of SCs have been classified following their origin and their potential of differentiation: Embryonic tem cells (ESCs) and somatic SCs.

The ESCs derive from the early blastocyst and the inner cell mass of the embryo and are able to differentiate into cell types of the three germ layers[30]. Even if they represent the most powerful tool for cell therapy in animal models, their application is associated to ethical issue and to high degree of variation with regard to differentiation potential due to their genetic and epigenetic instability.

The somatic SCs are obtained from fetal (after gastrulation) or adult tissues and traditionally differentiate only toward cell types that belong to the tissue which

they originate from. Among the adult tissues, somatic SC have been isolated from bone marrow[31], brain[32], blood[33], epidermis[34], skeletal muscle[35] and fat[10]. In each tissue, somatic SCs guarantee the maintaining of tissue homeostasis, but their action in replacing damaged cells after intense insults is limited by a mostly quiescent status or a weak activity. This is the case of neural stem cells (NSCs), located in adult mammals within a cellular niche[36] in the sub-ependymal layer of the ventricular zone and in the dentate gyrus of the hippocampus[37]. However, even if these differentiate *in vitro* into neurons, astrocytes and oligodendrocytes[38], they are not effective in containing neurodegenerative process.

The adult SCs offer the potential for autologous stem cell donation, reducing the risk of immune rejection and complications[39] and are additionally far from ethical and religious debates. We underscore that these advantages represent a solid basis for cultural renaissance and for scientific efforts to define the best source of adult SCs and to optimize methods for a safe, controlled and long-lasting differentiation.

According to our experience, the first description of a population of cells derived from human adipose tissue with a multilineage differentiation and high proliferation capacity *in vitro*[9] represents the milestone for scientific awakening and for overcoming specific tissue-linked limitations. Compared to bone marrow, adipose tissue is obtained with a not invasive, well-tolerated and safe procedure such as liposuction surgery. Moreover, the yield of obtained cells is relatively higher than other stem cell sources[40] and the digestion of lipoaspirate permits to isolate approximately from 0.5×10^4 to 2×10^5 stem cells per gram of adipose tissue[41]. Furthermore, ASCs can be cultured for several months *in vitro* with low levels of senescence[10]. The latter aspect is essential because it turns into a reduction of permanent post-mitotic states and the cells remain viable and proliferative over extensive periods during which the terminal differentiation could be stimulated. Thus, the critical point is the induction of a stable phenotype not restricted to mesodermal cells but including the ectodermal ones.

There is a diffuse disagreement about pluripotential properties of ASCs, but in our experience the phenotype of ASCs can be addressed toward mesodermal[12,14] and non-mesodermal lineages[20]. In these observations reside the scientific efforts in the evaluation of ASCs as tools for generation of neural cells to apply in cell therapy strategies and in cell models for various neurodegenerative disorders[42].

Because non-neural differentiation potential falls outside of the scope of the present review, we focus on *in vitro* methods to induce neural differentiation. A systematic literature search was conducted using PubMed, WoS, and Scopus. Studies providing only results for *in vitro* neural phenotype induction from ASCs and preclinical examination were included. When preliminary tests on animal model of diseases have been performed, the major relevant findings were discussed.

IN VITRO METHODS TO INDUCE NEURAL DIFFERENTIATION OF ASCs

The experimental conditions of ASCs neural induction and differentiation contemplate at least three main categories or microenvironment factors: (1) The elaboration of chemically-defined or growth factors-enriched media; (2) the creation of a functionalized tree-dimensional structure by biomaterials; and (3) the application of appropriate biophysical forces.

GROWTH FACTORS AND CHEMICALS FOR ASCs NEURAL DIFFERENTIATION

The most applied protocols for neural differentiation of ASCs are designed as "run-through" procedures, in which ASCs are sequentially propagated in different media enriched by growth factors or chemicals until they transdifferentiate into a desired phenotype. These approaches should be defined "physiological-inspired" or "chemical-based" as they try to mimic *in vitro* the complex environment of nervous system by adding growth factors or chemicals.

In the earlier reports, a two-steps method has been adopted and a phase of cellular preconditioning or induction was followed by application of differentiation stimuli. As preconditioning media, Safford *et al*[43] tested the enrichment with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), whereas Zuk *et al*[10] used DMEM supplemented by 20% of fetal bovine serum and β-mercaptoethanol. After this step, the neuronal differentiation was performed by medium composed of DMEM plus butylated hydroxyanisole, KCl, valproic acid, forskolin, hydrocortisone, and insulin or by a serum-free and β-mercaptoethanol-enriched medium, respectively. In both experimental conditions, ASCs developed to an early neuronal stage, as no expression of established oligodendrocyte and astrocyte markers or mature neuronal markers were observed. These two works are milestones for neuronal differentiation of ASCs, but they lacked in electrophysiological tests. Indeed, a delayed-rectifier type K^+ current (an early developmental ion channel) concomitantly with morphologic changes and increased expression of neural-specific markers suggested that ASCs differentiate toward early progenitors of neurons and/or glia after 2 wk in differentiating medium with isobutylmethylxanthine, indomethacin, and insulin[44]. The pre-induction was also performed by bFGF for seven days[19] or for twenty-four hours[45], following the incubation with forskolin alone[19] or in combination with N2 supplement, butylated hydroxyanisole, KCl, valproic acid[45]. Despite the similar protocol, the relevant findings were different. Krampera *et al*[45] reported a transient and reversible differentiation within 48-72 h

Table 1 Functions of growth factors on neural induction of adipose stem cells

Growth factors	Profile	Physiological activity	Effect on ASCs	Remarks
EGF	Small polypeptide of 53 amino acid residues and a molecular mass of approximately 6000 daltons[57]	Development of the oral cavity, lungs, gastrointestinal tract, epidermis, derma, eyelids and central nervous system[56]	Promotion of proliferation with delays of senescence and insurance of differentiation potency[55]	EGF and bFGF co-administration limits ASCs differentiation abilities by inducing ASCs into an ectodermal lineage rather than the mesodermal one[53]
bFGF	Non-glycosylated polypeptide of 18 kDa and 155 amino acid in length (heparin-binding growth factor family)	Stimulator of tissue repair and cellular viability released from an injured extracellular matrix[64]	Enhancement of proliferation, differentiation and hepatocyte growth factor expression ability[58]. Induction of the adipogenic[59] and chondrogenic[60] potential, with inhibition of osteogenic differentiation[61]	
PDGF	Dimeric glycoprotein	Potent mitogen for cell of mesodermal lineage and stimulator of tissue repair released from activated platelets on bleeding[65]	Supporting of cell proliferation *in vitro*: It increases ASCs yield. Promotion of neural differentiation in an antioxidant microenvironment[48]	Receptor-β signalling is involved primarily in ASCs stimulation[62]. ASCs stimulation with autologous platelet-rich plasma reduces the cost of differentiation[48]

EGF: Epidermal growth factor; bEGF: Basic fibroblast growth factor; PDGF: Platelet-derived growth factor; ASCs: Adipose stem cells.

of culture with basal medium. Indeed, in the protocol of Jang *et al*[19], the acquired neuron-like functions were demonstrated by evaluation of voltage-dependent tetrodotoxin (TTX)-sensitive sodium currents, outward potassium currents, and prominent negative resting membrane potentials. These events underscore that the *in vitro* microenvironment is capable to infer with the multiple functional ion channel currents that are physiologically present in undifferentiated ASCs[46].

Another approach showed morphological, immunocytochemical and electrophysiological evidences of stable neuronal differentiation of ASCs. It is based on the induction of floating sphere in serum-free medium in presence of bFGF, and EGF. The spheres were dissociated in single cells and cultured with brain derived neurotrophic factor (BDNF) and retinoic acid[47]. It was also investigated the possibility to transdifferentiate ASCs by using neural induction medium (high glucose DMEM, β-mercaptoethanol, and butylated hydroxyanisole) supplemented with and 10% of autologous platelet-rich plasma (PRP) isolated and prepared from venous blood of the same patient underwent liposuction[48]. Some reports showed an induction toward a neural-like phenotype by media previously conditioned thought incubation with neuroblastoma or olfactory ensheathing cells (OECs)[49] or with ASCs induced to secrete neurotrophic factors[50] in presence of estrogen[51], also.

All the major protocols considered for differentiation of ASCs to neural cells have been reviewed by linking them to the neural markers that should be used in each procedure and the possible pathways that are involved in this process[52]. Here we focus on the physiological input trying to define a profile that links chemicals and growth factors to ASCs fate.

According to the studies performed up to now, EGF and bFGF seem able to induce a useful pre-conditioning microenvironment for ASCs induction toward ectodermal lineage[53]. The co-administration of EGF and bFGF is essential because, as shown in Table 1, when tested alone on ASCs, EGF acts to promote ASCs proliferation by robust phosphorylation of SHC and ERK1/2[54], to induce migration, to delay senescence, and to maintain differentiation potency by EGF-induced activation of STAT signal pathway[55]. Indeed, bFGF alone enhances the proliferation, and the hepatocyte growth factor expression ability of ASCs[58], promoting the adipogenic[59] and chondrogenic[60] differentiation with the contemporary inhibition of the osteogenic one[61]. This biochemical event happens because ASCs express EGF and bFGF receptors[54]. Furthermore, they express PDGF receptors α and β. PDGFR-α is highly expressed, but its ligand only slightly increases the proliferation of ASCs. Therefore, it is reasonable to assume that PDGF-β and PDGF receptor-β signalling is involved primarily in the stimulation of ASCs[62]. PDGF is released from activated platelets on bleeding, thus the stimulation with autologous platelet-rich plasma (PRP) represents an effective method to mediate stimulatory effect on cell proliferation, to increase the yield of ASCs and to reduce the cost of ASCs differentiation. In the same manner, the incubation with conditioned media appears a good technique for introducing an enriched cocktail of growth factors with positive remarks at both financial and practical point of view. Specifically, the secretome of neuroblastoma B104 cells has been reported to contain PDGF-AA, bFGF and IGF-1[63], whereas brain derived neurotrophic factor (BDNF), nerve grown factor (NGF), neurotrophin-4/5 (NT-4/5), neuregulin, secreted

Table 2 Effects of chemicals-enriched microenvironment on adipose stem cells fate

Action	Chemicals/drugs	Profile	Recognized function	Effects on ASCs
Antioxidant	β-mercaptoethanol	Water-soluble thiol used as a reducing agent for disulfide bonds to protect sulfhydryl groups from oxidation	In peripheral intestinal nervous system increases the number of synapses and the vesicle population in the nerve terminals[69] Improve meiotic maturation *in vitro* cultured oocytes[70]	Key elements for the neural induction medium: Reduction of oxidative stress and reactive oxygen species production could support neural population
	Butylated hydroxyanisole	Mixture of two isomeric organic compounds	Inhibition of 17 β-estradiol(E2)-mediated oxidative stress and of oxidative DNA damage	
	N-acetyl-l-cysteine	Synthetic derivative of endogenous amino acid, L-cysteine, precursor of the antioxidant enzyme Glutathione	Stimulator of glutathione synthase Activator of NMDA1 receptor	When co-administrated, reduction of ASCs-doubling time and increase of cell number compared with b-FGF alone supplementation[66]
	Ascorbic acid-2-phosphate (Vitamin C)	Water-soluble essential vitamin	Reducing agent and coenzyme in several metabolic pathways	
Interference on DNA	Valproic acid	Branch-chained fatty acid, acting as a histone deacetylase inhibitor	Wide range of neuroprotection[71,72] Inhibitor of glycogen synthase kinase-3[73] Inducer of chromatin remodeling[74]	Promoter of neuron-like cells[75] *In vivo*, it improves homing of ASCs *via* overexpression of CXCR4 and CXCR6[76]
	5-azacytidine	Analog of cytidine nucleoside, acting as demethylating agent[77,78]	Inducer of cell plasticity and active molecule for cellular differentiation into multiple phenotype[79]	Stimulated-cells ameliorate neurological deficits when injected in rats after cerebral ischemia[80]
Anti-inflammation	Indomethacin	Synthetic nonsteroidal indole derivative	Inhibitor of COX1/2	Component of neural induction medium applied for two weeks[44]
Immuno modulation	Hydrocortisone	Glucocorticoid hormone	Suppressor of cell-mediate immunity	Form multi-nucleated myotubes, yielding protein markers for myocytes[9]
Energetic balance	N2 supplement	Chemically defined formulation containing insulin, transferrin, progesterone, putrescine and selenite	*In vitro* survival and expression of post-mitotic neurons in primary cultures from both the peripheral nervous system and the central nervous system	General promotion of neural cell survival
	B27 supplement	Mixture of vitamins (biotin, Tocopherol, Vitamin A), proteins (BSA, catalase, insulin transferrin, superoxide dismutase), Corticosterone, Galactose, Ethanolamine, Glutathione, Carnitine, Linoleic acid, linolenic acid, progesterone, putrescine, selenite, T3	Growth and maintenance of neurons. Differentiating Glial Precursor Cells into Astrocytes and Oligodendrocytes. Differentiating Neural Stem Cells into Neurons and Glial Cells	

ASCs: Adipose stem cells.

protein acidic rich in cysteine (SPARC) and matrix metalloproteinase-2 (MMP-2) have been reported as typical elements of OECs secretoma. Among these growth factors, nerve grown factor-β (NGF-β), BDNF, and neurotrophin-3 (NT-3) were applied on EGF plus bFGF-preconditioned ASCs to induce a neural-like phenotype[20].

Thus, the growth factors that physiologically act in tissue rapid turnover[56,64,65] seem to stimulate proliferation and to improve responsiveness of ASCs toward ectodermal-derived stimuli. From this basic speculation, the major question must be opened is addressed to define what happens in humans with neurodegenerative syndromes, or in which way the loss or the increase of a biochemical stimulus expression may interfere into ASCs phenotype by enhancing or limiting clinical application. Up to now, no scientific result can drive to answer.

Among the chemical reagents, the major applied ones were antioxidants or compounds active on DNA (Table 2).

An antioxidant microenvironment, obtained by *N*-acetyl-l-cysteine and ascorbic acid-2-phosphate, has been reported to reduce ASCs-doubling time and to increase cell number[66]. The β-mercaptoethanol sustained the induction of neural phenotype after pre-induction and differentiation[10], whereas the butylated hydroxyanisole promoted the neural stem cell survival.

Table 3 Biomaterials for neural phenotype of adipose stem cells

Biomaterials	Profile	Effect on ASCs	Test on animal	Limitation for clinical
Chitosan films	Naturally derived polysaccharide from chitin[81,82]	Spontaneous cell organization in a 3D architecture	Yes, higher cellular retention ratio of ASC spheroids after intramuscular injection in nude mouse[81]	Not declared
Chitosan and gelatin	Elastic-dominant, porous scaffold	Conditioning toward a neuron-like phenotype	Yes, better repair in a mouse model of traumatic brain injury[83]	Not declared
Chitosan and silk	Complex structural framework	Efficient as delivery vehicle for ASCs	Yes, proposed as nerve grafts in the regeneration of injured rat sciatic nerve[84]	Not declared
Collagen gel	Engineered neural tissue	Cells must be aligned to collagen fibres	Yes, supported robust neural regeneration of injured rat sciatic nerve[85]	Not declared
Albumin	Serum-derived porous scaffold	Promotion toward neurons	Yes, filler effect on the spinal cord cavity in animal models of spinal cord injury[86]	Not declared
Matrigel	Commercially available hydrogel	Good cell encapsulation and delivery[87]	Yes, mouse models of spinal cord injury	Not applicable for its isolation from the basement membrane of a mouse sarcoma
Alginate	Hydrogel	Neurospheres encapsulation and neural promotion[88,89]		Good biocompatible profile
Nanosized graphene oxide-laminin hybrid patterns	Engineered tissue	Efficient neuron-like cells differentiation[90]		

ASCs: Adipose stem cells; 3D: Three-dimensional.

All these beneficial effects of antioxidants toward a neural phenotype should be related to the essential role that a pro-oxidant microenvironment exerts on induction of adipogenic phenotype[67]. It has been recently demonstrated that oxidative stress and reactive oxygen species (ROS) overproduction could drive the activation of molecular pathways that are able to convert myoblasts into brown adipocytes[68]. Nevertheless, the B27 reagent, that is routinely applied in laboratory's procedures for the growth and maintenance of neurons or for differentiating of SCs into neurons and glial cells, contains tocopherol, Vitamin A, catalase, superoxide dismutase, glutathione, that, among the other effects, are largely described for their propriety of oxidative stress-limiting agents.

The drugs active on DNA usually applied for neural induction of ASCs are histone deacetylase (HDAC) inhibitors or methylation inhibitors, like the valproic acid and the 5-azacytidine, respectively. The valproic acid is commonly used for the treatment of seizures and bipolar disorder. Valproic acid demonstrated a wide range of neuroprotective properties in cellular and animal models of neurodegenerative diseases[71,72], probably for the activity as both toward the inhibition of glycogen synthase kinase-3 (GSK-3)[73] and the enhancement of CXCR4 expression[74]. In ASCs, *in vitro* treatment with valproic acid resulted in a promotion of neuron-like differentiation[75] and *in vivo* an enhanced homing of ASCs was reported *via* overexpression of CXCR4 and CXCR6[76]. Indeed, the demethylating agent 5-azacytidine is commonly employed to treat blood disorders such as myelodysplasia and leukemia[77]. It has been historically described as an inducer of cell plasticity and as an active molecule for cellular differentiation into multiple phenotypes[78,79]. The enrichment of ASCs microenvironment with 5-azacytidine has been effective to improve neural differentiation and to ameliorate neurological deficits after cerebral ischemia in rats[80].

Thus, the media formulation for ASCs neural differentiation is very far from a "magic recipe", but its definition, amelioration and reproducibility should necessary start not only from ASCs physiology but also from the analysis of their reactivity toward environmental stimuli. We think this aspect is essential especially with a clinical application in mind.

BIOMATERIALS FOR ASCs NEURAL DIFFERENTIATION

Chemicals and growth factors act as signal transducers to induce ASCs toward a neural-like phenotype, but the control of their differentiation toward a specific and stable lineage requires not only a controlled biochemical microenvironment, but also a milieu in which cell-cell and cell-environment interactions should be evaluated in a three-dimensional architecture. Biomaterials offer the possibility to deliver stem cell regulatory signals in a precise and near physiological manner without the exclusion of 3D space as parameter.

The effectiveness of biomaterials in driving ASCs differentiation has been already reported for their differentiation into epithelial cells. It was emblematic and noteworthy the geometric dependence of ASCs phenotype in fibrin culture[81]. In this well-conducted study, the experimental plan demonstrated clearly that the growth factor-enriched medium increased ASCs growth and chemotaxis, but the differentiation into epithelial cells was effective only in a 3D structure of fibrin. In this condition, the authors identified the formation of a bilayer of two segregate cell phenotypes: The superficial one with ASCs-derived epithelial cells and the deeper one with mesenchymal cells. This evidence strongly suggests that biomaterials may allow the control of proliferation and differentiation not only of ASCs, but also of their neural derivatives, because it is possible the hypothesis of biomaterials that should act by reducing ASCs propagation after differentiation and by maintaining a niche of ASCs with high level of stemness to be reprogrammed according to tissue necessity.

Table 3 summarizes the principal biomaterials for neural differentiation of ASCs investigated in preclinical studies. The spontaneous formation of three-dimensional spheroids has been reported by using chitosan, a naturally derived polysaccharide from chitin[82,83]. ASCs spheroids were formed on chitosan films because pure chitosan cannot support adequate cell adhesion for its biophysical parameter. This propriety enhanced spontaneous cell organization in a 3D architecture that permitted the close association of cells and a transmission of signal cues easier and faster than in monolayer cultures. Considering that chitosan lacks biological activity, the upregulation of pluripotency marker genes[82], the transdifferentiation efficiency into neural-like cells[83] or neuron[82] *in vitro,* and the higher cellular retention ratio of ASC spheroids after intramuscular injection in nude mouse[82] could be justified by the 3D organization of cells.

Moreover, chitosan combinations with gelatin or silk were tested in animal models of neurodegeneration. In the case of elastic-dominant, porous scaffolds from photocurable, chemically modified chitosan and gelatin, ASCs were conditioned toward neuron-like cells capable to better repair a traumatic brain injury mouse model[84]. Indeed, the chitosan/silk fibroin scaffold has been proposed as nerve grafts for its efficiency as delivery vehicle for ASCs and as structural framework in the regeneration of injured rat sciatic nerve[85]. In the same animal model, an engineered neural tissue (EngNT) composed by collagen gel and aligned rat ASCs supported robust neural regeneration across the gap and into the distal stump[86]. Similar efficiency was proved in animal models of spinal cord injury after implantation of ASCs seeded on serum-derived albumin scaffold, a porous biomaterial that completely filled the spinal cord cavity and permitted the passage of descending and ascending neurons[87]. Similar results have been obtained by using Matrigel[88], a commercially available hydrogel that, because of its isolation from the basement membrane of a mouse sarcoma, is unlikely to be approved for clinical use. To select the hydrogels, the patient safety should be always considered in order to perform translational researches. For example, the alginate hydrogel-that has been used to encapsulate neurospheres obtained from neural differentiation of ASCs[89,90]-had a good biocompatible profile.

More recently, nanosized graphene oxide (NGO)-laminin hybrid patterns were reported to be useful for ASCs transdifferentiation into neuron-like cells that were up to 30% higher than the control group. In the same work, it was proved that cells grown on NGO grid patterns were more differentiated than the other ones grown on PLL-coated Au or on NGO-coated Au[91].

Taken together, all these results strongly suggest that biomaterials provide a benefit for neural differentiation of ASCs: They should mimic the shape of interconnected neuronal network and the nanoscale topographical features of the extracellular matrix, as already reported for the nanoengineered polystyrene surface containing nanopore array-patterned substrate[92]. Moreover, biomaterials have been applied to realize an electrical cell culture system for selective induction of neurons. This *in vitro* technique is realized by cellular seeding on the conductive polypyrrole/chitosan membrane with a thickness of 0.4 mm. The membrane can be connected to an electric stimulator by two thin platinum electrodes. In this way, a defined electric intensity can be applied to cell culture (V/cm). It has been employed on Schwann cells[93], on OECs[94] and on mouse bone marrow stromal cells[95] to induce and sustain their phenotype regulation. Yang *et al*[96] applied it on ASCs; they tested the possibility to promote neuronal differentiation by using both electrical stimulation and *Nurr-1* gene transduction alone and in combination. The results clearly evidenced that both electrical extracellular microenvironments and intracellular patter profile were capable of promoting neuronal differentiation in ASCs, but the best result was achieved by a synergistically combination of electrical forces and genetic modification.

BIOPHYSICAL FORCES TO DRIVE NEURAL CELLS FROM ASCs

Biophysical forces, particularly the electro-mechanical coupling and the deformation forces are important physiological regulators of nervous systems. Actually, microgravity, as a mechanical factor, is more and more under investigation, especially for its implication in heath of spaceflights and astronauts in orbit. As summarized by Mariggiò and Fanò-Illic[97], the microgravity effects are not fully characterized and contrasting events have been reported: In some cases, cell differentiation and tissue assembly were not affected by microgravity, indeed in other cases alteration of cell morphology and function has been reported. For this reason, a three-dimensional glia–neuron co-culture cell model has been proposed as useful tool for the investigation of

microgravity as a new environment to successfully manipulate cell functions and phenotype. Generally, in monolayer tissue, an improvement of stem cell differentiation into neurons was reported for PC12 neuron-like cell[98] and for ESCs[99].

As about ASCs, the microgravity effect is very little known. Up to now, only a single and recent study tried to define a mechanistic link between microgravity and neural induction of ASCs[100]. In this experimental setup, it was found that microgravity stimulation with a clinostat instrument increased ASCs differentiation toward neural-like cells in presence of the classical chemically defined and growth factor enriched medium. Even if the differentiation was proved by evaluation of neural cell lineage markers, no data about the specific effect of microgravity have been produced. Thus, it remains unclear if microgravity alone can modify the cell phenotype in absence of growth factors and biochemical stimuli, also.

CONCLUSION

Since ASCs can be readily isolated, expanded and transplanted, their application in cell-based therapies is more and more under investigation. The differentiation of ASCs was initially considered restricted to mesodermal cells, but recent advances display ASCs ability to transdifferentiate, acquiring cell phenotype different from mesenchymal, including the ectodermal one. In the past decade, most of researches focused of the promotion of ASCs into neural-like cells for evaluating their potential application in neurodegenerative disorders. Different strategies were adopted. Among them, the cultures in chemically defined or growth factors-enriched media were applied to stimulate *in vitro* the physiological process of neural induction. This dynamic event involves many biological processes and signaling events that can be potentiated by the elaboration of an appropriate biophysical and biochemical microenvironment and should be evaluated in a 3D architecture. In this context, biomaterials provide a sophisticated microenvironment. Even if terminally differentiated, functional neurons have yet to be achieved, the reported data from animal tests have shown that some biomaterials have a great potential as nerve grafts. A synergic work between cell and tissue physiologists and biomaterial production experts seems to be useful for the future development of ASCs-based clinical therapeutics to be employed in neurodegenerative disorders.

REFERENCES

1 **Wee Yong V**. Inflammation in neurological disorders: a help or a hindrance? *Neuroscientist* 2010; **16**: 408-420 [PMID: 20817918 DOI: 10.1177/1073858410371379]

2 **Avola R**, Graziano AC, Pannuzzo G, Alvares E, Cardile V. Krabbe's leukodystrophy: Approaches and models in vitro. *J Neurosci Res* 2016; **94**: 1284-1292 [PMID: 27638610 DOI: 10.1002/jnr.23846]

3 **Parveen S**, Goberman AM. Comparison of self and proxy ratings for voice handicap index and motor-related quality-of-life of individuals with Parkinson's disease. *Int J Speech Lang Pathol* 2017; **19**: 174-183 [PMID: 27146109 DOI: 10.3109/17549507.2016.1167242]

4 **Avola R**, Graziano ACE, Pannuzzo G, Albouchi F, Cardile V. New insights on Parkinson's disease from differentiation of SH-SY5Y into dopaminergic neurons: An involvement of aquaporin4 and 9. *Mol Cell Neurosci* 2018; **88**: 212-221 [PMID: 29428877 DOI: 10.1016/j.mcn.2018.02.006]

5 **Garg H**, Bush S, Gappmaier E. Associations Between Fatigue and Disability, Functional Mobility, Depression, and Quality of Life in People with Multiple Sclerosis. *Int J MS Care* 2016; **18**: 71-77 [PMID: 27134580 DOI: 10.7224/1537-2073.2015-013]

6 **Block VA**, Pitsch E, Tahir P, Cree BA, Allen DD, Gelfand JM. Remote Physical Activity Monitoring in Neurological Disease: A Systematic Review. *PLoS One* 2016; **11**: e0154335 [PMID: 27124611 DOI: 10.1371/journal.pone.0154335]

7 **Graziano AC**, Cardile V. History, genetic, and recent advances on Krabbe disease. *Gene* 2015; **555**: 2-13 [PMID: 25260228 DOI: 10.1016/j.gene.2014.09.046]

8 **Baer PC**, Geiger H. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. *Stem Cells Int* 2012; **2012**: 812693 [PMID: 22577397 DOI: 10.1155/2012/812693]

9 **Zuk PA**, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001; **7**: 211-228 [PMID: 11304456 DOI: 10.1089/107632701300062859]

10 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13**: 4279-4295 [PMID: 12475952 DOI: 10.1091/mbc.E02-02-0105]

11 **Cawthorn WP**, Scheller EL, MacDougald OA. Adipose tissue stem cells: the great WAT hope. *Trends Endocrinol Metab* 2012; **23**: 270-277 [PMID: 22417866 DOI: 10.1016/j.tem.2012.01.003]

12 **Lo Furno D**, Graziano AC, Avola R, Giuffrida R, Perciavalle V, Bonina F, Mannino G, Cardile V. A Citrus bergamia Extract Decreases Adipogenesis and Increases Lipolysis by Modulating PPAR Levels in Mesenchymal Stem Cells from Human Adipose Tissue. *PPAR Res* 2016; **2016**: 4563815 [PMID: 27403151 DOI: 10.1155/2016/4563815]

13 **Musumeci G**, Mobasheri A, Trovato FM, Szychlinska MA, Graziano AC, Lo Furno D, Avola R, Mangano S, Giuffrida R, Cardile V. Biosynthesis of collagen I, II, RUNX2 and lubricin at different time points of chondrogenic differentiation in a 3D in vitro model of human mesenchymal stem cells derived from adipose tissue. *Acta Histochem* 2014; **116**: 1407-1417 [PMID: 25307495 DOI: 10.1016/j.acthis.2014.09.008]

14 **Graziano ACE**, Avola R, Pannuzzo G, Cardile V. Aquaporin1 and 3 modification as a result of chondrogenic differentiation of human mesenchymal stem cell. *J Cell Physiol* 2018; **233**: 2279-2291 [PMID: 28708257 DOI: 10.1002/jcp.26100]

15 **Halvorsen YD**, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO, Gimble JM. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. *Tissue Eng* 2001; **7**: 729-741 [PMID: 11749730 DOI: 10.1089/107632701753337681]

16 **Choi YS**, Matsuda K, Dusting GJ, Morrison WA, Dilley RJ. Engineering cardiac tissue in vivo from human adipose-derived stem cells. *Biomaterials* 2010; **31**: 2236-2242 [PMID: 20031204 DOI: 10.1016/j.biomaterials.2009.11.097]

17 **Choi SA**, Lee JY, Wang KC, Phi JH, Song SH, Song J, Kim SK. Human adipose tissue-derived mesenchymal stem cells: characteristics and therapeutic potential as cellular vehicles for prodrug gene therapy against brainstem gliomas. *Eur J Cancer* 2012; **48**: 129-137 [PMID: 21664124 DOI: 10.1016/j.ejca.2011.04.033]

18 **Lindroos B**, Suuronen R, Miettinen S. The potential of adipose stem cells in regenerative medicine. *Stem Cell Rev* 2011; **7**:

269-291 [PMID: 20853072 DOI: 10.1007/s12015-010-9193-7]
19 **Jang S**, Cho HH, Cho YB, Park JS, Jeong HS. Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. *BMC Cell Biol* 2010; **11**: 25 [PMID: 20398362 DOI: 10.1186/1471-2121-11-25]
20 **Avola R**, Graziano ACE, Pannuzzo G, Cardile V. Human Mesenchymal Stem Cells from Adipose Tissue Differentiated into Neuronal or Glial Phenotype Express Different Aquaporins. *Mol Neurobiol* 2017; **54**: 8308-8320 [PMID: 27921242 DOI: 10.1007/s12035-016-0312-6]
21 **Casteilla L**, Dani C. Adipose tissue-derived cells: from physiology to regenerative medicine. *Diabetes Metab* 2006; **32**: 393-401 [PMID: 17110894]
22 **Keung EZ**, Nelson PJ, Conrad C. Concise review: genetically engineered stem cell therapy targeting angiogenesis and tumor stroma in gastrointestinal malignancy. *Stem Cells* 2013; **31**: 227-235 [PMID: 23132810 DOI: 10.1002/stem.1269]
23 **Esfandiari B**, Soliemani M, Kaviani S, Parivar K. Rapid Neural Differentiation of Human Adipose Tissue derived Stem Cells Using NGF, Forskolin and bFGF. *Biomed Pharm J* 2016; **9**: 39-48 [DOI: 10.13005/bpj/906]
24 **Dai R**, Wang Z, Samanipour R, Koo KI, Kim K. Adipose-Derived Stem Cells for Tissue Engineering and Regenerative Medicine Applications. *Stem Cells Int* 2016; **2016**: 6737345 [PMID: 27057174 DOI: 10.1155/2016/6737345]
25 **Cukierman E**, Pankov R, Yamada KM. Cell interactions with three-dimensional matrices. *Curr Opin Cell Biol* 2002; **14**: 633-639 [PMID: 12231360]
26 **Edelman DB**, Keefer EW. A cultural renaissance: in vitro cell biology embraces three-dimensional context. *Exp Neurol* 2005; **192**: 1-6 [PMID: 15698613 DOI: 10.1016/j.expneurol.2004.10.005]
27 **Mikos AG**, Herring SW, Ochareon P, Elisseeff J, Lu HH, Kandel R, Schoen FJ, Toner M, Mooney D, Atala A, Van Dyke ME, Kaplan D, Vunjak-Novakovic G. Engineering complex tissues. *Tissue Eng* 2006; **12**: 3307-3339 [PMID: 17518671 DOI: 10.1089/ten.2006.12.3307]
28 **Herzog EL**, Chai L, Krause DS. Plasticity of marrow-derived stem cells. *Blood* 2003; **102**: 3483-3493 [PMID: 12893756 DOI: 10.1182/blood-2003-05-1664]
29 **Morrison SJ**, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* 2006; **441**: 1068-1074 [PMID: 16810241 DOI: 10.1038/nature04956]
30 **Schuldiner M**, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 2000; **97**: 11307-11312 [PMID: 11027332 DOI: 10.1073/pnas.97.21.11307]
31 **Friedenstein AJ**, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation* 1974; **17**: 331-340 [PMID: 4150881]
32 **Reynolds BA**, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992; **255**: 1707-1710 [PMID: 1553558 DOI: 10.1126/science.1553558]
33 **Domen J**, Weissman IL. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Mol Med Today* 1999; **5**: 201-208 [PMID: 10322312 DOI: 10.1016/S1357-4310(99)01464-1]
34 **Gandarillas A**, Watt FM. c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev* 1997; **11**: 2869-2882 [PMID: 9353256 DOI: 10.1101/gad.11.21.2869]
35 **Seale P**, Rudnicki MA. A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev Biol* 2000; **218**: 115-124 [PMID: 10656756]
36 **Rossi F**, Cattaneo E. Opinion: neural stem cell therapy for neurological diseases: dreams and reality. *Nat Rev Neurosci* 2002; **3**: 401-409 [PMID: 11988779 DOI: 10.1038/nrn809]
37 **Gage FH**. Neurogenesis in the adult brain. *J Neurosci* 2002; **22**: 612-613 [PMID: 11826087]
38 **McKay R**. Stem cells in the central nervous system. *Science* 1997; **276**: 66-71 [PMID: 9082987 DOI: 10.1126/science.276.5309.66]
39 **Reisman M**, Adams KT. Stem cell therapy: a look at current research, regulations, and remaining hurdles. *P T* 2014; **39**: 846-857 [PMID: 25516694]
40 **Illouz YG**. Body contouring by lipolysis: a 5-year experience with over 3000 cases. *Plast Reconstr Surg* 1983; **72**: 591-597 [PMID: 6622564]
41 **Zhu Y**, Liu T, Song K, Fan X, Ma X, Cui Z. Adipose-derived stem cell: a better stem cell than BMSC. *Cell Biochem Funct* 2008; **26**: 664-675 [PMID: 18636461 DOI: 10.1002/cbf.1488]
42 **Graziano ACE**. Is it Time to Study Leukodystrophies? *J Neurosci Rural Pract* 2017; **8**: S1-S2 [PMID: 28936061 DOI: 10.4103/jnrp.jnrp_146_17]
43 **Safford KM**, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun* 2002; **294**: 371-379 [PMID: 12051722 DOI: 10.1016/S0006-291X(02)00469-2]
44 **Ashjian PH**, Elbarbary AS, Edmonds B, DeUgarte D, Zhu M, Zuk PA, Lorenz HP, Benhaim P, Hedrick MH. In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. *Plast Reconstr Surg* 2003; **111**: 1922-1931 [PMID: 12711954 DOI: 10.1097/01.PRS.0000055043.62589.05]
45 **Krampera M**, Marconi S, Pasini A, Galiè M, Rigotti G, Mosna F, Tinelli M, Lovato L, Anghileri E, Andreini A, Pizzolo G, Sbarbati A, Bonetti B. Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. *Bone* 2007; **40**: 382-390 [PMID: 17049329 DOI: 10.1016/j.bone.2006.09.006]
46 **Bai X**, Ma J, Pan Z, Song YH, Freyberg S, Yan Y, Vykoukal D, Alt E. Electrophysiological properties of human adipose tissue-derived stem cells. *Am J Physiol Cell Physiol* 2007; **293**: C1539-C1550 [PMID: 17687001 DOI: 10.1152/ajpcell.00089.2007]
47 **Anghileri E**, Marconi S, Pignatelli A, Cifelli P, Galié M, Sbarbati A, Krampera M, Belluzzi O, Bonetti B. Neuronal differentiation potential of human adipose-derived mesenchymal stem cells. *Stem Cells Dev* 2008; **17**: 909-916 [PMID: 18564036 DOI: 10.1089/scd.2007.0197]
48 **Li H**, Han Z, Liu D, Zhao P, Liang S, Xu K. Autologous platelet-rich plasma promotes neurogenic differentiation of human adipose-derived stem cells in vitro. *Int J Neurosci* 2013; **123**: 184-190 [PMID: 23126279 DOI: 10.3109/00207454.2012.742077]
49 **Lo Furno D**, Pellitteri R, Graziano AC, Giuffrida R, Vancheri C, Gili E, Cardile V. Differentiation of human adipose stem cells into neural phenotype by neuroblastoma- or olfactory ensheathing cells-conditioned medium. *J Cell Physiol* 2013; **228**: 2109-2118 [PMID: 23589068 DOI: 10.1002/jcp.24386]
50 **Razavi S**, Razavi MR, Kheirollahi-Kouhestani M, Mardani M, Mostafavi FS. Co-culture with neurotrophic factor secreting cells induced from adipose-derived stem cells: promotes neurogenic differentiation. *Biochem Biophys Res Commun* 2013; **440**: 381-387 [PMID: 24064351 DOI: 10.1016/j.bbrc.2013.09.069]
51 **Razavi S**, Razavi MR, Ahmadi N, Kazemi M. Estrogen treatment enhances neurogenic differentiation of human adipose derived stem cells in vitro. *Iran J Basic Med Sci* 2015; **18**: 799-804 [PMID: 26557969]
52 **Salehi H**, Amirpour N, Niapour A, Razavi S. An Overview of Neural Differentiation Potential of Human Adipose Derived Stem Cells. *Stem Cell Rev* 2016; **12**: 26-41 [PMID: 26490462 DOI: 10.1007/s12015-015-9631-7]
53 **Hu F**, Wang X, Liang G, Lv L, Zhu Y, Sun B, Xiao Z. Effects of epidermal growth factor and basic fibroblast growth factor on the proliferation and osteogenic and neural differentiation of adipose-derived stem cells. *Cell Reprogram* 2013; **15**: 224-232 [PMID: 23713433 DOI: 10.1089/cell.2012.0077]
54 **Baer PC**, Schubert R, Bereiter-Hahn J, Plösser M, Geiger H. Expression of a functional epidermal growth factor receptor on human adipose-derived mesenchymal stem cells and its signaling

mechanism. *Eur J Cell Biol* 2009; **88**: 273-283 [PMID: 19167776 DOI: 10.1016/j.ejcb.2008.12.001]
55 **Ai G**, Shao X, Meng M, Song L, Qiu J, Wu Y, Zhou J, Cheng J, Tong X. Epidermal growth factor promotes proliferation and maintains multipotency of continuous cultured adipose stem cells via activating STAT signal pathway in vitro. *Medicine (Baltimore)* 2017; **96**: e7607 [PMID: 28746211 DOI: 10.1097/MD.0000000000007607]
56 **Carpenter G**, Goodman L, Shaver L. The Physiology of Epidermal Growth Factor. In: Kahn P, Graf T. Oncogenes and Growth Control. Springer, Berlin, Heidelberg 1986 [DOI: 10.1007/978-3-642-73325-3_9]
57 **Cohen S**. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem* 1962; **237**: 1555-1562 [PMID: 13880319]
58 **Tang WP**, Akahoshi T, Piao JS, Narahara S, Murata M, Kawano T, Hamano N, Ikeda T, Hashizume M. Basic fibroblast growth factor-treated adipose tissue-derived mesenchymal stem cell infusion to ameliorate liver cirrhosis via paracrine hepatocyte growth factor. *J Gastroenterol Hepatol* 2015; **30**: 1065-1074 [PMID: 25639333 DOI: 10.1111/jgh.12893]
59 **Kakudo N**, Shimotsuma A, Kusumoto K. Fibroblast growth factor-2 stimulates adipogenic differentiation of human adipose-derived stem cells. *Biochem Biophys Res Commun* 2007; **359**: 239-244 [PMID: 17543283 DOI: 10.1016/j.bbrc.2007.05.070]
60 **Kabiri A**, Esfandiari E, Hashemibeni B, Kazemi M, Mardani M, Esmaeili A. Effects of FGF-2 on human adipose tissue derived adult stem cells morphology and chondrogenesis enhancement in Transwell culture. *Biochem Biophys Res Commun* 2012; **424**: 234-238 [PMID: 22728881 DOI: 10.1016/j.bbrc.2012.06.082]
61 **Quarto N**, Longaker MT. FGF-2 inhibits osteogenesis in mouse adipose tissue-derived stromal cells and sustains their proliferative and osteogenic potential state. *Tissue Eng* 2006; **12**: 1405-1418 [PMID: 16846339 DOI: 10.1089/ten.2006.12.1405]
62 **Hye Kim J**, Gyu Park S, Kim WK, Song SU, Sung JH. Functional regulation of adipose-derived stem cells by PDGF-D. *Stem Cells* 2015; **33**: 542-556 [PMID: 25332166 DOI: 10.1002/stem.1865]
63 **Hu JG**, Wu XJ, Feng YF, Xi GM, Wang ZH, Zhou JS, Lü HZ. PDGF-AA and bFGF mediate B104CM-induced proliferation of oligodendrocyte precursor cells. *Int J Mol Med* 2012; **30**: 1113-1118 [PMID: 22922759 DOI: 10.3892/ijmm.2012.1110]
64 **Muthukrishnan L**, Warder E, McNeil PL. Basic fibroblast growth factor is efficiently released from a cytolsolic storage site through plasma membrane disruptions of endothelial cells. *J Cell Physiol* 1991; **148**: 1-16 [PMID: 1860889 DOI: 10.1002/jcp.1041480102]
65 **Werner S**, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 2003; **83**: 835-870 [PMID: 12843410 DOI: 10.1152/physrev.2003.83.3.835]
66 **Sun LY**, Pang CY, Li DK, Liao CH, Huang WC, Wu CC, Chou YY, Li WW, Chen SY, Liu HW, Chang YJ, Cheng CF. Antioxidants cause rapid expansion of human adipose-derived mesenchymal stem cells via CDK and CDK inhibitor regulation. *J Biomed Sci* 2013; **20**: 53 [PMID: 23915242 DOI: 10.1186/1423-0127-20-53]
67 **Han J**, Choi HY, Dayem AA, Kim K, Yang G, Won J, Do SH, Kim JH, Jeong KS, Cho SG. Regulation of Adipogenesis Through Differential Modulation of ROS and Kinase Signaling Pathways by 3,4'-Dihydroxyflavone Treatment. *J Cell Biochem* 2017; **118**: 1065-1077 [PMID: 27579626 DOI: 10.1002/jcb.25681]
68 **Morozzi G**, Beccafico S, Bianchi R, Riuzzi F, Bellezza I, Giambanco I, Arcuri C, Minelli A, Donato R. Oxidative stress-induced S100B accumulation converts myoblasts into brown adipocytes via an NF-κB/YY1/miR-133 axis and NF-κB/YY1/BMP-7 axis. *Cell Death Differ* 2017; **24**: 2077-2088 [PMID: 28885620 DOI: 10.1038/cdd.2017.132]
69 **Fehér E**, Pénzes L. Effect of an antioxidant compound (2-mercaptoethanol) on the nerve terminals of the aging small intestine. *Exp Gerontol* 1990; **25**: 135-140 [PMID: 2369928]
70 **Patel PA**, Chaudhary SS, Puri G, Singh VK, Odedara AB. Effects of β-mercaptoethanol on in vitro maturation and glutathione level of buffalo oocytes. *Vet World* 2015; **8**: 213-216 [PMID: 27047075 DOI: 10.14202/vetworld.2015.213-216]
71 **Chuang DM**, Leng Y, Marinova Z, Kim HJ, Chiu CT. Multiple roles of HDAC inhibition in neurodegenerative conditions. *Trends Neurosci* 2009; **32**: 591-601 [PMID: 19775759 DOI: 10.1016/j.tins.2009.06.002]
72 **Wang Z**, Tsai LK, Munasinghe J, Leng Y, Fessler EB, Chibane F, Leeds P, Chuang DM. Chronic valproate treatment enhances postischemic angiogenesis and promotes functional recovery in a rat model of ischemic stroke. *Stroke* 2012; **43**: 2430-2436 [PMID: 22811460 DOI: 10.1161/STROKEAHA.112.652545]
73 **Kim AJ**, Shi Y, Austin RC, Werstuck GH. Valproate protects cells from ER stress-induced lipid accumulation and apoptosis by inhibiting glycogen synthase kinase-3. *J Cell Sci* 2005; **118**: 89-99 [PMID: 15585578 DOI: 10.1242/jcs.01562]
74 **Gul H**, Marquez-Curtis LA, Jahroudi N, Lo J, Turner AR, Janowska-Wieczorek A. Valproic acid increases CXCR4 expression in hematopoietic stem/progenitor cells by chromatin remodeling. *Stem Cells Dev* 2009; **18**: 831-838 [PMID: 18847317 DOI: 10.1089/scd.2008.0235]
75 **Okubo T**, Hayashi D, Yaguchi T, Fujita Y, Sakaue M, Suzuki T, Tsukamoto A, Murayama O, Lynch J, Miyazaki Y, Tanaka K, Takizawa T. Differentiation of rat adipose tissue-derived stem cells into neuron-like cells by valproic acid, a histone deacetylase inhibitor. *Exp Anim* 2016; **65**: 45-51 [PMID: 26411320 DOI: 10.1538/expanim.15-0038]
76 **Hashemzadeh MA**, Seyedi Z, Rafiei S, Hassanzadeh-Moghaddam M, Edalatmanesh MA. Chemokine receptor's expression in human adipose derived mesenchymal stem cells primed with valproic acid. *Comp Clin Pathol* 2017; **26**: 115-120 [DOI: 10.1007/s00580-016-2352-8]
77 **Silverman LR**, Mufti GJ. Methylation inhibitor therapy in the treatment of myelodysplastic syndrome. *Nat Clin Pract Oncol* 2005; **2** Suppl 1: S12-S23 [PMID: 16341236]
78 **Taylor SM**, Jones PA. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* 1979; **17**: 771-779 [PMID: 90553]
79 **Jones PA**, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 1980; **20**: 85-93 [PMID: 6156004 DOI: 10.1016/0092-8674(80)90237-8]
80 **Kang SK**, Lee DH, Bae YC, Kim HK, Baik SY, Jung JS. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. *Exp Neurol* 2003; **183**: 355-366 [PMID: 14552877 DOI: 10.1016/S0014-4886(03)00089-X]
81 **Long JL**, Zuk P, Berke GS, Chhetri DK. Epithelial differentiation of adipose-derived stem cells for laryngeal tissue engineering. *Laryngoscope* 2010; **120**: 125-131 [PMID: 19856398 DOI: 10.1002/lary.20719]
82 **Cheng NC**, Wang S, Young TH. The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities. *Biomaterials* 2012; **33**: 1748-1758 [PMID: 22153870 DOI: 10.1016/j.biomaterials.2011.11.049]
83 **Hsueh YY**, Chiang YL, Wu CC, Lin SC. Spheroid formation and neural induction in human adipose-derived stem cells on a chitosan-coated surface. *Cells Tissues Organs* 2012; **196**: 117-128 [PMID: 22327282 DOI: 10.1159/000332045]
84 **Gao S**, Zhao P, Lin C, Sun Y, Wang Y, Zhou Z, Yang D, Wang X, Xu H, Zhou F, Cao L, Zhou W, Ning K, Chen X, Xu J. Differentiation of human adipose-derived stem cells into neuron-like cells which are compatible with photocurable three-dimensional scaffolds. *Tissue Eng Part A* 2014; **20**: 1271-1284 [PMID: 24251600 DOI: 10.1089/ten.TEA.2012.0773]
85 **Wei Y**, Gong K, Zheng Z, Wang A, Ao Q, Gong Y, Zhang X. Chitosan/silk fibroin-based tissue-engineered graft seeded with adipose-derived stem cells enhances nerve regeneration in a rat model. *J Mater Sci Mater Med* 2011; **22**: 1947-1964 [PMID: 21656031 DOI: 10.1007/s10856-011-4370-z]
86 **Georgiou M**, Golding JP, Loughlin AJ, Kingham PJ, Phillips JB. Engineered neural tissue with aligned, differentiated adipose-

derived stem cells promotes peripheral nerve regeneration across a critical sized defect in rat sciatic nerve. *Biomaterials* 2015; **37**: 242-251 [PMID: 25453954 DOI: 10.1016/j.biomaterials.2014.10.009]

87 **Ferrero-Gutierrez A**, Menendez-Menendez Y, Alvarez-Viejo M, Meana A, Otero J. New serum-derived albumin scaffold seeded with adipose-derived stem cells and olfactory ensheathing cells used to treat spinal cord injured rats. *Histol Histopathol* 2013; **28**: 89-100 [PMID: 23233062 DOI: 10.14670/HH-28.89]

88 **Park SS**, Lee YJ, Lee SH, Lee D, Choi K, Kim WH, Kweon OK, Han HJ. Functional recovery after spinal cord injury in dogs treated with a combination of Matrigel and neural-induced adipose-derived mesenchymal Stem cells. *Cytotherapy* 2012; **14**: 584-597 [PMID: 22348702 DOI: 10.3109/14653249.2012.658913]

89 **Razavi S**, Khosravizadeh Z, Bahramian H, Kazemi M. Time-Dependent Effect of Encapsulating Alginate Hydrogel on Neurogenic Potential. *Cell J* 2015; **17**: 304-311 [PMID: 26199909 DOI: 10.22074/cellj.2016.3736]

90 **Khosravizadeh Z**, Razavi S, Bahramian H, Kazemi M. The beneficial effect of encapsulated human adipose-derived stem cells in alginate hydrogel on neural differentiation. *J Biomed Mater Res B Appl Biomater* 2014; **102**: 749-755 [PMID: 24142904 DOI: 10.1002/jbm.b.33055]

91 **Kim TH**, Shah S, Yang L, Yin PT, Hossain MK, Conley B, Choi JW, Lee KB. Controlling differentiation of adipose-derived stem cells using combinatorial graphene hybrid-pattern arrays. *ACS Nano* 2015; **9**: 3780-3790 [PMID: 25840606 DOI: 10.1021/nn5066028]

92 **Jung AR**, Kim RY, Kim HW, Shrestha KR, Jeon SH, Cha KJ, Park YH, Kim DS, Lee JY. Nanoengineered Polystyrene Surfaces with Nanopore Array Pattern Alters Cytoskeleton Organization and Enhances Induction of Neural Differentiation of Human Adipose-Derived Stem Cells. *Tissue Eng Part A* 2015; **21**: 2115-2124 [PMID: 25919423 DOI: 10.1089/ten.TEA.2014.0346]

93 **Huang J**, Hu X, Lu L, Ye Z, Zhang Q, Luo Z. Electrical regulation of Schwann cells using conductive polypyrrole/chitosan polymers. *J Biomed Mater Res A* 2010; **93**: 164-174 [PMID: 19536828 DOI: 10.1002/jbm.a.32511]

94 **Qi F**, Wang Y, Ma T, Zhu S, Zeng W, Hu X, Liu Z, Huang J, Luo Z. Electrical regulation of olfactory ensheathing cells using conductive polypyrrole/chitosan polymers. *Biomaterials* 2013; **34**: 1799-1809 [PMID: 23228424 DOI: 10.1016/j.biomaterials.2012.11.042]

95 **Matsumoto M**, Imura T, Fukazawa T, Sun Y, Takeda M, Kajiume T, Kawahara Y, Yuge L. Electrical stimulation enhances neurogenin2 expression through β-catenin signaling pathway of mouse bone marrow stromal cells and intensifies the effect of cell transplantation on brain injury. *Neurosci Lett* 2013; **533**: 71-76 [PMID: 23142721 DOI: 10.1016/j.neulet.2012.10.023]

96 **Yang Y**, Ma T, Ge J, Quan X, Yang L, Zhu S, Huang L, Liu Z, Liu L, Geng D, Huang J, Luo Z. Facilitated Neural Differentiation of Adipose Tissue-Derived Stem Cells by Electrical Stimulation and Nurr-1 Gene Transduction. *Cell Transplant* 2016; **25**: 1177-1191 [PMID: 26337634 DOI: 10.3727/096368915X688957]

97 **Mariggiò MA**, Fanò-Illic G. The effects of simulated microgravity on the human nervous system: The proposal of a three-dimensional glia–neuron co-culture cell model. *Sci Proc* 2015; **2**: e892 [DOI: 10.14800/sp.892]

98 **Genchi GG**, Cialdai F, Monici M, Mazzolai B, Mattoli V, Ciofani G. Hypergravity stimulation enhances PC12 neuron-like cell differentiation. *Biomed Res Int* 2015; **2015**: 748121 [PMID: 25785273 DOI: 10.1155/2015/748121]

99 **Shinde V**, Brungs S, Henry M, Wegener L, Nemade H, Rotshteyn T, Acharya A, Baumstark-Khan C, Hellweg CE, Hescheler J, Hemmersbach R, Sachinidis A. Simulated Microgravity Modulates Differentiation Processes of Embryonic Stem Cells. *Cell Physiol Biochem* 2016; **38**: 1483-1499 [PMID: 27035921 DOI: 10.1159/000443090]

100 **Zarrinpour V**, Hajebrahimi Z, Jafarinia M. Expression pattern of neurotrophins and their receptors during neuronal differentiation of adipose-derived stem cells in simulated microgravity condition. *Iran J Basic Med Sci* 2017; **20**: 178-186 [PMID: 28293395 DOI: 10.22038/ijbms.2017.8244]

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Basic Study

NF-κB promotes the stem-like properties of leukemia cells by activation of LIN28B

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Author contributions: Zhou J and Chng WJ conceptualized the original idea, designed the experiments and analyzed the data; Zhou J performed the experiments, wrote the paper; Chooi JY, Ching YQ, Quah JY, Toh SHM and Ng Y performed the experiments; Tan TZ conducted the bioinformatics analysis; all authors read and approved the final manuscript.

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Abstract

AIM

To examine whether nuclear factor kappa B (NF-κB) activity regulates LIN28B expression and their roles in leukemia stem cell (LSC)-like properties.

METHODS

We used pharmacological inhibitor and cell viability assays to examine the relation between NF-κB and LIN28B. Western blot and qRT-PCR was employed to determine their protein and mRNA levels. Luciferase reporter was constructed and applied to explore the transcriptional regulation of LIN28B. We manipulated LIN28B level in acute myeloid leukemia (AML) cells and investigated LSC-like properties with colony forming and serial replating assays.

RESULTS

This study revealed the relationship between NF-κB

and LIN28B in AML cells through drug inhibition and overexpression experiments. Notably, inhibition of NF-κB by pharmacological inhibitors reduced LIN28B expression and decreased cell proliferation. We demonstrated that NF-κB binds to the -819 to -811 region of LIN28B promoter, and transcriptionally regulates LIN28B expression. LIN28B protein was significantly elevated in NFκB1 transfected cells compared to vector control. Importantly, ectopic expression of LIN28B partially rescued the self-renewal capacity impaired by pharmacological inhibition of NF-κB activity.

CONCLUSION

These results uncover a regulatory signaling, NF-κB/LIN28B, which plays a pivotal role in leukemia stem cell-like properties and it could serve as a promising intervening target for effective treatment of AML disease.

Key words: Nuclear factor kappa B; LIN28B; Leukemia stem cell; Acute myeloid leukemia

Core tip: In this study, we uncovered that LIN28B is regulated by nuclear factor kappa B (NF-κB) activity on transcriptional level and important for NF-κB-mediated leukemia-stem cell like properties of acute myeloid leukemia cells. NF-κB/LIN28B represents an attractive therapeutic target.

Zhou J, Chooi JY, Ching YQ, Quah JY, Toh SHM, Ng Y, Tan TZ, Chng WJ. NF-κB promotes the stem-like properties of leukemia cells by activation of LIN28B. *World J Stem Cells* 2018; 10(4): 34-42 Available from: URL: http://www.wjgnet.com/1948-0210/full/v10/i4/34.htm DOI: http://dx.doi.org/10.4252/wjsc.v10.i4.34

INTRODUCTION

The nuclear factor kappa B (NF-κB) is an well-studied dimeric transcription factor, which regulates the expression of a plethora of downstream targets in action to different cellular conditions, including cell viability, differentiation, proliferation, adhesion, cell cycle and immune response[1,2]. Cross-species analysis demonstrates the optimal DNA binding motif of the human NF-κB orthologous is highly evolutionarily conserved, suggesting its functional importance[3,4]. The Rel homology domain of NF-κB contains the nuclear localization signal (NLS), which determines the its subcellular localization. In inactivation state, Inhibitor of kappa-B (I-κB) binds to NF-κB, and covers-up NLS, preserving the NF-κB complexes in the cytoplasm[5]. Upon activation, an upstream IB kinase (IKKβ) phosphorylates I-κB, then the phosphorylated I-κB is degraded by proteasome system[6]. These NF-κB dimers with open NLS are translocated to the nucleus to binds consensus NF-κB binding motif. In the past a few decades, a substantial number of literatures underscored the key role of the NF-κB pathway in the pathogenesis of solid tumors, hematological malignancies[7-10]. Moreover, Constitutive activation of NF-κB has been implicated in the chemo-resistance and radiation resistance of cancer cells[11].

Acute myeloid leukemia (AML) consists of a group of clonal disorders characterized by different subtypes of genetic and epigenetic abnormalities, which has diverse response to treatment and prognosis[12-14]. Constitutive activity of NF-κB has been demonstrated in approximate 40%[15] to 70% of AML cases[16]. Aberrant NF-κB activity upregulates a long list of anti-apoptotic target genes, such as *MCL-1*, *XIAP*, *BCL-2*, *BCL-xL*, *FLIP*, enabling AML cells to evade apoptosis and increase proliferation. Notably, aberrant activation of NF-κB has also been detected in CD34+CD38-CD123+ subpopulation, which is defined as leukemia stem cell (LSC), but not unstimulated normal CD34+ progenitors[15,17]. LSC, also known as leukemia initiating cells (LIC), is the first reported and most studied cancer stem cells (CSC). Although LSCs account for a small fraction of leukemia cells, they are responsible for the treatment failure and disease relapse. The bone marrow microenvironment provides sanctuary to LSC, which is the root source of treatment failure and relapse. Taken together, these evidences suggest that selectively targeting LSC might represent an important strategy towards curing AML. However, the underlying mechanisms of transformation of LSC remain elusive.

The RNA-binding protein, LIN28B, is a microRNAs (miRNA) regulator, which negatively regulates let-7 miRNA family. LIN28B protein belongs to LIN28 family, which is used to reprogram induced pluripotent stem cells (iPSCs). A plethora of clinical and laboratory studies firmly established LIN28B as oncogene in a variety of cancers and hematological malignancies. A number of studies have revealed that LIN28B induces the transformation of CSCs and LSCs and predicts poor prognosis and advanced cancer stages. However, the connection of NF-κB and LIN28B in the pathology of AML remains unacquainted.

In this study, we demonstrate that NF-κB positively regulates LIN28B. We identify a consensus NF-κB binding motif on the LIN28B promoter, which is functionally important. We also show LIN28B expression is the key for the NF-κB-mediated stem cell properties of AML cells.

MATERIALS AND METHODS

Cell culture

TF-1a cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States) and grown in RPMI 1640 (Biowest, France), supplemented with 10% Fetal Bovine Serum (Biowest) and 1% of antibiotics (Biowest) in an incubator at 37 ℃ with 5% CO_2. HEK-293T cells were maintained in Dublecoo's modified Eagle's medium (DMEM) (Biowest)

with additional 10% FBS and 1% of antibiotics (Biowest).

LIN28B promoter constructs and site-specific mutagenesis

We first designed the 5' forward primers containing additional random sequence and 3' reverse primer containing additional random sequence and a Bag Ⅲ restriction site. Then, we used this pair of primers to amplify the human LIN28B promoter fragment containing potential NF-κB was amplified from genomic DNA and the purified PCR products were cloned into reporter pGL3.0 basic vector (Promega, Madison, WI, United States) to generate fusion genes. Sequencing analysis has confirmed these fusion genes. The consensus NF-κB binding motif GGCGATCCC (−819 to −811 relative to TSS) of the construct was mutated to GGCGATTTT using QuikChange Ⅱ XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, United States). These mutations were confirmed by sequencing analysis.

Transient transfection and dual luciferase assay

HEK-293T Cells (2 × 10^5 cells) were chemically transfected (PEI) with a mix containing 0.5 μg pGL3.0, 2 μg pCMV6-XL4 NFκB1, 2 μg NFκB reporter gene, 10 ng Renilla vector in 1ml of DMEM growth medium for one day. 293T cells were rinsed with 1 x PBS and harvested in 250 μL of Passive Lysis Buffer (Promega, WI, United States). We performed dual luciferase assay by using a kit from Promega according to the manufacturer's instruction. Briefly, firefly and renilla activity were recorded by a 20/20 luminomoter (Glomax). The relative intensity of firefly luciferase of each sample was normalized to its renilla luciferase. The reporter gene assays for the empty pGL3.0 vector as a control and LIN28N promoter vector were conducted in a similar fashion. These experiments were performed in triplication.

RNA extraction and qRT-PCR

RNeasy Mini Kit (Qiagen Hilden, Germany) was purchased for RNA extraction. In brief, cDNA was synthesized by using the i-Script™ Reverse Transcription Supermix (Biorad, Hercules, CA, United States) from 1 μg of total RNA. The RT reaction was carried under the following conditions: 5 min at 25 ℃, 30 min at 42 ℃ and 5 min at 85 ℃ in total 20 μL of reaction mixture. Quantitative real-time (qRT)-PCR was performed in a 7300 real-time PCR System (Thermo Fisher Scientific, Forster city, CA, United States). The reaction mixture consists of 0.8 μL of LIN28B or GAPDH primers in 10 μL of iTaq Universal SybrGreen mastermix (Biorad) to a total volume of 20 μL. The qRT-PCR cycle conditions are 2 min at 50 ℃, 10min at 95 ℃, followed by 40 cycles of 15 s at 95 ℃ and 1min at 60 ℃. The primer sequences were described as the following: LIN28B Forward: 5'-GGATTTGGATTC ATCTCCATGATAA-3', LIN28B Reverse: 5'-GAATTCCACTGGTTCTCCTTCTTTT-3'; GAPDH Forward: 5-GCACCACCAACTGCTTAGCA-3; and GAPDH Reverse: 5-GTCT TCTGGGTGGCAGTGATG-3. The relative quantification of *LIN28B* gene was determined by using the comparative C_T ($^{\Delta\Delta}C_T$) method as recommended by the manufacturer.

Drug treatment and cell viability assay

Bortezomib, MG-132, and IKK2 inhibitorⅣ were purchased form Sigma-Aldrich (St. Louis, MO, United States) were dissolved in either Dimethyl sulfoxide (DMSO) or PBS, depending on their solubility in the solvents. TF-1a cells were seeded at a density of 20000 viable cells per well in 96-well culture plates in triplication. As described previously, we used the CellTiter-Glo® Luminescent Cell Viability Assay, also known as CTG assay (Promega Corporation, Madison, WI) to study the effect of drugs on cell viability and proliferation[18]. The inhibitory concentration (IC_{50}) for the cell line was estimated using CTG assay and respective concentrations of each drug were used to treat TF-1a cells. For Bortezomib, the concentrations used were 12.5 nmol/L and 25 nmol/L, while for MG-132 were 312 nmol/L and 625 nmol/L and IKK2 Inhibitor Ⅳ were 5.31 μmol/L and 10.62 μmol/L. Cells were incubated at 24 h and 48 h prior protein extraction. Same batch of cells were added with either PBS or DMSO as controls. Each experiment was repeated 3 times.

Protein extraction and western blotting

Cells were lysed in Lysis buffer (1% Nonidet P-40, 50 mmol/L Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10% glycerol with protease and phosphatase inhibitors) and followed by protein extraction. The amount of proteins was quantified with Bradford assay (Biorad). The cell lysates were loaded into polyacrylamide denaturing gels (12%) and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Kenilworth, NJ, United States). These membranes were then blocked in 5% milk with 0.1% Tween 20-PBS (PBS-T) solution for one hour. These primary antibodies were used: anti-NFκB-p65 and anti-β-actin antibody (HRP-conjugated) from Santa Cruz Biotechnology (Santa Cruz, CA, United States), anti-phosphoNFκB-P65 antibody from Cell Signaling Technologies, Danvers, MA, United States. Respective second antibodies were applied and washed before exposure for Chemiluminescence (Santa Cruz Biotechnology).

Transfection

LIN28B gene was cloned into pEGFP vector (Clontech, Fremont, CA, United States) by standard method to create LIN28B-pEGFP vector. We transfected LIN28B-pEGPF and empty vector pEGFP into TF-1a cells by using a Neon™ transfection system (ThermoFisher Scientific, Waltham, MA, United States). The transfection was carried out as the following condition: 1 million cells with five microgram of each vector in 100

μL of Resuspension buffer R electroporation at 1200 V for 20 ms, three pulses. After transfection, cells were transferred into fresh culture medium and grown in the incubator.

Colony formation assay and serial replating assay

Trypan Blue Exclusion method was applied to determine the cell viability of TF-1a and TF-1a-LIN28B cells. Briefly, ten microliter of trypan blue dye (concentration: 0.4% from Sigma-Aldrich) was mixed with equal volume of the cell suspension to obtain a 1 to 2 dilution for one minute. The mixture was put on a hemacytometer and viable cells were counted under a light microscope. We then applied 2 x 10^4 viable TF-1a and TF-1a-LIN28B cells into in human StemMACS HSC-CFU basic medium without cytokines (130-091-275, Miltenyi Biotec, Germany) in 6-well plates for seven days. Normally, colonies comprises of greater than 50 cells which were counted under an inverted microscope. Total 5 random 4 x 10 magnification fields were counted for colony numbers. The average of colonies number was determined by total numbers divided into five. In serial replating assay, the colony number was determined , followed by harvesting and diluting, and 20000 cells were replated in fresh methylcellulose medium and subjected to replating every 7 d. All these experiments were repeated twice.

Statistical analysis

T-test was applied to compare the mean ± SD between two groups. Two-way analysis of variance (ANOVA) was used to analyse the statistical significance when there were more than two groups. We considered *P* values less than 0.05 as statistically significant.

RESULTS

Pharmacological inhibition of NF-κB reduces cell viability and LIN28B expression

We previously reported that human AML cell line TF-1a cells express abundant endogenous LIN28B protein[19]. In order to investigate the relationship between NF-κB and LIN28B, we treated the TF-1a cells with gradual concentrations of Bortezomib and MG-132, two different NF-κB inhibitors[20].

A significant decrease in cell viability could be observed when treated with increasing dose of Bortezomib and MG-132 (Figure 1A and D) and their IC_{50} values were about at 50 mol/L and 625 nmol/L, respectively. qRT-PCR analysis of TF-1a cells treated with 12.5 nmol/L and 25 nmol/L of Bortezomib showed a decrease in *LIN28B* mRNA by about 63% and 82%, respectively, compared to the control sample (Figure 1B). The reduction of LIN28B protein level was noticeable in cells treated with 25 nmol/L of Bortezomib, which coincides with decreased NF-κB p65, I-κBα (Figure 1C). Notably, a lower dose 12.5 nmol/L of Bortezomib didn't induce significant change of NF-κB p65, I-κBα. In concordance, LIN28B protein was not changed significantly either, indicating a specific correlation between NF-κB activity and LIN28B expression.

In agreement with the results from Bortezomib treatment, qRT-PCR analysis in MG-132-treated cells (312 and 625 nmol/L) showed significant reduction of *LIN28B* mRNA level (58.2% and 79%, respectively) relative to control (Figure 1E). Decline in NF-κB p65, I-κBα, as well as in LIN28B protein expression was observed in both of MG-132-treated cells as compared to control (Figure 1F). As Bortezomib and MG-132 are proteasome inhibitors, we also used an IKK2 inhibitor Ⅳ, a more specific and direct NF-κB inhibitor to treat the TF-1a cells. We observed increasing inhibition of cell proliferation starting from 0.625 μmol/L to 10 μmol/L, with 50% inhibition at around 5 μmol/L (Supplementary Figure S1A). In agreement with the data derived from Bortezomib and MG-132, IKK-2 inhibitor Ⅳ treatment significantly reduced the mRNA (Supplementary Figure S1B) and protein levels (Supplementary Figure S1C) of LIN28B. In conclusion, the regulation of *LIN28B* by NFκB activity is specific.

Taken together, our data suggest that inhibition of NF-κB decreases cell viability, accompanying reduction in *LIN28B* mRNA and protein, suggesting a possible role for NF-κB in regulation of LIN28B expression.

Overexpression of NF-κB increases LIN28B expression

We further examined the role of NF-κB in controlling *LIN28B* by overexpression of NF-κB in *HEK-293T*. The ability of pCMV6-XL4 NF-κB1 plasmid to overexpress NFκB protein was determined using Dual luciferase assay. *pCMV6-XL4 NFκB1* that encodes *p105* which can undergo proteolysis to produce *p50* that interacts with *p65* was overexpressed in HEK293T cells. Figure 2A showed that by overexpressing NF-κB1 could result in increase in activating NFκB reporter gene two times relative to cells with only expressed NF-κB reporter gene. This result validated the overexpression efficiency of this plasmid, which was subsequently used to overexpress in HEK293T cells for 48 h (Figure 2B). LIN28B protein was upregulated in NF-κB1 overexpression cells, suggesting the regulatory effect of NF-κB activity on LIN28B expression through the canonical pathway. Taken together, these data together with abovmentioned results derived from NF-κB inhibitors, firmly support the regulatory role of NF-κB in LIN28B expression.

NF-κB increase LIN28B promoter activity

We decided to explore the possible connection between NF-κB and *LIN28B* promoter. A detailed search of the -2.0 kb human *LIN28B* promoter region discovered one putative NF-κB -consensus-binding sequences[21], as decipted in Figure 3A, located at -819 to -811 relative to TSS (sequence: GGCGATCCC). Gene reporter assays revealed that luciferase activity increased by two times in 293T cells transfected with *LIN28B* constructs

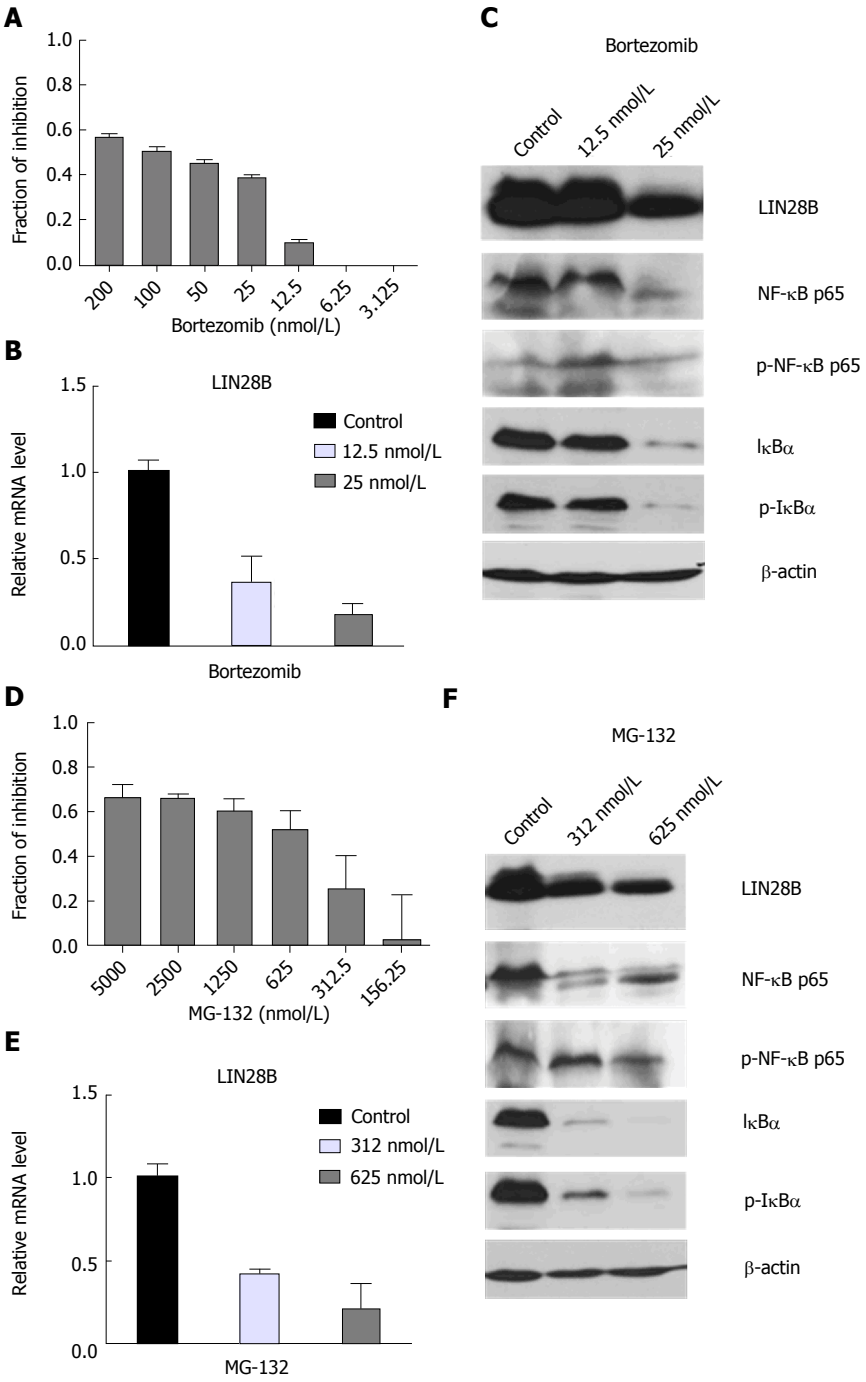

Figure 1 Effects of nuclear factor kappa B (NF-κB) inhibitors on acute myeloid leukemia cell viability and LIN28 mRNA and protein expression. TF-1a cells were treated with DMSO control, different doses of Bortezomib as indicated, followed by CellTiter-Glo® Luminescent Cell Viability Assay (CTG assays) (A) or RNA extraction for qRT-PCR (B) and protein extraction for Western blot analysis (C), TF-1 cells were treated with DMSO control, different doses of MG-132 as indicated, followed by CTG assays (D) or RNA extraction for qRT-PCR (E) and protein extraction for Western blot analysis (F). For CTG assays, luminescence of each drug concentration and their controls were quantified. The relative inhibition induced by drug treatment was calculated relative to DMSO controls. For qRT-PCR analysis, the LIN28B mRNA level in DMSO control samples was set as 1 and the relative fold changes of LIN28B in drug treated samples were normalized to DMSO control samples. The experiments were triplicated (n = 3, mean ± SD). For Western blot analysis, β-actin was used as loading control.

relative to the same cells transfected with *pGL3* empty vector (Figure 3B). Then, we determine whether NF-κB has the ability to activate the *LIN28B* promoter. To this end, we co-transfected an escalating doses of NF-κB p65 expression vector and the *LIN28B* promoter plasmid in 293T cells. We found that NF-κB p65 plasmid could dose-dependently increased activation of *LIN28B* promoter activity (Figure 3B). In order to validate that NF-κB binding motif on the *LIN28B* promoter is critical for the regulation of *LIN28B* by NF-κB, we employed site-directed mutagenesis method to mutate the consensus-binding sequence from GGCGATCCC to GGCGATTTT. These intended site mutations were validated by DNA sequencing method. As assessed by

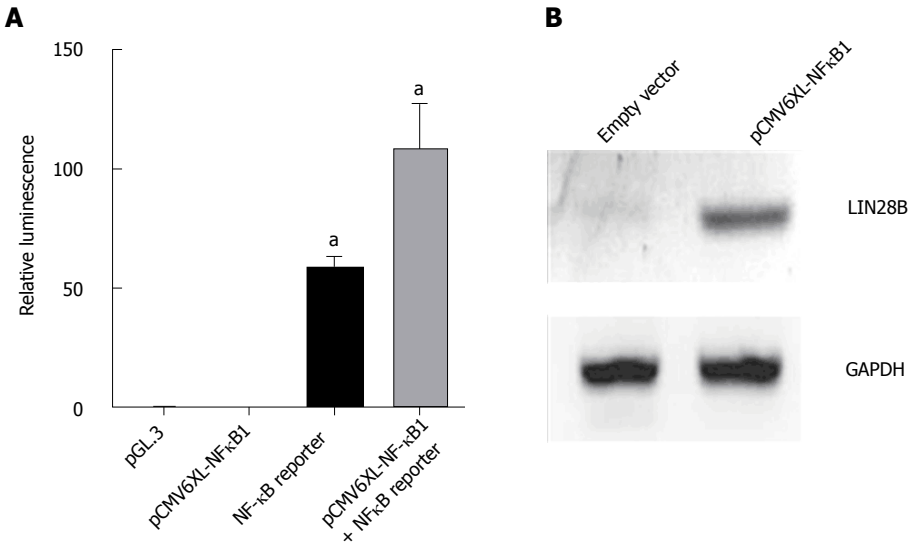

Figure 2 Overexpression of nuclear factor kappa B (NF-κB) in HEK293T cells. A: HEK293T cells were co-transfected with 0.5 μg of pGL3.0, 2 μg of pCMV6-XL4 NF-κB1, 2 μg of NF-κB reporter gene and 10 ng of Renilla vector as indicated on the x-axis in 1mL of DMEM growth medium for 24 h prior to lysis for measuring the luminescence. All samples were normalized with renilla luciferase to ensure equal transfection efficiency. Cells transfected with PGL3 vector was used as negative control. The experiments were repeated 6 times (mean ± SD); B: Western blot analysis of LIN28B protein level in HEK293 cells receiving empty vector and NFκB1 vector as indicated. Proteins were extracted after 48 h of transfection. GAPDH was used as loading control.[a]$P < 0.05$.

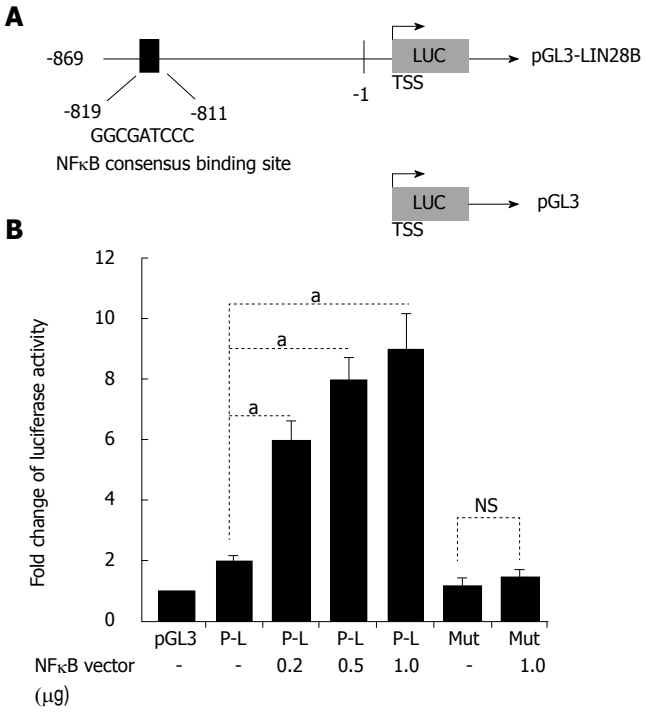

Figure 3 LIN28B is a target gene of nuclear factor kappa B (NF-κB). A: Schematic representation of the human LIN28B promoter region cloned into pGL3 reporter constructs (pGL3-LIN28B). Black blocks show NF-κB-consensus-binding sites. The sequences and positions of the NF-κB-consensus-binding site shown; B: The pGL3-LIN28B reporter constructs (P-L), as well as the mutant construct, were introduced into 293T cells together with or without NF-κB p65 vector. After 24 h, the cells were subjected to dual-luciferase reporter assay, as described. The experiments were repeated 6 times. The baseline luciferase activity of pGL3 was set as 1 and the luciferase readings of all other samples were normalized with the baseline (n = 6, mean ± SD). TSS: Transcription start site. NS: Not significant. [a]$P < 0.05$

dual-luciferase reporter assays, the luciferase activity of the mutated *LIN28B* promoter displayed 3 times less when compared to that of the wild-type *LIN28B* promoter (Figure 3B). Taken together, these data provide strong evidence that *LIN28B* promoter activity is positively regulated by NF-κB and the consensus NF-κB-binding motif is critical for the regulation.

LIN28B is critical for NF-κB-mediated stem-cell like property of AML cells

NF-κB is known to increase cell survival and growth, and promotes cells to less sensitive to drug treatment

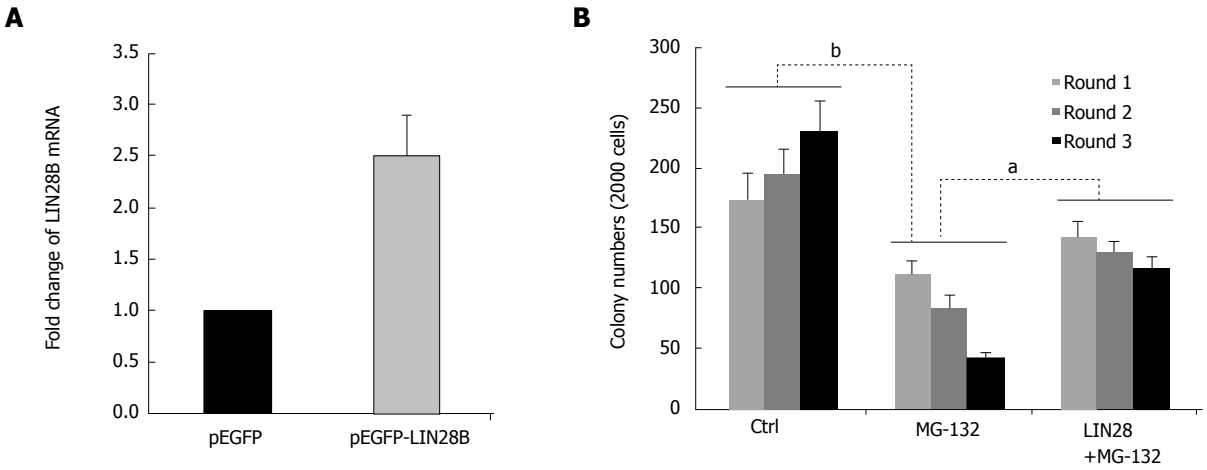

Figure 4 Role of LIN28B in nuclear factor kappa B (NF-B)-mediated leukemic stem cell-like properties. A: RNAs of pEGFP and pEGFP-LIN28B transfected TF-1a cells, followed by qRT-PCR comparing LIN28B transcription level. The expression of LIN28B in each sample was normalized with GAPDH respectively (n = 3, mean ± SD); B: A total of 2000 cells from TF-1a or LIN28B overexpression cells were initially plated in methylcellulose medium containing either DMSO as control or MG-132 at 50 nmol/L, followed by another two rounds of serial replating assays. Quantification of colonies of indicated cells over 3 rounds of replating in methylcellulose medium. Bar figures represented 3 separated experiments (n = 3, mean ± SD). $^{a}P < 0.05$; $^{b}P < 0.01$.

through upregulation of some important antiapoptotic molecules. Many studies have demonstrated that NF-κB is constitutively activated in human AML stem cells, but not in normal human CD34+ progenitor cells[15]. Notably, inhibition of NF-κB activity preferably kill LSC, while spare normal progenitor cells in AML patients[22,23]. To assess the functional involvement of *LIN28B* in NF-κB-mediated LSC property, we conducted serial replating assay to assess the self-renewal capacity, one of the intrinsic characteristic of LSC, in TF-1a cells. The increased of *LIN28B* mRNA by *pEGFP-LIN28B* was examined by qRT-PCR too. As shown in Figure 4A, the LIN28B expression was increased to 2.5-fold in TF-1a cells transfected with *pEGFP-LIN28B* than pEGFP transfected cells (Figure 4A). As expected, TF-1a cells were capable of being serially replated in basic methylcellulose without additional cytokines (Figure 4B), whereas the numbers of colonies were significantly reduced in TF-1a cells treated with NF-κB inhibitor, MG-132. Next, we overexpressed a GFP-tagged *LIN28B* gene in TF-1a cells, then followed by MG-132 treatment and serially replating assays. In fact, ectopic expression of LIN28B could partially rescue the colony formation capacity of TF-1a treated with MG-132 (Figure 4B). Overall, these data support a functional role for LIN28B in NF-κB-mediated LSC property of AML cells.

DISCUSSION

LIN28B has been associated with cancer progression metastasis. For example, overexpression of *LIN28B* was found to be associated with patients with colon and liver cancer who had a shorted survival and higher chance of tumor recurrence[24,25]. Increasing number of studies have shown that *LIN28B* plays an important role in the growth of CSCs[24,26], which could be the reason for recurrence of cancer. We previously reported that inhibition of *LIN28B* impairs the growth of AML cells through disrupting leukemia cell metabolism[19]. In this study, we uncovered that *LIN28B* is essential for NF-κB-mediated leukemic stem-cell like property of AML cells.

Constitutive activation of NFκB activity in TF-1a cells was shown to increase the expression of *LIN28B*, thus promoting cell proliferation. Meanwhile, it is well known that NF-κB activation could make the cancer cells resistant to apoptosis, enhanced proliferation and metastasis[27]. This study demonstrated the relationship between NF-κB signaling and *LIN28B* by treating TF-1a with NF-κB inhibitors, which acted on 26S proteasome to keep NFκB in an inactive state, thus reducing active NFκB *p65* binding onto the promoter for *LIN28B*. In order to further support the result that the decrease in NF-κB level after drug treatments were responsible for LIN28B's down regulation but not due to the effect of extensive apoptosis, an overexpression study of NF-κB1 in HEK-293T cells was carried out and showed positive increase in LIN28B expression. Together, these data further reinforces the finding that NF-κB regulates *LIN28B*.

One of the hallmarks of HSCs and most important characteristic of LSCs is their ability of sustainable self-renewal[28]. LSCs are regarded as the fundamental source of disease relapse and treatment failure[29]. Therefore, it is clinically desirable to targeting LSCs. In this study, we unveiled that *LIN28B* is transcriptionally regulated by NF-κB and *LIN28B* plays a key role in NF-κB-mediated leukemia stem cell-like properties. NF-κB inhibitors strongly decreases *LIN28B* expression and compromises LSC self-renew and abrogates its tumor-initiating capacity. This NF-κB/LIN28B regulatory axis could be the "Achilles' heel" of LSCs in AML, and inhibiting NF-κB activity may provide an opportunity to eradicate LSCs in some subtypes of AML in which NF-κB/LIN28B is essential for the disease progression,

offering a greater strategy in eradicating AML and LSCs to prevent relapse of the disease.

ARTICLE HIGHLIGHTS

Research background

Acute myeloid leukemia (AML) is a common blood cancer adult. The current standard chemotherapy can't cure the disease, as most of the patient relapse and become refractory to treatment. Leukemia stem cells (LSCs) are a small subpopulation that sustain the disease and often resistant to chemotherapy. LSCs are responsible for the disease relapse. So, a better understanding molecular biology of AML and novel therapies are urgently needed for AML patients.

Research motivation

The nuclear factor kappa B (NF-κB) is a pivotal transcription factor, playing different roles in all most all cellular functions. Aberrant activation of NF-κB has been found specifically in LSCs, but not in normal hematopoietic progenitor cells. LIN28 and LIN28B are RNA-binding protein and transcriptional regulators, which are used to create induced pluripotent stem cells (iPS). However, the detailed molecular basis of how NF-κB contributes to the LSC-like properties of AML cells is not well-understood.

Research objectives

In this study, we aim to explore the relationship between NF-κB and LIN28B expression, as well as to assess their roles in LSC-like properties. It will help us to better understand the formation of LSCs, and provide the opportunity to target LSCs.

Research methods

Several NF-κB inhibitors with different mode-of-actions was used to treat leukemia cells, then followed by assessment of cell viability. Western blot and qRT-PCR was employed to examine the correlation between NF-κB and LIN28B protein and mRNA levels. Luciferase reporter was constructed and applied to explore the transcriptional regulation of *LIN28B*. Colony forming and serial replating assays are functional assays for LSC-like properties.

Research results

Treatment of leukemia cells with direct and indirect NF-κB inhibitors significantly decreased LIN28B protein and mRNA levels and reduced cell viability. Mechanistically, the region of -819 to -811 region on the LIN28B promoter contains specific, consensus NF-κB binding motif, and mutations in this region compromised transcription activity and LIN28B expression. On contrast, transfection of NFκB1 increased LIN28B protein. Overexpression of LIN28B partially rescued the self-renewal capacity impaired by pharmacological inhibition of NF-κB activity. The functional role of NF-κB and LIN28B regulatory axis in LSCs was confirmed.

Research conclusions

Our data demonstrated the existing of NF-κB/LIN28B regulatory axis in AML, which plays a pivotal role in the formation of LSCs. This study provides a deep understanding of the previous finding that NF-κB is activated in CD34+CD38- AML cells. LIN28B is a critical downstream target of NF-κB pathway. This study also highlights the targeting NF-κB or LIN28B as an effective approach for eradication LSCs in AML.

Research perspectives

In summary, we characterized the NF-κB/LIN28B regulatory axis and its functional roles in maintenance of LSC-like properties of AML cells. We proposed that targeting either NF-κB or *LIN28B* could be an effective way to eradication of LSCs, which are known to resist to chemotherapy. Although NF-κB inhibitors are available, their side-effects should be carefully examined as NF-κB play important roles in multiple cellular processes, like immune defense. Furthermore, specific *LIN28B* inhibitor is currently not available. The development of novel class of small molecular inhibitors or drug-like compounds to inhibit *LIN28B* should be the focus of the future research.

REFERENCES

1 **Ghosh A**, Saginc G, Leow SC, Khattar E, Shin EM, Yan TD, Wong M, Zhang Z, Li G, Sung WK, Zhou J, Chng WJ, Li S, Liu E, Tergaonkar V. Telomerase directly regulates NF-κB-dependent transcription. *Nat Cell Biol* 2012; **14**: 1270-1281 [PMID: 23159929 DOI: 10.1038/ncb2621]

2 **Hayden MS**, Ghosh S. NF-κB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev* 2012; **26**: 203-234 [PMID: 22302935 DOI: 10.1101/gad.183434.111]

3 **Ryzhakov G**, Teixeira A, Saliba D, Blazek K, Muta T, Ragoussis J, Udalova IA. Cross-species analysis reveals evolving and conserved features of the nuclear factor κB (NF-κB) proteins. *J Biol Chem* 2013; **288**: 11546-11554 [PMID: 23508954 DOI: 10.1074/jbc.M113.451153]

4 **Cildir G**, Low KC, Tergaonkar V. Noncanonical NF-κB Signaling in Health and Disease. *Trends Mol Med* 2016; **22**: 414-429 [PMID: 27068135 DOI: 10.1016/j.molmed.2016.03.002]

5 **Finco TS**, Beg AA, Baldwin AS, Jr. Inducible phosphorylation of i kappa b alpha is not sufficient for its dissociation from nf-kappa b and is inhibited by protease inhibitors. *Proc Natl Acad Sci USA* 1994; **91**: 11884-11888 [DOI: 10.1073/pnas.91.25.11884]

6 **Wertz IE**, Dixit VM. Signaling to NF-kappaB: regulation by ubiquitination. *Cold Spring Harb Perspect Biol* 2010; **2**: a003350 [PMID: 20300215 DOI: 10.1101/cshperspect.a003350]

7 **Inoue J**, Gohda J, Akiyama T, Semba K. NF-kappaB activation in development and progression of cancer. *Cancer Sci* 2007; **98**: 268-274 [PMID: 17270016 DOI: 10.1111/j.1349-7006.2007.00389.x]

8 **Zhou J**, Ching YQ, Chng WJ. Aberrant nuclear factor-kappa b activity in acute myeloid leukemia: From molecular pathogenesis to therapeutic target. *Oncotarget* 2015; **6**: 5490-5500 [DOI: 10.18632/oncotarget.3545]

9 **Xia Y**, Shen S, Verma IM. NF-κB, an active player in human cancers. *Cancer Immunol Res* 2014; **2**: 823-830 [PMID: 25187272 DOI: 10.1158/2326-6066.CIR-14-0112]

10 **Chng WJ**, Goldschmidt H, Dimopoulos MA, Moreau P, Joshua D, Palumbo A, Facon T, Ludwig H, Pour L, Niesvizky R, Oriol A, Rosiñol L, Suvorov A, Gaidano G, Pika T, Weisel K, Goranova-Marinova V, Gillenwater HH, Mohamed N, Feng S, Aggarwal S, Hájek R. Carfilzomib-dexamethasone vs bortezomib-dexamethasone in relapsed or refractory multiple myeloma by cytogenetic risk in the phase 3 study ENDEAVOR. *Leukemia* 2017; **31**: 1368-1374 [PMID: 28025582 DOI: 10.1038/leu.2016.390]

11 **Li F**, Sethi G. Targeting transcription factor nf-kappab to overcome chemoresistance and radioresistance in cancer therapy. *Biochim Biophys Acta* 2010; **1805**: 167-180 [DOI: 10.1016/j.bbcan.2010.01.002]

12 **Valk PJ**, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM, Beverloo HB, Moorhouse MJ, van der Spek PJ, Löwenberg B, Delwel R. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* 2004; **350**: 1617-1628 [PMID: 15084694 DOI: 10.1056/NEJMoa040465]

13 **Zhou J**, Lu X, Tan TZ, Chng WJ. X-linked inhibitor of apoptosis inhibition sensitizes acute myeloid leukemia cell response to TRAIL and chemotherapy through potentiated induction of proapoptotic machinery. *Mol Oncol* 2018; **12**: 33-47 [PMID: 29063676 DOI: 10.1002/1878-0261.12146]

14 **Plesa A**, Dumontet C, Mattei E, Tagoug I, Hayette S, Sujobert P, Tigaud I, Pages MP, Chelghoum Y, Baracco F, Labussierre H, Ducastelle S, Paubelle E, Nicolini FE, Elhamri M, Campos L, Plesa C, Morisset S, Salles G, Bertrand Y, Michallet M, Thomas X. High frequency of CD34+CD38-/low immature leukemia cells is correlated with unfavorable prognosis in acute myeloid leukemia. *World J Stem Cells* 2017; **9**: 227-234 [PMID: 29321824 DOI: 10.4252/wjsc.v9.i12.227]

15 **Guzman ML**, Neering SJ, Upchurch D, Grimes B, Howard DS, Rizzieri DA, Luger SM, Jordan CT. Nuclear factor-kappab is constitutively activated in primitive human acute myelogenous

leukemia cells. *Blood* 2001; **98**: 2301-2307 [DOI: 10.1182/blood.V98.8.2301]
16 **Birkenkamp KU**, Geugien M, Schepers H, Westra J, Lemmink HH, Vellenga E. Constitutive NF-kappaB DNA-binding activity in AML is frequently mediated by a Ras/PI3-K/PKB-dependent pathway. *Leukemia* 2004; **18**: 103-112 [PMID: 14574326 DOI: 10.1038/sj.leu.2403145]
17 **Barreyro L**, Will B, Bartholdy B, Zhou L, Todorova TI, Stanley RF, Ben-Neriah S, Montagna C, Parekh S, Pellagatti A, Boultwood J, Paietta E, Ketterling RP, Cripe L, Fernandez HF, Greenberg PL, Tallman MS, Steidl C, Mitsiades CS, Verma A, Steidl U. Overexpression of IL-1 receptor accessory protein in stem and progenitor cells and outcome correlation in AML and MDS. *Blood* 2012; **120**: 1290-1298 [PMID: 22723552 DOI: 10.1182/blood-2012-01-404699]
18 **Zhou J**, Bi C, Cheong LL, Mahara S, Liu SC, Tay KG, Koh TL, Yu Q, Chng WJ. The histone methyltransferase inhibitor, DZNep, up-regulates TXNIP, increases ROS production, and targets leukemia cells in AML. *Blood* 2011; **118**: 2830-2839 [PMID: 21734239 DOI: 10.1182/blood-2010-07-294827]
19 **Zhou J**, Bi C, Ching YQ, Chooi JY, Lu X, Quah JY, Toh SH, Chan ZL, Tan TZ, Chong PS, Chng WJ. Inhibition of LIN28B impairs leukemia cell growth and metabolism in acute myeloid leukemia. *J Hematol Oncol* 2017; **10**: 138 [PMID: 28693523 DOI: 10.1186/s13045-017-0507-y]
20 **Gupta SC**, Sundaram C, Reuter S, Aggarwal BB. Inhibiting NF-κB activation by small molecules as a therapeutic strategy. *Biochim Biophys Acta* 2010; **1799**: 775-787 [PMID: 20493977 DOI: 10.1016/j.bbagrm.2010.05.004]
21 **Wan F**, Lenardo MJ. Specification of DNA binding activity of NF-kappaB proteins. *Cold Spring Harb Perspect Biol* 2009; **1**: a000067 [PMID: 20066093 DOI: 10.1101/cshperspect.a000067]
22 **Guzman ML**, Rossi RM, Karnischky L, Li X, Peterson DR, Howard DS, Jordan CT. The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. *Blood* 2005; **105**: 4163-4169 [PMID: 15687234 DOI: 10.1182/blood-2004-10-4135]
23 **Siveen KS**, Uddin S, Mohammad RM. Targeting acute myeloid leukemia stem cell signaling by natural products. *Mol Cancer* 2017; **16**: 13 [PMID: 28137265 DOI: 10.1186/s12943-016-0571-x]
24 **King CE**, Cuatrecasas M, Castells A, Sepulveda AR, Lee JS, Rustgi AK. LIN28B promotes colon cancer progression and metastasis. *Cancer Res* 2011; **71**: 4260-4268 [PMID: 21512136 DOI: 10.1158/0008-5472.CAN-10-4637]
25 **Cheng SW**, Tsai HW, Lin YJ, Cheng PN, Chang YC, Yen CJ, Huang HP, Chuang YP, Chang TT, Lee CT, Chao A, Chou CY, Chan SH, Chow NH, Ho CL. Lin28B is an oncofetal circulating cancer stem cell-like marker associated with recurrence of hepatocellular carcinoma. *PLoS One* 2013; **8**: e80053 [PMID: 24244607 DOI: 10.1371/journal.pone.0080053]
26 **Zhang WC**, Shyh-Chang N, Yang H, Rai A, Umashankar S, Ma S, Soh BS, Sun LL, Tai BC, Nga ME, Bhakoo KK, Jayapal SR, Nichane M, Yu Q, Ahmed DA, Tan C, Sing WP, Tam J, Thirugananam A, Noghabi MS, Pang YH, Ang HS, Mitchell W, Robson P, Kaldis P, Soo RA, Swarup S, Lim EH, Lim B. Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell* 2012; **148**: 259-272 [PMID: 22225612 DOI: 10.1016/j.cell.2011.11.050]
27 **Dolcet X**, Llobet D, Pallares J, Matias-Guiu X. NF-κB in development and progression of human cancer. *Virchows Arch* 2005; **446**: 475-482 [PMID: 15856292 DOI: 10.1007/s00428-005-1264-9]
28 **Wang X**, Huang S, Chen JL. Understanding of leukemic stem cells and their clinical implications. *Mol Cancer* 2017; **16**: 2 [PMID: 28137304 DOI: 10.1186/s12943-016-0574-7]
29 **Horton SJ**, Huntly BJ. Recent advances in acute myeloid leukemia stem cell biology. *Haematologica* 2012; **97**: 966-974 [PMID: 22511496 DOI: 10.3324/haematol.2011.054734]

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MINIREVIEWS

Stem cells as delivery vehicles for regenerative medicine-challenges and perspectives

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Abstract

The use of stem cells as carriers for therapeutic agents is an appealing modality for targeting tissues or organs of interest. Combined delivery of cells together with various information molecules as therapeutic agents has the potential to enhance, modulate or even initiate local or systemic repair processes, increasing stem cell efficiency for regenerative medicine applications. Stem-cell-mediated delivery of genes, proteins or small molecules takes advantage of the innate capability of stem cells to migrate and home to injury sites. As the native migratory properties are affected by *in vitro* expansion, the existent methods for enhancing stem cell targeting capabilities (modified culture methods, genetic modification, cell surface engineering) are described. The role of various nanoparticles in equipping stem cells with therapeutic small molecules is revised together with their class-specific advantages and shortcomings. Modalities to circumvent common challenges when designing a stem-cell-mediated targeted delivery system are described as well as future prospects in using this approach for regenerative medicine applications.

Key words: Stem cells; Delivery agents; Regenerative medicine; Nanoparticles; Targeted delivery

Core tip: The capability of stem cells to mobilize, home and target to inflammatory sites justifies their use as delivery agents for regenerative medicine purposes. Cell

and membrane engineering techniques can be used to increase the selective targeting potential of stem cells. Gene therapy and nanoparticle-mediated small-molecule delivery of informational cues have the potential to increase the efficiency of clinically relevant stem-cell-based regenerative therapies.

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INTRODUCTION

Stem cells, the natural reservoir for growth, development and repair in all multicellular organisms, have been the object of intense scrutiny in the last several decades for scientific reasons, but most of all, for their potential applications in biology and medicine. Stem cells are defined by their capability to self-renew and to differentiate into more specialized progeny according to their degree of potency[1]. The emerging domain of regenerative medicine (RM) aims to make possible the complete functional and structural restoration of tissues, organs or even bodily systems. Due to their unique properties in modulating local and systemic reparatory processes, stem cells are employed by RM as main the therapeutic tool[2]. Basic and preclinical research involving (stem) cell therapy to fight acute or chronic degenerative disease in various medical fields is currently underway. Stem-cell-based tissue engineering applications aim to produce implantable bio-substitutes that could waive the need for costly tissue and organ transplantation[3,4]. Some of the products of this new, research-intensive medical field are already in the stage of clinical trials, which promises to deliver revolutionary therapies in the near future.

Different types of stem cells are currently tested for RM applications: Embryonic stem cells (ESCs)[5] adult stem cells (ASCs)[6] or induced pluripotent stem cells (iPSCs)[7], each of them with specific advantages and risks. An outline of stem cell type-dependent advantages and shortcomings relative to their use as therapeutic agents is beyond the scope of this review; the reader is referred to excellent works in this respect[8,9].

Dependent on their type and therapeutic application, stem cells are expected to exert a certain effect, be it in recomposing the anatomy and/or function of a given structure, in restoring dysfunctional biological pathways or in modulating the local or systemic immune response. Stem-cell-based TE of various tissues (such as bone, cartilage, neural, cardiac or skeletal muscle and so on) is based on the cell capability to generate tissue specific progenies that directly recompose the given structure. TE strategies are largely based on directed differentiation and structural participation of ESCs, iPSCs or mesenchymal stem cells (MSCs). Due to their large availability, MSCs are the most commonly used ASCs for RM applications. MSCs were shown to release cytokines and growth factors (GFs) that exert paracrine effects upon transplantation within an injured structure, contributing to tissue repair by recruiting local cells to induce repair[10]. The expression of bioactive molecules with multiple trophic (regenerative) and immunomodulatory effects has prompted a call for changing the MSC denomination in medicinal signalling cells to more accurately reflect their potential for acting as therapeutic drugs[11]. Stem-cell-derived microvesicles containing mRNA, microRNA and proteins and released as exosomes into the intercellular milieu as a modality of cell-cell communication are increasingly recognized as potent therapeutic agents. ESC- and MSC-derived exosomes that contribute to restraining tissue injury and induce cell cycle re-entry of resident cells, leading to tissue self-repair, are being tested for different applications in cardiac, neural or musculoskeletal repair[12,13].

ESCs and MSCs were shown to home and engraft to the site of injury, facilitating tissue and organ repair following traumatic, degenerative, ischaemic or inflammatory processes[14,15]. The stem cell migratory potential enables the choice for systemic delivery whenever the direct, intra-lesion administration is invasive, implies associated morbidity or involves the situation of multiple and/or remote lesions[16]. In particular, MSCs are in focus as delivery vehicles due to their homing capabilities, low immunogenicity (not expressing class Ⅱ histocompatibility molecules) and immune-modulatory capabilities. Recently, the use of stem cells as delivery agents for artificially produced molecules that exert a local or systemic therapeutic effect has emerged as another field of cell therapy application. In the following, a brief outline of MSC native and enhanced homing mechanisms, methods of loading cells with therapeutic agents and several current applications of stem-cell-based delivery will be presented.

HOMING OF STEM CELLS

Tissues and organs possess, at least to a certain extent, the capability to repair or regenerate. The natural repair mechanisms are based on activation of the intrinsic progenitor population, trans-differentiation of local adult elements and the capability to recruit and home circulating stem cells[17]. Homing has been defined as the cell capability to arrest in the blood stream and localize and migrate through the endothelial walls of blood vessels within the tissue. This spontaneously occurring process enhances reparatory mechanisms, adding both cellular stock as well as signalling molecules, and normally leads to restoration of local structural and physiological parameters. The capability of self-repair can be overwhelmed by the severity of the injury combined with local or systemic particularities such as

inflammation, disease or ageing.

The use of stem cells as therapeutic agents is expected to increase or substitute regenerative processes. The use of stem cells as "biological regenerative supplementation" is largely based on their natural capability to mobilize, migrate and home. The mechanisms for cell migration and "nesting" to the sites of injuries have been described mostly in relation to MSCs, particularly regarding their migration to the bone marrow and other tissues. When mobilized from the host or therapeutically administered, similarly to leucocytes, MSCs first reach the blood stream, entering the systemic venous or arterial circulation. Reaching the target, the cells decelerate and come in contact with the endothelial walls by rolling and tethering. In this second step, MSC G-coupled protein receptors are activated followed by their arrest on the endothelial membrane within the target tissue by means of integrin receptor activation (third step)[18]. Transmigration through both the endothelium and the underlying basal extracellular matrix (ECM) membrane represent the fourth and final step of homing. The process normally takes a few hours and results in transient retention of stem cells within the tissue. The navigation process of homing only implies cell anchorage and transmigration from the blood stream to the tissue. Depending on the stem cell type and tissue characteristics, this process can be followed by division of the migrated cells, having as a consequence their engraftment and, eventually, tissue repopulation[19]. Homing is based on several constitutive abilities of stem cells to respond to external cues that enable their trafficking, guidance and migration. For therapeutic purposes, both stem cells as well as the external cues that govern homing can be modulated to enhance the targeting process and optimize the expected results.

MECHANISMS INVOLVED IN STEM CELL MIGRATION AND HOMING

Stem cells sense and are able to move according to a chemoattractant gradient by means of amoeboid cell migration. This highly conserved process, termed "chemotaxis" or "interstitial migration", can take place independently of blood flow. Chemoattractant gradients within the extracellular space or mechanical signals guide cell movement by directing cellular actin polymerization. Different cell types can migrate (such as leucocytes, progenitor and stem cells or metastatic cancer cells) displaying specific changes in their focal adhesion, myosin-based contractility and actin polymerization[20]. In naturally occurring circumstances, the first steps of the migration of progenitor cells require their mobilization by disruption of the ECM as well as cell adhesion to matrix proteins by means of proteolytic enzymes released by the injury and/or local inflammatory processes[21]. In turn, stem cells release proteases that contribute to ECM remodelling, also facilitating the migratory process. MSCs were shown to express several key components of the fibrinolytic cascade (including urokinase plasminogen activator receptor (uPAR) and plasminogen activator inhibitor (PAI-10) of the fibrinolytic system) known to exert crucial roles in cell migration, growth factor bioavailability during inflammation, tissue regeneration and cancer[22]. The involvement of an autocrine mechanism based on heat shock protein 70 (HSP70) released by murine mesangioblast interaction with Toll-like receptor 4 (TLR4) and CD91 has been proposed as another mechanism responsible for the stem cell ability to transverse the ECM and stimulate migration[23]. Adhesion molecules (such as E and P selectins) play an important role in the homing of haematopoietic stem cells (HSCs), being involved in the first step of tethering and rolling of those cells along the endothelial wall. Integrins such as CD49d/CD29 ($\alpha4\beta1$ or VLA-4) and CD11a/CD18 ($\alpha L\beta2$ or LFA-1) are involved in adhesion of HSCs to the endothelium and trans-endothelial migration[24]. VLA-4 binds to vascular cell adhesion molecule 1 (VCAM-1) within the endothelium and is functionally involved in MSC homing. MSCs express a large variety of chemokine membrane receptors that enable their migration towards wounds, inflammation and malignant-site-released chemokine gradients. It should be noted that chemokine receptors were shown to be species dependent, which is of importance when translating results from animal studies to clinical applications. For cultured MSCs, isolation and expansion methods are known to strongly influence the chemokine receptor repertoire inviting a thorough assessment when such cells are sought for targeting therapies[25]. Several chemokine axes are known to regulate the homing of a large variety of stem cells. The CXCL12 (also known as stromal derived factor 1α, SDF-1α)-CXCR4 axis may represent a general mechanism for the chemoattraction of MSCs, haematopoietic stem cells, and neural and endothelial progenitors to injury sites. CXCL12-CXCR4 is constitutively expressed in several cell populations such as dermal fibroblasts, endothelial cells or pericytes, increasing consistently during tissue injury. CXCR4 and CXCR7 receptors that bind SDF-1α have been detected on the surface of bone-marrow-derived MSCs and adipose-derived stem cells (ASDCs) that use an SDF1-α gradient to migrate to the site of acute and chronic injuries[26]. Constitutively expressed on ESCs, CXCR4 is a major contributor to organogenesis during development. SDF-1α/CXCR4 is also involved in pathological processes such as cancer stem cell migration and cancer metastasis[27]. Other chemokine axes such as CCL27-CCR10, CCL5-CCR5 and the more skin-specific CCL21-CCR7 are involved in natural processes of stem cell migration and homing. Different methods of enhancing chemokine-based gradient targeting are currently under scrutiny for regenerative purposes (see below)[28].

METHODS TO ENHANCE STEM CELL HOMING

Targeting injured or diseased tissues by stem cells largely depends on cell characteristics as well as on the existence of a chemoattractant gradient to guide their trafficking and homing. Stem cells and particularly MSCs are shown to possess migratory and targeting capabilities; however, only a small portion of therapeutically administered cells can home and engraft within the injured tissue[29]. Several strategies addressing cells such as modified culture conditions, specific preconditioning or manipulation before transplantation are currently being tested with the purpose of increasing their migratory capabilities. Methods that modify external conditions such as improved delivery methods or modified route of administration contribute to increasing the number of cells that reach their target.

Improving homing by manipulating stem cell characteristics

Depending on their tissue of origin and donor age and in the absence of natural stimuli, MSCs express low amounts of chemokine receptors[30]. *In vitro* expansion, which is required for obtaining a clinically relevant number of cells, further decreases the number of these receptors. Culturing stem cells under hypoxic conditions was shown to increase CXCR4, CXCR7 and SDF-1 expression by means of a hypoxia inducible factor-1α (HIF-1α) mechanism[31]. Hypoxia can increase chemokine receptor expression, induce the differential expression of MMPs, reduce reactive oxygen species by reduced mitochondrial respiration, and induce Notch signalling pathway-all factors known to be involved in sustaining proliferative and migratory capabilities in stem cells[32]. Some studies have raised concerns about the safety of MSCs cultured under hypoxic conditions[33], although other reports show that the method results in culture-expanded cells that grow faster and display an enhanced migratory capability while being non-oncogenic and retaining multipotency[34]. MSCs cultured at lower confluence were shown to possess superior migratory capabilities. It has been reported that highly confluent MSCs adapt to this condition by expressing higher amounts of tissue inhibitor of metalloproteinase-3 (TIMP-3), resulting in a decreased migratory potential[35].

Three-dimensional (3D) culture conditions were shown to increase differentiation and surface antigen expression, increasing their therapeutic potential in terms of cell viability and targeting capabilities[36]. Increased cell survival after transplantation and reduced replicative senescence was observed in spheroid cultured MSCs; however, a direct impact on the cell migratory potential and how this correlates with the drastic modification in the cell cytoskeleton under such conditions, needs to be further analysed[37].

An interesting approach exists for increasing CXCR4 expression in mouse bone marrow MSCs that have internalized magnetic nanoparticles (MNPs). MNP-loaded cells were shown to possess improved cell homing efficiency. MNP payload enabled cell tracking *in vivo* using magnetic resonance imaging (MRI), demonstrating this could be an efficient strategy for enhancing cell targeting and tracking capability[38].

Cell preconditioning

performed by exposing cultured cells to various soluble molecules is used to improve stem cell homing. Concern exists about the gradual decrease of chemokine receptors in cultivated MSCs; therefore, preconditioning could not only counteract this phenomenon but also enhance their innate targeting capability. Increased expression of cytokine membrane receptors (CXCR4) could be obtained using a cocktail of factors added to the culture media. Fms-like tyrosine kinase (Flt-3) ligand, stem cell factor (SCF), interleukin (IL) and hepatocyte growth factor (HGF) were shown to rapidly increase CXCR4 expression in human foetal-derived bone marrow stem cells and to increase their homing potential within the bone marrow of sub-lethally irradiated NOD/SCID mice[39]. Conditioned medium from tumour necrosis factor alpha (TNFα) pre-stimulated cord-blood-derived stem cells were shown to enhance intravenously and intramuscularly administered epithelial progenitor cells (EPCs) in a model of hind limb ischaemia by a mechanism involving interleukin-6 (IL-6) and interleukin 8 (IL-8)[40].

Preconditioning stem cells with insulin growth factor-1 (IGF-1) or with SDF-1 were shown to improve their migratory and homing capability *in vitro* and *in vivo*[41]. Other small molecules such as inhibitors of glycogen synthase kinase-3β (GSK-3β) or erythropoietin combined with granulocyte–colony stimulating factor (G-CSF) were shown to enhance MSC migratory capability by enhancing CXCR4 and MMP expression or by stimulating the extracellular signal related kinase (ERK)1/2 pathway, respectively[42,43]. MSC preconditioning with oxytocin was shown to increase the expression of protein kinase B (PKB or Akt) and phospho-ERK1/2 together with other proteins and genes such as vascular endothelial growth factor, thrombospondin, tissue inhibitor of metalloproteinase-(TIMP-) 1, TIMP-2, TIMP-3, and MMP-2, increasing their therapeutic potential[44,45]. Histone deacetylase (HDAC) inhibitor valproic acid combined with lithium was shown to be effective in enhancing the MSC migratory ability *in vitro* by means of a HADC-CXCR4, GSK-3β-MMP9 stimulatory mechanism[46].

Another strategy to improve stem cell homing is to increase the expression of targeting molecules in therapeutic cells by gene manipulation. CXCR4 overexpression has been reported by several groups to show a variable efficiency in increasing the targeting potential of MSCs. Non-viral methods are preferred, especially considering potential clinical applications, but are notorious for having a low transfection efficiency.

The use of several cationic liposomal agents (such as IBAfect, a polycationic liposomal transfection reagent) was shown to yield an improved transfection efficiency compared to that of adenoviral methods, resulting in a superior chemotactic index in transfected cord-blood-derived MSCs[47]. Overexpression of other chemokine receptors, such as CXCR7 and CXCR1, was shown to enhance the migratory and targeting properties in various stem cell populations[48].

Stem cell surface modification with rapid incorporation of recombinant CXCR4 protein on the membrane was shown to enhance stem cell migration towards an SDF-1 gradient[49]. Surface engineering aims to transiently modify cell membrane in order to improve their adhesion or endothelial transmigration. Several ingenious methods such as CD44 fucosylation to obtain P-selectin glycoprotein ligand-1 (PSGL-1) or HCELL on the surface of MSCs, biotinylation of MSC membranes or conjugating various antibodies against adhesion molecules [intercellular adhesion molecule (ICAM) or vascular adhesion molecule (VCAM-1)] were shown to increase the homing in surface-engineered cells[50].

Mode and route of administration is an important factor that influences therapeutic cell survival, migration and homing potential. Intravenous administration, the most commonly used modality of systemic cell delivery, poses the inconvenience of cell trapping within organs such as the lung, spleen or liver. This results in an important quantity of capillary-arrested cells and a decreased therapeutic effect if the target is located elsewhere. Pre-treatment of the host with vasodilatory substances or heparin administration pre-procedure was shown to diminish ADSC lung trapping and to increase hepatic targeting in a rat model of liver failure[51].

Genetically modified stem cells as therapeutic agents

Stem cells, and particularly MSCs, can be genetically engineered to release therapeutic proteins for regenerative purposes, with various applications in treating monogenic diseases (such as muscular dystrophy or haemophilia) or even degenerative diseases (such as inflammatory arthritis). Even if viral transduction methods were preferred in the beginning for their superior transfection rate, the improved efficiency of newer non-viral methods support their use for therapeutic purposes. Lower immunogenicity, increased scalability, decreased toxicity and considerable versatility of non-viral methods compared to viral methods recommend the use of the former when considering clinical applications[52]. Physical methods such as electroporation and nucleofection, which use an electric pulse to ferry nucleic acids through the cell membrane or nuclear membrane, respectively, as well as ultrasound-based gene delivery are methods that require intensive protocol optimization due to decreased cell viability[53]. Chemical non-viral methods employ liposomes, dendrimers, inorganic nanoparticles, magnetic nanoparticles (MNPs) or polymers as transfection agents. High transfection efficiency was reported using poly (ethylenimine) (PEI) in combination with gold or silica nanoparticles[54,55].

Genetically engineered stem cells are under scrutiny for the treatment of several monogenic diseases. Using systemically delivered MSCs, a high level of therapeutic protein production can be obtained using stem-cell-mediated gene delivery, which can substitute for the abnormal gene function at the tissue and organ level. Hydroxyapatite- polylactic-polyglycolic acid (PLGA) composites can be coated with biomineralized collagen 1 in combination with autologous gene-engineered factor IX (hFIX). MSCs were shown to deliver a consistent amount of hFIX in a mouse model of haemophilia B[56]. Autologous HSCs transduced with a viral vector containing a healthy copy of the mutated gene were shown to improve lysosomal storage diseases such as metachromatic leukodystrophy (MLD) or mucopolysaccharidosis type Ⅰ (MPS-I). The method allows production of the defective enzyme and cross correction of target cells in multiple target tissues[57].

Osteogenesis imperfecta (OI) represents a group of genetic disorders characterized by bone disease produced by missing or abnormal synthesis of type Ⅰ collagen, with different levels of severity[58]. Adeno-associated virus vector MSCs from OI patients were shown to disrupt dominant-negative mutant COL1A1 collagen genes and to produce normal collagen similar to that of wild-type cells-a fact that could be used to develop a therapeutic platform for addressing this incurable and disabling disease[59].

A particular field of interest is to use genetically modified stem cells for inducing immune tolerance against antigens of interest in several autoimmune diseases (such as diabetes type Ⅰ) or after organ or cell transplantation. Several strategies include transfection of a particular gene into HSCs or immunological precursor cells by educating the host immune system to recognize the therapeutic protein as "self". Another possibility is to induce the expression of a therapeutic protein in antigen-presenting cells such as immature dendritic cells or B cells that will induce immune tolerance for the respective antigen[60].

A large portion of genetic engineering strategies aim to improve stem cell survival and homing potential during cell transplantation for various cell therapy applications. A particular field of therapeutic protein delivery intends, however, to use genetic manipulation for providing repair and regeneration enhancers or to substitute for congenitally absent or abnormal protein production. Stem-cell-mediated gene delivery for RM is sought as a method for enhancing stem cell survival and/or targeting capabilities (see above), to increase local or systemic reparative processes or for steering immunomodulatory processes. Musculoskeletal tissue regeneration, cardiac diseases and brain traumatic injury are among the several domains in which this method is currently being tested.

Autologous or allogeneic MSCs engineered to overexpress bone morphogenetic protein (BMP) was

found to enable the repair of large bone defects in several animal models[61]. Systemically administered, viral transduced MSCs encoding BMP and vascular endothelial growth factor (VEGF) were able to promote the repair of large segmental bone defects in mice[62]. Scaffold-mediated locally delivered ADSCs overexpressing runx-2 were able to induce healing in large rat calvaria defects[63]. Bone marrow derived MSCS (BMSCs) cell sheets transfected with Lipofectamine 200 to overexpress antimiR-138 (a miRNA precursor) were shown to significantly increase osteogenesis *in vitro*. Forced expression of the transcription factor early growth response protein (Egr1)-programmed MSCs towards the tendon lineage promoted the formation of *in vitro*-engineered tendons, while Smad 8/BMP overexpressing MSCs locally delivered to injured Achilles tendons were shown to improve the histology and biomechanical properties[64,65]. However, caution needs to be exerted when assessing the therapeutic efficiency, as long-term results might differ from those of the immediate evaluation. MSCs engineered to overexpress basic fibroblast growth factor (bFGF) did not cause improvement in the histological appearance and biomechanical properties of the Achilles tendon 12 mo after transplantation in a rat model of tendon defect, questioning the overall impact of such therapies[66].

Cell sheets are versatile structures that can be easy manoeuvrable and accommodate a large variety of clinical defects[67]. MSCs overexpressing IL-8 binding protein (IL-BP8) were shown to decrease inflammation and enhance repair by means of enhanced vascularization in a rat model of global cardiac ischaemia[68]. ADSC sheets overexpressing VEGF were shown to reduce the infarct size and to improve cardiac functions to non-diseased levels in a rabbit model of cardiac infraction[69].

Adeno-associated virus (AAV)-transfected MSCs overexpressing IL-10 were proved to provide neuroprotection and enhance intravenously administered stem cell engraftment, improving brain recovery in a rat model of acute ischaemic stroke[70]. Human umbilical cord blood-derived mesenchymal stem cells (HUMBSCs) overexpressing VEGF were shown to reduce the clinical manifestation and the loss of dopaminergic neurons in the lesioned substantia nigra in hemi-parkinsonian rats. Enhanced HUMBSC differentiation to dopaminergic neurons was observed during this experiment, offering an improved modality for cell transplantation in Parkinson's disease[71].

Equipping stem cells with therapeutic agents: The role of nanoparticles

Genetic engineering of therapeutic cell populations can result in the release of only proteins that are intended to contribute to cell viability, proliferation, homing potential or to influence the regenerative process. Stem cells can also be engineered to deliver a larger variety of therapeutic molecules, making them veritable drug-delivery tools. Delivery of bioactive molecules is an important chapter for RM purposes. A group of information mediators and signalling molecules are grouped in three overlapping categories: (1) Mitogens (stimulate cell division); (2) growth factors (GFs) with multiple biological actions; and (3) morphogens (control the generation of tissue form) are crucial factors for initializing and sustaining regeneration and repair; therefore, the control of signalling molecules may potentially allow the control and modulation of regenerative processes. GFs are proteins or steroid hormones that stimulate cell growth, differentiation and healing[72]. Functional particularities of GF consist in their short-range diffusion through the extracellular matrix. To orchestrate regeneration, controlled local delivery coupled with precise timing is needed in order to obtain the desired action on target cells. Nanoparticles (NP) of different composition, shapes and physical properties are currently used to load native, primed or modified stem cells with information molecules intended to modulate regenerative processes. NP-drug complexes can be internalized by the cells or attached to their surface to be released at the target tissue following cell migration and targeted homing[73]. Such a procedure could be extended for enabling information-molecule-equipped stem cells to target regenerative sites (Figure 1).

One of the most straightforward procedures is to load stem cells with NPs by simply adding them within the cell culture media, taking advantage of cell membrane mechanisms responsible for foreign body internalization (Figure 2). NP complexes are internalized by cells *via* endocytosis, a largely polymorphic process that depends on the cell type, particle size, composition, culture media formulation, electrical charge, and surface modifications-to name only some of the factors. NPs intended to be used for regenerative purposes must possess several obligatory characteristics such as (1) Biocompatibility, *i.e.*, not being toxic to cells, tissues and organs and not eliciting an immune response at the local or systemic level; (2) biodegradability-capable of being decomposed by means of normal cellular metabolic pathways; and (3) stability of physical properties after surface modification; efficacy at therapeutic doses; chemical stability in physiological conditions[74]. Moreover, NP-loaded stem cells need to preserve critical stem cell characteristics such as proliferative, differentiation, and immune-modulatory as well as migratory and homing capabilities. Each type of NP-drug complex must be thoroughly characterized with regard to chemical and physical properties, effect on cell viability, phenotypic features, and migratory and homing potential after particle internalization.

Several classes of NPs, of which the most commonly used are briefly outlined below, can be employed for loading stem cells with small molecules, each of them with particular advantages and shortcomings (Table 1).

Polymeric NPs

Poly (D, L-lactide) (PLA) and poly (D, L-lactic acid-

Table 1 Advantages and disadvantages of the different types of nanoparticles used for stem cell loading

Composition	Type	Advantages	Disadvantages	Payload	Homing to	Ref.
Polymeric NP	Poly(D,L-lactide), poly(D,L-lactic acid-co-α,β-malic acid),Poly-L-lactic acid	Biocompatible, FDA approved, versatility, efficient upload by stem cells, human HSCs, MSCs retain differentiation potential	Biphasic and uncontrolled payload release	Antitumour drugs,	Glioma tumours	[77-79]
Silica NP	Mesoporous silica, amorphous silica	Fast uptake, negligible toxicity, long retention inside cells, lysosomal activation not associated with oxidative stress	Long term remanence within cells/tissues	Antitumour drug doxorubicin, fluorescent dye, paclitaxel	Mammary tumours, infarcted heart	[80-82,92]
Liposomal NP	Liposomes	Relatively facile manufacturing, versatility for drug delivery	Less-efficient uptake process, higher concentrations needed, which can be toxic to cells	6-coumarin	Glioma tumours	[83]
Magnetic nanoparticles	Iron oxide NPs, magnetite, maghemite	Cellular tracking potential, reduced cell toxicity, high loading efficiency	Can induce oxidative stress in carrier cells	NGF, FGF	Dorsal root ganglia, HUVECs	[84-86]

NP: Nanoparticle; NGF: Nerve growth factor; FGF: Fibroblast growth factor; HUVECs: Human umbilical endothelial vein cells.

co-α, β-malic acid) (PLMA) have the advantage of being already in clinical use and FDA approved, can be tuned in variable sizes (ranging from micrometres to several nanometres. Polymeric NPs are uploaded by cells by means of pinocytosis or clathrin-mediated endocytosis[75]. Particle upload is time and concentration dependent, with good loading efficiency, viability and phenotype preservation demonstrated in various cell types[76]. Polymeric NPs have a hydrophobic core with an important loading capability and a hydrophilic shell that provides stability and can be designed to encapsulate hydrophobic or hydrophilic molecules, as well as proteins and nucleic acid. After internalization by the cell, polymeric NPs such as PLA and polyglycolide (PLGA) are able to disrupt the lysosomal membrane, where they initially accumulate due to modifications in the surface charge, and escape into the cytoplasm[77]. While the majority of studies involving stem cell polymeric nanoparticle loaded drug delivery have aimed to develop anticancer therapies, the potential use for regenerative medicine purposes is promising[78]. Human HSCs and MSCs loaded with Poly-L-lactic acid (PLLA) and PLLA-Fe complexes were shown to retain viability and main phenotypic characteristics as well as differentiation potential, qualifying as a method for drug-enhanced cell therapy[79].

Negatively charged silica NPs are shown to be uptaken by clathrin-mediated endocytosis and to co-localize with the cell lysosomal system. Drug-loaded silica NPs were shown to be uploaded by decidual stem cells without affecting their viability and differentiation potential[80]. Human bone marrow MSCs loaded with amorphous silica NPs were shown to retain viability and differentiation and to engraft within the beating heart in a mouse model of cardiac ischaemia, offering a promising tool for cell tracking and potential drug delivery[81]. Mesoporous silica NPs conjugated with a fluorescent dye (cyanine) could be internalized by human MSCs, making possible the discrimination between viable and early apoptotic cells and pointing towards their potential role as cell-tracking agents in preclinical studies[82].

Liposomal NPs are uptaken by stem cells *via* endocytosis and release their cargo into the intracellular space after fusing with the endosomal membrane. Human MSCs loaded with lipid nano-capsules retained their viability and differentiation as well as migratory and targeting potential. Liposomal NPs can be manufactured to carry a large variety of small molecules. The loading process is, however, less efficient than in the case of polymeric nanoparticles. The higher concentration of liposomes required for efficient loading may interfere with cell viability, a process that is cell dependent[83].

Magnetic NPs (MNPs) have been used as such or in combination with other biomaterials for targeted delivery as well as controlled release of information molecules. MNPs incorporated into poly-l-lactic acid (PLLA) containing nerve growth factor (NGF) were shown to direct the extension of neurite outgrowth from the dorsal root ganglia (DRG) *in vitro* as a model for controlled axonal regeneration[84].

Fluorescently labelled heparin mesoporous silica MNPs were shown to being able to deliver bFGF to human umbilical endothelial vein cells (HUVECs) up to 6 d in culture conditions, suggesting their potential use as multimodal carriers[85]. Stem cells loaded with iron oxide MNPs can achieve various degrees of magnetization, being traceable *in vivo* using clinically approved methods such as magnetic resonance imaging (MRI). High-resolution tracking of MNP-loaded stem cells using MRI allows cellular imaging, which is an important advantage for therapeutic application as it allows observing the cell fate after implantation. MSCs loaded with ferucarbotran could be observed using MRI while differentiating to

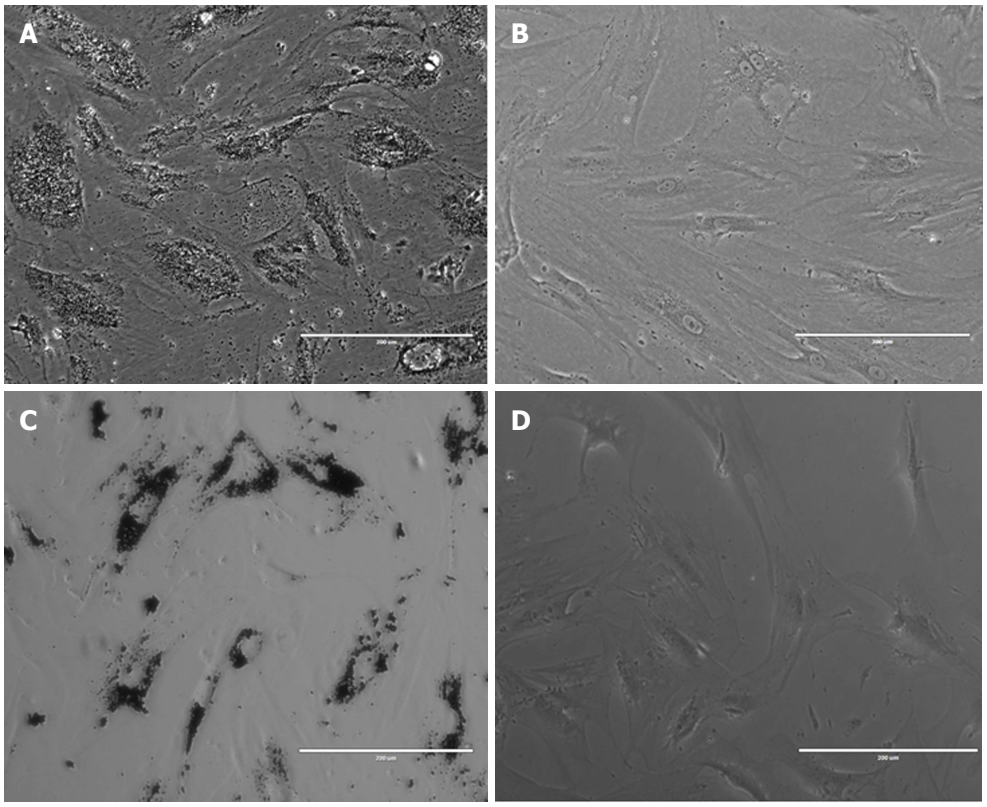

Figure 1 Stem and progenitor cells loaded with magnetic nanoparticles added to culture media. A: human osteoblasts loaded with Fe-Cr-Nb-B magnetic nanoparticles (MNPs); B: Non-loaded human osteoblasts; C: Human bone-marrow-derived mesenchymal stem cells loaded with Fe-Cr-Nb-B MNPs; D: Non-loaded human bone-marrow-derived mesenchymal stem cells.

functional neurons, a potential method for cellular tracking in stem-cell-based therapies[86].

Depending on the particle type, the upload process can extend from hours to several days of NP cell co-incubation. When considering potential clinical applications, the cell culture time needs to be decreased to a maximum. NP-dependent parameters such as NP colloidal stability, tendency to aggregate, surface charge or cell characteristics (*e.g.*, phagocytic or non-phagocytic cells, logarithmic growth phase in culture) are known to influence particle upload. Several methods to increase the efficiency of NP uptake have been proposed with variable efficiency depending on the cell and NP type. NP functionalization with antibodies against MSC surface antigens (receptor-mediated endocytosis) was shown to speed up the upload process in cultured cells[87]. Several transfection agents, commercially available poly-cationic polymer poly-L-lysine, protamine sulphate or lipofectamine are available for enhancing NP upload in therapeutic cells. Physical methods that produce a temporary disruption of the cell membrane, such as electroporation, pulsed ultrasonication or application of a magnetic field, the later applicable for MNPs (magnetoporation), have variable efficiency in increasing specific NP upload. However, such methods impose supplementary cell manipulation procedures and can affect the cell viability and phenotypic stability; therefore, their use needs to be carefully poised when designing clinical applications[88].

Attaching NP complexes to the cell membrane is another possibility to endow stem cells with therapeutic molecules. Cell surface engineering, a complex area in cell engineering, can rely on a large variety of procedures such as chemical modification based on native membrane functional molecules, metabolic or gene engineering of the cell membrane to express functional groups, adsorption, or insertion of hydrophobic coupling groups or ligand/receptor interaction at the membrane interface are currently exploited to attach NPs to various cell types[89]. Several reports exist regarding surface modifications that could allow the trafficking of NP-conjugated molecules using stem cells. Liposome-based non-covalent membrane modifications allow attaching a cargo to engineered binding sites on the cell surface. Liposome fusion was obtained by co-incubating MSCs with lipid vesicles containing ketones and oxyamine molecules. The liposomes underwent spontaneous membrane fusion to present the respective molecules from the cell surfaces serving as sites for chemo-selective ligation with oxyamine-conjugated molecules *in vitro*[90]. The majority of anchoring techniques aim to modify membrane characteristics for improved targeting capabilities (see above) and fewer NP delivery processes.

Release of therapeutic cargo from NP-equipped stem cells takes place mainly by passive diffusion or exocytosis. Release is dependent on the particle size, cell type and modality of cytoplasmic NP storage. Diffusion

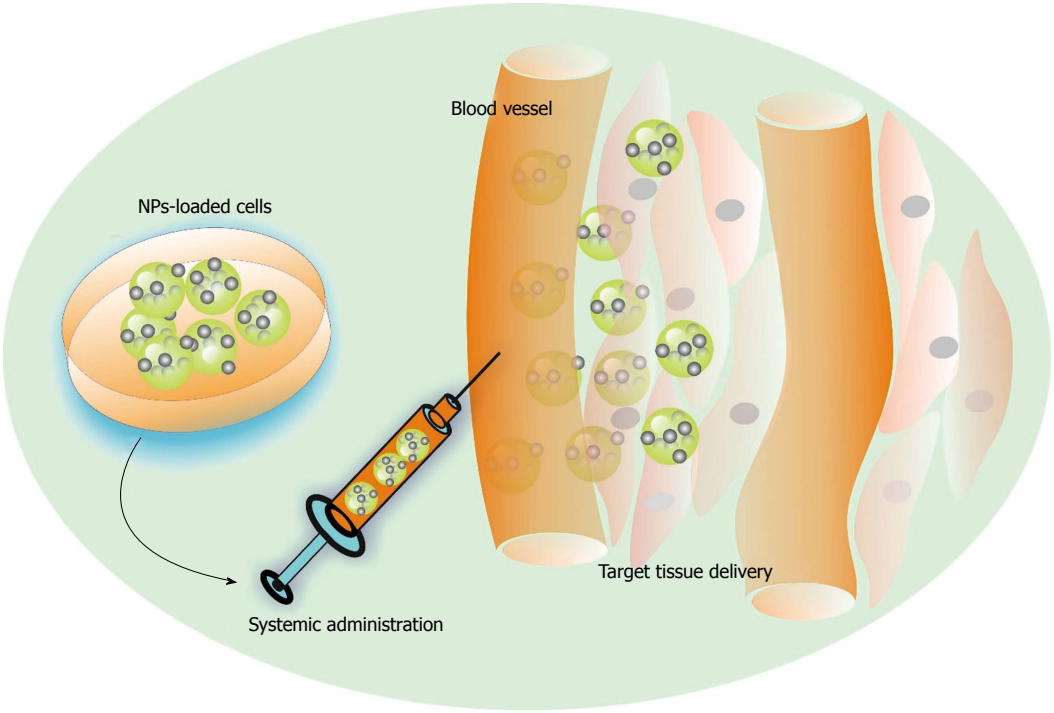

Figure 2 Possible scenario for nanoparticle-loaded stem cell delivery of bioactive molecules towards target organs. Stem cells loaded or decorated with nanoparticle attached to their membrane can be delivered via systemic infusion (intravenous or intraarterial) to migrate by means of blood flow, homing to regenerative sites.

and exocytosis can take place in time intervals ranging from minutes to several hours, while release from the cellular endosomal compartment, an energy consuming process, can take several days[73]. Ideally, a combination of the two mechanisms would be needed in order to enable uniform particle release. Pulsed and non-uniform payload release can impose hazards due to sudden drug or particle accumulation, not only compromising the therapeutic effect but also introducing supplementary hazards. In an ischaemic rat model subjected to MNP-loaded MSC injection, magnetic targeting resulted in vascular embolism and an inhomogeneous distribution of loaded cells, which prevented the intended targeted cell therapy from translating into a functional benefit[91]. Ingenious platforms for externally controlled release were shown to enhance the targeted release of a drug payload from decidua stem cells. Ultrasound-responsive mesoporous silica nanoparticles internalized by human decidua cells were shown to deliver their cargo under clinically available ultrasound frequencies both *in vitro* and in *vivo*, a promising strategy that could potentially be extended to the delivery of regenerative molecules[92].

For NP complexes attached to the cell membrane, the release profile can be engineered dependently on the type of receptor used for its conjugation to the cell membrane. A proposed solution is the design of slow-release systems by means of surface engineering as cell membrane biotinylation to obtain "synthetic biotin-avidin"-based cargo coupling[93].

Challenges for using stem cells as delivery agents in RM

Due to their targeting capabilities, stem cells are sought as biological "carriers" for the delivery of therapeutic molecules. Many research groups are proposing stem-cell-mediated drug delivery for targeting tumour tissues or for cancer theranostic applications. The interest in combining the intrinsic role of stem cells with their potential use as drug carriers is increasing; however, the procedure is not yet a road much travelled. Even though the possibility of delivering a supplement of growth factors or immune modulating agents together with various cell populations for either cell and/or gene therapy or for tissue engineering is appealing, and several challenges need to be addressed. Issues that challenge regenerative medicine product development generally apply to the field of stem-cell-mediated drug delivery. Improved scalable methods for stem cell isolation, expansion and culture combined with the use of serum-free media formulations are mandatory for large-scale clinical applications. Innovative methods for stem cell characterization and automated sorting for cell-based product characterization are expected to provide improved quality control tools. Particularly for cell-based carriers, a basic requirement is to ascertain that cargo loading and presence not only is non-toxic to cells but also preserves their phenotypic features. Stem cell type, origin, and culture method as well as NP size, surface, electric charge, and coating determine the modality of the interaction, impact the cell biological potential. Every (stem) cell type and NP-drug cargo

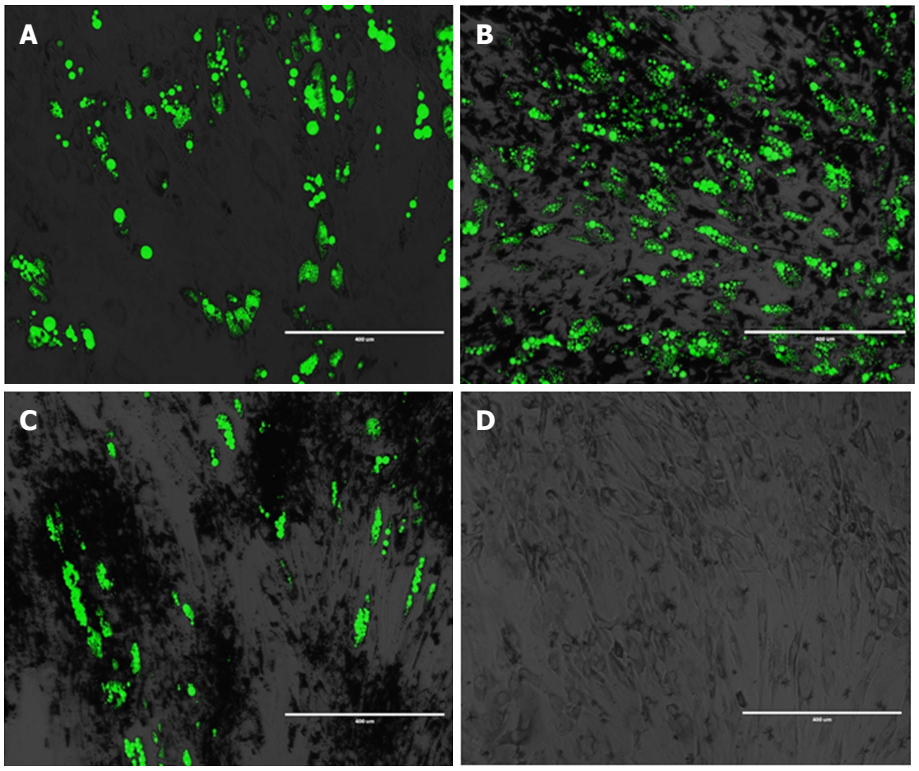

Figure 3 Adipogenic differentiation of human primary adipose-derived stem cells loaded with bare and chitosan-coated Fe-Cr-Nb-B magnetic nanoparticles. A: Adipose-derived stem cells (ADSCs) cells; B: ADSC-magnetic nanoparticles (MNPs); C: ADSC-C-MNPs; D: Control of differentiation (ADSCs grown in normal culture media).

combination needs to be tested in this respect. With several exceptions (such as titanium wear debris), biocompatible NP internalization or surface conjugation has been shown to preserve MSC proliferative, differentiation and immunomodulation capabilities in different cell types[94] (Figure 3). Moreover, NPs can be engineered to increase their differentiation capability towards a desired lineage. Silver nanoparticles were shown to increase bone healing by increasing mouse MSC proliferation, chemo-attraction and osteogenic potential in a long bone fracture model[95]. Internalized MNPs were shown to increase MSCs osteogenesis *via* a long non-coding RNA INZEB2 mechanism[96]. Superparamagnetic iron oxide nanoparticles (SPIOs) used as contrast agents were shown to transiently and reversibly affect chondrogenesis in some cell types but not in others, stressing the importance of testing each particular cell type for a specific application[97]. Membrane structure and composition after particle internalization or conjugation needs to be assessed, as changes in surface antibodies could affect stem cell migratory capabilities. Interesting, various types of MNP internalization were shown to enhance the expression of chemokine receptor CXCR4 in MSCs and to improve their homing to a brain injury and glioblastoma model compared to the findings in non-loaded cells[98]. Stem cells loaded with MNPs present a particular interest in drug delivery, as they may be remotely actuated using external magnetic fields in order to increase delivery to target tissues and/or organs. Challenges regarding the intensity of the magnetic field, distance from the target organ (which influences the tracking capability), and risk of cell agglutination within the blood stream still need to be addressed. Magnetic force actuation of MNP-loaded stem cells has proved efficient for directed cell localization and consecutive repair of arterial injuries in small animal models or cartilage defects in larger animals (swine), paving the way for future clinical applications[99].

For NP drug complexes attached to the cell membrane, enzymatic or hydrolytic drug degradation within the biological environment is a serious challenge that increases side effects and lowers the therapeutic efficiency. Relevant *in vivo* models must be employed for testing collateral particle release within the blood stream and the specific pharmacodynamics of these particles, especially the tendency to accumulate within non-target tissues such as lung, spleen or liver. Drug-induced immunogenicity needs to be ruled out as well as potential interactions with cellular and non-cellular immune effectors within the biological milieu.

It should be noted that both cargo and NP loading can affect cell migratory and homing capabilities. Genetic engineering can be used in this case to modify cell surface receptors, to improve their migratory capabilities and to increase survival. The efficiency of such cell manipulation in every phase of cell homing needs to be tested in relevant models *in vitro* as well as *in vivo*, in combination with the drug release efficiency.

FUTURE PROSPECTS

Stem cells are currently being tested by several groups as vehicles for targeting, imaging and treating tumours. For anticancer therapies, vehicle viability after successful cargo delivery is not an issue. In contrast, due to possible stem cell recruitment by tumours, it might prove safer to control cell proliferation and viability by means of cargo. Moreover, the demise of carrier cells triggers an immune response, contributing to tumour treatment[100]. For RM purposes, it would be beneficial not only to enable survival of these cells but also to improve their direct contribution to the regeneration process.

Controlling the stem cell fate by means of cargo could represent an appealing modality in steering regenerative processes**.** An MNP-based GF delivery and activation strategy was reported to be able to release TGF-β from its latent complex by application of an external magnetic field. Latent TGF-bioactive molecules such as GF, cytokines, DNA or miRNA could be delivered to control stem cell fate using MNPs. Magnetic core-shell MNPs composed of a highly magnetic core surrounded by a thin uniform gold shell enabled the delivery of controlling genetic materials [small interfering RNA (siRNA) and plasmid DNA (pDNA)] used to direct neural stem cell differentiation to neurons and oligodendrocytes. MNP-mediated RNA interference suppressed two key "neural switch" genes CAVEOLIN-1 and SOX9 that are responsible for oligodendrocyte and neuron differentiation, respectively. When conjugated to the surface of poly (ethylene glycol)-coated MNPs, molecules could be activated and released in a controlled manner[101]. Such a triggered GF-release strategy could be expanded to remote-control-delivered stem cell differentiation, increasing their participation in local healing.

Control of stem cell fate *via* the magnetic actuation of magnetic responsive cargo could be used to control the osteogenic differentiation of systemically delivered MSCs, preventing their adipogenic conversion for systemic osteoporosis treatment.

The large majority of current studies regarding stem-cell-mediated delivery of therapeutic agents are based on adult stem cells (MSCs or ESCs). MSCs and neural stem cells (NSCs) are promising targets for overcoming the blood-brain barrier to enable RNA and drug delivery for tumours or neurodegenerative disorders[102].

As is the case with any other stem-cell-based therapy, improved methods for cell collection and culture protocol standardization combined with innovative methods for donor and cell selection are expected to accelerate the transition into safe and efficient therapies. The stem-cell-mediated delivery of therapeutic agents for targeting regenerative sites poses special challenges. Cell procurement and culture that enables preservation and/or methods for augmenting the homing potential are needed for both native and genetically modified cells. The dynamic and phenotypic modification of NP-complex augmented cells needs to be described in detail as well as its effect in modifying the microenvironment of target tissues. Stem-cell-mediated delivery has the potential to consistently enhance the therapeutic effect of these cells for RM applications.

REFERENCES

1 **Mimeault M**, Hauke R, Batra SK. Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. *Clin Pharmacol Ther* 2007; **82**: 252-264 [PMID: 17671448 DOI: 10.1038/sj.clpt.6100301]

2 **Mahla RS**. Stem Cells Applications in Regenerative Medicine and Disease Therapeutics. *Int J Cell Biol* 2016; **2016**: 6940283 [PMID: 27516776 DOI: 10.1155/2016/6940283]

3 **Wobma H**, Vunjak-Novakovic G. Tissue Engineering and Regenerative Medicine 2015: A Year in Review. *Tissue Eng Part B Rev* 2016; **22**: 101-113 [PMID: 26714410 DOI: 10.1089/ten.TEB.2015.0535]

4 **Badowski MS**, Zhang T, Tsang TC, Harris DT. Chimeric antigen receptors for stem cell based immunotherapy. *J Exp Ther Oncol* 2009; **8**: 53-63 [PMID: 19827271]

5 **Schwartz SD**, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregori NZ, Hubschman JP, Davis JL, Heilwell G, Spirn M, Maguire J, Gay R, Bateman J, Ostrick RM, Morris D, Vincent M, Anglade E, Del Priore LV, Lanza R. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet* 2015; **385**: 509-516 [PMID: 25458728 DOI: 10.1016/S0140-6736(14)61376-3]

6 **Stoltz JF**, de Isla N, Li YP, Bensoussan D, Zhang L, Huselstein C, Chen Y, Decot V, Magdalou J, Li N, Reppel L, He Y. Stem Cells and Regenerative Medicine: Myth or Reality of the 21th Century. *Stem Cells Int* 2015; **2015**: 734731 [PMID: 26300923 DOI: 10.1155/2015/734731]

7 **Singh VK**, Kalsan M, Kumar N, Saini A, Chandra R. Induced pluripotent stem cells: applications in regenerative medicine, disease modeling, and drug discovery. *Front Cell Dev Biol* 2015; **3**: 2 [PMID: 25699255 DOI: 10.3389/fcell.2015.00002]

8 **Knoepfler PS**. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. *Stem Cells* 2009; **27**: 1050-1056 [PMID: 19415771 DOI: 10.1002/stem.37]

9 **Herberts CA**, Kwa MS, Hermsen HP. Risk factors in the development of stem cell therapy. *J Transl Med* 2011; **9**: 29 [PMID: 21418664 DOI: 10.1186/1479-5876-9-29]

10 **Gnecchi M**, Danieli P, Malpasso G, Ciuffreda MC. Paracrine Mechanisms of Mesenchymal Stem Cells in Tissue Repair. *Methods Mol Biol* 2016; **1416**: 123-146 [PMID: 27236669 DOI: 10.1007/978-1-4939-3584-0_7]

11 **Caplan AI**. Mesenchymal Stem Cells: Time to Change the Name! *Stem Cells Transl Med* 2017; **6**: 1445-1451 [PMID: 28452204 DOI: 10.1002/sctm.17-0051]

12 **Farber DB**, Katsman D. Embryonic Stem Cell-Derived Microvesicles: Could They be Used for Retinal Regeneration? *Adv Exp Med Biol* 2016; **854**: 563-569 [PMID: 26427460 DOI: 10.1007/978-3-319-17121-0_75]

13 **Biancone L**, Bruno S, Deregibus MC, Tetta C, Camussi G. Therapeutic potential of mesenchymal stem cell-derived microvesicles. *Nephrol Dial Transplant* 2012; **27**: 3037-3042 [PMID: 22851627 DOI: 10.1093/ndt/gfs168]

14 **Guo Y**, Hangoc G, Bian H, Pelus LM, Broxmeyer HE. SDF-1/CXCL12 enhances survival and chemotaxis of murine embryonic stem cells and production of primitive and definitive hematopoietic progenitor cells. *Stem Cells* 2005; **23**: 1324-1332 [PMID: 16210409 DOI: 10.1634/stemcells.2005-0085]

15 **De Becker A**, Riet IV. Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy? *World J Stem Cells* 2016; **8**: 73-87 [PMID: 27022438 DOI: 10.4252/wjsc.

v8.i3.73]
16 **Khaldoyanidi S**. Directing stem cell homing. *Cell Stem Cell* 2008; **2**: 198-200 [PMID: 18371444 DOI: 10.1016/j.stem.2008.02.012]
17 **Miller FD**, Kaplan DR. Mobilizing endogenous stem cells for repair and regeneration: are we there yet? *Cell Stem Cell* 2012; **10**: 650-652 [PMID: 22704501 DOI: 10.1016/j.stem.2012.05.004]
18 **Lapidot T**, Dar A, Kollet O. How do stem cells find their way home? *Blood* 2005; **106**: 1901-1910 [PMID: 15890683 DOI: 10.1182/blood-2005-04-1417]
19 **Karp JM**, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* 2009; **4**: 206-216 [PMID: 19265660 DOI: 10.1016/j.stem.2009.02.001]
20 **Lämmermann T**, Sixt M. Mechanical modes of 'amoeboid' cell migration. *Curr Opin Cell Biol* 2009; **21**: 636-644 [PMID: 19523798 DOI: 10.1016/j.ceb.2009.05.003]
21 **Yin Y**, Li X, He XT, Wu RX, Sun HH, Chen FM. Leveraging Stem Cell Homing for Therapeutic Regeneration. *J Dent Res* 2017; **96**: 601-609 [PMID: 28414563 DOI: 10.1177/0022034517706070]
22 **Heissig B**, Dhahri D, Eiamboonsert S, Salama Y, Shimazu H, Munakata S, Hattori K. Role of mesenchymal stem cell-derived fibrinolytic factor in tissue regeneration and cancer progression. *Cell Mol Life Sci* 2015; **72**: 4759-4770 [PMID: 26350342 DOI: 10.1007/s00018-015-2035-7]
23 **Barreca MM**, Spinello W, Cavalieri V, Turturici G, Sconzo G, Kaur P, Tinnirello R, Asea AA, Geraci F. Extracellular Hsp70 Enhances Mesoangioblast Migration via an Autocrine Signaling Pathway. *J Cell Physiol* 2017; **232**: 1845-1861 [PMID: 27925208 DOI: 10.1002/jcp.25722]
24 **Sahin AO**, Buitenhuis M. Molecular mechanisms underlying adhesion and migration of hematopoietic stem cells. *Cell Adh Migr* 2012; **6**: 39-48 [PMID: 22647939 DOI: 10.4161/cam.18975]
25 **Hocking AM**. The role of chemokines in mesenchymal stem cell homing to wounds. *Adv Skin Wound Ca* 2015; **4**: 623-630 [PMID: 26543676 DOI: 10.1089/wound.2014.0579]
26 **Stuermer EK**, Lipenksy A, Thamm O, Neugebauer E, Schaefer N, Fuchs P, Bouillon B, Koenen P. The role of SDF-1 in homing of human adipose-derived stem cells. *Wound Repair Regen* 2015; **23**: 82-89 [PMID: 25581571 DOI: 10.1111/wrr.12248]
27 **Miller RJ**, Banisadr G, Bhattacharyya BJ. CXCR4 signaling in the regulation of stem cell migration and development. *J Neuroimmunol* 2008; **198**: 31-38 [PMID: 18508132 DOI: 10.1016/j.jneuroim.2008.04.008]
28 **Alexeev V**, Donahue A, Uitto J, Igoucheva O. Analysis of chemotactic molecules in bone marrow-derived mesenchymal stem cells and the skin: Ccl27-Ccr10 axis as a basis for targeting to cutaneous tissues. *Cytotherapy* 2013; **15**: 171-184.e1 [PMID: 23321329 DOI: 10.1016/j.jcyt.2012.11.006]
29 **Leibacher J**, Henschler R. Biodistribution, migration and homing of systemically applied mesenchymal stem/stromal cells. *Stem Cell Res Ther* 2016; **7**: 7 [PMID: 26753925 DOI: 10.1186/s13287-015-0271-2]
30 **Bustos ML**, Huleihel L, Kapetanaki MG, Lino-Cardenas CL, Mroz L, Ellis BM, McVerry BJ, Richards TJ, Kaminski N, Cerdenes N, Mora AL, Rojas M. Aging mesenchymal stem cells fail to protect because of impaired migration and antiinflammatory response. *Am J Respir Crit Care Med* 2014; **189**: 787-798 [PMID: 24559482 DOI: 10.1164/rccm.201306-1043OC]
31 **Liu H**, Xue W, Ge G, Luo X, Li Y, Xiang H, Ding X, Tian P, Tian X. Hypoxic preconditioning advances CXCR4 and CXCR7 expression by activating HIF-1α in MSCs. *Biochem Biophys Res Commun* 2010; **401**: 509-515 [PMID: 20869949 DOI: 10.1016/j.bbrc.2010.09.076]
32 **Haque N**, Rahman MT, Abu Kasim NH, Alabsi AM. Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy. *ScientificWorldJournal* 2013; **2013**: 632972 [PMID: 24068884 DOI: 10.1155/2013/632972]
33 **Crowder SW**, Horton LW, Lee SH, McClain CM, Hawkins OE, Palmer AM, Bae H, Richmond A, Sung HJ. Passage-dependent cancerous transformation of human mesenchymal stem cells under carcinogenic hypoxia. *FASEB J* 2013; **27**: 2788-2798 [PMID: 23568779 DOI: 10.1096/fj.13-228288]
34 **Feng Y**, Zhu M, Dangelmajer S, Lee YM, Wijesekera O, Castellanos CX, Denduluri A, Chaichana KL, Li Q, Zhang H, Levchenko A, Guerrero-Cazares H, Quiñones-Hinojosa A. Hypoxia-cultured human adipose-derived mesenchymal stem cells are non-oncogenic and have enhanced viability, motility, and tropism to brain cancer. *Cell Death Dis* 2014; **5**: e1567 [PMID: 25501828 DOI: 10.1038/cddis.2014.521]
35 **De Becker A**, Van Hummelen P, Bakkus M, Vande Broek I, De Wever J, De Waele M, Van Riet I. Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3. *Haematologica* 2007; **92**: 440-449 [PMID: 17488654 DOI: 10.3324/haematol.10475]
36 **Frith JE**, Thomson B, Genever PG. Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. *Tissue Eng Part C Methods* 2010; **16**: 735-749 [PMID: 19811095 DOI: 10.1089/ten.TEC.2009.0432]
37 **Cesarz Z**, Tamama K. Spheroid Culture of Mesenchymal Stem Cells. *Stem Cells Int* 2016; **5**: 1-11 [PMID: 26649054 DOI: 10.1155/2016/9176357]
38 **Huang X**, Zhang F, Wang Y, SunX, Choi KY, Liu D, Choi J, Shin T-H Cheon J, Niu G, Chen X. Design considerations of iron-based nanoclusters for noninvasive tracking of mesenchymal stem cell homing. *ACS Nano* 2014; **8**: 4403-4414 [PMID: 24754735 DOI: 10.1021/nn4062726]
39 **Fernandez-Pernas P**, Rodríguez-Lesende I, de la Fuente A, Mateos J, Fuentes I, De Toro J, Blanco FJ, Arufe MC. CD105+-mesenchymal stem cells migrate into osteoarthritis joint: An animal model. *PLoS One* 2017; **12**: e0188072 [PMID: 29190645 DOI: 10.1371/journal.pone.0188072]
40 **Kwon YW**, Heo SC, Jeong GO, Yoon JW, Mo WM, Lee MJ, Jang IH, Kwon SM, Lee JS, Kim JH. Tumor necrosis factor-α-activated mesenchymal stem cells promote endothelial progenitor cell homing and angiogenesis. *Biochim Biophys Acta* 2013; **1832**: 2136-2144 [PMID: 23959047 DOI: 10.1016/j.bbadis.2013.08.002]
41 **Xinaris C**, Morigi M, Benedetti V, Imberti B, Fabricio AS, Squarcina E, Benigni A, Gagliardini E, Remuzzi G. A novel strategy to enhance mesenchymal stem cell migration capacity and promote tissue repair in an injury specific fashion. *Cell Transplant* 2013; **22**: 423-436 [PMID: 22889699 DOI: 10.3727/096368912X653246]
42 **Jones GN**, Moschidou D, Lay K, Abdulrazzak H, Vanleene M, Shefelbine SJ, Polak J, de Coppi P, Fisk NM, Guillot PV. Upregulating CXCR4 in human fetal mesenchymal stem cells enhances engraftment and bone mechanics in a mouse model of osteogenesis imperfecta. *Stem Cells Transl Med* 2012; **1**: 70-78 [PMID: 23197643 DOI: 10.5966/sctm.2011-0007]
43 **Kim YS**, Noh MY, Kim JY, Yu HJ, Kim KS, Kim SH, Koh SH. Direct GSK-3β inhibition enhances mesenchymal stromal cell migration by increasing expression of β-PIX and CXCR4. *Mol Neurobiol* 2013; **47**: 811-820 [PMID: 23288365 DOI: 10.1007/s12035-012-8393-3]
44 **Yu Q**, Chen L, You Y, Zou C, Zhang Y, Liu Q, Cheng F. Erythropoietin combined with granulocyte colony-stimulating factor enhances MMP-2expression in mesenchymal stem cells and promotes cell migration. *Mol Med Rep* 2011; **4**: 31-36 [PMID: 21461559 DOI: 10.3892/mmr.2010.387]
45 **Noiseux N**, Borie M, Desnoyers A, Menaouar A, Stevens LM, Mansour S, Danalache BA, Roy DC, Jankowski M, Gutkowska J. Preconditioning of stem cells by oxytocin to improve their therapeutic potential. *Endocrinology* 2012; **153**: 5361-5372 [PMID: 23024264 DOI: 10.1210/en.2012-1402]
46 **Tsai LK**, Leng Y, Wang Z, Leeds P, Chuang DM. The mood stabilizers valproic acid and lithium enhance mesenchymal stem cell migration via distinct mechanisms. *Neuropsychopharmacology* 2010; **35**: 2225-2237 [PMID: 20613717 DOI: 10.1038/npp.2010.97]
47 **Marquez-Curtis LA**, Janowska-Wieczorek A. Enhancing the migration ability of mesenchymal stromal cells by targeting the

sdf-1/cxcr4 axis. *BioMed Res Int* 2013; **2013**: 561098 [PMID: 24381939 DOI: 10.1155/2013/561098]
48 **Nowakowski A**, Walczak P, Lukomska B, and Janowski M. Genetic engineering of mesenchymal stem cells to induce their migration and survival. *Stem Cells Int* 2016; **1**: 1-9 [PMID: 27242906 DOI: 10.1155/2016/4956063]
49 **Won YW**, Patel AN, Bull DA. Cell surface engineering to enhance mesenchymal stem cell migration toward an SDF-1 gradient. *Biomaterials* 2014; **35**: 5627-5635 [PMID: 24731711 DOI: 10.1016/j.biomaterials.2014.03.070]
50 **Hassmann-Poznańska E**, Chodynicki S, Sulik M. [Tumor of the parapharyngeal space]. *Wiad Lek* 1989; **42**: 991-995 [PMID: 2640075 DOI: 10.1007/s12015-015-9625-5]
51 **Yukawa H**, Watanabe M, Kaji N, Okamoto Y, Tokeshi M, Miyamoto Y, Noguchi H, Baba Y, Hayashi S. Monitoring transplanted adipose tissue-derived stem cells combined with heparin in the liver by fluorescence imaging using quantum dots. *Biomaterials* 2012; **33**: 2177-2186 [PMID: 22192539 DOI: 10.1016/j.biomaterials.2011.12.009]
52 **Wang W**, Xu X, Li Z, Lendlein A, Ma N. Genetic engineering of mesenchymal stem cells by non-viral gene delivery. *Clin Hemorheol Microcirc* 2014; **58**: 19-48 [PMID: 25227201 DOI: 10.3233/CH-141883]
53 **Meacham JM**, Durvasula K, Degertekin FL, Fedorov AG. Physical methods for intracellular delivery: practical aspects from laboratory use to industrial-scale processing. *J Lab Autom* 2014; **19**: 1-18 [PMID: 23813915 DOI: 10.1177/2211068213494388]
54 **Das J**, Choi YJ, Yasuda H, Han JW, Park C, Song H, Bae H, Kim JH. Efficient delivery of C/EBP beta gene into human mesenchymal stem cells via polyethylenimine-coated gold nanoparticles enhances adipogenic differentiation. *Sci Rep* 2016; **6**: 33784 [PMID: 27677463 DOI: 10.1038/srep33784]
55 **Watermann A**, Brieger J. Mesoporous Silica Nanoparticles as Drug Delivery Vehicles in Cancer. *Nanomaterials* (Basel) 2017; **7**: [PMID: 28737672 DOI: 10.3390/nano7070189]
56 **Coutu DL**, Cuerquis J, El Ayoubi R, Forner KA, Roy R, François M, Griffith M, Lillicrap D, Yousefi AM, Blostein MD, Galipeau J. Hierarchical scaffold design for mesenchymal stem cell-based gene therapy of hemophilia B. *Biomaterials* 2011; **32**: 295-305 [PMID: 20864158 DOI: 10.1016/j.biomaterials.2010.08.094]
57 **Penati R**, Fumagalli F, Calbi V, Bernardo ME, Aiuti A. Gene therapy for lysosomal storage disorders: recent advances for metachromatic leukodystrophy and mucopolysaccaridosis I. *J Inherit Metab Dis* 2017; **40**: 543-554 [PMID: 28560469 DOI: 10.1007/s10545-017-0052-4]
58 **Chamberlain JR**, Schwarze U, Wang PR, Hirata RK, Hankenson KD, Pace JM, Underwood RA, Song KM, Sussman M, Byers PH, Russell DW. Gene targeting in stem cells from individuals with osteogenesis imperfecta. *Science* 2004; **303**: 1198-1201 [PMID: 14976317 DOI: 10.1126/science.1088757]
59 **Myers TJ**, Granero-Molto F, Longobardi L, Li T, Yan Y, Spagnoli A. Mesenchymal stem cells at the intersection of cell and gene therapy. *Expert Opin Biol Ther* 2010; **10**: 1663-1679 [PMID: 21058931 DOI: 10.1517/14712598.2010.531257]
60 **Sack BK**, Herzog RW, Terhorst C, Markusic DM. Development of gene transfer for induction of antigen-specific tolerance. *Mol Ther Methods Clin Dev* 2014; **1**: 14013 [PMID: 25558460 DOI: 10.1038/mtm.2014.13]
61 **Scarfì S**. Use of bone morphogenetic proteins in mesenchymal stem cell stimulation of cartilage and bone repair. *World J Stem Cells* 2016; **8**: 1-12 [PMID: 26839636 DOI: 10.4252/wjsc.v8.i1.1]
62 **Kumar S**, Wan C, Ramaswamy G, Clemens TL, Ponnazhagan S. Mesenchymal stem cells expressing osteogenic and angiogenic factors synergistically enhance bone formation in a mouse model of segmental bone defect. *Mol Ther* 2010; **18**: 1026-1034 [PMID: 20068549 DOI: 10.1038/mt.2009.315]
63 **Lee JM**, Kim EA, Im GI. Healing of tibial and calvarial bone defect using Runx-2-transfected adipose stem cells. *Tissue Eng Regen Med* 2015; **12**: 107 [DOI: 10.1007/s13770-014-0070-3]
64 **Guerquin MJ**, Charvet B, Nourissat G, Havis E, Ronsin O, Bonnin MA, Ruggiu M, Olivera-Martinez I, Robert N, Lu Y, Kadler KE, Baumberger T, Doursounian L, Berenbaum F, Duprez D. Transcription factor EGR1 directs tendon differentiation and promotes tendon repair. *J Clin Invest* 2013; **123**: 3564-3576 [PMID: 23863709 DOI: 10.1172/JCI67521]
65 **Pelled G**, Snedeker JG, Ben-Arav A, Rigozzi S, Zilberman Y, Kimelman-Bleich N, Gazit Z, Müller R, Gazit D. Smad8/BMP2-engineered mesenchymal stem cells induce accelerated recovery of the biomechanical properties of the Achilles tendon. *J Orthop Res* 2012; **30**: 1932-1939 [PMID: 22696396 DOI: 10.1002/jor.22167]
66 **Kraus TM**, Imhoff FB, Reinert J. Stem cells and bFGF in tendon healing: Effects of lentiviral gene transfer and long-term follow-up in a rat Achilles tendon defect model. *BMC Musculoskelet Disord* 2016; **17**:148 [PMID: 27048602 DOI: 10.1186/s12891-016-0999-6]
67 **Yan J**, Zhang C, Zhao Y, Cao C, Wu K, Zhao L, Zhang Y. Non-viral oligonucleotide antimiR-138 delivery to mesenchymal stem cell sheets and the effect on osteogenesis. *Biomaterials* 2014; **35**: 7734-7749 [PMID: 24952983 DOI: 10.1016/j.biomaterials.2014.05.089]
68 **Wang M**, Tan J, Wang Y, Meldrum KK, Dinarello CA, Meldrum DR. IL-18 binding protein-expressing mesenchymal stem cells improve myocardial protection after ischemia or infarction. *Proc Natl Acad Sci USA* 2009; **106**: 17499-17504 [PMID: 19805173 DOI: 10.1073/pnas.0908924106]
69 **Yeh TS**, Fang YH, Lu CH, Chiu SC, Yeh CL, Yen TC, Parfyonova Y, Hu YC. Baculovirus-transduced, VEGF-expressing adipose-derived stem cell sheet for the treatment of myocardium infarction. *Biomaterials* 2014; **35**: 174-184 [PMID: 24120047 DOI: 10.1016/j.biomaterials.2013.09.080]
70 **Nakajima M**, Nito C, Sowa K, Suda S, Nishiyama Y, Nakamura-Takahashi A, Nitahara-Kasahara Y, Imagawa K, Hirato T, Ueda M, Kimura K, Okada T. Mesenchymal Stem Cells Overexpressing Interleukin-10 Promote Neuroprotection in Experimental Acute Ischemic Stroke. *Mol Ther Methods Clin Dev* 2017; **6**: 102-111 [PMID: 28725658 DOI: 10.1016/j.omtm.2017.06.005]
71 **Xiong N**, Zhang Z, Huang J, Chen C, Zhang Z, Jia M, Xiong J, Liu X, Wang F, Cao X, Liang Z, Sun S, Lin Z, Wang T. VEGF-expressing human umbilical cord mesenchymal stem cells, an improved therapy strategy for Parkinson's disease. *Gene Ther* 2011; **18**: 394-402 [PMID: 21107440 DOI: 10.1038/gt.2010.152]
72 **Mitchell AC**, Briquez PS, Hubbell JA, Cochran JR. Engineering growth factors for regenerative medicine applications. *Acta Biomater* 2016; **30**: 1-12 [PMID: 26555377 DOI: 10.1016/j.actbio.2015.11.007]
73 **Park JS**, Suryaprakash S, Lao YH, Leong KW. Engineering mesenchymal stem cells for regenerative medicine and drug delivery. *Methods* 2015; **84**: 3-16 [PMID: 25770356 DOI: 10.1016/j.ymeth.2015.03.002]
74 **Markides H**, Rotherham M, El Haj AJ. Biocompatibility and Toxicity of Magnetic Nanoparticles in Regenerative Medicine. *J Nanomater* 2012; **5-6**: 11 [DOI: 10.1155/2012/614094]
75 **Van Rijt S**, Habibovic P. Enhancing regenerative approaches with nanoparticles. *J R S Interface* 2017; **14**: 20170093 [PMID: 28404870 DOI: 10.1098/rsif.2017.0093]
76 **Danhier F**, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. *J Control Release* 2012; **161**: 505-522 [PMID: 22353619 DOI: 10.1016/j.jconrel.2012.01.043]
77 **Roger M**, Clavreul A, Venier-Julienne MC, Passirani C, Sindji L, Schiller P, Montero-Menei C, Menei P. Mesenchymal stem cells as cellular vehicles for delivery of nanoparticles to brain tumors. *Biomaterials* 2010; **31**: 8393-8401 [PMID: 20688391 DOI: 10.1016/j.biomaterials.2010.07.048]
78 **Panyam J**, Zhou WZ, Prabha S, Sahoo SK, Labhasetwar V. Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. *FASEB J* 2002; **16**: 1217-1226 [PMID: 12153989 DOI: 10.1096/fj.02-0088com]
79 **Brüstle I**, Simmet T, Nienhaus GU, Landfester K, Mailänder V. Hematopoietic and mesenchymal stem cells: polymeric

nanoparticle uptake and lineage differentiation. *Beilstein J Nanotechnol* 2015; **6**: 383-395 [PMID: 25821678 DOI: 10.3762/bjnano.6.38]

80 **Paris JL**, de la Torre P, Manzano M, Cabañas MV, Flores AI, Vallet-Regí M. Decidua-derived mesenchymal stem cells as carriers of mesoporous silica nanoparticles. In vitro and in vivo evaluation on mammary tumors. *Acta Biomater* 2016; **33**: 275-282 [PMID: 26796209 DOI: 10.1016/j.actbio.2016.01.017]

81 **Gallina C**, Capelôa T, Saviozzi S, Accomaso L, Catalano F, Tullio F, Martra G, Penna C, Pagliaro P, Turinetto V, Giachino C. Human mesenchymal stem cells labelled with dye-loaded amorphous silica nanoparticles: long-term biosafety, stemness preservation and traceability in the beating heart. *J Nanobiotechnology* 2015; **13**: 77 [PMID: 26510588 DOI: 10.1186/s12951-015-0141-1]

82 **Accomasso L**, Cibrario Rocchietti E, Raimondo S, Catalano F, Alberto G, Giannitti A, Minieri V, Turinetto V, Orlando L, Saviozzi S, Caputo G, Geuna S, Martra G, Giachino C. Fluorescent silica nanoparticles improve optical imaging of stem cells allowing direct discrimination between live and early-stage apoptotic cells. *Small* 2012; **8**: 3192-3200 [PMID: 22821625 DOI: 10.1002/smll.201200882]

83 **Ying X**, Wang Y, Xu H, Li X, Yan H, Tang H, Wen C, Li Y. The construction of the multifunctional targeting ursolic acids liposomes and its apoptosis effects to C6 glioma stem cells. *Oncotarget* 2017; **8**: 64129-64142 [PMID: 28969057 DOI: 10.18632/oncotarget.19784]

84 **Wu Q**, Liu C, Fan L, Shi J, Liu Z, Li R, Sun L. Heparinized magnetic mesoporous silica nanoparticles as multifunctional growth factor delivery carriers. *Nanotechnology* 2012; **23**: 485703 [PMID: 23128185 DOI: 10.1088/0957-4484/23/48/485703]

85 **Zuidema JM**, Provenza C, Caliendo T, Dutz S, Gilbert RJ. Magnetic NGF-releasing PLLA/iron oxide nanoparticles direct extending neurites and preferentially guide neurites along aligned electrospun microfibers. *ACS Chem Neurosci* 2015; **6**: 1781-1788 [PMID: 26322376 DOI: 10.1021/acschemneuro.5b00189]

86 **Lu CW**, Hsiao JK, Liu HM, Wu CH. Characterization of an iron oxide nanoparticle labelling and MRI-based protocol for inducing human mesenchymal stem cells into neural-like cells. *Sci Rep* 2017; **7**: 3587 [PMID: 28620162 DOI: 10.1038/s41598-017-03863-x]

87 **Sarkar D**, Ankrum JA, Teo GS, Carman CV, Karp JM. Cellular and extracellular programming of cell fate through engineered intracrine-, paracrine-, and endocrine-like mechanisms. *Biomaterials* 2011; **32**: 3053-3061 [PMID: 21262537 DOI: 10.1016/j.biomaterials.2010.12.036]

88 **Lewis EE**, Child HW, Hursthouse A, Stirling D, McCully M, Paterson D, Mullin M, Berry CC. The influence of particle size and static magnetic fields on the uptake of magnetic nanoparticles into three dimensional cell-seeded collagen gel cultures. *J Biomed Mater Res B Appl Biomater* 2015; **103**: 1294-1301 [PMID: 25358626 DOI: 10.1002/jbm.b.33302]

89 **Stephan MT**, Irvine DJ. Enhancing Cell therapies from the Outside In: Cell Surface Engineering Using Synthetic Nanomaterials. *Nano Today* 2011; **6**: 309-325 [PMID: 21826117 DOI: 10.1016/j.nantod.2011.04.001]

90 **Dutta D**, Pulsipher A, Luo W, Mak H, Yousaf MN. Engineering cell surfaces via liposome fusion. *Bioconjug Chem* 2011; **22**: 2423-2433 [PMID: 22054009 DOI: 10.1021/bc200236m]

91 **Huang Z**, Shen Y, Pei N, Sun A, Xu J, Song Y, Huang G, Sun X, Zhang S, Qin Q,Zhu H, Yang S, Yang X, Zou Y, Qian J, Ge J. The effect of nonuniform magnetic targeting of intracoronary-delivering mesenchymal stem cells on coronary embolisation. *Biomaterials* 2013; **34**: 9905-9916 [PMID: 24055521 DOI: 10.1016/j.biomaterials.2013.08.092]

92 **Huang Z**, Shen Y, Pei N, Sun A, Xu J, Song Y, Huang G, Sun X, Zhang S, Qin Q, Zhu H, Yang S, Yang X, Zou Y, Qian J, Ge J. The effect of nonuniform magnetic targeting of intracoronary-delivering mesenchymal stem cells on coronary embolisation. *Biomaterials* 2013; **34**: 9905-9916 [PMID: 24055521 DOI: 10.1016/j.biomaterials.2013.08.092]

93 **Fliervoet LAL**, Mastrobattista E. Drug delivery with living cells. *Adv Drug Deliv Rev* 2016; **106**: 63-72 [PMID: 27129442 DOI: 10.1016/j.addr.2016.04.021]

94 **Farcal L**, Torres Andón F, Di Cristo L, Rotoli BM, Bussolati O, Bergamaschi E, Mech A, Hartmann NB, Rasmussen K, Riego-Sintes J, Ponti J, Kinsner-Ovaskainen A, Rossi F, Oomen A, Bos P, Chen R, Bai R, Chen C, Rocks L, Fulton N, Ross B, Hutchison G, Tran L, Mues S, Ossig R, Schnekenburger J, Campagnolo L, VecchioneL, Pietroiusti A, Fadeel B. Comprehensive in vitro toxicity testing of a panel of representative oxide nanomaterials: first steps towards an intelligent testing strategy. *PLoS One* 2015; **10**: e0127174 [PMID: 25996496 DOI: 10.1371/journal.pone.0127174]

95 **Zhang R**, Lee P, Lui VC, Chen Y, Liu X, Lok CN, To M, Yeung KW, Wong KK. Silver nanoparticles promote osteogenesis of mesenchymal stem cells and improve bone fracture healing in osteogenesis mechanism mouse model. *Nanomedicine* 2015; **11**: 1949-1959 [PMID: 26282383 DOI: 10.1016/j.nano.2015.07.016]

96 **Zhang W**, Dong R, Diao S, Du J, Fan Z, Wang F. Differential long noncoding RNA/mRNA expression profiling and functional network analysis during osteogenic differentiation of human bone marrow mesenchymal stem cells. *Stem Cell Res Ther* 2017; **8**: 30 [PMID: 28173844 DOI: 10.1186/s13287-017-0485-6]

97 **Saha S**, Yang XB, Tanner S, Curran S, Wood D, Kirkham J. The effects of iron oxide incorporation on the chondrogenic potential of three human cell types. *J Tissue Eng Regen M* 2013; **7**: 461-469 [PMID: 22396122 DOI: 10.1002/term.544]

98 **Riegler J**, Liew A, Hynes SO, Ortega D, O'Brien T, Day RM, Richards T, Sharif F, Pankhurst QA, Lythgoe MF. Superparamagnetic iron oxide nanoparticle targeting of MSCs in vascular injury. *Biomaterials* 2013; **34**: 1987-1994 [PMID: 23237516 DOI: 10.1016/j.biomaterials.2012.11.040]

99 **Ikuta Y**, Kamei N, Ishikawa M, Adachi N, Ochi M. In Vivo Kinetics of Mesenchymal Stem Cells Transplanted into the Knee Joint in a Rat Model Using a Novel Magnetic Method of Localization. *Cts Clin Transl Sci* 2015; **8**: 467-474 [PMID: 25963065 DOI: 10.1111/cts.12284]

100 **Hill BS**, Pelagalli A, Passaro N, Zannetti A. Tumor-educated mesenchymal stem cells promote pro-metastatic phenotype. *Oncotarget* 2017; **8**: 73296-73311 [PMID: 29069870 DOI: 10.18632/oncotarget.20265]

101 **Monsalve A**, Rodriguez AC, Rinaldi C, Dobson J. Remotely Triggered Activation of TGF- With Magnetic Nanoparticles. *IEEE Magn Lett* 2015; **6**: 1-4 [DOI: 10.1109/LMAG.2015.2477271]

102 **Aleynik A**, Gernavage KM, Mourad YS. Stem cell delivery of therapies for brain disorders. *Clin Transl Med* 2014; **3**: 24 [PMID: 25097727 DOI: 10.1186/2001-1326-3-24]

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REVIEW

Murine models based on acute myeloid leukemia-initiating stem cells xenografting

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Abstract

Acute myeloid leukemia (AML) is an aggressive malignant disease defined by abnormal expansion of myeloid blasts. Despite recent advances in understanding AML pathogenesis and identifying their molecular subtypes based on somatic mutations, AML is still characterized by poor outcomes, with a 5-year survival rate of only 30%-40%, the majority of the patients dying due to AML relapse. Leukemia stem cells (LSC) are considered to be at the root of chemotherapeutic resistance and AML relapse. Although numerous studies have tried to better characterize LSCs in terms of surface and molecular markers, a specific marker of LSC has not been found, and still the most universally accepted phenotypic signature remains the surface antigens CD34+CD38- that is shared with normal hematopoietic stem cells. Animal models provides the means to investigate the factors responsible for leukemic transformation, the intrinsic differences between secondary post-myeloproliferative neoplasm AML and *de novo* AML, especially the signaling pathways involved in inflammation and hematopoiesis. However, AML proved to be one of the hematological malignancies that is difficult to engraft even in the most immunodeficient mice strains, and numerous ongoing attempts are focused to develop "humanized mice" that can support the engraftment of LSC. This present review is aiming to in-

troduce the field of AML pathogenesis and the concept of LSC, to present the current knowledge on leukemic blasts surface markers and recent attempts to develop best AML animal models.

Key words: Acute myeloid leukemia; Leukemia-initiating stem cells; Antigen markers; Murine models; Xenografts

Core tip: The review is aiming to introduce the field of acute myeloid leukemia (AML) pathogenesis, the concept of leukemic stem cells, and also to present the current attempts to develop best AML animal models as means to investigate the factors responsible for leukemic transformation. Due to difficulties in engraftment of less aggressive AML samples, it is currently being attempted to develop humanized mice by introducing supporting human stromal cells as a source of proper cytokines, in a challenge to mimic an appropriate bone marrow niche able to support leukemic stem cells engraftment.

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INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive cancer characterized by unrestricted proliferation of functionally immature myeloid cells. High heterogeneity and variable expansion capacity of multiple clones within each patient[1,2], clinical and molecular differences between de novo and secondary AML, complicate even more treatment choices and make targeted therapy a goal yet to far to reach without using models that are able to simplify the multitude of mechanisms that might be involved in leukemogenesis.

In the last decades, murine models become very important tools in the field of preclinical research in oncology, hematology, and immunology, providing a platform for study of tumor biology and for *in vivo* evaluation of drugs in patient-derived xenograft tumors (PDX). Nowadays, a large variety of immunodeficient mice strains have emerged, able to support the xenografting and development of a complex human hemato-lymphoid system. The most difficult to reproduce is the immune system and the bone marrow (BM) microenvironment, mostly because of the differences between the signaling molecules responsible for the maturation of different hematopoietic cell populations[3]. Although the field of animal models has experienced a recent exponential growth through the development of IL2rg^{null} immunodeficiency mice, AML remains one of the hematologic malignancies difficult to engraft into the existing strains of mice due to the lack of a proper BM niche and absence of specific human growth factors and supporting stromal cells[4]. As a result several attempts were made to develop "humanized mice" that can better support myeloid leukemia-initiating stem cells xenografting.

This review is aiming to introduce AML pathogenesis and the concept of leukemic stem cells and the current most advanced strategies to overcome challenges in obtaining AML murine models.

AML PATHOGENESIS AND THE CONCEPT OF LEUKEMIC STEM CELLS

AML is a heterogeneous hematopoietic malignancy defined by clonal expansion of abnormally differentiated or undifferentiated myeloid progenitors (blasts) that accumulate in the BM and impair hematopoiesis, leading to multi-lineage cytopenias[5,6]. Blasts can also migrate from BM into peripheral blood and infiltrate other tissues[5].

AML can be divided in 3 categories taking into account their clinical ontogeny: Secondary AML (s-AML) occurred after leukemic transformation of a pre-existing myelodysplastic syndrome or myeloproliferative neoplasm, therapy-related AML (t-AML) developed in patients that received leukemogenic chemotherapy for antecedent non-myeloid malignancies and *de novo* AML generated in the absence of a previous stem cell disorder or a therapeutic exposure to cytotoxic drugs[7].

Despite recent progress in understanding AML pathogenesis and recognizing molecular subtypes of AML that have prognostic impact, AML is still characterized by poor outcomes, with a 5-year survival rate of only 30%-40%. The dismal prognosis is mainly related to high rate of relapse and refractory disease[2,8]. Patients with s-AML and t-AML display even a much worse prognosis, the median overall survival rate being 7 months. Notably, somatic mutations in *SRSF2, SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR*, or *STAG2* proved to be highly specific for s-AML. They are acquired early in leukemogenesis and tend to persist during clonal remissions[7].

Increasing evidence support the concept that a minor population of stem cells, named leukemia stem cells (LSCs), is responsible for leukemia initiation, disease progression and relapse, as well as drug resistance[9]. AML was among the first diseases in which the existence of cancer stem cells was documented using xenograft animal models[10].

LSCs are derived from transformed hematopoietic stem cells (HSCs) or downstream committed progenitors[11]. They are able to initiate the disease after transplantation into immunodeficient mice and are characterized by both unlimited self-renewal potential inducing disease in serial transplantation and capacity to

partially differentiate into non-LSC blasts that lack self-renewal properties and possibility of engraftment[12]. Although LSCs and non-LSC blasts harbor a common set of mutations there are epigenetic differences between them. A predominant hypo-methylation of *HOXA* gene cluster that has been involved in leukemogenesis represents a main feature of LSCs[13].

It is thought that, similarly to normal hematopoietic system, AML displays a hierarchical organization with LSCs on the top, being able to generate the whole population of AML blast cells[10]. Signaling pathways that control self-renewal of HSCs, such as Wnt/β-catenin, PI3K/Akt/mTOR, or Hedgehog, are also involved in LSC survival and expansion and can serve as therapeutic targets to facilitate eradication of LSCs[8,11]. Moreover, LSCs might escape apoptosis through up-regulation of NF-κB or downregulation of Fas/CD95. Additionally, CXCL12-CXCR4 axis promotes retaining of LSCs within the protective BM microenvironment[11].

The existence of a preleukemic stage in AML was proven by isolating from leukemia patients a population of HSCs that was found to bear some, but not all, of the mutations identified in the downstream leukemia. These preleukemic HSCs, that can be distinguished from LSCs by the surface antigen markers, TIM3 and CD99, are capable to generate bi-lineage engraftment in NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl} (NSG) mice and the derived lymphoid and myeloid progeny display the same preleukemia mutations[14]. According to the currently proposed model of preleukemic clonal evolution, the first leukemia-related mutation has to occur in a cell that possesses self-renewal capacity or, alternatively, must confer self-renewal properties to a more differentiated progenitor[15]. By single-cell analysis it was shown that during the process of leukemogenesis, the preleukemic HSCs gradually acquire somatic mutations in a nonrandom pattern[16]. Thus, in the early phases of AML evolution there is enrichment for mutations in epigenetic modifiers such as *TET2*, *DNMT3A, IDH1/2*, and *ASXL1*. On the other hand, mutations in genes involved in signaling pathways and proliferation, such as *FLT3* and *KRAS* occur in later stages. Other leukemogenic mutations in genes like *NPM1*, *CEBPA*, and *WT1* can be found in preleukemic phase as well as in later stages[15,16].

The preleukemic HSCs that eventually give rise to AML persist in patient samples at diagnosis and are resistant to current chemotherapy, thus representing a source of disease recurrence[17].

LEUKEMIA-INITIATING STEM CELLS AND BLASTS

The identification of LSCs in AML plays an important role in disease diagnosis, prognosis and AML therapy monitoring, and also represents an important step in development of targeted therapy and drug discovery[9]. Although initial studies suggested that LSCs were CD34+CD38- and did not expressed other lineage markers[18-21], later studies proved that the LSC phenotype was more complex and heterogeneous[22,23]. At present, it is established that LSCs are characterized by increased or decreased expression of surface markers of normal myeloid precursors (CD34, CD38, CD33, CD13, CD117, and CD123), asynchronous expression of antigens determined by AML morphological subtype and by the LSC stage of differentiation (CD4, CD11b, CD14, CD15, CD36, CD61, CD64, CD71, *etc*.), as well as by aberrant expression of lymphoid antigens (cross-lineage expression) (CD2, CD5, CD7, CD19, CD22, CD56, Tim3, *etc*.)[24-30]. LSCs reside mainly in CD34+CD38- population, but may be present also in other cellular fractions, usually CD34+CD38+, and in some cases, in CD34- population[12,31]. Additional markers, more specific for the advanced characterization of cellular subpopulations in AML, include: CD90[32], CD96[33], CD123[34,35], CD47[36], CD44, C-type lectin-like molecule-1 (CLL1)[37], aldehyde dehydrogenase, *etc*[38]. Currently, standard diagnosis and sub-classification of AML integrate the study of cell morphology, genetics/cytogenetics and multi-parametric immuno-phenotyping. The antibody panels for surface markers used for sub-classification of each AML group are showed in Figure 1.

Methods commonly used to assess HSC properties are colony-forming cell (CFC) assay, long term culture (LTC), flow cytometry and competitive repopulation.

The CFC assay is an important tool used to evaluate the ability of the progenitor cells to proliferate and differentiate into multiple lineages. In order to produce colonies, cells are cultured in a semisolid medium, in the presence of appropriate cytokines for 7-14 d. Colonies are counted and characterized according to morphologic and phenotypic criteria. Although this short-term colony assay can determine the frequencies of hematopoietic progenitor cells in analyzed populations, still, it is not able to detect more immature progenitors or HSCs/LSCs. To overcome this limitation, the cells can be cultured for 5-8 wk on a stromal feeder layer that can provide a substrate and a source of cytokines and growth factors, in effort to mimic the *in vivo* niche conditions[39]. The long-term culture-initiating cells (LTC-ICs) can be evaluated by their capacity to generate CFCs in culture supernatant after 5 wk. This period allows CFCs present in the inoculum to terminally differentiate and the remaining CFCs may represent the progeny of LTC-ICs. Subsequent limiting dilution tests can be performed to determine the LTC-IC frequency[40]. Although this method facilitates the detection of more immature progenitor cells, it is time consuming and the presence of stromal cells can induce procedure variations and different outcomes[41,42].

Competitive repopulation represents the best method to assess the functional abilities of immature progenitor/stem cells by serial transplants in immunocompromised mice. This method is based on the ability

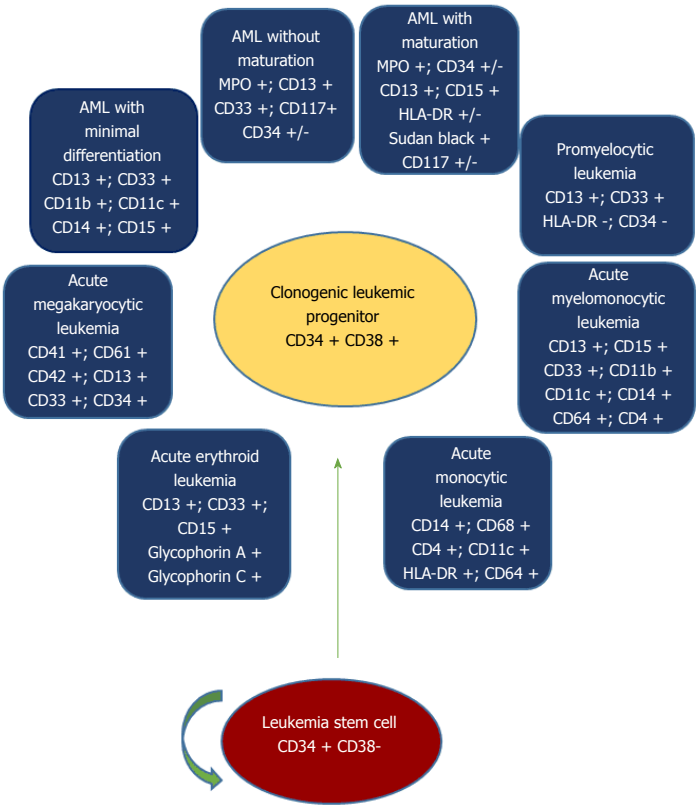

Figure 1 Advanced characterization of leukemic stem cell, clonogenic leukemic progenitors and various cellular subpopulations in acute myeloid leukemia. AML: Acute myeloid leukemia.

of cells that are investigated to compete with non-manipulated standard cells to repopulate the BM of an irradiated recipient[43].

MURINE MODELS - WHICH ARE THE BEST CHOICE?

Animal models are used as replacement for human biological niches due to ethical restrictions in the use of human tissue samples from donors. Moreover, animal models accurately recapitulate human disease and have been an important tool in advancing the understanding of human pathology, and development of pre-clinical therapy. Small animals, such as mice and rats, are often used as a model for various diseases because of their ease in breeding, maintenance, and manipulation. In spite of these many advantages, there are limitations due to the disparities between the murine and human biological systems. Human immune system and the BM microenvironment are the most difficult to be reproduced in mouse models because of the differences in the signaling molecules responsible for the maturation of various hematopoietic cell populations[3]. As a result, many malignant hematopoietic and other hematologic disorders do not successfully engraft in conventional mice models.

AML is one of these hematologic malignancies that fail to properly graft into the existing strains of mice due to the lack of a proper BM niche, homing elements, absence of specific human growth factors and supporting stromal cells[4]. As a result, several attempts have been made to develop murine models that reproduce with fidelity human hematopoiesis, particularly the development of the myeloid line.

Early attempts to increase the support for myelopoiesis involved the use of mice injected with IL-3, GM-CSF, SCF[44], mice producing human TPO[45] or MISTRG mice strain which produces human tumor necrosis factor and IL-6[46]. These confirmed that the introduction of human genes into mice led to the production of functional proteins capable of supporting engraftment and proliferation of human grafts.

The following attempts were aimed to develop next-generation mouse models genetically engineered to support myeloid differentiation from human HSC. Thus, it was necessary to act at three major levels in order to induce tolerance in the murine host, provide a supportive niche, and support hematopoiesis/proliferation with appropriate growth factors and cytokines.

The first request was fulfilled by the development of mice that lacked the adaptive and innate immune compartment like NSG and NODShi.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug} mice strains. These strains were developed on NOD *scid* immunodeficient mice by modifying them to bear mutations in the IL-2 receptor gamma chain gene th-

at induced either the absence or the presence of an nonfunctional truncated form of the receptor subunit. The gamma chain subunit is a major component of the IL2, IL4, IL7, IL9, IL15 and IL21 receptors, and is indispensable for binding and signaling of these cytokines[47,48].

The second condition, involved the ablation of mouse cells to create open niches for human transplanted cells. These were achieved through irradiation or depletion of mouse stroma *via* introducing mutations in *c-Kit* gene encoding for SCF receptor. SCF plays an important role in the maintenance and differentiation of HSCs[49]. The c-kit mutated mice strain, known as NODB6.SCID Il2rγ$^{-/-}$ Kit (W41/W41) (NBSGW) mice, supports engraftment studies with human HSCs without prior irradiation. McIntosh *et al*[49] showed that in peripheral blood, the median human CD45+ count in non-irradiated NBSGW mice was similar to the count in irradiated NSG. In BM a significant increase in CD45+ was observed in non-irradiated NBSGW (97%) compared to non-irradiated NSG (30%).

The third constraint regarding the need for supportive myeloid cytokines was overcome using animal models with transgenic expression of hSCF, hGM-CSF and hIL-3 on the NOD SCID background resulting the NSG-SGM3 mouse strain [also known as NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}Tg(CMV-IL3,CSF2,KITLG)/1Eav)]. Wunderlich *et al*[4] reported the development and use of NSG-SGM3 mouse for the engraftment of normal CD34+ and AML xenografts. The results showed an improvement in the expansion of normal human myeloid cells, as well as an enhanced engraftment of primary human AML samples. They injected five samples of primary AML in sub-lethally irradiated NGS and NSG-SGM3 mice. Only three samples from five engrafted in NSG mice, compared to five samples in NSG-SGM3 mice, showing variability in the engraftment potential across the AML samples, and that NSG-SGM3 strain was a better host for some subsets of AML, relative to NSG mice. Moreover, three of five samples with primary AML had higher BM engraftment level in NSG-SGM3[4]. Similar results regarding variable engraftment potential in immunodeficient mice strains were obtained by Klco *et al*[50] They injected blasts from six AML samples in tail vein of non-irradiated NSG and NSG-SGM3. The results showed that four samples had higher BM engraftment and CD34+ expression level in NSG-SGM3 than NSG mice.

Finally, we can conclude that next-generation humanized mouse models are able to support myeloid blast development and differentiation.

XENOGRAFT MOUSE MODELS USED FOR ACUTE MYELOID LEUKEMIA

Cell line derived xenografts

AML represents a heterogeneous disease including several subtypes which are characterized by specific fusion oncogenes as a result of chromosome abnormalities. The fusion oncogenes in AML are associated with different clinical and laboratory characteristics, highlighting the different ways of malignant transformation in this disease. A study focused on the evaluation of four important AML fusion oncogenes reported that MLL-AF9 and NUP98-HOXA9 had very similar effects *in vitro* on primary human CD34+ cells, resulting in erythroid hyperplasia and an obvious blockage in erythroid and myeloid maturation while AML1-ETO and PML-RARA produced only modest effects on myeloid and erythroid differentiation. Moreover, MLL-AF9, NUP98-HOXA9 and AML1-ETO fusion oncogenes generated a significant increase in long-term proliferation and self-renewal of CD34+ cells. The characterization of gene profiles determined by AML fusion oncogenes can be considered an important tool for the discovery of new potential drug targets. In this study, two different time patterns of gene deregulation as result of fusion of these oncogenes were observed: MLL-AF9 and NUP98-HOXA9, caused gene deregulation 3 d after transduction, while gene deregulation by AML1-ETO and PML-RARA appeared within 6 h. Interestingly, p53 inhibitor MDM2 was upregulated by AML1-ETO at 6 h suggesting that MDM2 upregulation was involved in cell transformation, being related to AML1-ETO[51].

Wei *et al*[52] evaluated the *in vitro* and *in vivo* effects of *MLL-AF9* gene fusion in human CD34+ cord blood cells using retroviral vectors. Thus, MA9 transduced cells became immortal and doubled in number every 2-3 d. The expression of CD33, CD11b, CD13, CD14 and CD15 suggested a myelo-monocytic lineage. Moreover, long-term cultured MA9 cells failed to differentiate towards the erythroid or B lymphoid lineages, remaining cytokine and FLT3L dependent for growth. In non-obese diabetic/severe combined immunodeficient [NOD/SCID (NS)], NS-β2M-/- (NS-B2M) and NS mice, MA9 cells induced acute myeloid, lymphoid, or mixed-lineage leukemia with blast cells present in the peripheral blood, BM, spleen and liver. Gene expression profile of MLL-AF9 transduced cells was similar to human AML with 11q23 translocations, Rac signaling pathway being the most affected pathway and a promising therapeutic target in MLL-rearranged AML[52].

Another AML subtype with a particularly poor outcome is characterized by the t(6;9)(p22;q34) chromosome rearrangement which generates DEK-NUP214 chimeric gene. Qin *et al*[53] developed an AML model harboring DEK-NUP214, using CD34+ human hematopoietic progenitor cells and MO7e cell lines xenografted into immunocompromised mice that expressed human myeloid cell growth factors. The M07e human megakaryoblastic leukemia cell line was strictly dependent on either IL-3 or GM-CSF for survival; retroviral expression of this fusion gene in IL-3 dependent M07e cell line induced a cytokine independence and increased colony formation ability in soft-agar. DEK-NUP214 expression also modified the differentiation of human cord blood CD34+ progenitor

cells, which expressed myeloid lineage markers (CD13+), with small subsets showing T- (CD3+) and B- (CD19+) cell linage markers. The obtained results suggested that DEK-NUP214 was involved in leukemic transformation and differentiation of myeloid cells. In this study, CD34+ progenitor cells obtained from three different umbilical cord blood samples and transduced with chimeric DEK-NUP214 were engrafted in NSG-SGM3 mice strain. Interestingly, two months after transplantation, almost 20% of peripheral blood cells from the transplanted mice displayed a human-specific CD45 immuno-phenotype with CD45+CD13+CD34+CD38+ cells. The analysis of peripheral blood smears also showed the typical human AML cell morphology with a larger nucleus and reduced cytoplasmic ratio. Therefore, the study demonstrated that DEK-NUP214 could transform human CD34+ progenitor cells and induced human AML *in vivo*. Gene profiling of this model revealed that several genes of HOX family (*HOXA9, 10, B3, B4* and *PBX3*) were highly upregulated. In this AML model pathways involving KRAS, BRCA1 and ALK were significantly dysregulated[53].

Similar results were obtained in case of t(8;13)-(p11;q12) chromosome translocation which led to ZMYM2-FGFR1 chimeric kinase, characteristic for another AML subtype. Human CD34+ cells harboring ZMYM2-FGFR1 transplanted into immune-compromised mice developed myeloproliferative disease that progressed to AML. Mice displayed hepatosplenomegaly, hypercellular BM and a CD45 + CD34 + CD13+ immunophenotype[54].

Preclinical cancer research remains essential for the discovery and the development of new therapies in case of the most advanced cancers. Various cancer cell lines have been developed and used for the study of cancer but with a great disadvantage that they do not really reflect the behavior of the original cancer cells, due to the artificial nature of their culture conditions.

Patient derived xenografts - patient stem-cells derived xenografts

PDX models established by transplanting patient cancer cells into immunocompromised mice represent an important tool in cancer research. They have a great potential to offer important information on cancer biology and to guide the therapeutic approach. Unlike cell lines derived from primary tumors that might have lost their original characteristics due to a prolonged *in vitro* growth, PDX mouse models seemed to be able to overcome this issue[55,56]. Many studies demonstrated that PDX models kept the most important features of the original tumor including histology, genomic pattern, cellular heterogeneity, and more important, drug responsiveness or personalized drug selection[57].

The development of PDX models of AML allows us to monitor *in vivo* the progression of the disease and to evaluate the efficacy of an experimental treatment on tumor growth using imaging techniques[58].

A first full study on the engraftment ability of a large cohort of AML samples in immunodeficient animal models was published by Kennedy *et al*[59] who transplanted BM or peripheral blood cells from 307 AML patients intra-femorally into sublethally irradiated NOD. SCID mice pre-treated with an anti-CD122 antibody. AML xenografts were obtained in 44% of cases, leukemic engraftment being associated with a higher white cell count in peripheral blood (mean of 92 x 10^9/L in engrafters *vs* 67 x 10^9/L in non-engrafters, $P = 0.01$). Moreover, results showed that complete remission was achieved in only 51% of patients whose diagnostic samples established AML xenografts, compared to 80% of non-engrafting samples ($P < 0.0001$). As a conclusion, AML xenografting was successful when using samples from AML patients with aggressive disease and with a poor response to standard induction therapy.

OVERCOMING CHALLENGES IN PATIENT-DERIVED XENOGRAFTS OF AML

Recent experiments are trying to improve mouse experimental models of AML, aiming to engraft with a higher success rate even less aggressive leukemia samples. Most of them are based on immunodeficient mice with humanized microenvironment created by injection of human mesenchymal stem cell (MSC) that provide a better niche for leukemic blast engraftment.

An interesting approach was that of Reinisch *et al*[60], who used human MSC grafts injected subcutaneously in NSG mice to form a humanized microenvironment named "ossicles", in which they subsequently injected (8 wk later) human HSCs and AML blasts. The final aim was to analyze the engraftment capacity the resulting niche. After 6-10 wk, the "ossicles" showed human BM–like functions and morphology and allowed enhanced engraftment of primary patient-derived AML.

A further attempt employed humanized niches based on genetically modified MSCs to express huIL-3 and TPO. Carretta *et al*[61] implanted subcutaneously these human MSCs in ceramic scaffolds or Matrigel in NGS mice, and 6 to 8 wk later transplanted CD34+ enriched AML blasts in the ectopically engineered BM niches. The engraftment capacity was then compared with the one from non-engineered MSC niches. The results showed that leukemic blasts efficiently engrafted in both models with no significant differences. An unexpected result was that CD33+-sorted myeloid clones from the animal model failed to self-renew in secondary recipients, probably due to overexpressed IL-3 and TPO cytokines from modified microenvironment that might have affected a proper self-renewal of myeloid blasts.

An important challenge for obtaining mouse models valuable as preclinical models is the capability of PDX cells to authentically mimic the heterogeneity of the initial disease. The xenograft mouse model of AML has been used mainly to study primary transplantation and further serial experiments were performed to

verify self-renewal competence or stability of gene expression profiles of engrafted cells. However, this model was rarely employed to investigate deeply AML biology or therapy[62]. Most of the published results revealed that PDX cells resembled the primary samples in terms of gene expression profiles but sub-clonal profiles were often not reflecting the primary sample. Another important drawback was the inability of the most proposed models to sensitively and repetitively monitor disease progression or drug effects. These were determined at single time points by invasive procedures or post mortem. However, researchers tried to overcome these challenges by proposing a better control of PDX cells. This control aimed to check the pattern of alterations in mutational or antigen expression possibly occurred during engraftment. For better monitoring disease progression or drug effects, recombinant luciferase enabled bioluminescence *in vivo* imaging has been proposed to facilitate *in vivo* monitoring of PDX AML cells as a quantitative, sensitive, reliable method for quantifying leukemia initiating cells[62].

CONCLUSION

Mouse models were of tremendous importance for understanding the molecular etiology of leukemia, proven to be valuable tools to facilitate preclinical *in vivo* studies.

Most of the studies verified that PDX models kept the most important features of the original tumor. However, mouse models should be controlled more carefully before and after xenotransplantation, especially in serial transplantation experiments, in order to ensure that the heterogeneity of the original sample is conserved and genetic drift is not modifying genetic, phenotypic or functional characteristics of the original disease.

Prospectively, advancements allowing repetitive, reliable, sensitive and fast studies, able to evaluate the efficacy of an experimental treatment in well genetically defined and heterogeneous subgroups of AML, will represent valuable tools to improve the individualized xenograft mouse model of AML and drastically reduce the number of mice to be used in these kind of experiments.

REFERENCES

1 **Bowman RL**, Busque L, Levine RL. Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies. *Cell Stem Cell* 2018; **22**: 157-170 [PMID: 29395053 DOI: 10.1016/j.stem.2018.01.011]

2 **Papaemmanuil E**, Döhner H, Campbell PJ. Genomic Classification in Acute Myeloid Leukemia. *N Engl J Med* 2016; **375**: 900-901 [PMID: 27579651 DOI: 10.1056/NEJMc1608739]

3 **Walsh NC**, Kenney LL, Jangalwe S, Aryee KE, Greiner DL, Brehm MA, Shultz LD. Humanized Mouse Models of Clinical Disease. *Annu Rev Pathol* 2017; **12**: 187-215 [PMID: 27959627 DOI: 10.1146/annurev-pathol-052016-100332]

4 **Wunderlich M**, Chou FS, Link KA, Mizukawa B, Perry RL, Carroll M, Mulloy JC. AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. *Leukemia* 2010; **24**: 1785-1788 [PMID: 20686503 DOI: 10.1038/leu.2010.158]

5 **Estey EH**. Acute myeloid leukemia: 2014 update on risk-stratification and management. *Am J Hematol* 2014; **89**: 1063-1081 [PMID: 25318680 DOI: 10.1002/ajh.23834]

6 **Döhner H**, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. *N Engl J Med* 2015; **373**: 1136-1152 [PMID: 26376137 DOI: 10.1056/NEJMra1406184]

7 **Lindsley RC**, Mar BG, Mazzola E, Grauman PV, Shareef S, Allen SL, Pigneux A, Wetzler M, Stuart RK, Erba HP, Damon LE, Powell BL, Lindeman N, Steensma DP, Wadleigh M, DeAngelo DJ, Neuberg D, Stone RM, Ebert BL. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood* 2015; **125**: 1367-1376 [PMID: 25550361 DOI: 10.1182/blood-2014-11-610543]

8 **Stahl M**, Kim TK, Zeidan AM. Update on acute myeloid leukemia stem cells: New discoveries and therapeutic opportunities. *World J Stem Cells* 2016; **8**: 316-331 [PMID: 27822339 DOI: 10.4252/wjsc.v8.i10.316]

9 **Ding Y**, Gao H, Zhang Q. The biomarkers of leukemia stem cells in acute myeloid leukemia. *Stem Cell Investig* 2017; **4**: 19 [PMID: 28447034 DOI: 10.21037/sci.2017.02.10]

10 **Pollyea DA**, Gutman JA, Gore L, Smith CA, Jordan CT. Targeting acute myeloid leukemia stem cells: a review and principles for the development of clinical trials. *Haematologica* 2014; **99**: 1277-1284 [PMID: 25082785 DOI: 10.3324/haematol.2013.085209]

11 **Wang X**, Huang S, Chen JL. Understanding of leukemic stem cells and their clinical implications. *Mol Cancer* 2017; **16**: 2 [PMID: 28137304 DOI: 10.1186/s12943-016-0574-7]

12 **Thomas D**, Majeti R. Biology and relevance of human acute myeloid leukemia stem cells. *Blood* 2017; **129**: 1577-1585 [PMID: 28159741 DOI: 10.1182/blood-2016-10-696054]

13 **Jung N**, Dai B, Gentles AJ, Majeti R, Feinberg AP. An LSC epigenetic signature is largely mutation independent and implicates the HOXA cluster in AML pathogenesis. *Nat Commun* 2015; **6**: 8489 [PMID: 26444494 DOI: 10.1038/ncomms9489]

14 **Mitchell GH**. A vaccine for ovine cysticercosis. *Vaccine* 1989; **7**: 379 [PMID: 2815974 DOI: 10.1016/0264-410X(89)90146-1]

15 **Corces MR**, Chang HY, Majeti R. Preleukemic Hematopoietic Stem Cells in Human Acute Myeloid Leukemia. *Front Oncol* 2017; **7**: 263 [PMID: 29164062 DOI: 10.3389/fonc.2017.00263]

16 **Corces-Zimmerman MR**, Hong WJ, Weissman IL, Medeiros BC, Majeti R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proc Natl Acad Sci U S A* 2014; **111**: 2548-2553 [PMID: 24550281 DOI: 10.1073/pnas.1324297111]

17 **Shlush LI**, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, Kennedy JA, Schimmer AD, Schuh AC, Yee KW, McLeod JL, Doedens M, Medeiros JJ, Marke R, Kim HJ, Lee K, McPherson JD, Hudson TJ; HALT Pan-Leukemia Gene Panel Consortium, Brown AM, Yousif F, Trinh QM, Stein LD, Minden MD, Wang JC, Dick JE. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* 2014; **506**: 328-333 [PMID: 24522528 DOI: 10.1038/nature13038]

18 **Bonnet D**, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730-737 [PMID: 9212098 DOI: 10.1038/nm0797-730]

19 **Blair A**, Hogge DE, Sutherland HJ. Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(-)/HLA-DR-. *Blood* 1998; **92**: 4325-4335 [PMID: 9834239]

20 **Bhatia M**, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A* 1997; **94**: 5320-5325 [PMID: 9144235 DOI: 10.1073/pnas.94.10.5320]

21 **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105-111 [PMID: 11689955 DOI: 10.1038/35102167]

22 **Sarry JE**, Murphy K, Perry R, Sanchez PV, Secreto A, Keefer C, Swider CR, Strzelecki AC, Cavelier C, Récher C, Mansat-De Mas V, Delabesse E, Danet-Desnoyers G, Carroll M. Human acute myelogenous leukemia stem cells are rare and heterogeneous when

assayed in NOD/SCID/IL2Rγc-deficient mice. *J Clin Invest* 2011; **121**: 384-395 [PMID: 21157036 DOI: 10.1172/JCI41495]
23 **Taussig DC**, Vargaftig J, Miraki-Moud F, Griessinger E, Sharrock K, Luke T, Lillington D, Oakervee H, Cavenagh J, Agrawal SG, Lister TA, Gribben JG, Bonnet D. Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. *Blood* 2010; **115**: 1976-1984 [PMID: 20053758 DOI: 10.1182/blood-2009-02-206565]
24 **Campos L**, Guyotat D, Archimbaud E, Devaux Y, Treille D, Larese A, Maupas J, Gentilhomme O, Ehrsam A, Fiere D. Surface marker expression in adult acute myeloid leukaemia: correlations with initial characteristics, morphology and response to therapy. *Br J Haematol* 1989; **72**: 161-166 [PMID: 2757962 DOI: 10.1111/j.1365-2141.1989.tb07677.x]
25 **Ouyang J**, Goswami M, Tang G, Peng J, Ravandi F, Daver N, Routbort M, Konoplev S, Lin P, Medeiros LJ, Jorgensen JL, Wang SA. The clinical significance of negative flow cytometry immunophenotypic results in a morphologically scored positive bone marrow in patients following treatment for acute myeloid leukemia. *Am J Hematol* 2015; **90**: 504-510 [PMID: 25732229 DOI: 10.1002/ajh.23988]
26 **Saultz JN**, Garzon R. Acute Myeloid Leukemia: A Concise Review. *J Clin Med* 2016; **5**: pii: E33 [PMID: 26959069 DOI: 10.3390/jcm5030033]
27 **Wolach O**, Stone RM. How I treat mixed-phenotype acute leukemia. *Blood* 2015; **125**: 2477-2485 [PMID: 25605373 DOI: 10.1182/blood-2014-10-551465]
28 **Ho TC**, LaMere M, Stevens BM, Ashton JM, Myers JR, O'Dwyer KM, Liesveld JL, Mendler JH, Guzman M, Morrissette JD, Zhao J, Wang ES, Wetzler M, Jordan CT, Becker MW. Evolution of acute myelogenous leukemia stem cell properties after treatment and progression. *Blood* 2016; **128**: 1671-1678 [PMID: 27421961 DOI: 10.1182/blood-2016-02-695312]
29 **Ossenkoppele GJ**, van de Loosdrecht AA, Schuurhuis GJ. Review of the relevance of aberrant antigen expression by flow cytometry in myeloid neoplasms. *Br J Haematol* 2011; **153**: 421-436 [PMID: 21385170 DOI: 10.1111/j.1365-2141.2011.08595.x]
30 **Grimwade D**, Freeman SD. Defining minimal residual disease in acute myeloid leukemia: which platforms are ready for "prime time"? *Blood* 2014; **124**: 3345-3355 [PMID: 25049280 DOI: 10.1182/blood-2014-05-577593]
31 **Kreso A**, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell* 2014; **14**: 275-291 [PMID: 24607403 DOI: 10.1016/j.stem.2014.02.006]
32 **Buccisano F**, Rossi FM, Venditti A, Del Poeta G, Cox MC, Abbruzzese E, Rupolo M, Berretta M, Degan M, Russo S, Tamburini A, Maurillo L, Del Principe MI, Postorino M, Amadori S, Gattei V. CD90/Thy-1 is preferentially expressed on blast cells of high risk acute myeloid leukaemias. *Br J Haematol* 2004; **125**: 203-212 [PMID: 15059143 DOI: 10.1111/j.1365-2141.2004.04883.x]
33 **Hosen N**, Park CY, Tatsumi N, Oji Y, Sugiyama H, Gramatzki M, Krensky AM, Weissman IL. CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proc Natl Acad Sci USA* 2007; **104**: 11008-11013 [PMID: 17576927 DOI: 10.1073/pnas.0704271104]
34 **Vergez F**, Green AS, Tamburini J, Sarry JE, Gaillard B, Cornillet-Lefebvre P, Pannetier M, Neyret A, Chapuis N, Ifrah N, Dreyfus F, Manenti S, Demur C, Delabesse E, Lacombe C, Mayeux P, Bouscary D, Recher C, Bardet V. High levels of CD34+CD38low/-CD123+ blasts are predictive of an adverse outcome in acute myeloid leukemia: a Groupe Ouest-Est des Leucemies Aigues et Maladies du Sang (GOELAMS) study. *Haematologica* 2011; **96**: 1792-1798 [PMID: 21933861 DOI: 10.3324/haematol.2011.047894]
35 **Zhi L**, Wang M, Rao Q, Yu F, Mi Y, Wang J. Enrichment of N-Cadherin and Tie2-bearing CD34+/CD38-/CD123+ leukemic stem cells by chemotherapy-resistance. *Cancer Lett* 2010; **296**: 65-73 [PMID: 20444543 DOI: 10.1016/j.canlet.2010.03.021]
36 **Majeti R**, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD Jr, van Rooijen N, Weissman IL. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell* 2009; **138**: 286-299 [PMID: 19632179 DOI: 10.1016/j.cell.2009.05.045]
37 **van Rhenen A**, van Dongen GA, Kelder A, Rombouts EJ, Feller N, Moshaver B, Stigter-van Walsum M, Zweegman S, Ossenkoppele GJ, Jan Schuurhuis G. The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood* 2007; **110**: 2659-2666 [PMID: 17609428 DOI: 10.1182/blood-2007-03-083048]
38 **Zhou J**, Chng WJ. Identification and targeting leukemia stem cells: The path to the cure for acute myeloid leukemia. *World J Stem Cells* 2014; **6**: 473-484 [PMID: 25258669 DOI: 10.4252/wjsc.v6.i4.473]
39 **Coulombel L**. Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. *Oncogene* 2004; **23**: 7210-7222 [PMID: 15378081 DOI: 10.1038/sj.onc.1207941]
40 **Bock TA**. Assay systems for hematopoietic stem and progenitor cells. *Stem Cells* 1997; **15** Suppl 1: 185-195 [PMID: 9368340 DOI: 10.1002/stem.5530150824]
41 **Frisch BJ**, Calvi LM. Hematopoietic stem cell cultures and assays. *Methods Mol Biol* 2014; **1130**: 315-324 [PMID: 24482184 DOI: 10.1007/978-1-62703-989-5_24]
42 **Clarke CJ**, Holyoake TL. Preclinical approaches in chronic myeloid leukemia: from cells to systems. *Exp Hematol* 2017; **47**: 13-23 [PMID: 28017647 DOI: 10.1016/j.exphem.2016.11.005]
43 **Ramos CA**, Venezia TA, Camargo FA, Goodell MA. Techniques for the study of adult stem cells: be fruitful and multiply. *Biotechniques* 2003; **34**: 572-578, 580-584, 586-591 [PMID: 12661162]
44 **Kamel-Reid S**, Dick JE. Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science* 1988; **242**: 1706-1709 [PMID: 2904703 DOI: 10.1126/science.2904703]
45 **Rongvaux A**, Willinger T, Takizawa H, Rathinam C, Auerbach W, Murphy AJ, Valenzuela DM, Yancopoulos GD, Eynon EE, Stevens S, Manz MG, Flavell RA. Human thrombopoietin knockin mice efficiently support human hematopoiesis in vivo. *Proc Natl Acad Sci USA* 2011; **108**: 2378-2383 [PMID: 21262827 DOI: 10.1073/pnas.1019524108]
46 **Rongvaux A**, Willinger T, Martinek J, Strowig T, Gearty SV, Teichmann LL, Saito Y, Marches F, Halene S, Palucka AK, Manz MG, Flavell RA. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol* 2014; **32**: 364-372 [PMID: 24633240 DOI: 10.1038/nbt.2858]
47 **Shultz LD**, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol* 2007; **7**: 118-130 [PMID: 17259968 DOI: 10.1038/nri2017]
48 **Shultz LD**, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, Greiner DL, Handgretinger R. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* 2005; **174**: 6477-6489 [PMID: 15879151 DOI: 10.4049/jimmunol.174.10.6477]
49 **McIntosh BE**, Brown ME, Duffin BM, Maufort JP, Vereide DT, Slukvin II, Thomson JA. Nonirradiated NOD,B6.SCID Il2rγ-/-Kit(W41/W41) (NBSGW) mice support multilineage engraftment of human hematopoietic cells. *Stem Cell Reports* 2015; **4**: 171-180 [PMID: 25601207 DOI: 10.1016/j.stemcr.2014.12.005]
50 **Klco JM**, Spencer DH, Miller CA, Griffith M, Lamprecht TL, O'Laughlin M, Fronick C, Magrini V, Demeter RT, Fulton RS, Eades WC, Link DC, Graubert TA, Walter MJ, Mardis ER, Dipersio JF, Wilson RK, Ley TJ. Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. *Cancer Cell* 2014; **25**: 379-392 [PMID: 24613412 DOI: 10.1016/j.ccr.2014.01.031]
51 **Abdul-Nabi AM**, Yassin ER, Varghese N, Deshmukh H, Yaseen NR. In vitro transformation of primary human CD34+ cells by AML fusion oncogenes: early gene expression profiling reveals possible drug target in AML. *PLoS One* 2010; **5**: e12464 [PMID: 20805992 DOI: 10.1371/journal.pone.0012464]
52 **Wei J**, Wunderlich M, Fox C, Alvarez S, Cigudosa JC, Wilhelm JS, Zheng Y, Cancelas JA, Gu Y, Jansen M, Dimartino JF, Mulloy JC. Microenvironment determines lineage fate in a human model

of MLL-AF9 leukemia. *Cancer Cell* 2008; **13**: 483-495 [PMID: 18538732 DOI: 10.1016/j.ccr.2008.04.020]

53 **Qin H**, Malek S, Cowell JK, Ren M. Transformation of human CD34+ hematopoietic progenitor cells with DEK-NUP214 induces AML in an immunocompromised mouse model. *Oncogene* 2016; **35**: 5686-5691 [PMID: 27065320 DOI: 10.1038/onc.2016.118]

54 **Ren M**, Qin H, Wu Q, Savage NM, George TI, Cowell JK. Development of ZMYM2-FGFR1 driven AML in human CD34+ cells in immunocompromised mice. *Int J Cancer* 2016; **139**: 836-840 [PMID: 27005999 DOI: 10.1002/ijc.30100]

55 **Aparicio S**, Hidalgo M, Kung AL. Examining the utility of patient-derived xenograft mouse models. *Nat Rev Cancer* 2015; **15**: 311-316 [PMID: 25907221 DOI: 10.1038/nrc3944]

56 **Yada E**, Wada S, Yoshida S, Sasada T. Use of patient-derived xenograft mouse models in cancer research and treatment. *Future Sci OA* 2017; **4**: FSO271 [PMID: 29568561 DOI: 10.4155/fsoa-2017-0136]

57 **Cho SY**, Kang W, Han JY, Min S, Kang J, Lee A, Kwon JY, Lee C, Park H. An Integrative Approach to Precision Cancer Medicine Using Patient-Derived Xenografts. *Mol Cells* 2016; **39**: 77-86 [PMID: 26831452 DOI: 10.14348/molcells.2016.2350]

58 **Gelebart P**, Popa M, McCormack E. Xenograft Models of Primary Acute Myeloid Leukemia for the Development of Imaging Strategies and Evaluation of Novel Targeted Therapies. *Curr Pharm Biotechnol* 2016; **17**: 42-51 [PMID: 26278528 DOI: 10.2174/1389201016666150817095703]

59 **Mitchell A**, Chen WC, McLeod J, Popescu AC, Arruda A, Minden MD, Dick JE, Wang JCY. Leukemic Engraftment In NOD.*SCID* Mice Is Correlated With Clinical Parameters and Predicts Outcome In Human AML. *Blood* 2013; **122**: 50

60 **Reinisch A**, Hernandez DC, Schallmoser K, Majeti R. Generation and use of a humanized bone-marrow-ossicle niche for hematopoietic xenotransplantation into mice. *Nat Protoc* 2017; **12**: 2169-2188 [PMID: 28933777 DOI: 10.1038/nprot.2017.088]

61 **Carretta M**, de Boer B, Jaques J, Antonelli A, Horton SJ, Yuan H, de Bruijn JD, Groen RWJ, Vellenga E, Schuringa JJ. Genetically engineered mesenchymal stromal cells produce IL-3 and TPO to further improve human scaffold-based xenograft models. *Exp Hematol* 2017; **51**: 36-46 [PMID: 28456746 DOI: 10.1016/j.exphem.2017.04.008]

62 **Vick B**, Rothenberg M, Sandhöfer N, Carlet M, Finkenzeller C, Krupka C, Grunert M, Trumpp A, Corbacioglu S, Ebinger M, André MC, Hiddemann W, Schneider S, Subklewe M, Metzeler KH, Spiekermann K, Jeremias I. An advanced preclinical mouse model for acute myeloid leukemia using patients' cells of various genetic subgroups and in vivo bioluminescence imaging. *PLoS One* 2015; **10**: e0120925 [PMID: 25793878 DOI: 10.1371/journal.pone.0120925]

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REVIEW

Applications of stem cells in orthodontics and dentofacial orthopedics: Current trends and future perspectives

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Abstract

A simple overview of daily orthodontic practice involves use of brackets, wires and elastomeric modules. However, investigating the underlying effect of orthodontic forces shows various molecular and cellular changes. Also, orthodontics is in close relation with dentofacial orthopedics which involves bone regeneration. In this review current and future applications of stem cells (SCs) in orthodontics and dentofacial orthopedics have been discussed. For craniofacial anomalies, SCs have been applied to regenerate hard tissue (such as treatment of alveolar cleft) and soft tissue (such as treatment of hemifacial macrosomia). Several attempts have been done to reconstruct impaired temporomandibular joint. Also, SCs with or without bone scaffolds and growth factors have been used to regenerate bone following distraction osteogenesis of mandibular bone or maxillary expansion. Current evidence shows that SCs also have potential to be used to regenerate infrabony alveolar defects and move the teeth into regenerated areas. Future application of SCs in orthodontics could involve accelerating tooth movement, regenerating resorbed roots and expanding tooth movement limitations. However, evidence supporting these roles is weak and further studies are required to evaluate the possibility of these ideas.

Key words: Alveolar bone grafting; Dentofacial deformities; Distraction osteogenesis; Guided tissue regeneration; Orthodontics; Orthodontic tooth movement; Orthognathic surgery; Periodontitis; Root resorption; Stem cells

Core tip: Stem cell therapy has multiple applications in the field of orthodontics and dentofacial orthopedics. Recent researches have demonstrated advantageous use of stem cells (SCs) for correction of craniofacial anomalies, rapid consolidation phase of distraction osteogenesis, reconstruction of temporomandibular joint and stability of palatal expansion. SCs also could be used to regenerate infrabony alveolar defects and move

the teeth into regenerated areas. Future application of SCs in orthodontics could involve accelerating tooth movement, regenerating resorbed roots and expanding tooth movement limitations.

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INTRODUCTION

Orthodontics involves treatment of dental malocclusions and correction of dentofacial deformities. The aim of orthodontic treatment is to achieve facial aesthetics and improve oral health related quality of life[1,2]. The prevalence of dental malocclusion varies in different communities and have been reported to be 22.5% to 93%[3-6]. Orthodontic treatment of malocclusions has several shortcomings such as prolonged treatment time, apical root resorption, tooth movement limited to alveolar bone and difficulties to overcome periodontal defects.

Although facial anomalies and jaw base deformities are less frequent compared to simple dental malocclusions, they are more burdensome[7]. About 5% of orthodontic patients could be considered as handicapped and need multidisciplinary treatments[8]. Current treatment modalities of craniofacial deformities can reduce the severity of these deformities but their final aesthetic outcomes are still not pleasing.

Stem cells (SCs) are self-renewal cells that could differentiate toward various cells under suitable conditions[9]. Various sources for harvesting SCs have been introduced such as muscle, dermis, bone marrow, adipose tissue, periosteum, blood, umbilical cord, synovial membrane and teeth[10,11]. Among these sources, some are easily accessible in orthodontics. As extraction of primary teeth or permanent premolar or wisdom teeth is common interventions in orthodontic treatment of malocclusions, SCs sources from the teeth could be gained without extra morbidity. Several studies have revealed differentiation and proliferation potential of mesenchymal stem cells (MSCs) obtained from dental pulp, periodontal ligament or human exfoliated deciduous teeth[12-15].

Nowadays, MSCs could be considered as "research trends" in the field of biology and medicine and their application in regenerative medicine is growing. Some modalities involve direct plantation of MSCs into the defect site while others use proper scaffolds to support the cells. In bone tissue engineering, MSCs are carried by an osteoconductive scaffold and differentiated toward osteogenic cells using osteoinductive growth factors[16]. Several types of scaffolds and growth factors have been used for regeneration of craniofacial bone defects including orthodontic related bone defects[17-19]. The aim of the current study was to review applications of SCs in treatment of dentofacial defects and deformities and to propose possible advantages of SC therapy in enhancing orthodontic treatments.

APPLICATIONS IN DENTOFACIAL ORTHOPEDICS

To evaluate the uses of SCs in dentofacial orthopedics, application of SCs in treatment of dentofacial anomalies and temporomandibular joint (TMJ) disorders as well as their possible role in distraction osteogenesis (DO) and maxillary expansion have been discussed (Figure 1).

Dentofacial anomalies

Craniofacial deformities such as congenital and developmental malformation and those resulting from trauma, tumor resection and nonunion of fractures, are common clinical problems in craniofacial surgery, which are difficult to remedy. Current surgical techniques in various combinations, autogenous, allogeneic, and prosthetic materials have been used to achieve bone and soft tissue reconstruction[20]. These approaches have several complications such as insufficient autogenous resources, donor site morbidity, contour irregularities, postoperative pain, additional cost, long surgical time and postsurgical reabsorption, disease transmission, major histoincompatibility, graft versus-host disease (GVHD), immunosuppression, unpredictable outcome for tissue formation and infection of foreign material[21-24]. In order to overcome these complications, stem cell-based tissue regeneration offers a promising approach to provide an advanced and reliable therapeutic strategy for craniofacial tissue reconstruction[25]. In the current review, regenerative approaches for two types of craniofacial anomalies are presented; cleft lip and palate (CLP) (for hard tissue regeneration) and hemifacial microsomia (HFM) (for soft tissue regeneration).

CLP is one of the most prevalent congenital anomalies which results from fusion failure of nasal process and oropalatal shelves. The prevalence of this malformation is 0.36-0.83 in 1000 live-born infants[26]. Alveolar bone defect, problem in swallowing and pronunciation, facial deformity, missing teeth, and maxillary deformity can be seen in CLP patients[27]. Repair of the malformed alveolar bone is critical for oronasal fistula closure, maxilla unification, tooth eruption, and support of the alar base[28,29]. The gold standard treatment for alveolar reconstruction in CLP patients is autogenous cancellous bone grafts[30] since they are immunologically inert and potential suppliers of cells with osteoconductive and osteoinductive properties[31,32]. The commonest site for acquiring autogenous bone for grafting is the anterior iliac crest[33]. An overall success rate for iliac crest bone grafting to the alveolar cleft with respect to bone resorption is 88%[34]. With the advent of tissue en-

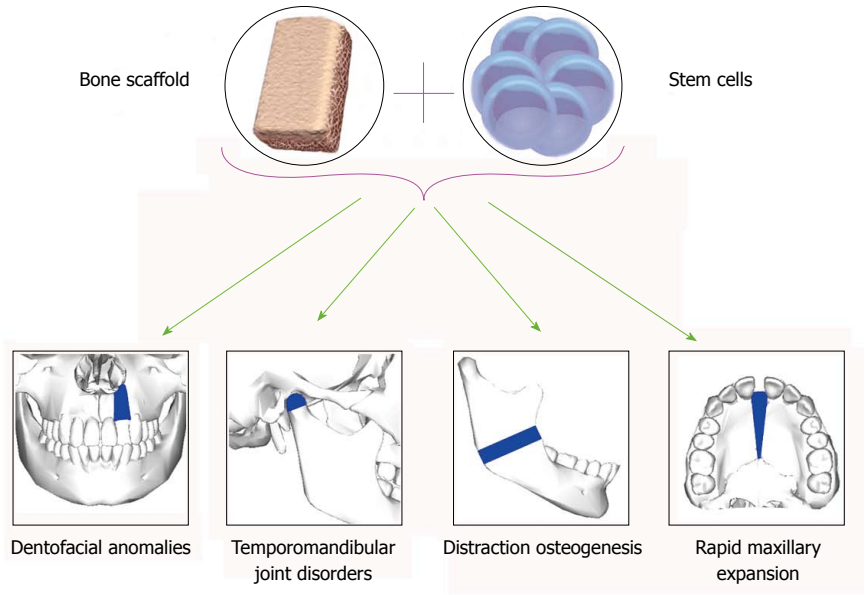

Figure 1 Applications of stem cells (alone or in conjugation with bone scaffolds) in dentofacial orthopedics.

gineering techniques, alternatives to the traditional iliac crest bone grafting techniques are available. MSCs have been shown to have the ability to form new bone when transplanted[35].

Some case reports and case series studies reported results of MSCs usage to regenerate alveolar cleft[36]. Composite scaffold of demineralized bone mineral and calcium phosphate loaded with MSCs showed 34.5% regenerated bone in the cleft area in one case and in the other there was 25.6% presentation of bone integrity[37]. About 50% fill of the bone defect was measured after placement of the scaffold, growth factor and MSCs in cleft area[38], whereas 79.1% bone regeneration has been reported in the another study[22]. Autogenous osteoblasts cultured on demineralized bone matrix showed more reduction in defect size in comparison to control group[39]. About 90% defect correction of soft palate defect has been reported 14 d after injection of autologous MSCs[40]. Biomaterial seeded with autogenous osteogenic cells into the alveolar cleft resulted in spontaneously eruption of canine in its proper place after eighteen months[41]. Poly-L-lactic acid with osteogenically differentiated fat-derived stem cells showed substantial bone regeneration in palatal defect[42]. The mean pain score, including both intensity and pain frequency and donor site morbidity was greatest at all-time points in traditional iliac crest bone graft and least at all-time points in tissue engineering[31].

Thus, it can be concluded that SCs seem to possess favorable potential for bone regeneration in oral and maxillofacial region and use of them in alveolar defect repair, reduce defect size by bone formation[37,39,42], have less postoperative morbidity compared to autogenous bone grafting[31] and help the teeth in the defect area to erupt in their proper position[41].

HFM is a rare, multi-systemic congenital disease. It is considered to be the product of unilateral abnormal morphogenesis of the first and second pharyngeal arches. HFM is a frequently encountered form of congenital facial malformation, ranking second only to cleft lip and palate[43]. The fundamental features of HFM include unilateral hypoplasia of the craniofacial skeleton and its overlying soft tissue[44]. Autologous fat grafting is considered to reconstruct soft tissue defect in the treatment of congenital malformations as well as post-traumatic malformations[45]. To overcome problems associated with fat grafting, such as unpredictable clinical results and a low rate of graft survival, many innovative efforts and refinements of surgical techniques have been reported[46]. Use of adipose derived stromal cells (ASCs) for tissue regeneration has attracted attention recently.

Patients with HFM which have been grafted with supplementation of ASCs Showed 88% of fat volume surviving after 6 mo in comparison to control group which was 54%[46]. Also, residual graft volumes of ASCs enriched grafts was significantly higher in comparison to control group[45].

Studies are ongoing, and as results are reported, it will be crucial to evaluate the long term outcome of such procedures. The current evidence suggests that use of ASCs for soft tissue reconstruction may enhance angiogenesis[47], improve the survival of grafts[45,46] and thus reduce atrophy[47].

Temporomandibular joint disorders

The temporomandibular joint (TMJ) is comprised of both osseous and cartilaginous structures. It is enclosed in a capsule that is lubricated with synovial fluid and serves as an important growth site during postnatal development with two articular surfaces that can adapt to changing environment conditions[48,49]. The mandibular condyle grows by proliferation of the progenitor/SCs that differentiate into chondrocytes[49,50] leading to formation

and increase of cartilage matrix, which will be replaced with lamellar trabecular bone[51]. As SCs possess the ability to differentiate into chondrogenic and osteogenic cells, they could be used for both maintenance of mandible in new position and repair of TMJ lesions.

Forward positioning of mandible, for example in functional therapy, leads to increase in the number of mesenchymal cells (stem/progenitor cells) in the temporal fossa, which resulted in new cortical bone formation[52]. Thus, the question arises as to whether the injection of SCs into articular space accelerates bone formation in the temporal fossa? This issue requires Further targeted researches.

TMJ is prone to injuries, tumors, osteoarthritis, rheumatoid arthritis and congenital anomalies. Approximately 10 million individuals in the United States have been affected by temporomandibular disorders (TMD)[53]. TMD manifest as pain, myalgia, headaches, and structural destruction, collectively known as degenerative joint disease[54]. The primary methods used to reconstruct the TMJ includes autogenous bone grafting such as harvesting from the rib, or the use of alloplastic materials, with neither being ideally suited for the task and sometimes leading to unwanted adverse effects. The major and final option for those patients with advanced degenerative diseases is surgical replacement of the mandibular condyle[55]. These approaches have complications such as immunorejection, infection, implant wear, dislocation, suboptimal biocompatibility, donor site limitation and morbidity, and potential pathogen transmission[56,57]. To overcome these disadvantages, strategies have been found to engineer osteochondral tissue, such as that found in the TMJ, will produce tissue that is both biologically and mechanically functional used. Recently, these cells have attracted much interest to joint reconstruction.

Engineering a TMJ-like osteochondral graft has been studied in several studies. The culture of human umbilical cord matrix (HUCM) SCs in growth medium containing chondrogenic factors, showed the HUCM SCs can outperform the TMJ condylar cartilage cells[58]. Rat bone marrow MSCs which encapsulated in poly (ethylene glycol)-based hydrogel molded into the shape of a cadaver human mandibular condyle, demonstrated two stratified layers of histogenesis of cartilaginous and osseous phenotypes[59,60]. Porcine MSCs which had been cultured in osteogenic induction medium and were seeded onto a poly DL-lactic–co-glycolic acid scaffold, formed the construct had a shape that closely resembled to the model condyle and it's radiodensity was between that of the normal condyle and that of control scaffolds[61].

Because of fibrocartilaginous structure of disk, there has been little success in the manufacture of synthetic TMJ discs rather than bone and cartilage and attention has turned to tissue engineering to reconstruct the disc[62]. In one study, Combination of polylactide acid discs with adipose tissue stem cell demonstrated the potential to development a tissue-engineered TMJ disc[63].

While animal studies are in progress to replicate bone the osteochondral interface to engineer TMJ, yet no clinical trials on humans have been done. These data revealed possibility of application of SCs in combination with different scaffolds as a promising approach to regenerate osteochondral tissues of TMJ and ultimately the joint disk.

Distraction osteogenesis

DO which is regarded as "endogenous bone tissue engineering" has been widely applied in orthopedic surgery for correction of limb length and also in the treatment of many craniofacial deformities[64]. DO is done by creating a corticotomy, placing a rigid distractor across the cut bone and gradually activating the device[65]. The mechanism of osteogenesis and gap repair initiated by an immediate inflammatory response that leads to the recruitment of MSCs and subsequent differentiation into chondrocytes that produce cartilage and osteoblasts which form bone[66]. Despite its great advantages, long treatment periods and fibrous union or even non-union of bone are possible major draw backs impeding its widespread clinical application[67,68].

Efforts have been made to accelerate osteogenesis in the distraction Gap, shorten the consolidation period and reduce complications such as the development of nonunion, infection, or fracture.

Recently, because of the role of MSCs in osteogenesis, many researchers have successfully documented the ability of SCs on promoting bone formation and shortening the consolidation period during DO. For this purpose various sources of SCs such as human exfoliated deciduous teeth (SHED)[69], bone marrow[70-77] and adipose tissue[78-80] have been used in studies. In some studies, alone MSCs[71,79,81,82], in the others, gene transferred MSCs[72,76-78,83] and factors[75,84,85] have been used to enhance bone regeneration following distraction osteogenesis. The modifications such as use of scaffolds[75], demineralized bone matrix[74] and Platelet-rich Plasma[73] have been done in some studies.

The injection of MSCs 1 d before onset of distraction resulted in increase in new bone volume in the distracted callus and the bone mineral density (BMD)[81], MSCs injection after distraction was complete showed higher radiodensity of the distraction zone and grater histologically callus, new bone volume and thickness of the new trabeculae[71] and doing this intervention on the first day of consolidation resulted in greater biomechanical strength and increase in total and compact bone ratio in regenerate bone[82]. The injection of SHED during osteotomy period showed higher percentage of newly formed bone after 2, 4, and 6 wk[69]. One study revealed that callus density, the ossification rate, quality of newly formed bone and the number of active cells in bone formation were higher in group which osteoblast-differentiated stem cell were injected to distraction site compared to control group and stem cell group[79]. Addition of MSCs sheet fragments yielded significant increases in bony union, more intensive

bone formation on histomorphometric analysis and higher peak load on biomechanical testing[70]. MSCs transfected with bFGF showed excellent bone formation and higher BMD and bone mineral content (BMC) in the distracted callus[76]. The use of MSCs osteogenic differentiation using FGF-2 and confirm cell integration with a gelatin-based Gelfoam scaffold, demonstrated less interfragmentary mobility, more advanced gap obliteration, higher mineral content and faster mineral apposition[75]. One study suggested that gene therapy using rhRunx2-modified ASCs promoted new bone formation during osteoporotic mandibular DO[78]. Application of ASCs transfected with pEGFP-OSX showed the highest BMD, thickness of new trabecula (TNT), and the volumes of the newly generated cortical bone (NBV1) and the cancellous bone (NBV2) in the distraction zones[78]. Excellent bone formation and highest BMD, TNT and NBV in the distraction zones was observed in groups that MSCs transfected with OSX[72]. The injection of MSCs transfected with Bone Morphogenic Protein (BMP) showed greater bone formation and earlier mineralization in the distracted callus[77], more mature medullary cavity[83], better bone quality and higher trabecular parameters (trabecular thickness, trabecular number, volumetric bone mineral density at tissue, and bone volume fraction) at the second and fourth weeks of the consolidation period[86] and acceleration of osteogenesis[87]. The use of stromal cell-derived factor-1 (SDF-1) facilitated migration of MSCs into osteogenesis site[84]. The addition of MSCs transfected with recombinant plasmids pIRES-hBMP2-hVEGF165 at the beginning of distraction is more ideal than the start of latency period[85].

These data shows that SCs from Various sources, alone or in combination of genes and factors, in different phases of treatment can lead to an increase in new bone volume and quality[69,71,72,77,78,81,86], bone mineral density[71,72,76,78,81], trabecular thickness[71,78,86], biomechanical strength[70,82].

Rapid maxillary expansion

Maxillary constriction can be associated with several problems that include occlusal disharmony and esthetics as well as such functional difficulties as narrowing of the pharyngeal airway, increased nasal resistance, and alterations in tongue posture, resulting in retroglossal airway narrowing and mouth breathing[88-90]. Maxillary constriction can be corrected with slow orthodontic expansion, rapid maxillary expansion (RME), surgically assisted rapid palatal expansion or a two-segmented Le Fort I-type osteotomy with expansion[91]. RME is indicated in patients younger than 12 years, who have lateral discrepancies involving several teeth, whether the constriction is skeletal, dental or a combination of both[92]. It is an effective orthopedic procedure to open the midpalatal suture, providing appropriate and stable maxillary width increase and re-stablish balance between the width of the jaws[93,94].

RME is similar to DO histologically. During RME, a gap in the midpalatal suture is created which is filled with blood and granulated tissue and followed by active bone formation. The expanded arch width relapses unless followed by an appropriate retention period. Therefore, providing a strategy to accelerate bone formation in the midpalatal suture might shorten treatment and retention period, achieve stability and prevent relapse. Because of the ability of SCs to differentiate into osteogenic cells, injection of SCs seems to have the ability to accelerate the process of bone formation. This was studies in one study by Ekizer *et al*[95]. In their animal study, local injection of MSCs into intermaxillary suture after force application resulted in increased new bone formation in the suture by increasing the number of osteoblasts and new vessel formation[95]. Thus, locally applied MCSs to the expanded maxilla might be a useful and practical treatment strategy to accelerate new bone formation in midpalatal suture and to shorten the treatment and retention period for patients undergoing orthopedic maxillary expansion.

APPLICATIONS IN ORTHODONTICS

To evaluate the uses of SCs in orthodontics, current evidence regarding application of SCs in expanding the limitations of orthodontic tooth movement (OTM), tooth movement into periodontal defects, accelerating OTM and treatment of external root resorption (ERR) have been reviewed (Figure 2).

Expanded envelope of discrepancy

The extent of OTM is limited by several factors including the anatomy of the alveolar bone, pressures exerted by soft tissues, periodontal tissue attachment levels, neuromuscular forces and lip–tooth relationships[96,97]. The anteroposterior, vertical, and transverse millimetric range of treatment possibilities in orthodontics can be expressed as an "envelope of discrepancy"[98] Gingival recession occurs secondarily to an alveolar bone dehiscence, if overlying tissues are stressed during OTM beyond this envelope. Sites in which the buccal or lingual bone cortex and covering gingival tissue are thin, such as lower incisors in patients with a prominent chin and compensation in the form of lingual tipping of these teeth are at particular risk of bone defects like fenestrations and dehiscence[99,100].

SCs have the potential to generate different tissues, including bone, thereby stem cell therapy is a promising approach to alveolar bone regeneration[101]. Some researches have applied stem cell therapy in case of bone ridge augmentation in humans and mainly used bone marrow cells[102-104]. The outcome of alveolar bone regeneration showed a tendency to enhance bone formation[105]. Hence, bone regeneration methods using SCs might provide an approach for expanding limitations of envelope of discrepancy.

As a hypothesis, relying on the results of alveolar

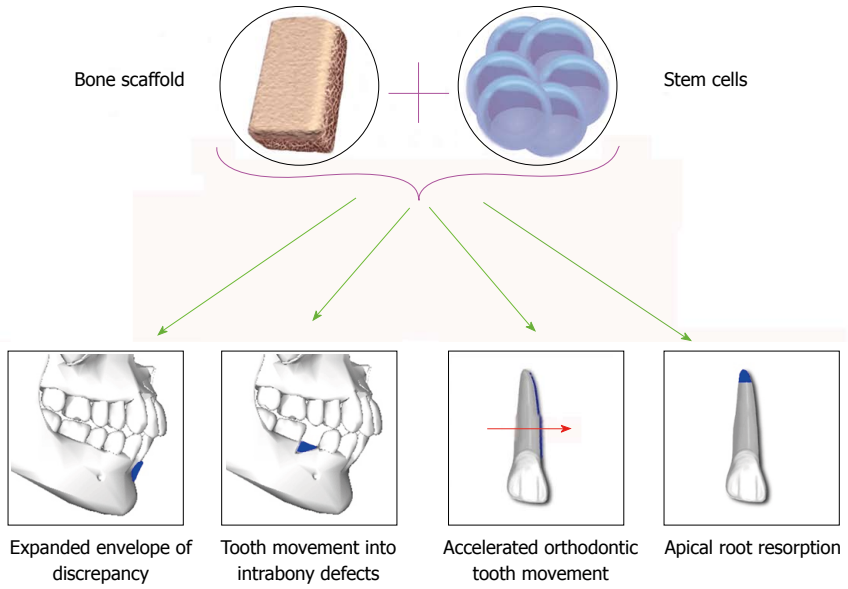

Figure 2 Possible applications of stem cells (alone or in conjugation with bone scaffolds) in orthodontics.

bone augmentation studies, it might be possible with the aid of stem cell based osteogenesis to horizontally augment the ridge in order to extend the tooth movement extent and to overcome some anatomical boundaries.

Periodontal regeneration

Periodontal complications are one of the most actual side effects linked to the orthodontics. It can be found in various forms, from gingivitis to periodontitis, dehiscence, fenestrations, interdental fold, gingival recession or overgrowth, black triangles[100]. Periodontal regeneration has been defined as the formation of new cementum, alveolar bone, and a functional periodontal ligament on a previously diseased root surface[106]. The current treatment approaches include the use of surgery, guided tissue regeneration (GTR), bone fillers and growth factors and application of bioactive molecules to induce regeneration[107,108]. Based on the differential potential capability of SCs and their ability of renewal *via* mitosis[109], they have the quality to regenerate damaged tissues, hence they can be used for regeneration of periodontium.

Periodontal defects could be a challenging situation both pre and post orthodontic treatment. On one hand, because of the increasing number of adult patients seeking orthodontic treatment, encountering the periodontally involved patients may be a potential problem for every practitioner. It has been suggested that, by moving the teeth into infrabony defects, we can achieve the regeneration of the attachment apparatus[110]. Accordingly with the combination of periodontal regeneration treatments such as GTR and OTM, it might be possible to reduce infrabony defect and upgrade periodontal health[111]. On the other hand, periodontal defects such as fenestration, dehiscence and attachment loss are among common complications of orthodontic treatments[112].

Several reports on application of SCs for regeneration of periodontal tissues have been published. In a study, induced pluripotent SCs have been implanted into a mouse periodontal fenestration defect model with a silk fibroin scaffold in combination with enamel matrix derivative gel. As a result, higher rate of cementum and alveolar bone formation was observed[113]. Also, it has been shown that the bone marrow derived mesenchymal stem cells (BM-MSC)-treated wounds exhibited significantly accelerated wound closure, with increased re-epithelialization, cellularity, and angiogenesis[114]. In another study conditioned medium (CM) obtained from PDLSCs were transplanted into a rat periodontal defect model and consequently PDLSC-CM enhanced periodontal regeneration by suppressing the inflammatory response *via* TNF-α production[115]. Incubation of induced PDLSCs with dentin non collagenous proteins *in vivo* revealed that cementum-like tissues formed along the chemical-conditioned root dentin surface, enhanced alkaline phosphatase (ALP) activity, increased matrix mineralization, and upregulated expression of mineralization-associated genes[116]. One study has revealed that autologous PDLSCs obtained from extracted teeth of the miniature pigs which were transplanted into the surgically created periodontal defect areas were capable of regenerating periodontal tissues, leading to a favorable treatment for periodontitis[117]. PDLSCs were delivered onto suitable collagen sponges and implanted into periodontal defects of immunodeficient nude rats in an *in vivo* study, as a result reformation of periodontal ligament-like tissue, collagen fibers, and elements of bone was observed[118]. In another *in vivo* study, PDLSCs sheet were transferred to a miniature pig periodontitis model. Significant peri-

odontal tissue regeneration was achieved in both the autologous and the allogeneic PDLSCs transplantation[119]. Using amniotic membrane for transferring PDLSCs for periodontal regeneration in a rat periodontal model as a new method of transplantation is also being suggested in a study[120].

According to aforesaid studies, human adult PDLSCs are capable of regenerating elements of bone and collagen, since the periodontitis is a chronic disease, it may benefit from such stem cell based therapies[114,117-119]. Thus the use of PDLSC transplantation in periodontal therapies can reduce treatment time and better outcomes followed by patient comfort, however, due to complex structure of periodontium, regeneration is a feasible and yet complicated procedure and may need pluripotent SCs and more investigations.

Accelerated OTM

OTM is achieved by the remodeling of periodontal ligament (PDL) and alveolar bone in response to mechanical loading[121,122]. The initiating inflammatory event at compression sites is caused by constriction of the PDL microvasculature, resulting in a focal necrosis, followed by recruiting of osteoclasts from the adjacent marrow spaces[123]. These osteoclasts are mostly derived from hematopoietic SCs[124]. Hence, SCs could be used to accelerate OTM by providing progenitor cells.

The development of new methods to accelerate OTM has been sought by clinicians as a way to shorten treatment times, reduce adverse effects such as pain, discomfort, dental caries, and periodontal diseases, and minimize iatrogenic damages such as root resorption and the subsequent development of non-vital teeth[125]. There are surgical methods like surgically-facilitated orthodontic therapy or corticotomy[126], periodontally accelerated osteogenic orthodontics[127] and some nonsurgical procedures such as systemic/local administration of chemical substances like epidermal growth factor, parathyroid hormone, 1,25-dihydroxyvitamin d 3, osteocalcin and prostaglandins, resonance vibration, static or pulsed magnetic field, low-intensity laser irradiation therapy[128].

In a study, increased PDL progenitor cells with suppressed expression of type I collagen (Col-I) were observed during orthodontic force application, whilst after force withdrawal they increase in Col-I expression, which suggests that PDLSCs are able to respond to orthodontic mechanical forces with suppressed collagen expression[129]. This ability of SCs could be used to accelerate OTM in response to orthodontic forces. When orthodontic force is applied, tooth movement is hindered until the necrosis is removed, leading to the clinical manifestation of a delay period. Hypothetically, transplantation of SCs in pressure sites may speed up the process, resulting in accelerated OTM.

ERR

ERR is a common and unfavorable side effect of orthodontic treatment[130,131], which any specialist may encounter. Many factors seems to be involved in ERR such as genetics, individual biological variability, age, sex, and orthodontic forces and treatment duration[132,133]. Orthodontic forces yet seem to be the main etiologic factors. ERR may lead to loss of tooth structure such as cementum and in more advanced stages, dentin, however no specific treatment has been introduced so far. One possible treatment modality could be regeneration of resorbed roots by application SCs and tissue engineering.

In severe cases ERR may cause poor prognosis of tooth, resulting in tooth loss. Regeneration of these lesions increases the longevity of tooth and may play an important role in facilitating the treatment. In a study designed to induce de novo cementum formation by SC therapy, MSCs driven from periodontal ligament in *in vivo* transplantation were able to form cellular cementum-like hard tissue containing embedded osteocalcin-positive cells[134]. According to studies in which the whole tooth structure has been bioengineered and transplanted into Rodent[135,136] and beagle dogs[137] models, it might be possible to regenerate the damaged tooth structure such as dentin and cementum and in the future to achieve a bioengineered functional human tooth structure.

Although it seems that there is a long way until regeneration of the teeth materials, cementogenesis and regeneration of dental structures through stem cell based therapies could be anticipated.

CONCLUSION

The current review showed application of SCs alone or in conjugation with bone scaffold or growth factors in surgical correction of dentofacial deformities, TMJ defects, and alveolar bone lesions. Recent studies show that SCs could improve treatment results and reduce treatment duration. Use of SCs is associated with accelerated healing and less morbidity compared to current surgical approached. Also, SCs could be used in DO surgeries and RME to increase consolidation rate and reduce relapse.

The contemporary evidence reveals feasibility of use of SCs for accelerating OTM, regenerating resorbed roots, expanding limitations of OTM while preserving periodontal health. In addition, SCs could be used for regeneration of periodontal tissues both pre and post OTM. *In vivo* studies are required to assess the possibility of such interventions.

REFERENCES

1 **Kiyak HA**. Does orthodontic treatment affect patients' quality of life? *J Dent Educ* 2008; **72**: 886-894 [PMID: 18676797]
2 **Silvola AS**, Varimo M, Tolvanen M, Rusanen J, Lahti S, Pirttiniemi P. Dental esthetics and quality of life in adults with severe malocclusion before and after treatment. *Angle Orthod* 2014; **84**: 594-599 [PMID: 24308529 DOI: 10.2319/060213-417.1]
3 **Silva RG**, Kang DS. Prevalence of malocclusion among Latino

adolescents. *Am J Orthod Dentofacial Orthop* 2001; **119**: 313-315 [PMID: 11244426 DOI: 10.1067/mod.2001.110985]
4 **Tausche E**, Luck O, Harzer W. Prevalence of malocclusions in the early mixed dentition and orthodontic treatment need. *Eur J Orthod* 2004; **26**: 237-244 [PMID: 15222706 DOI: 10.1093/ejo/26.3.237]
5 **Krooks L**, Pirttiniemi P, Kanavakis G, Lähdesmäki R. Prevalence of malocclusion traits and orthodontic treatment in a Finnish adult population. *Acta Odontol Scand* 2016; **74**: 362-367 [PMID: 26940248 DOI: 10.3109/00016357.2016.1151547]
6 **Akbari M**, Lankarani KB, Honarvar B, Tabrizi R, Mirhadi H, Moosazadeh M. Prevalence of malocclusion among Iranian children: A systematic review and meta-analysis. *Dent Res J* (Isfahan) 2016; **13**: 387-395 [PMID: 27857763 DOI: 10.4103/1735-3327.192269]
7 **Salzmann JA**. Editorial: Seriously handicapping orthodontic conditions. *Am J Orthod* 1976; **70**: 329-330 [PMID: 1066972 DOI: 10.1016/0002-9416(76)90340-7]
8 **Kelly JE**, Sanchez M, Van Kirk LE. An Assessment of the Occlusion of the Teeth of Children 6-11Years, United States. *Vital Health Stat 11* 1973: 1-60 [PMID: 25209689]
9 **Khojasteh A**, Motamedian SR. Mesenchymal Stem Cell Therapy for Treatment of Craniofacial Bone Defects: 10 Years of Experience. *Reg Reconst Restor* 2016; **1**: 1-7 [DOI: 10.7508/rrr.2015.01.001]
10 **Mafi R**, Hindocha S, Mafi P, Griffin M, Khan WS. Sources of adult mesenchymal stem cells applicable for musculoskeletal applications - a systematic review of the literature. *Open Orthop J* 2011; **5** Suppl 2: 242-248 [PMID: 21886689 DOI: 10.2174/1874325001105010242]
11 **Hass R**, Kasper C, Böhm S, Jacobs R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal* 2011; **9**: 12 [PMID: 21569606 DOI: 10.1186/1478-811X-9-12]
12 **Motamedian SR**, Tabatabaei FS, Akhlaghi F, Torshabi M, Gholamin P, Khojasteh A. Response of Dental Pulp Stem Cells to Synthetic, Allograft, and Xenograft Bone Scaffolds. *Int J Periodontics Restorative Dent* 2017; **37**: 49-59 [PMID: 27977818 DOI: 10.11607/prd.2121]
13 **Gay IC**, Chen S, MacDougall M. Isolation and characterization of multipotent human periodontal ligament stem cells. *Orthod Craniofac Res* 2007; **10**: 149-160 [PMID: 17651131 DOI: 10.1111/j.1601-6343.2007.00399.x]
14 **Khojasteh A**, Motamedian SR, Rad MR, Shahriari MH, Nadjmi N. Polymeric vs hydroxyapatite-based scaffolds on dental pulp stem cell proliferation and differentiation. *World J Stem Cells* 2015; **7**: 1215-1221 [PMID: 26640621 DOI: 10.4252/wjsc.v7.i10.1215]
15 **Miura M**, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 2003; **100**: 5807-5812 [PMID: 12716973 DOI: 10.1073/pnas.0937635100]
16 **Motamedian SR**, Iranparvar P, Nahvi G, Khojasteh A. Bone Tissue Engineering: A Literature Review. *Regen Reconst Restor* 2016; **1**: 103-120
17 **Motamedian SR**, Hosseinpour S, Ahsaie MG, Khojasteh A. Smart scaffolds in bone tissue engineering: A systematic review of literature. *World J Stem Cells* 2015; **7**: 657-668 [PMID: 25914772 DOI: 10.4252/wjsc.v7.i3.657]
18 **Jafari M**, Paknejad Z, Rad MR, Motamedian SR, Eghbal MJ, Nadjmi N, Khojasteh A. Polymeric scaffolds in tissue engineering: a literature review. *J Biomed Mater Res B Appl Biomater* 2017; **105**: 431-459 [PMID: 26496456 DOI: 10.1002/jbm.b.33547]
19 **Hosseinpour S**, Ghazizadeh Ahsaie M, Rezai Rad M, Baghani MT, Motamedian SR, Khojasteh A. Application of selected scaffolds for bone tissue engineering: a systematic review. *Oral Maxillofac Surg* 2017; **21**: 109-129 [PMID: 28194530 DOI: 10.1007/s10006-017-0608-3]
20 **Cowan CM**, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, Quarto N, Contag CH, Wu B, Longaker MT. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. *Nat Biotechnol* 2004; **22**: 560-567 [PMID: 15077117 DOI: 10.1038/nbt958]
21 **Warren SM**, Fong KD, Chen CM, Loboa EG, Cowan CM, Lorenz HP, Longaker MT. Tools and techniques for craniofacial tissue engineering. *Tissue Eng* 2003; **9**: 187-200 [PMID: 12740082 DOI: 10.1089/107632703764664666]
22 **Hibi H**, Yamada Y, Ueda M, Endo Y. Alveolar cleft osteoplasty using tissue-engineered osteogenic material. *Int J Oral Maxillofac Surg* 2006; **35**: 551-555 [PMID: 16584868 DOI: 10.1016/j.ijom.2005.12.007]
23 **Younger EM**, Chapman MW. Morbidity at bone graft donor sites. *J Orthop Trauma* 1989; **3**: 192-195 [PMID: 2809818 DOI: 10.1097/00005131-198909000-00002]
24 **Bayerlein T**, Proff P, Heinrich A, Kaduk W, Hosten N, Gedrange T. Evaluation of bone availability in the cleft area following secondary osteoplasty. *J Craniomaxillofac Surg* 2006; **34** Suppl 2: 57-61 [PMID: 17071393 DOI: 10.1016/S1010-5182(06)60013-9]
25 **Miura M**, Miura Y, Sonoyama W, Yamaza T, Gronthos S, Shi S. Bone marrow-derived mesenchymal stem cells for regenerative medicine in craniofacial region. *Oral Dis* 2006; **12**: 514-522 [PMID: 17054762 DOI: 10.1111/j.1601-0825.2006.01300.x]
26 **Luaces-Rey R**, Arenaz-Búa J, Lopez-Cedrún-Cembranos JL, Herrero-Patiño S, Sironvalle-Soliva S, Iglesias-Candal E, Pombo-Castro M. Is PRP useful in alveolar cleft reconstruction? Platelet-rich plasma in secondary alveoloplasty. *Med Oral Patol Oral Cir Bucal* 2010; **15**: e619-e623 [PMID: 20038881 DOI: 10.4317/medoral.15.e619]
27 **Le BT**, Woo I. Alveolar cleft repair in adults using guided bone regeneration with mineralized allograft for dental implant site development: a report of 2 cases. *J Oral Maxillofac Surg* 2009; **67**: 1716-1722 [PMID: 19615587 DOI: 10.1016/j.joms.2009.04.012]
28 **Brito LA**, Paranaiba LM, Bassi CF, Masotti C, Malcher C, Schlesinger D, Rocha KM, Cruz LA, Bárbara LK, Alonso N, Franco D, Bagordakis E, Martelli H Jr, Meyer D, Coletta RD, Passos-Bueno MR. Region 8q24 is a susceptibility locus for nonsyndromic oral clefting in Brazil. *Birth Defects Res A Clin Mol Teratol* 2012; **94**: 464-468 [PMID: 22511506 DOI: 10.1002/bdra.23011]
29 **Ramalingam M**, Haidar Z, Ramakrishna S, Kobayashi H, Haikely. Integrated Biomaterials in Tissue Engineering. Wiley, 2012: 183-234 [DOI: 10.1002/9781118371183]
30 **Nwoku AL**, Al Atel A, Al Shlash S, Oluyadi BA, Ismail S. Retrospective analysis of secondary alveolar cleft grafts using iliac of chin bone. *J Craniofac Surg* 2005; **16**: 864-868 [PMID: 16192872 DOI: 10.1097/01.scs.0000179742.45424.0a]
31 **Gimbel M**, Ashley RK, Sisodia M, Gabbay JS, Wasson KL, Heller J, Wilson L, Kawamoto HK, Bradley JP. Repair of alveolar cleft defects: reduced morbidity with bone marrow stem cells in a resorbable matrix. *J Craniofac Surg* 2007; **18**: 895-901 [PMID: 17667684 DOI: 10.1097/scs.0b013e3180a771af]
32 **Ishaug-Riley SL**, Crane GM, Gurlek A, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG. Ectopic bone formation by marrow stromal osteoblast transplantation using poly(DL-lactic-co-glycolic acid) foams implanted into the rat mesentery. *J Biomed Mater Res* 1997; **36**: 1-8 [PMID: 9212383 DOI: 10.1002/(SICI)1097-4636(19970707)36:1<1::AID-JBM1>3.0.CO;2-P]
33 **Eufinger H**, Leppänen H. Iliac crest donor site morbidity following open and closed methods of bone harvest for alveolar cleft osteoplasty. *J Craniomaxillofac Surg* 2000; **28**: 31-38 [PMID: 10851671 DOI: 10.1054/jcms.2000.0105]
34 **Schultze-Mosgau S**, Nkenke E, Schlegel AK, Hirschfelder U, Wiltfang J. Analysis of bone resorption after secondary alveolar cleft bone grafts before and after canine eruption in connection with orthodontic gap closure or prosthodontic treatment. *J Oral Maxillofac Surg* 2003; **61**: 1245-1248 [PMID: 14613077 DOI: 10.1016/S0278-2391(03)00722-5]
35 **Horswell BB**, Henderson JM. Secondary osteoplasty of the alveolar cleft defect. *J Oral Maxillofac Surg* 2003; **61**: 1082-1090 [PMID: 12966485 DOI: 10.1016/S0278-2391(03)00322-7]
36 **Khojasteh A**, Kheiri L, Motamedian SR, Nadjmi N. Regenerative medicine in the treatment of alveolar cleft defect: A systematic review of the literature. *J Craniomaxillofac Surg* 2015; **43**:

1608-1613 [PMID: 26302939 DOI: 10.1016/j.jcms.2015.06.041]
37 **Behnia H**, Khojasteh A, Soleimani M, Tehranchi A, Khoshzaban A, Keshel SH, Atashi R. Secondary repair of alveolar clefts using human mesenchymal stem cells. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009; **108**: e1-e6 [PMID: 19615638 DOI: 10.1016/j.tripleo.2009.03.040]
38 **Behnia H**, Khojasteh A, Soleimani M, Tehranchi A, Atashi A. Repair of alveolar cleft defect with mesenchymal stem cells and platelet derived growth factors: a preliminary report. *J Craniomaxillofac Surg* 2012; **40**: 2-7 [PMID: 21420310 DOI: 10.1016/j.jcms.2011.02.003]
39 **Pradel W**, Lauer G. Tissue-engineered bone grafts for osteoplasty in patients with cleft alveolus. *Ann Anat* 2012; **194**: 545-548 [PMID: 22776088 DOI: 10.1016/j.aanat.2012.06.002]
40 **Carstanjen B**, Desbois C, Hekmati M, Behr L. Successful engraftment of cultured autologous mesenchymal stem cells in a surgically repaired soft palate defect in an adult horse. *Can J Vet Res* 2006; **70**: 143-147 [PMID: 16639947]
41 **Pradel W**, Tausche E, Gollogly J, Lauer G. Spontaneous tooth eruption after alveolar cleft osteoplasty using tissue-engineered bone: a case report. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008; **105**: 440-444 [PMID: 18206405 DOI: 10.1016/j.tripleo.2007.07.042]
42 **Conejero JA**, Lee JA, Parrett BM, Terry M, Wear-Maggitti K, Grant RT, Breitbart AS. Repair of palatal bone defects using osteogenically differentiated fat-derived stem cells. *Plast Reconstr Surg* 2006; **117**: 857-863 [PMID: 16525276 DOI: 10.1097/01.prs.0000204566.13979.c1]
43 **Cohen N**, Cohen E, Gaiero A, Zecca S, Fichera G, Baldi F, Giordanetto JF, Mercier JM, Cohen A. Maxillofacial features and systemic malformations in expanded spectrum Hemifacial Microsomia. *Am J Med Genet A* 2017; **173**: 1208-1218 [PMID: 28319315 DOI: 10.1002/ajmg.a.38151]
44 **Gougoutas AJ**, Singh DJ, Low DW, Bartlett SP. Hemifacial microsomia: clinical features and pictographic representations of the OMENS classification system. *Plast Reconstr Surg* 2007; **120**: 112e-120e [PMID: 18090735 DOI: 10.1097/01.prs.0000287383.35963.5e]
45 **Kølle SF**, Fischer-Nielsen A, Mathiasen AB, Elberg JJ, Oliveri RS, Glovinski PV, Kastrup J, Kirchhoff M, Rasmussen BS, Talman ML, Thomsen C, Dickmeiss E, Drzewiecki KT. Enrichment of autologous fat grafts with ex-vivo expanded adipose tissue-derived stem cells for graft survival: a randomised placebo-controlled trial. *Lancet* 2013; **382**: 1113-1120 [PMID: 24075051 DOI: 10.1016/S0140-6736(13)61410-5]
46 **Tanikawa DY**, Aguena M, Bueno DF, Passos-Bueno MR, Alonso N. Fat grafts supplemented with adipose-derived stromal cells in the rehabilitation of patients with craniofacial microsomia. *Plast Reconstr Surg* 2013; **132**: 141-152 [PMID: 23806916 DOI: 10.1097/PRS.0b013e3182910a82]
47 **Tabit CJ**, Slack GC, Fan K, Wan DC, Bradley JP. Fat grafting versus adipose-derived stem cell therapy: distinguishing indications, techniques, and outcomes. *Aesthetic Plast Surg* 2012; **36**: 704-713 [PMID: 22069062 DOI: 10.1007/s00266-011-9835-4]
48 **Roberts WE**, Huja S, Roberts JA, editors. Bone modeling: biomechanics, molecular mechanisms, and clinical perspectives. *Semin Orthod* 2004: 123-161
49 **Carlson DS**. Biological rationale for early treatment of dentofacial deformities. *Am J Orthod Dentofacial Orthop* 2002; **121**: 554-558 [PMID: 12080297 DOI: 10.1067/mod.2002.124164]
50 **Rabie AB**, She TT, Hägg U. Functional appliance therapy accelerates and enhances condylar growth. *Am J Orthod Dentofacial Orthop* 2003; **123**: 40-48 [PMID: 12532062 DOI: 10.1067/mod.2003.45]
51 **St-Jacques B**, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 1999; **13**: 2072-2086 [PMID: 10465785 DOI: 10.1101/gad.13.16.2072]
52 **Rabie AB**, Wong L, Hägg U. Correlation of replicating cells and osteogenesis in the glenoid fossa during stepwise advancement. *Am J Orthod Dentofacial Orthop* 2003; **123**: 521-526 [PMID: 12750670 DOI: 10.1016/S0889-5406(02)57033-5]
53 **LeResche L**. Epidemiology of temporomandibular disorders: implications for the investigation of etiologic factors. *Crit Rev Oral Biol Med* 1997; **8**: 291-305 [PMID: 9260045 DOI: 10.1177/10454411970080030401]
54 **Okeson JP**. The American Academy of Orofacial Pain: Orofacial Pain Guidelines for assessment, diagnosis, and management. Quintessence Publishing Co. Inc: Chicago, 1996: 113-84
55 **Ta LE**, Phero JC, Pillemer SR, Hale-Donze H, McCartney-Francis N, Kingman A, Max MB, Gordon SM, Wahl SM, Dionne RA. Clinical evaluation of patients with temporomandibular joint implants. *J Oral Maxillofac Surg* 2002; **60**: 1389-1399 [PMID: 12464999 DOI: 10.1053/joms.2002.36089]
56 **Jacobs JJ**, Gilbert JL, Urban RM. Corrosion of metal orthopaedic implants. *J Bone Joint Surg Am* 1998; **80**: 268-282 [PMID: 9486734 DOI: 10.2106/00004623-199802000-00015]
57 **Shanti RM**, Li WJ, Nesti LJ, Wang X, Tuan RS. Adult mesenchymal stem cells: biological properties, characteristics, and applications in maxillofacial surgery. *J Oral Maxillofac Surg* 2007; **65**: 1640-1647 [PMID: 17656295 DOI: 10.1016/j.joms.2007.04.008]
58 **Bailey MM**, Wang L, Bode CJ, Mitchell KE, Detamore MS. A comparison of human umbilical cord matrix stem cells and temporomandibular joint condylar chondrocytes for tissue engineering temporomandibular joint condylar cartilage. *Tissue Eng* 2007; **13**: 2003-2010 [PMID: 17518722 DOI: 10.1089/ten.2006.0150]
59 **Alhadlaq A**, Mao JJ. Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells. *J Dent Res* 2003; **82**: 951-956 [PMID: 14630893 DOI: 10.1177/154405910308201203]
60 **Alhadlaq A**, Elisseeff JH, Hong L, Williams CG, Caplan AI, Sharma B, Kopher RA, Tomkoria S, Lennon DP, Lopez A, Mao JJ. Adult stem cell driven genesis of human-shaped articular condyle. *Ann Biomed Eng* 2004; **32**: 911-923 [PMID: 15298429 DOI: 10.1023/B:ABME.0000032454.53116.ee]
61 **Abukawa H**, Terai H, Hannouche D, Vacanti JP, Kaban LB, Troulis MJ. Formation of a mandibular condyle in vitro by tissue engineering. *J Oral Maxillofac Surg* 2003; **61**: 94-100 [PMID: 12524615 DOI: 10.1053/joms.2003.50015]
62 **Allen KD**, Athanasiou KA. Tissue Engineering of the TMJ disc: a review. *Tissue Eng* 2006; **12**: 1183-1196 [PMID: 16771633 DOI: 10.1089/ten.2006.12.1183]
63 **Mäenpää K**, Ellä V, Mauno J, Kellomäki M, Suuronen R, Ylikomi T, Miettinen S. Use of adipose stem cells and polylactide discs for tissue engineering of the temporomandibular joint disc. *J R Soc Interface* 2010; **7**: 177-188 [PMID: 19474082 DOI: 10.1098/rsif.2009.0117]
64 **McCarthy JG**, Stelnicki EJ, Mehrara BJ, Longaker MT. Distraction osteogenesis of the craniofacial skeleton. *Plast Reconstr Surg* 2001; **107**: 1812-1827 [PMID: 11391207 DOI: 10.1097/00006534-200106000-00029]
65 **Lawler ME**, Tayebaty FT, Williams WB, Troulis MJ, Kaban LB. Histomorphometric analysis of the porcine mandibular distraction wound. *J Oral Maxillofac Surg* 2010; **68**: 1543-1554 [PMID: 20561467 DOI: 10.1016/j.joms.2010.02.048]
66 **Ai-Aql ZS**, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. *J Dent Res* 2008; **87**: 107-118 [PMID: 18218835 DOI: 10.1177/154405910808700215]
67 **McCarthy JG**, Katzen JT, Hopper R, Grayson BH. The first decade of mandibular distraction: lessons we have learned. *Plast Reconstr Surg* 2002; **110**: 1704-1713 [PMID: 12447053 DOI: 10.1097/00006534-200212000-00013]
68 **Li G**. New developments and insights learned from distraction osteogenesis. *Curr Orthop Pract* 2004; **15**: 325-330 [DOI: 10.1097/01.bco.0000134434.61307.4e]
69 **Alkaisi A**, Ismail AR, Mutum SS, Ahmad ZA, Masudi S, Abd Razak NH. Transplantation of human dental pulp stem cells: enhance bone consolidation in mandibular distraction osteogenesis.

J Oral Maxillofac Surg 2013; **71**: 1758.e1-1758.13 [PMID: 24040948 DOI: 10.1016/j.joms.2013.05.016]
70 **Ma D**, Ren L, Yao H, Tian W, Chen F, Zhang J, Liu Y, Mao T. Locally injection of cell sheet fragments enhances new bone formation in mandibular distraction osteogenesis: a rabbit model. *J Orthop Res* 2013; **31**: 1082-1088 [PMID: 23494761 DOI: 10.1002/jor.22336]
71 **Qi M**, Hu J, Zou S, Zhou H, Han L. Mandibular distraction osteogenesis enhanced by bone marrow mesenchymal stem cells in rats. *J Craniomaxillofac Surg* 2006; **34**: 283-289 [PMID: 16777427 DOI: 10.1016/j.jcms.2006.02.002]
72 **Lai QG**, Yuan KF, Xu X, Li DR, Li GJ, Wei FL, Yang ZJ, Luo SL, Tang XP, Li S. Transcription factor osterix modified bone marrow mesenchymal stem cells enhance callus formation during distraction osteogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011; **111**: 412-419 [PMID: 20813560 DOI: 10.1016/j.tripleo.2010.05.012]
73 **Lee DH**, Ryu KJ, Kim JW, Kang KC, Choi YR. Bone marrow aspirate concentrate and platelet-rich plasma enhanced bone healing in distraction osteogenesis of the tibia. *Clin Orthop Relat Res* 2014; **472**: 3789-3797 [PMID: 24599650 DOI: 10.1007/s11999-014-3548-3]
74 **Hatzokos I**, Stavridis SI, Iosifidou E, Karataglis D, Christodoulou A. Autologous bone marrow grafting combined with demineralized bone matrix improves consolidation of docking site after distraction osteogenesis. *J Bone Joint Surg Am* 2011; **93**: 671-678 [PMID: 21471421 DOI: 10.2106/JBJS.J.00514]
75 **Sun Z**, Tee BC, Kennedy KS, Kennedy PM, Kim DG, Mallery SR, Fields HW. Scaffold-based delivery of autologous mesenchymal stem cells for mandibular distraction osteogenesis: preliminary studies in a porcine model. *PLoS One* 2013; **8**: e74672 [PMID: 24040314 DOI: 10.1371/journal.pone.0074672]
76 **Jiang X**, Zou S, Ye B, Zhu S, Liu Y, Hu J. bFGF-Modified BMMSCs enhance bone regeneration following distraction osteogenesis in rabbits. *Bone* 2010; **46**: 1156-1161 [PMID: 20036345 DOI: 10.1016/j.bone.2009.12.017]
77 **Hu J**, Qi MC, Zou SJ, Li JH, Luo E. Callus formation enhanced by BMP-7 ex vivo gene therapy during distraction osteogenesis in rats. *J Orthop Res* 2007; **25**: 241-251 [PMID: 17089407 DOI: 10.1002/jor.20288]
78 **Lai QG**, Sun SL, Zhou XH, Zhang CP, Yuan KF, Yang ZJ, Luo SL, Tang XP, Ci JB. Adipose-derived stem cells transfected with pEGFP-OSX enhance bone formation during distraction osteogenesis. *J Zhejiang Univ Sci B* 2014; **15**: 482-490 [PMID: 24793766 DOI: 10.1631/jzus.B1300203]
79 **Sunay O**, Can G, Cakir Z, Denek Z, Kozanoglu I, Erbil G, Yilmaz M, Baran Y. Autologous rabbit adipose tissue-derived mesenchymal stromal cells for the treatment of bone injuries with distraction osteogenesis. *Cytotherapy* 2013; **15**: 690-702 [PMID: 23522867 DOI: 10.1016/j.jcyt.2013.02.004]
80 **Sun JJ**, Zheng XH, Wang LY, Liu L, Jing W, Lin YF, Tian W, Tang W, Long J. New bone formation enhanced by ADSCs overexpressing hRunx2 during mandibular distraction osteogenesis in osteoporotic rabbits. *J Orthop Res* 2014; **32**: 709-720 [PMID: 24522890 DOI: 10.1002/jor.22590]
81 **Kim IS**, Cho TH, Lee ZH, Hwang SJ. Bone regeneration by transplantation of human mesenchymal stromal cells in a rabbit mandibular distraction osteogenesis model. *Tissue Eng Part A* 2013; **19**: 66-78 [PMID: 23083133 DOI: 10.1089/ten.tea.2011.0696]
82 **Aykan A**, Ozturk S, Sahin I, Gurses S, Ural AU, Oren NC, Isik S. Biomechanical analysis of the effect of mesenchymal stem cells on mandibular distraction osteogenesis. *J Craniofac Surg* 2013; **24**: e169-e175 [PMID: 23524827 DOI: 10.1097/SCS.0b013e31827c8706]
83 **Zhang WB**, Zheng LW, Chua DT, Cheung LK. Treatment of irradiated mandibles with mesenchymal stem cells transfected with bone morphogenetic protein 2/7. *J Oral Maxillofac Surg* 2012; **70**: 1711-1716 [PMID: 22580096 DOI: 10.1016/j.joms.2012.01.022]
84 **Cao J**, Wang L, Du ZJ, Liu P, Zhang YB, Sui JF, Liu YP, Lei DL. Recruitment of exogenous mesenchymal stem cells in mandibular distraction osteogenesis by the stromal cell-derived factor-1/chemokine receptor-4 pathway in rats. *Br J Oral Maxillofac Surg* 2013; **51**: 937-941 [PMID: 23747231 DOI: 10.1016/j.bjoms.2013.05.003]
85 **Wu G**, Hu C, He X, Yin K, Lan Y, Zhou B, Li S, Guo L. Effect of gene transfecting at different times on mandibular distraction osteogenesis. *J Craniofac Surg* 2013; **24**: 232-236 [PMID: 23348291 DOI: 10.1097/SCS.0b013e31826cff2a]
86 **Long J**, Li P, Du HM, Liu L, Zheng XH, Lin YF, Wang H, Jing W, Tang W, Chen WH, Tian WD. Effects of bone morphogenetic protein 2 gene therapy on new bone formation during mandibular distraction osteogenesis at rapid rate in rabbits. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011; **112**: 50-57 [PMID: 21194991 DOI: 10.1016/j.tripleo.2010.09.065]
87 **Castro-Govea Y**, Cervantes-Kardasch VH, Borrego-Soto G, Martínez-Rodríguez HG, Espinoza-Juarez M, Romero-Díaz V, Marino-Martínez IA, Robles-Zamora A, Álvarez-Lozano E, Padilla-Rivas GR, Ortiz-López R, Lara-Arias J, Vázquez-Juárez J, Rojas-Martínez A. Human bone morphogenetic protein 2-transduced mesenchymal stem cells improve bone regeneration in a model of mandible distraction surgery. *J Craniofac Surg* 2012; **23**: 392-396 [PMID: 22421833 DOI: 10.1097/SCS.0b013e318240fe9b]
88 **Vidya V**, Sumathi F. Rapid maxillary expansion as a standard treatment for obstructive sleep apnea syndrome: a systematic review. *J Dental Med Sci* 2015; **14**: 51-55
89 **Aloufi F**, Preston CB, Zawawi KH. Changes in the upper and lower pharyngeal airway spaces associated with rapid maxillary expansion. *ISRN Dent* 2012; **2012**: 290964 [PMID: 22778973 DOI: 10.5402/2012/290964]
90 **De Rossi M**, De Rossi A, Hallak JE, Vitti M, Regalo SC. Electromyographic evaluation in children having rapid maxillary expansion. *Am J Orthod Dentofacial Orthop* 2009; **136**: 355-360 [PMID: 19732669 DOI: 10.1016/j.ajodo.2007.08.027]
91 **Mommaerts MY**. Transpalatal distraction as a method of maxillary expansion. *Br J Oral Maxillofac Surg* 1999; **37**: 268-272 [PMID: 10475647 DOI: 10.1054/bjom.1999.0127]
92 **Bishara SE**, Staley RN. Maxillary expansion: clinical implications. *Am J Orthod Dentofacial Orthop* 1987; **91**: 3-14 [PMID: 3541577 DOI: 10.1016/0889-5406(87)90202-2]
93 **Lagravere MO**, Major PW, Flores-Mir C. Long-term dental arch changes after rapid maxillary expansion treatment: a systematic review. *Angle Orthod* 2005; **75**: 155-161 [PMID: 15825776]
94 **Baratieri C**, Alves M Jr, de Souza MM, de Souza Araújo MT, Maia LC. Does rapid maxillary expansion have long-term effects on airway dimensions and breathing? *Am J Orthod Dentofacial Orthop* 2011; **140**: 146-156 [PMID: 21803251 DOI: 10.1016/j.ajodo.2011.02.019]
95 **Ekizer A**, Yalvac ME, Uysal T, Sonmez MF, Sahin F. Bone marrow mesenchymal stem cells enhance bone formation in orthodontically expanded maxillae in rats. *Angle Orthod* 2015; **85**: 394-399 [PMID: 25054344 DOI: 10.2319/031114-177.1]
96 **Ackerman JL**, Proffit WR. Soft tissue limitations in orthodontics: treatment planning guidelines. *Angle Orthod* 1997; **67**: 327-336 [PMID: 9347106]
97 **Proffit WR**. Equilibrium theory revisited: factors influencing position of the teeth. *Angle Orthod* 1978; **48**: 175-186 [PMID: 280125]
98 **Proffit WR**, Fields H, Sarver D, Ackerman J. Contemporary Orthodontics. 5th ed. Mosbey Philadelphia, 2012: 691
99 **Proffit WR**. Special considerations in treatment for adults. Contemporary orthodontics: Mosby, St Louis, 2007: 635-685
100 **Preoteasa CT**, Ionescu E, Preoteasa E. Risks and complications associated with orthodontic treatment. Orthodontics-Basic Aspects and Clinical Consideration. InTech, 2012: 31-35
101 **Black CR**, Goriainov V, Gibbs D, Kanczler J, Tare RS, Oreffo RO. Bone Tissue Engineering. *Curr Mol Biol Rep* 2015; **1**: 132-140 [PMID: 26618105 DOI: 10.1007/s40610-015-0022-2]
102 **Rickert D**, Sauerbier S, Nagursky H, Menne D, Vissink A,

Raghoebar GM. Maxillary sinus floor elevation with bovine bone mineral combined with either autogenous bone or autogenous stem cells: a prospective randomized clinical trial. *Clin Oral Implants Res* 2011; **22**: 251-258 [PMID: 20831758 DOI: 10.1111/j.1600-0501.2010.01981.x]

103 **Payer M**, Lohberger B, Strunk D, Reich KM, Acham S, Jakse N. Effects of directly autotransplanted tibial bone marrow aspirates on bone regeneration and osseointegration of dental implants. *Clin Oral Implants Res* 2014; **25**: 468-474 [PMID: 23701676 DOI: 10.1111/clr.12172]

104 **Yamada Y**, Nakamura S, Ueda M, Ito K. Osteotome technique with injectable tissue-engineered bone and simultaneous implant placement by cell therapy. *Clin Oral Implants Res* 2013; **24**: 468-474 [PMID: 22150696 DOI: 10.1111/j.1600-0501.2011.02353.x]

105 **Miguita L**, Mantesso A, Pannuti CM, Deboni MCZ. Can stem cells enhance bone formation in the human edentulous alveolar ridge? A systematic review and meta-analysis. *Cell Tissue Bank* 2017; **18**: 217-228 [PMID: 28233169 DOI: 10.1007/s10561-017-9612-y]

106 **Reynolds MA**, Aichelmann-Reidy ME, Branch-Mays GL, Gunsolley JC. The efficacy of bone replacement grafts in the treatment of periodontal osseous defects. A systematic review. *Ann Periodontol* 2003; **8**: 227-265 [PMID: 14971256 DOI: 10.1902/annals.2003.8.1.227]

107 **Reynolds MA**, Kao RT, Camargo PM, Caton JG, Clem DS, Fiorellini JP, Geisinger ML, Mills MP, Nares S, Nevins ML. Periodontal regeneration - intrabony defects: a consensus report from the AAP Regeneration Workshop. *J Periodontol* 2015; **86**: S105-S107 [PMID: 25315019 DOI: 10.1902/jop.2015.140378]

108 **Khojasteh A**, Kheiri L, Motamedian SR, Khoshkam V. Guided Bone Regeneration for the Reconstruction of Alveolar Bone Defects. *Ann Maxillofac Surg* 2017; **7**: 263-277 [PMID: 29264297 DOI: 10.4103/ams.ams_76_17]

109 **Bianco P**, Robey PG, Saggio I, Riminucci M. "Mesenchymal" stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease. *Hum Gene Ther* 2010; **21**: 1057-1066 [PMID: 20649485 DOI: 10.1089/hum.2010.136]

110 **Geraci TF**. Orthodontic movement of teeth into artificially produced infrabony defects in the rhesus monkey. A histological report. *J Periodontol* 1973; **44**: 116

111 **Ghezzi C**, Masiero S, Silvestri M, Zanotti G, Rasperini G. Orthodontic treatment of periodontally involved teeth after tissue regeneration. *Int J Periodontics Restorative Dent* 2008; **28**: 559-567 [PMID: 19146051]

112 **Trossello VK**, Gianelly AA. Orthodontic treatment and periodontal status. *J Periodontol* 1979; **50**: 665-671 [PMID: 294480 DOI: 10.1902/jop.1979.50.12.665]

113 **Duan X**, Tu Q, Zhang J, Ye J, Sommer C, Mostoslavsky G, Kaplan D, Yang P, Chen J. Application of induced pluripotent stem (iPS) cells in periodontal tissue regeneration. *J Cell Physiol* 2011; **226**: 150-157 [PMID: 20658533 DOI: 10.1002/jcp.22316]

114 **Wu Y**, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 2007; **25**: 2648-2659 [PMID: 17615264 DOI: 10.1634/stemcells.2007-0226]

115 **Nagata M**, Iwasaki K, Akazawa K, Komaki M, Yokoyama N, Izumi Y, Morita I. Conditioned Medium from Periodontal Ligament Stem Cells Enhances Periodontal Regeneration. *Tissue Eng Part A* 2017; **23**: 367-377 [PMID: 28027709 DOI: 10.1089/ten.tea.2016.0274]

116 **Ma Z**, Li S, Song Y, Tang L, Ma D, Liu B, Jin Y. The biological effect of dentin noncollagenous proteins (DNCPs) on the human periodontal ligament stem cells (HPDLSCs) in vitro and in vivo. *Tissue Eng Part A* 2008; **14**: 2059-2068 [PMID: 18939934 DOI: 10.1089/ten.tea.2008.0021]

117 **Liu Y**, Zheng Y, Ding G, Fang D, Zhang C, Bartold PM, Gronthos S, Shi S, Wang S. Periodontal ligament stem cell-mediated treatment for periodontitis in miniature swine. *Stem Cells* 2008; **26**: 1065-1073 [PMID: 18238856 DOI: 10.1634/stemcells.2007-0734]

118 **Grimm WD**, Dannan A, Becher S, Gassmann G, Arnold W, Varga G, Dittmar T. The ability of human periodontium-derived stem cells to regenerate periodontal tissues: a preliminary in vivo investigation. *Int J Periodontics Restorative Dent* 2011; **31**: e94-e101 [PMID: 22140674]

119 **Ding G**, Liu Y, Wang W, Wei F, Liu D, Fan Z, An Y, Zhang C, Wang S. Allogeneic periodontal ligament stem cell therapy for periodontitis in swine. *Stem Cells* 2010; **28**: 1829-1838 [PMID: 20979138 DOI: 10.1002/stem.512]

120 **Iwasaki K**, Komaki M, Yokoyama N, Tanaka Y, Taki A, Honda I, Kimura Y, Takeda M, Akazawa K, Oda S, Izumi Y, Morita I. Periodontal regeneration using periodontal ligament stem cell-transferred amnion. *Tissue Eng Part A* 2014; **20**: 693-704 [PMID: 24032400 DOI: 10.1089/ten.tea.2013.0017]

121 **Masella RS**, Meister M. Current concepts in the biology of orthodontic tooth movement. *Am J Orthod Dentofacial Orthop* 2006; **129**: 458-468 [PMID: 16627170 DOI: 10.1016/j.ajodo.2005.12.013]

122 **Meikle MC**. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. *Eur J Orthod* 2006; **28**: 221-240 [PMID: 16687469 DOI: 10.1093/ejo/cjl001]

123 **Rody WJ Jr**, King GJ, Gu G. Osteoclast recruitment to sites of compression in orthodontic tooth movement. *Am J Orthod Dentofacial Orthop* 2001; **120**: 477-489 [PMID: 11709665 DOI: 10.1067/mod.2001.118623]

124 **Miyamoto T**, Suda T. Differentiation and function of osteoclasts. *Keio J Med* 2003; **52**: 1-7 [PMID: 12713016 DOI: 10.2302/kjm.52.1]

125 **Zainal Ariffin SH**, Yamamoto Z, Zainol Abidin IZ, Megat Abdul Wahab R, Zainal Ariffin Z. Cellular and molecular changes in orthodontic tooth movement. *ScientificWorldJournal* 2011; **11**: 1788-1803 [PMID: 22125437 DOI: 10.1100/2011/761768]

126 **Suya H**. Corticotomy in orthodontics. Mechanical and biological basics in orthodontic therapy. Heidelberg, Germany: Huthig Buch Verlag, 1991: 207-226

127 **Wilcko WM**, Wilcko T, Bouquot JE, Ferguson DJ. Rapid orthodontics with alveolar reshaping: two case reports of decrowding. *Int J Periodontics Restorative Dent* 2001; **21**: 9-19 [PMID: 11829041]

128 **Almpani K**, Kantarci A. Nonsurgical Methods for the Acceleration of the Orthodontic Tooth Movement. *Front Oral Biol* 2016; **18**: 80-91 [PMID: 26599121 DOI: 10.1159/000382048]

129 **Feng L**, Yang R, Liu D, Wang X, Song Y, Cao H, He D, Gan Y, Kou X, Zhou Y. PDL Progenitor-Mediated PDL Recovery Contributes to Orthodontic Relapse. *J Dent Res* 2016; **95**: 1049-1056 [PMID: 27161015 DOI: 10.1177/0022034516648604]

130 **Pizzo G**, Licata ME, Guiglia R, Giuliana G. Root resorption and orthodontic treatment. Review of the literature. *Minerva Stomatol* 2007; **56**: 31-44 [PMID: 17287705]

131 **Mohanty P**, Prasad NK, Sahoo N, Kumar G, Mohanty D, Sah S. Reforming craniofacial orthodontics via stem cells. *J Int Soc Prev Community Dent* 2015; **5**: 13-18 [PMID: 25767761 DOI: 10.4103/2231-0762.151966]

132 **Zahrowski J**, Jeske A. Apical root resorption is associated with comprehensive orthodontic treatment but not clearly dependent on prior tooth characteristics or orthodontic techniques. *J Am Dent Assoc* 2011; **142**: 66-68 [PMID: 21193769 DOI: 10.14219/jada.archive.2011.0030]

133 **Guo Y**, He S, Gu T, Liu Y, Chen S. Genetic and clinical risk factors of root resorption associated with orthodontic treatment. *Am J Orthod Dentofacial Orthop* 2016; **150**: 283-289 [PMID: 27476361 DOI: 10.1016/j.ajodo.2015.12.028]

134 **Shinagawa-Ohama R**, Mochizuki M, Tamaki Y, Suda N, Nakahara T. Heterogeneous Human Periodontal Ligament-Committed Progenitor and Stem Cell Populations Exhibit a Unique Cementogenic Property Under In Vitro and In Vivo Conditions. *Stem Cells Dev* 2017; **26**: 632-645 [PMID: 28136695 DOI: 10.1089/scd.2016.0330]

135 **Oshima M**, Mizuno M, Imamura A, Ogawa M, Yasukawa M,

Yamazaki H, Morita R, Ikeda E, Nakao K, Takano-Yamamoto T, Kasugai S, Saito M, Tsuji T. Functional tooth regeneration using a bioengineered tooth unit as a mature organ replacement regenerative therapy. *PLoS One* 2011; **6**: e21531 [PMID: 21765896 DOI: 10.1371/journal.pone.0021531]

136 **Ikeda E**, Morita R, Nakao K, Ishida K, Nakamura T, Takano-Yamamoto T, Ogawa M, Mizuno M, Kasugai S, Tsuji T. Fully functional bioengineered tooth replacement as an organ replacement therapy. *Proc Natl Acad Sci USA* 2009; **106**: 13475-13480 [PMID: 19666587 DOI: 10.1073/pnas.0902944106]

137 **Ono M**, Oshima M, Ogawa M, Sonoyama W, Hara ES, Oida Y, Shinkawa S, Nakajima R, Mine A, Hayano S, Fukumoto S, Kasugai S, Yamaguchi A, Tsuji T, Kuboki T. Practical whole-tooth restoration utilizing autologous bioengineered tooth germ transplantation in a postnatal canine model. *Sci Rep* 2017; **7**: 44522 [PMID: 28300208 DOI: 10.1038/srep44522]

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EDITORIAL

Ectopic expression of the osteogenic master gene *RUNX2* in melanoma

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Abstract

The transcription factor *RUNX2* is the osteogenic master gene expressed in mesenchymal stem cells during osteogenic commitment as well as in pre-osteoblasts and early osteoblasts. However, *RUNX2* is also ectopically expressed in melanoma and other cancers. Malignant melanoma (MM) is a highly metastatic skin cancer. The incidence of MM has increased considerably in the past half-century. The expression levels and mutation rates of genes such as *BRAF*, *KIT*, *NRAS*, *PTEN*, *P53*, *TERT* and *MITF* are higher in melanoma than in other solid malignancies. Additionally, transcription factors can affect cellular processes and induce cellular transformation since they control gene expression. Recently, several studies have identified alterations in *RUNX2* expression. In particular, the regulation of *KIT* by *RUNX2* and the increased expression of *RUNX2* in melanoma specimens have been shown. Melanocytes, whose transformation results in melanoma, arise from the neural crest and therefore show "stemness" features. *RUNX2* plays an important role in the re-activation of the MAPK and PI-3K/AKT pathways, thus endowing melanoma cells with a high metastatic potential. In melanoma, the most frequent metastatic sites are the lung, liver, brain and lymph nodes. In addition, bone metastatic melanoma has been described. Notably, studies focusing on *RUNX2* may contribute to the identification of an appropriate oncotarget in melanoma.

Key words: *RUNX2*; Mesenchymal stem cells; Epitelial mesenchymal transition; Bone; Melanoma

Core tip: In addition to its physiological expression in osteogenic cells, *RUNX2* ectopic expression is also reported in several cancers. Melanoma cells, which arise from the neural crest, are not typical epithelial cells and

exhibit "stemness" features. For this reason the epithelial mesenchymal transition process may be enhanced in melanoma cells. *RUNX2* expression in such context increases the migration and invasiveness of melanoma cells; it can therefore be considered a new oncotarget in melanoma.

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INTRODUCTION

In recent times, the incidence of malignant melanoma (MM) has increased. The increased number of MM patients may be ascribed to lifestyle and environmental changes. Unfortunately, the mortality rate of MM is very high, as MM is highly metastatic and genetically resistant to apoptosis. The surgical treatment of skin lesions before cell spreading is considered a successful therapeutic approach. However, after metastasis and dissemination of cells has occurred, the therapeutic tools are limited. Despite an initial positive response to treatment, melanoma cells become resistant to chemotherapy[1]. Many studies performed in animal models and in cell culture have identified the complex mechanism involved in the metastatic progression of melanoma. Mutations in master transcription regulators such as *BRAF, KIT, NRAS, PTEN, P53, TERT*, and *MITF* have been found frequently[2]. In particular, the microphthalmia-associated transcription factor (*MITF*) is an important oncogene, and it plays a critical role in melanoma transformation due to its role in melanocyte proliferation and differentiation[3]. However, transcription factors in general may affect cellular processes and thereby induce cellular transformation. Among them, *RUNX2*, the master gene of osteogenic differentiation, has been shown to be involved in the transformation and metastatic progression of melanoma[4-6]. *RUNX2*, together with *RUNX1* and *RUNX3*, belongs to the *RUNT*-related gene family[7]. These heterodimeric transcription factors have a DNA-binding "A" subunit and a non-DNA binding "B" subunit. The genomic and cDNA structures of the *RUNT* family members are evolutionarily conserved[8]. *RUNX2* is located on human chromosome 6; its coding sequence is organized in eight exons and controlled by two promoters. Isoforms may originate by the alternative use of promoters or by the alternative splicing of exons; however, the DNA-binding domain remains invariant[7]. *RUNX2* induces the commitment of mesenchymal stem cells to the osteogenic lineage by acting either as a monomer or as a heterodimeric complex, which shows a higher DNA binding activity[7]. Interestingly, differences in *RUNX2* expression are associated with the skeletal differences between modern humans and Neanderthals[9]. Otto *et al*[10] using an in vivo model, showed that the ossification process is lacking in *RUNX2* KO mice. *RUNX2* is expressed not only in mesenchymal stem cells to commit these cells to osteogenic differentiation but also in pre-osteoblasts and early osteoblasts. It is then downregulated, as its continuous expression would prevent osteoblast maturation. This aspect is important for bone remodelling. In acromegaly patients, who are characterized by an excessive production of GH and IGF-1, a high fracture risk occurs; we have demonstrated that RUNX2 gene overexpression induces bone loss and impairs bone remodelling in these patients[11].

RUNX2 OVEREXPRESSION IN CANCER

The *RUNX* family members are frequently deregulated in human cancer. Runx proteins are involved in critical cellular processes such DNA repair, apoptosis, hypoxia, the inflammatory response, EMT, stem cell functions, and oncogene-induced senescence, and they interact with different signalling pathways such as the RAS-ERK, WNT, TGFβ, BMP and Notch pathways[12]. In addition, *RUNX2* is involved in osteolytic diseases, neoangiogenic processes, and invasion and metastasis of solid tumours[4]. *RUNX2* can be associated with HDA6 and can thereby prevent the pro-apoptotic activity of P53 through its deacetylation[13]. *RUNX2* is ectopically expressed in several solid tumours such as breast cancer, pancreatic cancer, prostate cancer, lung cancer, and ovarian epithelial cancer; therefore, we have proposed *RUNX2* as a mesenchymal stem marker for cancer[14,15]. Interestingly, we observed higher levels of *RUNX2* expression in cancer patients with bone metastases compared to patients without bone metastases[15]. In thyroid cancer patients, we observed higher expression of *RUNX2* in cancer tissue as well as in circulating mRNA compared to those levels of *RUNX2* expression in controls[16]. In addition, *RUNX2* expression was higher in cancer patients with microcalcifications compared to patients without microcalcifications[16]. Finally, it has been reported that *RUNX2* induces its target genes SDF-1, CXCR7 and BSP, promoting bone homing[17].

RUNX2 IN MELANOMA

Ectopic expression of *RUNX2* in melanoma tissue and in melanoma cell lines has been shown to be associated with bone sialoprotein (BSP)[18]. BSP has been associated with tumour invasiveness[18]. *RUNX2* also regulates several matrix metalloproteinases that are involved in melanoma progression; therefore, *RUNX2* may be regarded as a common regulator of both BSP and metalloproteinases[19]. This finding could also explain the expression of other bone proteins, such as osteopontin and osteocalcin, in melanoma[20,21]. TGFβ, which induces metastasis formation in advanced melanoma stages[22], can upregulate the expression of *RUNX2* in melanoma

cell lines[23]. In contrast, the tumour suppressor p14ARF reduces *RUNX2* expression[24]. Loss-of-function *p14ARF* mutations lead to increased expression of *RUNX2* and melanoma progression[24]. Ectopic expression of *RUNX2* in melanoma has been associated with invasiveness; it has been demonstrated that *RUNX2* overexpression mediates the migration ability of melanoma cells[6]. Notably *RUNX2* stimulates *VEGF* gene expression[25] and the upregulation of angiogenesis, consequent to *RUNX2* overexpression, may represent a critical step for tumor metastasis.

Furthermore, *RUNX2* overexpression upregulates *SOX9, SNAI2* and *SMAD3*. These transcription factors are involved in EMT as well as in cytoskeletal remodelling and thus enhance the motility and invasive potential of cancer cells[17]. In melanoma, the overexpression of *EGF-R, PDGFRβ, AXL* and *IGF-1R* induces the reactivation of the MAPK and PI3K/AKT pathways, which are involved in migration and invasion processes[26]. The reactivation of these pathways is due to tyrosine kinase receptor-based autocrine loops, in which *RUNX2* plays a pivotal role[5]. The MAPK and AKT pathways regulate most physiological processes, such as proliferation, differentiation and cell survival. It has been suggested that the MAPK and AKT pathways are strongly associated with each other in melanoma; the link between the PI3K/AKT (AKT pathway) and MAPK/ERK1/2 (MAPK pathway) cascades is well known[27]. AKT enhances RUNX2 expression either directly or by inactivating RUNX2 regulators. Similarly, RUNX2 activates the PI3K/AKT pathway by regulating different components[4]. This reciprocal activation induces tumour progression and aggressiveness. In addition, RUNX2 promotes the crosstalk between MAPK and AKT through EGFR, as has been shown in human mammary epithelial cells[28] through the reduction of ERK-mediated inhibition of EGFR and the AKT pathway. The lung, liver, brain and lymph nodes are the most frequent metastatic sites for melanoma cells[29]. Since melanocytes arise from the neural crest, they are not typical epithelial cells; they show "stemness" features. This last finding could partially explain the high metastatic potential of melanoma[29]. Solid malignancies such as breast, prostate and lung cancers form metastases in bone. However, metastatic bone diseases from MM are under-investigated. The data in the literature report an incidence of skeletal metastases in MM patients that ranges from 0.85% to 18.6%[30]. Recently, Zekri *et al*[31], managing MM patients at two referral cancer centres in England, showed that bone metastases occur in 4.1% of patients at all stages of MM. In addition, another study showed that 17.2% of metastatic melanoma patients had metastases in bone tissue.

CONCLUSION

MM accounts for less than 5% of cutaneous malignancies. However, its incidence has been increasing considerably in the past half-century. Therefore, extensive research focusing on the genes involved in melanoma transformation and on the modulation of these genes is needed. Several studies have highlighted the involvement of RUNX2, the master gene of osteogenic differentiation, in melanoma. The invasiveness and metastatic features conferred by RUNX2 seem to be related to its ability to promote EMT. In addition, melanocytes are not typical epithelial cells, and their stemness features could explain melanoma invasiveness. Since metastatic melanoma has a poor prognosis, new and more effective therapeutic tools should be developed in order to implement current therapies. In this context, the exploitation of molecules actually involved in melanoma, such as RUNX2, represents an important frontier for the identification of new oncotargets. Transcription factors have been ignored thus far by the pharmaceutical industry; nonetheless, many studies have identified their central role in cellular transformation. We believe that future efforts directed towards unravelling the complex roles of RUNX2 may contribute to the identification of new therapeutic tools that improve the quality of life of melanoma patients.

REFERENCES

1 **Whiteman DC**, Pavan WJ, Bastian BC. The melanomas: a synthesis of epidemiological, clinical, histopathological, genetic, and biological aspects, supporting distinct subtypes, causal pathways, and cells of origin. *Pigment Cell Melanoma Res* 2011; **24**: 879-897 [PMID: 21707960 DOI: 10.1111/j.1755-148X.2011.00880.x]

2 **Damsky WE**, Bosenberg M. Melanocytic nevi and melanoma: unraveling a complex relationship. *Oncogene* 2017; **36**: 5771-5792 [PMID: 28604751 DOI: 10.1038/onc.2017.189]

3 **Levy C**, Khaled M, Fisher DE. MITF: master regulator of melanocyte development and melanoma oncogene. *Trends Mol Med* 2006; **12**: 406-414 [PMID: 16899407 DOI: 10.1016/j.molmed.2006.07.008]

4 **Cohen-Solal KA**, Boregowda RK, Lasfar A. RUNX2 and the PI3K/AKT axis reciprocal activation as a driving force for tumor progression. *Mol Cancer* 2015; **14**: 137 [PMID: 26204939 DOI: 10.1186/s12943-015-0404-3]

5 **Boregowda RK**, Medina DJ, Markert E, Bryan MA, Chen W, Chen S, Rabkin A, Vido MJ, Gunderson SI, Chekmareva M, Foran DJ, Lasfar A, Goydos JS, Cohen-Solal KA. The transcription factor RUNX2 regulates receptor tyrosine kinase expression in melanoma. *Oncotarget* 2016; **7**: 29689-29707 [PMID: 27102439 DOI: 10.18632/oncotarget.8822]

6 **Boregowda RK**, Olabisi OO, Abushahba W, Jeong BS, Haenssen KK, Chen W, Chekmareva M, Lasfar A, Foran DJ, Goydos JS, Cohen-Solal KA. RUNX2 is overexpressed in melanoma cells and mediates their migration and invasion. *Cancer Lett* 2014; **348**: 61-70 [PMID: 24657655 DOI: 10.1016/j.canlet.2014.03.011]

7 **Dalle Carbonare L**, Innamorati G, Valenti MT. Transcription factor Runx2 and its application to bone tissue engineering. *Stem Cell Rev* 2012; **8**: 891-897 [PMID: 22139789 DOI: 10.1007/s12015-011-9337-4]

8 **Otto F**, Lübbert M, Stock M. Upstream and downstream targets of RUNX proteins. *J Cell Biochem* 2003; **89**: 9-18 [PMID: 12682904 DOI: 10.1002/jcb.10491]

9 **Benítez-Burraco A**, Boeckx C. Possible functional links among brain- and skull-related genes selected in modern humans. *Front Psychol* 2015; **6**: 794 [PMID: 26136701 DOI: 10.3389/fpsyg.2015.00794]

10 **Otto F**, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 1997; **89**: 765-771 [PMID: 9182764 DOI: 10.1016/S0092-8674(00)80259-7]

11 **Valenti MT**, Mottes M, Cheri S, Deiana M, Micheletti V, Cosaro E, Davì MV, Francia G, Dalle Carbonare L. Runx2 overexpression compromises bone quality in acromegalic patients. *Endocr Relat Cancer* 2018; **25**: 269-277 [PMID: 29295822 DOI: 10.1530/ERC-17-0523]

12 **Ito Y**. RUNX genes in development and cancer: regulation of viral gene expression and the discovery of RUNX family genes. *Adv Cancer Res* 2008; **99**: 33-76 [PMID: 18037406 DOI: 10.1016/S0065-230X(07)99002-8]

13 **Ozaki T**, Nakamura M, Shimozato O. Novel Implications of DNA Damage Response in Drug Resistance of Malignant Cancers Obtained from the Functional Interaction between p53 Family and RUNX2. *Biomolecules* 2015; **5**: 2854-2876 [PMID: 26512706 DOI: 10.3390/biom5042854]

14 **Valenti MT**, Serafini P, Innamorati G, Gili A, Cheri S, Bassi C, Dalle Carbonare L. Runx2 expression: A mesenchymal stem marker for cancer. *Oncol Lett* 2016; **12**: 4167-4172 [PMID: 27895787 DOI: 10.3892/ol.2016.5182]

15 **Valenti MT**, Mori A, Malerba G, Dalle Carbonare L. Mesenchymal stem cells: A new diagnostic tool? *World J Stem Cells* 2015; **7**: 789-792 [PMID: 26131309 DOI: 10.4252/wjsc.v7.i5.789]

16 **Dalle Carbonare L**, Frigo A, Francia G, Davì MV, Donatelli L, Stranieri C, Brazzarola P, Zatelli MC, Menestrina F, Valenti MT. Runx2 mRNA expression in the tissue, serum, and circulating non-hematopoietic cells of patients with thyroid cancer. *J Clin Endocrinol Metab* 2012; **97**: E1249-E1256 [PMID: 22511796 DOI: 10.1210/jc.2011-2624]

17 **Baniwal SK**, Khalid O, Gabet Y, Shah RR, Purcell DJ, Mav D, Kohn-Gabet AE, Shi Y, Coetzee GA, Frenkel B. Runx2 transcriptome of prostate cancer cells: insights into invasiveness and bone metastasis. *Mol Cancer* 2010; **9**: 258 [PMID: 20863401 DOI: 10.1186/1476-4598-9-258]

18 **Riminucci M**, Corsi A, Peris K, Fisher LW, Chimenti S, Bianco P. Coexpression of bone sialoprotein (BSP) and the pivotal transcriptional regulator of osteogenesis, Cbfa1/Runx2, in malignant melanoma. *Calcif Tissue Int* 2003; **73**: 281-289 [PMID: 14667142 DOI: 10.1007/s00223-002-2134-y]

19 **Hofmann UB**, Westphal JR, Zendman AJ, Becker JC, Ruiter DJ, van Muijen GN. Expression and activation of matrix metalloproteinase-2 (MMP-2) and its co-localization with membrane-type 1 matrix metalloproteinase (MT1-MMP) correlate with melanoma progression. *J Pathol* 2000; **191**: 245-256 [PMID: 10878545 DOI: 10.1002/1096-9896(2000)9999:9999<::AID-PATH632>3.0.CO;2-#]

20 **Kiss T**, Ecsedi S, Vizkeleti L, Koroknai V, Emri G, Kovács N, Adany R, Balazs M. The role of osteopontin expression in melanoma progression. *Tumour Biol* 2015; **36**: 7841-7847 [PMID: 25944164 DOI: 10.1007/s13277-015-3495-y]

21 **Salvatierra E**, Alvarez MJ, Leishman CC, Rivas Baquero E, Lutzky VP, Chuluyan HE, Podhajcer OL. SPARC Controls Melanoma Cell Plasticity through Rac1. *PLoS One* 2015; **10**: e0134714 [PMID: 26248315 DOI: 10.1371/journal.pone.0134714]

22 **Lasfar A**, Cohen-Solal KA. Resistance to transforming growth factor β-mediated tumor suppression in melanoma: are multiple mechanisms in place? *Carcinogenesis* 2010; **31**: 1710-1717 [PMID: 20656791 DOI: 10.1093/carcin/bgq155]

23 **Mohammad KS**, Javelaud D, Fournier PG, Niewolna M, McKenna CR, Peng XH, Duong V, Dunn LK, Mauviel A, Guise TA. TGF-beta-RI kinase inhibitor SD-208 reduces the development and progression of melanoma bone metastases. *Cancer Res* 2011; **71**: 175-184 [PMID: 21084275 DOI: 10.1158/0008-5472.CAN-10-2651]

24 **Packer LM**, Pavey SJ, Boyle GM, Stark MS, Ayub AL, Rizos H, Hayward NK. Gene expression profiling in melanoma identifies novel downstream effectors of p14ARF. *Int J Cancer* 2007; **121**: 784-790 [PMID: 17450523 DOI: 10.1002/ijc.22725]

25 **Zelzer E**, Glotzer DJ, Hartmann C, Thomas D, Fukai N, Soker S, Olsen BR. Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. *Mech Dev* 2001; **106**: 97-106 [PMID: 11472838 DOI: 10.1016/S0925-4773(01)00428-2]

26 **Yadav V**, Denning MF. Fyn is induced by Ras/PI3K/Akt signaling and is required for enhanced invasion/migration. *Mol Carcinog* 2011; **50**: 346-352 [PMID: 21480388 DOI: 10.1002/mc.20716]

27 **Pappalardo F**, Russo G, Candido S, Pennisi M, Cavalieri S, Motta S, McCubrey JA, Nicoletti F, Libra M. Computational Modeling of PI3K/AKT and MAPK Signaling Pathways in Melanoma Cancer. *PLoS One* 2016; **11**: e0152104 [PMID: 27015094 DOI: 10.1371/journal.pone.0152104]

28 **Tandon M**, Chen Z, Pratap J. Role of Runx2 in crosstalk between Mek/Erk and PI3K/Akt signaling in MCF-10A cells. *J Cell Biochem* 2014; **115**: 2208-2217 [PMID: 25147082 DOI: 10.1002/jcb.24939]

29 **Braeuer RR**, Watson IR, Wu CJ, Mobley AK, Kamiya T, Shoshan E, Bar-Eli M. Why is melanoma so metastatic? *Pigment Cell Melanoma Res* 2014; **27**: 19-36 [PMID: 24106873 DOI: 10.1111/pcmr.12172]

30 **Fon GT**, Wong WS, Gold RH, Kaiser LR. Skeletal metastases of melanoma: radiographic, scintigraphic, and clinical review. *AJR Am J Roentgenol* 1981; **137**: 103-108 [PMID: 6787858 DOI: 10.2214/ajr.137.1.103]

31 **Zekri J**, Marples M, Taylor D, Kandukurti K, McParland L, Brown JE. Complications of bone metastases from malignant melanoma. *J Bone Oncol* 2017; **8**: 13-17 [PMID: 28856087 DOI: 10.1016/j.jbo.2017.08.003]

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REVIEW

Stem cell therapy for faecal incontinence: Current state and future perspectives

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Abstract

Faecal continence is a complex function involving different organs and systems. Faecal incontinence is a common disorder with different pathogeneses, disabling consequences and high repercussions for quality of life. Current management modalities are not ideal, and the development of new treatments is needed. Since 2008, stem cell therapies have been validated, 36 publications have appeared (29 in preclinical models and seven in clinical settings), and six registered clinical trials are currently ongoing. Some publications have combined stem cells with bioengineering technologies. The aim of this review is to identify and summarise the existing published knowledge of stem cell utilization as a treatment for faecal incontinence. A narrative or descriptive review is presented. Preclinical studies have demonstrated that cellular therapy, mainly in the form of local injections of muscle-derived (muscle derived stem cells or myoblasts derived from them) or mesenchymal (bone-marrow- or adipose-derived) stem cells, is safe. Cellular therapy has also been shown to stimulate

the repair of both acute and subacute anal sphincter injuries, and some encouraging functional results have been obtained. Stem cells combined with normal cells on bioengineered scaffolds have achieved the successful creation and implantation of intrinsically-innervated anal sphincter constructs. The clinical evidence, based on adipose-derived stem cells and myoblasts, is extremely limited yet has yielded some promising results, and appears to be safe. Further investigation in both animal models and clinical settings is necessary to drawing conclusions. Nevertheless, if the preliminary results are confirmed, stem cell therapy for faecal incontinence may well become a clinical reality in the near future.

Key words: Faecal incontinence; Anal sphincter; Cell implantation; Tissue engineering; Cell therapy; Stem cells

Core tip: Faecal incontinence is very frequent and is associated with severe consequences for patients. Available treatment outcomes are not optimal, particularly in the long-term. Stem cells, with or without bioengineering, could improve these results, as demonstrated in other clinical settings. We present a descriptive review of the published literature about faecal incontinence and stem cells, and discuss the existing limitations and concerns. Preclinical studies have confirmed the feasibility and safety of stem cells, and show some interesting results; the limited clinical experience confirms the safety and potential efficacy. However, further studies are needed to obtain clear conclusions.

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INTRODUCTION

Faecal incontinence (FI) is a highly prevalent nonfatal illness associated with considerable embarrassment, anxiety and poor quality of life. In a systematic review by McMillan, it was estimated to occur in 11%-15% of adults[1], and Nelson found it in 2.2% of the general population and 47% of institutionalised individuals[2]. It is difficult to know its real prevalence due to its psychosocial repercussions, as patients tend not to report it to their physicians and physicians rarely ask about it.

The physical repercussions are limited, but psychosocial ones are devastating, which include the loss of self-confidence, disability, body image alteration, social isolation, anxiety, depression, *etc.* A British study observed a four-fold increase in anxiety and five-fold increase in depression, with a significant association of both with faecal incontinence[3]. Furthermore, it could also lead to job loss and is the second highest cause of institutionalisation. Studies focusing on the quality of life reflect significant repercussions in multiple components, such as physical[4,5] and mental[5]; generally, the greater the incontinence, the greater the deterioration it causes ($P < 0.01$)[5]. A Spanish study showed an independent association between quality of life and declining mental health (OR 2.088 and $P = 0.017$)[6].

The economic impact is high and very difficult to estimate, but it consists of direct (diagnostic test, treatments, care, *etc*.) and indirect (job production, secondary treatments, such as for psychological consequences, *etc.*) costs. Indirect costs are harder to calculate and account for more than half[7]. In a Seattle study, annual healthcare costs increased up to $2897 in 2005 according to multivariate analyses (pads, barriers or institutionalisation not included)[4]. In a Dutch study, global costs increased yearly by 2169€ for each patient[7].

The prevalence is higher among women, the elderly, people with poor health or physical limitations, and those residing in nursing homes. Other risk factors include pelvic radiation, pregnancy, pelvic injury associated with vaginal delivery, anorectal surgery, diarrhoea, faecal impaction, some neurological conditions, and diabetes.

Bowel continence is a very developed function that depends on complex sensory and motor interactions between the rectum, anus, external anal sphincter (EAS), internal anal sphincter (IAS), puborectal muscle, and their vascularisation and innervation. When one or more of these structures or interactions are disrupted to such a degree that the others are unable to compensate, incontinence appears. Therefore, FI is a multifactorial disease. The most frequent morphological alteration found in almost 60% of patients is a sphincter lesion, with most of them being obstetric (30%-40%). Sphincter lesions during delivery range from 11%[8] to 26.9%[9], increase with every pregnancy, and cause incontinence in 76.8%-82.8% of patients[9].

Although sacral neuromodulation has been growing exponentially in recent years, surgery remains the treatment of choice for the most severe or refractory cases, mainly when sphincter lesions are present. There are a lot of surgical techniques, but sphincter repair is the most successful for sphincter injuries. Sphincter repair has shown good results in the short-term: Excellent-good in 66%, moderate in 22%, and poor in 12% of patients[10]. However, these outcomes do not persist in the long-term; Halverson and Hull found that 54% were fully incontinent, and only 14% were fully continent 69 mo later[11]. Similarly, the review by Glasgow and Lowry, with 16 publications comprising almost 900 reparations, observed an almost constant decline in initially subjective "good" outcomes in the long-term. Despite these worsening results over time, most patients remained satisfied (also in their quality of life). No failure predictive factors were found[12]. The

reasons for this decay are not well understood.

Stem cell (SC) therapy has been demonstrated to be safe and obtain promising results in a wide variety of clinical and experimental settings: Haematological, cardiovascular, neurological, digestive, traumatic, endocrine, renal and metabolic conditions are some examples. The most commonly used are haematopoietic stem cells (HSCs)[13], mesenchymal stem cells (MSCs)[14-16] or adipose-derived stem cells (ASCs)[17-19]. For example, ASCs have been tried and had favourable outcomes in environments that are particularly unfavourable for wound healing, such as experimental colitis[20], sepsis[21], anal fistula[22-29], Crohn's patients[30], experimental colonic[31,32], and tracheal[33] anastomoses.

Based on the published literature, as well as on our group's experience with FI treatment and using ASCs in experimental and clinical settings (having conducted or participated in more than six clinical trials with autologous or allogeneic ASCs for digestive fistula), our aim was to review published literature related to stem cell therapy for FI, , and currently ongoing clinical trials. To the best of our knowledge, there is only one review on this field from Gräs *et al*[34], which also includes tissue engineering studies published prior to June 2015.

SEARCH

We performed an exhaustive search of the published literature in the United States National Library of Medicine database ("PubMed") using the following terms: "faecal incontinence", "anal incontinence", "stem cells", "progenitor cells", "cellular therapy" and "cell therapy". Only studies published in indexed peer-reviewed journals were selected. "Similar articles" in PubMed and references of the selected studies were also analysed to detect potentially includable articles. Related to bioengineering, only publications combining it with SCs were considered for this review. The United States National Library of Medicine official registry of clinical trials "ClinicalTrials.gov" (http://www.clinicaltrials.gov) and the EU Clinical Trials Register (http://www.clinicaltrialsregister.eu) were searched using the same terms to detect ongoing registered clinical trials. Both searches were performed on April 1st 2018.

The high variability of FI models, the cellular products employed, and the methodology of applying it or evaluating their results, make it impossible to perform a meta-analysis. Therefore, a narrative or descriptive review is presented.

STEM CELLS APPLIED FOR FAECAL INCONTINENCE: A BRIEF OVERVIEW

The pioneering report in this field was in an animal model from Lorenzi *et al*[35] in 2008. From that point onwards, several articles have been published, mostly using animal models. However, only a few experiences with humans have gradually appeared since 2010.

We have identified a total of 36 publications eligible for a deeper analysis. Twenty-nine are preclinical studies on animal models, some combining SCs with bioengineering strategies, that try to create a biocompatible and implantable EAS or IAS construct. Seven publications are on humans. Also, six registered clinical trials were found that are "active" or apparently "ongoing". In the following sections, we are going to analyse and summarise the publications, ordering them using the internet publication date or "Epub" date.

ANIMAL STUDIES PUBLISHED

In the 29 selected preclinical animal studies, high heterogeneity on employed animals, faecal incontinence models, type of repair, kind of SC applied, and response evaluation system were applied. Overviews of the following aspects are presented in Tables 1-3: injury model and repair (Table 1), kind of SC employed (Table 2) and bioengineering strategies combined with SCs (Table 3). The types of animal used and the adjuvants employed to SCs are mentioned later.

The first publication was by Lorenzi *et al*[35] in 2008. They performed a left lateral selective sphincterotomy in male rats without specifying its length. The authors divided the animals into four groups of eight. Two received sphincter-injected BM-MSCs after non-overlapping repair (autologous: group C, or allogeneic associated with immunosuppressive drugs: group D) and were compared with groups of sham injury and saline injection (A), and injury, repair and saline injection (B). There were no relevant complications or *exitus*. After 30 d under histologic examination, a significant decrease in muscle tissue was observed at the site of repair, but morphometric analysis of groups C and D revealed a significantly greater muscle area than in group B ($P < 0.05$), but a significantly lower area than in group A. In functional assays, with *in vitro* contractility, a significantly better response to electrical stimulation and relaxing capability appeared in groups C and D compared with B ($P < 0.05$). No significant differences were found between groups C and D.

In the same year, Kang *et al*[36] published an investigation using cryoinjury in Sprague-Dawley female rats, without specifying the damaged volume (although the probe is applied against the right sphincter hemisphere). The authors studied injection with microscopic guidance of 3×10^6 autologous muscle-derived stem cells (MDSCs) into the sphincter damaged zone. Fifteen rats were divided into three groups: control (A); cryoinjury (B); and cryoinjury and cell therapy (C). Evaluations were performed one week after the injury. In muscle strip *in vitro* contractility assays, cryoinjury significantly decreased contractility and MDSCs increased its amplitude without reaching statistical significance. Upon histological examination, they found labelled cells in all animals at the MDSC

Table 1 Faecal incontinence models employed in published preclinical studies and their types of reparation systems

Surgical injury	Crioinjury	Pudendal nerve crush	No injury
Section	2	1[43]	3
Anterior: 2			
Left lateral: 9[43]			
Posterior subtotal: 3			
Proctoepisiotomy: 1			
25% excision: 4			
50% excision (IAS: 1) (both: 3)			
Total excision EAS: 1			
Type of reparations employed			
Surgical repair	**No repair**	**Substitution**	**Not applicable**
6	Crioinjury: 2	2	3
Randomized 2[39,64]	Section: 9[39,43,64]		
	Excision: 7		

Numbers indicate the number of published studies. EAS: External anal sphincter; IAS: Internal anal sphincter.

Table 2 Origin of stem cells used in published preclinical studies, classified by organ origin and transplant type

Kind of stem cells employed				
Muscle progenitors	**Bone marrow SCs**	**ASCs**	**Enteric neural**	**USCs**
Myoblasts: 6	BM-MSCs: 10[35,38,60]	Aut: 1	Aut: 1	Xenog: 1[38]
Muscle SCs: 9	Mononuclear: 1[60]	Xenog: 1	Xenog: 1	
Autologous/syngeneic	**Allogeneic**		**Xenogeneic**	
11[35]	17[35,38]		3[38]	

Numbers indicate the number of published studies. ASCs: Adipose-derived stem cells; USCs: Umbilical cord stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; Aut: Autologous; Xenog: Xenogeneic.

Table 3 Bioengineering strategies used with stem cells in published preclinical studies, and scaffolds employed as stem cell carriers to improve their function

Bioengineering models	
[46,55,56]	Polycaprolactone beads
[51]	IAS muscle cells + human ENPC + bilayer collagen and laminin hydrogel
[57]	Polyethylene glycol-based hydrogel matrix scaffold
[58]	Decellularized EAS
[76]	IAS cells + enteric neural progenitor cells (biosphincter)
[65]	Polyacrylamide hydrogel carrier (Bulkamid)
[61,63]	Gelatin scaffold

ENPC: Enteric neural progenitor cells; EAS: External anal sphincter; IAS: Internal anal sphincter.

injection site, confirming survival and tolerability (there were no immune responses in any animal), and also found differentiated muscle masses with variable orientations, suggesting partial myofiber (smooth and skeletal muscle) regeneration.

In 2009, Saihara *et al*[37] isolated allogeneic myoblasts from female F344 rats (at 1-4 wk), implanted them into nude mice, and evaluated myoblast evolution in subcutaneous tissue, damaged thigh muscles and healthy levator ani. Myoblasts were most efficiently obtained from more juvenile rats. SCs were capable of forming myotubes *in vitro* and in subcutaneous fat at 3 wk, and became integrated into damaged muscles with myofiber formation at 4 wk. Nevertheless, in healthy muscle, myoblasts survive in smaller numbers, surround the muscle without integrating into it, and form myotubes but not myofibers. Therefore, injury stimulus may be fundamental to myofiber formation.

Aghaee-Afshar *et al*[38] published the first rabbit model in the same year, applying surgical damage (EAS lateral sectioning) without repair. Two weeks later, seven animals per group received human umbilical cord stem cells (uSCs, 10^4), allogeneic rabbit BM-MSCs (10^4), culture medium or saline solution. These groups were also compared with three non-injured animals, all of which were evaluated before damage, before treatment and two weeks later. Clinical results: complete sphincter competence was found in four out of seven patients with BM-MSCs compared with two out of seven with uSCs, and partial competence in two out of seven with culture medium. On the electromyograph, there was a significant decrease in peaks per second

after the injury, and a significant increase in BM-MSCs compared with pre-treatment values and controls; an insignificant increase appeared in uSCs, and no increase appeared in other groups. Both kinds of SCs were able to survive at the injury site. Histopathologic evaluation showed a normal or muscle-dominant sphincter structure in all animals receiving BM-MSCs, and a fibrous-dominant structure in most animals receiving hUCM as well as in all animals without SCs. Authors do not mention the percentage of implanted cells that survived, and do not confirm their differentiation into myofibers or their "normal" histology.

In 2010, White *et al*[39] published the first randomised study with 120 Sprague-Dawley virginal female rats. The authors performed a transection of EAS with a 7 mm incision (in this species, EAS is about 3-4 mm in longitudinal length). Animals were first randomly allocated to repair or no repair groups, and then each group received injected allogeneic pre-confluence myogenic stem cells (3.2×10^6 on saline) or saline solution. If a repair was performed, a two-layer 5-0 braided polyglactin interrupted suture (1 mm apart) was applied to the rectal mucosa, and EAS were approximated with two single interrupted stitches. Injections were applied under microscopic guidance in EAS ends (before repair, if scheduled). Animals were sacrificed at 1, 3 and 13 wk, and EAS contractility was studied in muscle strips *in vitro*. Seven days after injury, contractile function had severely declined, which was independent of repair. Twitch tension, maximal tetanic contraction, and maximal force in response to electrical stimulation improved significantly with time after sphincter repair. Injected SCs in repaired sphincters resulted in significantly superior ($P < 0.001$) contractile function at both 7 d and 90 d compared with saline. In non-repaired animals, contractile function did not improve with or without SCs. Repair and surgery could cause short-term functional deterioration, and indicators of denervation did not change between groups. The authors propose that SCs need some favourable conditions to work (preserved innervation, muscle apposition), as demonstrated by the minimal effect on non-repaired animals.

In the same year, Craig *et al*[40] analysed the feasibility and safety of allogeneic rat myoblasts injected into the intact EAS of four non-pregnant female Sprague Dawley rodents. Here, 1.5 or 4.5×10^6 labelled cells, divided across three sites, were injected under electromyographic guidance between three and nine o'clock. Ten days later, the authors detected labelled cells within the EAS using immunofluorescence assays. To them, this demonstrated that myoblasts integrate into the host tissue.

Additionally in 2010, Kajbafzadeh *et al*[41] published a paper on rabbits. A surgical subtotal external sphincterotomy (9 mm longitudinal) was performed in the posterior part through an 8 mm longitudinal incision, with only the skin sutured. Three weeks later, autologous MDSCs (7×10^7, nine animals) or saline buffer (12 animals) were injected into section borders. Sphincter electromyography (EMG) and manometry (ARM) were performed immediately before injury, as well as 14, 28, and 60 d after injection in three animals per group. Animals were sacrificed at every interval for histology studies. The three remaining animals from the control group received EMG and ARM after 6 mo. Related to clinical presentation, after the injury, all rabbits demonstrated a flaccid sphincter and occasional loose faecal consistency; this persisted during the 6 mo follow-up in the control group, but recovered after four weeks in the SC group. Upon histological evaluation, circular fibers around lesions in the control group became atrophied, and inflammatory infiltrate, fibrosis and a muscular gap persisted at all of the follow-ups. With SCs, myotubes appeared at 2 wk, and myofibers with variable disposition at 4 wk. At two and 4 wk, labelled cells were detected in all of the grafted sphincters, and there was less CD3+ cell infiltration at 4 wk (null at eight) with very few CD34+ cells appearing. These two last results confirm that findings cannot be explained by bone marrow-derived cell infiltration). A higher proliferative index was also identified with SCs. Upon functional examinations, injury promoted a decrease of approximately 87% in basal pressures. ARM and EMG showed a significant ($P > 0.001$) improvement in the mean anal canal pressure and electrical activity, both at rest and after stimulation, since 4 wk after cell injection (74.8% and 60%-80% of normal values, respectively), which did not appear in the saline group. These values grew in the SC group during the evaluated period. No significant differences were noted in the control group with regard to functional and pathological parameters over time.

The following publication was issued by Pathi *et al*[42] in 2012, and first compared local and systemic SCs. They performed the same injury and repair as White *et al*[39] for 204 nulliparous Lewis rats compared to 20 non-injured animals. Operated rats randomly received one of the following: Local and intravenous phosphate buffered saline (PBS), local allogeneic BM-MSCs (4×10^6 labelled SCs injected on each side of the reparation) with intravenous PBS and intravenous allogeneic BM-MSCs (4×10^6 labelled) with local PBS. Animals were studied at 24 and 48 h and seven and 21 postoperative days, using genetic sphincter expression by quantitative reverse transcription polymerase chain reaction (IL-10, IL-6, TGFβ1, TNFα, CPH-A, COX2, LOX) and with histology and neurophysiology results at 21 d. Upon functional evaluation at 21 d, there was a significant decay in maximal contractile pressure, and an increase in fatigue with PBS; those values were equal to those in non-operated animals in the group receiving local BM-MSCs, and reached intermediate values when systemic BM-MSCs were applied. Upon histological evaluation, when PBS was injected locally (independent from the systemic product), there was a muscular gap replaced by an inflammatory area with fibrosis, and skeletal muscle fibres lost their orientation in the injury borders.

With local MSCs, the correct orientation appeared, and fibers crossed the fibrous area. Labelled cells were detected at 24 and 48 h, but not at seven and 21 d. In wound-healing parameters, pro-inflammatory (COX-2 and IL-6 during 48 h) and anti-inflammatory (IL-10 and TNFα during 21 d) increased transiently after injury in all groups, whereas TFG-β1 (an important mediator of matrix deposition by MSCs) and lysyl oxidase (related with collagen and elastin synthesis) increased significantly at earlier time points with direct MSCs, and in an intermediate manner with systemic MSCs. It was of note that there was a nearly significant (P = 0.057) mortality increase with systemic MSCs related to pulmonary embolisms. The authors concluded that local, but not intravenous, MSCs improved contractility, matrix deposition, and both TGF-β1 and LOX in the acute phase.

In 2013, Salcedo *et al*[43] published the first study that considered pudendal nerve injury using 70 virgin female Sprague-Dawley rats. They applied Zutshi' s surgical injury model, consisting of an incision of EAS and IAS through a precise 3-4 mm incision in the perianal skin[44] and pudendal nerve crush (comprising 30 seconds with a Castroviejo needle holder) as FI models. Animals were randomly assigned to: Surgical sphincterotomy (n = 20), pudendal nerve crush (n = 20), sham sphincterotomy (n = 10, five seconds pressure) and sham pudendal nerve crush (n = 20, dissection only). Then, when they had previously demonstrated significant cytokine level changes (24 h after injury)[45], they applied 2 × 10^6 labelled allogeneic BM-MSCs in PBS that were either injected into each of the four sphincter quadrants or intravenously (five animals for each delivery system per experimental group) and compared them with the same volume of local or intravenous PBS. ARM and EMG were recorded immediately after injury and 10 d after treatment. The authors found that Ⅳ MSCs resulted in a significant increase in resting and peak pressure, as well as EMG amplitude and frequency at 10 d compared to PBS. Local MSCs significantly increased resting pressure and EMG frequency, but not amplitude. There were no improvements with MSCs or PBS after pudendal nerve crush, possibly due to the prompt SC administration prior to denervation changes. With sham surgery, no changes appeared in any group. Labelled cells were not found in MSC-treated animals. The authors concluded that MSCs (local or systemic) could significantly improve ARM and that Ⅳ MSCs significantly improved EM–G after sphincterotomy, but not after pudendal nerve crush.

In 2013, Kang *et al*[46] published the first experiments combining bioengineering and SCs in a dog model of FI. Sphincter injury was induced by the partial extraction of 25% of the posterior IAS/EAS using electrocautery. The dogs were randomly allocated to either the control group, or to the experimental group where they received an injection of porous polycaprolactone beads containing autologous myoblasts into the injury three months later (five dogs per group). The authors evaluated compound muscle action potentials (CMAPs) of the pudendal nerve, ARM, and histopathology three mo after treatment. CMAPs significantly decreased with injury (P = 0.04), but there were no differences between experimental groups (P = 0.49). Resting and squeezing pressures also significantly decreased with injury (P = 0.04) and were higher in the SC group, but without statistical significance. In histological analysis of the control group, there was extensive damage to the muscle fibers with atrophy, cytoplasmic fibrosis and focal interstitial inflammatory cell infiltration. In the therapeutic group, there was a marked foreign body reaction (numerous giant cells and foamy macrophages), with weak staining for α-smooth muscle actin. Therefore, the results did not show firm evidence that injection could improve sphincter function. In the discussion, the authors mentioned that the physical properties of some beads could elicit an adverse immune response or foreign body reaction. These authors also insisted on the advantages of a large animal model to study effects (especially *in vivo*), mentioned the necessity of reinnervation, and emphasised the study's limitation due to the very low number of studied animals.

Also in 2013, Jacobs *et al*[47] published the first study with a safety concern. Here, 33 female virgin Sprague Dawley rats received surgical anal sphincter transection and repair, after which 24 underwent the injection of 5.0 × 10^6 allogeneic MDSCs and nine served as the sham control. SC migration to the liver and lung, as well as sphincter histology, were evaluated at 30 d. No evidence of SC migration to the liver or lung was found, but two local growth foci were noted in two animals receiving SCs. Further evaluations of them were consistent with a benign nature; there were no nuclear abnormalities or proliferation. The authors consider that this finding could be explained by the high dose employed, cell trapping, SC overgrowth, and/or paracrine factors. Finally, they concluded that more studies on safety are needed, which could be focused locally since no migration appeared.

Furthermore, Bisson *et al*[48] published in 2013 a cryoinjury study on Fischer rats. The authors verified that the minimal lesion that caused sustainable deficiency was done from 90 degrees, which was repeated after a 24 h interval. Evaluations relied on both an electro-stimulated ARM as well as histology. The experimental groups were: Uninjured controls (n = 11), cryoinjured + PBS (n = 8), and cryoinjured + labelled syngeneic myoblasts injected with microscopic guidance. The novelties included the analysis of different doses and injection sites, and the first long-term follow-up (6 mo); three individual injections of 1 × 10^5 (n = 6), 1 × 10^6 (n = 8), or 1 × 10^7 (n = 6), two at the borders and the last within the lesion; alternatively, a single dose of 1 × 10^6 (n = 6) was injected into a unique site, within or opposite the lesion. Injections were well-tolerated. In the histology, EAS reconstitution

was observed and SCs became integrated and differentiated into mature myofibers. Related to manometry, pressuresincreased over time; after day 30, the SC group had significantly higher pressures compared to PBS controls ($P < 0.001$), and equal pressures to normal rats at day 60. The therapeutic effect persisted over a period of 6 mo. A three-injection system was equally as effective as a single intra-lesion administration at day 60, but an injection opposite the lesion was unable to restore sphincter pressures.

The last publication from 2013 was from Lane *et al*[49], which used Sprague-Dawley rodents. They first established normative EMG EAS parameters. A more radical procedure named proctoepisiotomy, which involved an incision length of 5 mm to include transection of the IAS and EAS, was designed. Then, a layered repair was performed with 6-0 delayed absorbable sutures in a running fashion, followed by an interrupted layer. Animals were randomly assigned to receive myogenic SCs ($n = 24$, 5×10^6 injected under direct visualisation with a dissecting microscope one half to each side of the EAS) or PBS (control group, $n = 9$). The authors evaluated the efficacy by EMG (basal, two and 4 wk) and ARM (basal and 2 wk post-intervention), and measurements of IAS, EAS and total sphincter thickness (millimetres) were also calculated. They found a significant difference between the experimental groups inEMG ($P < 0.01$) and ARM at 2 wk (the SC group recovered basal values), but there were no differences in EMG at 4 wk (both groups returned to baseline). Notably, there were no relevant complications, and measurements of sphincter muscle thickness did not differ between transplant and control rats.

The group of Elmi *et al*[50] published a study in 2014 focusing on SC homing and tracing, employing magnetic resonance imaging (MRI) for the first time in this field. They employed the Kazbafzadeh[41] model of FI in 12 rabbits. Animals were randomly assigned to receive either ultra-small superparamagnetic iron oxide (USPIO)-labelled 9×10^7 autologous MDSCs (experimental group) or saline (control group) at the site of damage 3 wk later. Evaluations were performed with *in vivo* MRI, EMG, and ARM before, 1 h after, and one, two, and 4 wk after the injection. At 4 wk, sphincter sections were obtained for histology; the semi-quantitative analysis of fibrosis, desmin, iron, CD3, and CD68 was performed in two distinct regions according to either the presence (zone Ⅰ) or absence (zone Ⅱ) of signal loss (related to USPIO) on the MRI. Regarding MRI results, signal loss was significant at 1 h, 1 wk, and 2 wk when compared with the pre-injection signal intensity in the SC group, and the maximum signal loss was detected at 1 h followed by a gradual increase during the follow-up (statistical differences at 4 wk appeared compared with those at 1 h). In the control group, there was no statistically significant difference in signal intensity at each time point. In a functional evaluation, a significant improvement in pressure and electrical activity was found in the SC group after 4 wk ($P < 0.001$, 76% of basal values). In the histological studies, atrophic thin circular muscle fibres with fibrosis were seen in the control group, whereas regenerating myofibers staining positively for desmin as well as clusters of iron-positive particles were detectable in the experimental group, mainly in zone Ⅰ areas. A significant decrease in the fibrotic area in zone Ⅰ of the therapeutic group was identified ($P = 0.004$). Minimal infiltration of CD68+ cells and mild CD3+ was reported in both groups. Therefore, iron oxide-enhanced MRI can monitor transplanted SCs.

In the same year, Raghavan *et al*[51] published the development and successful implantation of a bioengineered IAS by employing SCs in rats. Following their studies of bioengineered IAS since 2005[52], the authors created human IAS tissue constructs combining IAS circular smooth muscle cells and human enteric neuronal progenitor cells on a collagen and laminin bilayer hydrogel. Then, constructs were implanted in the perianal region of athymic rats, optimising the implantation with platelet-derived growth factor that was delivered through a microosmotic pump. The implantation was feasible and safe; there were no complications or rejection during the 4 wk follow-up. Implants were viable and had normal morphology, relevant neovascularisation, and normal contractility both *in vitro* and *in vivo*. Treated animals had also normal stooling.

The group of Salcedo and Zutshi from the Cleveland Clinic in Ohio, one of the most important in this field, published a randomized study in 2014[53]. They randomly divided 50 Sprague-Dawley rats into two groups: non-injured ($n = 15$) or injured ($n = 35$). The authors modified their prior injury model to a more aggressive one: An excision of 25% of IAS and EAS through an incision in the ventral aspect, and excision from the ten to two o'clock position under a dissecting microscope. They evaluated the delay to injury administration (24 h or 3 wk) of allogeneic MSCs. Non-injured animals were divided into groups that received either intrasphincteric MSCs ($n = 8$, evaluated at 10 d -5- and 5 wk -3-) or MSCs by serial i.v. infusions ($n = 7$, evaluated at 10 d -5- and 5 wk -2-) 24 h later. Twenty-four hours later, the injury group was divided into groups that received: (1) saline ($n = 10$), either locally ($n = 5$) or by serial i.v. infusions ($n = 5$); (2) MSCs ($n = 10$) into the sphincter ($n = 5$) or by serial i.v. infusions ($n = 5$); or (3) no treatment ($n = 5$). Rats were evaluated with ARM and immunofluorescence 10 d after treatment and at 5 wk. An additional group of ten rats underwent local (five rats) or i.v. (five rats) application of MSCs 3 wk after injury to test the hypothesis that delayed administration will not produce SC homing because of the loss of cytokine signalling. SC administration consisted of the delivery of 5×10^5 labelled allogeneic BM-MSCs in PBS; in i.v. treatments, the same dosage was delivered daily for six consecutive days *via* the tail vein. Related to function, ten days after IM/IV MSC treatment, pressures were significantly increased compared with both the

PBS group and pre-treatment ($P < 0.001$). At 5 wk, there were no significant differences between injury and non-injury, independent of treatment, but pressures were significantly increased after systemic or local MSC administration compared with PBS ($P < 0.001$). Related to histology, when MSCs were supplied, less of a muscular gap, and a marked decrease in fibrosis and scar tissue appeared, with the i.v. infusion showing the least scarring. When MSCs were administered three weeks after injury, there were significant differences only with the pre-treatment values and not with the other experimental groups.

The last publication of 2014 is from Fitzwater *et al*[54], and was a continuation of the White investigation using the same injury and repair procedures[39] in 40 young female Sprague-Dawley rats. Animals were randomised to receive an injection of either PBS or allogeneic MDSCs at the transection site (two injections of 1.6×10^6 at each side) and then euthanised at seven or 90 d (a half each period) for histological evaluation. The authors found sphincter disruption in 100% of the animals in both groups 7 d after injection, but 89% of controls and 78% of SCs had intact sphincters at 90 d. Striated muscle volume increased significantly from 7 to 90 d in both groups, without statistical differences between them at 7 or 90 d. Significant inflammatory infiltrate was seen in both groups at 7 d, and persisted at 90 d, without any differences between groups. However, White *et al*[39] observed a substantial temporal improvement in the contractility of the SC group compared with PBS, so the authors suggest that SCs might improve function without modifying histology.

In 2015, Oh *et al*[55] contributed with two publications about an FI model in mongrel dogs, which consisted of resecting 25% of the posterior part of both sphincters through a perianal incision; no repair was performed and treatments were administered 1 mo after injury. In the first one[55], the authors compared a control group of sham surgery (only skin incision, $n = 5$) with ten injured dogs receiving polycaprolactone beads with PKH-26-labelled autologous myoblasts ($n = 5$) or PBS solution ($n = 5$) injected locally. Three months later, ARM and histopathological studies were performed. Anal pressures were significantly higher in SC-treated dogs than in control dogs, and the PBS group had significantly lower pressures than sham surgery dogs ($P < 0.05$). Contractile pressure in SCs dogs was 49.5% of the average before surgery, whereas it was only 32.8% in the PBS group at the same time. Immunofluorescence confirmed that some myoblasts were differentiated in all animals because labelled cells were detected, as well as some expressed smooth and skeletal muscle markers. In their second publication[56], they randomised ten injured dogs to receive either PKH-26-labeled autologous myoblasts (group A, five dogs) or autologous myoblasts and bFGF-loaded (basic Fibroblast Growth Factor, a muscle differentiation regulator) polycaprolactone beads (group B, five dogs). ARM, pudendal nerve CMAPs and histology were evaluated at 3 mo. They found a significant improvement in ARM and CMAPs in group B compared to A ($P = 0.002$ and 0.001, respectively; in fact, both decreased in group A compared to basal values) and labelled cells were detected in 2/5 (40%) and 5/5 (100%) dogs in the A and B groups, respectively. Therefore, group B treatment improved the recovery, outcomes and SC implantation compared to cell-based therapy alone.

In the same year, Montoya *et al*[57] published a bioengineering investigation with Sprague-Dawley female rats. Eighty rats underwent midline transection of both AS by a 7 mm full-thickness incision without repair. After 2 wk, the edges were re-exposed and animals were randomly assigned to receive the following treatment by injection (20 animals per treatment): (1) nothing (non-repaired control, NRC); (2) a polyethylene glycol-based hydrogel matrix scaffold combined with PBS (PBS/hydrogel); (3) a hydrogel matrix scaffold combined with allogeneic pre-confluence MDSCs (3.2×10^6 cells, a half in each edge, SC/hydrogel); and (4) type I collagen. Then, animals were sacrificed 4 or 12 wk later (ten and ten animals from each group, respectively), and their sphincters were analysed for contractile function, disruption, and striated muscle volume. Time-matched unoperated controls were utilised for each of the two time points ($n = 10$ each period). In functional analysis, after 4 wk, maximal electrical field-stimulated contractions were significantly decreased in all four non-repaired groups compared with non-injured; however, contractions were improved in SC/hydrogel group relative to NRC (significant), PBS/hydrogel, or collagen groups. NRC and PBS/hydrogel deteriorated at 12 wk, while SC/hydrogel maintained improvement. Related to morphology, striated muscle volume increased significantly *vs* NRC from 4 to 12 wk for PBS/hydrogel (65%) and SC/hydrogel animals (63%). At 12 wk, SC/hydrogel animals had greater striated muscle volumes than all other treatment groups ($P = 0.001$); no differences appeared at 4 wk. At 12 wk, all NRC showed disruption, while only 20% of SC/hydrogel ($P = 0.048$) and 0% of collagen-treated ($P = 0.008$) were disrupted. There was also remarkably little inflammation at 4 and 12 wk with SC/hydrogel or collagen, with occasional giant multinucleated cells and small vascular channels on intervening fibrosis between muscular endings. Therefore, a compatible matrix may facilitate SC survival, differentiation, or function, leading to functional recovery despite morphological disruption.

In 2015, Kajbafzadeh *et al*[58] published another bioengineering model with rabbits. The EAS of 16 rabbits were resected, decellularised and transplanted into the terminal rectum of the incontinent rabbits 6 mo later. Animals were divided into two groups: 1 ($n = 8$) receiving injected 7×10^7 autologous myogenic satellite cells into the implant; and 2 ($n = 8$) without injection. Histological evaluation at 3-mo intervals and EMG with electrical stimulation after two years (the longest follow-up published) were performed. In the

histological evaluation, no evidence of inflammation or rejection was observed and the transplanted EAS appeared normal; there were no morphological differences, but all immunohistochemical markers in the SC group revealed significant enhancement three and 6 mo after surgery ($P < 0.001$) without significant differences between 12 and 24 mo. In the functional evaluation of both groups, grafted EAS contracted in response to needle and electrical signals to both the muscle and pudendal nerve; more signals were always detected in group 1, but no statistical study about this issue was provided.

In 2016, Sun *et al*[59], also from Zutshi's team, further expanded the concepts of delayed repair and SC homing. First, the authors investigated the best electrical stimulation parameters in an SD rat model; secondly, they evaluated the most efficient delivery route for allogeneic BM-MSCs, randomly allocating SD rats into three groups: Intravascular ($n = 20$), intraperitoneal ($n = 8$), or direct (intramuscular) injection ($n = 14$). In both experiments, *in vivo* cytokine expression and luciferase-labelled sphincter cell imaging were employed. A significant ($P = 0.03$) increase was found in MSC retention at the site of electrical stimulation with direct intramuscular injection (not in the other groups) compared to sham-stimulated animals. Finally, 16 SD rats underwent a ventral excision of 50% circumference of AS and then randomly received (four animals each group): (1) no treatment; (2) daily electrical stimulation for 3 d; (3) 3 d stimulation followed by 10^6 MSCs at the injury site the third day; and (4) 3 d stimulation with two injections of 10^6 MSCs the first and third days three weeks later. Function was assessed before and 4 wk after intervention when histologic assessment was also done. In the results, there was significantly more new muscle in the injured area four weeks after intervention, and there was also a significantly improved anal resting pressure in group 3 compared with all other groups.

Also in 2016, Mazzanti *et al*[60] (from Lorenzi' s group) published a study with 32 Lewis rats using Lorenzi's injury and primary repair models[35]. There were four experimental groups: Sphincterotomy and repair plus intrasphincteric injection of saline (A), *in vitro*-expanded allogeneic BM-MSCs (B), minimally-manipulated allogeneic BM mononuclear cells (MNCs, C) and the fourth underwent sham operation (D). At day 30, histologic, morphometric, *in vitro* contractility, and functional analyses were performed. Both SCs improved muscle regeneration: A large gap in the muscular layer filled with dense connective tissue and mast cells appeared in group A, which was almost completely repaired in the SC groups that contained numerous small clusters of smooth muscle cells irregularly interspersed in the fibrosis. Moreover, SC groups showed increased contractile function compared to saline ($P < 0.05$). No significant difference was observed between the two SCs used. GFP+ (Green Fluorescent Protein) cells remained in the injury proximity for up to 30 d post-injection. The authors concluded that both kinds of SCs are similar in terms of efficacy.

In 2017, Sun *et al*[61] published an interesting paper combining cytokines, bioengineering and SCs in an attempt to mimic acute injury conditions by homing SCs with cytokines, since healing at a time distant to injury, as in clinical situations, is a huge challenge. Thirty-two female Sprague Dawley rats underwent 50% excision of the AS complex; three weeks later, four interventions were randomly allocated ($n = 8$): (1) no intervention; (2) 100 μg plasmid -expressing stromal derived factor 1 (SDF-1); (3) plasmid and 800000 allogeneic BM-MSCs (injected at injury area); and (4) plasmid with a gelatine scaffold mixed with cells (same dose) injected 3 d later. The authors analysed ARM before and 4 wk after intervention, when histology was also studied. Related to function, the three intervention groups had a significantly greater change in resting pressure compared with the control group. In histology, plasmid and plasmid with cells groups showed increased muscle mass and architectural organisation, whereas controls showed disorganised architecture and less muscle. There was also significantly less fibrosis at the injury sites in the plasmid and plasmid plus cells groups compared with the control group. Therefore, the local delivery of the SDF-1 plasmid with or without local MSCs enhanced sphincter muscle regeneration long after injury, thereby improving functional outcome.

Also in 2017, Bohl *et al*[76] developed a passive FI model in rabbits and studied bioengineered IAS. The injury consisted of an IAS hemicircumferencial sphincterectomy through a ventral curvilinear incision. Autologous biosphincter innervated constructs were produced using IAS biopsy and small bowel biopsy to obtain enteric neural SCs, employing the methodology of Gilmont *et al*[62]. Six constructs were obtained from each animal and were supplemented with neural differentiation medium (Neurobasal-A). Each rabbit received four biosphincters (with two million smooth muscle cells and 800000 neural progenitors). Twenty female rabbits divided into three groups were used: Non-treated (6): Injury without treatment; Treated (10): Injury followed by the implantation of biosphincters conforming a ring in the intersphincteric space 6-8 wk later (only eight were finally evaluated); and sham group (4): Injury followed by re-accessing the surgical site without more manoeuvres. ARM was used before and after injury and one and 3 mo after treatment; histology was also analysed. After the injury, all rabbits had significantly decreased basal tone and loss of both Recto-Anal Inhibitory Reflex (RAIR) and anal hygiene; these findings were sustained at 3 mo in groups A and C. In group B, both parameters were restored and significantly higher at one and 3 mo. In histological evaluation, smooth muscle reconstruction and continuity were observed in group B compared with the others; innervation and vascularisation of implants were also observed.

The same year, Sun *et al*[63] hypothesised that

regenerating at a time remote from injury requires the re-expression of cytokines to attract SCs. Here, 56 female Sprague-Dawley animals underwent the same procedure as in their previous paper (50% ventral excision)[59,61] and three weeks later were randomly allocated to four groups (14 animals per group): (1) no treatment; (2) 100 μg of SDF-1 plasmid injected locally; (3) local injection of plasmid and 8×10^5 BM-MSCs 3 d later; and (4) plasmid and a gelatine scaffold mixed with BM-MSCs 3 d later. The protein expression of cytokines CXCR4 and Myf5 was investigated 1 wk after treatment (n = 6 per group) and the resting animals received ARM, histology, immunohistochemistry and morphometry 8 wk after treatment. Related to functional results, all of the groups receiving the plasmid had significantly higher anal pressures than controls, with no differences between groups receiving the plasmid. In morphology, all of the groups receiving the plasmid had significantly more organised muscle architecture than controls, with no differences between therapeutic groups. Also, animals receiving plasmid alone had significantly greater muscle (smooth and skeletal) in the defect (P = 0.03) than either animals with injury alone (P = 0.02) or those receiving the plasmid, cells, and scaffold (P = 0.03). Significantly less fibrosis appeared with plasmid alone. There were no differences in CXCR4 or Myf5 levels at 1 wk. The authors concluded that an SDF-1 plasmid may be sufficient to repair an injured anal sphincter, even long after the injury and without either MSCs or scaffold treatments.

In the first 3 mo of 2018, three publications have appeared. The first is from our research team, and is the pioneer study employing both autologous (syngenic) ASCs and biosutures for FI[64]. First, anorectal normal anatomy was studied on Wistar and BDIX female rats. Then, an injury model consisting of a 1 cm extra-mucosal myotomy beginning at the anal verge in the anterior middle line was defined and characterised histologically and functionally (ARM). After injury, 36 BDIX rats were randomised to three groups for: (1) cell injection (10^6 labelled ASCs) without repair; (2) biosuture repair (two sutures with 1.5×10^6 GFP-ASCs); and (3) conventional suture repair and cell injection. Functional, safety and morphological studies were conducted during 1 wk. Biosutures became covered with 820000-860000 ASCs, with 100% viability, but some ASCs remained adhered after suture use. ARM showed spontaneous, consistent, rhythmic contractions, taking the form of "plateaus" with multiple twitches that were very heterogeneous in their frequency, mean duration and mean number of peaks. With the injury, both sphincters were completely sectioned, and in ARM, the described activity was replaced by a gentle oscillation of basal line without a pattern. Surprisingly, these findings appeared irrespective of repair or treatment received. ASCs survived in this potentially septic area for at least 7 d: 84% of animals had GFP+ cells, mainly in the muscular section area or in the interposed tissue, forming "conglomerates" with the injections (groups 1 and 3) or wrapping the biosutures. ASCs were also able to migrate to the damaged zone. No relevant adverse events, mortality or unexpected tissue growths were found.

The following publication was from Kuismanen *et al*[65] with Sprague-Dawley rats and with the novelty of employing xenogeneic human ASCs supplemented with human platelet lysate. For injury, the authors mimicked an acute fourth grade sphincter tear by sectioning both AS and anal mucosa, and then repaired them plane by plane with 6-0 poliglecaprone running sutures using magnifying loupes. Injections (at 30° and 330° on a superimposed clock face) were administered prior to perianal skin closure. They also tested whether ASC efficacy could be improved by adding a polyacrylamide hydrogel carrier called Bulkamid. Female virgin rats were randomised into four groups (n = 14-15/group): hASCs (3×10^5) in saline, or Bulkamid and saline, or Bulkamid alone. Evaluation methods: ARM before and two (n = 58) and four weeks after injury (n = 33), micro-computed tomography, and histology. In functional evaluation, both the median resting and peak pressure were significantly higher at 2 and 4 wk in the ASC groups compared with the other groups, and both grew more during the evaluation period; there was no difference between the ASC-carriers (saline *vs* Bulkamid). In the morphological evaluation, no ASCs were recognised at either 2 or 4 wk, and there was no difference in muscle continuity, fibrosis, or collagen formation between the four groups. Bulkamid-hydrogel was well integrated with minor foreign body reaction. The inflammation was scored considering cell infiltration, oedema, haemorrhage and necrosis, as described by Nolte *et al*[66], and there was significantly more inflammation in the hASC-groups, especially in the saline-ASCs. The authors also found a good correlation between histology and micro-CT, so they suggested this for non-destructive morphometric analysis on the whole injured area.

The most recent publication is from Li *et al*[67], the pioneer evaluating electroacupuncture (with a galvanic stimulation) combined with SC therapy. The authors employed Zutshi's surgical injury[44] without repair. Sixty Sprague-Dawley rats were randomly divided into five groups of 12: (1) sham-operated control; (2) injured; (3) injury plus electroacupuncture (EA); (4) injury plus allogeneic BM-MSCs; and (5) injury plus BM-MSCs and EA. EA was performed once a day for six consecutive days by inserting an acupuncture needle bilaterally 5 mm at the ST36 point and connecting them to a low-frequency electronic pulse instrument. BM-MSCs were administered with a single injection of 9.6×10^6 SCs in the caudal vein. Animals not receiving EA underwent needling at ST36 connected to an acupuncture apparatus and animals not receiving BM-MSCs were given a normal saline injection. Only morphological analyses were performed on days 1, 3, 7 and 14. In histology, BM-MSCs and EA associated with

neovascularisation, fibroplasia and less inflammation, and both combined obtained the strongest effects; also BM-MSCs and EA significantly increased capillary density, with the BM-MSC + EA group having the highest values. Sarcomeric α-actinin expression was significantly higher at day 14 in groups 3–5 compared to 2 (injury only), and in group 5 compared with 3 and 4 (P = 0.009 and P = 0.005, respectively), suggesting that tissue repair was higher in the BM-MSC+EA group. Similar results were observed for SDF-1 and MCP-3 expression, suggesting the promotive effects of EA on the homing of BM-MSCs. The authors concluded that the combination of EA and BMSC is more effective.

In a brief analysis, there is high heterogeneity in faecal incontinence models (different surgical sections, variable partial excisions, total excision, cryoinjuries and pudendal nerve crush) and in injury managements (repair or not, substitution). The two most employed SCs include: muscle progenitors (including MDSCs and myoblasts, more committed and derived from the previous, 15 studies) and bone marrow cells (10); allogeneic or autologous use is similar (17 and 11 studies, respectively, one uses both types). Muscle progenitors are less well-defined in the literature compared with MSCs; there is no consensus defining MDSCs and myoblasts as opposed to MSCs and ASCs, so the cellular products employed in publications could be more heterogeneous and could combine different cell lines. Thirteen studies randomly assigned treatments. Murine models are primarily employed (mainly for accessibility and lower cost: 21 studies), however bigger animal models have grown in the last years (looking for greater human similarity: five studies with rabbits and three with dogs have been published). More than one third of published studies have combined SCs and bioengineering with favourable results, and eight have employed different adjuvants to enhance SC function, implantation or survival (2 SDF-1 and one study for each one of the following: human platelet lysate, PDGF, bFGF, anal electrical stimulation, electroacupuncture, and neural differentiation medium). The publications are summarised in Table 4.

All investigations, except two, confirm the safety and absence of relevant adverse events. There is one alert with local injection (two local benign foci of growth in nearly 400 published injected animals)[47] and another with systemic (mortality increment associated with pulmonary embolisms)[42], possibly due to the high doses employed.

In general, good and encouraging morphological and functional results have been observed, as well as data suggesting regeneration aspects. There are only three studies[54,63,64] that find no differences using SCs or control products (placebo[54,64] or active[63]), and another one putting it in doubt[61]. The majority have confirmed SC survival in this potentially septic area, but some have not been able to find cells that retain SC labelling[42,43]. Most publications only perform short or at least medium-term follow-up (three-6 mo), with only one long-term follow-up (2 yr) published[58]. There are also many doubts concerning the mechanisms of action of SCs in this field.

We think that many more studies are needed to draw concrete conclusions. To date, publications indicate safety and suggest a very interesting potential efficacy, but more are required to confirm these promising results.

HUMAN STUDIES PUBLISHED

There are seven publications regarding SC administration in humans for FI, including 89 patients (55 receiving SCs). There was one study not focused on FI, one case report, three observational studies (two with the same patient cohort) and two randomised controlled trials. Employed SCs have been myoblasts (five studies, all autologous) and ASCs (one autologous and one allogeneic). An overview of these published investigations is presented in Table 5.

A Phase Ⅱ study for complex perianal fistula by García-Olmo *et al*[24] analysed FI in patients operated upon at their centre. Five out of 13 (38.46%) from the experimental group (fistulae treated with ASCs plus fibrin glue) had FI and three improved (60%), compared to three out of 13 (23.08%) in the control group (fibrin glue) who did not improve[24]. The evaluation was purely subjective, and the study was not designed to accomplish this objective. These results should therefore be evaluated with caution.

The first specific publication was the observational study from Frudinger *et al*[68]. The authors injected autologous myoblasts into the EAS from ten female patients with non-operated anterior lesions that were refractory to conservative treatment. Attempting to optimise SC integration, patients received anal electrical stimulation 15 min per day for 10 wk prior to implantation and 28 d after it. Cell dosage is not perfectly described; the authors performed 12-14 0.5 mL injections of a solution containing 20.16 × 10^6 SC/mL under ultrasonic guidance in a semi-circular array, including EAS divided ends and the intervening scar. No adverse events appeared. There were significant decreases in the Wexner scale (13.7 unities), daily defecations (0.4), and incontinence episodes per week (8) at the one year follow-up. Related to function, voluntary pressure grew significantly at one and 6 mo, but later decayed to basal values at 12 mo; maximal and median resting pressure also significantly decreased (7 and 6mmHg respectively) between six and 12 mo. Morphologically, there were no important changes in ultrasonography during the follow-up. Quality of life improved significantly during all the studies. The authors concluded that the treatment is feasible, safe, well-tolerated, and improved symptomatology

Table 4 Overview and concise review of different published studies related to faecal incontinence and stem cell therapy in animal models

Ref.	Animal	*N*	Randomized	Type of SC	Compared to	FI model	Repair?	Treatment	Effect measure	Follow up	Principal Results	Security concerns
[35]	Rats	32	No	AUT/ALLOG BM-MSCs	Sham injury Injury + SSF	Surg section	Surg	Inj IE	Histology *In vitro* contractility	30 d	↑ muscular area ↑ Electric response and relaxing	No
[36]	Rats	15	No	MDSC AUT	No injury Crioinj/crioinj + SCs	Crioinjury	No	Inj IE	Histology *In vitro* contractility	7 d	SC survive + myofibre differentiation ↑ contractility (NSS)	No
[37]	Rats	??	No	Myoblast ALLOG	Subcutaneous levator ani thig muscle	No	No	Inj levator ani	Histology	??	SC survivor injury necessary for myofibre formation	No
[38]	Rab-bits	31	No	hUSCs SYNG BM-MSCs ALLOG	Culture medium Saline	Section	No	Inj IE 2 wk later	Clinic EMG Histology	2 wk	BM-MSC: better continence ↑ act SS ↑ muscle	No
[39]	Rats	120	Yes	MDSC ALLOG	Saline	Surg section EAS	Surg	Inj IE	Contractility	13 wk	↑ SS contractility 7/90 d only repaired	No
[40]	Rats	4	No	Myoblasts ALLOG	None	No	No	Inj IE	Histology	10 d	SC survival and integration in sane host tissue	No
[41]	Rabbits	21	No	MDSC AUT	Saline	Surg section EAS	No	Inj IE 3 wk later	Clinic Histology EMG + MAR	2 mo 6 mo (control)	↑ continence since 4w Miotube + myofibre (4wk), SC Survival, ↓ Cd3 and cd34 cells, ↑ proliferate ↑ SS MAR and EMG since 4wk and grew	No
[42]	Rats	224	No	BM-MSCs ALLOG local/systemic	PBS local/Syst	Surg section EAS	Surg	Inj IE/systemic	Mollecular Histology Neurophisiology	21 d	Local: ↑ECM acute phase ↑ fibers SS detected 24-48 h (no later) ↑ activity	↑ mortality nearly SS systemic
[43]	Rats	70	Yes	BM-MSCs ALLOG local/systemic	PBS local/Syst/ Sham injuries	Surg section PNC	No	Inj IE/systemic	MAR + EMG	10 d	IM/IV improve MAR, IV MAR non after PNC No SC survivor	No
[46]	Dogs	10	No	Myoblast AUT + bioengineering	SC/nothing	Excision 25% AS	No	Inj IE 3 mo later	CMAP/MAR Histology	3 mo	↑ MAR (non SS) Foreign body reaction	No
[47]	Rats	33	No	MDSCs ALLOG	Sham control (9 *vs* 24 rats)	Surg section	Surg	Inj IE	Migration lung-liver AS histology	30 d	No migration	2 benign local foci

[48]	Rats	45	No	Myoblast SYNG	Uninjured crioinj + PBS	Crioinjury	No	Inj IE	Histology/MAR	2 mo (histo) 6 mo (function)	Restitutio (60 d), SC integrated ↑ MAR 30 d, SS from 60 d	No
[49]	Rats	33	Yes	MDSC ALLOG	PBS	Surg section (Proctoepisio)	Surg	Inj IE	MAR + EMG Histology	4 wk	Improve SS EMG + MAR 2wk not 4wk No differences in sphincter thickness	No
[50]	Rabbits	12	Yes	MDSC AUT	Saline	Surg section EAS	No	Inj IE 3wk later	MRI/MAR + EMG Histology	4 wk	Labelled cells in MRI + areas, iron + myofibre ↑ ES MAR y EMG	No
[51]	Rats	??	No	Neural enteric progenitors XENOG	No injury/Crio/ Crio + SCs	NO	No	BE: NPC + IAS cells + bilayer	Histology/EMG	4 wk	↑ neovascularization normal functioning	No
[53]	Rats	50	Yes	BM-MSCs ALLOG local/systemic	Saline Uninjured	Excision 25% AS	No	Inj IE/serial Ⅳ 24 h/3 wk later	MAR Histology (immunofluoresc)	5 wk	-↑ P 10d MSCs, 5wk MSC > Saline but no differences with uninjured Histology: ↓gap, fibrosis, scar/ Delayed 3wk no efficacy	No
[54]	Rats	40	Yes	MDSC ALLOG	PBS	Surg section	Surg	Inj IE	Histology	3 mo	No differences between groups	No
[55]	Dogs	15	No	Myobl AUT + PCL beads	PBS Uninjured	Excision 25% AS	No	Inj IE 1mo later	MAR Histology	3 mo	↑ SS MAR (50% basal) SC survival + differentiation	No
[56]	Dogs	10	Yes	Myoblast AUT (A)	(B) Myob aut + PCL beads with bFGF	Excision 25% AS	No	Inj IE 1 mo later	MAR/CMAP Histology	3 mo	↑ SS MAR + CMAP B > A SC en 40% (A) *vs* 100% (B)	No
[57]	Rats	80+ 20	Yes	MDSC ALLOG + hidrogel	Nothing PBS-hydrogel Collagen/No injury	Surg Section	No	Inj IE	Contractility Histology	3 mo	↑Contract and ↑ all F-U in SC-Hydrogel ↑ SS Muscle SC-Hydrogel; ↓ inflammation SC-Hydrogel and collagen	No

[58]	Rab-bits	16	No	MDSC AUT	Only EAS scafold	Total EAS excision	No	EAS sustitution	Histol (every 3 mo) EMG 2 yr	2 yr	No inflammation-reject, improve SS 3-6mo Improve EMG (no statistics provided)	No
[59]	Rats	58	Yes	BM-MSC ALLOG + electrostim	No treatment Elecrostimulation	Excision 50%	No	Inj IE + electrostim	Histology/MAR	4 wk	4wk, electrostimulation + 1 dose MSCs: ↑ muscle in injury area ↑ resting P compared with other groups	No
[60]	Rats	32	No	BM-MSCs ALLOG BM mononuclear	Sham surgery SSF	Surg section	Surg	Inj IE	Histol/ morphometry/ MAR *In vitro* contractility	30 d	SC ↑ regen and SS contractility No differences between SC SC survive 30 d	No
[61]	Rats	32	Yes	BM-MSCs ALLO + SDF-1 (simult/ deferred)	No treatment SDF-1	Excision 50%	No	Inj IE + SDF-1 ± gelatin scaffold	Histology/MAR	4 wk	SDF-1 +/- SCs: ↑ resting P and %muscle and muscle organization and ↓ fibrosis (SS)	No
[76]	Rabbits	20	No	Neural enteric Progenitors AUT	No treatment Sham injury	Excision 50% IAS	No	Sustitution (biosphincter) 6-8 wk later	Histology/MAR	3 mo	Functional improvement since 1mo, SS with others Regeneration, neovascularization and innervation	No
[63]	Rats	56	Yes	BM-MSCs ALLOG + SDF-1 (deferred)	No treatment SDF-1	Excision 50%	No	Inj IE + SDF-1 ± gelatin scaffold	Histology Morphometry MAR Cytoquines	8 wk	Plasmid +/- SCS: ↑ MAR, muscle organization Plasmid: ↑ muscle mass SDF-1 sufficient for repairing without SC+ / -scaffold	No
[64]	Rats	36	Yes	ASCs SYNG	Conventional suture	Surg section	Yes/No	Inj IE biosuture	Histology/MAR	7 d	No functional differences SC survivor and migration to injury	No

[65]	Rats	58	Yes	Human ASCs	SSF Bulkamid (hydrogel)	Surg section	Surg	Inj IE	MAR micro-CT Histology	4 wk	Functional: ↑ SS ASCs and grew: no differences between carriers Morphology: no differences in muscle, > inflammation if ASCs, micro-CT correlation	No
[67]	Rats	60	No	BM-MSCs ALLOG ± electroacupunct	Sham injury Elecroacupunture SSF acupuncture	Surg section	No	Inj IV	Morphology	14 d	SC+EA ↑ vessels, fibroplasia and ↓ inflammation ↑ muscle SS and homing growth factors (SS). Electroacupunct promotes homing	No

AUT: Autologous; ALLOG: Allogeneic; SYNG: Syngeneic; XENOG: Xenogeneic; SSF: Saline solution; Surg: Surgical; Inj: Injection; IE: Intrasphincteric; Crioinj: Cryoinjury; NSS: Non-statistically significant; SS: Statistically significant; ??: Unknown; ECM: Extracellular matrix; AS: Anal sphincter; Proctoepisio: Proctoepisiotomy; NPC: Neural progenitor cells; Immunofluoresc: Immunofluorescence; P: Pressure; PCL: Polycaprolactone; F-U: Followup; Histol: Histology; Simult: Simultaneous; Electrostim: Electrostimulation; Electroacupunt: Electroacupuncture.

with a functional correlation. Five years later, a long-term evaluation was published[69] that analyzed defecatory diaries, blood analyses, quality of life and function annually. No adverse events or changes in blood analyses appeared. Wexner, resting and voluntary contraction pressures, as well as the overall and sub-measures of quality of life, improved significantly ($P < 0.001$) for the entire evaluated period. Reduced defecation frequency and the number of FI episodes also persisted for five years.

Romaniszyn *et al*[70] initially published, as a proof-of concept, a case of autologous myoblast implantation in EAS. Cells were obtained from the quadriceps, and the patient had a traumatic AS rupture refractory to both sphincyteroplasty and biofeedback; an 8-10 mm scar on both AS persisted, and the Faecal Incontinence Severity Index (FISI) score was 20 points. Here, 6×10^8 myoblasts were transplanted under ultrasonographic guidance and distributed on both sides of the muscle scar, on the remaining EAS, and directly into the scar. The procedure was uneventful. Controls took place every 6 wk for three visits, and then after one year. FI improved from the 6th week: it disappeared to solids and soiling but persisted to flatus. Squeezing pressure also improved, and activity in the EMG started to register on the scar area, where there was no activity before implantation. These results motivated them to perform a prospective study on ten patients that was published in 2015[71]. They included patients with FI of different origins with a Wexner (CCI) > ten, as well as low pressures with preserved reflex and innervation. In addition, they excluded patients with Wexner = 20, EAS defects > 90° and denervation. They implanted 3×10^8 myoblasts distributed into three injections: if a defect existed, 0.5 mL for each EAS border, 1 mL in the scar and the remaining volume in normal EAS, and if there was no defect, 3 mL was distributed around the EAS ring. The follow-up was the same as in the pilot study and was completed by nine patients. No muscle biopsies or implantation procedures generated complications. Regarding ARM, no changes appeared at 6 wk, but values gradually increased at 12 and 18 wk (significantly at 18). After 18 wk, significant subjective improvement was obtained in six patients (66.7%), and all patients improved in ARM, five very significantly (55.6%). Upon EMG evaluation, improvement was found in all visits, with the highest values at 12 and 18 wk. Twelve months later, a deterioration of continence was reported by two of the six patients, with good results at 18 wk (also present in ARM and EMG); nevertheless, mean values were

Table 5 Overview of published clinical experience in stem cell therapy for faecal incontinence

Ref.	Study type	*N*	Stem cell	Treat-ment	Compared	Other treatments	Effect measure	F-U	Principal results
[102]	Phase Ⅱ RCT	26	AUT ASCs	Injection fistula	Fibrin glue	No	Subjective	1 yr	Improvement 60% *vs* 23%
[68]	Obser-vational	10	AUT MB	Injection EAS	No	Anal electrical estimulation 10 + 4 wk	Clinical MAR Morphology	1 yr	↓ Wexner and episodes 1 yr, ↑QoL ↑ Voluntary P 1, 6 mo no at 12 Morphology no changes
[69]	Obser-vational	=						5 yr	↓ Wexner and episodes, ↑QoL ↑ P
[70]	Case report	1	AUT MB	Injection EAS	No	No	Clinical MAR + EMG	1 yr	Improved since 6 wk ↑ P and EMG on scar area
[71]	Obser-vational	10	AUT MB	Injection EAS	No	No	Clinical MAR	1 yr	MAR SS 18 wk Clinical: 66% 18 wk and 44.4% 1 yr EMG improvement all F-U
[72]	RCT double-blind	18	ALLO ASCs	Injection EAS	PBS	Surgery	Wexner US EMG	2 mo	No differences on Wexner ↑ SS muscle area and EMG
[73]	Phase II RCT	24	AUT MB	Injection EAS	Placebo	Biofeedback 15 d	Wexner, FIQL MAR, NPS US, MRI	1 yr	SS improve wexner 1 yr, response 60% Partial improvement FIQL 6-12 mo No morphologic differences at 12 mo Transient placebo effect

AUT: Autologous; ALLO: Allogeneic; RCT: Randomized controlled trial; MB: Myoblast; NPS: Neurophysiology; SS: Statistically significant; QoL: Quality of life; P: Pressure; F-U: Follow-up.

still significantly better than before implantation. The remaining four (44.4%) continued with satisfactory results. The authors concluded that more studies are needed to obtain a longer response.

In 2017, a double-blind randomised clinical trial with allogeneic ASCs for sphincter defects was published by Sarveazad *et al*[72]. Twenty patients were randomised, but 18 were analysed (one exclusion by cancer diagnosis before treatment, and one lost in follow-up) in two groups: Both received a non-overlapping sphincteroplasty with 3-0 PDS and then received either 6×10^6 ASCs (nine patients, one-half injected into each end of the muscle) or PBS (nine patients). Two months later, the CCI score, endorectal sonography, and EMG were recorded. No adverse events related to SC were detected. Both groups improved their Wexner scores without differences. In echography and EMG, the ratio of the area occupied by the muscle to the total lesion area showed a significant (P = 0.002) 7.91% increase in the SC group. EMG activity was significantly higher in the therapeutic group (P = 0.002). The authors conclude that ASCs may act as an adjuvant for surgeries that replace fibrous tissue with muscle. The trial was registered at the Iranian Registry of Clinical Trials with the code IRCT2016022826316N2.

Finally This year, Boyer *et al*[73] published a phase Ⅱ randomised placebo-controlled study using autologous myoblasts. They included patients with severe FI (CCI ⩾ 10) due to sphincter deficiency (single defect, multiple disruption or degeneration of EAS; lesions > 30% circumference are excluded) and refractory to medical treatment and biofeedback. In total, 24 patients were included, 12 receiving intrasphincteric injections of SCs and 12 receiving placebo. Eight injections of 100 ± 20 $\times 10^6$ SCs or placebo were made into both the remnant EAS and circumferentially as an outpatient procedure under echography guidance. A seven-day course of antibiotics and a biofeedback re-education program of 15 d were employed, and patients in the placebo group were eligible to receive frozen SCs after one year. The follow-up consisted of visits at six and 12 mo, as well as the completion of CCI (primary endpoint), FIQL scores, ARM, perineal electrophysiological tests, anal sonography, and MRI. Related to the primary endpoint, the median CCI at 6 mo significantly decreased from baseline in both the therapeutic (9 *vs* 15, P = 0.02) and placebo (10 *vs* 15, P = 0.01) groups without differences between them. However, at 12 mo, the median CCI continued to ameliorate in the SC group (6.5 *vs* 15, P = 0.006), while the effect was lost in the placebo group (14 *vs* 15, P = 0.35), with a higher response rate observed in the SC arm (58% *vs* 8%, P = 0.03). After delayed rescue SC injection in the placebo group, the response rate was 60% (6/10) at 12 mo. In secondary endpoints, FIQL did not improve in the placebo arm at both six- and 12-mo, and one and two of its components significantly ameliorated in the therapeutic arm at six and 12 mo, respectively.

No change was evident for either arm on sonography, MRI or electrophysiological tests at 12 mo. No relevant adverse events were identified relatable to treatment. Therefore, SCs have shown tolerance, safety, and clinical benefits at 12 mo, despite a transient placebo effect at 6 mo.

In a brief analysis of these few publications, all of them confirm the implant safety, the absence of relevant adverse events, and the feasibility of employing SCs; of the 89 patients, 55 received SC-based therapies. Regarding results, encouraging clinical, morphological and functional results have been observed, and data suggesting muscle increase have appeared. Only ten patients from one study have surpassed a long-term evaluation[69]; the habitual follow-up is one year. More randomised and comparative studies, as well as long-term evaluations, are needed to draw conclusions about efficacy.

ONGOING CLINICAL TRIALS

According to both the United States National Institutes of Health worldwide clinical trials registry (accessible from www.clinicaltrials.gov) and the EU Clinical Trials Register (www.clinicaltrialsregister.eu) on 1st April 2018, there were six registered clinical trials about stem cell therapy for FI. Surprisingly, there are no new records since previously performed search one year earlier, which is unusual for SC therapies since they are so extensively studied. We will describe them briefly here:

NCT02292628

A Spanish phase Ⅰ/Ⅱ triple-blinded randomised trial comparing autologous injected ASCs (40×10^6) with placebo in 16 patients.

Inclusion: A unique IAS defect and/or EAS ($\leqslant 100°$). CCI $\geqslant$ 12 and/or at least six episodes per month. FI for at least two years.

Outcome measures: The primary is serious adverse events during 12 mo, and the secondary are changes in FI diary, ARM, CCI or FI quality of life during 12 mo.

Actual situation: Active but not recruiting.

NCT02161003

An Egyptian phase Ⅰ/Ⅱ non-masked single group trial for children with FI after surgery for high imperforate anus.

Treatment: Unspecified dose of autologous MSCs injected all around the sphincter (12 points). Estimated enrolment: 50 patients.

Outcome measures: the primary is FI Score at 24 wk, and the secondary is clinical assessment at 12 wk; maximum daily dry intervals (days 1, 30, 90); pelvic MRI and EMG at 90 and 180 d.

Actual situation: Unknown recruitment status, estimated completion date surpassed and not actualised since June 2014.

NCT01011686

Phase Ⅰ trial in South Korea focused on the security of local autologous ASCs (the registry does not specify the dose or implantation method).

Eligible patients: CCI $\geqslant$ 5, FI for more than 6 mo, AS continuity (ultrasound) and abnormal ARM.

Outcome measures: in primary, there is one about efficacy (CCI) and another about safety (abnormality of laboratory and adverse events), and the secondary consists of ARM and ultrasound. All these measures are evaluated at 4 wk.

Actual situation: Appears as "terminated" without obtaining the desired recruitment for unknown reasons (last data update in 2011). No related results have been published.

NCT02384499

Phase Ⅰ randomised placebo-controlled, unicentric and single-blinded trial with allogeneic ASCs from South Korea and with two phases. Safety study: a dose escalation study: three groups of three patients receive 3×10^7, 6×10^7 or 9×10^7 cells/mL, respectively. Follow-up: physical examination, serologic and immunologic response test, CCI, satisfaction survey, WHO toxicity scale, adverse events, ARM and ultrasound at 1, 4, 8 wk, 4, 6, 9, and 12 mo. Response is assessed at 8 wk to select the best dosage. Efficacy test: It compares the efficacy of ASCs *vs* placebo (0.9% normal saline plus fibrin glue) with six patients in each group. Employs randomised, open-label and single-blind design. Clinical assessment and follow-up are identical to the safety study.

Eligible patients: Failed medical therapy or biofeedback for more than 2 mo with CCI $\geqslant$ 8, continuous sphincter on sonography with decreased pressures on ARM. Cell therapy procedure: 6 mL of fibrin glue plus ASCs solution are prepared; 4 mL are injected at four points in IAS (3, 6, 9 and 12 h), and the other third in the EAS intermediate four positions.

Actual situation: The authors published the study protocol in 2017[74], but the recruitment status is "unknown". The estimated study completion date has been surpassed by more than one year, and the last update of the registry was on March 2015. No related

results have been published.

NCT01949922

A non-masked Danish pilot study in 15 patients. It is not a pure SC trial because it analyzes the injection of autologous muscle fibres and not SCs. However, a small part of the fibres is used for analysing MDSCs number and, therein, the regenerative potential of the sample.

Eligible patients: Patients with CCI ⩾ 9 or affected quality of life after 3 mo with pelvic floor muscle training.

Outcome measures: The primary is efficacy (CCI), and the secondary is safety both at one year. Other: efficacy of pelvic floor muscle training (3 mo); improvements in quality of life, anal reflectometry, 3D ultrasound (1 yr), and correlation between the regenerative potential and effects of the tissue samples (1 yr).

Actual situation: The recruitment status is "unknown", the completion date has passed, and the data has not been actualised within the past two years.

NCT02687672

A phase Ⅰ/Ⅱ trial in Jordan that is unrelated to FI. Designed to treat chronic complete spinal cord injuries by autologous, purified CD34+ and CD133+ HSCs using bone marrow or leukapheresis as sources. The study focuses on safety and efficacy over five years, and includes FI evaluation with a questionnaire as a secondary outcome. Currently active but non-recruiting, and the estimated completion is in December 2021.

In a critical analysis of these "ongoing" trials, it draws attention that some of them are in a non-updated state, have been closed or cancelled without completing recruitment, or for no well-defined causes. This generates some doubt about the methodology, or even worse, the efficacy and safety. No alerts have been publicised about safety, so it therefore cannot be a real concern, however it is better to wait for new trials as well as the completion and publication of the ongoing trials' results.

DISCUSSION

FI is a frequent, chronic and highly limiting condition that mainly affects quality of life and has very important economic implications. Its current treatment is multimodal and progressive. If conservative and pharmacological management fails, a variety of invasive procedures are available: sacral or tibial nerve stimulation, the injection of bulking agents, sphincter repairs, sphincter substitution using the gracilis muscle or an artificial device, and finally, in totally refractory patients, a proximal stoma may be useful. To summarise, these procedures have moderate short-term efficacy and decreasing or unknown long-term efficacy, and many have high morbidity rates and compromised cost-effectiveness. In this context, a cellular therapy based in SCs is an attractive potential alternative.

One of the first problems to be solved in this field is how to obtain an FI model, and its correlation with the clinical problem. Published literature shows a high variability of models, with the most frequently applied being surgery (23 publications), including 15 sections and eight excisions (from 25% to 100% of the sphincter complex).

In section models, Lorenzi *et al*[35] described a left lateral selective sphincterotomy without specifying the length. Zutshi's model[44] consisted of "a precise 3-4 mm incision", which might not cause a total sphincter section. White *et al*[39] performed a selective EAS lesion by a total section of 7 mm followed by rectal mucosa repair. We have described an anterior section of both sphincters of 1 cm in length[64], one of the most extensive sections. Going further, Lane *et al*[49] performed a more aggressive injury defined as a "proctoepisiotomy", but did not describe the technical details or extension. Similar or minor modified procedures have been employed by Mazzanti[60], Salcedo[75], Elmi[50], Pathi[42], Fitzwater[54], Montoya[57], Kuismanen[65] and Li[67].

A Salcedo publication in 2010[75] found that rats receiving Zutshi's injury without repair or treatment presented anal pressure recovery 14 d later, comparable registries to controls after 28 d and bridging fibrosis in histology; these findings were not observed with pudendal nerve transection. This publication made Zutshi's team from Cleveland Clinic Ohio turn to more aggressive procedures. Salcedo *et al*[53] described an excision of 25% of both sphincters (through an incision in the ventral aspect, from the ten to two o' clock position) and later, Sun extended it to an excision of 50%[59]. A similar model of partial excision was employed in dogs by Kang[46] and Oh[55,56] and in rabbits by Bohl[76], and a total excision was employed by Kajbafzadeh[58]. Aiming to minimise the effects of the poorly developed sphincters in rats, some authors have employed bigger animals such as rabbits[38,41,50,58,76] and dogs[46,55,56], but there are only eight published papers compared to twenty-one in rats. Other FI models include cryoinjuries employed by Kang *et al*[36] (they did not specify the muscular volume damaged) and by Bisson *et al*[48], who published that the minimal significant cryoinjury must be of at least 90°.

We can discuss the clinical relevance of these models. Human obstetric trauma is more complex than a simple section during episiotomy or a perineum tear. Sphincter injury may be related to muscle injury, prolonged regional hypoxia, denervation, faulty repair, or a combination of them. Other factors could be added later in life: aging, hormonal changes, surgeries, *etc.* In an effort to reproduce these complex effects, simulated childbirth injury models have been designed. This was first described for urinary incontinence by Resplande *et al*[77] and by Sievert *et al*[78] where an inflated 10F Foley catheter is inserted inside the vagina for 3-4 h; to

simulate labour, an episiotomy and balloon extraction was performed. Later, Healy *et al*[79] published a model for FI using two intrapelvic, retrouterine balloons (six Fr urinary catheters) placed through a 3 cm laparotomy for one hour. More studies on simulated childbirth models are possibly needed.

On the other hand, it is known in clinical settings that immediate repair offers better results, but the most frequent scenario is a repair indicated years later and with chronic local conditions (fibrosis, denervation, atrophies, no inflammation). Thus, in preclinical papers, there are nine delayed treatments (repairing or injections delayed 2[38], 3[41,46,50,53] or 4 wk[55,56] and substitutions delayed 6-8 wk[76] or 6 mo[58]). We may discuss whether the considered periods are sufficient to mimic the chronic setting, as it is likely that only acute and subacute conditions have been tested. In the acute setting, some potential confounding factors have been observed[64] (mucosal tears, faecal contamination, *etc.*) that could compromise SCs survivorship or effects. However, there are also cytokines that are fundamental for SC homing and activation, as has been demonstrated for acute myocardial infarction with the SDF-1 factor[80]. In this field, Salcedo *et al*[45] made some relevant contributions; they studied Stromal derived factor-1 (SDF-1 or CXCL12) and monocyte chemotactic protein-3 (MCP-3), known signals that force homing of BM-MSCs to ischaemic tissue, and their expression following direct injury to the AS and pudendal nerve. They found that direct injury resulted in higher levels soon after injury and for 3 wk, whereas denervation resulted in an overexpression for only 10 d, which may lead to more fibrosis[45]. Therefore, in chronic conditions, these molecules will have normal values and thus could make it difficult for SCs to act. To increase these factors, SCs could be transfected with plasmids or the local production could be stimulated using surgical injury or electrical stimulation, such as in the paper by Sun[59]. The previously mentioned publications[61,63] open up an interesting new research field; the combination of SCs or their vehicles (for example sutures with Vascular Endothelial Growing Factor[81]) with cytokines and growth factors.

Another problem to be solved is how to obtain better SC delivery, survival and function in tissues. All studies except ours have employed cell injections; we thought that biosutures[31-33] could be useful for depositing SCs at the focus of the injury. Other authors have made different modifications to biosutures: Yao *et al*[82] added poly-L-lysine and fibronectin to improve cell adherence, and Horváthy *et al*[83] observed better BM-MSC adherence if the suture was previously covered with albumin and SC survivor in implanted tissues at 5 wk. No evidence exists about the best dose, or at least the minimal "clinically-active" dose. With 1.5×10^6 ASCs, SCs were found to form "clusters" both over the suture and in culture medium, and remained adhered after biosuture usage[64]. Therefore, more studies on suture preparation are needed. Delivered doses could be more controlled by injection, but a similar phenomenon can sometimes be observed; similar clusters appear outside the muscle layer with consequent cellular loss[64]. Injected doses have been very variable in the published literature. To improve survival and function in tissues, the employed strategies have been the use of growth factors and cytokines, as mentioned before; this field will be very interesting in the future.

Related to the mechanism of SC actions, there are many remaining questions. The first to be solved is whether SCs survive, integrate and participate in regeneration. More studies to identify critical pathways that are dysregulated in tissue repair are needed. Studies with myogenic cells have detected the labelling on muscle in acute and subacute phases, but medium- or long-term incorporation, or the differentiation of BM-derived cells, has not been clearly identified, and regeneration is at least doubtful. It is possible that myogenic SCs have a greater role based on differentiation, but MSCs likely base their role much more on immunomodulation, as well as on anti-inflammatory and angiogeneic capabilities. There is growing evidence of the immunomodulation capability of MSCs, which is thought to be largely based on inhibition of T cell and B cell proliferation and dendritic cell maturation[84], as well as on the secretion of a large number of cytokines and growth factors[85]. Németh *et al*[86] observed MSC sepsis attenuation by macrophage reprogramming to increase IL-10, a cytokine that decreases neutrophil migration. Our research team has added contributions to that evidence: Georgiev-Hristov found an early shift from acute to chronic inflammation in the presence of ASCs (neutrophil descent and macrophage increment) after tracheal anastomosis[33], and Riera observed less acute and chronic inflammation during 3 mo, with the increasing fibrosis of the aneurysm sac in pigs[87]. Regeneration is not clearly demonstrated in many studies and is very difficult to observe; it may be that more complex morphometric or molecular analyses are needed to confirm it. Similar studies would also be applied for another mechanism like immunomodulation (studying the amount of different cells and molecules). In fact, there are some remaining barriers to achieve "regeneration" with SCs. We need to teach them how to differentiate in an efficient manner, then, possibly with tissue engineering, we need to integrate them into an appropriate delivery system. Finally, we also need to generate a blood supply and innervation that is sufficient to allow their engraftment and survival.

The last critical question is about safety; although there are other potential side-effects, the most worrisome is possible carcinogenicity. SCs have surpassed preclinical studies on biodistribution and toxicity, but investigations into tumour formation are still ongoing. Some publications have observed that MSCs that are cultured for a long time may develop malignant changes and even promote tumours in mice[88]. However,

subsequent publications, including those from the same authors, attributed those findings to tumour cell cross-contamination[89,90]. Furthermore, other studies did not detect tumourigenesis under extreme culture conditions and it has never been observed *in vivo*. In fact, the relationship between SCs and tumours is contradictory. No direct MSC transformation has been observed, but there is a consensus that MSCs have enhanced tropism toward tumours and have pro-tumour (growing, angiogenesis, participation in the microenvironment, immunomodulation)[91,92] and anti-tumour (apoptosis, proliferation inhibition)[93,94] properties. This relationship depends on a lot of factors, including the type of MSCs, source, type of cancer cell line, *in vivo* or *in vitro* conditions, factors secreted by MSCs, and interactions between MSCs, host immune cells and cancer cells. A possible key factor of these effects is time. When MSCs are administered with an existing tumour, a suppressive effect has been observed[95], but in some studies with co-administration of SCs and tumour cells, tumour growth was higher compared to tumour cells alone[96]. These complex interactions have been studied by several authors and reviewed by Ramdasi *et al*[97]. Tropism to tumours has been exploited to treat tumours in experimental models, as reviewed by Chulpanova *et al*[98]. Moreover, a recent NEJM paper published the first severe adverse event potentially relatable to ASCs. Three women suffering from macular degeneration after undergoing ASC therapies developed complications, including vision loss, detached retinas and bleeding, leaving all with complete blindness (although the ASCs were mixed with blood plasma and large numbers of platelets)[99]. In conclusion, cumulated experience seems to support the oncogenic safety of SCs, but more studies and long-term follow-ups are needed to definitively exclude all the risks.

An in-depth analysis about published literature has been provided at the end of each chapter. The 29 published animal investigations confirm the safety (except one), and generally good morphological and functional results appeared with questions remaining about SCs survival, effect, long-term results, efficacy on chronic conditions, *etc.* In human research, there is one unrelated study[24], six studies involving 55 patients receiving SCs with promising results[68-73] and six ongoing clinical trials. More highly rigorous investigations (related to SC type, dosage, delivery system, adjuvant factors, and safety) are needed before SC therapy for FI becomes a clinical reality.

Related to economy, regenerative strategies use costly culture-expansion procedures that require Good Manufacture Practice laboratories compromising cost-effectiveness, as has been demonstrated in a recent survey of clinicians about SC therapy adoption[100]. It is very difficult to estimate the real potential cost of this kind of therapy for humans because there is no consensus in the type of SC, autologous or allogeneic use, the required dose, *etc*. The real efficacy needs yet to be clarified. If a cure could be achieved, direct and indirect costs mentioned before could disappear, and hospitalization costs might be lower due to less invasive procedures to implant SCs compared with FI surgery. Based on our previous experience in clinical trials for anal fistulae[22-29], approximated costs in Spain are the following: 1500-2500€ (1727.8 to 2879.73 USD) for closed system SVF, 2800-4000€ (3225.48-4607.83$) for $40x10^6$ autologous ASCs and 3500-5000€ (4032.88-5761.26$) for 100×10^6 allogeneic ASCs; the costs for other MSCs are equivalent. It must be taken into account that these costs are for SCs produced and dedicated to research, and not for commercial use (maybe higher at least during the first years). The first allogeneic ASC medicine product for fistula marketing is expected between 2018 and 2019, so we will be able to know the real costs of large-scale production. Moreover, some publications have reported acceptable results with free autologous muscle grafts in FI in children[101] (grafts also contain SCs such as satellite cells, but the processing is easier and cheaper), opening up a new field for study.

CONCLUSION

FI is frequent and the available treatments need to be improved, so alternative treatments are therefore needed. Regenerative therapies have exciting potential to improve patient outcomes, and different strategies have been explored (with or without biomaterials) in preclinical and clinical studies.

In preclinical studies, SCs derived from muscle, bone marrow and adipose tissue have been most intensively investigated. In general, safety seems to be guaranteed and some encouraging results have been observed. Clinical evidence is very limited, but the therapy appears to be safe and may be effective. More data are necessary; to date, no SC-based therapy is yet ready for ordinary clinical use, as both short-term and long-term efficacy and safety have to be firmly established. More knowledge about SC, healing biology, and bioengineering principles is needed before regenerative medicine for FI can become really implemented.

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REFERENCES

1 **Macmillan AK**, Merrie AE, Marshall RJ, Parry BR. The prevalence of fecal incontinence in community-dwelling adults: a systematic review of the literature. *Dis Colon Rectum* 2004; **47**: 1341-1349

[PMID: 15484348 DOI: 10.1007/s10350-004-0593-0]
2 **Nelson R**, Norton N, Cautley E, Furner S. Community-based prevalence of anal incontinence. *JAMA* 1995; **274**: 559-561 [PMID: 7629985 DOI: 10.1001/jama.1995.03530070057030]
3 **Edwards NI**, Jones D. The prevalence of faecal incontinence in older people living at home. *Age Ageing* 2001; **30**: 503-507 [PMID: 11742780 DOI: 10.1093/ageing/30.6.503]
4 **Dunivan GC**, Heymen S, Palsson OS, von Korff M, Turner MJ, Melville JL, Whitehead WE. Fecal incontinence in primary care: prevalence, diagnosis, and health care utilization. *Am J Obstet Gynecol* 2010; **202**: 493.e1-493.e6 [PMID: 20223447 DOI: 10.1016/j.ajog.2010.01.018]
5 **Varma MG**, Brown JS, Creasman JM, Thom DH, Van Den Eeden SK, Beattie MS, Subak LL; Reproductive Risks for Incontinence Study at Kaiser (RRISK) Research Group. Fecal incontinence in females older than aged 40 years: who is at risk? *Dis Colon Rectum* 2006; **49**: 841-851 [PMID: 16741640 DOI: 10.1007/s10350-006-0535-0]
6 **Parés D**, Vial M, Bohle B, Maestre Y, Pera M, Roura M, Comas M, Sala M, Grande L. Prevalence of faecal incontinence and analysis of its impact on quality of life and mental health. *Colorectal Dis* 2011; **13**: 899-905 [PMID: 20394640 DOI: 10.1111/j.1463-1318.2010.02281.x]
7 **Deutekom M**, Dobben AC, Dijkgraaf MG, Terra MP, Stoker J, Bossuyt PM. Costs of outpatients with fecal incontinence. *Scand J Gastroenterol* 2005; **40**: 552-558 [PMID: 16036507 DOI: 10.1080/00365520510012172]
8 **Dudding TC**, Vaizey CJ, Kamm MA. Obstetric anal sphincter injury: incidence, risk factors, and management. *Ann Surg* 2008; **247**: 224-237 [PMID: 18216527 DOI: 10.1097/SLA.0b013e318142cdf4]
9 **Oberwalder M**, Connor J, Wexner SD. Meta-analysis to determine the incidence of obstetric anal sphincter damage. *Br J Surg* 2003; **90**: 1333-1337 [PMID: 14598410 DOI: 10.1002/bjs.4369]
10 **Madoff RD**. Surgical treatment options for fecal incontinence. *Gastroenterology* 2004; **126**: S48-S54 [PMID: 14978638 DOI: 10.1053/j.gastro.2003.10.015]
11 **Halverson AL**, Hull TL. Long-term outcome of overlapping anal sphincter repair. *Dis Colon Rectum* 2002; **45**: 345-348 [PMID: 12068192 DOI: 10.1007/s10350-004-6180-6]
12 **Glasgow SC**, Lowry AC. Long-term outcomes of anal sphincter repair for fecal incontinence: a systematic review. *Dis Colon Rectum* 2012; **55**: 482-490 [PMID: 22426274 DOI: 10.1097/DCR.0b013e3182468c22]
13 **Chivu-Economescu M**, Rubach M. Hematopoietic Stem Cells Therapies. *Curr Stem Cell Res Ther* 2017; **12**: 124-133 [PMID: 26496888 DOI: 10.2174/1574888X10666151026114241]
14 **García-Gómez I**, Elvira G, Zapata AG, Lamana ML, Ramírez M, Castro JG, Arranz MG, Vicente A, Bueren J, García-Olmo D. Mesenchymal stem cells: biological properties and clinical applications. *Expert Opin Biol Ther* 2010; **10**: 1453-1468 [PMID: 20831449 DOI: 10.1517/14712598.2010.519333]
15 **Tsuchiya A**, Kojima Y, Ikarashi S, Seino S, Watanabe Y, Kawata Y, Terai S. Clinical trials using mesenchymal stem cells in liver diseases and inflammatory bowel diseases. *Inflamm Regen* 2017; **37**: 16 [PMID: 29259715 DOI: 10.1186/s41232-017-0045-6]
16 **Majka M**, Sułkowski M, Badyra B, Musiałek P. Concise Review: Mesenchymal Stem Cells in Cardiovascular Regeneration: Emerging Research Directions and Clinical Applications. *Stem Cells Transl Med* 2017; **6**: 1859-1867 [PMID: 28836732 DOI: 10.1002/sctm.16-0484]
17 **Mizuno H**, Tobita M, Uysal AC. Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem Cells* 2012; **30**: 804-810 [PMID: 22415904 DOI: 10.1002/stem.1076]
18 **Trebol Lopez J**, Georgiev Hristov T, García-Arranz M, García-Olmo D. Stem cell therapy for digestive tract diseases: current state and future perspectives. *Stem Cells Dev* 2011; **20**: 1113-1129 [PMID: 21187000 DOI: 10.1089/scd.2010.0277]
19 **Ma T**, Sun J, Zhao Z, Lei W, Chen Y, Wang X, Yang J, Shen Z. A brief review: adipose-derived stem cells and their therapeutic potential in cardiovascular diseases. *Stem Cell Res Ther* 2017; **8**: 124 [PMID: 28583198 DOI: 10.1186/s13287-017-0585-3]
20 **González MA**, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009; **136**: 978-989 [PMID: 19135996 DOI: 10.1053/j.gastro.2008.11.041]
21 **Gonzalez-Rey E**, Anderson P, González MA, Rico L, Büscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009; **58**: 929-939 [PMID: 19136511 DOI: 10.1136/gut.2008.168534]
22 **García-Olmo D**, García-Arranz M, García LG, Cuellar ES, Blanco IF, Prianes LA, Montes JA, Pinto FL, Marcos DH, García-Sancho L. Autologous stem cell transplantation for treatment of rectovaginal fistula in perianal Crohn's disease: a new cell-based therapy. *Int J Colorectal Dis* 2003; **18**: 451-454 [PMID: 12756590 DOI: 10.1007/s00384-003-0490-3]
23 **García-Olmo D**, García-Arranz M, Herreros D, Pascual I, Peiro C, Rodríguez-Montes JA. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. *Dis Colon Rectum* 2005; **48**: 1416-1423 [PMID: 15933795 DOI: 10.1007/s10350-005-0052-6]
24 **Garcia-Olmo D**, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. *Dis Colon Rectum* 2009; **52**: 79-86 [PMID: 19273960 DOI: 10.1007/DCR.0b013e3181973487]
25 **Herreros MD**, Garcia-Arranz M, Guadalajara H, De-La-Quintana P, Garcia-Olmo D; FATT Collaborative Group. Autologous expanded adipose-derived stem cells for the treatment of complex cryptoglandular perianal fistulas: a phase III randomized clinical trial (FATT 1: fistula Advanced Therapy Trial 1) and long-term evaluation. *Dis Colon Rectum* 2012; **55**: 762-772 [PMID: 22706128 DOI: 10.1097/DCR.0b013e318255364a]
26 **Guadalajara H**, Herreros D, De-La-Quintana P, Trebol J, Garcia-Arranz M, Garcia-Olmo D. Long-term follow-up of patients undergoing adipose-derived adult stem cell administration to treat complex perianal fistulas. *Int J Colorectal Dis* 2012; **27**: 595-600 [PMID: 22065114 DOI: 10.1007/s00384-011-1350-1]
27 **de la Portilla F**, Alba F, García-Olmo D, Herrerías JM, González FX, Galindo A. Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: results from a multicenter phase I/IIa clinical trial. *Int J Colorectal Dis* 2013; **28**: 313-323 [PMID: 23053677 DOI: 10.1007/s00384-012-1581-9]
28 **Panés J**, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi L, Grimaud JC, de la Portilla F, Goldin E, Richard MP, Leselbaum A, Danese S; ADMIRE CD Study Group Collaborators. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet* 2016; **388**: 1281-1290 [PMID: 27477896 DOI: 10.1016/S0140-6736(16)31203-X]
29 **García-Arranz M**, Herreros MD, González-Gómez C, de la Quintana P, Guadalajara H, Georgiev-Hristov T, Trébol J, Garcia-Olmo D. Treatment of Crohn's-Related Rectovaginal Fistula With Allogeneic Expanded-Adipose Derived Stem Cells: A Phase I-IIa Clinical Trial. *Stem Cells Transl Med* 2016; **5**: 1441-1446 [PMID: 27412883 DOI: 10.5966/sctm.2015-0356]
30 **Oyama Y**, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. *Gastroenterology* 2005; **128**: 552-563 [PMID: 15765390]
31 **Pascual I**, de Miguel GF, Gómez-Pinedo UA, de Miguel F, Arranz MG, García-Olmo D. Adipose-derived mesenchymal stem cells in biosutures do not improve healing of experimental colonic anastomoses. *Br J Surg* 2008; **95**: 1180-1184 [PMID: 18690635 DOI: 10.1002/bjs.6242]
32 **Pascual I**, Fernández de Miguel G, García Arranz M, García-

Olmo D. Biosutures improve healing of experimental weak colonic anastomoses. *Int J Colorectal Dis* 2010; **25**: 1447-1451 [PMID: 20544210 DOI: 10.1007/s00384-010-0952-3]

33 **Georgiev-Hristov T**, García-Arranz M, García-Gómez I, García-Cabezas MA, Trébol J, Vega-Clemente L, Díaz-Agero P, García-Olmo D. Sutures enriched with adipose-derived stem cells decrease the local acute inflammation after tracheal anastomosis in a murine model. *Eur J Cardiothorac Surg* 2012; **42**: e40-e47 [PMID: 22689184 DOI: 10.1093/ejcts/ezs357]

34 **Gräs S**, Tolstrup CK, Lose G. Regenerative medicine provides alternative strategies for the treatment of anal incontinence. *Int Urogynecol J* 2017; **28**: 341-350 [PMID: 27311602 DOI: 10.1007/s00192-016-3064-y]

35 **Lorenzi B**, Pessina F, Lorenzoni P, Urbani S, Vernillo R, Sgaragli G, Gerli R, Mazzanti B, Bosi A, Saccardi R, Lorenzi M. Treatment of experimental injury of anal sphincters with primary surgical repair and injection of bone marrow-derived mesenchymal stem cells. *Dis Colon Rectum* 2008; **51**: 411-420 [PMID: 18224375 DOI: 10.1007/s10350-007-9153-8]

36 **Kang SB**, Lee HN, Lee JY, Park JS, Lee HS, Lee JY. Sphincter contractility after muscle-derived stem cells autograft into the cryoinjured anal sphincters of rats. *Dis Colon Rectum* 2008; **51**: 1367-1373 [PMID: 18536965 DOI: 10.1007/s10350-008-9360-y]

37 **Saihara R**, Komuro H, Urita Y, Hagiwara K, Kaneko M. Myoblast transplantation to defecation muscles in a rat model: a possible treatment strategy for fecal incontinence after the repair of imperforate anus. *Pediatr Surg Int* 2009; **25**: 981-986 [PMID: 19690871 DOI: 10.1007/s00383-009-2454-3]

38 **Aghaee-Afshar M**, Rezazadehkermani M, Asadi A, Malekpour-Afshar R, Shahesmaeili A, Nematollahi-mahani SN. Potential of human umbilical cord matrix and rabbit bone marrow-derived mesenchymal stem cells in repair of surgically incised rabbit external anal sphincter. *Dis Colon Rectum* 2009; **52**: 1753-1761 [PMID: 19966609 DOI: 10.1007/DCR.0b013e3181b55112]

39 **White AB**, Keller PW, Acevedo JF, Word RA, Wai CY. Effect of myogenic stem cells on contractile properties of the repaired and unrepaired transected external anal sphincter in an animal model. *Obstet Gynecol* 2010; **115**: 815-823 [PMID: 20308844 DOI: 10.1097/AOG.0b013e3181d56cc5]

40 **Craig JB**, Lane FL, Nistor G, Motakef S, Pham QA, Keirstead H. Allogenic myoblast transplantation in the rat anal sphincter. *Female Pelvic Med Reconstr Surg* 2010; **16**: 205-208 [PMID: 22453342 DOI: 10.1097/SPV.0b013e3181ec1edd]

41 **Kajbafzadeh AM**, Elmi A, Talab SS, Esfahani SA, Tourchi A. Functional external anal sphincter reconstruction for treatment of anal incontinence using muscle progenitor cell auto grafting. *Dis Colon Rectum* 2010; **53**: 1415-1421 [PMID: 20847624 DOI: 10.1007/DCR.0b013e3181e53088]

42 **Pathi SD**, Acevedo JF, Keller PW, Kishore AH, Miller RT, Wai CY, Word RA. Recovery of the injured external anal sphincter after injection of local or intravenous mesenchymal stem cells. *Obstet Gynecol* 2012; **119**: 134-144 [PMID: 22183221 DOI: 10.1097/AOG.0b013e3182397009]

43 **Salcedo L**, Mayorga M, Damaser M, Balog B, Butler R, Penn M, Zutshi M. Mesenchymal stem cells can improve anal pressures after anal sphincter injury. *Stem Cell Res* 2013; **10**: 95-102 [PMID: 23147650 DOI: 10.1016/j.scr.2012.10.002]

44 **Zutshi M**, Salcedo LB, Zaszczurynski PJ, Hull TL, Butler RS, Damaser MS. Effects of sphincterotomy and pudendal nerve transection on the anal sphincter in a rat model. *Dis Colon Rectum* 2009; **52**: 1321-1329 [PMID: 19571711 DOI: 10.1007/DCR.0b013e31819f746d]

45 **Salcedo L**, Sopko N, Jiang HH, Damaser M, Penn M, Zutshi M. Chemokine upregulation in response to anal sphincter and pudendal nerve injury: potential signals for stem cell homing. *Int J Colorectal Dis* 2011; **26**: 1577-1581 [PMID: 21706136 DOI: 10.1007/s00384-011-1269-6]

46 **Kang SB**, Lee HS, Lim JY, Oh SH, Kim SJ, Hong SM, Jang JH, Cho JE, Lee SM, Lee JH. Injection of porous polycaprolactone beads containing autologous myoblasts in a dog model of fecal incontinence. *J Korean Surg Soc* 2013; **84**: 216-224 [PMID: 23577316 DOI: 10.4174/jkss.2013.84.4.216]

47 **Jacobs SA**, Lane FL, Pham QA, Nistor G, Robles R, Chua C, Boubion B, Osann K, Keirstead H. Safety assessment of myogenic stem cell transplantation and resulting tumor formation. *Female Pelvic Med Reconstr Surg* 2013; **19**: 362-368 [PMID: 24165451 DOI: 10.1097/SPV.0000000000000035]

48 **Bisson A**, Fréret M, Drouot L, Jean L, Le Corre S, Gourcerol G, Doucet C, Michot F, Boyer O, Lamacz M. Restoration of anal sphincter function after myoblast cell therapy in incontinent rats. *Cell Transplant* 2015; **24**: 277-286 [PMID: 24143883 DOI: 10.3727/096368913X674053]

49 **Lane FL**, Jacobs SA, Craig JB, Nistor G, Markle D, Noblett KL, Osann K, Keirstead H. In vivo recovery of the injured anal sphincter after repair and injection of myogenic stem cells: an experimental model. *Dis Colon Rectum* 2013; **56**: 1290-1297 [PMID: 24105005 DOI: 10.1097/DCR.0b013e3182a4adfb]

50 **Elmi A**, Kajbafzadeh AM, Oghabian MA, Talab SS, Tourchi A, Khoei S, Rafie B, Esfahani SA. Anal sphincter repair with muscle progenitor cell transplantation: serial assessment with iron oxide-enhanced MRI. *AJR Am J Roentgenol* 2014; **202**: 619-625 [PMID: 24555600 DOI: 10.2214/AJR.13.11146]

51 **Raghavan S**, Miyasaka EA, Gilmont RR, Somara S, Teitelbaum DH, Bitar KN. Perianal implantation of bioengineered human internal anal sphincter constructs intrinsically innervated with human neural progenitor cells. *Surgery* 2014; **155**: 668-674 [PMID: 24582493 DOI: 10.1016/j.surg.2013.12.023]

52 **Hecker L**, Baar K, Dennis RG, Bitar KN. Development of a three-dimensional physiological model of the internal anal sphincter bioengineered in vitro from isolated smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G188-G196 [PMID: 15774939 DOI: 10.1152/ajpgi.00335.2004]

53 **Salcedo L**, Penn M, Damaser M, Balog B, Zutshi M. Functional outcome after anal sphincter injury and treatment with mesenchymal stem cells. *Stem Cells Transl Med* 2014; **3**: 760-767 [PMID: 24797828 DOI: 10.5966/sctm.2013-0157]

54 **Fitzwater JL**, Grande KB, Sailors JL, Acevedo JF, Word RA, Wai CY. Effect of myogenic stem cells on the integrity and histomorphology of repaired transected external anal sphincter. *Int Urogynecol J* 2015; **26**: 251-256 [PMID: 25253391 DOI: 10.1007/s00192-014-2496-5]

55 **Oh HK**, Lee HS, Lee JH, Oh SH, Lim JY, Ahn S, Hwang JY, Kang SB. Functional and histological evidence for the targeted therapy using biocompatible polycaprolactone beads and autologous myoblasts in a dog model of fecal incontinence. *Dis Colon Rectum* 2015; **58**: 517-525 [PMID: 25850839 DOI: 10.1097/DCR.0000000000000346]

56 **Oh HK**, Lee HS, Lee JH, Oh SH, Lim JY, Ahn S, Kang SB. Coadministration of basic fibroblast growth factor-loaded polycaprolactone beads and autologous myoblasts in a dog model of fecal incontinence. *Int J Colorectal Dis* 2015; **30**: 549-557 [PMID: 25592048 DOI: 10.1007/s00384-015-2121-1]

57 **Montoya TI**, Acevedo JF, Smith B, Keller PW, Sailors JL, Tang L, Word RA, Wai CY. Myogenic stem cell-laden hydrogel scaffold in wound healing of the disrupted external anal sphincter. *Int Urogynecol J* 2015; **26**: 893-904 [PMID: 25644049 DOI: 10.1007/s00192-014-2620-6]

58 **Kajbafzadeh AM**, Kajbafzadeh M, Sabetkish S, Sabetkish N, Tavangar SM. Tissue-Engineered External Anal Sphincter Using Autologous Myogenic Satellite Cells and Extracellular Matrix: Functional and Histological Studies. *Ann Biomed Eng* 2016; **44**: 1773-1784 [PMID: 26424474 DOI: 10.1007/s10439-015-1468-3]

59 **Sun L**, Yeh J, Xie Z, Kuang M, Damaser MS, Zutshi M. Electrical Stimulation Followed by Mesenchymal Stem Cells Improves Anal Sphincter Anatomy and Function in a Rat Model at a Time Remote From Injury. *Dis Colon Rectum* 2016; **59**: 434-442 [PMID: 27050606 DOI: 10.1097/DCR.0000000000000548]

60 **Mazzanti B**, Lorenzi B, Borghini A, Boieri M, Ballerini L, Saccardi R, Weber E, Pessina F. Local injection of bone marrow progenitor cells for the treatment of anal sphincter injury: in-vitro expanded

versus minimally-manipulated cells. *Stem Cell Res Ther* 2016; **7**: 85 [PMID: 27328811 DOI: 10.1186/s13287-016-0344-x]
61 **Sun L**, Xie Z, Kuang M, Penn M, Damaser MS, Zutshi M. Regenerating the Anal Sphincter: Cytokines, Stem Cells, or Both? *Dis Colon Rectum* 2017; **60**: 416-425 [PMID: 28267010 DOI: 10.1097/DCR.0000000000000783]
62 **Gilmont RR**, Raghavan S, Somara S, Bitar KN. Bioengineering of physiologically functional intrinsically innervated human internal anal sphincter constructs. *Tissue Eng Part A* 2014; **20**: 1603-1611 [PMID: 24328537 DOI: 10.1089/ten.TEA.2013.0422]
63 **Sun L**, Kuang M, Penn M, Damaser MS, Zutshi M. Stromal Cell-Derived Factor 1 Plasmid Regenerates Both Smooth and Skeletal Muscle After Anal Sphincter Injury in the Long Term. *Dis Colon Rectum* 2017; **60**: 1320-1328 [PMID: 29112569 DOI: 10.1097/DCR.0000000000000940]
64 **Trébol J**, Georgiev-Hristov T, Vega-Clemente L, García-Gómez I, Carabias-Orgaz A, García-Arranz M, García-Olmo D. Rat model of anal sphincter injury and two approaches for stem cell administration. *World J Stem Cells* 2018; **10**: 1-14 [PMID: 29391927 DOI: 10.4252/wjsc.v10.i1.1]
65 **Kuismanen K**, Juntunen M, Narra Girish N, Tuominen H, Huhtala H, Nieminen K, Hyttinen J, Miettinen S. Functional Outcome of Human Adipose Stem Cell Injections in Rat Anal Sphincter Acute Injury Model. *Stem Cells Transl Med* 2018; **7**: 295-304 [PMID: 29383878 DOI: 10.1002/sctm.17-0208]
66 **Nolte T**, Brander-Weber P, Dangler C, Deschl U, Elwell MR, Greaves P, Hailey R, Leach MW, Pandiri AR, Rogers A, Shackelford CC, Spencer A, Tanaka T, Ward JM. Nonproliferative and Proliferative Lesions of the Gastrointestinal Tract, Pancreas and Salivary Glands of the Rat and Mouse. *J Toxicol Pathol* 2016; **29**: 1S-125S [PMID: 26973378 DOI: 10.1293/tox.29.1S]
67 **Li X**, Guo X, Jin W, Lu J. Effects of electroacupuncture combined with stem cell transplantation on anal sphincter injury-induced faecal incontinence in a rat model. *Acupunct Med* 2018; **pii**: acupmed-2016-011262 [PMID: 29519860 DOI: 10.1136/acupmed-2016-011262]
68 **Frudinger A**, Kölle D, Schwaiger W, Pfeifer J, Paede J, Halligan S. Muscle-derived cell injection to treat anal incontinence due to obstetric trauma: pilot study with 1 year follow-up. *Gut* 2010; **59**: 55-61 [PMID: 19875391 DOI: 10.1136/gut.2009.181347]
69 **Frudinger A**, Pfeifer J, Paede J, Kolovetsiou-Kreiner V, Marksteiner R, Halligan S. Autologous skeletal-muscle-derived cell injection for anal incontinence due to obstetric trauma: a 5-year follow-up of an initial study of 10 patients. *Colorectal Dis* 2015; **17**: 794-801 [PMID: 25773013 DOI: 10.1111/codi.12947]
70 **Romaniszyn M**, Rozwadowska N, Nowak M, Malcher A, Kolanowski T, Walega P, Richter P, Kurpisz M. Successful implantation of autologous muscle-derived stem cells in treatment of faecal incontinence due to external sphincter rupture. *Int J Colorectal Dis* 2013; **28**: 1035-1036 [PMID: 23549961 DOI: 10.1007/s00384-013-1692-y]
71 **Romaniszyn M**, Rozwadowska N, Malcher A, Kolanowski T, Walega P, Kurpisz M. Implantation of autologous muscle-derived stem cells in treatment of fecal incontinence: results of an experimental pilot study. *Tech Coloproctol* 2015; **19**: 685-696 [PMID: 26266767 DOI: 10.1007/s10151-015-1351-0]
72 **Sarveazad A**, Newstead GL, Mirzaei R, Joghataei MT, Bakhtiari M, Babahajian A, Mahjoubi B. A new method for treating fecal incontinence by implanting stem cells derived from human adipose tissue: preliminary findings of a randomized double-blind clinical trial. *Stem Cell Res Ther* 2017; **8**: 40 [PMID: 28222801 DOI: 10.1186/s13287-017-0489-2]
73 **Boyer O**, Bridoux V, Giverne C, Bisson A, Koning E, Leroi AM, Chambon P, Déhayes J, Le Corre S, Jacquot S, Bastit D, Martinet J, Houivet E, Tuech JJ, Benichou J, Michot F; and the Study Group of Myoblast Therapy for Faecal Incontinence. Autologous Myoblasts for the Treatment of Fecal Incontinence: Results of a Phase 2 Randomized Placebo-controlled Study (MIAS). *Ann Surg* 2018; **267**: 443-450 [PMID: 28426476 DOI: 10.1097/SLA.0000000000002268]
74 **Park EJ**, Kang J, Baik SH. Treatment of faecal incontinence using allogeneic-adipose-derived mesenchymal stem cells: a study protocol for a pilot randomised controlled trial. *BMJ Open* 2016; **6**: e010450 [PMID: 26888731 DOI: 10.1136/bmjopen-2015-010450]
75 **Salcedo L**, Damaser M, Butler R, Jiang HH, Hull T, Zutshi M. Long-term effects on pressure and electromyography in a rat model of anal sphincter injury. *Dis Colon Rectum* 2010; **53**: 1209-1217 [PMID: 20628287 DOI: 10.1007/DCR.0b013e3181de7fe0]
76 **Bohl JL**, Zakhem E, Bitar KN. Successful Treatment of Passive Fecal Incontinence in an Animal Model Using Engineered Biosphincters: A 3-Month Follow-Up Study. *Stem Cells Transl Med* 2017; **6**: 1795-1802 [PMID: 28678378 DOI: 10.1002/sctm.16-0458]
77 **Resplande J**, Gholami SS, Graziottin TM, Rogers R, Lin CS, Leng W, Lue TF. Long-term effect of ovariectomy and simulated birth trauma on the lower urinary tract of female rats. *J Urol* 2002; **168**: 323-330 [PMID: 12050564 DOI: 10.1016/S0022-5347(05)64915-4]
78 **Sievert KD**, Bakircioglu ME, Tsai T, Nunes L, Lue TF. The effect of labor and/or ovariectomy on rodent continence mechanism--the neuronal changes. *World J Urol* 2004; **22**: 244-250 [PMID: 15365750 DOI: 10.1007/s00345-004-0444-6]
79 **Healy CF**, O'Herlihy C, O'Brien C, O'Connell PR, Jones JF. Experimental models of neuropathic fecal incontinence: an animal model of childbirth injury to the pudendal nerve and external anal sphincter. *Dis Colon Rectum* 2008; **51**: 1619-1626; discussion 1626 [PMID: 18779998 DOI: 10.1007/s10350-008-9283-7]
80 **Ghadge SK**, Mühlstedt S, Ozcelik C, Bader M. SDF-1α as a therapeutic stem cell homing factor in myocardial infarction. *Pharmacol Ther* 2011; **129**: 97-108 [PMID: 20965212 DOI: 10.1016/j.pharmthera.2010.09.011]
81 **Bigalke C**, Luderer F, Wulf K, Storm T, Löbler M, Arbeiter D, Rau BM, Nizze H, Vollmar B, Schmitz KP, Klar E, Sternberg K. VEGF-releasing suture material for enhancement of vascularization: development, in vitro and in vivo study. *Acta Biomater* 2014; **10**: 5081-5089 [PMID: 25204522 DOI: 10.1016/j.actbio.2014.09.002]
82 **Yao J**, Korotkova T, Riboh J, Chong A, Chang J, Smith RL. Bioactive sutures for tendon repair: assessment of a method of delivering pluripotential embryonic cells. *J Hand Surg Am* 2008; **33**: 1558-1564 [PMID: 18984338 DOI: 10.1016/j.jhsa.2008.06.010]
83 **Horváthy DB**, Vácz G, Cselenyák A, Weszl M, Kiss L, Lacza Z. Albumin-coated bioactive suture for cell transplantation. *Surg Innov* 2013; **20**: 249-255 [PMID: 22717700 DOI: 10.1177/1553350612451353]
84 **Nauta AJ**, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 2007; **110**: 3499-3506 [PMID: 17664353 DOI: 10.1182/blood-2007-02-069716]
85 **Chen L**, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* 2008; **3**: e1886 [PMID: 18382669 DOI: 10.1371/journal.pone.0001886]
86 **Németh K**, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009; **15**: 42-49 [PMID: 19098906 DOI: 10.1038/nm.1905]
87 **Riera del Moral L**, Largo C, Ramirez JR, Vega Clemente L, Fernández Heredero A, Riera de Cubas L, Garcia-Olmo D, Garcia-Arranz M. Potential of mesenchymal stem cell in stabilization of abdominal aortic aneurysm sac. *J Surg Res* 2015; **195**: 325-333 [PMID: 25592273 DOI: 10.1016/j.jss.2014.12.020]
88 **Røsland GV**, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwietz J, Tonn JC, Goldbrunner R, Lønning PE, Bjerkvig R, Schichor C. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res* 2009; **69**: 5331-5339 [PMID: 19509230 DOI: 10.1158/0008-5472.CAN-08-4630]
89 **Garcia S**, Bernad A, Martín MC, Cigudosa JC, Garcia-Castro J, de la Fuente R. Pitfalls in spontaneous in vitro transformation of human mesenchymal stem cells. *Exp Cell Res* 2010; **316**: 1648-1650 [PMID: 20171963 DOI: 10.1016/j.yexcr.2010.02.016]
90 **Torsvik A**, Røsland GV, Svendsen A, Molven A, Immervoll

H, McCormack E, Lønning PE, Primon M, Sobala E, Tonn JC, Goldbrunner R, Schichor C, Mysliwietz J, Lah TT, Motaln H, Knappskog S, Bjerkvig R. Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track - letter. *Cancer Res* 2010; **70**: 6393-6396 [PMID: 20631079 DOI: 10.1158/0008-5472.CAN-10-1305]

91 **Rhodes LV**, Muir SE, Elliott S, Guillot LM, Antoon JW, Penfornis P, Tilghman SL, Salvo VA, Fonseca JP, Lacey MR, Beckman BS, McLachlan JA, Rowan BG, Pochampally R, Burow ME. Adult human mesenchymal stem cells enhance breast tumorigenesis and promote hormone independence. *Breast Cancer Res Treat* 2010; **121**: 293-300 [PMID: 19597705 DOI: 10.1007/s10549-009-0458-2]

92 **Xu WT**, Bian ZY, Fan QM, Li G, Tang TT. Human mesenchymal stem cells (hMSCs) target osteosarcoma and promote its growth and pulmonary metastasis. *Cancer Lett* 2009; **281**: 32-41 [PMID: 19342158 DOI: 10.1016/j.canlet.2009.02.022]

93 **Cousin B**, Ravet E, Poglio S, De Toni F, Bertuzzi M, Lulka H, Touil I, André M, Grolleau JL, Péron JM, Chavoin JP, Bourin P, Pénicaud L, Casteilla L, Buscail L, Cordelier P. Adult stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both in vitro and in vivo. *PLoS One* 2009; **4**: e6278 [PMID: 19609435 DOI: 10.1371/journal.pone.0006278]

94 **Sun N**, Panetta NJ, Gupta DM, Wilson KD, Lee A, Jia F, Hu S, Cherry AM, Robbins RC, Longaker MT, Wu JC. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc Natl Acad Sci USA* 2009; **106**: 15720-15725 [PMID: 19805220 DOI: 10.1073/pnas.0908450106]

95 **Khakoo AY**, Pati S, Anderson SA, Reid W, Elshal MF, Rovira II, Nguyen AT, Malide D, Combs CA, Hall G, Zhang J, Raffeld M, Rogers TB, Stetler-Stevenson W, Frank JA, Reitz M, Finkel T. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J Exp Med* 2006; **203**: 1235-1247 [PMID: 16636132 DOI: 10.1084/jem.20051921]

96 **Klopp AH**, Gupta A, Spaeth E, Andreeff M, Marini F 3rd. Concise review: Dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? *Stem Cells* 2011; **29**: 11-19 [PMID: 21280155 DOI: 10.1002/stem.559]

97 **Ramdasi S**, Sarang S, Viswanathan C. Potential of Mesenchymal Stem Cell based application in Cancer. *Int J Hematol Oncol Stem Cell Res* 2015; **9**: 95-103 [PMID: 25922650]

98 **Chulpanova DS**, Kitaeva KV, Tazetdinova LG, James V, Rizvanov AA, Solovyeva VV. Application of Mesenchymal Stem Cells for Therapeutic Agent Delivery in Anti-tumor Treatment. *Front Pharmacol* 2018; **9**: 259 [PMID: 29615915 DOI: 10.3389/fphar.2018.00259]

99 **Kuriyan AE**, Albini TA, Townsend JH, Rodriguez M, Pandya HK, Leonard RE 2nd, Parrott MB, Rosenfeld PJ, Flynn HW Jr, Goldberg JL. Vision Loss after Intravitreal Injection of Autologous "Stem Cells" for AMD. *N Engl J Med* 2017; **376**: 1047-1053 [PMID: 28296617 DOI: 10.1056/NEJMoa1609583]

100 **Davies BM**, Smith J, Rikabi S, Wartolowska K, Morrey M, French A, MacLaren R, Williams D, Bure K, Pinedo-Villanueva R, Mathur A, Birchall M, Snyder E, Atala A, Reeve B, Brindley D. A quantitative, multi-national and multi-stakeholder assessment of barriers to the adoption of cell therapies. *J Tissue Eng* 2017; **8**: 2041731417724413 [PMID: 28835816 DOI: 10.1177/2041731417724413]

101 **Danielson J**, Karlbom U, Graf W, Wester T. Long-term outcome after free autogenous muscle transplantation for anal incontinence in children with anorectal malformations. *J Pediatr Surg* 2010; **45**: 2036-2040 [PMID: 20920725 DOI: 10.1016/j.jpedsurg.2010.06.009]

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REVIEW

Stem cell-derived exosomes - an emerging tool for myocardial regeneration

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Abstract

Cardiovascular diseases (CVDs) continue to represent the number one cause of death and disability in industrialized countries. The most severe form of CVD is acute myocardial infarction (AMI), a devastating disease associated with high mortality and disability. In a substantial proportion of patients who survive AMI, loss of functional cardiomyocytes as a result of ischaemic injury leads to ventricular failure, resulting in significant alteration to quality of life and increased mortality. Therefore, many attempts have been made in recent years to identify new tools for the regeneration of functional cardiomyocytes. Regenerative therapy currently represents the ultimate goal for restoring the function of damaged myocardium by stimulating the regeneration of the infarcted tissue or by providing cells

that can generate new myocardial tissue to replace the damaged tissue. Stem cells (SCs) have been proposed as a viable therapy option in these cases. However, despite the great enthusiasm at the beginning of the SC era, justified by promising initial results, this therapy has failed to demonstrate a significant benefit in large clinical trials. One interesting finding of SC studies is that exosomes released by mesenchymal SCs (MSCs) are able to enhance the viability of cardiomyocytes after ischaemia/reperfusion injury, suggesting that the beneficial effects of MSCs in the recovery of functional myocardium could be related to their capacity to secrete exosomes. Ten years ago, it was discovered that exosomes have the unique property of transferring miRNA between cells, acting as miRNA nanocarriers. Therefore, exosome-based therapy has recently been proposed as an emerging tool for cardiac regeneration as an alternative to SC therapy in the post-infarction period. This review aims to discuss the emerging role of exosomes in developing innovative therapies for cardiac regeneration as well as their potential role as candidate biomarkers or for developing new diagnostic tools.

Key words: Acute myocardial infarction; Exosome; Stem cell; Cardiac regeneration; Cardiovascular diseases

Core tip: Regenerative therapy represents the ultimate goal for restoring the function of damaged myocardium by stimulating the regeneration of infarcted tissue. Exosomes are small microvesicles released by living cells that act as miRNA nanocarriers, and exosomes can stimulate and modulate cellular proliferation and regeneration. Elevated exosome levels have been detected in human plasma in various cardiovascular diseases. Furthermore, myocardium-derived exosomes are potentially associated with myocardial healing. Given their paracrine properties, myocardium-derived exosomes have been proposed as a potential therapeutic option for myocardial regeneration. This review discusses the emerging roles of exosomes as candidate biomarkers and innovative therapies for cardiac regeneration.

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INTRODUCTION

Cardiovascular diseases (CVDs) continue to be the number one cause of death and disability in industrialized countries. Despite many efforts to increase the rate of early diagnosis for acute coronary syndromes (ACS) and decrease associated mortality, acute myocardial infarction (AMI) is associated with mortality as high as 12%[1].

In a substantial proportion of patients who survive AMI, loss of functional cardiomyocytes as a result of ischaemic injury leads to ventricular failure, resulting in significant alteration to quality of life and increased mortality. Therefore, many attempts have been made in recent years to identify new tools to regenerate functional cardiomyocytes.

Regenerative therapy represents the ultimate goal for restoring the function of damaged myocardium by stimulating the regeneration of the infarcted tissue or providing cells that can generate new myocardial tissue to replace the damaged tissue. Stem cells (SCs) have been proposed to represent a viable therapy option in these cases. However, despite the great enthusiasm at the beginning of the SC era, justified by promising initial results, this therapy has failed to demonstrate a significant benefit in large clinical trials[2]. This lack of significant clinical benefit was initially attributed to the different origins of the SCs and the different routes of delivery used in clinical trials[3-8].

One interesting finding of the SC studies was that exosomes released by mesenchymal SCs (MSCs) are able to enhance the viability of cardiomyocytes after ischaemia/reperfusion injury, suggesting that the beneficial effects of MSCs in the recovery of functional myocardium could be related to their capacity to secrete exosomes[9]. Therefore, exosome-based therapy has recently been proposed as an emerging tool for cardiac regeneration as an alternative to SC therapy in the post-infarction period.

The role of exosome vesicles in different cardiovascular applications was discovered several decades ago. However, major interest in exosomes began in 2007, when it was discovered that they have the unique property of transferring miRNA between cells and acting as miRNA nanocarriers[10].

This review aims to discuss the emerging role of exosomes in developing innovative therapies for cardiac regeneration, as well as their potential role as candidate biomarkers for developing new diagnostic tools.

EXOSOMES - DEFINITION AND ROLES

Exosomes are nanosized vesicles (30-150 nm diameter) of endosomal origin that are released by various cells and contain proteins, lipids, and genetic material[11]. Exosomes are present in enormous quantities in the blood, estimated to be 10^{10}/mL of plasma in healthy individuals[12,13].

It has been demonstrated that living cells are able to secrete vesicles of different sizes and intracellular origins. The main types of cell-generated vesicles are exosomes (diameter between 30 and 150 nm), microvesicles (diameter range 50-1000 nm) and apoptosomes (diameter range 50-5000 nm). The main differences between these populations of vesicles are

not only their diameter but also their mechanism of generation. While exosomes are generated by internal budding of plasma membranes, microvesicles arise from direct budding of injured cell plasma membranes, and apoptosomes originate as fragments of cells undergoing programmed death[14].

Exosomes result from inward budding of cell membrane ligands, a process associated with internalization of extracellular membrane ligands to the surface of the small vesicles generated by inward budding. This inward budding allows the internalization of small proteins, mRNAs, miRNA and DNA into the exosomes[15]. In the next stage, these small bodies are fused with the cell membrane and released through an exocytotic process, carrying various molecules, proteins, mRNAs, ncRNAs and enzymes[16]. After the exosomes are released into the circulation, they migrate to recipient cells. Once the exosomes are absorbed by the recipient, the molecules and RNA carried by the exosomes from the parent cells are transferred to the recipient cells. From the entire spectrum of microvesicles generated by living cells, exosomes are the category richest in miRNAs, thus representing an ideal nanocarrier for transferring miRNA molecules to target tissues.

Exosomes as intercellular communication messengers

Exosomes are able to transfer activated receptors to recipient cells and act as transfer molecules, generating signalling pathways[14]. They have the ability to transmit functional signals between cells (such as miRNAs), which are involved in various pathophysiological processes related to atheromatous plaque instability and ischaemic injury[10].

One fundamental property of exosomes is their ability to transfer non-coding RNA (including miRNA and lncRNA) from the parent cells to the recipient cells, thereby modulating the phenotype and protein expression of recipient cells[16]. As a result, exosome-mediated intercellular communication has been demonstrated to play a substantial role in two major mechanisms involved in acute cardiovascular events: (1) ensuring vascular integrity to prevent atheromatous plaque progression and rupture; and (2) ensuring a significant level of cardioprotection following AMI.

SOURCES OF EXOSOMES WITH POTENTIAL APPLICATIONS IN MYOCARDIAL REGENERATION

Cardiomyocytes are able to generate exosomes functionalized with heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) at their surface, while cardiac fibroblasts are able to secrete exosomes stimulating an giotensin Ⅱ production, thus promoting cardiomyocyte hypertrophy[17]. At the same time, exosomes obtained from healthy controls have been shown to exert a cardioprotective action on ischaemic myocardium from patients with coronary artery disease by releasing cardioprotective HSP70 and other protective signals. Therefore, there is a potential therapeutic role of these promising microparticles in clinical applications[13]. However, endothelial cells may be the most relevant source of exosomes under ischaemic conditions. It has been demonstrated that endothelial cell-derived exosomes express increased levels of intercellular adhesion molecules (ICAM-1 or VCAM-1), which are involved in the complex mechanisms of coronary atheromatous plaque vulnerabilisation[14].

SCs-derived exosomes

Different populations of SCs are able to generate exosomes that will serve as transfer mediators. The potential sources of SC-derived cardioprotective exosomes include MSCs, cardiac stem cells (CSCs), embryonic SCs, haematopoietic SCs, cardiosphere-derived SCs and plasma.

MSCs appear to have relevant immunosuppressive properties; therefore, MSC-generated exosomes may play a role in immune-mediated responses with immunosuppressive properties[18]. More than 700 proteins have been identified in the proteome of MSc exosomes[11,19], and these proteins are involved in the stimulation of vascular endothelial growth factor (VEGF) and hepatocyte-growth factor (HGF).

Arslan *et al*[20] demonstrated that injection of MSc-derived exosomes can decrease infarct size by 45% and reduce systemic inflammation. At the same time, intramyocardial infusion of MSc-derived exosomes improved contractility of cardiomyocytes and reduced infarct size in a rat model of AMI[21], demonstrating that exosomes definitely play a cardioprotective role by preventing cardiac remodelling during the post-AMI period.

CSC-derived exosomes have been isolated from the right atrial appendage of patients undergoing bypass surgery and have shown an increased capacity to stimulate endothelial tube formation[22]. Under hypoxic conditions, antifibrotic miRNA-enriched exosomes are transferred from cardiac progenitor cells to fibroblasts, thereby decreasing cardiac fibrosis and apoptosis and increasing angiogenesis[22,23]. Embryonic SC-derived exosomes have also been demonstrated to induce neovascularization, increase cardiomyocyte survival, and reduce fibrosis during the post-infarction period[24]. Haematopoietic SC-derived exosomes were demonstrated to increase tube formation as well as endothelial cell viability and proliferation[11,25].

Cardiosphere-derived exosomes can have a delayed protective effect on cardiomyocytes as a result of their action on cardiac macrophages. This induces a specific cardioprotective phenotype at this level[26] and stimulates both angiogenesis and proliferation of cardiomyocytes[27]. Interestingly, human cardiosphere-derived exosomes have been shown to reduce infarct size after intramyocardial administration, but without any

significant benefit following intracoronary administration.

Isolation and purification of exosomes

Exosomes can be isolated from various cell cultures, such as cells from haematologic origin (B-, T-lymphocytes, mast cells, dendritic cells and platelets), colorectal cells, tumour cells, neurons and body fluids (blood, urine, bronchial lavage, breast milk, sperm, ascites and synovial fluid). The challenge of obtaining high yields of pure exosomes arises from the fact that the cultures are frequently contaminated by shedding microvesicles (SMVs) and apoptotic blebs (ABs). A comparative analysis of studies that have investigated exosomes has proven to be difficult due to the various purification techniques that were implemented. Contamination can be avoided by proper isolation and purification procedures. Exosomes exhibit smaller sizes (30-150 nm diameter *vs* 100-1000 nm for SMV and 50-500 nm for AB), different densities (1.10-1.21 g/mL *vs* 1.16-1.28 g/mL) and cell type-specific proteins. Based on these biophysical properties, pure exosomes can be obtained using differential centrifugation with membrane filters, rate zonal centrifugation and immunoaffinity capture with magnetic beads using specific antibodies/proteins[28,29].

EMERGING ROLE OF EXOSOMES IN CVD

It has been demonstrated that exosomes have beneficial effects on injured hearts, protecting cardiomyocytes in both acute and chronic models of ischaemia or in acute ischaemia/reperfusion injury[11]. Their beneficial effects have been related to a significant decrease in infarct size, reduction of fibrosis and associated remodelling, stimulation of angiogenesis and alteration of immune function[11].

Exosomes as a source of biomarkers in CVD

The potential of exosomes to serve as reliable biomarkers for CV diseases relies on their ability to incorporate miRNAs, RNAs, proteins and lipids for various clinical conditions. Bioinformatics tools are currently able to differentiate the composition of a large number of miRNAs. As a result, specific mRNAs/miRNAs have been discovered in exosomes isolated from patients with AMI or with atheromatous plaques. Patients with CAD exhibit increased levels of circulating exosomes, especially a subpopulation rich in miR-199a and miR-126, thus showing a great potential to serve as biomarkers for CAD[30]. At the same time, elevated levels of miR-1 and miR-133 have been identified in the serum of patients with acute coronary syndromes and have been shown to correlate well with troponin values[31]. Several studies have demonstrated increased levels of miR-1 and miR-133 in the peripheral circulation of patients with various types of ACS, including unstable angina, AMI or Takotsubo cardiomyopathy[31], while patients with troponin-positive ACS exhibited increased levels of miR-133a and miR-499[32]. However, very few studies have attempted to validate the role of exosomes as biomarkers in coronary artery disease (CAD).

Cardiomyocytes produce a large number of miRNAs. From these, four types are specifically related to AMI - miRNA-1, miRNA-133a and b, miRNA-208a and miRNA-499. During AMI, these miRNAs rapidly increase in the peripheral blood up to 3000-fold compared to healthy individuals, indicating myocardial damage. Therefore, such a panel of miRNA biomarkers can serve as reliable markers of myocardial necrosis with a higher specificity than traditional biomarkers. Furthermore, their elevation occurs much earlier than the increase in troponin, thus representing a promising tool for an immediate and accurate diagnosis of AMI.

It has also been demonstrated that in patients with ACS, injured cardiomyocyte-released exosomes are rich in cardiac-specific miRNAs, such as miRNA-1, miRNA-208 and miRNA-133. At the same time, miRNA-133 present in exosomes can serve as a reliable biomarker for myocardial damage in AMI[16]. Elevated serum levels of exosome-derived miR-208a were correlated with deterioration of the hemodynamic status, as expressed by an increase in the Killip class (class Ⅰ: no evidence of heart failure, class Ⅱ: mild to moderate heart failure, with rales less half way up the lung fields, class Ⅲ: pulmonary oedema, and class Ⅳ: cardiogenic shock) and reduced survival in AMI patients[33]. Interestingly, in patients with AMI, various miRNAs inside exosomes have been associated with the occurrence of heart failure (HF) during the post-infarction period. Matsumoto *et al*[34] showed that exosomal-derived miRNA-192, miRNA-194 and miRNA-34a were significantly increased in patients with AMI who developed HF and ventricular remodelling.

Exosomes as therapeutic tools in CVD

The use of exosomes as therapeutic tools is based on the premise that the use of paracrine mediators of SCs could be more effective than the use of whole SCs. It has been demonstrated that only a small proportion of injected SCs are retained at the site of infusion and that cell engraftment is rare. This observation raises serious doubts about the capability of the SCs to act as a reliable regeneration tool and led to a hypothesis about the paracrine-mediated effects of the SCs. However, reliable *in vivo* tracing of exosomes is not currently feasible, and it is difficult to explain why the paracrine factor (exosome) would be more effective than the parent cell[11]. Therefore, a new hypothesis could rely on the capacity of exosomes to reprogram immune cells to confer a cardioprotective effect.

MSC-derived exosomes recapitulate the properties of their parent cells in terms of immunomodulation and cardioprotection[35,36]. The advantages of using exosomes instead of SC therapy for myocardial regeneration are several. First, this new therapy can provide active molecules, such as mRNA, miRNA and proteins, to target cells, and these molecules can be modified by source cell manipulation or by external means. Second, this source of therapy is associated with very low immunogenicity.

However, the disadvantages of this approach are the very labourious and inefficient isolation techniques as well as the exosomes' short-term use and inability to regenerate[37].

A promising application of exosomes is represented by their potential to act as vehicles for the delivery of specific miRNAs to target tissues. The therapeutic effect of SC-derived exosomes has been attributed to the delivery of specific microRNAs, such as miR-146a, miR-22, miR-21, miR-126 or miR-210, to the ischaemic myocardium[38]. It has been shown that treatment with MSC-derived exosomes significantly changed the miRNA expression profile in CSCs, suggesting that the miRNAs play a major role in mediating the beneficial effects of MSC-derived exosomes[39]. The fact that MSC-derived exosomes have a therapeutic effect that is superior to that of MSCs can also be explained by the increased expression of several miRNAs, such as miR-15 and miR-21, in MSC-derived exosomes compared to their expression in MSCs[40]. Similarly, a significant enrichment of mi-294 in ESC-derived exosomes compared to the level in ESCs was recorded, suggesting that the beneficial effects of exosomes can be attributed to the increased delivery of miR-294 to cardiac cells[41].

Another use for exosomes in cardiovascular applications is related to the treatment of SCs with exosomes. In a recent study, miR-133 transfection of MSCs improved cardiac function in a rat model of myocardial infarction[42]. CSC pretreatment with exosomes showed upregulation of miR-147 and miR-503-3p and downregulation of miR-207, miR-326-5p and miR-702-5p, leading to improved cardiac function and increased vessel density at the site of infarction[43,44]. Additionally, Zhang *et al*[39] demonstrated that pretreatment of CSCs with MSC-derived exosomes stimulated proliferation, migration and tube formation of CSCs in a rat model of myocardial infarction. This pretreatment was also associated with improved survival, enhanced capillary density and reduced cardiac fibrosis[39].

Exosomes as drug delivery carriers

Exosomes can be modified to become an effective delivery tool for transferring bioactive molecules to specific cells[45]. They have been demonstrated to represent effective targeted drug delivery systems. Personalized exosome-mimetic nanovesicles could represent a promising emerging application in the future as a novel drug delivery system.

An emerging therapeutic field of exosome-based therapy is nanotherapy. This new field of exosome-related treatment is based on the incorporation of miRNA into exosomes to deliver miRNAs to recipient tissues for their cardioprotective effect or for the reduction of inflammation and atheromatous plaque formation.

Exosomes can be used as nanoparticles for targeted delivery of miRNAs to promote angiogenesis and myocardial regeneration. Interestingly, exosomes have been proposed to serve as an efficient nanocarrier for transporting protein regulators such as Shh protein regulators, morphogenic proteins involved in cardioprotection and in promoting neovascularization in the post-MI heart, with significant anti-apoptotic and vasculoprotective properties[46].

The main approaches proposed thus far for using exosomes as nanocarriers include loading exosomes isolated from parental cells with different drugs, loading parental cells with drugs that will be released into the exosomes, or transfecting parental cells with active compounds to be released into the exosomes[47]. However, none of these approaches has so far been validated in clinical trials.

EXOSOMES AND ATHEROSCLEROSIS

Exosomes, inflammation and atheromatous plaque progression

Atherosclerosis is associated with augmented systemic inflammation, the release of inflammatory cytokines, increased oxidative stress and endothelial cell activation. It is well known that cardiomyocytes and endothelial cells interact with each other *via* exosome-mediated transfers. MiR-223 secreted by activated macrophages and included in the exosomes released by these macrophages is involved in the inflammatory response associated with atherosclerosis development. Some recent data suggest that exosomes containing the HSP70 protein may be involved in the migration of monocytes in the subendothelial space[16]. At the same time, exosomes released by cells associated with atheromatous plaques stimulate the expression of adhesion molecules (ICAM and VCAM) and trigger local inflammation[48].

The role of exosomes in CAD is related to their effect on inflammation, thrombosis, neoangiogenesis and cell survival. They can also promote the adhesion of monocytes to the endothelium, increase the endothelial expression of adhesion molecules and increase the expression of adhesion molecule receptors in monocytes[30]. miRNA-222, which is present in exosomes, can also regulate ICAM-1 expression[49]. Interestingly, exosomes from atheromatous plaques can also transfer ICAM-1 directly to recipient cells[48], favouring early atherosclerotic processes.

Shear stress has been shown to represent a vulnerability factor associated with atheromatous plaque progression. Some reports shown the role of increased sub-endothelial stress in determining particular types of acute coronary syndromes[50]. It has been demonstrated that exosomes containing miR-143/145 are increased in cells exposed to increased shear stress[51-53]. Activation of Kruppel-like factor 2, which is largely dependent on the level of shear stress, can lead to the release of exosomes containing miR-143 and miR-145, which inhibit smooth muscle cell de-differentiation and thus support a potential cardioprotective effect[30,54].

At the same time, platelet-derived exosomes may make a substantial contribution to the atherosclerotic

process by prompting pro-inflammatory activation of endothelial smooth muscle cells[55]. Alternately, exosomes released by monocytes activate macrophages, and endothelial cells favour the progression of atherogenesis[56]. Interestingly, platelet-derived exosomes have both protective and detrimental effects. The protective effects result from their capacity to stimulate angiogenesis, while their detrimental effects are related to their pro-thrombotic activity.

Exosomes are also involved in the development of arterial calcification. It has been demonstrated that vascular smooth muscle releases exosomes that promote vascular calcification. Additionally, the injection of exosomes into apoE- mice was associated with a reduction in atherosclerotic lesion development in the aorta[57].

Exosomes and atheromatous plaque vulnerability

In the case of atheromatous plaque rupture, the contents of the vascular wall are exposed to procoagulant components of the blood, thereby leading to thrombotic occlusion of the vessels. The mechanisms which exosomes act in vulnerable plaques (VP) are various. First, VPs contain a large number of microvesicles with advanced procoagulant properties, especially at the level of the necrotic core. VPs are characterized by a large amount of low density cholesterol and a thin fibrous cap[58-60]. When the fibrous cap of the VP ruptures, this procoagulant content is exposed to the components of the blood, favouring immediate thrombus formation. At the same time, microvesicles promote local inflammation at the site of the VP, which favours plaque rupture[61].

Unlike microvesicles, platelet-derived exosomes have been shown to play a major antithrombotic role and act rather as anticoagulants, inhibiting platelet aggregation in a murine model of carotid artery injury[62]. Platelet-derived exosomal miR-320 demonstrated a clear atheroprotective effect by reducing endothelial expression of adhesion molecules such as ICAM-1, reducing inflammation, and inhibiting thrombus formation[63]. This is consistent with the conclusions of a pilot study suggesting that SCs could play a protective role in the vascular endothelium by reducing atherosclerosis progression and calcium accumulation in coronary arteries[64].

EXOSOMES AS EMERGING TOOLS FOR POST-MYOCARDIAL INFARCTION REGENERATION

Exosomes and cardioprotection

In the case of AMI, reperfusion of an occluded coronary vessel can lead to reperfusion injury, which adds to the initial injury caused by the abrupt occlusion. Therefore, the reducing reperfusion injury is crucial for improving the long-term evolution of AMI survivors.

The acute cardioprotective effects of exosomes were demonstrated in 2010, following the observation that exosomes injected into mice suffering a 30 min ischaemia led to a significant reduction of infarct size within 20 h[37]. However, exosomes have been demonstrated to play a significant cardioprotective role in models of continuous ischaemia without reperfusion. In a study by Zhao *et al*[65], after ligation of the left anterior descending artery in rats, injection of exosomes was associated with a significant improvement in systolic function at 4 wk, concomitant with a significant reduction in cardiac fibrosis and apoptosis.

Plasma exosomes originating from various cells demonstrated significant cardioprotective effects in the post-AMI period, reducing infarct size after intravenous administration. At the same time, the release of cytoprotective HSP70 and HSP90 from exosomes has been identified in mouse cardiomyocytes[66]. HSP70 present at the surface of plasma exosomes stimulates the activation of several cardioprotective pathways[13].

The effects of exosomes on ischaemic hearts can be mediated through various types of receptor cells. In macrophages and other cells, exosomes are involved in immunosuppression mechanisms. Alternately, they stimulate angiogenesis at the level of endothelial cells, inhibition of fibrosis at the level of fibroblasts, and cardioprotection at the level of cardiomyocytes[11].

Post-myocardial infarction release of exosomes containing cardiac-specific miRNA is essential to ensure an adequate level of cardioprotection, as cardiac-specific miRNA exhibits significant protective effects: miRNA-133 has anti-apoptotic and anti-fibrotic effects; miRNA-1 has a specific anti-oxidant role; and miRNA-499 has anti-apoptotic properties[67]. In another study, microRNA analysis of CPC-derived exosomes indicated the presence of increased levels of miR-210, miR-132 and miR-146a-3p in a myocardial infarction model, inducing a sustained anti-apoptotic and pro-angiogenic response[68].

Hypoxic exosomes contain higher amounts of pro-angiogenic miRNAs, showing a more pronounced angiogenic potential[23]. Interestingly, exosomes from the pericardial fluid during the post-infarction period also exhibited cardioprotective effects by decreasing apoptosis and enhancing arteriogenesis[69].

An interesting finding was the role of exercise in further increasing the number of circulating exosomes in healthy individuals but not in patients with CAD[70]. At the same time, cardiomyocyte-derived exosomes from exercised mice expressed higher levels of miR-29b and miR-455 compared to sedentary ones, and these miRNAs had the capacity to downregulate matrix-metalloprotease 9 and reduce cardiac fibrosis[71]. Thus, we can conclude that in the post-MI period, cardiac cells release exosomes with augmented cardioprotective effects to promote myocardial regeneration.

Exosomes as myocardial regenerative tools

The efforts to regenerate myocardium *via* injecting various types of SCs into the myocardium or into the infarct-

affected coronary arteries did not lead to significant evidence of their potential to generate new myocardium. However, the benefits of SCs have been attributed to their paracrine effects, which could be mediated by exosomes[64,72]. Following the observation that the SCs remain at the site of injection release factors mediating this paracrine effect, exosomes have been proposed as important potential paracrine mediators for myocardial regeneration. Given their carrier capacity, exosomes exhibit the potential for delivering biologics containing proteins or small interfering RNA (siRNA). Experimental studies have demonstrated that engineered $CD34^+$ SCs were able to excrete manipulated exosomes containing a proangiogenic factor, which was delivered to infarcted mouse myocardium and led to decreased infarct size, increased angiogenesis and improved long-term regeneration[11,73].

In AMI, myocardial tissue is exposed to increased ischaemic stress signals. As a result, cardiomyocytes respond by increasing the secretion of exosomes, which has been identified in different amounts in peri-infarcted areas and in healthy myocardium. Exosomes released by the damaged myocardium transfer proteins and miRNAs that send ischaemic signals to distant tissues or organs, such as bone marrow (BM), and stimulate the production of SC from the BM. In turn, BM releases SCs and exosomes that travel back to the ischaemic myocardium to stimulate the repair process and trigger myocardial regeneration[38]. Injured myocardium exhibits a multitude of responses to injury, including necrosis, inflammation, apoptosis, remodelling and fibrosis. Paracrine effects of exosomes released by non-injured myocardium from peri-infarcted areas can reprogram cardiomyocytes and rescue the peri-infarcted region from these deleterious mechanisms. This is mediated by the specific transfer of RNAs, peptides and small molecules[74].

Several preclinical studies have demonstrated the beneficial role of SC-derived exosomes in the repair of ischaemic tissues and myocardial regeneration[27,75-77]. Therefore, exosomes can represent a new line of cell-free therapy for myocardial regeneration in AMI. However, their translation into clinical application is still far away.

Arslan *et al*[20] showed that exosome treatment in the post-MI period enhanced myocardial viability and reduced adverse ventricular remodelling by decreasing oxidative stress and activating the PI3K/Akt pathway. Lai *et al*[77] also demonstrated that the administration of MSC-derived exosomes significantly reduced infarct size in mice. Intramyocardial injection of CSC-derived exosomes in mice undergoing ischaemia-reperfusion injury led to a 53% reduction in cardiomyocyte-related apoptosis[78]. Furthermore, Barile *et al*[22] found that intramyocardial injection of CSC-derived exosomes reduced the amount of scar tissue, increased vessel density *via* angiogenic effects and significantly decreased apoptosis of cardiomyocytes.

In a study on acute myocardial ischaemic injury, Luo *et al*[79] demonstrated that exosomes derived from adipose-derived stem cells (ADSCs) overexpressing miR-126 decreased myocardial injury by reducing the expression of inflammation factors. This suggests that ADSC-derived exosomes can also protect myocardial cells from apoptosis, inflammation and fibrosis, thus preventing myocardial damage and favouring angiogenesis and myocardial repair[79]. These findings were demonstrated in both *in vitro* and in *vivo* environments; thus, the administration of miR-126–enriched exosome treatment may serve as a potential therapeutic alternative where SC therapy fails to reduce myocardial injury or promote the regeneration process after myocardial infarction.

Exosomes may also play a role in vascular regeneration. Endothelial cells, monocytes and vascular smooth muscle cells also possess the ability to secrete exosomes, which stimulate and mediate angiogenesis, vascular healing, and remodelling by promoting cell migration, adhesion, and proliferation[80]. Experimental studies also suggest that due to their autocrine and paracrine effects, exosomes are implicated in the modulation of physiological processes such as thrombus formation by binding coagulation factors. In contrast to the protective effects, vascular smooth muscle cell exosomes can play a detrimental role in vascular calcification and atherogenesis[81]. These findings open the way for therapeutic approaches targeting inhibition of exosome secretion, thus preventing excessive coagulation and vascular calcification. Inhibiting exosome secretion may be extremely challenging considering the fine line between the physiological role of exosomes in healing processes and the harmful effect in pathological conditions.

It is interesting to note that different cells release exosomes that can exhibit a dual role in CVD: On the one hand, a protective role, especially with respect to their cardioprotective properties, and on the other hand, a destructive role, with respect to their role in mediating inflammatory responses.

CONCLUSION

Exosomes offer unique opportunities for the development of new therapies, representing promising cell-free therapeutic options for myocardial repair. However, because of their ubiquitous presence and effect on both physiological and pathological processes, the role in cardiac regeneration needs further investigation to validate them as both biomarkers and as a therapeutic option. The results of recent experimental studies suggest that exosomes possess great therapeutic potential that might overcome the shortcomings of SC therapy and could open new frontiers in regenerative cardiovascular medicine; however, this hope needs to be validated by further clinical studies.

REFERENCES

1 **Kristensen SD**, Laut KG, Fajadet J, Kaifoszova Z, Kala P, Di Mario C, Wijns W, Clemmensen P, Agladze V, Antoniades L,

Alhabib KF, De Boer MJ, Claeys MJ, Deleanu D, Dudek D, Erglis A, Gilard M, Goktekin O, Guagliumi G, Gudnason T, Hansen KW, Huber K, James S, Janota T, Jennings S, Kajander O, Kanakakis J, Karamfiloff KK, Kedev S, Kornowski R, Ludman PF, Merkely B, Milicic D, Najafov R, Nicolini FA, Noč M, Ostojic M, Pereira H, Radovanovic D, Sabaté M, Sobhy M, Sokolov M, Studencan M, Terzic I, Wahler S, Widimsky P; European Association for Percutaneous Cardiovascular Interventions. Reperfusion therapy for ST elevation acute myocardial infarction 2010/2011: current status in 37 ESC countries. *Eur Heart J* 2014; **35**: 1957-1970 [PMID: 24419804 DOI: 10.1093/eurheartj/eht529]

2 **Gyöngyösi M**, Lukovic D, Zlabinger K, Mandic L, Winkler J, Gugerell A. Cardiac Stem Cell-based Regenerative Therapy for the Ischemic Injured Heart - a Short Update 2017. *Journal of Cardiovascular Emergencies* 2017; **3**: 81-83 [DOI: 10.1515/jce-2017-0009]

3 **Wollert KC**, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A, Drexler H. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet* 2004; **364**: 141-148 [PMID: 15246726 DOI: 10.1016/S0140-6736(04)16626-9]

4 **Leistner DM**, Fischer-Rasokat U, Honold J, Seeger FH, Schächinger V, Lehmann R, Martin H, Burck I, Urbich C, Dimmeler S, Zeiher AM, Assmus B. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy. *Clin Res Cardiol* 2011; **100**: 925-934 [PMID: 21633921 DOI: 10.1007/s00392-011-0327-y]

5 **Gyöngyösi M**, Wojakowski W, Lemarchand P, Lunde K, Tendera M, Bartunek J, Marban E, Assmus B, Henry TD, Traverse JH, Moyé LA, Sürder D, Corti R, Huikuri H, Miettinen J, Wöhrle J, Obradovic S, Roncalli J, Malliaras K, Pokushalov E, Romanov A, Kastrup J, Bergmann MW, Atsma DE, Diederichsen A, Edes I, Benedek I, Benedek T, Pejkov H, Nyolczas N, Pavo N, Bergler-Klein J, Pavo IJ, Sylven C, Berti S, Navarese EP, Maurer G; ACCRUE Investigators. Meta-Analysis of Cell-based CaRdiac stUdiEs (ACCRUE) in patients with acute myocardial infarction based on individual patient data. *Circ Res* 2015; **116**: 1346-1360 [PMID: 25700037 DOI: 10.1161/CIRCRESAHA.116.304346]

6 **Pavo N**, Charwat S, Nyolczas N, Jakab A, Murlasits Z, Bergler-Klein J, Nikfardjam M, Benedek I, Benedek T, Pavo IJ, Gersh BJ, Huber K, Maurer G, Gyöngyösi M. Cell therapy for human ischemic heart diseases: critical review and summary of the clinical experiences. *J Mol Cell Cardiol* 2014; **75**: 12-24 [PMID: 24998410 DOI: 10.1016/j.yjmcc.2014.06.016]

7 **Gyöngyösi M**, Hemetsberger R, Posa A, Charwat S, Pavo N, Petnehazy O, Petrasi Z, Pavo IJ, Hemetsberger H, Benedek I, Benedek T, Benedek I Jr, Kovacs I, Kaun C, Maurer G. Hypoxia-inducible factor 1-alpha release after intracoronary versus intramyocardial stem cell therapy in myocardial infarction. *J Cardiovasc Transl Res* 2010; **3**: 114-121 [PMID: 20560024 DOI: 10.1007/s12265-009-9154-1]

8 **Gyöngyösi M**, Hemetsberger R, Wolbank S, Pichler V, Kaun C, Posa A, Petrasi Z, Petnehazy Ö, Hofer-Warbinek R, de Martin R, Gruber F, Benedek I, Benedek T, Kovacs I, Benedek I Jr, Plass CA, Charwat S, Maurer G. Delayed recovery of myocardial blood flow after intracoronary stem cell administration. *Stem Cell Rev* 2011; **7**: 616-623 [PMID: 21153508 DOI: 10.1007/s12015-010-9213-7]

9 **Feng Y**, Huang W, Wani M, Yu X, Ashraf M. Ischemic preconditioning potentiates the protective effect of stem cells through secretion of exosomes by targeting Mecp2 *via* miR-22. *PLoS One* 2014; **9**: e88685 [PMID: 24558412 DOI: 10.1371/journal.pone.0088685]

10 **Valadi H**, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007; **9**: 654-659 [PMID: 17486113 DOI: 10.1038/ncb1596]

11 **Davidson SM**, Yellon DM. Exosomes and cardioprotection - A critical analysis. *Mol Aspects Med* 2018; **60**: 104-114 [PMID: 29122678 DOI: 10.1016/j.mam.2017.11.004]

12 **Dragovic RA**, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJ, Hole P, Carr B, Redman CW, Harris AL, Dobson PJ, Harrison P, Sargent IL. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine* 2011; **7**: 780-788 [PMID: 21601655 DOI: 10.1016/j.nano.2011.04.003]

13 **Vicencio JM**, Yellon DM, Sivaraman V, Das D, Boi-Doku C, Arjun S, Zheng Y, Riquelme JA, Kearney J, Sharma V, Multhoff G, Hall AR, Davidson SM. Plasma exosomes protect the myocardium from ischemia-reperfusion injury. *J Am Coll Cardiol* 2015; **65**: 1525-1536 [PMID: 25881934 DOI: 10.1016/j.jacc.2015.02.026]

14 **Barile L**, Moccetti T, Marbán E, Vassalli G. Roles of exosomes in cardioprotection. *Eur Heart J* 2017; **38**: 1372-1379 [PMID: 27443883 DOI: 10.1093/eurheartj/ehw304]

15 **Ottaviani L**, De Windt LJ, da Costa Martins PA. Exosomes: scytales in the damaged heart. *Ann Transl Med* 2016; **4**: 222 [PMID: 27384882 DOI: 10.21037/atm.2016.05.17]

16 **Sun HJ**, Zhu XX, Cai WW, Qiu LY. Functional roles of exosomes in cardiovascular disorders: a systematic review. *Eur Rev Med Pharmacol Sci* 2017; **21**: 5197-5206 [PMID: 29228434 DOI: 10.26355/eurrev_201711_13840]

17 **Bang C**, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, Just A, Remke J, Zimmer K, Zeug A, Ponimaskin E, Schmiedl A, Yin X, Mayr M, Halder R, Fischer A, Engelhardt S, Wei Y, Schober A, Fiedler J, Thum T. Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. *J Clin Invest* 2014; **124**: 2136-2146 [PMID: 24743145 DOI: 10.1172/JCI70577]

18 **De Miguel MP**, Fuentes-Julián S, Blázquez-Martínez A, Pascual CY, Aller MA, Arias J, Arnalich-Montiel F. Immunosuppressive properties of mesenchymal stem cells: advances and applications. *Curr Mol Med* 2012; **12**: 574-591 [PMID: 22515979 DOI: 10.2174/156652412800619950]

19 **Kim HS**, Choi DY, Yun SJ, Choi SM, Kang JW, Jung JW, Hwang D, Kim KP, Kim DW. Proteomic analysis of microvesicles derived from human mesenchymal stem cells. *J Proteome Res* 2012; **11**: 839-849 [PMID: 22148876 DOI: 10.1021/pr200682z]

20 **Arslan F**, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguor EN, Timmers L, van Rijen HV, Doevendans PA, Pasterkamp G, Lim SK, de Kleijn DP. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res* 2013; **10**: 301-312 [PMID: 23399448 DOI: 10.1016/j.scr.2013.01.002]

21 **Yu B**, Kim HW, Gong M, Wang J, Millard RW, Wang Y, Ashraf M, Xu M. Exosomes secreted from GATA-4 overexpressing mesenchymal stem cells serve as a reservoir of anti-apoptotic microRNAs for cardioprotection. *Int J Cardiol* 2015; **182**: 349-360 [PMID: 25590961 DOI: 10.1016/j.ijcard.2014.12.043]

22 **Barile L**, Lionetti V, Cervio E, Matteucci M, Gherghiceanu M, Popescu LM, Torre T, Siclari F, Moccetti T, Vassalli G. Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction. *Cardiovasc Res* 2014; **103**: 530-541 [PMID: 25016614 DOI: 10.1093/cvr/cvu167]

23 **Gray WD**, French KM, Ghosh-Choudhary S, Maxwell JT, Brown ME, Platt MO, Searles CD, Davis ME. Identification of therapeutic covariant microRNA clusters in hypoxia-treated cardiac progenitor cell exosomes using systems biology. *Circ Res* 2015; **116**: 255-263 [PMID: 25344555 DOI: 10.1161/CIRCRESAHA.116.304360]

24 **Khan M**, Nickoloff E, Abramova T, Johnson J, Verma SK, Krishnamurthy P, Mackie AR, Vaughan E, Garikipati VN, Benedict C, Ramirez V, Lambers E, Ito A, Gao E, Misener S, Luongo T, Elrod J, Qin G, Houser SR, Koch WJ, Kishore R. Embryonic stem cell-derived exosomes promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction. *Circ Res* 2015; **117**: 52-64 [PMID: 25904597 DOI: 10.1161/CIRCRESAHA.117.305990]

25 **Sahoo S**, Klychko E, Thorne T, Misener S, Schultz KM, Millay M, Ito A, Liu T, Kamide C, Agrawal H, Perlman H, Qin G, Kishore R, Losordo DW. Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. *Circ Res* 2011; **109**: 724-728

[PMID: 21835908 DOI: 10.1161/CIRCRESAHA.111.253286]
26 **Kanazawa H**, Tseliou E, Malliaras K, Yee K, Dawkins JF, De Couto G, Smith RR, Kreke M, Seinfeld J, Middleton RC, Gallet R, Cheng K, Luthringer D, Valle I, Chowdhury S, Fukuda K, Makkar RR, Marbán L, Marbán E. Cellular postconditioning: allogeneic cardiosphere-derived cells reduce infarct size and attenuate microvascular obstruction when administered after reperfusion in pigs with acute myocardial infarction. *Circ Heart Fail* 2015; **8**: 322-332 [PMID: 25587096 DOI: 10.1161/CIRCHEARTFAILURE.114.001484]
27 **Ibrahim AG**, Cheng K, Marbán E. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. *Stem Cell Reports* 2014; **2**: 606-619 [PMID: 24936449 DOI: 10.1016/j.stemcr.2014.04.006]
28 **Simpson RJ**, Jensen SS, Lim JW. Proteomic profiling of exosomes: current perspectives. *Proteomics* 2008; **8**: 4083-4099 [PMID: 18780348 DOI: 10.1002/pmic.200800109]
29 **Mathivanan S**, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics* 2010; **73**: 1907-1920 [PMID: 20601276 DOI: 10.1016/j.jprot.2010.06.006]
30 **Boulanger CM**, Loyer X, Rautou PE, Amabile N. Extracellular vesicles in coronary artery disease. *Nat Rev Cardiol* 2017; **14**: 259-272 [PMID: 28150804 DOI: 10.1038/nrcardio.2017.7]
31 **Kuwabara Y**, Ono K, Horie T, Nishi H, Nagao K, Kinoshita M, Watanabe S, Baba O, Kojima Y, Shizuta S, Imai M, Tamura T, Kita T, Kimura T. Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. *Circ Cardiovasc Genet* 2011; **4**: 446-454 [PMID: 21642241 DOI: 10.1161/CIRCGENETICS.110.958975]
32 **De Rosa S**, Fichtlscherer S, Lehmann R, Assmus B, Dimmeler S, Zeiher AM. Transcoronary concentration gradients of circulating microRNAs. *Circulation* 2011; **124**: 1936-1944 [PMID: 21969012 DOI: 10.1161/CIRCULATIONAHA.111.037572]
33 **Bei Y**, Das S, Rodosthenous RS, Holvoet P, Vanhaverbeke M, Monteiro MC, Monteiro VVS, Radosinska J, Bartekova M, Jansen F, Li Q, Rajasingh J, Xiao J. Extracellular Vesicles in Cardiovascular Theranostics. *Theranostics* 2017; **7**: 4168-4182 [PMID: 29158817 DOI: 10.7150/thno.21274]
34 **Matsumoto S**, Sakata Y, Suna S, Nakatani D, Usami M, Hara M, Kitamura T, Hamasaki T, Nanto S, Kawahara Y, Komuro I. Circulating p53-responsive microRNAs are predictive indicators of heart failure after acute myocardial infarction. *Circ Res* 2013; **113**: 322-326 [PMID: 23743335 DOI: 10.1161/CIRCRESAHA.113.301209]
35 **Baglio SR**, Pegtel DM, Baldini N. Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. *Front Physiol* 2012; **3**: 359 [PMID: 22973239 DOI: 10.3389/fphys.2012.00359]
36 **Chen TS**, Yeo RWY, Arslan F, Yin Y, Tan SS, Lai RC, Choo A, Padmanabhan J, Lee CN, de Kleijn DPV, Tan KH, Lim SK. Efficiency of exosome production correlates inversely with the development al maturity of MSC donor. *J Stem Cell Res Ther* 2013; **3**: 145 [DOI: 10.4172/2157-7633.1000145]
37 **Dougherty JA**, Mergaye M, Kumar N, Chen CA, Angelos MG, Khan M. Potential Role of Exosomes in Mending a Broken Heart: Nanoshuttles Propelling Future Clinical Therapeutics Forward. *Stem Cells Int* 2017; **2017**: 5785436 [PMID: 29163642 DOI: 10.1155/2017/5785436]
38 **Huang P**, Tian X, Li Q, Yang Y. New strategies for improving stem cell therapy in ischemic heart disease. *Heart Fail Rev* 2016; **21**: 737-752 [PMID: 27459850 DOI: 10.1007/s10741-016-9576-1]
39 **Zhang Z**, Yang J, Yan W, Li Y, Shen Z, Asahara T. Pretreatment of Cardiac Stem Cells With Exosomes Derived From Mesenchymal Stem Cells Enhances Myocardial Repair. *J Am Heart Assoc* 2016; **5**: pii: e002856 [PMID: 26811168 DOI: 10.1161/JAHA.115.002856]
40 **Shao L**, Zhang Y, Lan B, Wang J, Zhang Z, Zhang L, Xiao P, Meng Q, Geng YJ, Yu XY, Li Y. MiRNA-Sequence Indicates That Mesenchymal Stem Cells and Exosomes Have Similar Mechanism to Enhance Cardiac Repair. *Biomed Res Int* 2017; **2017**: 4150705 [PMID: 28203568 DOI: 10.1155/2017/4150705]
41 **Blin G**, Nury D, Stefanovic S, Neri T, Guillevic O, Brinon B, Bellamy V, Rücker-Martin C, Barbry P, Bel A, Bruneval P, Cowan C, Pouly J, Mitalipov S, Gouadon E, Binder P, Hagège A, Desnos M, Renaud JF, Menasché P, Pucéat M. A purified population of multipotent cardiovascular progenitors derived from primate pluripotent stem cells engrafts in postmyocardial infarcted nonhuman primates. *J Clin Invest* 2010; **120**: 1125-1139 [PMID: 20335662 DOI: 10.1172/JCI40120]
42 **Chen Y**, Zhao Y, Chen W, Xie L, Zhao ZA, Yang J, Chen Y, Lei W, Shen Z. MicroRNA-133 overexpression promotes the therapeutic efficacy of mesenchymal stem cells on acute myocardial infarction. *Stem Cell Res Ther* 2017; **8**: 268 [PMID: 29178928 DOI: 10.1186/s13287-017-0722-z]
43 **Maring JA**, Beez CM, Falk V, Seifert M, Stamm C. Myocardial Regeneration *via* Progenitor Cell-Derived Exosomes. *Stem Cells Int* 2017; **2017**: 7849851 [PMID: 29333167 DOI: 10.1155/2017/7849851]
44 **Chong JJ**, Yang X, Don CW, Minami E, Liu YW, Weyers JJ, Mahoney WM, Van Biber B, Cook SM, Palpant NJ, Gantz JA, Fugate JA, Muskheli V, Gough GM, Vogel KW, Astley CA, Hotchkiss CE, Baldessari A, Pabon L, Reinecke H, Gill EA, Nelson V, Kiem HP, Laflamme MA, Murry CE. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. *Nature* 2014; **510**: 273-277 [PMID: 24776797 DOI: 10.1038/nature13233]
45 **Loyer X**, Vion AC, Tedgui A, Boulanger CM. Microvesicles as cell-cell messengers in cardiovascular diseases. *Circ Res* 2014; **114**: 345-353 [PMID: 24436430 DOI: 10.1161/CIRCRESAHA.113.300858]
46 **Soleti R**, Martinez MC. Sonic Hedgehog on microparticles and neovascularization. *Vitam Horm* 2012; **88**: 395-438 [PMID: 22391314 DOI: 10.1016/B978-0-12-394622-5.00018-3]
47 **Batrakova EV**, Kim MS. Using exosomes, naturally-equipped nanocarriers, for drug delivery. *J Control Release* 2015; **219**: 396-405 [PMID: 26241750 DOI: 10.1016/j.jconrel.2015.07.030]
48 **Rautou PE**, Leroyer AS, Ramkhelawon B, Devue C, Duflaut D, Vion AC, Nalbone G, Castier Y, Leseche G, Lehoux S, Tedgui A, Boulanger CM. Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. *Circ Res* 2011; **108**: 335-343 [PMID: 21164106 DOI: 10.1161/CIRCRESAHA.110.237420]
49 **Jansen F**, Yang X, Baumann K, Przybilla D, Schmitz T, Flender A, Paul K, Alhusseiny A, Nickenig G, Werner N. Endothelial microparticles reduce ICAM-1 expression in a microRNA-222-dependent mechanism. *J Cell Mol Med* 2015; **19**: 2202-2214 [PMID: 26081516 DOI: 10.1111/jcmm.12607]
50 **Benedek I**. Atherosclerosis-Triggered Hypertension or Hypertension-Triggered Atherosclerosis? A Challenging Hypothesis. *Journal of Cardiovascular Emergencies* 2017; **3**: 5-8 [DOI: 10.1515/jce-2017-0001]
51 **Vengrenyuk Y**, Carlier S, Xanthos S, Cardoso L, Ganatos P, Virmani R, Einav S, Gilchrist L, Weinbaum S. A hypothesis for vulnerable plaque rupture due to stress-induced debonding around cellular microcalcifications in thin fibrous caps. *Proc Natl Acad Sci USA* 2006; **103**: 14678-14683 [PMID: 17003118 DOI: 10.1073/pnas.0606310103]
52 **Criqui MH**, Denenberg JO, Ix JH, McClelland RL, Wassel CL, Rifkin DE, Carr JJ, Budoff MJ, Allison MA. Calcium density of coronary artery plaque and risk of incident cardiovascular events. *JAMA* 2014; **311**: 271-278 [PMID: 24247483 DOI: 10.1001/jama.2013.282535]
53 **Martin SS**, Blaha MJ, Blankstein R, Agatston A, Rivera JJ, Virani SS, Ouyang P, Jones SR, Blumenthal RS, Budoff MJ, Nasir K. Dyslipidemia, coronary artery calcium, and incident atherosclerotic cardiovascular disease: implications for statin therapy from the multi-ethnic study of atherosclerosis. *Circulation* 2014; **129**: 77-86 [PMID: 24141324 DOI: 10.1161/CIRCULATIONAHA.113.003625]
54 **Pfeifer P**, Werner N, Jansen F. Role and Function of MicroRNAs in Extracellular Vesicles in Cardiovascular Biology. *Biomed Res Int* 2015; **2015**: 161393 [PMID: 26558258 DOI: 10.1155/2015/161393]
55 **Liu ML**, Scalia R, Mehta JL, Williams KJ. Cholesterol-induced membrane microvesicles as novel carriers of damage-associated molecular patterns: mechanisms of formation, action, and detoxification. *Arterioscler Thromb Vasc Biol* 2012; **32**: 2113-2121 [PMID: 22814745 DOI: 10.1161/ATVBAHA.112.255471]
56 **Hoyer FF**, Giesen MK, Nunes França C, Lütjohann D, Nickenig G, Werner N. Monocytic microparticles promote atherogenesis by modulating inflammatory cells in mice. *J Cell Mol Med* 2012; **16**: 2777-2788 [PMID: 22697268 DOI: 10.1111/j.1582-4934.2012.01595.x]

57 **Hergenreider E**, Heydt S, Tréguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, Braun T, Urbich C, Boon RA, Dimmeler S. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nat Cell Biol* 2012; **14**: 249-256 [PMID: 22327366 DOI: 10.1038/ncb2441]

58 **Benedek T**, Gyöngyösi M, Benedek I. Multislice computed tomographic coronary angiography for quantitative assessment of culprit lesions in acute coronary syndromes. *Can J Cardiol* 2013; **29**: 364-371 [PMID: 23333164 DOI: 10.1016/j.cjca.2012.11.004]

59 **Benedek T,** Bucur O, Pascanu I, Benedek I. Analysis of coronary plaque morphology by 64-multislice computed tomography coronary angiography and calcium scoring in patients with type 2 diabetes mellitus. *Acta Endocrinologica* 2011; **7**: 59-68 [DOI: 10.4183/aeb.2011.59]

60 **Benedek T**, Jako B, Benedek I. Plaque quantification by coronary CT and intravascular ultrasound identifies a low CT density core as a marker of plaque instability in acute coronary syndromes. *Int Heart J* 2014; **55**: 22-28 [PMID: 24463925 DOI: 10.1536/ihj.13-213]

61 **Falk E**, Nakano M, Bentzon JF, Finn AV, Virmani R. Update on acute coronary syndromes: the pathologists' view. *Eur Heart J* 2013; **34**: 719-728 [PMID: 23242196 DOI: 10.1093/eurheartj/ehs411]

62 **Srikanthan S**, Li W, Silverstein RL, McIntyre TM. Exosome poly-ubiquitin inhibits platelet activation, downregulates CD36 and inhibits pro-atherothombotic cellular functions. *J Thromb Haemost* 2014; **12**: 1906-1917 [PMID: 25163645 DOI: 10.1111/jth.12712]

63 **Gidlöf O**, van der Brug M, Ohman J, Gilje P, Olde B, Wahlestedt C, Erlinge D. Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression. *Blood* 2013; **121**: 3908-3917, S1-26 [PMID: 23493781 DOI: 10.1182/blood-2012-10-461798]

64 **Benedek I**, Bucur O, Benedek T. Intracoronary infusion of mononuclear bone marrow-derived stem cells is associated with a lower plaque burden after four years. *J Atheroscler Thromb* 2014; **21**: 217-229 [PMID: 24126180 DOI: 10.5551/jat.19745]

65 **Zhao Y**, Sun X, Cao W, Ma J, Sun L, Qian H, Zhu W, Xu W. Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells Relieve Acute Myocardial Ischemic Injury. *Stem Cells Int* 2015; **2015**: 761643 [PMID: 26106430 DOI: 10.1155/2015/761643]

66 **Gupta S**, Knowlton AA. HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. *Am J Physiol Heart Circ Physiol* 2007; **292**: H3052-H3056 [PMID: 17307989 DOI: 10.1152/ajpheart.01355.2006]

67 **Chistiakov DA**, Orekhov AN, Bobryshev YV. Cardiac Extracellular Vesicles in Normal and Infarcted Heart. *Int J Mol Sci* 2016; **17**: pii: E63 [PMID: 26742038 DOI: 10.3390/ijms17010063]

68 **Kishore R**, Khan M. More Than Tiny Sacks: Stem Cell Exosomes as Cell-Free Modality for Cardiac Repair. *Circ Res* 2016; **118**: 330-343 [PMID: 26838317 DOI: 10.1161/CIRCRESAHA.115.307654]

69 **Foglio E**, Puddighinu G, Fasanaro P, D'Arcangelo D, Perrone GA, Mocini D, Campanella C, Coppola L, Logozzi M, Azzarito T, Marzoli F, Fais S, Pieroni L, Marzano V, Germani A, Capogrossi MC, Russo MA, Limana F. Exosomal clusterin, identified in the pericardial fluid, improves myocardial performance following MI through epicardial activation, enhanced arteriogenesis and reduced apoptosis. *Int J Cardiol* 2015; **197**: 333-347 [PMID: 26159041 DOI: 10.1016/j.ijcard.2015.06.008]

70 **Bei Y**, Xu T, Lv D, Yu P, Xu J, Che L, Das A, Tigges J, Toxavidis V, Ghiran I, Shah R, Li Y, Zhang Y, Das S, Xiao J. Exercise-induced circulating extracellular vesicles protect against cardiac ischemia-reperfusion injury. *Basic Res Cardiol* 2017; **112**: 38 [PMID: 28534118 DOI: 10.1007/s00395-017-0628-z]

71 **Chaturvedi P**, Kalani A, Medina I, Familtseva A, Tyagi SC. Cardiosome mediated regulation of MMP9 in diabetic heart: role of mir29b and mir455 in exercise. *J Cell Mol Med* 2015; **19**: 2153-2161 [PMID: 25824442 DOI: 10.1111/jcmm.12589]

72 **Madonna R**, Van Laake LW, Davidson SM, Engel FB, Hausenloy DJ, Lecour S, Leor J, Perrino C, Schulz R, Ytrehus K, Landmesser U, Mummery CL, Janssens S, Willerson J, Eschenhagen T, Ferdinandy P, Sluijter JP. Position Paper of the European Society of Cardiology Working Group Cellular Biology of the Heart: cell-based therapies for myocardial repair and regeneration in ischemic heart disease and heart failure. *Eur Heart J* 2016; **37**: 1789-1798 [PMID: 27055812 DOI: 10.1093/eurheartj/ehw113]

73 **Yellon DM**, Davidson SM. Exosomes: nanoparticles involved in cardioprotection? *Circ Res* 2014; **114**: 325-332 [PMID: 24436428 DOI: 10.1161/CIRCRESAHA.113.300636]

74 **Walsh DG**, Kaplan LR, Burney RE, Topol EJ, O'Neill WW. Use of tissue plasminogen activator in the emergency department for acute myocardial infarction. *Ann Emerg Med* 1987; **16**: 243-247 [PMID: 2949679 DOI: 10.1161/CIRCRESAHA.118.312657]

75 **Ong SG**, Lee WH, Huang M, Dey D, Kodo K, Sanchez-Freire V, Gold JD, Wu JC. Cross talk of combined gene and cell therapy in ischemic heart disease: role of exosomal microRNA transfer. *Circulation* 2014; **130**: S60-S69 [PMID: 25200057 DOI: 10.1161/CIRCULATIONAHA.113.007917]

76 **Mackie AR**, Klyachko E, Thorne T, Schultz KM, Millay M, Ito A, Kamide CE, Liu T, Gupta R, Sahoo S, Misener S, Kishore R, Losordo DW. Sonic hedgehog-modified human CD34+ cells preserve cardiac function after acute myocardial infarction. *Circ Res* 2012; **111**: 312-321 [PMID: 22581926 DOI: 10.1161/CIRCRESAHA.112.266015]

77 **Lai RC**, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010; **4**: 214-222 [PMID: 20138817 DOI: 10.1016/j.scr.2009.12.003]

78 **Chen L**, Wang Y, Pan Y, Zhang L, Shen C, Qin G, Ashraf M, Weintraub N, Ma G, Tang Y. Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury. *Biochem Biophys Res Commun* 2013; **431**: 566-571 [PMID: 23318173 DOI: 10.1016/j.bbrc.2013.01.015]

79 **Luo Q**, Guo D, Liu G, Chen G, Hang M, Jin M. Exosomes from MiR-126-Overexpressing Adscs Are Therapeutic in Relieving Acute Myocardial Ischaemic Injury. *Cell Physiol Biochem* 2017; **44**: 2105-2116 [PMID: 29241208 DOI: 10.1159/000485949]

80 **Deng L**, Blanco FJ, Stevens H, Lu R, Caudrillier A, McBride M, McClure JD, Grant J, Thomas M, Frid M, Stenmark K, White K, Seto AG, Morrell NW, Bradshaw AC, MacLean MR, Baker AH. MicroRNA-143 Activation Regulates Smooth Muscle and Endothelial Cell Crosstalk in Pulmonary Arterial Hypertension. *Circ Res* 2015; **117**: 870-883 [PMID: 26311719 DOI: 10.1161/CIRCRESAHA.115.306806]

81 **Kapustin AN**, Shanahan CM. Emerging roles for vascular smooth muscle cell exosomes in calcification and coagulation. *J Physiol* 2016; **594**: 2905-2914 [PMID: 26864864 DOI: 10.1113/JP271340]

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EDITORIAL

Gingival-derived mesenchymal stem cells: An endless resource for regenerative dentistry

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Abstract

The gingiva, the masticatory portion of the oral mucosa, is excised and discarded frequently during routine dental treatments and following tooth extraction, dental crown lengthening, gingivectomy and periodontal surgeries. Subsequent to excision, healing eventually happens in a short time period after gingival surgery. Clinically, the gingival tissue can be collected very easily and, in the laboratory, it is also very easy to isolate gingival-derived mesenchymal stem cells (GMSCs) from this discarded gingival tissue. GMSCs, a stem cell population within the lamina propria of the gingival tissue, can be isolated from attached and free gingiva, inflamed gingival tissues, and from hyperplastic gingiva. Comparatively, they constitute more attractive alternatives to other dental-derived mesenchymal stem cells due to the availability and accessibility of gingival tissues. They have unique immunomodulatory functions and well-documented self-renewal and multipotent differentiation properties. They display positive signals for Stro-1, Oct-4 and SSEA-4 pluripotency-associated markers, with some co-expressing Oct4/Stro-1 or Oct-4/SSEA-4. They should be considered as the best stem cell source for cell-based therapies and regenerative dentistry. The clinical use of GMSCs for regenerative dentistry represents an attractive therapeutic modality. However, numerous biological and technical challenges need to be addressed prior to considering transplantation approaches of GMSCs as clinically realistic therapies for humans.

Key words: Gingival-derived mesenchymal stem cells; Regenerative dentistry; Lamina propria of the gingiva; Gingiva; Stem cell therapy

Core tip: Current therapeutic interventions in dentistry depend on biomaterials such as metals, polymers, ceramics, and composites. These restorative synthetic dental materials cannot restore the physiological architecture and function of the tissue. Thus, dentistry should move from restorative to regenerative dentistry, with the ability to regrow damaged or missing teeth with their own dental stem cells. Regenerating an entire tooth or individual parts of the tooth require a suitable number

of specific stem cell populations for use and implantation. Considering their neural crest origin and ease of availability, gingival-derived mesenchymal stem cells should be considered as an attractive source for stem cells that can be used in regenerative dentistry.

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INTRODUCTION

New directions for biomaterials research in dentistry is focused mainly on two different aspects. The first field of investigation involves the use of existing technology, such as conventional dental materials with the use Polyethylene fiber (ribbond) and Panavia F cement to give additional strength to the reattached tooth fragment of vital maxillary anterior teeth and obtaining fracture resistance equal to an intact tooth[1]. This involves machineries that use the ER:Yag laser, which is a more conservative alternative to conventional acid-etching for aesthetic brackets[2]. The second field of investigation involves research about new features, such as biomimetic materials that use fiber reinforced composite and polyethylene fibers with nanohybrid composite as alternates to crown coverage for endodontically-treated molars[3]. In addition, computer-aided design/computer-aided manufacturing of customized devices is used to improve the standardization process in the evaluation of cell behavior on different biomaterials for *in vitro* research on biomedical scaffolds[4]. Furthermore, nanomaterials with the use of nanofillers are used to improve the mechanical properties of fiber-reinforced composites that are polymerized with light-curing and additional postcuring[5]. Lastly, stem cells are also used as a source for regenerative therapies in dental research and practice[6].

Gingiva is the band from the masticatory mucosal tissue that encircles the necks of erupted teeth, and is considered as one of the constituents of the periodontium. Anatomically, the gingiva is divided into free, attached and interdental areas. The attached gingiva is tightly bound to the cementum of the root and to the underlying periosteum of the maxillary and mandibular alveolar bone. Histologically, the gingiva is composed of stratified squamous epithelial tissue supported by a matrix of dense fibrous connective tissue stroma termed lamina propria. Developmentally, the connective tissue of the gingiva is derived from both the neural crest and the mesenchyme. In cranial regions, neural crest cells are thought to differentiate into a wide variety of ectomesenchymal and non-ectomesenchymal derivatives[7]. The formed ectomesenchyme plays a pivotal role in the formation of the soft and hard tissues of the head and neck region, such as the majority of facial connective tissues and the facial skeleton, while the non-ectomesenchymal derivatives consist of pigment cells, glia and neurons[8].

Consequently, stem cells have been recognized in different oral tissues, such as stem cells isolated from exfoliated deciduous teeth, bone marrow-derived stem cells isolated from orofacial bones, stem cells from the apical papilla and dental follicle, dental pulp stem cells isolated from dental pulp tissue, periodontal ligament stem cells, progenitor/stem cells from oral epithelium, periosteum-derived stem cells, salivary gland-derived stem cell and gingival-derived mesenchymal stem cells (GMSCs) from gingival lamina propria[9]. The gingiva represents the most accessible, abundant, conservative and minimally-invasive source for stem/progenitor cell isolation from the oral cavity[10]. GMSCs can be isolated from normal or inflamed gingiva, from the attached and free gingiva, and from hyperplastic gingiva. Periodontal lesions, albeit inflamed, retain healing potential as inferred by the presence of MSC-like cells with similar immunophenotypic characteristics to those found in healthy periodontal tissue[11]. These stem cells can be isolated through enzymatic digestion or explant culture, have the liability to differentiate into different mesenchymal lineages, and are also associated with immunomodulatory properties. Therapeutically, these cells were used for skin wound repair, tendon periodontal, and bone defect regeneration. They were also used to treat peri-implantitis, oral mucositis, experimental colitis, collagen-induced arthritis, and contact hypersensitivity. In addition, they also are known to have antitumor effects[12].

STUDY ANALYSIS

Our study, along with others, have launched the earliest appraisal on GMSCs and carried out several biological research investigations. In the head and neck region, GMSCs can be used as the cellular components for 3D bio-printing of scaffold-free nerve constructs to meet the increasing clinical demand for peripheral nerve repair and regeneration[13]. They could also be used as a strategy to treat accidental or surgery trauma, especially for cranial bones[14], as well as to treat gingival defects with a safe and effective innovative treatment method[15]. They also may help ameliorate the regeneration of partially-dissected submandibular salivary gland, especially when combined with fibrin glue[16], and have shown significant potential for periodontal tissue regeneration[17]. Although neither full nor partial biological tooth regeneration has been achievable, emerging opportunities in stem cell therapy may shift the paradigm in the future. The quality of stem cells is extremely important, as cells obtained from younger patients are of exceptionally higher value *vs.* older ones. In addition, their differentiation capacity, accessibility and possible immunomodulatory properties should be considered. Most of the regenerative studies have been done *in vitro* or in animal models,

and data from human clinical research remains scarce. The successful application of stem cells in the clinical practice of dentistry remains an elusive and challenging objective.

PERSPECTIVE

Mesenchymal stem cells from adult gingival mucosa retain unique features, including multipotent differentiation capacity, neural crest origin, potent immunemodulatory properties, and fetal-like phenotypes. These features, with their ease of availability, noninvasive access to gingival tissue, and fast tissue regeneration after gingival excision, make gingiva a fascinating source for cell isolation and regenerative dentistry. These cells are attractive to treat diseases like dental caries and periodontitis, or to improve the regeneration of craniofacial bone[6]. In contrast to bone marrow-derived mesenchymal stem cells, these cells are more closely related to dental tissues. To achieve this goal, experimental animal studies should be accomplished to ensure the ability of these cells to form such dental structures. This step should then be followed up with clinical trials that involve an adequate population number.

REFERENCES

1 **Hiremath H**, Kulkarni S, Saikalyan S, Chordhiya R. Use of ribbond and panavia F cement in reattaching fractured tooth fragments of vital maxillary anterior teeth. *Contemp Clin Dent* 2012; **3**: 478-480 [PMID: 23633814 DOI: 10.4103/0976-237X.107446]

2 **Sfondrini MF**, Calderoni G, Vitale MC, Gandini P, Scribante A. Is laser conditioning a valid alternative to conventional etching for aesthetic brackets? *Eur J Paediatr Dent* 2018; **19**: 61-66 [PMID: 29569456 DOI: 10.23804/ejpd.2018.19.01.11]

3 **Hiremath H**, Kulkarni S, Hiremath V, Kotipalli M. Evaluation of different fibers and biodentine as alternates to crown coverage for endodontically treated molars: An in vitro study. *J Conserv Dent* 2017; **20**: 72-75 [PMID: 28855750 DOI: 10.4103/0972-0707.212248]

4 **Marrelli M**, Pujia A, Palmieri F, Gatto R, Falisi G, Gargari M, Caruso S, Apicella D, Rastelli C, Nardi GM, Paduano F, Tatullo M. Innovative approach for the in vitro research on biomedical scaffolds designed and customized with CAD-CAM technology. *Int J Immunopathol Pharmacol* 2016; **29**: 778-783 [PMID: 27106276 DOI: 10.1177/0394632016646121]

5 **Scribante A**, Massironi S, Pieraccini G, Vallittu P, Lassila L, Sfondrini MF, Gandini P. Effects of nanofillers on mechanical properties of fiber-reinforced composites polymerized with light-curing and additional postcuring. *J Appl Biomater Funct Mater* 2015; **13**: e296-e299 [PMID: 26108426 DOI: 10.5301/jabfm.5000226]

6 **Aly LA**. Stem cells: Sources, and regenerative therapies in dental research and practice. *World J Stem Cells* 2015; **7**: 1047-1053 [PMID: 26328020 DOI: 10.4252/wjsc.v7.i7.1047]

7 **Cho MI**, Garant PR. Development and general structure of the periodontium. *Periodontol 2000* 2000; **24**: 9-27 [PMID: 11276876 DOI: 10.1034/j.1600-0757.2000.2240102.x]

8 **Cordero DR**, Brugmann S, Chu Y, Bajpai R, Jame M, Helms JA. Cranial neural crest cells on the move: their roles in craniofacial development. *Am J Med Genet A* 2011; **155A**: 270-279 [PMID: 21271641 DOI: 10.1002/ajmg.a.33702]

9 **Ercal P**, Pekozer GG, Kose GT. Dental Stem Cells in Bone Tissue Engineering: Current Overview and Challenges. *Adv Exp Med Biol* 2018; 1-15 [PMID: 29498025 DOI: 10.1007/5584_2018_171]

10 **Fawzy El-Sayed KM**, Dörfer C, Fändrich F, Gieseler F, Moustafa MH, Ungefroren H. Adult mesenchymal stem cells explored in the dental field. *Adv Biochem Eng Biotechnol* 2013; **130**: 89-103 [PMID: 22936399 DOI: 10.1007/10_2012_151]

11 **Apatzidou DA**, Nile C, Bakopoulou A, Konstantinidis A, Lappin DF. Stem cell-like populations and immunoregulatory molecules in periodontal granulation tissue. *J Periodontal Res* 2018 [PMID: 29687448 DOI: 10.1111/jre.12551]

12 **Fawzy El-Sayed KM**, Dörfer CE. Gingival Mesenchymal Stem/Progenitor Cells: A Unique Tissue Engineering Gem. *Stem Cells Int* 2016; **2016**: 7154327 [PMID: 27313628 DOI: 10.1155/2016/7154327]

13 **Zhang Q**, Nguyen PD, Shi S, Burrell JC, Cullen DK, Le AD. 3D bioprinted scaffold-free nerve constructs with human gingiva-derived mesenchymal stem cells promote rat facial nerve regeneration. *Sci Rep* 2018; **8**: 6634 [PMID: 29700345 DOI: 10.1038/s41598-018-24888-w]

14 **Diomede F**, Gugliandolo A, Cardelli P, Merciaro I, Ettorre V, Traini T, Bedini R, Scionti D, Bramanti A, Nanci A, Caputi S, Fontana A, Mazzon E, Trubiani O. Three-dimensional printed PLA scaffold and human gingival stem cell-derived extracellular vesicles: a new tool for bone defect repair. *Stem Cell Res Ther* 2018; **9**: 104 [PMID: 29653587 DOI: 10.1186/s13287-018-0850-0]

15 **Li J**, Xu SQ, Zhang K, Zhang WJ, Liu HL, Xu Z, Li H, Lou JN, Ge LH, Xu BH. Treatment of gingival defects with gingival mesenchymal stem cells derived from human fetal gingival tissue in a rat model. *Stem Cell Res Ther* 2018; **9**: 27 [PMID: 29402326 DOI: 10.1186/s13287-017-0751-7]

16 **Abd El-Latif N**, Abdulrahman M, Helal M, Grawish ME. Regenerative capacity of allogenic gingival margin- derived stem cells with fibrin glue on albino rats' partially dissected submandibular salivary glands. *Arch Oral Biol* 2017; **82**: 302-309 [PMID: 28688332 DOI: 10.1016/j.archoralbio.2017.06.030]

17 **Fawzy El-Sayed KM**, Paris S, Becker ST, Neuschl M, De Buhr W, Sälzer S, Wulff A, Elrefai M, Darhous MS, El-Masry M, Wiltfang J, Dörfer CE. Periodontal regeneration employing gingival margin-derived stem/progenitor cells: an animal study. *J Clin Periodontol* 2012; **39**: 861-870 [PMID: 22694281 DOI: 10.1111/j.1600-051X.2012.01904.x]

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REVIEW

Regulatory role of sphingosine kinase and sphingosine-1-phosphate receptor signaling in progenitor/stem cells

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Abstract

Balanced sphingolipid signaling is important for the maintenance of homeostasis. Sphingolipids were demonstrated to function as structural components, second messengers, and regulators of cell growth and survival in normal and disease-affected tissues. Particularly, sphingosine kinase 1 (SphK1) and its product sphingosine-1-phosphate (S1P) operate as mediators and facilitators of proliferation-linked signaling. Unlimited proliferation (self-renewal) within the regulated environment is a hallmark of progenitor/stem cells that was recently associated with the S1P signaling network in vasculature, nervous, muscular, and immune systems. S1P was shown to regulate progenitor-related characteristics in normal and cancer stem cells (CSCs) *via* G-protein coupled receptors S1P*n* (*n* = 1 to 5). The SphK/S1P axis is crucially involved in the regulation of embryonic development of vasculature and the nervous system, hematopoietic stem cell migration, regeneration of skeletal muscle, and development of multiple sclerosis. The ratio of the S1P receptor expression, localization, and specific S1P receptor-activated downstream effectors influenced the rate of self-renewal and should be further explored as regeneration-related targets. Considering malignant transformation, it is essential to control the level of self-renewal capacity. Proliferation of the progenitor cell should be synchronized with differentiation to provide healthy lifelong function of blood, immune systems, and replacement of damaged or

dead cells. The differentiation-related role of SphK/S1P remains poorly assessed. A few pioneering investigations explored pharmacological tools that target sphingolipid signaling and can potentially confine and direct self-renewal towards normal differentiation. Further investigation is required to test the role of the SphK/S1P axis in regulation of self-renewal and differentiation.

Key words: Sphingosine-1-phosphate; Sphingolipids; Embryonic stem cells; Mesenchymal stem cells; Bone marrow hematopoietic stem cells; Sphingosine kinase; Progenitor

Core tip: The aim of this study is to review the role of sphingosine kinase, sphingosine-1-phosphate (S1P), and its receptors in the regulation of stem/progenitor cell function. Our analysis indicates that S1P receptor expression, localization, and specific downstream effectors influence the rate of self-renewal and differentiation of myogenic, hematopoietic, endothelial, neural, and cancer progenitor cells.

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INTRODUCTION

During organism growth, development, and adaptation to changed environmental conditions, orchestrated functioning of multiple processes supports physiological homeostasis. The synchronization should occur at the level of a single cell, such as controlled cell division and apoptosis, and at the level of organs and systems, such as directed angiogenesis, immune responses, and regeneration. Many of those biological processes have been shown to rely on the sphingolipid signalling cascade. An important member of the sphingolipid family, sphingosine-1-phosphate (S1P), is a bioactive signalling molecule. S1P effects are essential for structural and functional regulation of cell growth and survival. The main source of S1P is catabolic degradation of membrane glycosphingolipids and sphingomyelin, which results in production of sphingosine which, in turn, is phosphorylated by sphingosine kinases (SphK1 and SphK2)[1,2]. Supported by experimental evidence observed in *Sphk* knockout mice *in vivo*, SphK isozymes can partially balance metabolism for each other, although there are some SphK1- and SphK2-specific non-overlapping functions[3,4].

Suggestively, S1P is generated during membrane restructuring in all types of cells. Locally produced S1P can act either intracellularly or extracellularly. S1P can be released to the extracellular environment by erythrocytes[5], platelets[6], and endothelial cells[7]. Circulating S1P is an important signalling mediator and ligand for specific G protein-coupled receptors S1P*n* (*n* = 1 to 5)[8,9]. S1P1 is highly expressed in various tissues, but specifically in endothelial cells and vasculature. S1P2 and S1P3 are also broadly expressed, although their levels of expression demonstrated some function specificity. Activated S1P receptors trigger distinctive downstream effectors and respective responses[10,11]. Intracellularly produced S1P can be utilized in two different metabolic pathways[8,12]. Firstly, S1P can be recycled through ceramide synthesis by S1P-specific phosphatases[13]. Secondly, S1P can be irreversibly degraded by S1P lyase into phospho-ethanolamine and hexadecenal linked to a variety of intracellular signalling cascades[14].

Various growth stimulating agents, hormones, and cytokines, the canonical regulators of cell proliferation and survival, can activate SphK and stimulate S1P production. Hormones, cytokines, and growth factors including EGF[15], PDGF[16], IGF[17], VEGF[18], NGF[19], TGF[20], TNF[21], and the steroid hormone estrogen[15,22,23] were shown to trigger SphK1/S1P signaling in different cells. Supporting the global role of the sphingolipid network in regulation of proliferation, the list of SphK/S1P-inducing agents keeps growing. Recent experimental findings demonstrate that S1P and its network play a complex role in the regulation of stem/progenitor cell signalling in normal and malignant tissues.

Stem or progenitor cells are defined as undifferentiated cells with specific clonogenic potential, unlimited self-renewal capacity that is accompanied by directed differentiation into multiple (often limited to a specific number) cell lineages[24,25]. According to their programmed differentiation potential, stem cells are encoded for particular tissue regeneration and cell replacement. For instance, pluripotent embryonic stem cells (ESCs) can differentiate into cell-types of all the primary germ layers. Bone marrow (BM)-located adult stem cells are considered multipotent[26] or pluripotent[27,28]. Other groups of adult stem cells are oligopotent, bipotent, or unipotent and are represented by basal cells in the epidermis, spermatogonial stem cells, and satellite cells in skeletal muscles[28]. The cells with limited potency are often referred to as progenitor cells and include, for instance, endothelial progenitor cells (EPCs)[29] and pancreatic progenitor cells[30]. Progenitor cells are marked not only by limited number of divisions, but also higher levels of directed lineage differentiation.

The core properties of ESC pluripotency are maintained by a group of lineage-specific transcription factors (TFs) such as Nanog, Oct4, and Sox2-NOS and their regulatory networks[31]. Recently, it was demonstrated that high intracellular levels of S1P is associated with

increased mouse ESC proliferation and higher expression of the cell surface pluripotency markers SSEA1 and Oct4[31]. The authors found that ESCs express high level of sphingosine phosphate lyase (SPL), an enzyme that catalyzes the S1P degradation, thus, keeping the intracellular level of S1P under tight control[32]. During the last decade, besides the detected effects in ESCs, the regulatory role of sphingolipids has been assessed in several types of precursor multipotent cells including neural, muscle, hematopoietic, endothelial, and mesenchymal progenitor/stem cells. S1P was suggested to functions as a trophic factor for many stem cell types. However, the role of sphingolipids in the regulation of cell renewal and differentiation remains only partially addressed. Here, we review and discuss recent advancement and development about the functional role of sphingosine kinase, S1P and S1P receptors in stem/progenitor cells.

SPHK/S1P/S1P RECEPTORS SIGNALLING IN HEMATOPOIETIC AND ENDOTHELIAL STEM/PROGENITOR CELLS

Hematopoietic stem cells (HSCs) represent the rare population of precursor cells that defines the blood composition and homeostasis. HSCs are characterized by their unique capacity for self-renewal and multi-lineage differentiation. HSCs and downstream partially lineage-committed progenitor cell functions are tightly linked to their migratory properties, especially during fetal development[33,34]. Although the majority of postnatal and adult HSCs/progenitors stay in BM specialized niches or cavities[35], some of the HSCs/progenitors belong to a highly migratory subpopulation that recirculates between BM and blood[36,37]. Suggestively, the HSC/progenitor trafficking mechanism supports full occupancy of stem cell niches in all BM cavities[37,38]. HSC trafficking is directed by an S1P blood/lymph/tissue gradient that is mostly maintained by SphK/S1P receptor and S1P-degrading enzyme S1P lyase network[38]. Notably, another well-studied sphingolipid, ceramide-1-phosphate (C1P), can also enhance the migration of endothelial and lymphoid progenitor cells[39], suggesting that other sphingolipid family members should be tested for potential involvement in the regulation of hematopoietic stem/progenitor cell functions.

S1P concentration in peripheral blood and lymph regulates HSC and lymphocyte egress from lymphoid organs[34,37]. HSCs and progenitors express S1P1 receptor(s) that can sense blood plasma S1P and direct stem cell migration[40]. Although water-insoluble S1P binds apolipoprotein M and circulates in peripheral blood mostly as a part of high-density lipoprotein (HDL) particles, the level of S1P is always higher in plasma and lymph compared to the S1P level in interstitial fluids of all organs, including thymus and lymph nodes[41,42]. The concentration gradient serves as a chemoattractant to direct the migration of S1P1-expressing cells from BM to peripheral blood[37,40]. Similarly, lymphocyte egress from lymph nodes is directed by the S1P gradient between lymphoid tissue and lymph[38]. The level of S1P1 receptor expression is a critical factor that regulates sensitivity to circulating S1P. Expressed in blood or lymph-circulating cell, S1P1 receptors are rapidly internalized and downregulated through G-protein coupled receptor kinase-2–mediated phosphorylation[43]. Inside of tissue, S1P1 is up-regulated under the condition of low-S1P concentration in interstitial fluids. The high level of S1P1 on tissue-located cells supports the traversing of cells from tissues towards high S1P in blood plasma or lymphatics. Animal HSCs also express S1P1 receptors that mediate stem cell trafficking from BM into peripheral blood[44]. The mouse model has allowed for the use of the specific S1P1 inhibitor W146, which confirmed a key role for the receptor in BM retention of hematopoietic cells[44].

Three different S1P receptors, including S1P1, S1P2, and S1P3, influence the development and function of the embryonic vasculature[45,46]. The S1P/S1P1 axis plays a leading role during embryonic vascularization and angiogenesis. Supporting a functional link between the endothelial and red blood cell network, S1P synthesis and release from erythrocytes is required for embryonic vascular development[47]. S1P2 and S1P3 effects are considered important, although as accessory or partially redundant in some studies[11,12].

Activated S1P1 receptor has been found to stimulate the proliferation of endothelial vascular (outgrowth) progenitors[48] and colony-forming cells[49]. Morphogenesis of the kidney vasculature is also mediated by S1P1 signalling. A hypothetical endothelial and hematopoietic precursor has been shown to express S1P1 receptor in the kidney. The receptor activation maintains an appropriate development of glomerular capillaries, arterial mural cell coating, and lymphatic vessel development[50]. Besides S1P1, S1P3 receptor positively directs capillary-like formation and EPC migration. Notably, S1P2 partially blocks the migratory capacity of mesenchymal progenitor cells, mesangioblasts, whereas SphK and/or S1P1/S1P3 are involved in the positive regulation of angiogenesis *in vivo*[20,45]. S1P2 signalling is clearly tissue-specific and can promote proliferation in different cells similarly to S1P1 and/or S1P3. Accordingly, small hepatocyte-progenitor and stem (oval) cell proliferation is positively associated with S1P2 and S1P4 expression during liver injury[51]. Furthermore, S1P2 promotes the growth of primary $CD34^+$ mononuclear cells obtained from chronic myeloid leukemia (CML) patients[52].

The S1P-producing enzyme SphK1 is partially responsible for the maintenance of the EPC-specific phenotype. SphK1 controls the rate and direction of EPC differentiation, although the enzyme expression level did not affect the hematopoietic compartment[53]. The authors detected high levels of SphK1 activity in EPCs, which gradually decreased in more differentiated endothelial

cells. Notably, *SphK1* knockout mice demonstrated higher levels of circulating EPCs[53]. The data suggests a potential negative role of the enzyme in the regulation of vascular regeneration when the presence of EPCs in circulation is highly desirable, although the question requires further investigation. Overexpression of SphK1 facilitates the retention of EPCs at the progenitor stage with probable delay in the following differentiation program, but that was not tested. Suggestively, SphK1 function in EPCs can be replaced by SphK2. The role of SphK2 in the regulation of EPC function remains unclear.

The S1P3 receptor axis influences some specific S1P responses in EPCs. Patient-derived EPCs were tested for the activation of G protein-coupled protein receptor C-X-C chemokine receptor 4 (CXCR4) signalling. CXCR4 axis is an important regulator of pluripotent cell development and function, as it is involved in the interaction between HSCs cells or hematologic and solid tumor cells and their protective microenvironment[54]. It was detected that the S1P/S1P3 axis positively induced the CXCR4-dependent signalling pathway[55]. Furthermore, the specific S1P1 receptor antagonist FTY720 increased CXCR4-dependent chemotactic responsiveness and migration of human $CD34^+$ lineage-committed progenitor cells[56]. Similarly, besides EPCs, S1P1 and S1P3 activation was required in the regulation of CSCs migration[57,58].

S1P1 effects were tested in megakaryocytes, the thrombocyte lineage-specific progenitors. S1P1 is involved in the initiation of the elongation of trans-endothelial pro-thrombocyte extensions into BM sinusoids and activates the subsequent shedding of thrombocytes[59]. During activation, platelets can release considerable amounts of S1P and further increase S1P concentration in blood plasma besides the release of the lipid from erythrocytes and endothelial cells[60]. The sudden local increase in S1P concentration is potentially associated with activation of immune cell migration. The role of platelet-derived S1P in the regulation of HSCs and/or progenitor trafficking requires further testing.

SPHK/S1P/S1P RECEPTORS SIGNALLING IN MUSCLE STEM/PROGENITOR CELLS

Skeletal muscles are formed by myoblasts, muscle cell progenitors. The multistage process of myogenesis is preceded by myoblast division, which is followed by terminal differentiation, cell merging into multinucleated myofibers, and maturation[61-63]. The initiation of the myoblast differentiation process is marked by progenitor cell cycle cessation accompanied by vigorous synthesis of myogenin and expression of muscle-specific proteins, including sarcomeric components and creatine kinase[61]. Represented by quiescent mononucleated satellite cells, adult muscle cell progenitors employ a similar differentiation program as developing myoblasts[62]. The satellite cells/myoblasts, although mitotically quiescent, can be induced to proliferate by physical trauma, weight bearing, or inflammation-induced trauma[64]. After multiple rounds of satellite cell divisions, the cell cycle stops and the newly produced cells fuse onto the existing damaged muscle fibers. This process was observed *in vitro* using C2C12 cells, a skeletal myoblast cell line derived from murine satellite cells[65-67]. Despite significant progress, the molecular mechanisms of myogenesis remain only partially explored. For instance, molecular regulation of muscle progenitor signalling and associated repair mechanisms remain largely unclear, although growth factors and cytokines have been confirmed to regulate skeletal muscle biology[63]. Sphingolipids can transduce signalling from growth factor and cytokine receptors as messengers and amplifiers in a large variety of cells[11]. Consequently, the role of sphingolipids has been examined in the regulation of myogenesis.

Traumatic tissue injury and subsequent inflammatory activation of leukocytes and macrophages are marked by the release of cytokines and growth factors that can stimulate skeletal muscle regeneration and remodelling[63,68]. One of the keystone recent discoveries demonstrated a direct link between sphingolipid signalling and trauma/inflammation-provoked responses in muscle progenitor cells[67]. Bradykinin and its related peptides are pro-inflammatory molecules and muscle-specific growth factors that mediate exudative and inflammatory phases of muscle healing[69,70]. Bradykinin is also the leading member of the kinin/kallikrein system, which directs inflammation-linked responses in mesenchymal cells including fibroblasts, myofibroblasts, and smooth muscle cells[70]. Bradykinin has been shown to induce myogenic differentiation in C2C12 myoblasts through SphK1, the specific S1P-transporter spinster homolog 2 (Spns2), and S1P2 receptor. Specific pharmacological inhibition and/or protein expression silencing was used to confirm the involvement of the S1P axis in bradykinin-induced myogenic differentiation[67].

Serving as a muscle trophic factor, S1P has been suggested to play a leading role in the stimulation of myogenesis and regeneration provoked by various agents *via* transactivation of the $S1P_2$ receptor pathway[65,66,71,72]. Extracellular S1P reduced serum-induced cell proliferation, promoted cell cycle exit, and up-regulated the expression of various differentiation markers in myoblasts. The S1P-dependent myogenic differentiation is mediated by S1P2, activation of ERK1/ERK2 and p38 MAPK, both identified as downstream effectors of S1P2[71]. Furthermore, insulin growth factor 1 (IGF-1) increased SphK activity and induced transactivation of the S1P2 receptor in C2C12 murine myoblasts. The activation was linked to the IGF-1 myogenic differentiation effect. Pharmacological inhibition of SphK, specific silencing of SphK1 or SphK2, and S1P2 receptor downregulation resulted in reduction of the IGF-1 pro-differentiating effect in myoblasts[66]. Interestingly, IGF-1 also activated S1P1/S1P3 receptors. Contrary to S1P2, S1P1/S1P3 negatively regulated the IGF-1-induced

mitogenic differentiation. Specific silencing of S1P1/S1P3 receptors notably stimulated myoblast proliferation[66]. The data correspond to the growth-stimulating signalling of S1P3 in tumors where the ability of sphingolipids to mediate IGF-1 effects is well recognized[11]. The myogenesis-stimulating role of S1P2 is partially unexpected, as S1P2 anti-proliferative effects were previously observed in various, although mostly not stem-like cells[11,12]. S1P2 was shown to inhibit Rac signalling and related cell migration contrary to its demonstrated effects in myoblasts[73]. However, the divergence might be associated with the high specialization of stem cells and adjustments to pluripotency of the S1P2 signalling network. Notably, S1P2 induced the myogenic differentiation program independently of acute S1P treatment[67]. Conclusively, the pleiotropic role of SphK/S1P receptor axis in skeletal myoblasts reflects the association of S1P receptor expression pattern with contrasting biological responses.

Proliferative and chemotactic effects of vascular endothelial growth factor (VEGF) signalling are also transduced by SphK/S1P network in muscle progenitor cells[74]. Previously, VEGF signalling pathway was shown to interact with the SphK/S1P axis in several types of normal[75] and malignant cells[12,18]. SphK activation and S1P1 expression can be induced by VEGF. S1P1 and VEGF receptor-2 (VEGFR-2) proteins were found to interact and form a signalling complex[11,18]. The interaction was described as mutual, as S1P enhanced the levels of VEGF expression and transactivated VEGFR-2[11,18,75]. The role of S1P in the mediation of VEGF myogenesis-related effects was confirmed in another study that tested the role of bone-marrow-derived mesenchymal stromal cells (MSCs) as regulators of myogenesis. MSCs synthesize and release a large amount of S1P, which assists in skeletal muscle healing[74]. Conditioned media with MSC-secreted S1P stimulated C2C12 myoblast and satellite cell proliferation. A similar effect was reached by exposure to VEGF, as the myoblast growth response to MSC-secreted VEGF also induced S1P release from C2C12 cells[74,75].

Notably, the involvement of $S1P_2$ and $S1P_3$ receptors in the regulation of myogenesis was detected more than a decade ago[76]. Meacci *et al*[76] observed that myogenic differentiation was accompanied by a significant variation in S1P receptor expression levels. The authors also suggested that the S1P signalling axis is a key component required for sphingolipid effects in proliferating muscle cells[76]. However, the authors observed that the S1P2 receptor is down-regulated during myogenesis, while SphK was enhanced in differentiating C2C12 myoblasts[77]. Suggestively, S1P2 and S1P3 can be activated during different stages of myogenesis and stimulate alternative biological responses in regular and progenitor muscle cells. For instance, S1P3 levels are high in quiescent murine myogenic cells, but decrease during cell cycle initiation[78]. Constitutive expression of S1P3 resulted in the suppression of satellite cell cycle progression. S1P3-null satellite cells exhibited enhanced proliferation. Acute cardiotoxin-induced muscle regeneration was promoted in S1P3-null myoblasts *in vivo,* marked by bigger muscle fibers compared to control mice. The data are supported by experimental observations in the mdx mouse model of Duchenne muscular dystrophy. S1P3 knockdown produced a less severe muscle dystrophic phenotype, indicating that the S1P3-linked pathway represses cell cycle progression to direct myoblast functions[77].

Myoblasts and fully differentiated muscle cells are marked by a heterogeneous expression pattern of S1P receptor subtypes. S1P1 has been mostly found in cardiomyocytes, while S1P2/S1P3 receptors are expressed by cardiac progenitor cells[78]. S1P receptor activates Rho signalling, which in turn, switches in the proliferation of cardiac myoblasts. Notably, both S1P2 and S1P3 induce RhoA activation through $G\alpha12/13$ during myocardial regeneration, indicating some redundancy of signalling pathways[78]. However, there is some specificity for different S1P receptor subtypes. For instance, during construction of the primary heart tube in zebrafish, S1P2 controls proper endoderm formation and cardiac myoblast migration[79]. Notably, in another study, S1P2 negatively regulated satellite cell migration, while S1P1/S1P4 facilitated the S1P migratory effect in myoblast cells[72].

Considering muscle-specific cytoskeletal remodeling, the role of specific S1P receptors is unclear. Myoblasts and satellite cell differentiation capacity depends on cytoskeletal remodelling and can be controlled by gap junction proteins, particularly connexin (Cx) 43[80]. It has been shown that S1P induces p38 MAPK activation, phosphorylation of Cx43 and association of Cx43 with cortactin and F-actin, followed by murine C2C12 myoblasts differentiation[81]. S1P-induced C2C12 myoblast differentiation and transient receptor potential canonical 1 (TRPC1) channel activity have been linked to Cx43 expression/function *via* calpain/PKCα axis[76], although the involvement of S1P receptor has not been demonstrated.

Transforming growth factor beta 1 (TGFβ1), inflammation-associated pleiotropic cytokine, was shown to control skeletal muscle regeneration *via* S1P3 receptor signalling[65]. TGFβ1 increased levels of SphK1 in C2C12 myoblasts in a Smad-dependent manner and stimulated the expression of S1P3 receptors that resulted in induction of fibrosis. The study demonstrated the involvement of Rho/Rho kinase signalling downstream of S1P as profibrotic TGFβ1 effect[65]. Notably, S1P receptors are linked to various downstream effectors in myoblasts. For instance, S1P2 myogenic signalling is mediated by activated phosphatidylinositol 3-kinase (PI3K)[72] and signal transducer and activator of transcription 3 (STAT3)-dependent pathways[82]. The biological meaning of the divergence of S1P receptor signalling requires further clarification (Figure 1). For instance, Rho signalling that can mediate S1P effects in non-pluripotent cells[73] is also activated in cardiac myoblasts[76], suggesting that S1P isoforms might be linked to similar downstream

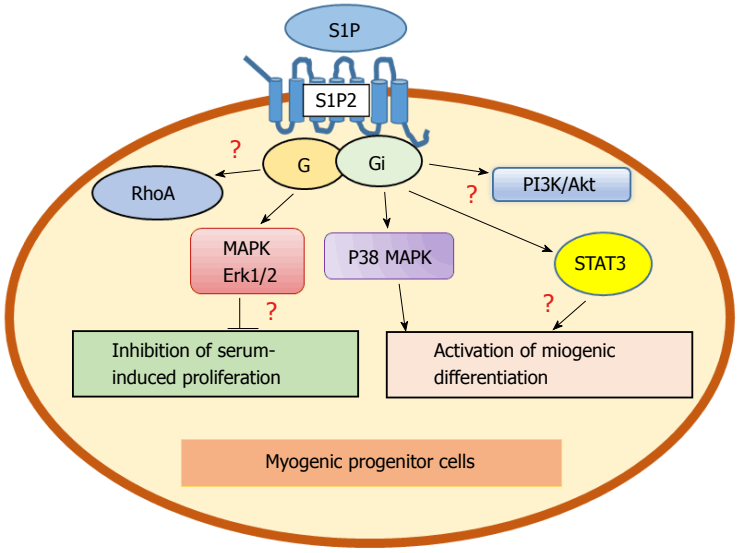

Figure 1 Diversion of myogenic stimulation at the S1P2 receptor level (hypothetical scheme). Activation of S1P2 receptor signaling results in dual effects in muscle progenitor cells: Erk1/2 mediates inhibition of serum-induced proliferation, while p38MAPK[78,79], phosphatidylinositol 3-kinase (PI3K)[72] and activator of transcription 3 (STAT3)-dependent pathways[82] are required for S1P-trigerred activation of myogenic differentiation. The role of RhoA signaling is unclear. Question marks indicate unclear signaling regulation. S1P: Sphingosine-1-phosphate.

effectors independently of pluripotency. Future studies should clarify how S1P receptors induce different effects in normal, malignant, and progenitor cells using similar downstream effectors.

It has been shown that myogenesis is regulated not only by SphK/S1P receptors, but also by other S1P metabolizing enzymes, including S1P lyase. The lyase irreversibly catabolizes S1P at carbon bond C(2-3), producing hexadecenal and ethanolamine-phosphate. The lyase enhances apoptosis induced by chemotherapy, radiation and ischemia in different cells[82]. Undetectable in resting skeletal muscle, the S1P lyase level is upregulated after muscle injury[82]. The mdx mouse model for muscular dystrophy was marked by skeletal muscle S1P lyase upregulation and S1P deficiency *in vivo*. Accordingly, pharmacological S1P lyase inhibition stimulated an increase in muscle S1P levels and myoblast recruitment, thus advancing mdx skeletal muscle regeneration[82]. S1P lyase knockdown cells demonstrated increased levels of intra- and extra-cellular S1P, but decreased myotube formation and delayed induction of three myogenic microRNAs (miRNAs) including miR-1, miR-206, and miR-486[83]. Myotube formation was recovered in cells treated with an S1P1 agonist, S1P2 antagonist, and combination treatments. Transfection with miR-1 or miR-206 allowed the S1P lyase knockdown cells to reverse the inhibition of differentiation[83]. Considering that stem cell resistance to apoptosis is a keystone of regeneration, pharmacological inhibition of S1P lyase during specific stages of myogenesis seems like an attractive therapeutic approach to enhance muscular remodelling after injury[78,82].

Another sphingolipid C1P has been implicated in the regulation of skeletal muscle regeneration. C1P induced myoblast proliferation and myoblast cell cycle progression without activation of a putative G(i)-coupled C1P receptor[84]. C1P stimulated the phosphorylation of glycogen synthase kinase-3β and the production of retinoblastoma gene, and enhanced cyclin D1 protein levels. Furthermore, various downstream target proteins, including phosphatidylinositol 3-kinase/Akt, ERK1/2, and the mammalian target of rapamycin, mediated C1P signalling in myoblasts. Interestingly, C1P did not influence the induction of myoblast apoptosis or myogenic differentiation[84]. Previous knowledge of C1P signalling is limited to the demonstrated effects in fibroblasts and macrophages, thus, demanding further investigation of C1P role in progenitor cells.

According to the recently developed theory, brown adipose cells are derived from a mesenchymal progenitor that shares some similarity with muscle cell precursor cells and expresses Myf5-Cre proteins, while white adipocytes originate from a Myf5-negative precursor[85]. According to another theory, adipocytes arise from a vascular bed and originate from a subset of endothelial cells[86]. While the theory is clarified, S1P was revealed to promote differentiation of C3H10T1/2 multipotent mesenchymal stem cells into osteogenic rather than adipogenic progenitors[87]. Furthermore, adipose tissue itself was shown to contain stem cell progenitors. The adipose stromal-vascular cell fraction is an abundant source of both multipotent and pluripotent progenitor cells, defined as adipose-derived stem cells. S1P1 is involved in the induction of adipose-derived stem cell growth by HDL[88].

Obesity and metabolic disorders might be associated with dysfunctional adipose progenitor cells and diabetes. Notably, multipotent pancreatic progenitor cells (MPCs)

have been suggested as a promising target for the treatment of type-1 diabetes mellitus[30]. MPCs are stem cells with limited potency that proliferate and differentiate into three distinct lineages, including insulin-producing β cells, acinar cells, and ductal cells. The early MPCs were classified by the expression of the TFs Pdx1, Ptf1a, and Sox9, some of them known as mesenchymal progenitor markers[89]. High levels of Notch and Hippo/YES signalling are also required to maintain tree-like branched epithelium and block early MPC differentiation[90,91]. The SphK/S1P/S1P2 axis was found to support pancreatic progenitor differentiation[92]. S1P2, SphK1, and SphK2 expression levels were upregulated during pancreas second transition in the developing epithelium and co-localized with both trunk and tip progenitors[92]. S1P2 receptor activated YES-associated protein (YAP) and up-regulated connective tissue growth factor signalling important for the survival of endocrine and acinar pancreatic progenitors. S1P signalling decreased Notch regulation of lineage allocation necessary for endocrine and acinar specification[92]. *S1P2 receptor*-null embryos demonstrated high perinatal mortality marked by pathological hematopoietic and vascular system phenotypes[46]. Expression of a negative posttranscriptional regulator of Notch signalling protein Sel1l was also influenced by S1P2 signalling. S1pr2 inhibition resulted in the loss of the Sel1l protein, which in turn is required to maintain normal Notch signalling and proper acinar and endocrine differentiation[92]. Given such an important role of S1P2 in the regulation of MPC differentiation, the role of S1R receptors in the regulation of differentiation should be further explored in future studies.

Conclusively, the sphingolipid signalling network is a potential therapeutic target to influence myogenesis, adipogenesis, and associated metabolic pathologies including diabetes. Pharmacological control over sphingolipid signalling should be tested during induction of muscle regeneration, aging, inflammation and trauma-associated muscle fibrosis. The role of the SphK/S1P axis in the regulation of adipose cell precursor function and adipose-derived stem cell differentiation remains to be clarified in future studies.

SPHK/S1P/S1P RECEPTORS SIGNALLING IN NEURAL STEM/PROGENITOR CELLS

S1P signalling exhibited neuro-protective effects as a mediator of nerve growth factor (NGF) in hippocampal neurons and pheochromocytoma PC12 cells[93,94]. During the last couple of decades, several groups have addressed the role of sphingolipids in neural progenitor cells. Neural progenitor/stem cells (NPSCs) have limited potency, though they are still promising to for the treatment of Alzheimer's disease[95] and brain or spinal cord injuries[96]. Besides insufficient proliferation rates, NPSCs maintain slow differentiation and migration characteristics. However, similar to its effects on circulating immune cells in blood and lymph, extracellular S1P is a powerful chemoattractant for microglial cells in the brain. More effective than fibroblast growth factor (FGF), S1P is a powerful stimulator of neurogenesis[97] (Figure 2). S1P can mediate FGF signalling in different neural cells. It was found that FGF coordinates S1P release from astrocytes. The extracellular S1P, through autocrine or paracrine mechanisms, stimulates astrocyte differentiation mediated by S1P receptors[98]. Previously, it was demonstrated that cerebellar astrocytes express S1P1, S1P2, and S1P3 receptors[99]. Another study indicated that S1P3 is overexpressed in astrocytes under pro-inflammatory conditions[100]. However, astrocytes are not true progenitor cells, but rather precursor cells. Furthermore, S1P receptor demonstrates heterogeneous expression in neural and NPSCs[101,102]. For instance, up-regulation of S1P1 was noted in NPSCs that migrate out of the embryoid body/ESCs[103]. However, the level and role of S1P receptor subtypes in NPSCs remains controversial, as different studies have demonstrated noticeable variations in S1P receptor expression[104].

In the presence of activated astrocytes, S1P further enhances NPSCs differentiation, indicated by neurite outgrowth and arborization[97,105]. Notably, neural precursors derived from ESCs express all five S1P receptor mRNAs, although S1P2 and S1P3 mRNA levels are the highest[97,105]. The effect of S1P on NPSCs is mediated by increased laminin expression and extracellular matrix (ECM) interactions with progenitor integrins. However, the role of particular S1P receptor subtypes has not been verified. For instance, the role of S1P2 remains unclear. Kimura *et al*[106] demonstrated that NPSC migration to sites of injury was inhibited by S1P2 activation.

The role of SphK/S1P in neural stem cells has been explored by Meng *et al*[107]. The authors detected SphK1 expression in neuron and progenitor cells of nascent trigeminal and dorsal root ganglia of the mouse embryo. The enzyme was found to increase NPSC proliferation and survival during early sensory ganglia development[107]. Embryos with both *Sphk1* and *Sphk2* genes knocked out displayed clear developmental defects marked by fewer neurons and progenitor cells in trigeminal and dorsal root ganglia[107]. This finding supports the previously shown data of crucial involvement of SphK1/S1P axis in the regulation of cell growth and survival in the developing neural system[107-110]. According to the proposed mechanism, sphingolipids are involved in neural cell signalling downstream of p75 and/or neurotrophin receptor pathways[107,111-117].

To establish a role for S1P in the regulation of neural cell survival, Saini *et al*[118] tested the involvement of SphK1 in the neurotrophin-3 (NT-3) signalling pathway. It was found that SphK1 mediates NT-3–dependent activation of cAMP-response element binding protein (CREB) in cultured oligodendrocyte progenitors. NT-3 increased SphK1 activation and translocation from the cytoplasm to the plasma membrane of oligodendrocytes.

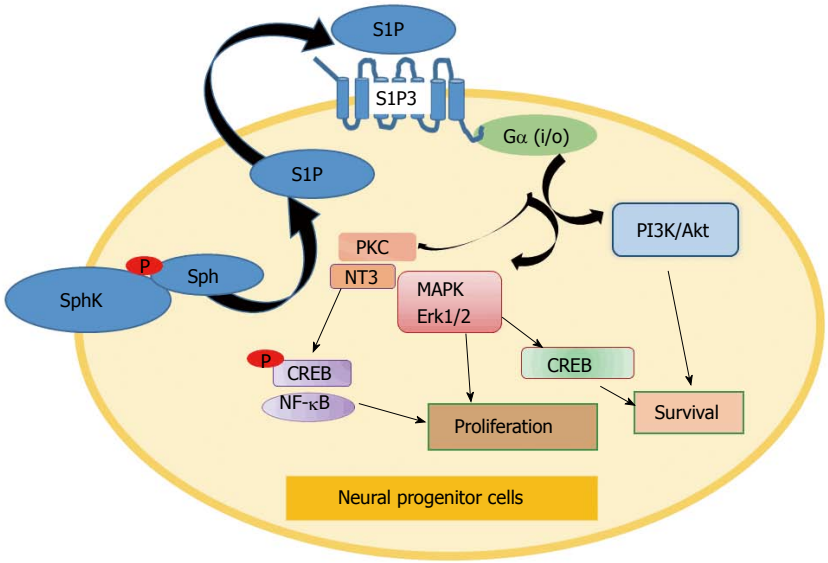

Figure 2 Sphingosine kinase/sphingosine-1-phosphate signaling axis in neural stem/progenitor cells (hypothetical scheme). Activation of S1P3 receptor signaling and activation of neural cell progenitor differentiation are mediated by various downstream effectors including PKC, PI3K/Akt, MAPK/Erk1/2, NT3, and CREB/NF-κB TFs. SphK: Sphingosine kinase; S1P: Sphingosine-1-phosphate; CREB: cAMP-response element binding protein; NT3: Neurotrophin 3; TF: Transcription factor.

The effects coincided with enhanced S1P accumulation at the membrane. Downregulation of SphK1 facilitated apoptosis in oligodendrocyte progenitors induced by growth factor deprivation. Inhibition of Erk1/2 and PKC also blocked NT3- and S1P-induced CREB phosphorylation, indicating a concerted interaction among NT-3, SphK, Erk1/2 and PKC pathways[115,119]. Crosstalk between NT-3 and SphK1 has been also demonstrated in animal models of multiple sclerosis[120]. Notably, PTEN and Notch signalling mediate the anti-fibrotic effects of dihydro-S1P in systemic sclerosis[121]. However, a complexity of functional crosstalk between NT-3 and SphK signalling requires further clarification during oligodendrocyte development.

Survival-related mechanisms of S1P effects were linked to multiple signalling pathways. For instance, S1P can activate membrane S1P receptor(s) to induce CREB phosphorylation in oligodendroglial progenitors[122-124]. Downstream S1P receptor effects were mediated by activation of Erk1/2 and PKC-dependent pathways in progenitor cells[102,122]. Another signalling mechanism was associated with activation of growth factor signalling[93]. For instance, platelet-derived growth factor (PDGF) receptor was shown to activate SphK1 in oligodendrocytes[123]. In turn, SphK1 mediated PDGF-dependent up-regulation of mRNAs encoding the Kv1.5 and Kv1.6 K^+ channels during oligodendrocyte proliferation[124]. Furthermore, SphK1 promoted survival of oligodendrocyte progenitors *via* upregulation of the anti-apoptotic protein Bcl-2 and downregulation of the pro-apoptotic protein Bim in a CREB-dependent manner. The mechanism is based on the established SphK1-dependent regulation of a balance between pro-apoptotic and anti-apoptotic Bcl-2 proteins shown in normal and cancer cells[11]. Considering genomic and epigenetic regulation, SphK1 can trigger the activation of various TFs, including AP-1 and NF-κB, which were shown to promote anti-apoptotic signalling[125-129]. Involvement of SphK/S1P/S1P3 receptor-dependent signalling in the regulation of survival and differentiation of neural stem/progenitor cells is summarized in Figure 2.

S1P receptor demonstrates heterogeneous expression in neural cells. Brain white matter cells contain the highest expression of S1P2[130]. S1P5 is expressed by mature oligodendrocytes where the receptor regulates survival and cell process retraction[131]. Higher expression level and activation of S1P1 facilitates survival of oligodendrocyte progenitors and induces oligodendroglial differentiation[132]. NPSCs derived from ESCs express all five S1P receptor mRNAs, although the actual protein expression level was not tested[105].

The level of S1P receptor subtype requires utter attention, as a large group of S1P receptor agonists/antagonists has been developed to target the receptor signalling in the central nervous system. For instance, FTY720, a S1P receptor agonist, can cross the blood-brain barrier and target NPSCs[133]. FTY720 advanced clinical trials were efficient for the treatment of multiple sclerosis. Restoration and protection of neural cells by FTY720 signalling has been shown for astrocytes and oligodendrocytes[132,134]. Moreover, FTY720 increased the viability and neurogenicity of irradiated NPSCs from the hippocampus[135], thus promising to serve as a healing agent for neurological diseases[133] (Figure 3).

SPHK/S1P/S1P RECEPTOR SIGNALLING IN BREAST CSCS

Involvement of the SphK/S1P signalling axis in CSC

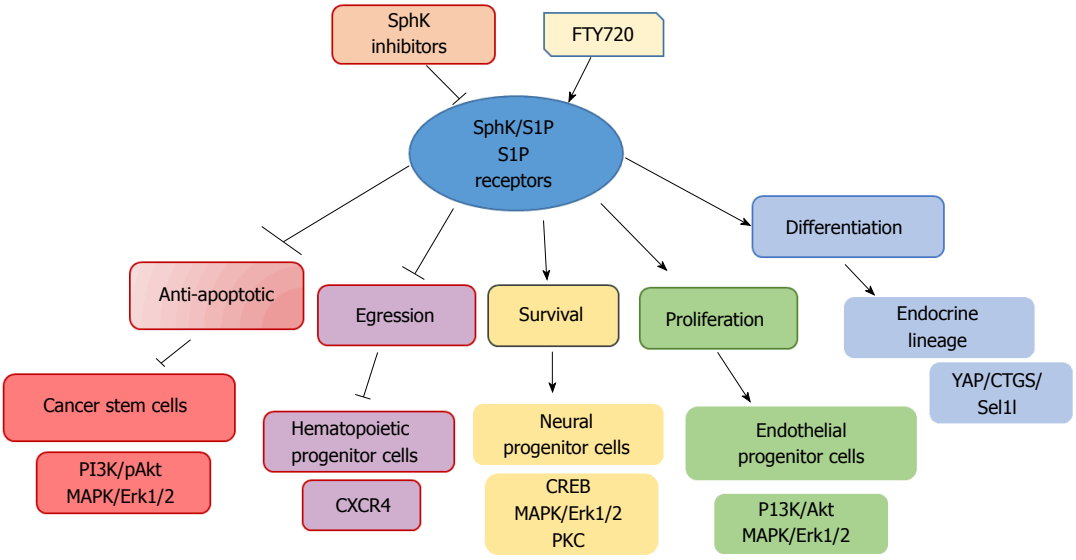

Figure 3 Differentiating effects of sphingosine kinase/sphingosine-1-phosphate inhibition and S1P receptor activation on downstream signaling pathways in various stem/progenitor cells. SphK: Sphingosine kinase; S1P: Sphingosine-1-phosphate; CREB: cAMP-response element binding protein; YAP: YES-associated protein.

functioning has been recently investigated in several malignancies, including glioblastoma[136], melanoma[137], hepatocellular carcinoma[138], and breast adenocarcinoma[139,140]. Considering the established role of sphingolipid signalling in mammary carcinomas, this study addressed the role of S1P receptors only in breast CSCs.

According to a cancer progenitor theory, mammary cancers originate from a small population of tumour-initiating cells. Marked by strong survival characteristics and a high level of heterogeneity, CSCs yield the majority of cancers through continuous self-renewal and very limited differentiation. CSCs have been reported to utilize similar molecular mechanisms as embryonic and normal adult stem cells. For instance, CSC self-renewal capacity has been associated with Notch, Hedgehog and Wnt signalling pathways[139]. Sphingolipid and particularly the S1P receptor signalling network has been recently explored in breast CSC models[140,141].

The stimulatory role of S1P and its effect on CSC proliferation was tested after the treatment of breast cancer cells with environmental carcinogens phthalate and benzyl butyl phthalate. These agents activate aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that is known to regulate quiescence, self-renewal, and differentiation of HSCs[142]. Activated AhR stimulated SphK1/S1P/S1PR3 signalling and promoted CSC-induced metastasis *in vivo*[141]. The study suggests that toxic agents and AhR triggered epigenetic activity (histone modification) in CSCs, which in turn, induced transcriptional activation of S1Pr3. Increased release of S1P was also observed because of SphK1 activation. S1P3 knockdown strongly decreased $CD44^{high}/CD24^{low}$ (supposedly stem) MCF-7 cell populations[141].

Another group used different CSC markers and demonstrated a key regulatory role of S1P3 in mammary CSCs[140]. S1P enhanced the mammosphere-forming capacity of aldehyde dehydrogenase (ALDH)-positive CSCs *via* S1P3 and associated induction of the Notch signalling pathway. SphK1-overexpressing CSCs demonstrated an increased ability to develop tumors in nude mice *in vivo*. The tumorigenicity of these CSCs was also blocked by S1P3 knockdown and the specific S1P3 antagonists TY52156 and CAY10444[140]. The study detected high expression levels of S1P3, but lower S1P2 in the ALDH-positive CSC population. S1P activated Notch-dependent proliferation, employing ligand-independent activation of Notch *via* p38MAPK[140]. Notably, breast cancer patient-derived CSCs contained SphK1+/ALDH1+ cells or S1PR3+/ALDH1+ cells[140], indicating a leading role for this receptor in the maintenance of self-renewal potential.

Conclusively, inhibition of S1P3 signaling seems like an attractive clinical target in the treatment of breast cancers. One of the S1P receptor inhibitors, FTY720, might be a beneficial clinical agent. FTY720 can provoke global cytoskeletal change that results in deformed and decreased filopodia formation, reduced expression of integrins, apoptosis, decreased cancer cell adhesion, and prevention of metastasis[143]. These diverse multifunctional effects of FTY720 suggest an ability to interact with more than one specific target in tested cells (Figure 3). Thus, the exact mechanisms of FTY720 signaling was not tested in breast CSCs. FTY720 reactivated expression of the silenced estrogen receptor α (ERα) and sensitized them to tamoxifen, the widely used chemotherapy agent in mammary cancer patients[144]. However, the potential interaction of FTY720 and tamoxifen signaling remains unclear in CSCs. Tamoxifen is the tissue-specific ER agonist/antagonist/modulator shown to inhibit proliferation of ER-positive breast cancer cells. However, prolonged

tamoxifen treatment up-regulates Wnt signaling and promotes survival of CSCs. Notably, ER signaling and tamoxifen resistance were mediated by SphK1/S1P3 receptor signaling in MCF-7 cells[11]. Moreover, estrogen was found to regulate breast CSC numbers through the FGF/Tbx3 signaling pathway, which is also responsible for the regulation of normal embryonic breast stem cell function[145]. An additive effect of tamoxifen and FTY720 in mammary CSCs remains to be explored in future studies.

CONCLUSION

The SphK/S1P receptor network has emerged as a key mediator of stem cell proliferation, survival and differentiation. The essential function of S1P receptor(s) for vascular and neural development has been proven in genetic knockout mice[146]. Considering the very high survival capacity of stem/progenitor cells, the activation of SphK/S1P signaling in normal progenitor and CSCs seems highly likely. S1P regulates cell proliferation and survival mainly through increased phosphorylation of p42/44-MAPK/Erk1/2 and PI3K/Akt, the two major chain reaction arms responsible for anti-apoptotic effects (Figures 2 and 3). In neurodegenerative disease, the S1P receptor agonist FTY720 may exert protective effects on oligodendrocyte survival, counteracting ceramide-induced apoptosis[103,147] (Figure 3). The role of SphK/S1P receptor signaling in the regulation of normal progenitor function looks very attractive. SphK/S1P/S1P receptor signaling should be explored as a promising strategy to promote tissue regeneration in acute myocardial infarction, muscular degeneration, and various neurological pathologies. While induction of SphK/S1P signaling might be useful to boost regeneration and survival of normal stem/progenitor cells, inhibition of the SphK/S1P-dependent survival pathway should be considered for cancer treatment/prevention[1,11,108,140]. Suggesting potential useful application of S1P receptor inhibitors in various CSCs, an increase in SphK/S1P3 signaling correlated with poor prognosis in breast cancer patients[1,11] and promoted mammary CSCs expansion[140,141].

REFERENCES

1 **Spiegel S**, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 2003; **4**: 397-407 [PMID: 12728273 DOI: 10.1038/nrm1103]

2 **Proia RL**, Hla T. Emerging biology of sphingosine-1-phosphate: its role in pathogenesis and therapy. *J Clin Invest* 2015; **125**: 1379-1387 [PMID: 25831442 DOI: 10.1172/JCI76369]

3 **Allende ML**, Sasaki T, Kawai H, Olivera A, Mi Y, van Echten-Deckert G, Hajdu R, Rosenbach M, Keohane CA, Mandala S, Spiegel S, Proia RL. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J Biol Chem* 2004; **279**: 52487-52492 [PMID: 15459201 DOI: 10.1074/jbc.M406512200]

4 **Song DD**, Zhou JH, Sheng R. Regulation and function of sphingosine kinase 2 in diseases. *Histol Histopathol* 2018; **33**: 433-445 [PMID: 29057430 DOI: 10.14670/HH-11-939]

5 **Yatomi Y**, Ozaki Y, Ohmori T, Igarashi Y. Sphingosine 1-phosphate: synthesis and release. *Prostaglandins Other Lipid Mediat* 2001; **64**: 107-122 [PMID: 11324700 DOI: 10.1016/S0090-6980(01)00103-4]

6 **Pappu R**, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, Camerer E, Zheng YW, Huang Y, Cyster JG, Coughlin SR. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* 2007; **316**: 295-298 [PMID: 17363629 DOI: 10.1126/science.1139221]

7 **Hisano Y**, Kobayashi N, Yamaguchi A, Nishi T. Mouse SPNS2 functions as a sphingosine-1-phosphate transporter in vascular endothelial cells. *PLoS One* 2012; **7**: e38941 [PMID: 22723910 DOI: 10.1371/journal.pone.0038941]

8 **Saba JD**, Hla T. Point-counterpoint of sphingosine 1-phosphate metabolism. *Circ Res* 2004; **94**: 724-734 [PMID: 15059942 DOI: 10.1161/01.RES.0000122383.60368.24]

9 **Blaho VA**, Hla T. An update on the biology of sphingosine 1-phosphate receptors. *J Lipid Res* 2014; **55**: 1596-1608 [PMID: 24459205 DOI: 10.1194/jlr.R046300]

10 **Chae SS**, Proia RL, Hla T. Constitutive expression of the S1P1 receptor in adult tissues. *Prostaglandins Other Lipid Mediat* 2004; **73**: 141-150 [PMID: 15165038 DOI: 10.1016/j.prostaglandins.2004.01.006]

11 **Sukocheva OA**. Expansion of Sphingosine Kinase and Sphingosine-1-Phosphate Receptor Function in Normal and Cancer Cells: From Membrane Restructuring to Mediation of Estrogen Signaling and Stem Cell Programming. *Int J Mol Sci* 2018; **19**: pii: E420 [PMID: 29385066 DOI: 10.3390/ijms19020420]

12 **Kihara A**. Sphingosine 1-phosphate is a key metabolite linking sphingolipids to glycerophospholipids. *Biochim Biophys Acta* 2014; **1841**: 766-772 [PMID: 23994042 DOI:10.1016/j.bbalip.2013.08.014]

13 **Schulze H**, Sandhoff K. Sphingolipids and lysosomal pathologies. *Biochim Biophys Acta* 2014; **1841**: 799-810 [PMID: 24184515 DOI: 10.1016/j.bbalip.2013.10.015]

14 **Zhou J**, Saba JD. Identification of the first mammalian sphingosine phosphate lyase gene and its functional expression in yeast. *Biochem Biophys Res Commun* 1998; **242**: 502-507 [PMID: 9464245 DOI: 10.1006/bbrc.1997.7993]

15 **Sukocheva O**, Wadham C, Holmes A, Albanese N, Verrier E, Feng F, Bernal A, Derian CK, Ullrich A, Vadas MA, Xia P. Estrogen transactivates EGFR via the sphingosine 1-phosphate receptor Edg-3: the role of sphingosine kinase-1. *J Cell Biol* 2006; **173**: 301-310 [PMID: 16636149 DOI: 10.1083/jcb.200506033]

16 **Waters C**, Sambi B, Kong KC, Thompson D, Pitson SM, Pyne S, Pyne NJ. Sphingosine 1-phosphate and platelet-derived growth factor (PDGF) act via PDGF beta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. *J Biol Chem* 2003; **278**: 6282-6290 [PMID: 12480944 DOI: 10.1074/jbc.M208560200]

17 **El-Shewy HM**, Johnson KR, Lee MH, Jaffa AA, Obeid LM, Luttrell LM. Insulin-like growth factors mediate heterotrimeric G protein-dependent ERK1/2 activation by transactivating sphingosine 1-phosphate receptors. *J Biol Chem* 2006; **281**: 31399-31407 [PMID: 16926156 DOI: 10.1074/jbc.M605339200]

18 **Shu X**, Wu W, Mosteller RD, Broek D. Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and mitogen-activated protein kinases. *Mol Cell Biol* 2002; **22**: 7758-7768 [PMID: 12391145 DOI: 10.1128/MCB.22.22.7758-7768.2002]

19 **Toman RE**, Payne SG, Watterson KR, Maceyka M, Lee NH, Milstien S, Bigbee JW, Spiegel S. Differential transactivation of sphingosine-1-phosphate receptors modulates NGF-induced neurite extension. *J Cell Biol* 2004; **166**: 381-392 [PMID: 15289497 DOI: 10.1083/jcb.200402016]

20 **Kono Y**, Nishiuma T, Nishimura Y, Kotani Y, Okada T, Nakamura S, Yokoyama M. Sphingosine kinase 1 regulates differentiation of human and mouse lung fibroblasts mediated by TGF-beta1. *Am J Respir Cell Mol Biol* 2007; **37**: 395-404 [PMID: 17641298 DOI: 10.1165/rcmb.2007-0065OC]

21 **Pettus BJ**, Bielawski J, Porcelli AM, Reames DL, Johnson KR, Morrow J, Chalfant CE, Obeid LM, Hannun YA. The sphingosine

kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha. *FASEB J* 2003; **17**: 1411-1421 [PMID: 12890694 DOI: 10.1096/fj.02-1038com]

22 **Sukocheva O**, Wang L, Verrier E, Vadas MA, Xia P. Restoring endocrine response in breast cancer cells by inhibition of the sphingosine kinase-1 signaling pathway. *Endocrinology* 2009; **150**: 4484-4492 [PMID: 19706837 DOI: 10.1210/en.2009-0391]

23 **Sukocheva OA**, Wang L, Albanese N, Pitson SM, Vadas MA, Xia P. Sphingosine kinase transmits estrogen signaling in human breast cancer cells. *Mol Endocrinol* 2003; **17**: 2002-2012 [PMID: 12881510 DOI: 10.1210/me.2003-0119]

24 **Weissman IL**. Stem cells are units of natural selection for tissue formation, for germline development, and in cancer development. *Proc Natl Acad Sci USA* 2015; **112**: 8922-8928 [PMID: 26195745 DOI: 10.1073/pnas.1505464112]

25 **Dulak J**, Szade K, Szade A, Nowak W, Józkowicz A. Adult stem cells: hopes and hypes of regenerative medicine. *Acta Biochim Pol* 2015; **62**: 329-337 [PMID: 26200199 DOI: 10.18388/abp.2015_1023]

26 **Zou T**, Fan J, Fartash A, Liu H, Fan Y. Cell-based strategies for vascular regeneration. *J Biomed Mater Res A* 2016; **104**: 1297-1314 [PMID: 26864677 DOI: 10.1002/jbm.a.35660]

27 **Jaramillo-Ferrada PA**, Wolvetang EJ, Cooper-White JJ. Differential mesengenic potential and expression of stem cell-fate modulators in mesenchymal stromal cells from human-term placenta and bone marrow. *J Cell Physiol* 2012; **227**: 3234-3242 [PMID: 22105866 DOI: 10.1002/jcp.24014]

28 **Visvader JE**, Clevers H. Tissue-specific designs of stem cell hierarchies. *Nat Cell Biol* 2016; **18**: 349-355 [PMID: 26999737 DOI: 10.1038/ncb3332]

29 **Melchiorri AJ**, Bracaglia LG, Kimerer LK, Hibino N, Fisher JP. In Vitro Endothelialization of Biodegradable Vascular Grafts Via Endothelial Progenitor Cell Seeding and Maturation in a Tubular Perfusion System Bioreactor. *Tissue Eng Part C Methods* 2016; **22**: 663-670 [PMID: 27206552 DOI: 10.1089/ten.TEC.2015.0562]

30 **Tremblay JR**, LeBon JM, Luo A, Quijano JC, Wedeken L, Jou K, Riggs AD, Tirrell DA, Ku HT. In Vitro Colony Assays for Characterizing Tri-potent Progenitor Cells Isolated from the Adult Murine Pancreas. *J Vis Exp* 2016 [PMID: 27340914 DOI: 10.3791/54016]

31 **Wang J**, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH. A protein interaction network for pluripotency of embryonic stem cells. *Nature* 2006; **444**: 364-368 [PMID: 17093407 DOI: 10.1038/nature05284]

32 **Smith GS**, Kumar A, Saba JD. Sphingosine Phosphate Lyase Regulates Murine Embryonic Stem Cell Proliferation and Pluripotency through an $S1P^2$/STAT3 Signaling Pathway. *Biomolecules* 2013; **3**: 351-368 [PMID: 24619572 DOI: 10.3390/biom3030351]

33 **Cumano A**, Godin I. Ontogeny of the hematopoietic system. *Annu Rev Immunol* 2007; **25**: 745-785 [PMID: 17201678 DOI: 10.1146/annurev.immunol.25.022106.141538]

34 **Massberg S**, Schaerli P, Knezevic-Maramica I, Köllnberger M, Tubo N, Moseman EA, Huff IV, Junt T, Wagers AJ, Mazo IB, von Andrian UH. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell* 2007; **131**: 994-1008 [PMID: 18045540 DOI: 10.1016/j.cell.2007.09.047]

35 **Adams GB**, Scadden DT. The hematopoietic stem cell in its place. *Nat Immunol* 2006; **7**: 333-337 [PMID: 16550195 DOI: 10.1038/ni1331]

36 **Wright DE**, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. *Science* 2001; **294**: 1933-1936 [PMID: 11729320 DOI: 10.1126/science.1064081]

37 **Ogle ME**, Olingy CE, Awojoodu AO, Das A, Ortiz RA, Cheung HY, Botchwey EA. Sphingosine-1-Phosphate Receptor-3 Supports Hematopoietic Stem and Progenitor Cell Residence Within the Bone Marrow Niche. *Stem Cells* 2017; **35**: 1040-1052 [PMID: 28026131 DOI: 10.1002/stem.2556]

38 **Schwab SR**, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* 2005; **309**: 1735-1739 [PMID: 16151014 DOI: 10.1126/science.1113640]

39 **Kim C**, Schneider G, Abdel-Latif A, Mierzejewska K, Sunkara M, Borkowska S, Ratajczak J, Morris AJ, Kucia M, Ratajczak MZ. Ceramide-1-phosphate regulates migration of multipotent stromal cells and endothelial progenitor cells--implications for tissue regeneration. *Stem Cells* 2013; **31**: 500-510 [PMID: 23193025 DOI: 10.1002/stem.1291]

40 **Blaho VA**, Galvani S, Engelbrecht E, Liu C, Swendeman SL, Kono M, Proia RL, Steinman L, Han MH, Hla T. HDL-bound sphingosine-1-phosphate restrains lymphopoiesis and neuroinflammation. *Nature* 2015; **523**: 342-346 [PMID: 26053123 DOI: 10.1038/nature14462]

41 **Cyster JG**. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol* 2005; **23**: 127-159 [PMID: 15771568 DOI: 10.1146/annurev.immunol.23.021704.115628]

42 **Schwab R**, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D. Specific effects of microRNAs on the plant transcriptome. *Dev Cell* 2005; **8**: 517-527 [PMID: 15809034 DOI: 10.1016/j.devcel.2005.01.018]

43 **Arnon TI**, Xu Y, Lo C, Pham T, An J, Coughlin S, Dorn GW, Cyster JG. GRK2-dependent S1PR1 desensitization is required for lymphocytes to overcome their attraction to blood. *Science* 2011; **333**: 1898-1903 [PMID: 21960637 DOI: 10.1126/science.1208248]

44 **Liu J**, Zhang C, Tao W, Liu M. Systematic review and meta-analysis of the efficacy of sphingosine-1-phosphate (S1P) receptor agonist FTY720 (fingolimod) in animal models of stroke. *Int J Neurosci* 2013; **123**: 163-169 [PMID: 23167788 DOI: 10.3109/00207454.2012.749255]

45 **Kono M**, Mi Y, Liu Y, Sasaki T, Allende ML, Wu YP, Yamashita T, Proia RL. The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. *J Biol Chem* 2004; **279**: 29367-29373 [PMID: 15138255 DOI: 10.1074/jbc.M403937200]

46 **Mendelson K**, Evans T, Hla T. Sphingosine 1-phosphate signalling. *Development* 2014; **141**: 5-9 [PMID: 24346695 DOI: 10.1242/dev.094805]

47 **Xiong Y**, Yang P, Proia RL, Hla T. Erythrocyte-derived sphingosine 1-phosphate is essential for vascular development. *J Clin Invest* 2014; **124**: 4823-4828 [PMID: 25250575 DOI: 10.1172/JCI77685]

48 **Williams PA**, Stilhano RS, To VP, Tran L, Wong K, Silva EA. Hypoxia augments outgrowth endothelial cell (OEC) sprouting and directed migration in response to sphingosine-1-phosphate (S1P). *PLoS One* 2015; **10**: e0123437 [PMID: 25875493 DOI: 10.1371/journal.pone.0123437]

49 **Poitevin S**, Cussac D, Leroyer AS, Albinet V, Sarlon-Bartoli G, Guillet B, Hubert L, Andrieu-Abadie N, Couderc B, Parini A, Dignat-George F, Sabatier F. Sphingosine kinase 1 expressed by endothelial colony-forming cells has a critical role in their revascularization activity. *Cardiovasc Res* 2014; **103**: 121-130 [PMID: 24743591 DOI: 10.1093/cvr/cvu104]

50 **Hu Y**, Belyea BC, Li M, Göthert JR, Gomez RA, Sequeira-Lopez ML. Identification of cardiac hemo-vascular precursors and their requirement of sphingosine-1-phosphate receptor 1 for heart development. *Sci Rep* 2017; **7**: 45205 [PMID: 28338096 DOI: 10.1038/srep45205]

51 **Svetlov SI**, Sautin YY, Crawford JM. EDG receptors and hepatic pathophysiology of LPA and S1P: EDG-ology of liver injury. *Biochim Biophys Acta* 2002; **1582**: 251-256 [PMID: 12069835 DOI: 10.1016/S1388-1981(02)00178-6]

52 **Salas A**, Ponnusamy S, Senkal CE, Meyers-Needham M, Selvam SP, Saddoughi SA, Apohan E, Sentelle RD, Smith C, Gault CR, Obeid LM, El-Shewy HM, Oaks J, Santhanam R, Marcucci G, Baran Y, Mahajan S, Fernandes D, Stuart R, Perrotti D, Ogretmen B. Sphingosine kinase-1 and sphingosine 1-phosphate receptor 2 mediate Bcr-Abl1 stability and drug resistance by modulation

of protein phosphatase 2A. *Blood* 2011; **117**: 5941-5952 [PMID: 21527515 DOI: 10.1182/blood-2010-08-300772]
53 **Bonder CS**, Sun WY, Matthews T, Cassano C, Li X, Ramshaw HS, Pitson SM, Lopez AF, Coates PT, Proia RL, Vadas MA, Gamble JR. Sphingosine kinase regulates the rate of endothelial progenitor cell differentiation. *Blood* 2009; **113**: 2108-2117 [PMID: 19109558 DOI: 10.1182/blood-2008-07-166942]
54 **Roato I**, Ferracini R. Cancer Stem Cells, Bone and Tumor Microenvironment: Key Players in Bone Metastases. *Cancers* (Basel) 2018; **10**: pii: E56 [PMID: 29461491 DOI: 10.3390/cancers10020056]
55 **Walter DH**, Rochwalsky U, Reinhold J, Seeger F, Aicher A, Urbich C, Spyridopoulos I, Chun J, Brinkmann V, Keul P, Levkau B, Zeiher AM, Dimmeler S, Haendeler J. Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. *Arterioscler Thromb Vasc Biol* 2007; **27**: 275-282 [PMID: 17158356 DOI: 10.1161/01.ATV.0000254669.12675.70]
56 **Kimura T**, Boehmler AM, Seitz G, Kuçi S, Wiesner T, Brinkmann V, Kanz L, Möhle R. The sphingosine 1-phosphate receptor agonist FTY720 supports CXCR4-dependent migration and bone marrow homing of human CD34+ progenitor cells. *Blood* 2004; **103**: 4478-4486 [PMID: 14988150 DOI: 10.1182/blood-2003-03-0875]
57 **Yamashita H**, Kitayama J, Shida D, Yamaguchi H, Mori K, Osada M, Aoki S, Yatomi Y, Takuwa Y, Nagawa H. Sphingosine 1-phosphate receptor expression profile in human gastric cancer cells: differential regulation on the migration and proliferation. *J Surg Res* 2006; **130**: 80-87 [PMID: 16183075 DOI: 10.1016/j.jss.2005.08.004]
58 **Li MH**, Sanchez T, Yamase H, Hla T, Oo ML, Pappalardo A, Lynch KR, Lin CY, Ferrer F. S1P/S1P1 signaling stimulates cell migration and invasion in Wilms tumor. *Cancer Lett* 2009; **276**: 171-179 [PMID: 19131156 DOI: 10.1016/j.canlet.2008.11.025]
59 **Zhang L**, Orban M, Lorenz M, Barocke V, Braun D, Urtz N, Schulz C, von Brühl ML, Tirniceriu A, Gaertner F, Proia RL, Graf T, Bolz SS, Montanez E, Prinz M, Müller A, von Baumgarten L, Billich A, Sixt M, Fässler R, von Andrian UH, Junt T, Massberg S. A novel role of sphingosine 1-phosphate receptor S1pr1 in mouse thrombopoiesis. *J Exp Med* 2012; **209**: 2165-2181 [PMID: 23148237 DOI: 10.1084/jem.20121090]
60 **Vito CD**, Hadi LA, Navone SE, Marfia G, Campanella R, Mancuso ME, Riboni L. Platelet-derived sphingosine-1-phosphate and inflammation: from basic mechanisms to clinical implications. *Platelets* 2016; **27**: 393-401 [PMID: 26950429 DOI: 10.3109/09537104.2016.1144179]
61 **Lassar AB**, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschka SD, Weintraub H. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* 1989; **58**: 823-831 [PMID: 2550138 DOI: 10.1016/0092-8674(89)90935-5]
62 **Grounds MD**. Reasons for the degeneration of ageing skeletal muscle: a central role for IGF-1 signalling. *Biogerontology* 2002; **3**: 19-24 [PMID: 12014835 DOI: 10.1023/A:1015234709314]
63 **Donati C**, Cencetti F, Bruni P. Sphingosine 1-phosphate axis: a new leader actor in skeletal muscle biology. *Front Physiol* 2013; **4**: 338 [PMID: 24324439 DOI: 10.3389/fphys.2013.00338]
64 **Seale P**, Rudnicki MA. A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev Biol* 2000; **218**: 115-124 [PMID: 10656756 DOI: 10.1006/dbio.1999.9565]
65 **Cencetti F**, Bernacchioni C, Nincheri P, Donati C, Bruni P. Transforming growth factor-beta1 induces transdifferentiation of myoblasts into myofibroblasts via up-regulation of sphingosine kinase-1/S1P3 axis. *Mol Biol Cell* 2010; **21**: 1111-1124 [PMID: 20089836 DOI: 10.1091/mbc.E09-09-0812]
66 **Bernacchioni C**, Cencetti F, Blescia S, Donati C, Bruni P. Sphingosine kinase/sphingosine 1-phosphate axis: a new player for insulin-like growth factor-1-induced myoblast differentiation. *Skelet Muscle* 2012; **2**: 15 [PMID: 22788716 DOI: 10.1186/2044-5040-2-15]
67 **Bruno G**, Cencetti F, Bernacchioni C, Donati C, Blankenbach KV, Thomas D, Meyer Zu Heringdorf D, Bruni P. Bradykinin mediates myogenic differentiation in murine myoblasts through the involvement of SK1/Spns2/S1P^2 axis. *Cell Signal* 2018; **45**: 110-121 [PMID: 29408301 DOI: 10.1016/j.cellsig.2018.02.001]
68 **Sprague AH**, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem Pharmacol* 2009; **78**: 539-552 [PMID: 19413999 DOI: 10.1016/j.bcp.2009.04.029]
69 **Frimm Cde C**, Sun Y, Weber KT. Wound healing following myocardial infarction in the rat: role for bradykinin and prostaglandins. *J Mol Cell Cardiol* 1996; **28**: 1279-1285 [PMID: 8782069 DOI: 10.1006/jmcc.1996.0118]
70 **Ricciardolo FLM**, Folkerts G, Folino A, Mognetti B. Bradykinin in asthma: Modulation of airway inflammation and remodelling. *Eur J Pharmacol* 2018; **827**: 181-188 [PMID: 29548973 DOI: 10.1016/j.ejphar.2018.03.017]
71 **Donati C**, Meacci E, Nuti F, Becciolini L, Farnararo M, Bruni P. Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor. *FASEB J* 2005; **19**: 449-451 [PMID: 15625079 DOI: 10.1096/fj.04-1780fje]
72 **Calise S**, Blescia S, Cencetti F, Bernacchioni C, Donati C, Bruni P. Sphingosine 1-phosphate stimulates proliferation and migration of satellite cells: role of S1P receptors. *Biochim Biophys Acta* 2012; **1823**: 439-450 [PMID: 22178384 DOI: 10.1016/j.bbamcr.2011.11.016]
73 **Sugimoto N**, Takuwa N, Okamoto H, Sakurada S, Takuwa Y. Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Mol Cell Biol* 2003; **23**: 1534-1545 [PMID: 12588974 DOI: 10.1128/MCB.23.5.1534-1545.2003]
74 **Sassoli C**, Nosi D, Tani A, Chellini F, Mazzanti B, Quercioli F, Zecchi-Orlandini S, Formigli L. Defining the role of mesenchymal stromal cells on the regulation of matrix metalloproteinases in skeletal muscle cells. *Exp Cell Res* 2014; **323**: 297-313 [PMID: 24631289 DOI: 10.1016/j.yexcr.2014.03.003]
75 **Tanimoto T**, Jin ZG, Berk BC. Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine 1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS). *J Biol Chem* 2002; **277**: 42997-43001 [PMID: 12226078 DOI: 10.1074/jbc.M204764200]
76 **Meacci E**, Donati C, Farnararo M, Bruni P. Sphingosine 1-phosphate signal transduction in muscle cells. *Ital J Biochem* 2003; **52**: 25-27 [PMID: 12833634]
77 **Fortier M**, Figeac N, White RB, Knopp P, Zammit PS. Sphingosine-1-phosphate receptor 3 influences cell cycle progression in muscle satellite cells. *Dev Biol* 2013; **382**: 504-516 [PMID: 23911934 DOI: 10.1016/j.ydbio.2013.07.006]
78 **Castaldi A**, Chesini GP, Taylor AE, Sussman MA, Brown JH, Purcell NH. Sphingosine 1-phosphate elicits RhoA-dependent proliferation and MRTF-A mediated gene induction in CPCs. *Cell Signal* 2016; **28**: 871-879 [PMID: 27094722 DOI: 10.1016/j.cellsig.2016.04.006]
79 **Fukui H**, Terai K, Nakajima H, Chiba A, Fukuhara S, Mochizuki N. S1P-Yap1 signaling regulates endoderm formation required for cardiac precursor cell migration in zebrafish. *Dev Cell* 2014; **31**: 128-136 [PMID: 25313964 DOI: 10.1016/j.devcel.2014.08.014]
80 **Araya R**, Eckardt D, Maxeiner S, Krüger O, Theis M, Willecke K, Sáez JC. Expression of connexins during differentiation and regeneration of skeletal muscle: functional relevance of connexin43. *J Cell Sci* 2005; **118**: 27-37 [PMID: 15601660 DOI: 10.1242/jcs.01553]
81 **Squecco R**, Sassoli C, Nuti F, Martinesi M, Chellini F, Nosi D, Zecchi-Orlandini S, Francini F, Formigli L, Meacci E. Sphingosine 1-phosphate induces myoblast differentiation through Cx43 protein expression: a role for a gap junction-dependent and -independent function. *Mol Biol Cell* 2006; **17**: 4896-4910 [PMID: 16957055 DOI: 10.1091/mbc.E06-03-0243]
82 **Saba JD**, de la Garza-Rodea AS. S1P lyase in skeletal muscle regeneration and satellite cell activation: exposing the hidden lyase. *Biochim Biophys Acta* 2013; **1831**: 167-175 [PMID: 22750505 DOI: 10.1016/j.bbalip.2012.06.009]

83 **de la Garza-Rodea AS**, Baldwin DM, Oskouian B, Place RF, Bandhuvula P, Kumar A, Saba JD. Sphingosine phosphate lyase regulates myogenic differentiation via S1P receptor-mediated effects on myogenic microRNA expression. *FASEB J* 2014; **28**: 506-519 [PMID: 24158395 DOI: 10.1096/fj.13-233155]

84 **Gangoiti P**, Bernacchioni C, Donati C, Cencetti F, Ouro A, Gómez-Muñoz A, Bruni P. Ceramide 1-phosphate stimulates proliferation of C2C12 myoblasts. *Biochimie* 2012; **94**: 597-607 [PMID: 21945811 DOI: 10.1016/j.biochi.2011.09.009]

85 **Rodeheffer MS**, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells in vivo. *Cell* 2008; **135**: 240-249 [PMID: 18835024 DOI: 10.1016/j.cell.2008.09.036]

86 **Gupta RK**, Mepani RJ, Kleiner S, Lo JC, Khandekar MJ, Cohen P, Frontini A, Bhowmick DC, Ye L, Cinti S, Spiegelman BM. Zfp423 expression identifies committed preadipocytes and localizes to adipose endothelial and perivascular cells. *Cell Metab* 2012; **15**: 230-239 [PMID: 22326224 DOI: 10.1016/j.cmet.2012.01.010]

87 **Hashimoto Y**, Matsuzaki E, Higashi K, Takahashi-Yanaga F, Takano A, Hirata M, Nishimura F. Sphingosine-1-phosphate inhibits differentiation of C3H10T1/2 cells into adipocyte. *Mol Cell Biochem* 2015; **401**: 39-47 [PMID: 25445169 DOI: 10.1007/s11010-014-2290-1]

88 **Shen H**, Zhou E, Wei X, Fu Z, Niu C, Li Y, Pan B, Mathew AV, Wang X, Pennathur S, Zheng L, Wang Y. High density lipoprotein promotes proliferation of adipose-derived stem cells via S1P1 receptor and Akt, ERK1/2 signal pathways. *Stem Cell Res Ther* 2015; **6**: 95 [PMID: 25976318 DOI: 10.1186/s13287-015-0090-5]

89 **Zhou Q**, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA. A multipotent progenitor domain guides pancreatic organogenesis. *Dev Cell* 2007; **13**: 103-114 [PMID: 17609113 DOI: 10.1016/j.devcel.2007.06.001]

90 **Apelqvist A**, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl U, Edlund H. Notch signalling controls pancreatic cell differentiation. *Nature* 1999; **400**: 877-881 [PMID: 10476967 DOI: 10.1038/23716]

91 **Cebola I**, Rodríguez-Seguí SA, Cho CH, Bessa J, Rovira M, Luengo M, Chhatriwala M, Berry A, Ponsa-Cobas J, Maestro MA, Jennings RE, Pasquali L, Morán I, Castro N, Hanley NA, Gomez-Skarmeta JL, Vallier L, Ferrer J. TEAD and YAP regulate the enhancer network of human embryonic pancreatic progenitors. *Nat Cell Biol* 2015; **17**: 615-626 [PMID: 25915126 DOI: 10.1038/ncb3160]

92 **Serafimidis I**, Rodriguez-Aznar E, Lesche M, Yoshioka K, Takuwa Y, Dahl A, Pan D, Gavalas A. Pancreas lineage allocation and specification are regulated by sphingosine-1-phosphate signalling. *PLoS Biol* 2017; **15**: e2000949 [PMID: 28248965 DOI: 10.1371/journal.pbio.2000949]

93 **Edsall LC**, Pirianov GG, Spiegel S. Involvement of sphingosine 1-phosphate in nerve growth factor-mediated neuronal survival and differentiation. *J Neurosci* 1997; **17**: 6952-6960 [PMID: 9278531 DOI: 10.1523/JNEUROSCI.17-18-06952.1997]

94 **Rius RA**, Edsall LC, Spiegel S. Activation of sphingosine kinase in pheochromocytoma PC12 neuronal cells in response to trophic factors. *FEBS Lett* 1997; **417**: 173-176 [PMID: 9395290 DOI: 10.1016/S0014-5793(97)01277-5]

95 **Tincer G**, Mashkaryan V, Bhattarai P, Kizil C. Neural stem/progenitor cells in Alzheimer's disease. *Yale J Biol Med* 2016; **89**: 23-35 [PMID: 27505014]

96 **Tashiro S**, Nishimura S, Iwai H, Sugai K, Zhang L, Shinozaki M, Iwanami A, Toyama Y, Liu M, Okano H, Nakamura M. Functional Recovery from Neural Stem/Progenitor Cell Transplantation Combined with Treadmill Training in Mice with Chronic Spinal Cord Injury. *Sci Rep* 2016; **6**: 30898 [PMID: 27485458 DOI: 10.1038/srep30898]

97 **Harada J**, Foley M, Moskowitz MA, Waeber C. Sphingosine-1-phosphate induces proliferation and morphological changes of neural progenitor cells. *J Neurochem* 2004; **88**: 1026-1039 [PMID: 14756825 DOI: 10.1046/j.1471-4159.2003.02219.x]

98 **Bassi R**, Anelli V, Giussani P, Tettamanti G, Viani P, Riboni L. Sphingosine-1-phosphate is released by cerebellar astrocytes in response to bFGF and induces astrocyte proliferation through Gi-protein-coupled receptors. *Glia* 2006; **53**: 621-630 [PMID: 16470810 DOI: 10.1002/glia.20324]

99 **Anelli V**, Bassi R, Tettamanti G, Viani P, Riboni L. Extracellular release of newly synthesized sphingosine-1-phosphate by cerebellar granule cells and astrocytes. *J Neurochem* 2005; **92**: 1204-1215 [PMID: 15715670 DOI: 10.1111/j.1471-4159.2004.02955.x]

100 **Fischer I**, Alliod C, Martinier N, Newcombe J, Brana C, Pouly S. Sphingosine kinase 1 and sphingosine 1-phosphate receptor 3 are functionally upregulated on astrocytes under pro-inflammatory conditions. *PLoS One* 2011; **6**: e23905 [PMID: 21887342 DOI: 10.1371/journal.pone.0023905]

101 **Terai K**, Soga T, Takahashi M, Kamohara M, Ohno K, Yatsugi S, Okada M, Yamaguchi T. Edg-8 receptors are preferentially expressed in oligodendrocyte lineage cells of the rat CNS. *Neuroscience* 2003; **116**: 1053-1062 [PMID: 12617946 DOI: 10.1016/S0306-4522(02)00791-1]

102 **Yu N**, Lariosa-Willingham KD, Lin FF, Webb M, Rao TS. Characterization of lysophosphatidic acid and sphingosine-1-phosphate-mediated signal transduction in rat cortical oligodendrocytes. *Glia* 2004; **45**: 17-27 [PMID: 14648542 DOI: 10.1002/glia.10297]

103 **Bieberich E**. There is more to a lipid than just being a fat: sphingolipid-guided differentiation of oligodendroglial lineage from embryonic stem cells. *Neurochem Res* 2011; **36**: 1601-1611 [PMID: 21136155 DOI: 10.1007/s11064-010-0338-5]

104 **Spohr TC**, Dezonne RS, Nones J, Dos Santos Souza C, Einicker-Lamas M, Gomes FC, Rehen SK. Sphingosine 1-phosphate-primed astrocytes enhance differentiation of neuronal progenitor cells. *J Neurosci Res* 2012; **90**: 1892-1902 [PMID: 22588662 DOI: 10.1002/jnr.23076]

105 **Blanc CA**, Grist JJ, Rosen H, Sears-Kraxberger I, Steward O, Lane TE. Sphingosine-1-phosphate receptor antagonism enhances proliferation and migration of engrafted neural progenitor cells in a model of viral-induced demyelination. *Am J Pathol* 2015; **185**: 2819-2832 [PMID: 26435414 DOI: 10.1016/j.ajpath.2015.06.009]

106 **Kimura A**, Ohmori T, Kashiwakura Y, Ohkawa R, Madoiwa S, Mimuro J, Shimazaki K, Hoshino Y, Yatomi Y, Sakata Y. Antagonism of sphingosine 1-phosphate receptor-2 enhances migration of neural progenitor cells toward an area of brain. *Stroke* 2008; **39**: 3411-3417 [PMID: 18757288 DOI: 10.1161/STROKEAHA.108.514612]

107 **Meng H**, Yuan Y, Lee VM. Loss of sphingosine kinase 1/S1P signaling impairs cell growth and survival of neurons and progenitor cells in the developing sensory ganglia. *PLoS One* 2011; **6**: e27150 [PMID: 22096531 DOI: 10.1371/journal.pone.0027150]

108 **Maceyka M**, Payne SG, Milstien S, Spiegel S. Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *Biochim Biophys Acta* 2002; **1585**: 193-201 [PMID: 12531554 DOI: 10.1016/S1388-1981(02)00341-4]

109 **Madhunapantula SV**, Hengst J, Gowda R, Fox TE, Yun JK, Robertson GP. Targeting sphingosine kinase-1 to inhibit melanoma. *Pigment Cell Melanoma Res* 2012; **25**: 259-274 [PMID: 22236408 DOI: 10.1111/j.1755-148X.2012.00970.x]

110 **Maceyka M**, Milstien S, Spiegel S. Sphingosine-1-phosphate: the Swiss army knife of sphingolipid signaling. *J Lipid Res* 2009; **50** Suppl: S272-S276 [PMID: 18987387 DOI: 10.1194/jlr.R800065-JLR200]

111 **Dobrowsky RT**, Carter BD. Coupling of the p75 neurotrophin receptor to sphingolipid signaling. *Ann N Y Acad Sci* 1998; **845**: 32-45 [PMID: 9668341 DOI: 10.1111/j.1749-6632.1998.tb09660.x]

112 **Barrett GL**. The p75 neurotrophin receptor and neuronal apoptosis. *Prog Neurobiol* 2000; **61**: 205-229 [PMID: 10704998 DOI: 10.1016/S0301-0082(99)00056-8]

113 **Barrett GL**, Bartlett PF. The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc Natl Acad Sci USA* 1994; **91**: 6501-6505 [PMID: 8022812 DOI: 10.1073/pnas.91.14.6501]

114 **Ginty DD**, Bonni A, Greenberg ME. Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription

via phosphorylation of CREB. *Cell* 1994; **77**: 713-725 [PMID: 8205620 DOI: 10.1016/0092-8674(94)90055-8]
115 **Bonni A**, Ginty DD, Dudek H, Greenberg ME. Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. *Mol Cell Neurosci* 1995; **6**: 168-183 [PMID: 7551568 DOI: 10.1006/mcne.1995.1015]
116 **Xing J**, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* 1996; **273**: 959-963 [PMID: 8688081 DOI: 10.1126/science.273.5277.959]
117 **Finkbeiner S**. CREB couples neurotrophin signals to survival messages. *Neuron* 2000; **25**: 11-14 [PMID: 10707967 DOI: 10.1016/S0896-6273(00)80866-1]
118 **Saini HS**, Coelho RP, Goparaju SK, Jolly PS, Maceyka M, Spiegel S, Sato-Bigbee C. Novel role of sphingosine kinase 1 as a mediator of neurotrophin-3 action in oligodendrocyte progenitors. *J Neurochem* 2005; **95**: 1298-1310 [PMID: 16313513 DOI: 10.1111/j.1471-4159.2005.03451.x]
119 **Johnson JR**, Chu AK, Sato-Bigbee C. Possible role of CREB in the stimulation of oligodendrocyte precursor cell proliferation by neurotrophin-3. *J Neurochem* 2000; **74**: 1409-1417 [PMID: 10737596 DOI: 10.1046/j.1471-4159.2000.0741409.x]
120 **Brinkmann V**, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov* 2010; **9**: 883-897 [PMID: 21031003 DOI: 10.1038/nrd3248]
121 **Bu S**, Asano Y, Bujor A, Highland K, Hant F, Trojanowska M. Dihydrosphingosine 1-phosphate has a potent antifibrotic effect in scleroderma fibroblasts via normalization of phosphatase and tensin homolog levels. *Arthritis Rheum* 2010; **62**: 2117-2126 [PMID: 20309867 DOI: 10.1002/art.27463]
122 **Hida H**, Nagano S, Takeda M, Soliven B. Regulation of mitogen-activated protein kinases by sphingolipid products in oligodendrocytes. *J Neurosci* 1999; **19**: 7458-7467 [PMID: 10460252 DOI: 10.1523/JNEUROSCI.19-17-07458.1999]
123 **Fatatis A**, Miller RJ. Platelet-derived growth factor (PDGF)-induced Ca2+ signaling in the CG4 oligodendroglial cell line and in transformed oligodendrocytes expressing the beta-PDGF receptor. *J Biol Chem* 1997; **272**: 4351-4358 [PMID: 9020156 DOI: 10.1074/jbc.272.7.4351]
124 **Soliven B**, Ma L, Bae H, Attali B, Sobko A, Iwase T. PDGF upregulates delayed rectifier via Src family kinases and sphingosine kinase in oligodendroglial progenitors. *Am J Physiol Cell Physiol* 2003; **284**: C85-C93 [PMID: 12475761 DOI: 10.1152/ajpcell.00145.2002]
125 **Wang F**, Buckley NE, Olivera A, Goodemote KA, Su Y, Spiegel S. Involvement of sphingolipids metabolites in cellular proliferation modulated by ganglioside GM1. *Glycoconj J* 1996; **13**: 937-945 [PMID: 8981085 DOI: 10.1007/bf01053189]
126 **Su Y**, Rosenthal D, Smulson M, Spiegel S. Sphingosine 1-phosphate, a novel signaling molecule, stimulates DNA binding activity of AP-1 in quiescent Swiss 3T3 fibroblasts. *J Biol Chem* 1994; **269**: 16512-16517 [PMID: 8206962]
127 **Shatrov VA**, Lehmann V, Chouaib S. Sphingosine-1-phosphate mobilizes intracellular calcium and activates transcription factor NF-kappa B in U937 cells. *Biochem Biophys Res Commun* 1997; **234**: 121-124 [PMID: 9168973 DOI: 10.1006/bbrc.1997.6598]
128 **Xia P**, Gamble JR, Rye KA, Wang L, Hii CS, Cockerill P, Khew-Goodall Y, Bert AG, Barter PJ, Vadas MA. Tumor necrosis factor-alpha induces adhesion molecule expression through the sphingosine kinase pathway. *Proc Natl Acad Sci USA* 1998; **95**: 14196-14201 [PMID: 9826677 DOI: 10.1073/pnas.95.24.14196]
129 **Takeshita A**, Watanabe A, Takada Y, Hanazawa S. Selective stimulation by ceramide of the expression of the alpha isoform of retinoic acid and retinoid X receptors in osteoblastic cells. A role of sphingosine 1-phosphate-mediated AP-1 in the ligand-dependent transcriptional activity of these receptors. *J Biol Chem* 2000; **275**: 32220-32226 [PMID: 10915783 DOI: 10.1074/jbc.M002569200]
130 **Im DS**, Clemens J, Macdonald TL, Lynch KR. Characterization of the human and mouse sphingosine 1-phosphate receptor, S1P5 (Edg-8): structure-activity relationship of sphingosine1-phosphate receptors. *Biochemistry* 2001; **40**: 14053-14060 [PMID: 11705398 DOI: 10.1021/bi011606i]
131 **Jaillard C**, Harrison S, Stankoff B, Aigrot MS, Calver AR, Duddy G, Walsh FS, Pangalos MN, Arimura N, Kaibuchi K, Zalc B, Lubetzki C. Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. *J Neurosci* 2005; **25**: 1459-1469 [PMID: 15703400 DOI: 10.1523/JNEUROSCI.4645-04.2005]
132 **Miron VE**, Jung CG, Kim HJ, Kennedy TE, Soliven B, Antel JP. FTY720 modulates human oligodendrocyte progenitor process extension and survival. *Ann Neurol* 2008; **63**: 61-71 [PMID: 17918267 DOI: 10.1002/ana.21227]
133 **Tan B**, Luo Z, Yue Y, Liu Y, Pan L, Yu L, Yin Y. Effects of FTY720 (Fingolimod) on Proliferation, Differentiation, and Migration of Brain-Derived Neural Stem Cells. *Stem Cells Int* 2016; **2016**: 9671732 [PMID: 27829841 DOI: 10.1155/2016/9671732]
134 **Choi JW**, Gardell SE, Herr DR, Rivera R, Lee CW, Noguchi K, Teo ST, Yung YC, Lu M, Kennedy G, Chun J. FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. *Proc Natl Acad Sci USA* 2011; **108**: 751-756 [PMID: 21177428 DOI: 10.1073/pnas.1014154108]
135 **Stessin AM**, Gursel DB, Schwartz A, Parashar B, Kulidzhanov FG, Sabbas AM, Boockvar J, Nori D, Wernicke AG. FTY720, sphingosine 1-phosphate receptor modulator, selectively radioprotects hippocampal neural stem cells. *Neurosci Lett* 2012; **516**: 253-258 [PMID: 22507238 DOI: 10.1016/j.neulet.2012.04.004]
136 **Mahajan-Thakur S**, Bien-Möller S, Marx S, Schroeder H, Rauch BH. Sphingosine 1-phosphate (S1P) signaling in glioblastoma multiforme-A systematic review. *Int J Mol Sci* 2017; **18**: pii: E2448 [PMID: 29149079 DOI: 10.3390/ijms18112448]
137 **Mukherjee N**, Lu Y, Almeida A, Lambert K, Shiau CW, Su JC, Luo Y, Fujita M, Robinson WA, Robinson SE, Norris DA, Shellman YG. Use of a MCL-1 inhibitor alone to de-bulk melanoma and in combination to kill melanoma initiating cells. *Oncotarget* 2017; **8**: 46801-46817 [PMID: 27086916 DOI: 10.18632/oncotarget.8695]
138 **Luo J**, Wang P, Wang R, Wang J, Liu M, Xiong S, Li Y, Cheng B. The Notch pathway promotes the cancer stem cell characteristics of CD90+ cells in hepatocellular carcinoma. *Oncotarget* 2016; **7**: 9525-9537 [PMID: 26848615 DOI: 10.18632/oncotarget.6672]
139 **Takebe N**, Ivy SP. Controversies in cancer stem cells: targeting embryonic signaling pathways. *Clin Cancer Res* 2010; **16**: 3106-3112 [PMID: 20530695 DOI: 10.1158/1078-0432.CCR-09-2934]
140 **Hirata N**, Yamada S, Shoda T, Kurihara M, Sekino Y, Kanda Y. Sphingosine-1-phosphate promotes expansion of cancer stem cells via S1PR3 by a ligand-independent Notch activation. *Nat Commun* 2014; **5**: 4806 [PMID: 25254944 DOI: 10.1038/ncomms5806]
141 **Wang YC**, Tsai CF, Chuang HL, Chang YC, Chen HS, Lee JN, Tsai EM. Benzyl butyl phthalate promotes breast cancer stem cell expansion via SPHK1/S1P/S1PR3 signaling. *Oncotarget* 2016; **7**: 29563-29576 [PMID: 27129165 DOI: 10.18632/oncotarget.9007]
142 **Gasiewicz TA**, Singh KP, Bennett JA. The Ah receptor in stem cell cycling, regulation, and quiescence. *Ann N Y Acad Sci* 2014; **1310**: 44-50 [PMID: 24495120 DOI: 10.1111/nyas.12361]
143 **Azuma H**, Takahara S, Ichimaru N, Wang JD, Itoh Y, Otsuki Y, Morimoto J, Fukui R, Hoshiga M, Ishihara T, Nonomura N, Suzuki S, Okuyama A, Katsuoka Y. Marked prevention of tumor growth and metastasis by a novel immunosuppressive agent, FTY720, in mouse breast cancer models. *Cancer Res* 2002; **62**: 1410-1419 [PMID: 11888913]
144 **Hait NC**, Avni D, Yamada A, Nagahashi M, Aoyagi T, Aoki H, Dumur CI, Zelenko Z, Gallagher EJ, Leroith D, Milstien S, Takabe K, Spiegel S. The phosphorylated prodrug FTY720 is a histone deacetylase inhibitor that reactivates ERα expression and enhances hormonal therapy for breast cancer. *Oncogenesis* 2015; **4**: e156 [PMID: 26053034 DOI: 10.1038/oncsis.2015.16]

145 **Fillmore CM**, Gupta PB, Rudnick JA, Caballero S, Keller PJ, Lander ES, Kuperwasser C. Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling. *Proc Natl Acad Sci USA* 2010; **107**: 21737-21742 [PMID: 21098263 DOI: 10.1073/pnas.1007863107]
146 **Mizugishi K**, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol* 2005; **25**: 11113-11121 [PMID: 16314531 DOI: 10.1128/MCB.25.24.11113-11121.2005]
147 **Bieberich E**. Smart drugs for smarter stem cells: making SENSe (sphingolipid-enhanced neural stem cells) of ceramide. *Neurosignals* 2008; **16**: 124-139 [PMID: 18253053 DOI: 10.1159/000111558]

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EDITORIAL

Hematopoietic stem cell transplantation for Crohn's disease: Gaps, doubts and perspectives

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Abstract

Crohn's disease (CD) is an inflammatory bowel disease that can affect any site of the digestive system. It occurs due to an immunological imbalance and is responsible for intestinal mucosal lesions and complications such as fistulas and stenoses. Treatment aims to stabilize the disease, reducing the symptoms and healing intestinal lesions. Surgical procedures are common in patients. Cell therapy was initially used to treat this disease in patients who also suffered from lymphoma and leukemia and were considered to be good candidates for autologous and allogeneic transplantation. After transplantation, an improvement was also observed in their CD. In 2003, the procedure began to be used to treat the disease itself, and several case series and randomized studies have been published since then; this approach currently comprises a new option in the treatment of CD. However, considerable doubt along with significant gaps in our knowledge continue to exist in relation to cell therapy for CD. Cell therapy is currently restricted to the autologous modality of hematopoietic stem cell transplantation and, experimentally, to mesenchymal stromal cells to directly treat lesions of the anal mucosa. This article presents the supporting claims for transplantation as well as aspects related to the mobilization regime, conditioning and perspectives of cell therapy.

Key words: Stem cell therapy; Hematopoietic stem cell transplantation; Treatment; Crohn's disease

Core tip: Crohn's disease (CD) is an inflammatory bowel disease that can affect any part of the digestive tract. Hematopoietic stem cell transplantation is considered an option in cases of severe disease refractory to conventional treatment. To date, the results are promising, however many gaps and doubts remain regarding procedures for and indications of cell therapy, which still require improvement. The aim of this editorial is to discuss these aspects and the future of cell therapy in CD.

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INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease that can affect any section of the digestive tract[1]. Althought more common in the United States, Western Europe, Australia and New Zealand, there has recently been an increase in the frequency of cases in Asia, Eastern Europe and South America[2]. These increases are attributed to the globalization of diet and customs[3]. CD is a chronic, heterogeneous disease of unknown etiology that may occur with extra-intestinal manifestations associated with other autoimmune diseases[1,2]. The Genome-wide Association Study Project identified hereditary and genetic factors as possible indicators of susceptibility for the disease, as well as the triggers of immunological imbalance found in patients[4].

Treatment aims to stabilize the disease, reduce symptoms and heal the patient's intestinal lesions. Anti-inflammatory drugs, immunosuppressive agents, corticosteroids and biological agents are prescribed alone or in combination. Drugs are usually administered in a step-wise sequence, called "Step Down". Nevertheless, controversies and doubts remain regarding early indications of biological agents associated with immunosuppressants in cases considered to be more serious ("Top Down" treatment plan)[1].

Surgical treatment is common in CD cases and depends on the extent and location of the disease. There is a need for surgical procedures of varying complexity in more than 50% of patients within five years of diagnosis[1,5].

STEM CELL THERAPY

Cell therapy emerged as a form of CD treatment due to the chronicity of the disease, lack of therapeutic options in refractory patients, and the description of disease improvements in cases with concomitant leukemia or lymphoma that were submitted for hematopoietic stem cell transplantation (HSCT)[6-8]. This was the first modality of cell therapy exclusively used for the treatment of CD. It was initially described in sporadic cases, yet a number of long-term and randomized studies of autologous HSCT has since placed the procedure on the map as an appropriate disease treatment for similar autoimmune diseases[9-11].

HSCT refers to any procedure that uses hematopoietic stem cells from any donor or recipient to repopulate or replace hematopoietic tissue in part or completely. The goal of this CD treatment procedure is to reprogram the immune system.

Despite the existence of established standard treatments, according to the European Bone Marrow Transplant Society, the indication of autologous HSCT for CD is the same as for other serious, progressive and refractory autoimmune diseases as a Level Ⅱ clinical option. This states that the procedure should be recommended only after careful consideration of the risks and benefits to patients. Allogeneic HSCT is generally not recommended for CD because of the inherent toxicity risks of the procedure as well as the risk of graft-*vs*-host disease[12].

Thus, the criteria for the indication of HSCT for CD always includes: (1) patients refractory to immunosuppressive and biological agents; (2) the persistence of disease activity proven by endoscopy, colonoscopy or magnetic resonance enterography; and (3) extensive disease for which an imminent surgical procedure exposes the patient to the risk of short bowel syndrome or refractory colonic disease. A fourth criterion is the presence of a persistent perianal lesion where coloproctectomy with a definitive stoma implant is not accepted by the patient[13].

Even so, doubts persist in the medical and academic communities regarding HSCT for the treatment of autoimmune diseases like CD. The main fears regarding HSCT is the toxicity related to chemotherapeutic and immunosuppressive agents, the risk of infections due to the period of aplasia that commonly occurs after the conditioning regimen, and the transplant itself (when hematopoietic progenitor cells are infused). In the past, the morbidity rate was much higher in relation to toxicity. Today, although death as a result is practically nonexistent, it still occurs due to complications or infections caused by resistant germs, which often exist in immunosuppressed patients within a hospital environment[14].

Thus, the selection of cases for elective HSCT should be rigorous, and the patients who are evaluated must be monitored and followed-up meticulously throughout the procedure. Patient selection should rule out comorbidities such as cardiac and pulmonary diseases, as well as other preexisting anomalies, such as clinical situations that add risk to the procedure. In short, the procedure should be carried out under the care of a multidisciplinary team and within an institution that meets national and international legal criteria with a

history of good medical practices[12].

The standard mobilization regimen in CD patients is cyclophosphamide (Cy), which is associated with granulocyte colony stimulating factor (G-CSF). Until recently, there was contention over whether the administered dose of Cy should be 4 g/m^2 or 2 g/m^2. High doses of Cy were shown to correlate with an increased risk of cardiac toxicity, in addition to risks of bladder toxicity. In addition, no benefit is gained from the use of high doses, in terms of obtaining a higher number of cells for HSCT either in CD or other autoimmune diseases[15]. CD patients are often super-mobilizers and rapidly recover with low toxicity after HSCT. These conditions improve the quality of life soon after the procedure. In relation to Cy, there are already proposals to reduce the mobilization regime dose to 1 g/m^2.

Another question concerns the manipulation or selection of cells for HSCT. Several reports used the selection or enrichment of CD34$^+$ cells to reduce the volume and increase the efficacy of the product to be infused. From a study with four patients where manipulation was not used, due to the technical difficulty of selecting and enriching cells), manipulation is no longer performed and several authors have reported successful treatment without affecting the results of HSCT[16]. Generally, the dose of G-CSF for mobilization is 10 μg/kg per day from the 5th day after Cy administration. It is not clear which day is optimal for starting administration of the cytokine, nor are there any reports of its use alone in the mobilization of CD patients. This has likely not been tried to date due to reports of flares or disease exacerbation in other autoimmune diseases[17]. However, it should be noted that there are references claiming that G-CSF provides benefits to CD patients[18].

The standard conditioning regimen for CD is the association of Cy with rabbit or horse antithymocyte globulin (GAT). The doses of Cy, rabbit GAT and horse GAT are 200 mg/kg, 6.5 mg/kg and 90 mg/kg, respectively, split over four consecutive days. This regimen usually leads to peripheral pancytopenia, which often occurs one to seven days after cell infusion. In this period, the patient is subject to the possibility of infectious complications, so care should be doubled depending on the patient's previous alterations, such as perianal disease, fistulas or the presence of an implanted colostomy. Cy and GAT should be carefully administered to avoid the inherent and habitual adverse effects of these medications.

There is now doubt as to whether it is a good idea to reduce the dose of CY, or to introduce another chemotherapeutic or immunosuppressant agent instead of GAT in the conditioning regimen for HSCT.

The results of HSCT have an impact on the patient's immediate and long-term quality of life[19]. However, the clinical evaluation of patients submitted to HSCT is mandatory, and understanding the signs that indicate that the patient will benefit long-term from HSCT is very important.

There are also no specific reports of patients who relapse after HSCT, or their evolution after the reintroduction of biological agents or other treatments. There are vague citations reporting that patients who were previously refractory to certain biological agents prior to HSCT cease to be refractory after HSCT. Furthermore, doubts exist regarding the selection of cases, which as already mentioned, are restricted to severe cases without other therapeutic options. It is not clear whether an early indication of HSCT would be beneficial to newly diagnosed patients before they become dependent on corticosteroids and develop severe perianal disease. Thus, the prognostic factors related to HSCT have not yet been determined.

Another relevant aspect is the need for studies to determine the minimum immunological screening necessary prior to HSCT. It is essential to first evaluate the immunological reconstitution of patients submitted to HSCT, and then to determine possible markers and predictive factors of relapse after the procedure.

Another type of experimental cell therapy that has been advocated is the administration of mesenchymal stromal stem cells systemically, directly, or to perianal lesions[13]. A systematic review and meta-analysis concluded that, in spite of the heterogeneity of the selected studies, the administration of mesenchymal stromal stem cells provides benefits to patients by improving lesions without causing adverse effects[20].

CONCLUSION

Thus, 25 years after the first reported use of HSCT in CD, current results allow us to conclude that cellular therapy has a place in the treatment of CD, a heterogeneous disease with multiple facets. However, the systematization with stratification of cases is necessary in order to determine the proper place and time for its implementation.

REFERENCES

1 **Baumgart DC**, Sandborn WJ. Crohn's disease. *Lancet* 2012; **380**: 1590-1605 [PMID: 22914295 DOI: 10.1016/S0140-6736(12)60026-9]

2 **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 2018; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]

3 **Kaplan GG**, Ng SC. Understanding and Preventing the Global Increase of Inflammatory Bowel Disease. *Gastroenterology* 2017; **152**: 313-321.e2 [PMID: 27793607 DOI: 10.1053/j.gastro.2016.10.020]

4 **Verstockt B**, Smith KG, Lee JC. Genome-wide association studies in Crohn's disease: Past, present and future. *Clin Transl Immunology* 2018; **7**: e1001 [PMID: 29484179 DOI: 10.1002/cti2.1001]

5 **Baumgart BC**, Daniel C. The natural history of inflammatory bowel disease. In Crohn's Disease and Ulcerative Colitis. New York, NY: Springer US, 2012 [DOI: 10.1007/978-1-4614-0998-4]

6 **Drakos PE**, Nagler A, Or R. Case of Crohn's disease in bone marrow transplantation. *Am J Hematol* 1993; **43**: 157-158 [PMID: 8342550 DOI: 10.1002/ajh.2830430223]

7 **Quevedo FJL**, López PL. Vacunas en Peditria. 3rd ed. Madrid: Editorial Medica Panamericana, 2008: 306

8 **Kashyap A**, Forman SJ. Autologous bone marrow transplantation for non-Hodgkin's lymphoma resulting in long-term remission of coincidental Crohn's disease. *Br J Haematol* 1998; **103**: 651-652 [PMID: 9858212 DOI: 10.1046/j.1365-2141.1998.01059.x]

9 **Oyama Y**, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. *Gastroenterology* 2005; **128**: 552-563 [PMID: 15765390 DOI: 10.1053/j.gastro.2004.11.051]

10 **Burt RK**, Craig RM, Milanetti F, Quigley K, Gozdziak P, Bucha J, Testori A, Halverson A, Verda L, de Villiers WJ, Jovanovic B, Oyama Y. Autologous nonmyeloablative hematopoietic stem cell transplantation in patients with severe anti-TNF refractory Crohn disease: long-term follow-up. *Blood* 2010; **116**: 6123-6132 [PMID: 20837778 DOI: 10.1182/blood-2010-06-292391]

11 **Hawkey CJ**, Allez M, Clark MM, Labopin M, Lindsay JO, Ricart E, Rogler G, Rovira M, Satsangi J, Danese S, Russell N, Gribben J, Johnson P, Larghero J, Thieblemont C, Ardizzone S, Dierickx D, Ibatici A, Littlewood T, Onida F, Schanz U, Vermeire S, Colombel JF, Jouet JP, Clark E, Saccardi R, Tyndall A, Travis S, Farge D. Autologous Hematopoetic Stem Cell Transplantation for Refractory Crohn Disease: A Randomized Clinical Trial. *JAMA* 2015; **314**: 2524-2534 [PMID: 26670970 DOI: 10.1001/jama.2015.16700]

12 **Sureda A**, Bader P, Cesaro S, Dreger P, Duarte RF, Dufour C, Falkenburg JH, Farge-Bancel D, Gennery A, Kröger N, Lanza F, Marsh JC, Nagler A, Peters C, Velardi A, Mohty M, Madrigal A. Indications for allo- and auto-SCT for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2015. *Bone Marrow Transplant* 2015; **50**: 1037-1056 [PMID: 25798672 DOI: 10.1038/bmt.2015.6]

13 **Snowden JA**, Panés J, Alexander T, Allez M, Ardizzone S, Dierickx D, Finke J, Hasselblatt P, Hawkey C, Kazmi M, Lindsay JO, Onida F, Salas A, Saccardi R, Vermeire S, Rovira M, Ricart E; European Crohn's and Colitis Organisation (ECCO); European Society for Blood and Marrow Transplantation (EBMT); Autoimmune Diseases Working Party (ADWP); Joint Accreditation Committee of the International Society for Cellular Therapy (ISCT) and EBMT (JACIE). Autologous Haematopoietic Stem Cell Transplantation (AHSCT) in Severe Crohn's Disease: A Review on Behalf of ECCO and EBMT. *J Crohns Colitis* 2018; **12**: 476-488 [PMID: 29325112 DOI: 10.1093/ecco-jcc/jjx184]

14 **Daikeler T**, Tichelli A, Passweg J. Complications of autologous hematopoietic stem cell transplantation for patients with autoimmune diseases. *Pediatr Res* 2012; **71**: 439-444 [PMID: 22430379 DOI: 10.1038/pr.2011.57]

15 **Blank N**, Lisenko K, Pavel P, Bruckner T, Ho AD, Wuchter P. Low-dose cyclophosphamide effectively mobilizes peripheral blood stem cells in patients with autoimmune disease. *Eur J Haematol* 2016; **97**: 78-82 [PMID: 26381040 DOI: 10.1111/ejh.12686]

16 **Cassinotti A**, Annaloro C, Ardizzone S, Onida F, Della Volpe A, Clerici M, Usardi P, Greco S, Maconi G, Porro GB, Deliliers GL. Autologous haematopoietic stem cell transplantation without CD34+ cell selection in refractory Crohn's disease. *Gut* 2008; **57**: 211-217 [PMID: 17895357 DOI: 10.1136/gut.2007.128694]

17 **Openshaw H**, Stuve O, Antel JP, Nash R, Lund BT, Weiner LP, Kashyap A, McSweeney P, Forman S. Multiple sclerosis flares associated with recombinant granulocyte colony-stimulating factor. *Neurology* 2000; **54**: 2147-2150 [PMID: 10851379 DOI: 10.1212/WNL.54.11.2147]

18 **Mannon PJ**, Leon F, Fuss IJ, Walter BA, Begnami M, Quezado M, Yang Z, Yi C, Groden C, Friend J, Hornung RL, Brown M, Gurprasad S, Kelsall B, Strober W. Successful granulocyte-colony stimulating factor treatment of Crohn's disease is associated with the appearance of circulating interleukin-10-producing T cells and increased lamina propria plasmacytoid dendritic cells. *Clin Exp Immunol* 2009; **155**: 447-456 [PMID: 19094118 DOI: 10.1111/j.1365-2249.2008.03799.x]

19 **Ruiz MA**, Kaiser RL Jr, de Quadros LG, Piron-Ruiz L, Peña-Arciniegas T, Faria MAG, Siqueira RC, Pirozzi FF, Kaiser FSL, Burt RK. Low toxicity and favorable clinical and quality of life impact after non-myeloablative autologous hematopoietic stem cell transplant in Crohn's disease. *BMC Res Notes* 2017; **10**: 495 [PMID: 28985769 DOI: 10.1186/s13104-017-2824-1]

20 **Lightner AL**, Wang Z, Zubair AC, Dozois EJ. A Systematic Review and Meta-analysis of Mesenchymal Stem Cell Injections for the Treatment of Perianal Crohn's Disease: Progress Made and Future Directions. *Dis Colon Rectum* 2018; **61**: 629-640 [PMID: 29578916 DOI: 10.1097/DCR.0000000000001093]

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ORIGINAL ARTICLE

Retrospective Cohort Study

Efficacy and safety of autologous stem cell transplantation for decompensated liver cirrhosis: A retrospective cohort study

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Abstract

AIM

To evaluate the long-term efficacy and safety of autologous stem cell transplantation (SCT) for decompensated liver cirrhosis.

METHODS

Consecutive patients with decompensated liver cirrhosis were included and assigned into the SCT group and non-transplantation (non-SCT) group according to whether they received SCT treatment. Patients were

followed up for ten years. The long-term survival rate and incidence of hepatocellular carcinoma (HCC) were compared between groups.

RESULTS

A total of 159 patients were enrolled, including 27 cases in the SCT group and 132 cases in the non-SCT group. The baseline characteristics were significantly different between the two groups. Propensity score matching (PSM) was used to match SCT and non-SCT patients. After PSM, 92 subjects were enrolled in the final analysis, including 23 cases in the SCT group and 69 cases in the non-SCT group. The overall mortality was 73.9% and 55.1%, and the median survival period was 48 and 64 mo, respectively. However, no significant difference was found in the long-term survival rate between the two groups ($P > 0.05$). In addition, the incidence of HCC was higher in the SCT group than in the non-SCT group (47.8% *vs* 21.7%, $P < 0.05$). After adjusting for other covariates, SCT (OR = 3.065, 95%CI: 1.378-6.814) and age (OR = 1.061, 95%CI: 1.021-1.102) were independently correlated with the development of HCC in this decompensated liver cirrhosis cohort.

CONCLUSION

Autologous SCT may fail to improve the long-term efficacy and increase the incidence of HCC for decompensated liver cirrhosis. Close monitoring of HCC is strongly recommended in patients undergoing autologous SCT.

Key words: Decompensated liver cirrhosis; Stem cell transplantation; Hepatocellular carcinoma; Propensity score matching

Core tip: Stem cell therapy has shown short-term efficacy and safety for treatment of liver cirrhosis. However, the tumorigenicity of stem cells requires increased attention.

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INTRODUCTION

Liver cirrhosis is a diffuse hepatic process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules[1]. Patients with decompensated liver cirrhosis usually have symptoms of portal hypertension and hepatic dysfunction, which greatly affects patients' quality of life and has a high mortality[2]. Currently, there is still a lack of effective treatments for decompensated liver cirrhosis, and symptomatic and supportive therapy and protection of residual hepatocytes remain the predominant strategy for the management of decompensated liver cirrhosis[3].

Orthotopic liver transplantation has been recognized as the best option for the treatment of decompensated liver cirrhosis, which improves both the quality of life and survival[1]. However, this treatment suffers from problems of huge shortage of donor livers, post-surgical complications, immune rejection, high medical expenditure and moral and ethical issues[4,5], which greatly limits its wide application in clinical practices. Development of regenerative treatment strategies for decompensated liver cirrhosis is therefore urgently needed[6,7].

Recently, stem cell-based therapy has become a novel strategy for the treatment of decompensated liver cirrhosis[8], and results from phase Ⅰ/Ⅱ clinical trials have shown generalized functional improvements and may be slightly superior to current conventional treatments[9]. It has been demonstrated that stem cells, such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), may be induced to differentiate into hepatocytes under certain conditions, which may promote liver renewal, alleviate hepatic fibrosis and be involved in the repair and reconstruction of the damaged liver. In particular, bone marrow (BM)-MSCs have been prevalently utilized[10]. Results from clinical studies showed that the liver disease patients had alleviation of clinical symptoms following autologous stem cell therapy, suggesting that stem cell therapy has short-term efficacy and safety[11-15]. However, stem cells have multi-lineage differentiation capability[10,16], and stem cell tumorigenicity has been paid increasing attention to the identification of liver cancer stem cells[17-19]. In addition, a limited follow-up period and no controls were assigned in most of the previous clinical studies[11,13], and there is little knowledge on the long-term clinical efficacy and safety of stem cell transplantation (SCT) to date.

In this retrospective cohort study, we aimed to compare the survival rate and incidence of hepatocellular carcinoma (HCC) in decompensated liver cirrhosis patients with and without SCT, so as to evaluate the long-term efficacy and safety of SCT.

MATERIALS AND METHODS

Ethical statement

This study was approved by the Ethics Review Committee of the First Affiliated Hospital of Fujian Medical University, Permission No. 2015[084]. All methods were performed in accordance with the Declaration of Helsinki regarding ethical standards for research involving human subjects.

Subjects

In this retrospective cohort study, patients with decompensated liver cirrhosis admitted to the First Affiliated Hospital of Fujian Medical University (Fuzhou, China) during the period from January 2008 through December 2010 were included. Decompensated liver cirrhosis was diagnosed by previous medical history, blood and imaging examinations or liver biopsy. Those who met the following criteria were excluded from the study: (1) subjects with HCC or cancers in other organs; (2) pregnancy; (3) subjects with severe heart, lung, renal or hematologic diseases; (4) subjects died within a month; (5) subjects with HIV infection, sepsis or other life-threatening infectious diseases; and (6) subjects without any follow-up after discharge from the hospital. All subjects were assigned into the SCT group and non-SCT group according to whether they have received SCT.

Laboratory examinations

Upon admission, all subjects received blood examinations for a prothrombin time (PT) test, a routine blood test, liver and kidney function tests and a blood glucose test, and for determining serum hepatitis B virus (HBV) markers, HBV DNA viral load and serum alpha fetoprotein (AFP) concentration. The liver function was quantified using the Child-Pugh classification and the model for end-stage liver disease (MELD) score. HCC was diagnosed according to the Expert Consensus on Standardization of the Management of HCC in China[20]. Abdominal B ultrasonography, CT or MRI scans were performed to exclude other disorders, including HCC.

SCT

Autologous bone marrow mesenchymal stem cell (BMSC) transplantation or peripheral HSC transplantation was performed according to the patients' willingness. All subjects signed the informed consent of SCT. For autologous BMSC transplantation, after skin sterilization and local anesthesia, marrow aspiration was performed in bilateral posterior-superior iliac crests. Approximately 100 mL of bone marrow (BM) was mixed evenly with BM storage buffer in a total volume of 180 mL and stored at 4 ℃ for subsequent experiments. For peripheral HSC transplantation, patients were given 300 μg (1.2 mL) recombinant human granulocyte colony stimulating factor injection for seven successive days to mobilize HSCs before transplantation, which has been demonstrated to be feasible and effective in previous studies[21,22]. HSCs were separated by a stem cell separator, and then 100 mL of HSCs were collected. The number of $CD34^+$ stem cells was counted using flow cytometry, and $CD34^+$ stem cells were obtained at a density of $(2.81 \pm 1.03) \times 10^6$ cells/mL.

Digital subtraction angiography-guided femoral artery puncture and catheterization was performed using the Seldinger technique, and hepatic angiography was conducted to identify the distribution of intrahepatic blood vessels. The catheter was inserted into the hepatic artery, and 100 mL of peripheral HSC (at a speed of 1.5 mL/min) or 180 mL of autologous BMSC (at 3 mL/min) were slowly injected with a microinfusion pump.

Follow-up

The survival and development of HCC was observed through outpatient follow-up visits. The follow up was performed during the period from the time of SCT to December 31, 2017 or death.

Statistical analysis

The patients' gender, age, cause and Child-Pugh classification were adjusted using propensity score matching (PSM)[23], and the number of cases and controls were matched at a ratio of 1:3 by means of the nearest neighborhood matching and caliper matching, with a caliper width set as 0.2. Non-normally distributed data were described as quartiles and compared using the rank-sum test. Normally distributed data were expressed as mean ± SD and compared using a Student's *t*-test. Differences of proportions were tested for statistical significance with a χ^2 test. Survival analysis was performed using the Kaplan-Meier method, and the survival rate and incidence of HCC were compared between groups with the log-rank test. The risk factors of HCC were identified using a Cox proportional hazards regression model. All data were analyzed by SPSS 18.0 software (SPSS Inc., Chicago, IL, United States), and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of baseline characteristics of overall cases between groups

A total of 218 patients with decompensated liver cirrhosis were admitted to the hospital during the period from January 2008 through December 2010, and 59 patients were excluded; finally, a total of 159 subjects were enrolled, including 27 patients undergoing SCT and 132 patients without transplantation (Figure 1). Of the 27 subjects undergoing SCT, there were 15 cases undergoing autologous bone-marrow SCT and 12 cases of peripheral hematopoietic SCT. There were significant differences in the prevalence of severe liver cirrhosis (Child-Pugh class C), PT, total bilirubin (TBIL) concentration, the prevalence of HBV infection and seropositive rate of HBsAg between the SCT group and the non-SCT group ($P < 0.05$) before PSM (Table 1). The overall mortality was 47.8% (76/159) in all study subjects, with 77.8% (21/27) mortality in the SCT group and 41.7% (55/132) in the non-SCT group ($P < 0.05$), and the overall incidence of HCC was 27.0% (43/159), with 40.7% (11/27) incidence in the SCT group and 24.2% (32/132) in the non-SCT group ($P < 0.05$).

Baseline characteristics of patients after PSM

Since the Child-Pugh classification, HBV infection, gender and age were reported to correlate with the pro-

Table 1 Comparison of the baseline demographic and clinical characteristics between the stem cell transplantation group and the non-transplantation group

Characteristic	Before propensity score matching			After propensity score matching		
	Stem cell transplantation group (n = 27)	Non-transplantation group (n = 132)	P-value	Stem cell transplantation group (n = 23)	Non-transplantation group (n = 69)	P-value
No. of male (%)	18 (66.7%)	100 (75.8%)	0.325	15 (65.2%)	45 (65.2%)	1
Age (yr)	53.7 ± 9.7	53.3 ± 10.8	0.84	53.0 ± 9.7	55.3 ± 9.8	0.351
History of smoking (%)	6 (22.2)	33 (25.0)	0.76	4 (17.4)	15 (21.7)	0.656
History of alcohol drinking, n (%)	8 (29.6)	33 (25.0)	0.616	6 (26.1)	20 (29)	0.789
Diabetes, n (%)	6 (22.2)	23 (17.4)	0.556	6 (26.1)	16 (23.2)	0.778
Family history of liver cancer, n (%)	3 (11.1)	10 (7.6)	0.541	3 (13)	3 (4.3)	0.144
Child-Pugh B to C ratio	4:23	67:65	0.001	4:19	17:52	0.473
MELD score	14.36 (9.33-18.69)	10.43 (7.22-15.91)	0.051	15.55 ± 7.66	14.21 ± 8.32	0.498
HBsAg positive, n (%)	20 (74.1)	127 (96.2)	0	20 (87)	66 (95.7)	0.144
PT (s)	21.5 (18.2-26.7)	18.2 (16.0-20.7)	0.003	22.0 (18.1-27.8)	19.3 (16.8-22.6)	0.143
TBIL (μmol/L)	88.7 (46.6-141.1)	38.4 (23.1-111.6)	0.012	73.2 (46.6-137.9)	46.4 (26.6-155.6)	0.21
ALB (g/L)	26.89 ± 4.36	28.16 ± 5.63	0.268	27.08 ± 4.59	26.27 ± 6.02	0.56
ALT (U/L)	45 (30-60)	54 (35-113)	0.055	47 (36-63)	48 (30-93)	0.701
PLT (× 10^9/L)	79.11 ± 40.39	92.66 ± 58.39	0.252	81.78 ± 41.33	90.49 ± 65.35	0.551
AFP (ng/mL)	7.62 (3.68-20.80)	6.90(2.73-33.87)	0.889	7.93 (3.9-29.37)	6.3 (2.47-23.88)	0.564
HBeAg titer (s/copies)	0.45 (0.34-11.26)	0.5 (0.34-24.68)	0.808	0.45 (0.34-11.26)	0.48 (0.34-22.13)	0.906
LogHBV DNA (IU/mL)	4.45 ± 1.21	4.47 ± 1.44	0.966	4.45 ± 1.21	4.65 ± 1.48	0.097
Cause, n (%)						
HBV infection	20 (74.1)	127 (96.2)	0	20 (87)	66 (95.7)	0.154
Alcohol drinking	4 (14.8)	1 (0.8)		1 (4.3)	0	
Others	3 (11.1)	4 (3)		2 (8.7)	3 (4.3)	

MELD: Model for end-stage liver disease; HBsAg: Hepatitis B surface antigen; PT: Prothrombin time; TBIL: Total bilirubin; ALB: Albumin; ALT: Alanine aminotransferase; PLT: Platelet; AFP: Alpha fetoprotein; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

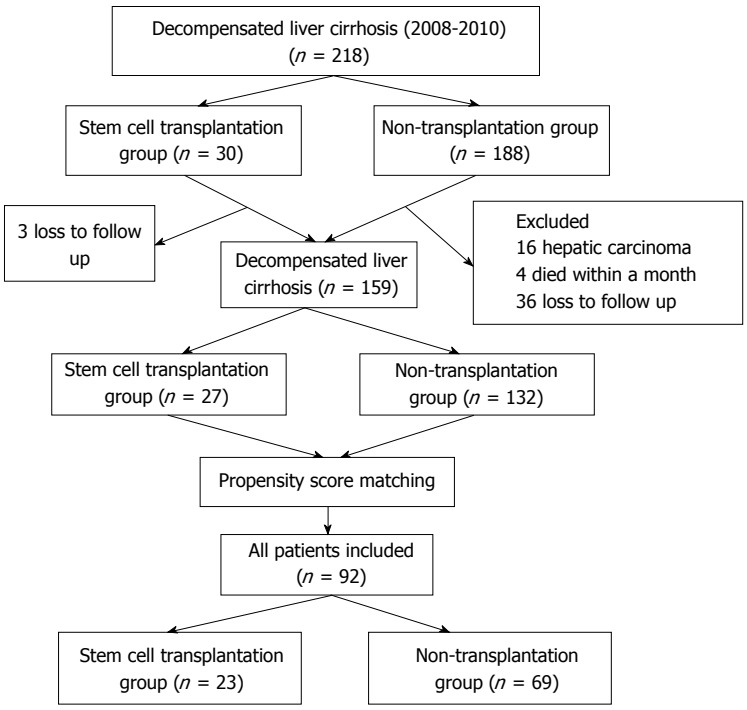

Figure 1 Flowchart of the study subject enrollment.

gnosis of liver cirrhosis[24,25], the subjects' gender, age, cause and Child-Pugh classification were adjusted using PSM at a caliper width of 0.2 and a ratio of 1:3, and finally 92 subjects were enrolled in the final analysis (Figure 1). The subjects in the SCT group after PSM (n = 23) had a mean age of 53.0 ± 9.7 years and included 12 cases undergoing autologous bone-marrow stem-cell transplantation and 11 cases of peripheral hemato-

Table 2 Comparison of the survival rate and incidence of liver cancer between the stem cell transplantation group and the non-transplantation group

	Time	Stem cell transplantation group (n = 23)	Non-transplantation group (n = 69)	χ^2 value	P-value
Survival rate	3-mo	95.70%	84.10%	0.951	0.33
	6-mo	91.30%	82.60%		
	1-yr	87.00%	73.80%		
	2-yr	73.90%	62.90%		
	3-yr	50.80%	58.10%		
	4-yr	46.20%	54.50%		
	5-yr	41.60%	50.70%		
	6-yr	32.30%	44.20%		
	7-yr	24.30%	39.50%		
Incidence of liver cancer	3-mo	0	0	6.3	0.011
	6-mo	4.80%	1.70%		
	1-yr	9.50%	5.50%		
	2-yr	20.80%	14.10%		
	3-yr	41.40%	19.30%		
	4-yr	49.80%	22.40%		
	5-yr	66.50%	29.00%		
	6-yr	66.50%	36.30%		
	7-yr	83.30%	42.70%		

poietic stem-cell transplantation, and the subjects in the non-SCT group (n = 69) had a mean age of 55.3 ± 9.8 years. The subjects in both groups had a median follow-up period of 42 mo (range: 1-118 mo). Following PSM, no significant differences were detected in the demographic and clinical features between the two groups (P > 0.05) (Table 1).

Impact of SCT on survival

Of the 92 patients with decompensated liver cirrhosis, there were 55 deaths during the study period, with an overall mortality rate of 59.8%. There were 17 deaths in the SCT group (73.9% mortality), including five cases dying of gastrointestinal bleeding, seven cases dying of end-stage HCC, three cases dying of hepatic failure and two cases dying of cerebrovascular accidents. There were 38 deaths in the non-SCT group (55.1% mortality), including 13 cases dying of gastrointestinal bleeding, 13 cases dying of hepatic failure, five cases dying of HCC, and seven cases dying of other causes (lung cancer, laryngeal cancer, arrhythmia, electrolyte disorders and infection). The median survival period was 48 mo in the SCT group and 64 mo in the non-SCT group (Figure 2). No significant difference was found in the survival rate between the two groups (P > 0.05) (Table 2 and Figure 2A).

Impact of SCT on the incidence of HCC

Of the 92 patients with decompensated liver cirrhosis, 26 patients developed HCC during the study period, with an incidence rate of 28.3%. There were 11 and 15 cases that developed HCC in the SCT group and non-SCT group, with 47.8% and 21.7% incidence, respectively, and a significant difference was observed between the two groups (P < 0.05). In addition, the 1-, 3-, 5- and 7-year incidence of HCC were all significantly higher in the SCT group than in the non-SCT group (P < 0.05) (Table 2 and Figure 2B).

Risk factors of HCC

In the univariate Cox regression analysis, SCT and age were found to correlate with the development of HCC (P < 0.05), while the medical history of diabetes, history of smoking, history of alcohol drinking, HBV infection, sex, Child-Pugh classification and family history of HCC in the first-degree relatives were not associated with the development of HCC (P > 0.05) (Table 3).

In the multivariate Cox regression, SCT (OR = 3.065, 95%CI: 1.378-6.814) and age (OR = 1.061, 95%CI: 1.021-1.102) were independently correlated with the development of HCC in this decompensated cirrhotic cohort (Table 3).

DISCUSSION

Recently, SCT has achieved great successes in the treatment of liver diseases[8]. However, there are still a large number of unsolved problems to date, such as the long-term efficacy and safety of SCT, which remain to be investigated[26].

Results from the clinical studies have shown that SCT achieves a satisfactory short-term efficacy for the treatment of decompensated liver cirrhosis[11,12]; however, the transplantation does not seem to increase the long-term efficacy[12,27,28]. In 53 liver failure patients caused by hepatitis B, a single transplantation with autologous BMSCs did not result in significant differences in liver function or MELD score between the transplantation group and controls three years after transplantation, and the 192-wk follow-up revealed no significant difference in the survival rate between the two groups, suggesting no marked improvements in long-term efficacy[12]. A recent meta-analysis to examine the clinical outcomes of the transplantation of stem cells

Table 3 Cox regression analysis of risk factors of hepatocellular carcinoma

Variable	Univariate Cox regression analysis			Multivariate Cox regression analysis		
	HR	95%CI	*P*	HR	95%CI	*P*
Stem cell transplantation	2.664	1.211-5.859	0.015	3.065	1.378-6.814	0.006
Age	1.055	1.016-1.096	0.006	1.055	1.016-1.096	0.006
Sex	1.588	0.728-3.467	0.246			
History of diabetes	1.098	0.439-2.741	0.842			
History of smoking	1.475	0.675-3.223	0.330			
History of alcohol consumption	1.546	0.698-3.423	0.283			
HBsAg positivity	0.664	0.086-5.117	0.694			
Child-Pugh classification	1.301	0.522-3.246	0.573			
Family history of liver cancer	1.283	0.303-5.444	0.735			

HBsAg: Hepatitis B surface antigen.

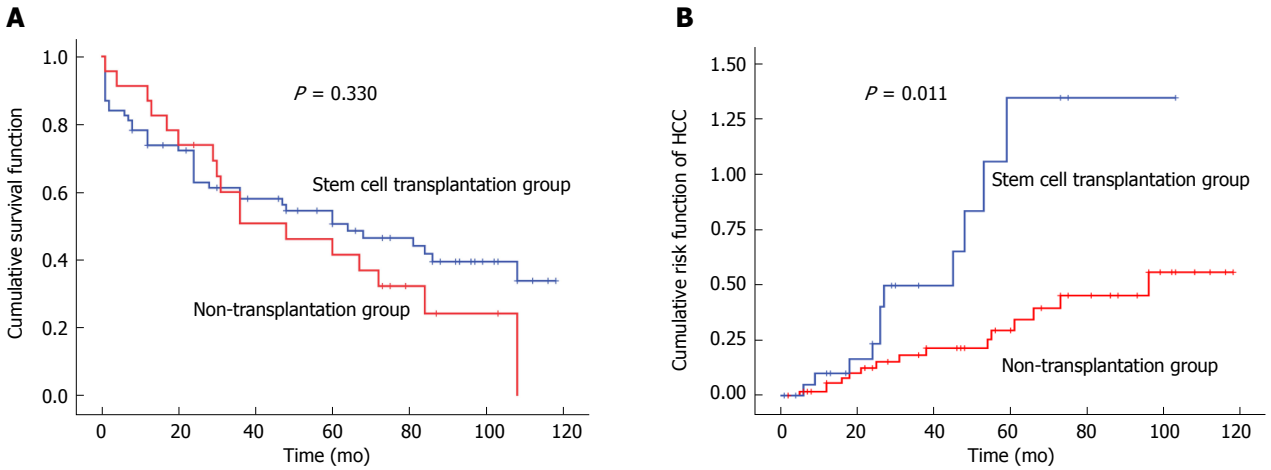

Figure 2 Long-term outcomes of the study subjects. A: Survival curve of the study subjects; B: Risk curve for hepatocellular carcinoma in the study subjects. HCC: Hepatocellular carcinoma.

from various human tissue sources in cirrhotic patients showed no significant difference in the mortality between the treatment and control groups, and concluded that SCT could improve liver function but appeared to not be significant in increasing the survival in cirrhotic patients[29]. In the current study, a 10-year follow-up revealed 73.9% (17/23) deaths in the decompensated liver cirrhosis cases undergoing SCT. We did not find a significant difference in the survival rate between the two groups, which was similar to previous reports[12,29]. The plausible explanation is that SCT can minimally reverse portal hypertension and the development of cancer in decompensated cirrhosis, as most causes of death were due to gastrointestinal bleeding and HCC in patients undergoing SCT in this cohort.

Previous studies have demonstrated the short-term safety of SCT[11,13,29,30]; however, its long-term safety has not been fully demonstrated. Results from previous clinical studies have demonstrated that SCT does not increase the risk of developing HCC[11-13,29,31]; however, the follow-up periods (no more than two years) in those studies were not long enough to observe the development of cancer. In this study, the 10-year follow-up showed a gradual increase in the incidence of HCC in the SCT group with the extension of the follow-up period. This significant difference between two groups suggested a possible tumorigenicity of SCT in patients with decompensated liver cirrhosis. Stem cells have a strong self-renewal capability and multi-lineage differentiation potential[16], and tumorigenicity of BM stem cell has been observed in animal experiments[32,33], which provides theoretical evidence for the findings from the present study. As reported by a recent review, the 5-year cumulative incidence of all second malignancies after autologous SCT for hematological disorders is 4.3%, and the 15-year cumulative incidence is 8%-15.3%[17], indicating a gradually increased risk for malignancy in patients with SCT therapy. The risk of HCC in cirrhosis patients might be associated with the activation of hepatic stellate cells and secretion of multiple growth factors and cytokines[34,35]. This may produce a microenvironment for developing HCC, thereby promoting the development and progression of HCC[35]. Follow-up of the fate of administered stem cells using combined imaging methods has been proposed as a method to discriminate tumorigenic transformation. In the future, this technology can be used to monitor liver cancer after SCT[36,37].

Approximately 80% of HCC develops from liver cirrhosis[1]. Multiple factors have been identified as the risk factors of HCC in liver fibrotic patients[31,38,39]. In

the current study, only two factors, SCT and age, were included in the multivariate Cox regression model. Multiple risk factors of HCC were excluded during PSM, such as HBV infection, resulting in no statistical significance of conventional risk factors during the univariate Cox regression analysis. However, this did not deny the significance of these variables. In addition, Cox hazard regression analysis identified age as the risk factor of HCC in patients with liver fibrosis, which may be attributed to the longer duration of liver fibrosis in older patients.

The current study has some limitations: (1) Considering the likelihood of tumorigenicity of stem cells, cancers may occur in both the liver and other organs[17,18]; however, we only found four cancers in organs other than the liver, which cannot be analyzed; and (2) This is a single-center retrospective cohort study, although we tried to match patients with and without SCT by PSM. However the selection bias and confounding bias cannot be completely excluded. Further randomized, prospective clinical trials with larger sample sizes and extension of follow-up period are required to evaluate the long-term efficacy and safety of stem cell therapy for decompensated liver cirrhosis.

In summary, the results of the present study demonstrate that SCT fails to increase the long-term survival rate and increase the incidence of HCC in patients with decompensated liver cirrhosis, indicating an unsatisfactory long-term efficacy and safety. It is suggested that close monitoring of HCC is required in patients with decompensated liver cirrhosis undergoing SCT.

ARTICLE HIGHLIGHTS

Research background

Decompensated liver cirrhosis greatly affects patients' life quality and expectancy. However, the tumorigenicity of stem cells impedes them as a basis for regenerative medicine treatment.

Research motivation

This study evaluates the long-term efficacy and safety of autologous stem cell transplantation (SCT) for decompensated liver cirrhosis based on ten years of follow-up.

Research objectives

We aimed to compare the survival rate and incidence of hepatocellular carcinoma (HCC) in decompensated liver cirrhosis patients with and without SCT, so as to evaluate the long-term efficacy and safety of SCT.

Research methods

Consecutive patients with decompensated liver cirrhosis were included and assigned into the SCT group and non-transplantation (non-SCT) group according to whether they received SCT treatment. Patients were followed up for ten years.

Research results

The incidence of HCC was higher in the SCT group than in the non-SCT group. After adjusting for other covariates, SCT and age were independently correlated with the development of HCC in this decompensated liver cirrhosis cohort.

Research conclusions

Autologous SCT may fail to improve the long-term efficacy and increase the incidence of HCC for decompensated liver cirrhosis.

Research perspectives

Close monitoring of HCC is strongly recommended in patients undergoing autologous SCT.

REFERENCES

1 **Tsochatzis EA**, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014; **383**: 1749-1761 [PMID: 24480518 DOI: 10.1016/S0140-6736(14)60121-5]

2 **Poordad FF**. Presentation and complications associated with cirrhosis of the liver. *Curr Med Res Opin* 2015; **31**: 925-937 [PMID: 25697811 DOI: 10.1185/03007995.2015.1021905]

3 **Lerschmacher O**, Koch A, Streetz K, Trautwein C, Tacke F. [Management of decompensated liver cirrhosis in the intensive care unit]. *Med Klin Intensivmed Notfmed* 2013; **108**: 646-656 [PMID: 24030843 DOI: 10.1007/s00063-013-0259-6]

4 **El-Masry M**, Puig CA, Saab S. Recurrence of non-viral liver disease after orthotopic liver transplantation. *Liver Int* 2011; **31**: 291-302 [PMID: 21281429 DOI: 10.1111/j.1478-3231.2010.02434.x]

5 **Abdeldayem HM**, Allam NA, Salah E, Mostafa Aziz A, Kashkoush S, Adawy NM, Gad H, Helmy A. Moral and ethical issues in living-donor liver transplant in Egypt. *Exp Clin Transplant* 2009; **7**: 18-24 [PMID: 19364307]

6 **Giri S**, Bader A. Personalized and Regenerative Medicine for Liver Diseases. *Curr Stem Cell Res Ther* 2016; **11**: 692-705 [PMID: 26496884 DOI: 10.2174/1574888X10666151026115128]

7 **Kholodenko IV**, Yarygin KN. Cellular Mechanisms of Liver Regeneration and Cell-Based Therapies of Liver Diseases. *Biomed Res Int* 2017; **2017**: 8910821 [PMID: 28210629 DOI: 10.1155/2017/8910821]

8 **Shiota G**, Itaba N. Progress in stem cell-based therapy for liver disease. *Hepatol Res* 2017; **47**: 127-141 [PMID: 27188253 DOI: 10.1111/hepr.12747]

9 **Kwak KA**, Cho HJ, Yang JY, Park YS. Current Perspectives Regarding Stem Cell-Based Therapy for Liver Cirrhosis. *Can J Gastroenterol Hepatol* 2018; **2018**: 4197857 [PMID: 29670867 DOI: 10.1155/2018/4197857]

10 **Tsolaki E**, Yannaki E. Stem cell-based regenerative opportunities for the liver: State of the art and beyond. *World J Gastroenterol* 2015; **21**: 12334-12350 [PMID: 26604641 DOI: 10.3748/wjg.v21.i43.12334]

11 **Suk KT**, Yoon JH, Kim MY, Kim CW, Kim JK, Park H, Hwang SG, Kim DJ, Lee BS, Lee SH, Kim HS, Jang JY, Lee CH, Kim BS, Jang YO, Cho MY, Jung ES, Kim YM, Bae SH, Baik SK. Transplantation with autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: Phase 2 trial. *Hepatology* 2016; **64**: 2185-2197 [PMID: 27339398 DOI: 10.1002/hep.28693]

12 **Peng L**, Xie DY, Lin BL, Liu J, Zhu HP, Xie C, Zheng YB, Gao ZL. Autologous bone marrow mesenchymal stem cell transplantation in liver failure patients caused by hepatitis B: short-term and long-term outcomes. *Hepatology* 2011; **54**: 820-828 [PMID: 21608000 DOI: 10.1002/hep.24434]

13 **Spahr L**, Chalandon Y, Terraz S, Kindler V, Rubbia-Brandt L, Frossard JL, Breguet R, Lanthier N, Farina A, Passweg J, Becker CD, Hadengue A. Autologous bone marrow mononuclear cell transplantation in patients with decompensated alcoholic liver disease: a randomized controlled trial. *PLoS One* 2013; **8**: e53719 [PMID: 23341981 DOI: 10.1371/journal.pone.0053719]

14 **Tsuchiya A**, Kojima Y, Ikarashi S, Seino S, Watanabe Y, Kawata Y, Terai S. Clinical trials using mesenchymal stem cells in liver diseases and inflammatory bowel diseases. *Inflamm Regen* 2017; **37**: 16 [PMID: 29259715 DOI: 10.1186/s41232-017-0045-6]

15 **Amer ME**, El-Sayed SZ, El-Kheir WA, Gabr H, Gomaa AA, El-Noomani N, Hegazy M. Clinical and laboratory evaluation of patients with end-stage liver cell failure injected with bone marrow-derived hepatocyte-like cells. *Eur J Gastroenterol Hepatol* 2011; **23**: 936-941 [PMID: 21900788 DOI: 10.1097/MEG.0b013e32834-

88b00]
16 **Trounson A**, McDonald C. Stem Cell Therapies in Clinical Trials: Progress and Challenges. *Cell Stem Cell* 2015; **17**: 11-22 [PMID: 26140604 DOI: 10.1016/j.stem.2015.06.007]
17 **Danylesko I**, Shimoni A. Second Malignancies after Hematopoietic Stem Cell Transplantation. *Curr Treat Options Oncol* 2018; **19**: 9 [PMID: 29423555 DOI: 10.1007/s11864-018-0528-y]
18 **Ben-David U**, Benvenisty N. The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nat Rev Cancer* 2011; **11**: 268-277 [PMID: 21390058 DOI: 10.1038/nrc3034]
19 **Sell S**, Leffert HL. Liver cancer stem cells. *J Clin Oncol* 2008; **26**: 2800-2805 [PMID: 18539957 DOI: 10.1200/JCO.2007.15.5945]
20 **Ye SL**. [Expert consensus on standardization of the management of primary liver cancer]. *Zhonghua Ganzangbing Zazhi* 2009; **17**: 403-410 [PMID: 19645134]
21 **Sharma M**, Rao PN, Sasikala M, Kuncharam MR, Reddy C, Gokak V, Raju B, Singh JR, Nag P, Nageshwar Reddy D. Autologous mobilized peripheral blood CD34(+) cell infusion in non-viral decompensated liver cirrhosis. *World J Gastroenterol* 2015; **21**: 7264-7271 [PMID: 26109814 DOI: 10.3748/wjg.v21.i23.7264]
22 **Salama H**, Zekri AR, Bahnassy AA, Medhat E, Halim HA, Ahmed OS, Mohamed G, Al Alim SA, Sherif GM. Autologous CD34+ and CD133+ stem cells transplantation in patients with end stage liver disease. *World J Gastroenterol* 2010; **16**: 5297-5305 [PMID: 21072892 DOI: 10.3748/wjg.v16.i42.5297]
23 **Pingault JB**, Côté SM, Petitclerc A, Vitaro F, Tremblay RE. Assessing the independent contribution of maternal educational expectations to children's educational attainment in early adulthood: a propensity score matching analysis. *PLoS One* 2015; **10**: e0119638 [PMID: 25803867 DOI: 10.1371/journal.pone.0119638]
24 **Piekarska A**, Zboinska J, Szymczak W, Kuydowicz J. Independent prognostic factors in patients with liver cirrhosis. *Hepatogastroenterology* 2008; **55**: 1034-1040 [PMID: 18705324]
25 **D'Amico G**, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. *J Hepatol* 2006; **44**: 217-231 [PMID: 16298014 DOI: 10.1016/j.jhep.2005.10.013]
26 **Di Nardo P**, Singla D, Li RK. The challenges of stem cell therapy. *Can J Physiol Pharmacol* 2012; **90**: 273-274 [PMID: 22338594 DOI: 10.1139/y2012-016]
27 **Liu Z**, Li J, Li P, Bai M, Guo Y, Han M, Zhang F, Ahmed R, Jin S. Stem cell transplantation for the treatment of liver diseases: A systematic review and meta-analysis. *Turk J Gastroenterol* 2016; **27**: 499-508 [PMID: 27852540 DOI: 10.5152/tjg.2016.16398]
28 **Mohamadnejad M**, Alimoghaddam K, Bagheri M, Ashrafi M, Abdollahzadeh L, Akhlaghpoor S, Bashtar M, Ghavamzadeh A, Malekzadeh R. Randomized placebo-controlled trial of mesenchymal stem cell transplantation in decompensated cirrhosis. *Liver Int* 2013; **33**: 1490-1496 [PMID: 23763455 DOI: 10.1111/liv.12228]
29 **Qi X**, Guo X, Su C. Clinical outcomes of the transplantation of stem cells from various human tissue sources in the management of liver cirrhosis: a systematic review and meta-analysis. *Curr Stem Cell Res Ther* 2015; **10**: 166-180 [PMID: 25391380 DOI: 10.2174/1574888X09666141112114011]
30 **Nakamura T**, Torimura T, Iwamoto H, Kurogi J, Inoue H, Hori Y, Sumie S, Fukushima N, Sakata M, Koga H, Abe M, Ikezono Y, Hashimoto O, Ueno T, Oho K, Okamura T, Okuda S, Kawamoto A, Ii M, Asahara T, Sata M. CD34(+) cell therapy is safe and effective in slowing the decline of hepatic reserve function in patients with decompensated liver cirrhosis. *J Gastroenterol Hepatol* 2014; **29**: 1830-1838 [PMID: 24731186 DOI: 10.1111/jgh.12622]
31 **Mohamadnejad M**, Ashrafi M, Alimoghaddam K, Vosough M, Mardpour S, Azimian V, Aghdami N, Bagheri M, Abdollahzadeh L, Bashtar M, Akhlaghpoor S, Ghavamzadeh A, Baharvand H, Malekzadeh R. Surveillance for hepatocellular carcinoma after autologous stem cell transplantation in cirrhosis. *Middle East J Dig Dis* 2012; **4**: 145-149 [PMID: 24829648]
32 **Nussbaum J**, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *FASEB J* 2007; **21**: 1345-1357 [PMID: 17284483 DOI: 10.1096/fj.06-6769com]
33 **Petersen BE**, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. Bone marrow as a potential source of hepatic oval cells. *Science* 1999; **284**: 1168-1170 [PMID: 10325227 DOI: 10.1126/science.284.5417.1168]
34 **Thompson AI**, Conroy KP, Henderson NC. Hepatic stellate cells: central modulators of hepatic carcinogenesis. *BMC Gastroenterol* 2015; **15**: 63 [PMID: 26013123 DOI: 10.1186/s12876-015-0291-5]
35 **Carloni V**, Luong TV, Rombouts K. Hepatic stellate cells and extracellular matrix in hepatocellular carcinoma: more complicated than ever. *Liver Int* 2014; **34**: 834-843 [PMID: 24397349 DOI: 10.1111/liv.12465]
36 **Labusca LS**, Herea DD, Radu E, Danceanu C, Chiriac H, Lupu N. Human Adipose Derived Stem Cells and Osteoblasts Interaction with Fe-Cr-Nb-B Magnetic Nanoparticles. *J Nanosci Nanotechnol* 2018; **18**: 5143-5153 [PMID: 29442706 DOI: 10.1166/jnn.2018.15330]
37 **Labusca L**, Herea DD, Mashayekhi K. Stem cells as delivery vehicles for regenerative medicine-challenges and perspectives. *World J Stem Cells* 2018; **10**: 43-56 [PMID: 29849930 DOI: 10.4252/wjsc.v10.i5.43]
38 **Takami T**, Terai S, Sakaida I. Novel findings for the development of drug therapy for various liver diseases: Current state and future prospects for our liver regeneration therapy using autologous bone marrow cells for decompensated liver cirrhosis patients. *J Pharmacol Sci* 2011; **115**: 274-278 [PMID: 21350310 DOI: 10.1254/jphs.10R13FM]
39 **Yi SW**, Choi JS, Yi JJ, Lee YH, Han KJ. Risk factors for hepatocellular carcinoma by age, sex, and liver disorder status: A prospective cohort study in Korea. *Cancer* 2018; **124**: 2748-2757 [PMID: 29669170 DOI: 10.1002/cncr.31406]

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REVIEW

Cytokines in adipose-derived mesenchymal stem cells promote the healing of liver disease

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Abstract

Adipose-derived mesenchymal stem cells (ADSCs) are a treatment cell source for patients with chronic liver injury. ADSCs are characterized by being harvested from the patient's own subcutaneous adipose tissue, a high cell yield (*i.e.*, reduced immune rejection response), accumulation at a disease nidus, suppression of excessive immune response, production of various

growth factors and cytokines, angiogenic effects, anti-apoptotic effects, and control of immune cells via cell-cell interaction. We previously showed that conditioned medium of ADSCs promoted hepatocyte proliferation and improved the liver function in a mouse model of acute liver failure. Furthermore, as found by many other groups, the administration of ADSCs improved liver tissue fibrosis in a mouse model of liver cirrhosis. A comprehensive protein expression analysis by liquid chromatography with tandem mass spectrometry showed that the various cytokines and chemokines produced by ADSCs promote the healing of liver disease. In this review, we examine the ability of expressed protein components of ADSCs to promote healing in cell therapy for liver disease. Previous studies demonstrated that ADSCs are a treatment cell source for patients with chronic liver injury. This review describes the various cytokines and chemokines produced by ADSCs that promote the healing of liver disease.

Key words: Cell transplantation therapy; Cytokine; Hepatocytes; Liquid chromatography with tandem mass spectrometry; Liver cirrhosis; Adipose-derived mesenchymal stem cells

Core tip: We previously showed that conditioned medium of adipose-derived mesenchymal stem cells (ADSCs) promoted hepatocyte proliferation and improved the liver function in a mouse model of acute liver failure. Furthermore, as reported by many other groups, the administration of ADSCs improved liver tissue fibrosis in a mouse model of liver cirrhosis. A comprehensive protein expression analysis by liquid chromatography with tandem mass spectrometry showed that the various cytokines and chemokines produced by ADSCs have the ability to promote the healing of liver disease. In this review, we examine the ability of the expressed protein components of ADSCs to promote healing in cell therapy for liver disease.

Nahar S, Nakashima Y, Miyagi-Shiohira C, Kinjo T, Toyoda Z, Kobayashi N, Saitoh I, Watanabe M, Noguchi H, Fujita J. Cytokines in adipose-derived mesenchymal stem cells promote the healing of liver disease. *World J Stem Cells* 2018; 10(11): 146-159 Available from: URL: http://www.wjgnet.com/1948-0210/full/v10/i11/146.htm DOI: http://dx.doi.org/10.4252/wjsc.v10.i11.146

INTRODUCTION

We and others have conducted clinical studies in patients with symptoms of autoimmune liver disease[1], hepatitis C[2,3], bacterial infection[4], acute liver failure liver[5], nonalcoholic fatty liver disease[6] and cirrhosis. We have also conducted translational research that bridges basic research using hematopoietic cells[7-14], hepatic stellate cells (HSCs)[15-18], embryonic stem cell-derived hepatocytes[19-22], bioartificial livers[23-26], animals[27-33] and clinical research in humans.

Thanks to their expected therapeutic efficacy, mesenchymal stem cells[34] are currently under clinical evaluation as a cell source for cell therapy in trials of regenerative medicine for a broad spectrum of diseases. Since adipose-derived mesenchymal stem cells (ADSCs)[35,36] are obtained from the patient's abdomen by liposuction, cell procurement is relatively easy (large numbers of cells can be obtained by minimally invasive treatment). ADSCs can avoid immune rejection if they are autografted[37-39]; however, similarly to other cell sources, they are subject to immune rejection if allogeneic or xeno cell transplantation is performed. Mesenchymal stem cells are used for medical treatment worldwide. Since ADSCs are not a mainstream therapeutic cell, we have been performing clinical studies of treatments using ADSCs. For this reason, cell therapy using ADSCs is now performed in many public and private hospitals worldwide. In this review, we examine the effects of ADSCs in cell therapy for liver diseases, focusing on the proteins secreted by ADSCs. We previously reported that the proteins expressed by human ADSCs cultured using Dulbecco's Modified Eagle's medium [10% fetal bovine serum (FBS)] and clinical-grade chemically-defined medium showed 98% similarity, demonstrating that the proteins expressed by ADSCs cultured in media for research and clinical use largely coincide[40,41]. However, using animal-derived components in the process of culturing cells for treatment is associated with a risk of transmitting pathogenic infections derived from animals (*e.g.*, bovine spongiform encephalopathy or swine fever). Furthermore, when animal-derived ingredients are used the risks and quality may vary in individual lots. Thus, the use of chemically defined media with recombinant protein is recommended for large-scale culture conditions, such as the manufacturing of therapeutic cells for industrial use. In this background, we use the medium containing FBS as the control medium for the cultivation of research cells, while chemically-defined media is the first choice for culturing therapeutic cells. In addition, we do not deny the option of using a supplement that uses infectious and highly safe virus-tested human serum as a raw material for culturing therapeutic cells.

Gene ontology (GO) facilitates[42,43] the development of a common vocabulary to describe biological concepts. Terms defined in GO are called GO terms (GO is a classification of biological phenomena that associates genes with their known [reported in the existing literature] biological role structured based on given criteria), which are divided into three categories: Biological processes, cellular components, and molecular functions. The Gene Ontology Consortium (http://www.

geneontology.org/) is a database of functional information that aims to describe biological phenomena in standardized terms. In recent years, liquid chromatography with tandem mass spectrometry (LC-MS/MS) has been used to perform a GO classification of comprehensive expression data using protein analysis software programs. Both a comprehensive expression analysis of proteins using LC-MS/MS and a protein GO analysis were performed according to methods that we reported previously[40,41,44].

In normal liver tissue, blood flows from the portal vein through the central veins, each of which supplies sufficient blood to the hepatocytes of a single hepatic lobule (Figure 1). The blood supply to hepatocytes remains sufficient in carbon tetrachloride (CCL4: 0.5 mL/kg)-administered acute liver failure model in mice[40,41]. Liver failure is a defined as a group of diseases associated with the development of symptoms such as jaundice, ascites, hepatic encephalopathy, bleeding tendency, or the like, due to a decrease in the number of hepatocytes or a decrease in their function. Acute liver failure is defined by the presence of necrosis and inflammation in normal liver tissue, with the period until the onset of symptoms of hepatic insufficiency being within 8 wk. Cases in which the onset of symptoms is 8-24 wk or > 24 wk are classified as delayed liver failure (late onset hepatic failure; LOHF) and chronic liver failure, respectively. The American Association for the Study of Liver Diseases published a position paper on acute liver failure in 2005 to unify the terms and disease concepts[45].

In contrast to this acute model, the preparation of the CCL4 (2.0 mL/kg)-administered cirrhosis model mouse requires more than 6 wk. The liver tissue in this mouse model of liver cirrhosis shows a marked increase in the fiber component of the fibrous septa. This results in the separation of some lobules by fiber components, and the creation of pseudo-lobules. At the same time, the blood supply to the lobules decreases and liver cell necrosis occurs (Figure 2). Chronic liver injury also results in the differentiation of astrocytes into myofibroblastoid cells, in turn causing the pathogenesis of fibrotic liver injury[46]. Given this background, the factor most necessary for the improvement of acute liver failure symptoms is hepatocyte growth factor (HGF). In contrast, the most important factors for the improvement of the symptoms of chronic liver failure (*i.e.,* liver cirrhosis) are: (1) growth factors; (2) inhibition of the inflammation of hepatic stellate cells; and (3) angiogenic factors.

When the disordered repair process is delayed or inhibited after liver damage from drugs, trauma, inflammation, or other insults, liver regeneration is insufficient and hepatic failure develops. In hepatic tissue repair, in addition to growth factors that promote hepatocyte proliferation, angiogenic factors that promote hepatic microvascular remodeling are important. In addition, the extracellular matrix in the liver is mainly produced in hepatic stellate cells. The ability of hepatic stellate cells to produce extracellular matrix is low in the normal liver. However, in the fibrous liver, it is known that hepatic stellate cells are activated to differentiate into myofibroblasts and their ability to produce extracellular matrix markedly increases. Interleukin-6 (IL-6), tumor necrosis factor-α, HGF, and other factors secreted from HSCs, hepatic sinusoidal endothelial cells and Kupffer cells are thought to have the greatest influence on hepatocyte proliferation[47]. Angiogenesis is a physiological phenomenon in which a new blood vessel branch is branched from an existing blood vessel to construct a vascular network. The various factors involved in angiogenesis include fibroblast growth factor, vascular endothelial cell growth factor (VEGF), angiopoietin, and platelet derived growth factor (PDGF).

GROWTH FACTORS IMPROVING THE SYMPTOMS OF LIVER CIRRHOSIS

HGF acts as a HGF and metabolic regulator and promotes hepatocyte proliferation[48]. The hepatic development of the liver is the origin of the gut tube, which is formed by the accumulation of hematopoietic cells[49]. Thus, the idea that HGF expressed by hematopoietic cells promotes the regeneration of hepatocytes is plausible. Liver tissue regeneration using HGF may accordingly be considered a treatment method that reproduces the original development of the liver. HGF is expressed by both HSCs and ADSCs.

Our group previously investigated the clinical application of organ preservation solution[50-56]. We found that the expression level of HGF mRNA did not decrease in ADSCs, even when they were stored in preservation solution for 16 h after separation from adipose tissue. In addition, we found no difference between the expression levels of HGF using glucose-free University of Wisconsin and glucose-containing (5.6 mmol/L) Hank's Balanced Salt Solution. This result shows that the expression of HGF by ADSCs does not decrease after separation from adipose tissue. Moreover, the expression is not affected by the glucose concentration. In addition, the expression of VEGF showed a similar tendency. In short, ADSCs constantly express HGF and VEGF both *in vivo* and *in vitro*[57]. A recent theory suggests that the biliary tree functions as a source of liver and pancreatic stem cells and progenitor cells. VEGF is secreted by the biliary tree as a response to stress[58]. From these developmental perspectives, HGF and VEGF secreted by ADSCs appear to have a marked promoting effect on hepatocyte proliferation.

Our experiments showed that the administration of ADSC conditioned medium (CM) from a single vein rapidly promotes the cellular proliferation of mouse hepatocytes (Figure 3). The proteins associated with a growth function (GO analysis), identified by the presen-

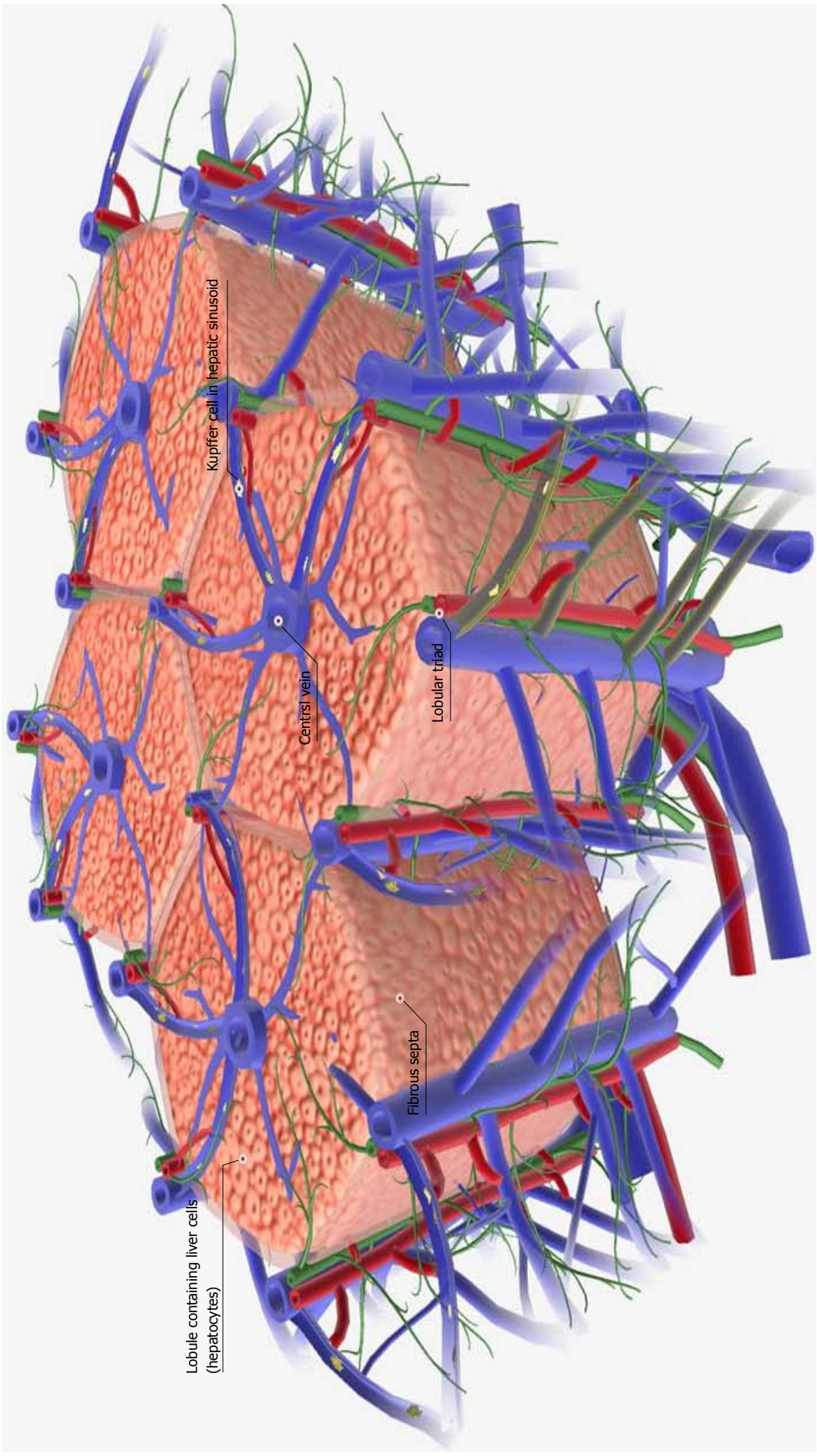

Figure 1 Schematic diagram of a normal liver tissue model. The liver is a digestive organ that filters and detoxifies blood from the digestive tract. It also produces proteins, such as albumin, and synthesizes cholesterol and bile. The functional portion of the liver tissue is organized into hexagonal columns called liver lobules. Each liver lobule contains hundreds of individual liver cells (hepatocytes) and a large central vein. Lobular portal triads, which contain branches from the hepatic portal vein, hepatic artery and bile duct, are located at the points of the hexagonal lobule. Blood from the branches of the hepatic artery joins the blood of the hepatic vein branches, forming hepatic sinusoids. Hepatic sinusoids are lined with specialized cells called Kupffer cells, which help collect debris and detoxify the blood. All hepatic sinusoids in the liver lobule drain into the central vein. Adjacent lobules are separated by a thin fibrous septa. Images were obtained from BIODIGITAL HUMAN 3.0 (https://human.biodigital.com/index.html) (BioDigital, Broadway, NY, United States).

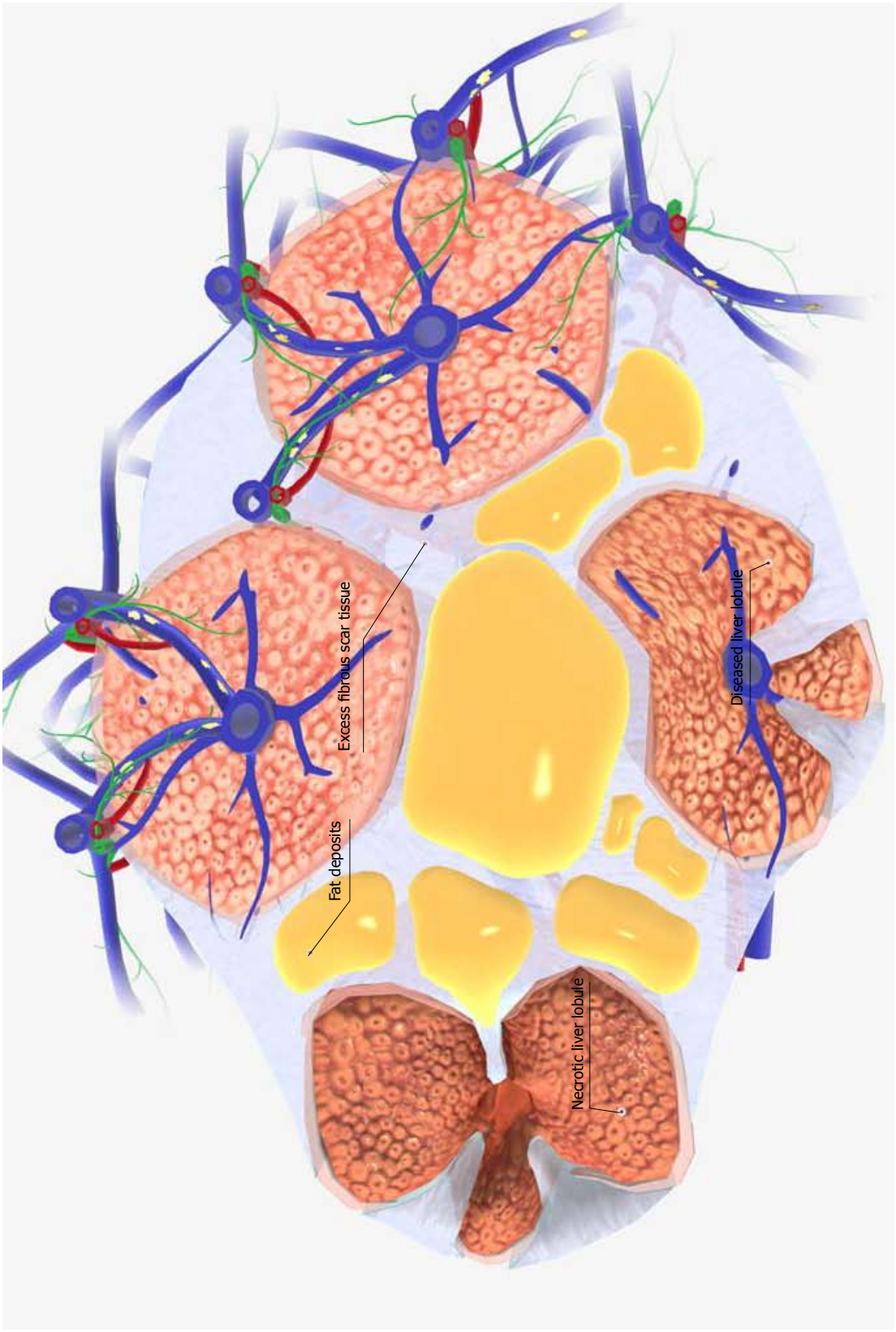

Figure 2 Schematic diagram of a liver cirrhosis tissue model. The functional portion of the liver tissue is organized into hexagonal columns called liver lobules. Each liver lobule contains hundreds of individual liver cells (hepatocytes). In healthy liver tissue, adjacent lobules are separated by thin fibrous septa. However, liver cirrhosis involves thickening of the fibrous septa that separate lobules, and the deposition of fat. As a result, the blood flow in the lobules is disturbed and hepatocyte necrosis occurs. Also, the fibrous septa that separate the lobules transform the lobules and produce pseudolobules. Although it has a wide variety of causes, liver cirrhosis is most commonly caused by chronic alcohol abuse, chronic hepatitis, and nonalcoholic fatty liver disease. Images were obtained from BIODIGITAL HUMAN 3.0 (https://human.biodigital.com/index.html) (BioDigital, Broadway, NY, United States).

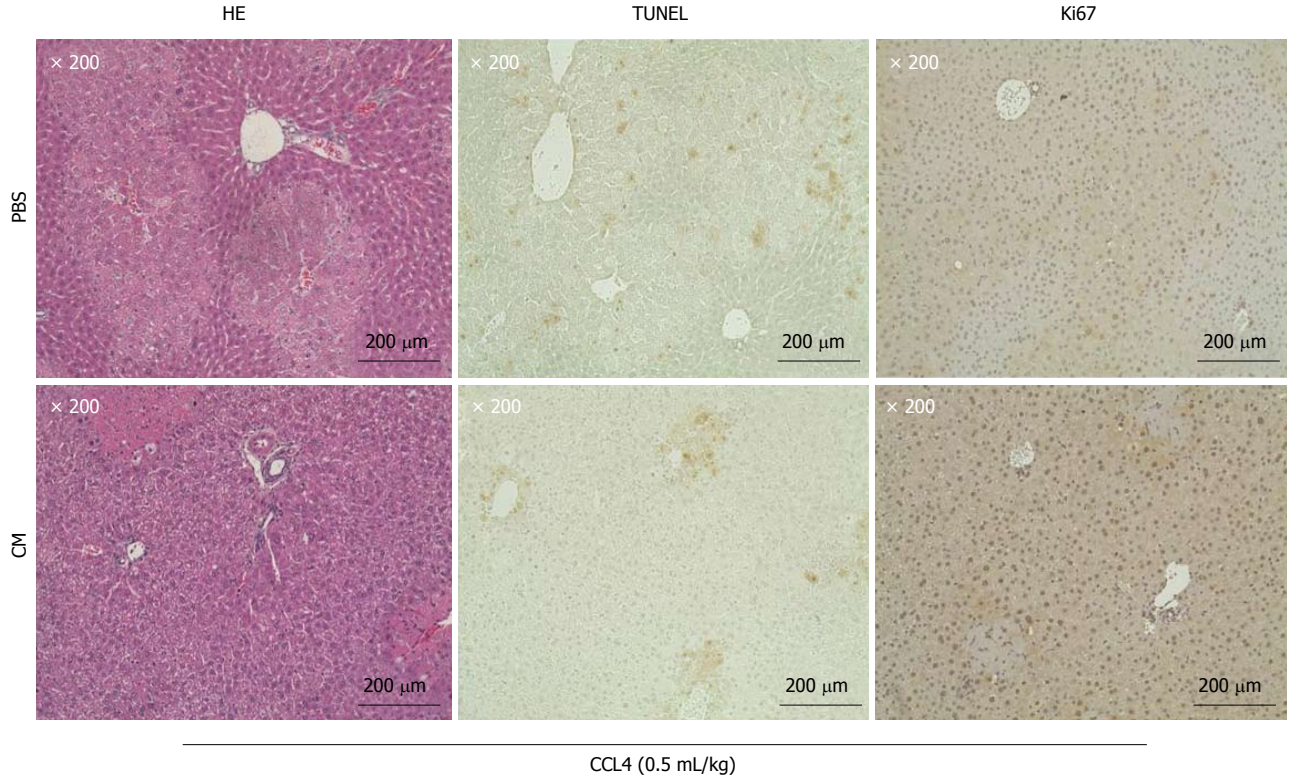

Figure 3 Culture supernatant concentrate significantly improved the symptoms of acute liver failure caused by the administration of CCL4. Micrographic images of Hematoxylin and Eosin (HE) staining (left panels), TUNEL assay (middle panels) and tissue immunostaining of Ki67 (right panels) of liver specimens. Microscopic images of liver specimens 20 h after the administration of PBS (upper panels) and CM (lower panels) *via* the mouse tail vein. Fragmented DNA generated in the process of apoptosis can be detected by the TUNEL (TdT-mediated UTP nick end labeling) method. Ki67 protein present in the nucleus of cells in G1, S, G2 and M cycles (cell growth phase) was detected using immunostaining to identify cells in the growth phase in liver tissue. It was also used to count the number of positively stained cells in images of TUNNEL-stained sections (× 200). The numbers of positively stained cells in the PBS and CM groups were 14.00 ± 4.54 and 8.25 ± 5.57, respectively (n = 4; P = 0.19). The numbers of cells with positively stained nuclei on images of Ki67-stained sections (× 200) were also counted. The numbers of cells with positively stained nuclei in the PBS and CM groups were 9.25 ± 7.61 and 116.25 ± 3.06, respectively (n = 4; P < 0.01).

ce of ADSC-CM, were Periostin (POSTN), P component (SAP), semaphorin 7A (SEM7A), and Inactive tyrosine-protein kinase 7[40,41]. Periostin, which is encoded by the POSTIN gene, has been reported to be an extracellular factor that promotes hepatosteatosis[59,60]. Nevertheless, much remains unknown about proteins with the ability to promote the cellular proliferation of hepatocytes. For example, SAP, a protein that is expressed in hepatocytes and secreted into serum, is known to be involved in processes associated with immune regulation, such as the action of opsonins[61], but whether SAP is involved in the cellular proliferation of hepatocytes is unknown. Further, SEM7A is known to contribute to transforming growth factor (TGF)-β-mediated hepatic fibrosis[62], but whether it promotes hepatocyte cell proliferation is unknown. Future studies should therefore investigate whether the growth-associated proteins that are newly identified by GO analyses promote the cellular proliferation of hepatocytes in CCL4-induced liver impairment. What is certain is that HGF and VEGF secreted by ADSCs are among the key factors promoting the proliferation of hepatocytes.

INFLAMMATION INHIBITOR OF HEPATIC STELLATE CELLS

The Jun amino-terminal kinases (JNK) signaling pathway is involved in the activation of HSCs[63,64]. JNK1 plays a major role in the upregulation of the α-SMA expression in HSCs under the stress conditions induced by TGF-β during liver fibrosis[65]. We previously reported the clinical application of organ preservation solution with a JNK inhibitory peptide (11R-JNKI)[66-68] and 8R-sJNKI(-9)[69]. The design of these cell-permeable inhibitory peptides is not only significant for *in vivo* studies, but also for future attempts to design inhibitors of liver fibrosis for the clinical treatment of liver cirrhosis. In addition, we previously reported that Arg-Gly-Asp (RGD) peptide[70] and Rho-kinase inhibitor[71] suppresses liver fibrosis. Our experiments show that the administration of ADSCs (1×10^6 cells) from a total of three veins at a twice weekly interval rapidly improves the fibrosis of excessive mesenchyme around mouse hepatocytes (Figure 4). When ADSCs are administered via the mouse tail vein, they cause pulmonary embolism, which has a high pro-

Table 1 Relationship between immunomodulatory protein secreted by adipose-derived mesenchymal stem cells and liver cirrhosis

UniProt/SWISS-PROT ID	Description		Reference
FINC	Fibronectin	Fibronectin expression is critical for liver fibrogenesis in vivo and in vitro	[74]
CO1A2	Collagen alpha-2(I) chain	N/A	
CO1A1	Collagen alpha-1(I) chain	Type I collagen has also been reported to be one of the components of hepatic fibrosis	[73]
CATB	Cathepsin B	N/A	
TSP1	Thrombospondin-1	TSP1 might contribute to liver fibrosis not only as an activator of TGF-β, but also as an modulator of angiogenesis	[75]
CFAH	Complement factor H	N/A	
GAS6	Growth arrest-specific protein 6	Gas6/Axl pathway is activated in chronic liver disease and its targeting reduces fibrosis *via* hepatic stellate cell inactivation	[77]
LEG1	Galectin-1	N/A	
PTX3	Pentraxin-related protein PTX3	The pentraxins PTX3 and SAP in innate immunity, regulation of inflammation and tissue remodelling	[78]
C1S	Complement C1s subcomponent	N/A	
SEM7A	Semaphorin-7A	N/A	
CLUS	Clusterin	N/A	
G3P	Glyceraldehyde-3-phosphate dehydrogenase	N/A	
PXDN	Peroxidasin homolog	N/A	
SRCRL	Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D	N/A	
CD248	Endosialin	CD248 reduces susceptibility to liver fibrosis *via* an effect on PDGF signalling	[79]
SPON2	Spondin-2	N/A	
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	N/A	
CD109	CD109 antigen	N/A	
CFAB	Complement factor B	N/A	
CATL1	Cathepsin L1	N/A	
MFAP5	Microfibrillar-associated protein 5	N/A	
MIF	Macrophage migration inhibitory factor	MIF exerts antifibrotic effects in experimental liver fibrosis *via* CD74	[80]
CXCL5	C-X-C motif chemokine 5	Plasma CXCL5 levels in patients with liver cirrhosis were lower than in healthy controls	[81,82]
ADAM9	Disintegrin and metalloproteinase domain-containing protein 9	N/A	
CATK	Cathepsin K	N/A	

bability of causing the death of the mouse. Our group developed a method to safely administer ADSCs using heparin[72]. The proteins associated with the immune system process (GO analysis) identified by the presence of ADSC-CM were FINC, CO1A2, CO1A1, CATB, TSP1, CFAH, GAS6, LEG1, PTX3, C1S, SEM7A, CLUS, G3P, PXDN, SRCRL, CD248, SPON2, ENPP2, CD109, CFAB, CATL1, MFAP5, MIF, CXCL5, ADA M9, and CATK (Table 1)[40,41]. Among these ADSC-secreted proteins, we found no studies reporting a relationship in the field of liver cirrhosis and hepatic stellate cells for FINC, CO1A2, CATB, CFAH, LEG1, C1S, SEM7A, CLUS, G3P, PXDN, SRCRL, SPON2, ENPP2, CD109, CFAB, CATL1, MFAP5, ADAM9, or CATK. It is expected that these proteins will be investigated in future studies.

Type I collagen and fibronectin are also reported to be components of hepatic fibrosis[73,74]. It is therefore unlikely that CO1A1 and CO1A2, which are secreted by ADSCs, suppress the excess activity of HSCs. Thrombospondin-1, a matricellular glycoprotein that is secreted by many cell types, modulates a variety of cellular functions by binding to extracellular proteins and/or cell surface receptors. Thrombospondin-1 might contribute to liver fibrosis not only as an activator of TGF-β, but also as a modulator of angiogenesis[75]. In the normal liver, growth arrest-specific gene 6 (Gas6) is mainly expressed in Kupffer cells. The expression of Gas6 increases in activated HSCs and

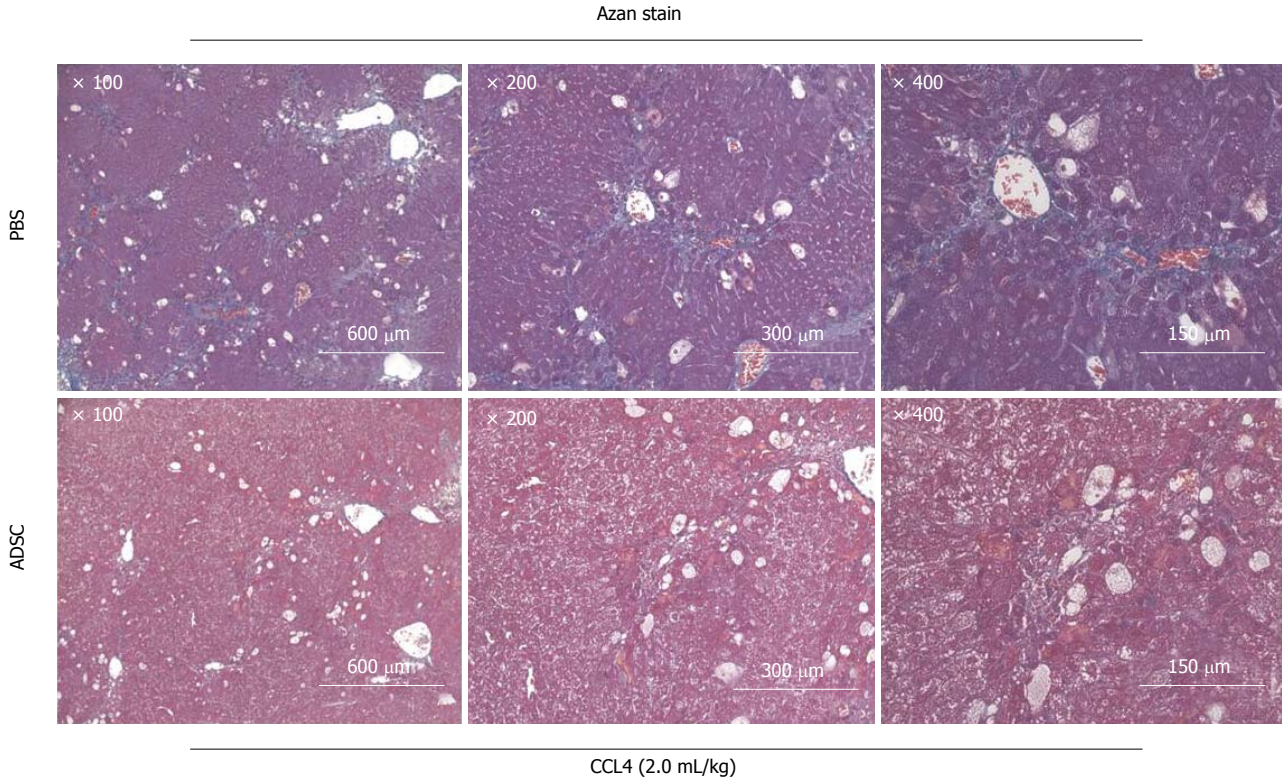

Figure 4 Adipose-derived mesenchymal stem cells improved symptoms of tissue fibrosis in cirrhosis caused by the administration of CCL4. Micrographic image of Azan staining of liver specimens. Azan staining is a fibrous connective tissue staining method that differentiates collagen fibers and muscle fibers. The fibrous connective tissue in the tissue section was stained blue. Microscopic images of the liver specimens 20 h after the administration of PBS (upper panels) and adipose-derived mesenchymal stem cells (ADSC) (lower panels) *via* the mouse tail vein. In the ADSC administration group, fibrosis and pseudolobule formation were ameliorated. Microscopic images (×100 - 400) of the same tissue section.

macrophages after acute CCL4 administration[76]. Given that Gas6 and Axl are reported to be necessary for HSC activation[77], Gas6 secreted by ADSCs seems to have no effect in inhibiting the activity of HSCs. Pentraxin 3 (PTX3) is expressed and released by hematopoietic cells and stromal cells and is an essential component of innate immunity. IL-1 induces the production of PTX3 by Kupffer cells, endothelial cells and biliary duct epithelial cells. PTX3 is reported to be a biomarker of liver fibrosis in response to hepatic injury[78]. These reports indicate that PTX3 secreted by ADSCs is unlikely to suppress the activation of HSCs. CD248 (endosialin) is a stromal cell marker expressed on fibroblasts and pericytes. During liver injury, myofibroblasts are the main source of fibrotic matrix. Liver fibrosis was reported to be suppressed in CD248 knockout mice[79], suggesting that it is unlikely that CD248 secreted by ADSCs inhibits hepatic fibrosis. Macrophage migration inhibitory factor (MIF) is a pleiotropic inflammatory cytokine that has been implicated in various inflammatory diseases. MIF knockout mice were reported to have strongly increased fibrosis in a mouse model of chronic liver injury model. This phenomenon was accompanied by no change in the infiltration of intrahepatic immune cells. MIF has an anti-fibrotic effect on the liver *via* the MIF receptor (CD74). In addition, recombinant MIF protein has a similar anti-fibrotic effect[80]. These results indicate that MIF secreted by ADSCs is a major component in the suppression of liver fibrosis. CXCL5 is the best known for its function as a neutrophil chemotactic factor and activator, with a molecular structure similar to that of IL-8, 4, 5. CXCL5 is released from monocytes, neutrophils, epithelial cells, fibroblasts and smooth muscle during inflammation. Interestingly, CXCL5 has a proliferative effect on rat hepatocytes. The use of a neutralizing antibody of CXCL5 slowed the liver regeneration rate after partial hepatectomy[81]. The plasma CXCL5 levels are low in patients with chronic liver disease, and CXCL5 may be involved in the pathogenesis of chronic liver disease[82]. These results strongly indicate the possibility that CXCL5 secreted by ADSCs also promotes hepatocyte proliferation. These findings indicated that MIF is one of the components that suppress liver fibrosis among the ADSC-secreted proteins that we identified. In addition, CXCL5 was identified as a component that promotes hepatocyte proliferation. Of course, the function of these proteins has been previously reported. Table 1 shows 26 different proteins classified as immunomodulatory (GO analysis). The 18 proteins indicated by N/A have not been reported in the liver field, and further research into them is anticipated. Note that proteins that were not classified as immunomodulatory (by a GO analysis) may

Table 2 Relationship between angiogenesis protein secreted by adipose-derived mesenchymal stem cells and liver cirrhosis

UniProt/SWISS-PROT ID	Description		Reference
PAI1	Plasminogen activator inhibitor 1	PAI-1 regulates angiogenesis via effects on extracellular matrix proteolysis and cell adhesion	[87]
FSTL1	Follistatin-related protein 1	Knockdown of Fstl1 attenuates hepatic stellate cell activation through the TGF-β1/Smad3 signaling pathway	[88]
POSTN	Periostin	POSTN, a ligand of αvβ3/5 integrins, as an effector protein in SULF2-induced angiogenesis	[89]
MMP2	72 kDa type IV collagenase	MMP2 has an important role in the preservation of liver vascular homeostasis	[91]
TSP1	Thrombospondin-1	TSP1 was reported to be increased in HSCs isolated from the liver of CCl4-induced cirrhosis model mice	[92]
TIMP1	Metalloproteinase inhibitor 1	Inhibition of TIMP1 was reported to promote angiogenesis by increasing cell motility during fibrovascular invasion	[93]
FBLN3	EGF-containing fibulin-like extracellular matrix protein 1	FBLN3 has been reported as an angiogenesis antagonist regulating cell morphology, growth, adhesion and motility	[95]
MFGM	Lactadherin	MFGM promote angiogenesis via enhanced PDGF-PDGFRβ signaling mediated by cross-talk of the integrin growth factor receptor	[97]

also have effects on the liver.

ANGIOGENIC FACTORS

EGF, VEGF and HGF, which are expressed by ADSCs, have a strong angiogenic effect on liver tissue[83-85]. Table 2 lists eight types of proteins [Plasminogen activator inhibitor 1 (PAI1), follistatin-related protein 1 (FSTL1), periostin (POSTN), matrix metalloproteinases 2 (MMP2), TSP1, metalloproteinase inhibitor 1 (TIMP1), Fibulins 3 (FBLN3) and Lactadherin (MFGM)] affecting angiogenesis from among 101 types of proteins secreted by ADSCs. PAI1 is a member of a family of proteins that inhibit plasminogen activators[86]. Although the binding of VEGF to vitronectin induces strong angiogenic signaling, this is inhibited by competitive binding to PAI1[87]. PAI1 secreted from ADSCs is therefore thought to inhibit angiogenesis by VEGF in liver tissue. FSTL1 is a secretory glycoprotein belonging to the follistatin and SPARC family. FSTL1 was reported to be highly expressed in fibrotic human liver tissue and activated HSCs[88]. FSTL1 has the effect of promoting the activity of HSCs. It is therefore unlikely that FSTL1 secreted by ADSCs affects angiogenesis. POSTN, an extracellular matrix (ECM) molecule of the fasciclin family, has roles in vascular cell differentiation and migration[89]. We therefore hypothesize that POSTN secreted by ADSCs may promote angiogenesis of the liver. MMPs are a family of over 24 zinc-dependent endopeptidases capable of degrading virtually any component of the ECM[90]. MMP2 plays an important role in the preservation of liver vascular homeostasis *via* its participation in the TGF-β activation process[91]. MMP2 secreted by ADSCs-and many other cell types-is therefore considered to be one of the main factors. TSP1 was reported to be increased in HSCs isolated from the liver in a mouse model of CCL4-induced cirrhosis. In liver samples of patients with alcohol cirrhosis and non-alcoholic steatohepatitis-related cirrhosis, TSP1 levels were reported to be increased[92]. It is thought that TSP1 expressed in the liver has the effect of promoting liver fibrosis. If so, it would be unlikely that TSP1 secreted by ADSCs promotes liver angiogenesis. TIMP1 is a widely expressed inhibitor of MMPs. Given that the inhibition of TIMP1 promotes angiogenesis by increasing cell motility during fibrovascular invasion[93], TIMP1 secreted by ADSCs may inhibit liver angiogenesis. FBLNs, a versatile family of extracellular matrix proteins, comprise a small family of widely expressed ECM proteins[94]. FBLN3 has been reported to be an angiogenesis antagonist that regulates cell morphology, growth, adhesion and motility[95]. It is therefore unlikely that FBLN3 secreted by ADSCs promotes liver angiogenesis. MFGM interacts with alphaV-beta3 and alphaV-beta5 integrins and alters both VEGF-dependent Akt phosphorylation and neovascularization[96]. MFGM was reported to promote angiogenesis *via* enhanced PDGF-PDGFRβ signaling mediated by cross-talk of the integrin growth factor receptor[97]. Thus, MFGM secreted by ADSCs is considered to be one of the main components promoting liver angiogenesis. We recently reported that ADSCs strongly express MFGM and that human MFGM protected dopamine neurons in a rat model of Parkinson's disease model[98]. At the present stage, there are no reports on a therapeutic method for the disease-specific selection of therapeutic cells that reference to the list of protein components expressed by therapeutic stem cells.

Regarding cell therapy using ADSCs, we believe that excellent effects on immune response control by cell adhesion can be expected based on reports in the literature. For treatment with ADSC-CM, we used a method to concentrate ADSC-CM 20 times using a 10k filter. ADSC-CM is a liquid and has the advantage of

being able to pass through both a 0.22-μm sterilizing filter and a 0.10-μm virus removal filter. We think that lowering the hurdles to cell therapy by taking advantage of the simple adjustment of the solution will contribute to a wide range of medical needs.

These results suggest that VEGF, HGF, EGF, MMP2, POSTN, and MFGM secreted by ADSCs promote hepatic angiogenesis. Among the 101 proteins expressed by ADSCs, we identified three proteins that promote angiogenesis from among eight proteins reported to be involved in angiogenesis. The further development of research into the 93 other proteins is expected.

A study of one-shot stem cell therapy reported the effects of bone marrow-derived mesenchymal stem cell treatment in 53 cirrhosis patients[99]. Although study period was sufficient to observe short-term symptomatic improvement over a period of days to weeks, it has been reported that there is no improvement in symptoms over the longer term (more than a few months). Although this study did not involve the use of ADSCs, it showed that stem cell treatment improves the symptoms of cirrhosis over the short term. However, it shows that one-shot stem cell therapy does not reset the pathological state of cirrhosis through natural healing power leading to recovery. In this paper, we reported that ADSC has three angiogenesis-inducing effects, which promoted the proliferation of hepatocytes, which suppressed the fibrosis of the liver tissue. We believe that medical stakeholders and patients will be more likely to challenge clinical studies of ADSCs in the treatment of cirrhosis. However, it is unlikely that ADSCs play a direct role in controlling all of the pathological conditions of cirrhosis in the body. Thus, we are of the opinion that cell therapy using ADSCs and treatment using ADSC-CM will be useful as supplementary treatments.

CONCLUSION

The factors necessary for improvement of the symptoms of chronic liver failure (*i.e.*, liver cirrhosis) are: (1) growth factors; (2) inhibitors of hepatic stellate cell inflammation; and (3) angiogenic factors. (1) It is certain that ADSC-secreted HGF and VEGF are among the factors that promote the proliferation of hepatocytes. In addition, CXCL5 was identified as a component that promotes hepatocyte proliferation; (2) MIF, which was one of the ADSC-secreted proteins that we identified that suppressed liver fibrosis; and finally (3) ASDC-secreted VEGF, HGF, EGF, MMP2, POSTN and MFGM are factors that promote hepatic angiogenesis. It seems that the therapeutic effect on the symptoms of cirrhosis is based on ADSC-secreted growth factors, anti-inflammatory effects on stellate cells, and the anti-fibrotic and angiogenic effects of ADSC-secreted proteins.

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REFERENCES

1 **Aoyama H**, Hirata T, Sakugawa H, Watanabe T, Miyagi S, Maeshiro T, Chinen T, Kawane M, Zaha O, Nakayoshi T, Kinjo F, Fujita J. An inverse relationship between autoimmune liver diseases and Strongyloides stercoralis infection. *Am J Trop Med Hyg* 2007; **76**: 972-976 [PMID: 17488925 DOI: 10.4269/ajtmh.2007.76.972]

2 **Hoshino K**, Sugiyama M, Date T, Maruwaka S, Arakaki S, Shibata D, Maeshiro T, Hokama A, Sakugawa H, Kanto T, Fujita J, Mizokami M. Phylogenetic and phylodynamic analyses of hepatitis C virus subtype 1a in Okinawa, Japan. *J Viral Hepat* 2018; **25**: 976-985 [PMID: 29577516 DOI: 10.1111/jvh.12898]

3 **Maeshiro T**, Arakaki S, Watanabe T, Aoyama H, Shiroma J, Yamashiro T, Hirata T, Hokama A, Kinjo F, Nakayoshi T, Nakayoshi T, Mizokami M, Fujita J, Sakugawa H. Different natural courses of chronic hepatitis B with genotypes B and C after the fourth decade of life. *World J Gastroenterol* 2007; **13**: 4560-4565 [PMID: 17729406 DOI: 10.3748/wjg.v13.i34.4560]

4 **Hibiya K**, Utsunomiya K, Yoshida T, Toma S, Higa F, Tateyama M, Fujita J. Pathogenesis of systemic Mycobacterium avium infection in pigs through histological analysis of hepatic lesions. *Can J Vet Res* 2010; **74**: 252-257 [PMID: 21197224]

5 **Tanaka K**, Kobayashi N, Gutierrez AS, Rivas-Carrillo JD, Navarro-Alvarez N, Chen Y, Narushima M, Miki A, Okitsu T, Noguchi H, Tanaka N. Prolonged survival of mice with acute liver failure with transplantation of monkey hepatocytes cultured with an antiapoptotic pentapeptide V5. *Transplantation* 2006; **81**: 427-437 [PMID: 16477231 DOI: 10.1097/01.tp.0000188693.48882.18]

6 **Obika M**, Noguchi H. Diagnosis and evaluation of nonalcoholic fatty liver disease. *Exp Diabetes Res* 2012; **2012**: 145754 [PMID: 22110476 DOI: 10.1155/2012/145754]

7 **Kobayashi N**, Noguchi H, Westerman KA, Watanabe T, Matsumura T, Totsugawa T, Fujiwara T, Leboulch P, Tanaka N. Successful Retroviral Gene Transfer of Simian Virus 40 T Antigen and Herpes Simplex Virus-Thymidine Kinase into Human Hepatocytes 1. *Cell Transplant* 2001; **10**: 377-381 [PMID: 28886299 DOI: 10.3727/000000001783986585]

8 **Kobayashi N**, Noguchi H, Westerman KA, Watanabe T, Matsumura T, Totsugawa T, Fujiwara T, Leboulch P, Tanaka N. Cre/loxP-Based Reversible Immortalization of Human Hepatocytes 1. *Cell Transplant* 2001; **10**: 383-386 [PMID: 28886302 DOI: 10.3727/000000001783986558]

9 **Shahid JM**, Iwamuro M, Sasamoto H, Kubota Y, Seita M, Kawamoto H, Nakaji S, Noguchi H, Yamamoto K, Kobayashi N. Establishment of an immortalized porcine liver cell line JSNK-1 with retroviral transduction of SV40T. *Cell Transplant* 2010; **19**: 849-856 [PMID: 20955660 DOI: 10.3727/096368910X508979]

10 **Miyamoto Y**, Ikeuchi M, Noguchi H, Yagi T, Hayashi S. Spheroid Formation and Evaluation of Hepatic Cells in a Three-Dimensional Culture Device. *Cell Med* 2015; **8**: 47-56 [PMID: 26858908 DOI: 10.3727/215517915X689056]

11 **Miyamoto Y**, Ikeuchi M, Noguchi H, Yagi T, Hayashi S. Three-Dimensional In Vitro Hepatic Constructs Formed Using Combinatorial Tapered Stencil for Cluster Culture (TASCL) Device. *Cell Med* 2014; **7**: 67-74 [PMID: 26858895 DOI: 10.3727/215517914X685187]

12 **Kobayashi N**, Westerman KA, Tanaka N, Fox IJ, Leboulch P. A reversibly immortalized human hepatocyte cell line as a source of

hepatocyte-based biological support. *Addict Biol* 2001; **6**: 293-300 [PMID: 11900607 DOI: 10.1080/13556210020077019]

13 **Kobayashi N**, Tanaka N. Engineering of Human Hepatocyte Lines for Cell Therapies in Humans: Prospects and Remaining Hurdles. *Cell Transplant* 2002; **11**: 417-420 [PMID: 28866930 DOI: 10.3727/000000002783985693]

14 **Sawada S**, Kinjo T, Makishi S, Tomita M, Arasaki A, Iseki K, Watanabe H, Kobayashi K, Sunakawa H, Iwamasa T, Mori N. Downregulation of citrin, a mitochondrial AGC, is associated with apoptosis of hepatocytes. *Biochem Biophys Res Commun* 2007; **364**: 937-944 [PMID: 18273444 DOI: 10.1016/j.bbrc.2007.10.105]

15 **Watanabe T**, Shibata N, Westerman KA, Okitsu T, Allain JE, Sakaguchi M, Totsugawa T, Maruyama M, Matsumura T, Noguchi H, Yamamoto S, Hikida M, Ohmori A, Reth M, Weber A, Tanaka N, Leboulch P, Kobayashi N. Establishment of immortalized human hepatic stellate scavenger cells to develop bioartificial livers. *Transplantation* 2003; **75**: 1873-1880 [PMID: 12811248 DOI: 10.1097/01.Tp.0000064621.50907.A6]

16 **Shibata N**, Watanabe T, Okitsu T, Sakaguchi M, Takesue M, Kunieda T, Omoto K, Yamamoto S, Tanaka N, Kobayashi N. Establishment of an immortalized human hepatic stellate cell line to develop antifibrotic therapies. *Cell Transplant* 2003; **12**: 499-507 [PMID: 12953924 DOI: 10.3727/000000003108747064]

17 **Higashi N**, Kohjima M, Fukushima M, Ohta S, Kotoh K, Enjoji M, Kobayashi N, Nakamuta M. Epigallocatechin-3-gallate, a green-tea polyphenol, suppresses Rho signaling in TWNT-4 human hepatic stellate cells. *J Lab Clin Med* 2005; **145**: 316-322 [PMID: 15976760 DOI: 10.1016/j.lab.2005.03.017]

18 **Nakamuta M**, Higashi N, Kohjima M, Fukushima M, Ohta S, Kotoh K, Kobayashi N, Enjoji M. Epigallocatechin-3-gallate, a polyphenol component of green tea, suppresses both collagen production and collagenase activity in hepatic stellate cells. *Int J Mol Med* 2005; **16**: 677-681 [PMID: 16142404]

19 **Soto-Gutiérrez A**, Kobayashi N, Rivas-Carrillo JD, Navarro-Alvarez N, Zhao D, Okitsu T, Noguchi H, Basma H, Tabata Y, Chen Y, Tanaka K, Narushima M, Miki A, Ueda T, Jun HS, Yoon JW, Lebkowski J, Tanaka N, Fox IJ. Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes. *Nat Biotechnol* 2006; **24**: 1412-1419 [PMID: 17086173 DOI: 10.1038/nbt1257]

20 **Soto-Gutierrez A**, Navarro-Alvarez N, Rivas-Carrillo JD, Chen Y, Yamatsuji T, Tanaka N, Kobayashi N. Differentiation of human embryonic stem cells to hepatocytes using deleted variant of HGF and poly-amino-urethane-coated nonwoven polytetrafluoroethylene fabric. *Cell Transplant* 2006; **15**: 335-341 [PMID: 16898227 DOI: 10.3727/000000006783981945]

21 **Soto-Gutiérrez A**, Navarro-Alvarez N, Zhao D, Rivas-Carrillo JD, Lebkowski J, Tanaka N, Fox IJ, Kobayashi N. Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines. *Nat Protoc* 2007; **2**: 347-356 [PMID: 17406596 DOI: 10.1038/nprot.2007.18]

22 **Basma H**, Soto-Gutiérrez A, Yannam GR, Liu L, Ito R, Yamamoto T, Ellis E, Carson SD, Sato S, Chen Y, Muirhead D, Navarro-Alvarez N, Wong RJ, Roy-Chowdhury J, Platt JL, Mercer DF, Miller JD, Strom SC, Kobayashi N, Fox IJ. Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. *Gastroenterology* 2009; **136**: 990-999 [PMID: 19026649 DOI: 10.1053/j.gastro.2008.10.047]

23 **Kobayashi N**, Okitsu T, Tanaka N. Cell choice for bioartificial livers. *Keio J Med* 2003; **52**: 151-157 [PMID: 14529147 DOI: 10.2302/kjm.52.151]

24 **Kobayashi N**, Okitsu T, Nakaji S, Tanaka N. Hybrid bioartificial liver: establishing a reversibly immortalized human hepatocyte line and developing a bioartificial liver for practical use. *J Artif Organs* 2003; **6**: 236-244 [PMID: 14691665 DOI: 10.1007/s10047-003-0235-7]

25 **Kobayashi N**. Life support of artificial liver: development of a bioartificial liver to treat liver failure. *J Hepatobiliary Pancreat Surg* 2009; **16**: 113-117 [PMID: 19110648 DOI: 10.1007/s00534-008-0022-1]

26 **Iwamuro M**, Shiraha H, Nakaji S, Furutani M, Kobayashi N, Takaki A, Yamamoto K. A preliminary study for constructing a bioartificial liver device with induced pluripotent stem cell-derived hepatocytes. *Biomed Eng Online* 2012; **11**: 93 [PMID: 23217363 DOI: 10.1186/1475-925X-11-93]

27 **Yonekawa Y**, Okitsu T, Wake K, Iwanaga Y, Noguchi H, Nagata H, Liu X, Kobayashi N, Matsumoto S. A new mouse model for intraportal islet transplantation with limited hepatic lobe as a graft site. *Transplantation* 2006; **82**: 712-715 [PMID: 16969298 DOI: 10.1097/01.tp.0000234906.29193.a6]

28 **Totsugawa T**, Yong C, Rivas-Carrillo JD, Soto-Gutierrez A, Navarro-Alvarez N, Noguchi H, Okitsu T, Westerman KA, Kohara M, Reth M, Tanaka N, Leboulch P, Kobayashi N. Survival of liver failure pigs by transplantation of reversibly immortalized human hepatocytes with Tamoxifen-mediated self-recombination. *J Hepatol* 2007; **47**: 74-82 [PMID: 17434229 DOI: 10.1016/j.jhep.2007.02.019]

29 **Yuasa T**, Yamamoto T, Rivas-Carrillo JD, Chen Y, Navarro-Alvarez N, Soto-Guiterrez A, Noguchi H, Matsumoto S, Tanaka N, Kobayashi N. Laparoscopy-assisted creation of a liver failure model in pigs. *Cell Transplant* 2008; **17**: 187-193 [PMID: 18468249 DOI: 10.3727/000000008783906973]

30 **Yukawa H**, Noguchi H, Oishi K, Takagi S, Hamaguchi M, Hamajima N, Hayashi S. Cell transplantation of adipose tissue-derived stem cells in combination with heparin attenuated acute liver failure in mice. *Cell Transplant* 2009; **18**: 611-618 [PMID: 19775523 DOI: 10.1177/096368970901805-617]

31 **Yamamoto T**, Navarro-Alvarez N, Soto-Gutierrez A, Yuasa T, Iwamuro M, Kubota Y, Seita M, Kawamoto H, Javed SM, Kondo E, Noguchi H, Kobayashi S, Nakaji S, Kobayashi N. Treatment of acute liver failure in mice by hepatocyte xenotransplantation. *Cell Transplant* 2010; **19**: 799-806 [PMID: 20573299 DOI: 10.3727/096368910X508915]

32 **Maruyama M**, Totsugawa T, Kunieda T, Okitsu T, Shibata N, Takesue M, Kurabayashi Y, Oshita M, Nakaji S, Kodama M, Tanaka N, Kobayashi N. Hepatocyte isolation and transplantation in the pig. *Cell Transplant* 2003; **12**: 593-598 [PMID: 14579927 DOI: 10.3727/000000003108747190]

33 **Chen Y**, Kobayashi N, Suzuki S, Soto-Gutierrez A, Rivas-Carrillo JD, Tanaka K, Navarro-Alvarez N, Fukazawa T, Narushima M, Miki A, Okitsu T, Amemiya H, Tanaka N. Transplantation of human hepatocytes cultured with deleted variant of hepatocyte growth factor prolongs the survival of mice with acute liver failure. *Transplantation* 2005; **79**: 1378-1385 [PMID: 15912107 DOI: 10.1097/01.TP.0000160813.37515.97]

34 **Wagers AJ**, Weissman IL. Plasticity of adult stem cells. *Cell* 2004; **116**: 639-648 [PMID: 15006347 DOI: 10.1016/S0092-8674(04)00208-9]

35 **Szöke K**, Brinchmann JE. Concise review: therapeutic potential of adipose tissue-derived angiogenic cells. *Stem Cells Transl Med* 2012; **1**: 658-667 [PMID: 23197872 DOI: 10.5966/sctm.2012-0069]

36 **Miyagi-Shiohira C**, Kurima K, Kobayashi N, Saitoh I, Watanabe M, Noguchi Y, Matsushita M, Noguchi H. Cryopreservation of Adipose-Derived Mesenchymal Stem Cells. *Cell Med* 2015; **8**: 3-7 [PMID: 26858903 DOI: 10.3727/215517915X689100]

37 **Seki A**, Sakai Y, Komura T, Nasti A, Yoshida K, Higashimoto M, Honda M, Usui S, Takamura M, Takamura T, Ochiya T, Furuichi K, Wada T, Kaneko S. Adipose tissue-derived stem cells as a regenerative therapy for a mouse steatohepatitis-induced cirrhosis model. *Hepatology* 2013; **58**: 1133-1142 [PMID: 23686813 DOI: 10.1002/hep.26470]

38 **Ullah I**, Subbarao RB, Rho GJ. Human mesenchymal stem cells - current trends and future prospective. *Biosci Rep* 2015; **35**: e00191 [PMID: 25797907 DOI: 10.1042/BSR20150025]

39 **Miyagi-Shiohira C**, Kobayashi N, Saitoh I, Watanabe M, Noguchi

Y, Matsushita M, Noguchi H. Evaluation of Serum-Free, Xeno-Free Cryopreservation Solutions for Human Adipose-Derived Mesenchymal Stem Cells. *Cell Med* 2016; **9**: 15-20 [PMID: 28174671 DOI: 10.3727/215517916X693122]

40 **Nakashima Y**, Nahar S, Miyagi-Shiohira C, Kinjo T, Kobayashi N, Saitoh I, Watanabe M, Fujita J, Noguchi H. A Liquid Chromatography with Tandem Mass Spectrometry-Based Proteomic Analysis of Cells Cultured in DMEM 10% FBS and Chemically Defined Medium Using Human Adipose-Derived Mesenchymal Stem Cells. *Int J Mol Sci* 2018; **19**: 2042 [PMID: 30011845 DOI: 10.3390/ijms19072042]

41 **Nakashima Y,** Nahar S, Miyagi-Shiohira C, Kinjo T, Toyoda Z, Kobayashi N, Saitoh I, Watanabe M, Fujita J, Noguchi H. A liquid chromatography with tandem mass spectrometry-based proteomic analysis of the proteins secreted by human adipose-derived mesenchymal stem cells. *Cell Transplantation* 2018; **2**: 1469-1494 [PMID: 30226075 DOI: 10.1177/0963689718795096]

42 **Ashburner M**, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000; **25**: 25-29 [PMID: 10802651 DOI: 10.1038/75556]

43 **Huntley RP**, Sawford T, Martin MJ, O'Donovan C. Understanding how and why the Gene Ontology and its annotations evolve: the GO within UniProt. *Gigascience* 2014; **3**: 4 [PMID: 24641996 DOI: 10.1186/2047-217X-3-4]

44 **Nakashima Y**, Miyagi-Shiohira C, Kobayashi N, Saitoh I, Watanabe M, Noguchi H. A proteome analysis of pig pancreatic islets and exocrine tissue by liquid chromatography with tandem mass spectrometry. *Islets* 2017; **9**: 159-176 [PMID: 29099648 DOI: 10.1080/19382014.2017.1389826]

45 **Polson J**, Lee WM; American Association for the Study of Liver Disease. AASLD position paper: the management of acute liver failure. *Hepatology* 2005; **41**: 1179-1197 [PMID: 15841455 DOI: 10.1002/hep.20703]

46 **Hautekeete ML**, Geerts A. The hepatic stellate (Ito) cell: its role in human liver disease. *Virchows Arch* 1997; **430**: 195-207 [PMID: 9099976 DOI: 10.1007/BF01324802]

47 **Zimmers TA**, McKillop IH, Pierce RH, Yoo JY, Koniaris LG. Massive liver growth in mice induced by systemic interleukin 6 administration. *Hepatology* 2003; **38**: 326-334 [PMID: 12883476 DOI: 10.1053/jhep.2003.50318]

48 **Fausto N**, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology* 2006; **43**: S45-S53 [PMID: 16447274 DOI: 10.1002/hep.20969]

49 **Soto-Gutierrez A**, Navarro-Alvarez N, Caballero-Corbalan J, Tanaka N, Kobayashi N. Endoderm induction for hepatic and pancreatic differentiation of ES cells. *Acta Med Okayama* 2008; **62**: 63-68 [PMID: 18464881 DOI: 10.18926/AMO/30961]

50 **Hamada E**, Ebi N, Miyagi-Shiohira C, Tamaki Y, Nakashima Y, Kobayashi N, Saitoh I, Watanabe M, Kinjo T, Noguchi H. Comparison Between Modified Extracellular-Type Trehalose-Containing Kyoto Solution and University of Wisconsin Solution in 18-Hour Pancreas Preservation for Islet Transplantation. *Pancreas* 2018; **47**: e46-e47 [PMID: 29985851 DOI: 10.1097/MPA.0000000000001104]

51 **Nakashima Y,** Miyagi-Shiohira C, Ebi N, Hamada E, Tamaki Y, Kuwae K, Kobayashi N, Saitoh I, Watanabe M, Kinjo T, Noguchi H. A Comparison of Pancreatic Islet Purification using Iodixanol with University of Wisconsin Solution and with Na-lactobionate and Histidine Solution. *Cell Med* 2018; **10**: 1-7 [DOI: 10.1177/2155179018775071]

52 **Miyagi-Shiohira C,** Nakashima Y, Ebi N, Hamada E, Tamaki Y, Kuwae K, Kobayashi N, Saitoh I, Watanabe M, Kinjo T, Noguchi H. Comparison of tissue loading before and after the creation of a continuous density gradient in porcine islet purification. *Cell Med* 2018; **10**: 1-7 [DOI: 10.1177/2155179018781343]

53 **Miyagi-Shiohira C**, Kobayashi N, Saitoh I, Watanabe M, Noguchi Y, Matsushita M, Noguchi H. Comparison of Purification Solutions With Different Osmolality for Porcine Islet Purification. *Cell Med* 2016; **9**: 53-59 [PMID: 28174675 DOI: 10.3727/215517916X693140]

54 **Takesue M**, Maruyama M, Shibata N, Kunieda T, Okitsu T, Sakaguchi M, Totsugawa T, Kosaka Y, Arata A, Ikeda H, Matsuoka J, Oyama T, Kodama M, Ohmoto K, Yamamoto S, Kurabayashi Y, Yamamoto I, Tanaka N, Kobayashi N. Maintenance of cold-preserved porcine hepatocyte function with UW solution and ascorbic acid-2 glucoside. *Cell Transplant* 2003; **12**: 599-606 [PMID: 14579928 DOI: 10.3727/000000003108747208]

55 **Kunieda T**, Maruyama M, Okitsu T, Shibata N, Takesue M, Totsugawa T, Kosaka Y, Arata T, Kobayashi K, Ikeda H, Oshita M, Nakaji S, Ohmoto K, Yamamoto S, Kurabayashi Y, Kodama M, Tanaka N, Kobayashi N. Cryopreservation of primarily isolated porcine hepatocytes with UW solution. *Cell Transplant* 2003; **12**: 607-616 [PMID: 14579929 DOI: 10.3727/000000003108747217]

56 **Arata T**, Okitsu T, Fukazawa T, Ikeda H, Kobayashi K, Yong C, Kosaka Y, Narushima M, Matsuoka J, Yamamoto I, Tanaka N, Lakey JR, Kobayashi N. Maintenance of glucose-sensitive insulin secretion of cryopreserved human islets with University of Wisconsin solution and ascorbic acid-2 glucoside. *Artif Organs* 2004; **28**: 529-536 [PMID: 15153144 DOI: 10.1111/j.1525-1594.2004.07296.x]

57 **Nahar S,** Nakashima Y, Miyagi-Shiohira C, Kinjo T, Toyoda Z, Kobayashi N, Saitoh I, Watanabe M, Fujita J, Noguchi H. Tissue-derived mesenchymal stem cells using the university of wisconsin solution and hank's balanced salt solution. *Stem Cells Int* 2018; **2018**: 1625464 [PMID: 30258463 DOI: 10.1155/2018/1625464]

58 **de Jong IEM**, van Leeuwen OB, Lisman T, Gouw ASH, Porte RJ. Repopulating the biliary tree from the peribiliary glands. *Biochim Biophys Acta* 2018; **1864**: 1524-1531 [PMID: 28778591 DOI: 10.1016/j.bbadis.2017.07.037]

59 **Wu T**, Wu S, Ouyang G. Periostin: a new extracellular regulator of obesity-induced hepatosteatosis. *Cell Metab* 2014; **20**: 562-564 [PMID: 25295785 DOI: 10.1016/j.cmet.2014.09.005]

60 **Lu Y**, Liu X, Jiao Y, Xiong X, Wang E, Wang X, Zhang Z, Zhang H, Pan L, Guan Y, Cai D, Ning G, Li X. Periostin promotes liver steatosis and hypertriglyceridemia through downregulation of PPARα. *J Clin Invest* 2014; **124**: 3501-3513 [PMID: 25003192 DOI: 10.1172/JCI74438]

61 **Zhou Z**, Xu MJ, Gao B. Hepatocytes: a key cell type for innate immunity. *Cell Mol Immunol* 2016; **13**: 301-315 [PMID: 26685902 DOI: 10.1038/cmi.2015.97]

62 **De Minicis S**, Rychlicki C, Agostinelli L, Saccomanno S, Trozzi L, Candelaresi C, Bataller R, Millán C, Brenner DA, Vivarelli M, Mocchegiani F, Marzioni M, Benedetti A, Svegliati-Baroni G. Semaphorin 7A contributes to TGF-β-mediated liver fibrogenesis. *Am J Pathol* 2013; **183**: 820-830 [PMID: 23850082 DOI: 10.1016/j.ajpath.2013.05.030]

63 **Kluwe J**, Pradere JP, Gwak GY, Mencin A, De Minicis S, Osterreicher CH, Colmenero J, Bataller R, Schwabe RF. Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. *Gastroenterology* 2010; **138**: 347-359 [PMID: 19782079 DOI: 10.1053/j.gastro.2009.09.015]

64 **Poulos JE**, Weber JD, Bellezzo JM, Di Bisceglie AM, Britton RS, Bacon BR, Baldassare JJ. Fibronectin and cytokines increase JNK, ERK, AP-1 activity, and transin gene expression in rat hepatic stellate cells. *Am J Physiol* 1997; **273**: G804-G811 [PMID: 9357821]

65 **Hong IH**, Park SJ, Goo MJ, Lee HR, Park JK, Ki MR, Kim SH, Lee EM, Kim AY, Jeong KS. JNK1 and JNK2 regulate α-SMA in hepatic stellate cells during CCl4 -induced fibrosis in the rat liver. *Pathol Int* 2013; **63**: 483-491 [PMID: 24134609 DOI: 10.1111/pin.12094]

66 **Noguchi H**, Nakai Y, Ueda M, Masui Y, Futaki S, Kobayashi N, Hayashi S, Matsumoto S. Activation of c-Jun NH2-terminal kinase (JNK) pathway during islet transplantation and prevention

of islet graft loss by intraportal injection of JNK inhibitor. *Diabetologia* 2007; **50**: 612-619 [PMID: 17225125 DOI: 10.1007/s00125-006-0563-2]

67 **Noguchi H**, Nakai Y, Matsumoto S, Kawaguchi M, Ueda M, Okitsu T, Iwanaga Y, Yonekawa Y, Nagata H, Minami K, Masui Y, Futaki S, Tanaka K. Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. *Am J Transplant* 2005; **5**: 1848-1855 [PMID: 15996231 DOI: 10.1111/j.1600-6143.2005.00985.x]

68 **Noguchi H**. Activation of c-Jun NH2-terminal kinase during islet isolation. *Endocr J* 2007; **54**: 169-176 [PMID: 17124365 DOI: 10.1507/endocrj.KR-87]

69 **Noguchi H**, Miyagi-Shiohira C, Nakashima Y, Ebi N, Hamada E, Tamaki Y, Kuwae K, Kobayashi N, Saitoh I, Watanabe M. Modified cell-permeable JNK inhibitors efficiently prevents islet apoptosis and improves the outcome of islet transplantation. *Sci Rep* 2018; **8**: 11082 [PMID: 30038242 DOI: 10.1038/s41598-018-29481-9]

70 **Kotoh K**, Nakamuta M, Kohjima M, Fukushima M, Morizono S, Kobayashi N, Enjoji M, Nawata H. Arg-Gly-Asp (RGD) peptide ameliorates carbon tetrachloride-induced liver fibrosis via inhibition of collagen production and acceleration of collagenase activity. *Int J Mol Med* 2004; **14**: 1049-1053 [PMID: 15547672 DOI: 10.3892/ijmm.14.6.1049]

71 **Fukushima M**, Nakamuta M, Kohjima M, Kotoh K, Enjoji M, Kobayashi N, Nawata H. Fasudil hydrochloride hydrate, a Rho-kinase (ROCK) inhibitor, suppresses collagen production and enhances collagenase activity in hepatic stellate cells. *Liver Int* 2005; **25**: 829-838 [PMID: 15998434 DOI: 10.1111/j.1478-3231.2005.01142.x]

72 **Yukawa H**, Watanabe M, Kaji N, Okamoto Y, Tokeshi M, Miyamoto Y, Noguchi H, Baba Y, Hayashi S. Monitoring transplanted adipose tissue-derived stem cells combined with heparin in the liver by fluorescence imaging using quantum dots. *Biomaterials* 2012; **33**: 2177-2186 [PMID: 22192539 DOI: 10.1016/j.biomaterials.2011.12.009]

73 **Koilan S**, Hamilton D, Baburyan N, Padala MK, Weber KT, Guntaka RV. Prevention of liver fibrosis by triple helix-forming oligodeoxyribonucleotides targeted to the promoter region of type I collagen gene. *Oligonucleotides* 2010; **20**: 231-237 [PMID: 20818932 DOI: 10.1089/oli.2010.0244]

74 **Liu XY**, Liu RX, Hou F, Cui LJ, Li CY, Chi C, Yi E, Wen Y, Yin CH. Fibronectin expression is critical for liver fibrogenesis in vivo and in vitro. *Mol Med Rep* 2016; **14**: 3669-3675 [PMID: 27572112 DOI: 10.3892/mmr.2016.5673]

75 **Li Y**, Turpin CP, Wang S. Role of thrombospondin 1 in liver diseases. *Hepatol Res* 2017; **47**: 186-193 [PMID: 27492250 DOI: 10.1111/hepr.12787]

76 **Lafdil F**, Chobert MN, Couchie D, Brouillet A, Zafrani ES, Mavier P, Laperche Y. Induction of Gas6 protein in CCl4-induced rat liver injury and anti-apoptotic effect on hepatic stellate cells. *Hepatology* 2006; **44**: 228-239 [PMID: 16799993 DOI: 10.1002/hep.21237]

77 **Bárcena C**, Stefanovic M, Tutusaus A, Joannas L, Menéndez A, García-Ruiz C, Sancho-Bru P, Marí M, Caballeria J, Rothlin CV, Fernández-Checa JC, de Frutos PG, Morales A. Gas6/Axl pathway is activated in chronic liver disease and its targeting reduces fibrosis via hepatic stellate cell inactivation. *J Hepatol* 2015; **63**: 670-678 [PMID: 25908269 DOI: 10.1016/j.jhep.2015.04.013]

78 **Bottazzi B**, Inforzato A, Messa M, Barbagallo M, Magrini E, Garlanda C, Mantovani A. The pentraxins PTX3 and SAP in innate immunity, regulation of inflammation and tissue remodelling. *J Hepatol* 2016; **64**: 1416-1427 [PMID: 26921689 DOI: 10.1016/j.jhep.2016.02.029]

79 **Wilhelm A**, Aldridge V, Haldar D, Naylor AJ, Weston CJ, Hedegaard D, Garg A, Fear J, Reynolds GM, Croft AP, Henderson NC, Buckley CD, Newsome PN. CD248/endosialin critically regulates hepatic stellate cell proliferation during chronic liver injury via a PDGF-regulated mechanism. *Gut* 2016; **65**: 1175-1185 [PMID: 26078290 DOI: 10.1136/gutjnl-2014-308325]

80 **Heinrichs D**, Knauel M, Offermanns C, Berres ML, Nellen A, Leng L, Schmitz P, Bucala R, Trautwein C, Weber C, Bernhagen J, Wasmuth HE. Macrophage migration inhibitory factor (MIF) exerts antifibrotic effects in experimental liver fibrosis via CD74. *Proc Natl Acad Sci U S A* 2011; **108**: 17444-17449 [PMID: 21969590 DOI: 10.1073/pnas.1107023108]

81 **Colletti LM**, Green M, Burdick MD, Kunkel SL, Strieter RM. Proliferative effects of CXC chemokines in rat hepatocytes in vitro and in vivo. *Shock* 1998; **10**: 248-257 [PMID: 9788656 DOI: 10.1097/00024382-199810000-00004]

82 **Tacke F**, Zimmermann HW, Trautwein C, Schnabl B. CXCL5 plasma levels decrease in patients with chronic liver disease. *J Gastroenterol Hepatol* 2011; **26**: 523-529 [PMID: 21332547 DOI: 10.1111/j.1440-1746.2010.06436.x]

83 **Elpek GÖ**. Angiogenesis and liver fibrosis. *World J Hepatol* 2015; **7**: 377-391 [PMID: 25848465 DOI: 10.4254/wjh.v7.i3.377]

84 **Amarapurkar AD**, Amarapurkar DN, Vibhav S, Patel ND. Angiogenesis in chronic liver disease. *Ann Hepatol* 2007; **6**: 170-173 [PMID: 17786144]

85 **Fernández M**, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. *J Hepatol* 2009; **50**: 604-620 [PMID: 19157625 DOI: 10.1016/j.jhep.2008.12.011]

86 **Huber K**, Christ G, Wojta J, Gulba D. Plasminogen activator inhibitor type-1 in cardiovascular disease. Status report 2001. *Thromb Res* 2001; **103** Suppl 1: S7-S19 [PMID: 11567664 DOI: 10.1016/S0049-3848(01)00293-6]

87 **Wu J**, Strawn TL, Luo M, Wang L, Li R, Ren M, Xia J, Zhang Z, Ma W, Luo T, Lawrence DA, Fay WP. Plasminogen activator inhibitor-1 inhibits angiogenic signaling by uncoupling vascular endothelial growth factor receptor-2-αVβ3 integrin cross talk. *Arterioscler Thromb Vasc Biol* 2015; **35**: 111-120 [PMID: 25378411 DOI: 10.1161/ATVBAHA.114.304554]

88 **Shang H**, Liu X, Guo H. Knockdown of Fstl1 attenuates hepatic stellate cell activation through the TGF-β1/Smad3 signaling pathway. *Mol Med Rep* 2017; **16**: 7119-7123 [PMID: 28901425 DOI: 10.3892/mmr.2017.7445]

89 **Lindner V**, Wang Q, Conley BA, Friesel RE, Vary CP. Vascular injury induces expression of periostin: implications for vascular cell differentiation and migration. *Arterioscler Thromb Vasc Biol* 2005; **25**: 77-83 [PMID: 15514205 DOI: 10.1161/01.ATV.0000149141.81230.c6]

90 **Kessenbrock K**, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 2010; **141**: 52-67 [PMID: 20371345 DOI: 10.1016/j.cell.2010.03.015]

91 **Duarte S**, Baber J, Fujii T, Coito AJ. Matrix metalloproteinases in liver injury, repair and fibrosis. *Matrix Biol* 2015; **44-46**: 147-156 [PMID: 25599939 DOI: 10.1016/j.matbio.2015.01.004]

92 **Smalling RL**, Delker DA, Zhang Y, Nieto N, McGuiness MS, Liu S, Friedman SL, Hagedorn CH, Wang L. Genome-wide transcriptome analysis identifies novel gene signatures implicated in human chronic liver disease. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G364-G374 [PMID: 23812039 DOI: 10.1152/ajpgi.00077.2013]

93 **Reed MJ**, Koike T, Sadoun E, Sage EH, Puolakkainen P. Inhibition of TIMP1 enhances angiogenesis in vivo and cell migration in vitro. *Microvasc Res* 2003; **65**: 9-17 [PMID: 12535866 DOI: 10.1016/S0026-2862(02)00026-2]

94 **Timpl R**, Sasaki T, Kostka G, Chu ML. Fibulins: a versatile family of extracellular matrix proteins. *Nat Rev Mol Cell Biol* 2003; **4**: 479-489 [PMID: 12778127 DOI: 10.1038/nrm1130]

95 **Wang R**, Zhang YW, Chen LB. Aberrant promoter methylation of FBLN-3 gene and clinicopathological significance in non-small cell lung carcinoma. *Lung Cancer* 2010; **69**: 239-244 [PMID: 19913326 DOI: 10.1016/j.lungcan.2009.10.009]

96 **Silvestre JS**, Théry C, Hamard G, Boddaert J, Aguilar B, Delcayre A, Houbron C, Tamarat R, Blanc-Brude O, Heeneman S, Clergue M, Duriez M, Merval R, Lévy B, Tedgui A, Amigorena S, Mallat

Z. Lactadherin promotes VEGF-dependent neovascularization. *Nat Med* 2005; **11**: 499-506 [PMID: 15834428 DOI: 10.1038/nm1233]

97 **Uchiyama A**, Yamada K, Ogino S, Yokoyama Y, Takeuchi Y, Udey MC, Ishikawa O, Motegi S. MFG-E8 regulates angiogenesis in cutaneous wound healing. *Am J Pathol* 2014; **184**: 1981-1990 [PMID: 24838098 DOI: 10.1016/j.ajpath.2014.03.017]

98 **Nakashima Y**, Miyagi-Shiohira C, Noguchi H, Omasa T. The Healing Effect of Human Milk Fat Globule-EGF Factor 8 Protein (MFG-E8) in A Rat Model of Parkinson's Disease. *Brain Sci* 2018; **8**: 167 [PMID: 30200351 DOI: 10.3390/brainsci8090167]

99 **Sanyal AJ**, Brunt EM, Kleiner DE, Kowdley KV, Chalasani N, Lavine JE, Ratziu V, McCullough A. Endpoints and clinical trial design for nonalcoholic steatohepatitis. *Hepatology* 2011; **54**: 344-353 [PMID: 21520200 DOI: 10.1002/hep.24376]

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MINIREVIEWS

Single-cell analysis of tumors: Creating new value for molecular biomarker discovery of cancer stem cells and tumor-infiltrating immune cells

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Abstract

Biomarker-driven individualized treatment in oncology has made tremendous progress through technological developments, new therapeutic modalities and a deeper understanding of the molecular biology for tumors, cancer stem cells and tumor-infiltrating immune cells. Recent technical developments have led to the establishment of a variety of cancer-related diagnostic, prognostic and predictive biomarkers. In this regard, different modern OMICs approaches were assessed in order to categorize and classify prognostically different forms of neoplasia. Despite those technical advancements, the extent of molecular heterogeneity at the individual cell level in human tumors remains largely uncharacterized. Each tumor consists of a mixture of heterogeneous cell types. Therefore, it is important to quantify the dynamic cellular variations in order to predict clinical parameters, such as a response to treatment and or potential for disease recurrence. Recently, single-cell based methods have been developed to characterize the heterogeneity in seemingly homogenous cancer cell populations prior to and during treatment. In this review, we highlight the recent advances for single-cell analysis and discuss the challenges and prospects for molecular characterization of cancer cells, cancer stem cells and tumor-infiltrating immune cells.

Key words: Cancer cells; Cancer stem cells; Cancer biomarkers; Tumor-infiltrating lymphocytes; Single-cell analysis

Core tip: Extensive heterogeneity in cancer cells negatively influences treatment efficacy and survival of patients. The existing molecular methods for biomarker discovery of cancer cells and cancer stem cells are often unsuited to capture the heterogeneous nature of cell populations. Recent advances in single-cell based profiling approaches allowed the detection of molecular changes in individual cancer cells. Therefore, single-cell analysis is leading to build a complete landscape of cell types within tumor cells and facilitating the study of complex molecular heterogeneity in cancer cell populations. This will improve the investigation of more specific biomarkers to identify and target cancer stem cells.

Radpour R, Forouharkhou F. Single-cell analysis of tumor cells: Creating new value for molecular biomarker discovery of cancer stem cells. *World J Stem Cells* 2018; 10(11): 160-171 Available from: URL: http://www.wjgnet.com/1948-0210/full/v10/i11/160.htm DOI: http://dx.doi.org/10.4252/wjsc.v10.i11.160

INTRODUCTION

In principle, three main classes of biomarkers are distinguished for cancer disease stratification: Diagnostic, prognostic and predictive biomarkers[1-4]. In oncology, the diagnostic biomarkers essentially serve to substantiate a specific entity association and suspected malignant disease-spreading pattern. Classical examples are the *in situ* immunophenotyping of a neoplasm such as lung cancer[5] by immunohistology as well as the specific representation of entity-defining molecules such as prostate-specific membrane antigen in prostate cancer[6].

By contrast, prognostic biomarkers have the function of predicting the natural course of a malignant disease. These include classical parameters such as clinical and pathological staging but also the collection of molecular factors, such as tumor specific genetic aberrations (chromosomal abnormalities, gene mutations, pathologic epigenetic changes or dysregulated genes/pathways) that may be associated with more aggressive disease progression. However, a prognostic biomarker has only a limited value for the patient, since mere knowledge about the prognosis of disease alone has little benefit[2,4,7].

The predictive biomarkers specifically describe the expected likelihood of a patient responding to an available therapy option based on the molecular properties of the tumor. This concept is currently used in the context of targeted drug-based tumor treatment with targeted drugs, *e.g.*, with inhibitors of the epidermal growth factor receptor (EGFR) in the presence of a tissue-based EGFR mutation in lung carcinomas[8] or CD70-CD27 signaling in leukemia including acute myeloid leukemia (AML)[9] or chronic myeloid leukemia (CML)[10]. However, there are only a few approved tissue-based predictive biomarkers available. Predictive analytics using molecular imaging and blood-based technologies are still at the stage of development.

The boundaries between these biomarker types can be blurred. For example, a pathologic genetic alteration in different situations may represent a diagnostic, a prognostic, and a predictive biomarker. This is illustrated by the *BRAF* mutation, as it can support the early diagnosis of a thyroid carcinoma[11], prognostically define an unfavorable subtype of colorectal carcinoma[4] and predictably provide therapy with a BRAF-specific small molecule inhibitor (*e.g.*, vemurafenib) in malignant melanoma[12].

Classical macroscopically assisted histomorphologic evaluation of a malignant tumor remains by far the most significant diagnostic, prognostic and in many respects predictive biomarker with the greatest impact on patient treatment. Nevertheless, in recent decades, a refinement of biomarker analysis by molecular methods has found its way into pathological diagnostics and shaped the new area of individualized medicine.

CANCER STEM CELLS

Cancer stem cells (CSCs) or tumor precursor cells are a minor fraction of cells within the bulk tumor population, which, because of their unique stem cell properties of relative quiescence and self-renewal, have been found to reconstitute and propagate the tumor and are considered to be essential for tumor neoplasm and metastasis[13]. The theory of CSCs was firstly postulated in the 1970s and was experimentally confirmed by the isolation of tumor-initiating cells in AML[14]. Furthermore, CSC has been demonstrated in a variety of solid tumors, such as tumors in brain, colorectal, hematopoietic malignancies (*e.g.*, myeloid or lymphoid leukemia), head and neck, mammary glands, lung, liver, melanoma and also prostate carcinomas[9,10,15,16]. Heterogeneity is a major hallmark of tumor cells including CSCs. Each cancer cell clone is characterized by harboring different combinations of mutations or genetic alterations, and subsequently the processes of tumorigenesis occur differently based on the type of genetic lesions[17].

CSCs are often resistant against standard therapies such as irradiation, chemotherapy, cytotoxic drugs and probably also against immune attack. This may be due to different escape mechanisms of CSCs and/or due to protective mechanisms of the microenvironment. Unravelling the function of the CSCs has been one of the main challenges of cancer research[16,18].

HIGH-THROUGHPUT TECHNOLOGIES FOR PROFILING OF CANCER CELLS AND CSCs

Over the past few decade, a variety of biomarkers for a wide range of solid tumors and hematopoietic malignancies has been identified[2,4]. The technologies for biomarker analysis are developing rapidly. The next generation sequencing (NGS) technology promptly follows some of the technologies mentioned above, which will lead to further dynamization of the biomarker discovery in oncology. In addition, blood-based assays using circulating cell free DNA that move beyond the classic tumor marker determination will become more important for the monitoring of disease processes and resistance as well as the prediction of therapy outcome[16,19-21]. Mutational analysis in *EGFR*-mutated lung carcinoma prior to therapy with Osimertinib is an example of a blood-based assay that has already found way into the routine diagnostic pipelines[22]. Further assays are being developed to trace and target circulating tumor cells (mainly CSCs) in the blood, urine, cerebrospinal fluid and other body fluids. The goal must be to transfer molecular markers from tissue diagnostics into non-invasive molecular profiling approaches.

MOLECULAR BIOMARKERS OF CANCER CELLS AND CSCs

Proteomics

Targeted proteomics using tissue-based *in situ* methods such as immunohistology has been developed as an important biomarker analysis tool in oncology[23]. This approach is used in many areas of pathology including pathological oncology, and the predictive biomarker analysis still relies significantly on this method. Examples include the analysis of human epidermal growth factor receptor 2 (HER2) expression prior to treatment with HER2 inhibitors (*e.g.,* trastuzumab) in gastric and breast carcinoma[24,25] as well as the stratifying assignment of treatment with immune checkpoint inhibitors in programmed death-ligand 1 (PD-L1)-positive advanced non-small cell lung cancer[26]. The development of multiplexable and quantitatively more precise proteomic methods promises new opportunities for biomarker discovery/analysis in the near future. These include using slice-based imaging mass spectrometry [*e.g.,* matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS)][27] or quantitative multiplex protein analysis using extract-based mass spectrometry (LC-MS)[28].

Genomics

The earliest clinically relevant genomic studies on predictive biomarker analysis used in routine diagnostics were the application of fluorescence *in situ* hybridizations (FISH) to determine the gene copy number of *ERBB2*, the *HER2* gene, in breast cancer, which could assign it to a positive or negative category for *HER2* expression[2,29,30]. One of the first examples of large solid tumor profiling is mutation screening for *KRAS* and *NRAS* genes in metastatic colorectal carcinoma as a predictive biomarker for using the EGFR inhibitor panitumumab[4,31]. Today, numerous individual examinations of gene mutations or chromosomal aberrations (*e.g.*, translocations or amplifications) are firmly anchored in the routine diagnostic of different tumors. Currently, new technologies such as massive parallel sequencing (MPS) have been priced into areas where routine diagnostic application has become possible. Those methods have already been adapted to high-throughput screening in routine applications[32]. Implementation of those high-throughput approaches has led to improvement of diagnosis and therapy of different cancer types[33,34].

Epigenomics

The first introduced epigenetic biomarker into the routine diagnostic was investigation of promoter methylation of the *MGMT* gene using sequence-based techniques to predict response to treatment with temozolomide in glioblastoma[35]. However, newer epigenetic screening approaches, which are still in the process of diagnostic development, focus on the simultaneous investigation of DNA methylation in a large number of coding genes using array-based or high-throughput sequencing methods (*e.g.*, Methyl-seq). Since it is postulated that pathologic methylation patterns in individual tumor entities are more stable and reproducible than transcriptome profiles, these technologies are currently being tested primarily in molecular entity assignment. Large studies have substantiated their overall suitability for cancer with unknown primary and for some rare tumor families but have not yet been implemented in the routine diagnostic pipelines[36-38].

Transcriptomics

The analysis of RNA expression signature within cancer cells and CSCs using quantitative polymerase chain reaction (qPCR), array-based capture, NanoString technology or massive parallel RNA sequencing (RNA-Seq) approaches has a long tradition in cancer molecular biomarker analysis. However, individual methods (*e.g.,* quantitative polymerase chain reaction) could never prevail over immunohistology despite partially superior precision. Initially, the parallel analysis of RNA expression patterns was assessed with the hope that diagnostic assignments could be made in unclear cases (*e.g.,* cancer with unknown primary)[39]. Despite their potential and some positive results, these applications could not establish themselves in the wide range of diagnostic services. In addition, many tumor entities

have been used to develop predictors for the efficacy of conventional chemotherapies based on transcriptomic profiles. Some success in this context has been gene expression tests in breast cancer, which can be used as an additional decision-making aid in the therapy stratification of breast cancer patients for adjuvant chemotherapy[40]. However, these tests are currently not being used consistently in clinical care.

As indicated, tumors are a pool of heterogeneous cells including CSCs. Inter- or intra-tumor heterogeneity may completely render CSC biomarkers inapt. Seemingly homogenous cell populations that are enriched and purified by a set of well-known surface markers often hide exceptional heterogeneity. This is more pronounced in the hematological malignancies[16]. Such tumor heterogeneity can be the result of different genetically distinct clones within the tumor due to having various genetic lesions or dysregulation of markers *via* pathologic epigenetic regulations[2,4,41-46].

SINGLE-CELL BASED APPROACHES

Different OMICs approaches have allowed for the discovery and characterization of a variety of cancer-related cell populations. However, those approaches are unsuited to capture the heterogeneous nature of cancer cell populations. Therefore, interest was shifted towards characterization of single-cells rather than cell populations. The technical advances that include single-cell imaging, genomics or transcriptomics assessed full characterization of different cell populations. The OMICs analysis is usually performed using samples of many cells. However, this type of analysis lacks the kind of detailed assessment needed for evaluating contribution of individual cells to the overall phenotype. In contrast, single-cell analysis allows comparing the captured OMICs data of thousands of individual cells (Figure 1). Applied methods for single-cell isolation have rapidly enhanced in the past few years from manual micromanipulation, cell-search antibody-based isolation or flow-sorting of cells to high-throughput isolation methods using dielectrophoresis (DEP) arrays, microfluidics, emulsion-based platforms or 10X genomics Chromium™ single cell controller system. This technical advance could provide massive advantages by significantly increasing the throughput sensitivity and accuracy of employed approaches (Figure 1B).

One of the prime reasons for using single-cell analysis is to evaluate heterogeneity in seemingly homogenous cell populations. Another reason is to detect small subpopulations that would otherwise be missed in bulk populations. In addition, by using single-cell analysis, it is possible to find CSCs and trace them in the circulation, investigate the clonal evolution and mutational rate of cancer cells, to study better the invasion and trace the metastatic dissemination and to understand the molecular mechanisms of therapy resistance of cancer cells and CSCs (Figure 2).

The first single-cell RNA-Seq study was published in 2009[47]. Since then the interest for the approach is growing[48,49]. Single-cell RNA sequencing is being used for identifying cellular intermediates during developmental processes. Different microfluidic systems have been proposed to isolate single cells and help in library preparation[50]. Several novel methods are available for single-cell analyses. Multiplexed error robust fluorescence (MERFISH), is a high-throughput method that uses sequential imaging with combinatorial labeling and multiplex single molecule FISH, allowing for robust detection of many genes at the same time in both tissues and cell culture conditions[51]. Another approach is quantitative hybridization chain reaction (qHCR), which uses probes harboring initiators for DNA interacting with fluorophore-labelled hairpins assembled into polymerase. Using this method, the mRNA expression of thousands of different genes can be captured simultaneously at a single-cell based resolution[52]. Single-cell linage tracking allows researchers to follow and trace the fate of individual cells over the time. This also includes the tracing of different cancer cells from primitive CSCs. Lineage tracing by nuclease-activated editing of ubiquitous sequences (LINNAEUS), is a novel method for cell type identification, characterization and massively parallel linage tracking. In this approach, a double strand break will be introduced to the cells using a CRISPR/Cas9 system, which upon repair, reacts as a unique heritable scar in the daughter cells in order to trace the cellular linages[53].

Pooled screenings rely on readouts that average properties of the cell population of interest. Although these approaches provide an assessment of gene function at the genome scale, they cannot identify the contribution of subpopulations to the bulk phenotype. Moreover, the consequences of distinct perturbations to the overall phenotype cannot be evaluated. To circumvent the problem, methods have been recently developed to study the impact of perturbations at the single-cell levels[54,55]. These approaches integrate parallel massive single-cell RNA-Seq and pooled screens to reconstruct the gene regulatory networks controlling particular biological processes. Perturb-Seq is a platform for multiplexed profiling of perturbations at the single-cell resolution[54]. Profiling the genomic perturbation and the transcriptome in the same cell provides a powerful means to simultaneously the function of multiple factors and their interactions.

Heterogeneity in the tumor cell population was recently evaluated in different forms of human cancers. In the ovarian cancer, single-cell analysis revealed two major subsets of cells characterized by stromal gene expression patterns [genes associated with epithelial-to-mesenchymal transition (EMT) and also extracellular

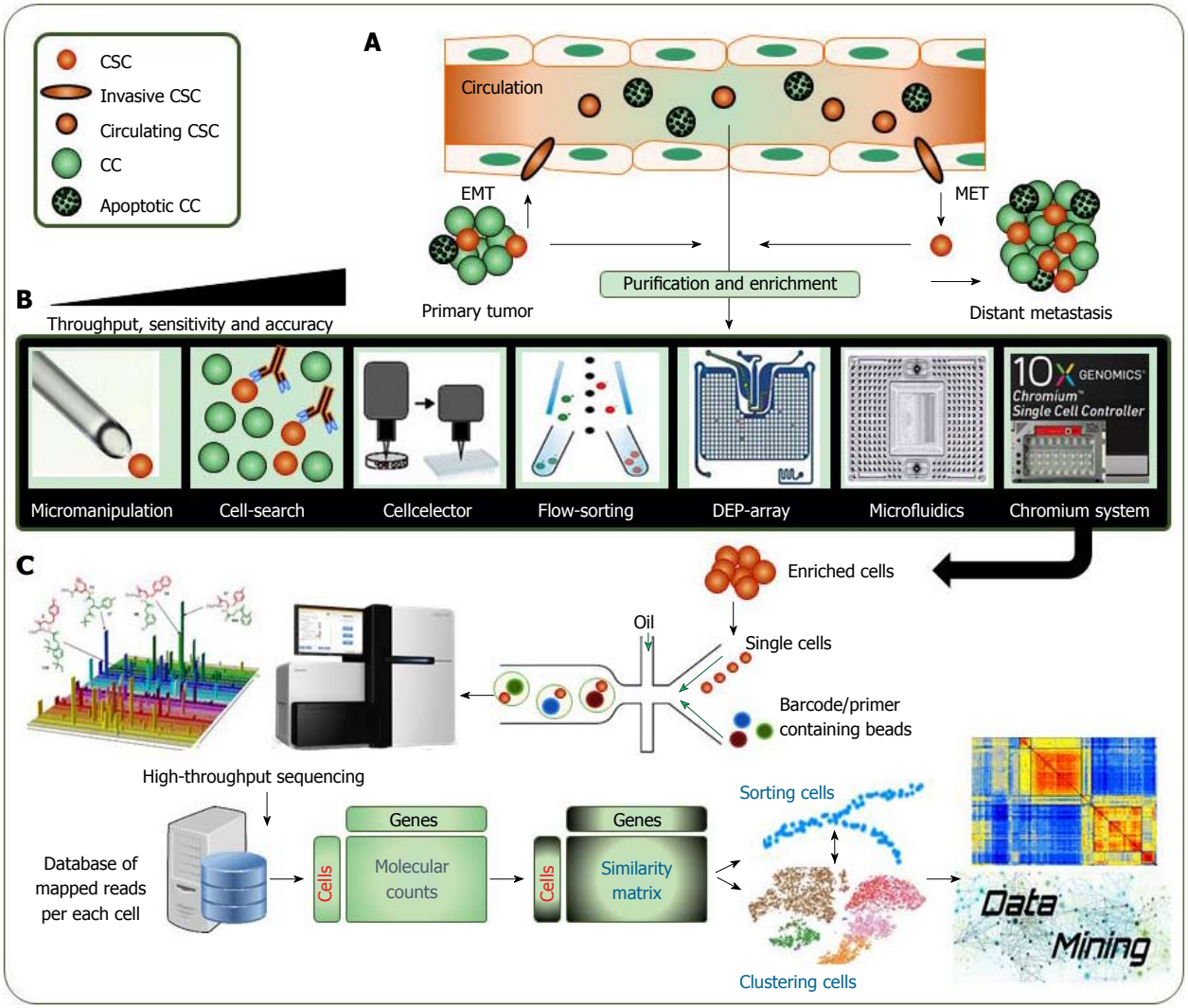

Figure 1 Single-cell analysis of cancer cells and cancer stem cells. A: Cancer cells, in particular CSCs, represent a complex process of invasion, EMT, shedding into the blood stream (intravasation), MET and invasion of circulating CSCs to the other tissues (extravasation); B: These CSCs can be isolated or also purified and enriched using different approaches based on their known molecular markers for variety of solid tumors or hematopoietic malignancies; C: Those enriched CSCs will be subjected to the single-cell based transcriptomic analysis. Upon sequencing, a pool of mapped reads will be analyzed based on the possible similarity to either sort the single cells to show how different cells are differentiated from more primitive ones, or will be sub-clustered according to their gene expression differences in order to dissect heterogeneous cell populations. CC: Cancer cell; CSC: Cancer stem cell; EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition.

matrix (ECM) genes] and epithelial gene expression signature (characterized by proliferation- and oxidative phosphorylation-related genes)[56]. Analysis of CSCs in CML, uncovered distinct molecular signatures of leukemia stem cells with a high level of heterogeneity in the seemingly homogenous cell populations of CSCs[57]. Single-cell whole exome sequencing (scWES), is a promising tool for detecting sub-clones and possibly leukemia stem cells in AML[58]. Furthermore, epigenetically distinct hematopoietic stem cell sub-populations have been detected by high-resolution single-cell DNA methylation analysis[59]. Single-cell sequencing of glioblastoma and glioma cells also detected a heterogeneous gene expression signature within the tumor population[60,61]. In breast cancer, regulatory networks influencing stemness, pluripotency, proliferation, differentiation and EMT have been identified using single-cell gene expression profiling. The analysis has shown that $ALDH^{-}CD44^{+}CD24^{-}$ and $ALDH^{+}$ human mammary cells have mesenchymal-like and epithelial-like characteristics, respectively. At the single-cell level, these cells express high levels of stemness- and EMT-associated gene signatures. In contrast, both detected populations had some co-expressing $ALDH^{+}$ and $CD44^{+}CD24^{-}$ by flow cytometry[62]. Important findings using single-cell sequencing studies on variety of human primary tumors, including bladder, blood, brain, breast, colorectal, kidney, lung and ovarian cancer, are summarized in Table 1.

SINGLE-CELL T CELL RECEPTOR SEQUENCING OF TUMOR-INFILTRATING LYMPHOCYTES

Most immune cell types can be present in a tumor, and the fraction of immune cells can vary greatly across

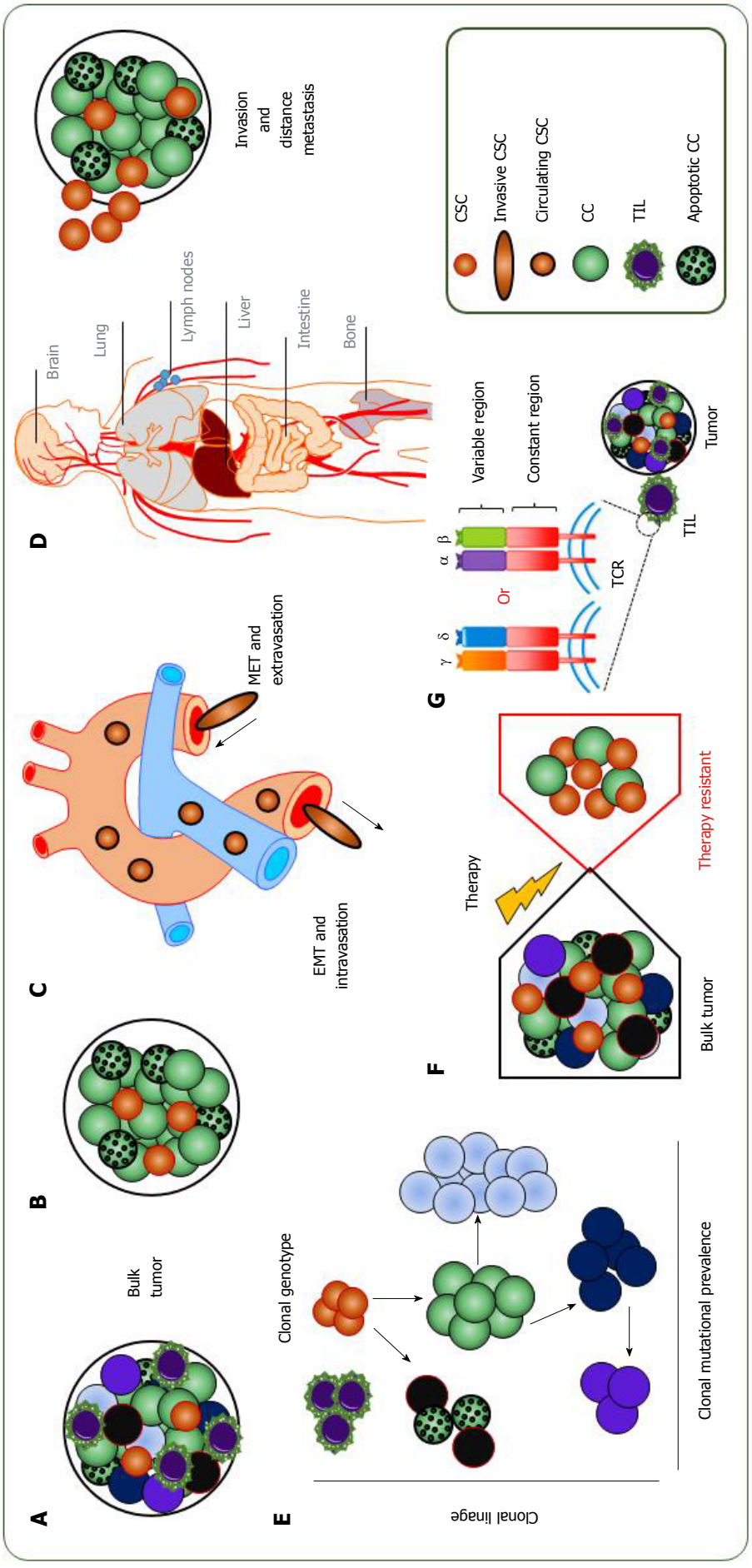

Figure 2 Main applications of single-cell based profiling in cancer research. A: Resolving intratumor heterogeneity; B: Finding and profiling CSCs within the bulk tumor; C: Tracing circulating CSCs; D: Study extravasation or intravasation and cell plasticity in invasive and metastatic cancer cells; E: Investigating clonal evolution in tumor cells based on their linage differentiation or mutational prevalence; F: Discovery the mechanism of therapy resistance at a single-cell level; G: Single-cell TCR sequencing of tumor-infiltrating lymphocyte. CC: Cancer cell; CSC: Cancer stem cell; EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; TCR: T cell receptor; TIL: Tumor-infiltrating lymphocyte.

different tumors and patients[63]. T lymphocytes are among the most studied tumor-infiltrating immune cells, since they have the potential to recognize mutated protein epitopes displayed by human lymphocyte antigen (HLA) molecules on cancer cells and CSCs, thereby allowing immune recognition of the tumor. Different types of tumor-infiltrating lymphocytes (TILs) have different effects. For instance, $CD4^+$ Tregs have been associated with poor survival and have been demonstrated to play an immune-suppressive role[64]. Conversely, $CD8^+$ T cells can mediate cytolytic activity against cancer cells or CSCs. However, cancer cells, particularly CSCs, evade immune recognition and elimination by TILs *via* various mechanisms, including loss of antigen and the expression of immune inhibitory molecules. Tumor-infiltrating $CD8^+$ T cell are often anergic,

Table 1 Single-cell sequencing studies on variety of human tumors

Tumor type	Source	Platform	Major finding	Ref.
Bladder cancer	Squamous cell carcinoma	RNA-seq	Cellular heterogeneity in the gene expression affects the disease outcome	[73]
	Muscle-invasive cell carcinoma	SNV-seq	Lineage-specific mutations are driving cancer initiation and progress	[74]
Blood cancer	B-cell ALL	CNV-seq	CNVs were developed as an impact of environmental stressors, which was only detectable at single-cell level	[75]
	Pediatric ALL	SNV-seq	Analysis revealed clonal somatic mutational prevalence at single-cell resolution	[76]
	Therapy resistant AML	RNA-seq	Identified molecular signature of resistant LSCs versus therapy-naive LSCs	[77]
	Secondary AML	SNV-seq	Genomic complexity was identified at single cells which was not seen at bulk leukemic populations	[78]
	CML	RNA-seq	Single-cell analysis uncovered molecular signature of LSCs	[57]
	JAK2 negative MPN	SNV-seq	Large genetic distances was observed between mono-clonal tumor cells	[79]
	JAK2V617F MPN	RNA-seq	Single-cell sequencing revealed the molecular networks driving self-renewal of CSCs	[80]
Brain cancer	*EGFR* amplified GBM	CNV-seq	Heterogeneity in *EGFR* mutations among different tumor cells leading to variation in therapy response	[81]
	GBM	RNA-seq	Heterogeneity in gene expression panthers was identified including *EGFR* gene	[82]
Breast cancer	ER^+	CNV-seq	Showed clonal evolution of tumor cells at single-cell resolution	[83]
	$HER2^+$	RNA-seq	404 differentially expressed gene signature was identified in CSCs, which had a prognostic value	[84]
	MDA-MB-231 and CN34 cell lines	RNA-seq	Gene expression profiling identifies small sub-population with more metastatic potential, which was therapy resistant.	[85]
	TNBC	CNV-seq RNA-seq	Showed clonal evolution of tumor cells at single-cell level. Also, chemo-resistance evolution in TNBC was identified	[86,87]
	TNBC or ER^+ $HER2^-$	SNV-seq CNV-seq	ER^+ $HER2^-$ tumors represented significantly less mutational rate compared to TNBC tumors	[88]
Colorectal cancer	Colon tumor and adjacent normal cells	SNV-seq	Different mutational profiles were identified among tumors' sub-populations	[89]
	Colon tumor	CNV-seq	CSCs ($EpCAM^{high}CD44^+$) and DTCs ($EpCAM^{high}CD44^-$) had similar somatic CNV pattern, while they had regional differences	[90]
	Rectal tumor	CNV-seq	Multi-region single-cell analysis showed somatic copy number alterations are an early event in cancer development	[91]
Kidney cancer	ccRCC primary carcinoma and paired metastasis	RNA-seq	Heterogeneity in the expression of targetable genes was identified. The finding highlights the necessity of multi-agent therapies	[92]
Lung cancer	NSCLC	RNA-seq	Characterization of tumor-infiltrating T cells revealed that inter-tissue effector T cells with a highly migratory nature	[93]
	Clear cell renal cell carcinoma	SNV-seq	A complex mutational pattern was observed at single-cells compared to bulk tumors	[94]
	Adenocarcinoma PDX	RNA-seq	Single-cell sequencing identified $KRAS^+$ drug resistant cell population within the tumor	[95]
	LC2/ad and LC2/ad-R cell lines	RNA-seq	Gene expression profiling identifies signature that is linked to therapy resistance	[96]
Ovarian cancer	HGSOC	RNA-seq	Single-cell analysis could distinguish two major sub-populations within the tumor based on their gene expression signature	[56]

ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; ccRCC: Clear cell renal cell carcinoma; CML: Chronic myeloid leukemia; CNV: Copy number variant; CSC: Cancer stem cell; DTC: Differentiated tumor cell; HER2: Human epidermal growth factor receptor 2; ER: Estrogen receptor; EGFR: Epidermal growth factor receptor; GBM: Glioblastoma; HGSOC: High grade serous ovarian carcinomas; JAK2: Janus kinase 2; KRAS: Kirsten rat sarcoma viral oncogene homolog; LSC: Leukemia stem cell; MPN: Myeloproliferative neoplasm; NSCLC: Non-small-cell lung carcinoma; PDX: Patient-derived xenograft; SNV: Single nucleotide variant; TNBC: Triple negative breast cancer.

as characterized by their exhaustion phenotype[65]. Overall, it is clear that complex relationships govern the interactions between immune cells and cancer cells or CSCs.

During T cell development in the thymus, they gain the ability to recognize many different foreign antigens. This ability is assessed by the expression of highly polymorphic surface T cell receptors (TCRs). The enormous diversity of TCRs is resulted by random combinations of genes' segments encoding TCR chains [including variable (V), diversity (D), and joining (J) segments][66]. Molecular profiling and characterization of TCRs in TILs could describe T cell dynamics in different tumors[67].

TILs are typically studied by immunohistochemistry or by flow cytometry, relying on a panel of antibodies targeting specific markers of immune cells. To

complement this approach, gene expression of whole tumors can be used and expression of the immune cell type markers can inform us about the presence of the corresponding cell types[68]. One promising aspect of this approach is that it provides information about the whole transcriptome and is not restricted by the availability of antibodies; however, it is not capable of overcoming the extensive heterogeneity among TILs.

The next generation sequencing approach using genomic DNA (gDNA) as starting material was first used to characterize the TCR diversity in healthy individuals[69] and rapidly adapted to TCR profiling in tumor immunology[70]. However, the use of gDNA was more challenging due to the fact that non-productive TCR rearrangements were also sequenced. In addition, the presence of introns can introduce more technical biases. Therefore, RNA-seq was selected as a better approach. Upon introducing more advanced single-cell analysis approaches like microfluidics or 10 × genomics, there was promise to couple RNA-seq and TCR sequencing from the same cell, which has the great advantage to identify and characterize very rare T cell populations. A recent work using different single-cell analysis methods investigated the T cell repertoire according to their TCR variability in both mice and human Treg cells[71]. The results of this comprehensive TCR single-cell sequencing revealed that Tregs with some highly activated subpopulations could display a broad heterogeneity, while Treg sharing the same antigen recognition specificity were more transcriptionally similar than those with different TCR sequence.

The coupled profiling of TCRs sequencing and single-cell gene expression analysis from the same cell provides an unbiased classification of T cells based of their TCR signature, which is association of the transcriptional landscape of individual cell[72]. This approach will provide a powerful tool to study the potential impact of TILs on CSCs and will yield valuable insights to personalized immunotherapy of cancer patients.

CONCLUSION

The determination of diagnostic, prognostic and predictive biomarkers forms the basis of individualized patient treatment in oncology. As a biomarker, it is demanded to be reproducible, robust and quality-assured. As of today, the collection of specific biomarkers are not be able to define the complete subsequent of oncological therapy for cancer patients. This affects the efficacy of a treatment, the side effects that a patient is exposed to and the cost of therapy.

Despite some developments in the field of blood-based tests and molecular imaging, biomarker analysis in oncology continues to rely essentially on molecular tissue analysis. An exact molecular characterization of CSCs in the tumor requires the development of specific markers and suitable enrichment methods. New genomics, epigenomics, transcriptomics and proteomics methods as well as the introduction of novel single-cell based approached will result in an accelerating identification of specific oncological biomarkers.

Single-cell technologies are allowing for the detection of molecular changes in individual cancer cells. This can improve investigation of more specific biomarkers with unprecedented resolution leading to build a complete landscape of different cell types within tumors. Single-cell analysis of CSCs is challenging mainly due to their rarity and the small amount of total RNA in a single cell. Using a combination of different cellular enrichment strategies, such as flow cytometry for rare cell population like CSCs with the single-cell analyzing methods, will improve the resolution in profiling and characterization of CSCs. Likewise, the ability to amplify and sequence other RNA molecules, such as micro RNAs and long non-coding RNAs, will provide valuable information on gene regulation. New methods to simultaneously profile genomic DNA variants, DNA methylation and gene expression from the same cell coupled with potential proteomic analysis, could provide powerful tools for assessing the effects of genomic variation and gene expression profiles or epigenetic modifications on cancer cell heterogeneity. Particularly, from high-throughput single-cell based technologies, we can expect valuable insights regarding suitable associated biomarkers to identify and target CSCs. Furthermore, cancer immunotherapy may also benefit from single-cell methods that define the role of TILs within the CSCs and monitor the individual response to the immune-regulatory agents. This would be an important step towards individualized cancer management.

REFERENCES

1 **Biomarkers Definitions Working Group**. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001; **69**: 89-95 [PMID: 11240971 DOI: 10.1067/mcp.2001.113989]

2 **Radpour R**, Barekati Z, Kohler C, Holzgreve W, Zhong XY. New trends in molecular biomarker discovery for breast cancer. *Genet Test Mol Biomarkers* 2009; **13**: 565-571 [PMID: 19814613 DOI: 10.1089/gtmb.2009.0060]

3 **Zhang B**, Barekati Z, Kohler C, Radpour R, Asadollahi R, Holzgreve W, Zhong XY. Proteomics and biomarkers for ovarian cancer diagnosis. *Ann Clin Lab Sci* 2010; **40**: 218-225 [PMID: 20689132]

4 **Aghagolzadeh P**, Radpour R. New trends in molecular and cellular biomarker discovery for colorectal cancer. *World J Gastroenterol* 2016; **22**: 5678-5693 [PMID: 27433083 DOI: 10.3748/wjg.v22.i25.5678]

5 **Warth A**, Muley T, Herpel E, Meister M, Herth FJ, Schirmacher P, Weichert W, Hoffmann H, Schnabel PA. Large-scale comparative analyses of immunomarkers for diagnostic subtyping of non-small-cell lung cancer biopsies. *Histopathology* 2012; **61**: 1017-1025 [PMID: 22882703 DOI: 10.1111/j.1365-2559.2012.04308.x]

6 **Maurer T**, Eiber M, Schwaiger M, Gschwend JE. Current use of PSMA-PET in prostate cancer management. *Nat Rev Urol* 2016; **13**: 226-235 [PMID: 26902337 DOI: 10.1038/nrurol.2016.26]

7 **Nalejska E**, Mączyńska E, Lewandowska MA. Prognostic and

predictive biomarkers: tools in personalized oncology. *Mol Diagn Ther* 2014; **18**: 273-284 [PMID: 24385403 DOI: 10.1007/s40291-013-0077-9]

8 **Tan DS**, Yom SS, Tsao MS, Pass HI, Kelly K, Peled N, Yung RC, Wistuba II, Yatabe Y, Unger M, Mack PC, Wynes MW, Mitsudomi T, Weder W, Yankelevitz D, Herbst RS, Gandara DR, Carbone DP, Bunn PA Jr, Mok TS, Hirsch FR. The International Association for the Study of Lung Cancer Consensus Statement on Optimizing Management of EGFR Mutation-Positive Non-Small Cell Lung Cancer: Status in 2016. *J Thorac Oncol* 2016; **11**: 946-963 [PMID: 27229180 DOI: 10.1016/j.jtho.2016.05.008]

9 **Riether C**, Schürch CM, Bührer ED, Hinterbrandner M, Huguenin AL, Hoepner S, Zlobec I, Pabst T, Radpour R, Ochsenbein AF. CD70/CD27 signaling promotes blast stemness and is a viable therapeutic target in acute myeloid leukemia. *J Exp Med* 2017; **214**: 359-380 [PMID: 28031480 DOI: 10.1084/jem.20152008]

10 **Riether C**, Schürch CM, Flury C, Hinterbrandner M, Drück L, Huguenin AL, Baerlocher GM, Radpour R, Ochsenbein AF. Tyrosine kinase inhibitor-induced CD70 expression mediates drug resistance in leukemia stem cells by activating Wnt signaling. *Sci Transl Med* 2015; **7**: 298ra119 [PMID: 26223302 DOI: 10.1126/scitranslmed.aab1740]

11 **Tennakoon TMPB**, Rushdhi M, Ranasinghe ADCU, Dassanayake RS. Values of molecular markers in the differential diagnosis of thyroid abnormalities. *J Cancer Res Clin Oncol* 2017; **143**: 913-931 [PMID: 28008451 DOI: 10.1007/s00432-016-2319-9]

12 **Bollag G**, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, Spevak W, Zhang C, Zhang Y, Habets G, Burton EA, Wong B, Tsang G, West BL, Powell B, Shellooe R, Marimuthu A, Nguyen H, Zhang KY, Artis DR, Schlessinger J, Su F, Higgins B, Iyer R, D'Andrea K, Koehler A, Stumm M, Lin PS, Lee RJ, Grippo J, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, Chapman PB, Flaherty KT, Xu X, Nathanson KL, Nolop K. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* 2010; **467**: 596-599 [PMID: 20823850 DOI: 10.1038/nature09454]

13 **Dawood S**, Austin L, Cristofanilli M. Cancer stem cells: implications for cancer therapy. *Oncology* (Williston Park) 2014; **28**: 1101-1107, 1110 [PMID: 25510809]

14 **Lapidot T**, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645-648 [PMID: 7509044 DOI: 10.1038/367645a0]

15 **Visvader JE**, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008; **8**: 755-768 [PMID: 18784658 DOI: 10.1038/nrc2499]

16 **Radpour R**. Tracing and targeting cancer stem cells: New venture for personalized molecular cancer therapy. *World J Stem Cells* 2017; **9**: 169-178 [PMID: 29104735 DOI: 10.4252/wjsc.v9.i10.169]

17 **Meacham CE**, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature* 2013; **501**: 328-337 [PMID: 24048065 DOI: 10.1038/nature12624]

18 **Murone M**, Radpour R, Attinger A, Chessex AV, Huguenin AL, Schürch CM, Banz Y, Sengupta S, Aguet M, Rigotti S, Bachhav Y, Massière F, Ramachandra M, McAllister A, Riether C. The Multikinase Inhibitor Debio 0617B Reduces Maintenance and Self-renewal of Primary Human AML CD34$^+$ Stem/Progenitor Cells. *Mol Cancer Ther* 2017; **16**: 1497-1510 [PMID: 28468777 DOI: 10.1158/1535-7163.MCT-16-0889]

19 **Wan JCM**, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R, Rosenfeld N. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* 2017; **17**: 223-238 [PMID: 28233803 DOI: 10.1038/nrc.2017.7]

20 **Zachariah RR**, Schmid S, Buerki N, Radpour R, Holzgreve W, Zhong X. Levels of circulating cell-free nuclear and mitochondrial DNA in benign and malignant ovarian tumors. *Obstet Gynecol* 2008; **112**: 843-850 [PMID: 18827127 DOI: 10.1097/AOG.0b013e3181867bc0]

21 **Zachariah R**, Schmid S, Radpour R, Buerki N, Fan AX, Hahn S, Holzgreve W, Zhong XY. Circulating cell-free DNA as a potential biomarker for minimal and mild endometriosis. *Reprod Biomed Online* 2009; **18**: 407-411 [PMID: 19298741 DOI: 10.1016/S1472-6483(10)60100-9]

22 **Mok TS**, Wu Y-L, Ahn M-J, Garassino MC, Kim HR, Ramalingam SS, Shepherd FA, He Y, Akamatsu H, Theelen WS, Lee CK, Sebastian M, Templeton A, Mann H, Marotti M, Ghiorghiu S, Papadimitrakopoulou VA; AURA3 Investigators. Osimertinib or Platinum-Pemetrexed in EGFR T790M-Positive Lung Cancer. *N Engl J Med* 2017; **376**: 629-640 [PMID: 27959700 DOI: 10.1056/NEJMoa1612674]

23 **Prichard JW**. Overview of automated immunohistochemistry. *Arch Pathol Lab Med* 2014; **138**: 1578-1582 [PMID: 25427039 DOI: 10.5858/arpa.2014-0083-RA]

24 **Cameron D**, Piccart-Gebhart MJ, Gelber RD, Procter M, Goldhirsch A, de Azambuja E, Castro G Jr, Untch M, Smith I, Gianni L, Baselga J, Al-Sakaff N, Lauer S, McFadden E, Leyland-Jones B, Bell R, Dowsett M, Jackisch C; Herceptin Adjuvant (HERA) Trial Study Team. 11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial. *Lancet* 2017; **389**: 1195-1205 [PMID: 28215665 DOI: 10.1016/S0140-6736(16)32616-2]

25 **Bang YJ**, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK; ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697 [PMID: 20728210 DOI: 10.1016/S0140-6736(10)61121-X]

26 **Hui R**, Garon EB, Goldman JW, Leighl NB, Hellmann MD, Patnaik A, Gandhi L, Eder JP, Ahn MJ, Horn L, Felip E, Carcereny E, Rangwala R, Lubiniecki GM, Zhang J, Emancipator K, Roach C, Rizvi NA. Pembrolizumab as first-line therapy for patients with PD-L1-positive advanced non-small cell lung cancer: a phase 1 trial. *Ann Oncol* 2017; **28**: 874-881 [PMID: 28168303 DOI: 10.1093/annonc/mdx008]

27 **Kriegsmann M**, Casadonte R, Kriegsmann J, Dienemann H, Schirmacher P, Hendrik Kobarg J, Schwamborn K, Stenzinger A, Warth A, Weichert W. Reliable Entity Subtyping in Non-small Cell Lung Cancer by Matrix-assisted Laser Desorption/Ionization Imaging Mass Spectrometry on Formalin-fixed Paraffin-embedded Tissue Specimens. *Mol Cell Proteomics* 2016; **15**: 3081-3089 [PMID: 27473201 DOI: 10.1074/mcp.M115.057513]

28 **Panis C**, Pizzatti L, Souza GF, Abdelhay E. Clinical proteomics in cancer: Where we are. *Cancer Lett* 2016; **382**: 231-239 [PMID: 27561426 DOI: 10.1016/j.canlet.2016.08.014]

29 **Piccart-Gebhart MJ**, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, Gianni L, Baselga J, Bell R, Jackisch C, Cameron D, Dowsett M, Barrios CH, Steger G, Huang CS, Andersson M, Inbar M, Lichinitser M, Láng I, Nitz U, Iwata H, Thomssen C, Lohrisch C, Suter TM, Rüschoff J, Suto T, Greatorex V, Ward C, Straehle C, McFadden E, Dolci MS, Gelber RD; Herceptin Adjuvant (HERA) Trial Study Team. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 2005; **353**: 1659-1672 [PMID: 16236737 DOI: 10.1056/NEJMoa052306]

30 **Radpour R**, Sikora M, Grussenmeyer T, Kohler C, Barekati Z, Holzgreve W, Lefkovits I, Zhong XY. Simultaneous isolation of DNA, RNA, and proteins for genetic, epigenetic, transcriptomic, and proteomic analysis. *J Proteome Res*2009; **8**: 5264-5274 [PMID: 19780627 DOI: 10.1021/pr900591w]

31 **Amado RG**, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman

DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 1626-1634 [PMID: 18316791 DOI: 10.1200/JCO.2007.14.7116]

32 **Endris V**, Stenzinger A, Pfarr N, Penzel R, Möbs M, Lenze D, Darb-Esfahani S, Hummel M, Sabine-Merkelbach-Bruse, Jung A, Lehmann U, Kreipe H, Kirchner T, Büttner R, Jochum W, Höfler G, Dietel M, Weichert W, Schirmacher P. NGS-based BRCA1/2 mutation testing of high-grade serous ovarian cancer tissue: results and conclusions of the first international round robin trial. *Virchows Arch* 2016; **468**: 697-705 [PMID: 27003155 DOI: 10.1007/s00428-016-1919-8]

33 **Jesinghaus M**, Pfarr N, Endris V, Kloor M, Volckmar AL, Brandt R, Herpel E, Muckenhuber A, Lasitschka F, Schirmacher P, Penzel R, Weichert W, Stenzinger A. Genotyping of colorectal cancer for cancer precision medicine: Results from the IPH Center for Molecular Pathology. *Genes Chromosomes Cancer* 2016; **55**: 505-521 [PMID: 26917275 DOI: 10.1002/gcc.22352]

34 **Haghighi MM**, Radpour R, Mahmoudi T, Mohebbi SR, Vahedi M, Zali MR. Association between MTHFR polymorphism (C677T) with nonfamilial colorectal cancer. *Oncol Res* 2009; **18**: 57-63 [PMID: 20066895 DOI: 10.3727/096504009789954636]

35 **Wick W**, Weller M, van den Bent M, Sanson M, Weiler M, von Deimling A, Plass C, Hegi M, Platten M, Reifenberger G. MGMT testing--the challenges for biomarker-based glioma treatment. *Nat Rev Neurol* 2014; **10**: 372-385 [PMID: 24912512 DOI: 10.1038/nrneurol.2014.100]

36 **Moran S**, Martínez-Cardús A, Sayols S, Musulén E, Balañá C, Estival-Gonzalez A, Moutinho C, Heyn H, Diaz-Lagares A, de Moura MC, Stella GM, Comoglio PM, Ruiz-Miró M, Matias-Guiu X, Pazo-Cid R, Antón A, Lopez-Lopez R, Soler G, Longo F, Guerra I, Fernandez S, Assenov Y, Plass C, Morales R, Carles J, Bowtell D, Mileshkin L, Sia D, Tothill R, Tabernero J, Llovet JM, Esteller M. Epigenetic profiling to classify cancer of unknown primary: a multicentre, retrospective analysis. *Lancet Oncol* 2016; **17**: 1386-1395 [PMID: 27575023 DOI: 10.1016/S1470-2045(16)30297-2]

37 **Röhrich M**, Koelsche C, Schrimpf D, Capper D, Sahm F, Kratz A, Reuss J, Hovestadt V, Jones DT, Bewerunge-Hudler M, Becker A, Weis J, Mawrin C, Mittelbronn M, Perry A, Mautner VF, Mechtersheimer G, Hartmann C, Okuducu AF, Arp M, Seiz-Rosenhagen M, Hänggi D, Heim S, Paulus W, Schittenhelm J, Ahmadi R, Herold-Mende C, Unterberg A, Pfister SM, von Deimling A, Reuss DE. Methylation-based classification of benign and malignant peripheral nerve sheath tumors. *Acta Neuropathol* 2016; **131**: 877-887 [PMID: 26857854 DOI: 10.1007/s00401-016-1540-6]

38 **Radpour R**, Barekati Z, Haghighi MM, Kohler C, Asadollahi R, Torbati PM, Holzgreve W, Zhong XY. Correlation of telomere length shortening with promoter methylation profile of p16/Rb and p53/p21 pathways in breast cancer. *Mod Pathol* 2010; **23**: 763-772 [PMID: 20081803 DOI: 10.1038/modpathol.2009.195]

39 **Stenzinger A**, Kriegsmann M, Weichert W. [The role of pathology in the diagnostics of CUP syndrome]. *Radiologe* 2014; **54**: 124-133 [PMID: 24463713 DOI: 10.1007/s00117-013-2546-x]

40 **Harris LN**, Ismaila N, McShane LM, Andre F, Collyar DE, Gonzalez-Angulo AM, Hammond EH, Kuderer NM, Liu MC, Mennel RG, Van Poznak C, Bast RC, Hayes DF; American Society of Clinical Oncology. Use of Biomarkers to Guide Decisions on Adjuvant Systemic Therapy for Women With Early-Stage Invasive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline. *J Clin Oncol* 2016; **34**: 1134-1150 [PMID: 26858339 DOI: 10.1200/JCO.2015.65.2289]

41 **Radpour R**, Haghighi MM, Fan AX, Torbati PM, Hahn S, Holzgreve W, Zhong XY. High-throughput hacking of the methylation patterns in breast cancer by in vitro transcription and thymidine-specific cleavage mass array on MALDI-TOF silicochip. *Mol Cancer Res* 2008; **6**: 1702-1709 [PMID: 19010818 DOI: 10.1158/1541-7786.MCR-08-0262]

42 **Radpour R**, Kohler C, Haghighi MM, Fan AX, Holzgreve W, Zhong XY. Methylation profiles of 22 candidate genes in breast cancer using high-throughput MALDI-TOF mass array. *Oncogene* 2009; **28**: 2969-2978 [PMID: 19503099 DOI: 10.1038/onc.2009.149]

43 **Barekati Z**, Radpour R, Kohler C, Zhang B, Toniolo P, Lenner P, Lv Q, Zheng H, Zhong XY. Methylation profile of TP53 regulatory pathway and mtDNA alterations in breast cancer patients lacking TP53 mutations. *Hum Mol Genet*2010; **19**: 2936-2946 [PMID: 20466735 DOI: 10.1093/hmg/ddq199]

44 **Radpour R**, Barekati Z, Kohler C, Schumacher MM, Grussenmeyer T, Jenoe P, Hartmann N, Moes S, Letzkus M, Bitzer J, Lefkovits I, Staedtler F, Zhong XY. Integrated epigenetics of human breast cancer: synoptic investigation of targeted genes, microRNAs and proteins upon demethylation treatment. *PLoS One* 2011; **6**: e27355 [PMID: 22076154 DOI: 10.1371/journal.pone.0027355]

45 **Radpour R**, Barekati Z, Kohler C, Lv Q, Bürki N, Diesch C, Bitzer J, Zheng H, Schmid S, Zhong XY. Hypermethylation of tumor suppressor genes involved in critical regulatory pathways for developing a blood-based test in breast cancer. *PLoS One* 2011; **6**: e16080 [PMID: 21283676 DOI: 10.1371/journal.pone.0016080]

46 **Barekati Z**, Radpour R, Lu Q, Bitzer J, Zheng H, Toniolo P, Lenner P, Zhong XY. Methylation signature of lymph node metastases in breast cancer patients. *BMC Cancer* 2012; **12**: 244 [PMID: 22695536 DOI: 10.1186/1471-2407-12-244]

47 **Tang F**, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, Wang X, Bodeau J, Tuch BB, Siddiqui A, Lao K, Surani MA. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods* 2009; **6**: 377-382 [PMID: 19349980 DOI: 10.1038/nmeth.1315]

48 **Haque A**, Engel J, Teichmann SA, Lönnberg T. A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genome Med* 2017; **9**: 75 [PMID: 28821273 DOI: 10.1186/s13073-017-0467-4]

49 **Macosko EZ**, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, Tirosh I, Bialas AR, Kamitaki N, Martersteck EM, Trombetta JJ, Weitz DA, Sanes JR, Shalek AK, Regev A, McCarroll SA. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell* 2015; **161**: 1202-1214 [PMID: 26000488 DOI: 10.1016/j.cell.2015.05.002]

50 **Ziegenhain C**, Vieth B, Parekh S, Reinius B, Guillaumet-Adkins A, Smets M, Leonhardt H, Heyn H, Hellmann I, Enard W. Comparative Analysis of Single-Cell RNA Sequencing Methods. *Mol Cell* 2017; **65**: 631-643.e4 [PMID: 28212749 DOI: 10.1016/j.molcel.2017.01.023]

51 **Moffitt JR**, Hao J, Bambah-Mukku D, Lu T, Dulac C, Zhuang X. High-performance multiplexed fluorescence in situ hybridization in culture and tissue with matrix imprinting and clearing. *Proc Natl Acad Sci U S A* 2016; **113**: 14456-14461 [PMID: 27911841 DOI: 10.1073/pnas.1617699113]

52 **Trivedi V**, Choi HMT, Fraser SE, Pierce NA. Multidimensional quantitative analysis of mRNA expression within intact vertebrate embryos. *Development* 2018; **145**: pii: dev156869 [PMID: 29311262 DOI: 10.1242/dev.156869]

53 **Spanjaard B**, Hu B, Mitic N, Olivares-Chauvet P, Janjuha S, Ninov N, Junker JP. Simultaneous lineage tracing and cell-type identification using CRISPR-Cas9-induced genetic scars. *Nat Biotechnol* 2018; **36**: 469-473 [PMID: 29644996 DOI: 10.1038/nbt.4124]

54 **Adamson B**, Norman TM, Jost M, Cho MY, Nuñez JK, Chen Y, Villalta JE, Gilbert LA, Horlbeck MA, Hein MY, Pak RA, Gray AN, Gross CA, Dixit A, Parnas O, Regev A, Weissman JS. A Multiplexed Single-Cell CRISPR Screening Platform Enables Systematic Dissection of the Unfolded Protein Response. *Cell* 2016; **167**: 1867-1882.e21 [PMID: 27984733 DOI: 10.1016/j.cell.2016.11.048]

55 **Datlinger P**, Rendeiro AF, Schmidl C, Krausgruber T, Traxler P, Klughammer J, Schuster LC, Kuchler A, Alpar D, Bock C. Pooled

CRISPR screening with single-cell transcriptome readout. *Nat Methods* 2017; **14**: 297-301 [PMID: 28099430 DOI: 10.1038/nmeth.4177]

56 **Winterhoff BJ**, Maile M, Mitra AK, Sebe A, Bazzaro M, Geller MA, Abrahante JE, Klein M, Hellweg R, Mullany SA, Beckman K, Daniel J, Starr TK. Single cell sequencing reveals heterogeneity within ovarian cancer epithelium and cancer associated stromal cells. *Gynecol Oncol* 2017; **144**: 598-606 [PMID: 28111004 DOI: 10.1016/j.ygyno.2017.01.015]

57 **Giustacchini A**, Thongjuea S, Barkas N, Woll PS, Povinelli BJ, Booth CAG, Sopp P, Norfo R, Rodriguez-Meira A, Ashley N, Jamieson L, Vyas P, Anderson K, Segerstolpe Å, Qian H, Olsson-Strömberg U, Mustjoki S, Sandberg R, Jacobsen SEW, Mead AJ. Single-cell transcriptomics uncovers distinct molecular signatures of stem cells in chronic myeloid leukemia. *Nat Med* 2017; **23**: 692-702 [PMID: 28504724 DOI: 10.1038/nm.4336]

58 **Walter C**, Pozzorini C, Reinhardt K, Geffers R, Xu Z, Reinhardt D, von Neuhoff N, Hanenberg H. Single-cell whole exome and targeted sequencing in NPM1/FLT3 positive pediatric acute myeloid leukemia. *Pediatr Blood Cancer* 2018; **65** [PMID: 29090521 DOI: 10.1002/pbc.26848]

59 **Hui T**, Cao Q, Wegrzyn-Woltosz J, O'Neill K, Hammond CA, Knapp DJHF, Laks E, Moksa M, Aparicio S, Eaves CJ, Karsan A, Hirst M. High-Resolution Single-Cell DNA Methylation Measurements Reveal Epigenetically Distinct Hematopoietic Stem Cell Subpopulations. *Stem Cell Reports* 2018; **11**: 578-592 [PMID: 30078558 DOI: 10.1016/j.stemcr.2018.07.003]

60 **Sen R**, Dolgalev I, Bayin NS, Heguy A, Tsirigos A, Placantonakis DG. Single-Cell RNA Sequencing of Glioblastoma Cells. *Methods Mol Biol* 2018; **1741**: 151-170 [PMID: 29392698 DOI: 10.1007/978-1-4939-7659-1_12]

61 **Johnson E**, Dickerson KL, Connolly ID, Hayden Gephart M. Single-Cell RNA-Sequencing in Glioma. *Curr Oncol Rep* 2018; **20**: 42 [PMID: 29637300 DOI: 10.1007/s11912-018-0673-2]

62 **Colacino JA**, Azizi E, Brooks MD, Harouaka R, Fouladdel S, McDermott SP, Lee M, Hill D, Madden J, Boerner J, Cote ML, Sartor MA, Rozek LS, Wicha MS. Heterogeneity of Human Breast Stem and Progenitor Cells as Revealed by Transcriptional Profiling. *Stem Cell Reports* 2018; **10**: 1596-1609 [PMID: 29606612 DOI: 10.1016/j.stemcr.2018.03.001]

63 **Fridman WH**, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; **12**: 298-306 [PMID: 22419253 DOI: 10.1038/nrc3245]

64 **Curiel TJ**, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10**: 942-949 [PMID: 15322536 DOI: 10.1038/nm1093]

65 **Baitsch L**, Baumgaertner P, Devêvre E, Raghav SK, Legat A, Barba L, Wieckowski S, Bouzourene H, Deplancke B, Romero P, Rufer N, Speiser DE. Exhaustion of tumor-specific $CD8^+$ T cells in metastases from melanoma patients. *J Clin Invest* 2011; **121**: 2350-2360 [PMID: 21555851 DOI: 10.1172/JCI46102]

66 **Schatz DG**, Ji Y. Recombination centres and the orchestration of V(D)J recombination. *Nat Rev Immunol* 2011; **11**: 251-263 [PMID: 21394103 DOI: 10.1038/nri2941]

67 **Kirsch IR**, Watanabe R, O'Malley JT, Williamson DW, Scott LL, Elco CP, Teague JE, Gehad A, Lowry EL, LeBoeuf NR, Krueger JG, Robins HS, Kupper TS, Clark RA. TCR sequencing facilitates diagnosis and identifies mature T cells as the cell of origin in CTCL. *Sci Transl Med* 2015; **7**: 308ra158 [PMID: 26446955 DOI: 10.1126/scitranslmed.aaa9122]

68 **Bindea G**, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, Angell H, Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman WH, Becker C, Pagès F, Speicher MR, Trajanoski Z, Galon J. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* 2013; **39**: 782-795 [PMID: 24138885 DOI: 10.1016/j.immuni.2013.10.003]

69 **Robins HS**, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O, Riddell SR, Warren EH, Carlson CS. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood* 2009; **114**: 4099-4107 [PMID: 19706884 DOI: 10.1182/blood-2009-04-217604]

70 **Sherwood AM**, Emerson RO, Scherer D, Habermann N, Buck K, Staffa J, Desmarais C, Halama N, Jaeger D, Schirmacher P, Herpel E, Kloor M, Ulrich A, Schneider M, Ulrich CM, Robins H. Tumor-infiltrating lymphocytes in colorectal tumors display a diversity of T cell receptor sequences that differ from the T cells in adjacent mucosal tissue. *Cancer Immunol Immunother* 2013; **62**: 1453-1461 [PMID: 23771160 DOI: 10.1007/s00262-013-1446-2]

71 **Zemmour D**, Zilionis R, Kiner E, Klein AM, Mathis D, Benoist C. Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. *Nat Immunol* 2018; **19**: 291-301 [PMID: 29434354 DOI: 10.1038/s41590-018-0051-0]

72 **De Simone M**, Rossetti G, Pagani M. Single Cell T Cell Receptor Sequencing: Techniques and Future Challenges. *Front Immunol* 2018; **9**: 1638 [PMID: 30072991 DOI: 10.3389/fimmu.2018.01638]

73 **Zhang X**, Zhang M, Hou Y, Xu L, Li W, Zou Z, Liu C, Xu A, Wu S. Single-cell analyses of transcriptional heterogeneity in squamous cell carcinoma of urinary bladder. *Oncotarget* 2016; **7**: 66069-66076 [PMID: 27602771 DOI: 10.18632/oncotarget.11803]

74 **Li Y**, Xu X, Song L, Hou Y, Li Z, Tsang S, Li F, Im KM, Wu K, Wu H, Ye X, Li G, Wang L, Zhang B, Liang J, Xie W, Wu R, Jiang H, Liu X, Yu C, Zheng H, Jian M, Nie L, Wan L, Shi M, Sun X, Tang A, Guo G, Gui Y, Cai Z, Li J, Wang W, Lu Z, Zhang X, Bolund L, Kristiansen K, Wang J, Yang H, Dean M, Wang J. Single-cell sequencing analysis characterizes common and cell-lineage-specific mutations in a muscle-invasive bladder cancer. *Gigascience* 2012; **1**: 12 [PMID: 23587365 DOI: 10.1186/2047-217X-1-12]

75 **Bakker B**, Taudt A, Belderbos ME, Porubsky D, Spierings DC, de Jong TV, Halsema N, Kazemier HG, Hoekstra-Wakker K, Bradley A, de Bont ES, van den Berg A, Guryev V, Lansdorp PM, Colomé-Tatché M, Foijer F. Single-cell sequencing reveals karyotype heterogeneity in murine and human malignancies. *Genome Biol* 2016; **17**: 115 [PMID: 27246460 DOI: 10.1186/s13059-016-0971-7]

76 **Gawad C**, Koh W, Quake SR. Dissecting the clonal origins of childhood acute lymphoblastic leukemia by single-cell genomics. *Proc Natl Acad Sci USA* 2014; **111**: 17947-17952 [PMID: 25425670 DOI: 10.1073/pnas.1420822111]

77 **Boyd AL**, Aslostovar L, Reid J, Ye W, Tanasijevic B, Porras DP, Shapovalova Z, Almakadi M, Foley R, Leber B, Xenocostas A, Bhatia M. Identification of Chemotherapy-Induced Leukemic-Regenerating Cells Reveals a Transient Vulnerability of Human AML Recurrence. *Cancer Cell* 2018; **34**: 483-498.e5 [PMID: 30205048 DOI: 10.1016/j.ccell.2018.08.007]

78 **Hughes AE**, Magrini V, Demeter R, Miller CA, Fulton R, Fulton LL, Eades WC, Elliott K, Heath S, Westervelt P, Ding L, Conrad DF, White BS, Shao J, Link DC, DiPersio JF, Mardis ER, Wilson RK, Ley TJ, Walter MJ, Graubert TA. Clonal architecture of secondary acute myeloid leukemia defined by single-cell sequencing. *PLoS Genet* 2014; **10**: e1004462 [PMID: 25010716 DOI: 10.1371/journal.pgen.1004462]

79 **Hou Y**, Song L, Zhu P, Zhang B, Tao Y, Xu X, Li F, Wu K, Liang J, Shao D, Wu H, Ye X, Ye C, Wu R, Jian M, Chen Y, Xie W, Zhang R, Chen L, Liu X, Yao X, Zheng H, Yu C, Li Q, Gong Z, Mao M, Yang X, Yang L, Li J, Wang W, Lu Z, Gu N, Laurie G, Bolund L, Kristiansen K, Wang J, Yang H, Li Y, Zhang X, Wang J. Single-cell exome sequencing and monoclonal evolution of a JAK2-negative myeloproliferative neoplasm. *Cell* 2012; **148**: 873-885 [PMID: 22385957 DOI: 10.1016/j.cell.2012.02.028]

80 **Shepherd MS**, Li J, Wilson NK, Oedekoven CA, Li J, Belmonte

M, Fink J, Prick JCM, Pask DC, Hamilton TL, Loeffler D, Rao A, Schröder T, Göttgens B, Green AR, Kent DG. Single-cell approaches identify the molecular network driving malignant hematopoietic stem cell self-renewal. *Blood* 2018; **132**: 791-803 [PMID: 29991556 DOI: 10.1182/blood-2017-12-821066]

81 **Francis JM**, Zhang CZ, Maire CL, Jung J, Manzo VE, Adalsteinsson VA, Homer H, Haidar S, Blumenstiel B, Pedamallu CS, Ligon AH, Love JC, Meyerson M, Ligon KL. EGFR variant heterogeneity in glioblastoma resolved through single-nucleus sequencing. *Cancer Discov* 2014; **4**: 956-971 [PMID: 24893890 DOI: 10.1158/2159-8290.CD-13-0879]

82 **Patel AP**, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, Cahill DP, Nahed BV, Curry WT, Martuza RL, Louis DN, Rozenblatt-Rosen O, Suvà ML, Regev A, Bernstein BE. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* 2014; **344**: 1396-1401 [PMID: 24925914 DOI: 10.1126/science.1254257]

83 **Baslan T**, Kendall J, Ward B, Cox H, Leotta A, Rodgers L, Riggs M, D'Italia S, Sun G, Yong M, Miskimen K, Gilmore H, Saborowski M, Dimitrova N, Krasnitz A, Harris L, Wigler M, Hicks J. Optimizing sparse sequencing of single cells for highly multiplex copy number profiling. *Genome Res* 2015; **25**: 714-724 [PMID: 25858951 DOI: 10.1101/gr.188060.114]

84 **Lei B**, Zhang XY, Zhou JP, Mu GN, Li YW, Zhang YX, Pang D. Transcriptome sequencing of HER2-positive breast cancer stem cells identifies potential prognostic marker. *Tumour Biol* 2016; **37**: 14757-14764 [PMID: 27629143 DOI: 10.1007/s13277-016-5351-0]

85 **Nguyen A**, Yoshida M, Goodarzi H, Tavazoie SF. Highly variable cancer subpopulations that exhibit enhanced transcriptome variability and metastatic fitness. *Nat Commun* 2016; **7**: 11246 [PMID: 27138336 DOI: 10.1038/ncomms11246]

86 **Gao R**, Davis A, McDonald TO, Sei E, Shi X, Wang Y, Tsai PC, Casasent A, Waters J, Zhang H, Meric-Bernstam F, Michor F, Navin NE. Punctuated copy number evolution and clonal stasis in triple-negative breast cancer. *Nat Genet*2016; **48**: 1119-1130 [PMID: 27526321 DOI: 10.1038/ng.3641]

87 **Kim C**, Gao R, Sei E, Brandt R, Hartman J, Hatschek T, Crosetto N, Foukakis T, Navin NE. Chemoresistance Evolution in Triple-Negative Breast Cancer Delineated by Single-Cell Sequencing. *Cell* 2018; **173**: 879-893.e13 [PMID: 29681456 DOI: 10.1016/j.cell.2018.03.041]

88 **Wang Y**, Waters J, Leung ML, Unruh A, Roh W, Shi X, Chen K, Scheet P, Vattathil S, Liang H, Multani A, Zhang H, Zhao R, Michor F, Meric-Bernstam F, Navin NE. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. *Nature* 2014; **512**: 155-160 [PMID: 25079324 DOI: 10.1038/nature13600]

89 **Yu C**, Yu J, Yao X, Wu WK, Lu Y, Tang S, Li X, Bao L, Li X, Hou Y, Wu R, Jian M, Chen R, Zhang F, Xu L, Fan F, He J, Liang Q, Wang H, Hu X, He M, Zhang X, Zheng H, Li Q, Wu H, Chen Y, Yang X, Zhu S, Xu X, Yang H, Wang J, Zhang X, Sung JJ, Li Y, Wang J. Discovery of biclonal origin and a novel oncogene SLC12A5 in colon cancer by single-cell sequencing. *Cell Res* 2014; **24**: 701-712 [PMID: 24699064 DOI: 10.1038/cr.2014.43]

90 **Liu M**, Di J, Liu Y, Su Z, Jiang B, Wang Z, Su X. Comparison of $EpCAM^{high}CD44^{+}$ cancer stem cells with $EpCAM^{high}CD44^{-}$ tumor cells in colon cancer by single-cell sequencing. *Cancer Biol Ther* 2018; **19**: 939-947 [PMID: 29580161 DOI: 10.1080/15384047.2018.1456605]

91 **Liu M**, Liu Y, Di J, Su Z, Yang H, Jiang B, Wang Z, Zhuang M, Bai F, Su X. Multi-region and single-cell sequencing reveal variable genomic heterogeneity in rectal cancer. *BMC Cancer* 2017; **17**: 787 [PMID: 29169336 DOI: 10.1186/s12885-017-3777-4]

92 **Kim KT**, Lee HW, Lee HO, Song HJ, Jeong da E, Shin S, Kim H, Shin Y, Nam DH, Jeong BC, Kirsch DG, Joo KM, Park WY. Application of single-cell RNA sequencing in optimizing a combinatorial therapeutic strategy in metastatic renal cell carcinoma. *Genome Biol* 2016; **17**: 80 [PMID: 27139883 DOI: 10.1186/s13059-016-0945-9]

93 **Guo X**, Zhang Y, Zheng L, Zheng C, Song J, Zhang Q, Kang B, Liu Z, Jin L, Xing R, Gao R, Zhang L, Dong M, Hu X, Ren X, Kirchhoff D, Roider HG, Yan T, Zhang Z. Global characterization of T cells in non-small-cell lung cancer by single-cell sequencing. *Nat Med* 2018; **24**: 978-985 [PMID: 29942094 DOI: 10.1038/s41591-018-0045-3]

94 **Xu X**, Hou Y, Yin X, Bao L, Tang A, Song L, Li F, Tsang S, Wu K, Wu H, He W, Zeng L, Xing M, Wu R, Jiang H, Liu X, Cao D, Guo G, Hu X, Gui Y, Li Z, Xie W, Sun X, Shi M, Cai Z, Wang B, Zhong M, Li J, Lu Z, Gu N, Zhang X, Goodman L, Bolund L, Wang J, Yang H, Kristiansen K, Dean M, Li Y, Wang J. Single-cell exome sequencing reveals single-nucleotide mutation characteristics of a kidney tumor. *Cell* 2012; **148**: 886-895 [PMID: 22385958 DOI: 10.1016/j.cell.2012.02.025]

95 **Kim KT**, Lee HW, Lee HO, Kim SC, Seo YJ, Chung W, Eum HH, Nam DH, Kim J, Joo KM, Park WY. Single-cell mRNA sequencing identifies subclonal heterogeneity in anti-cancer drug responses of lung adenocarcinoma cells. *Genome Biol* 2015; **16**: 127 [PMID: 26084335 DOI: 10.1186/s13059-015-0692-3]

96 **Suzuki A**, Matsushima K, Makinoshima H, Sugano S, Kohno T, Tsuchihara K, Suzuki Y. Single-cell analysis of lung adenocarcinoma cell lines reveals diverse expression patterns of individual cells invoked by a molecular target drug treatment. *Genome Biol* 2015; **16**: 66 [PMID: 25887790 DOI: 10.1186/s13059-015-0636-y]

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MINIREVIEWS

Pancreatic cancer stem cells: Perspectives on potential therapeutic approaches of pancreatic ductal adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma is one of the most aggressive solid tumours of the pancreas, characterised by a five-year survival rate less than 8%. Recent reports that pancreatic cancer stem cells (PCSCs) contribute to the tumorigenesis, progression, and chemoresistance of pancreatic cancer have prompted the investigation of new therapeutic approaches able to directly target PCSCs. In the present paper the non-cancer related drugs that have been proposed to target CSCs that could potentially combat pancreatic cancer are reviewed and evaluated. The role of some pathways and deregulated proteins in PCSCs as new therapeutic targets are also discussed with a focus on selected specific inhibitors. Finally, advances in the development of nanoparticles for targeting PCSCs and site-specific drug delivery are highlighted, and their limitations considered.

Key words: Pancreatic cancer stem cells; Pancreatic cancer; Therapeutic approaches; Pancreas; Treatment

Core tip: Pancreatic cancer is characterised by remarkable resistance to treatment conferred by pancreatic cancer stem cells (PCSCs). Unfortunately, most conventional treatments are unable to eradicate tumours. Recent research has focused on characterising PCSCs to accelerate the development of novel therapeutic strategies. In the present paper, we shed light on promising new strategies such as using non-cancer drugs as anticancer therapeutics, targeting of deregulated pathways and proteins of PCSCs, and using nanoparticles for improved drug delivery.

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INTRODUCTION

Pancreatic cancer comprises many types of cancers, of which the most common is an infiltrating neoplasm named pancreatic ductal adenocarcinoma (PDAC)[1], which derives from the pancreatic ductal tree[2]. PDAC is almost always fatal, it is refractory to conventional treatments, and consequently has a documented five-year survival rate as low as 8%. The major driver genes participating in the whole process of disease development include *KRAS, TP53, CDKN2A*, and *SMAD4*. With a near 100% *KRAS* mutation frequency, PDAC is considered the most RAS-addicted of all cancers[3]. PDAC is also characterised by a dense tumour microenvironment, perineural and vascular local growth, and early distant metastases. In particular, it typically has a tendency to metastasise preferentially to the liver where soluble factors and extracellular vesicles deriving from the primary tumour contribute to form a supportive niche[4]. Patients seldom exhibit symptoms. Therefore, early diagnosis of the tumour is very difficult. Indeed, the majority of patients are diagnosed when metastatic events have occurred or during advanced-stage disease. For this reason, primary prevention such as avoiding smoking and having a fat-poor diet is important[5]. Currently, surgery coupled with chemo or radiation therapy is the main treatment approach although it doesn't present satisfactory results[6]. Moreover, disease can persist or recur with local and distant metastases. Most patients subjected to resection of the tumour die from metastasis within five years[7]. Despite its low efficacy, gemcitabine (a pyrimidine analogue) was the first-choice chemotherapeutic strategy in advanced PDAC for many years[8]. It is effective in only 23.8% of PDAC cases[9] due to dense tumour stroma and scarce diffusion of drug and to subsequent development of gemcitabine chemoresistance[10].

Recently, understanding of pancreatic carcinogenesis has improved and some new therapeutic options have been suggested. For example, it has been demonstrated that FOLFIRINOX, a chemotherapy regimen made up of four drugs (folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin), or nab-paclitaxel plus gemcitabine provide a survival benefit over gemcitabine alone[11]. However, we are still far from a substantially better life expectancy for patients since these new therapeutic options increase the median survival by only a few months.

A growing body of evidence suggests that the drug resistance and metastasis of PDAC are mainly influenced by the presence of cancer stem cells (CSCs). In the present paper, we aim to summarise the current understanding of pancreatic cancer stem cells (PCSCs) and analyse and discuss therapeutic options for targeting PCSCs.

PCSCs

PCSCs characteristics

It has recently been demonstrated that CSCs play critical roles in resistance to anticancer treatment and are responsible for metastasis in several human malignancies, including PDAC[12]. CSCs are rare immortal tumour cells, which have the ability to self-renew, produce differentiated progeny, form tumours in mice, and form non-adherent spheroids called tumour-spheres *in vitro*[13,14]. CSCs are more resistant than non-CSCs to chemotherapy and radiotherapy treatments because they have higher expression levels of anti-apoptotic proteins, ABC transporters, and multidrug resistance genes[15]. These cells reside in a niche, a specific hypoxic/necrotic microenvironment that includes different cell types (each one possessing distinct metabolic properties), such as fibroblastic, immune, endothelial, and perivascular cells, as well as extracellular matrix components, cytokines, and growth factors. In this environment, CSCs protect and reprogramme their metabolism and respond to the metabolism of surrounding cells, increasing tumour growth and preserving phenotypic plasticity[14,16]. Induction and maintenance of CSC phenotypes are related to more than 20 different transcription factors, including NF-κB and the hypoxia inducible factors[13,17]. Moreover, CSCs adjust their metabolism to their microenvironment by acquiring intermediate metabolic phenotypes or shifting from oxidative phosphorylation (OXPHOS) to glycolysis/Warburg effect. CSCs are also characterised by a high autophagic flux, which is involved in resistance to microenvironment stresses, such as hypoxia, starvation, or anticancer treatment[18]. Thus, it has been supposed that autophagy plays a significant role in the resistance to CSCs related anticancer therapy[19].

Pancreatic CSCs, first described in 2007[20], represent less than 1% of all pancreatic cancer cells[21] and are responsible for PDAC tumour growth (initiation, progression, and recurrence), maintenance, metastasis, and chemoresistance. The origin of PCSCs remains unknown. The hypothesized sources are: Tissue stem cells or progenitor cells, stem cells derived from bone marrow, or dedifferentiated cells that result from genetic mutation[22]. PCSCs can be identified by markers, such as CD133, CD24, CD44, ESA/EpCAM (epithelial-specific antigen), c-Met, ALDH1, DclK1, CXCR4, and Lgr5. However, a universal signature is still lacking[13,23,24]. The main signalling pathways of PCSCs, which are essential for self-renewal, are the epithelial to mesenchymal transition (EMT) process, and resistance to conventional therapies include Wnt/β-catenin, Sonic Hedgehog (SHH), and Notch. In addition, other biological aspects, such as autophagy, forkhead box protein M1 (FoxM1), mammalian target of rapamycin (mTOR), Bmi-1, NODAL/ACTIVIN, NF-κB and PTEN pathways, have been shown

to be implicated in PCSC activity.

Importantly, PCSCs co-exist with other cellular and non-cellular components that constitute the tumour microenvironment (including cancer-associated fibroblasts, pancreatic stellate cells, and tumour-associated macrophages). Understanding the relationship between PCSCs and all these components is extremely important to improve the knowledge of the PCSC biology[12]. Recently, it has been demonstrated that PCSCs are involved in highly dynamic cross-talk with the PDAC parenchymal cells[25] by a symbiotic relationship that underlies the initiation and maintenance of early PDAC infiltration and metastasis. In particular, the secretome of PCSCs paracrinically inhibits parental cell growth and autocrinically stimulates their own growth and vascularity, while the secretome of parental cells both paracrinically inhibits PCSC growth and autocrinically inhibits their own growth. It is clear that to make a substantial impact on pancreatic cancer, it is necessary to eradicate PCSCs with targeted therapeutics[26]. For this reason, a complete molecular characterisation of PCSC biology is fundamental. Recently, we have characterised the proteome[7] and the secretome[27] of Panc1 CSCs, demonstrating the functional role of fatty acid synthesis and mevalonate pathways in PCSC viability and identifying secreted proteins involved in cancer differentiation, invasion, and metastasis. Through a combined proteomics and metabolomics approach we also found that Panc1 CSCs, as compared to the parental Panc1 cells, have induced expression of proteins and metabolites involved in glycolysis, pyruvate-malate cycle, folate cycle, pentose phosphate pathway, and lipid metabolism, and reduced expression of proteins and metabolites involved in the Krebs cycle, spliceosome, and non-homologous end joining pathway[7].

PCSCs chemoresistance

Chemoresistance is the major obstacle to successful cancer treatment. Many drugs are not able to eliminate PDAC, which represents the primary reason for tumour recurrence and metastasis. PCSCs are very resistant and can survive conventional treatments interfering with the total eradication of a tumour[16,23]. The mechanisms involved in the chemoresistance of CSCs include the metabolic inactivation of the drug and efflux of the drug from the cells, as well as mutation or deregulation of the drug targets[28]. In particular, an altered drug transport activity, as an over-expression of aldehyde dehydrogenase and proteasome, and a decreased expression of the human equilibrative nucleoside transporters (ENTs) and human concentrative nucleoside transporters (CNTs), play a key role in the chemoresistance of PCSCs[23].

As previously reported, PCSCs reside in niches that are responsible for the protection of cancer cells, tumour growth, and phenotypic plasticity. Critical components for the ever-changing tumour microenvironment and for construction of CSCs niche are Wnt/RSPO (R-spondin), c-Jun N-terminal protein kinase (JNK), Nodal/Activin, Notch, or Hedgehog proteins[23]. This specific CSCs microenvironment has also been proposed to contribute to drug resistance.

Chemoresistance is also related to the EMT process, which has a fundamental role in invasive and metastatic behaviour in PDAC. EMT, in pancreatic cancer cells, is controlled by several transcription factors, such as Zeb1, which suppresses the adhesion molecule E-cadherin by repressing the miR-203 (an inhibitor of stemness) and the miR-200 family members (which regulate expression of stem cell factors)[29]. Accordingly, it has been demonstrated that the class I HDAC inhibitor mocetinostat interferes with Zeb1 function, represses EMT, and restores the drug sensitivity of PDAC cells[30]. In particular, EMT contributes to enhanced resistance to gemcitabine because it leads to an increase in cancer cells with reduced expression of nucleoside transporters (ENT and CNT) that are involved in drug uptake[31]. Finally, it has been hypothesised that quiescence protects PCSCs from chemotherapeutic treatment, which usually targets rapidly proliferating cells.

POTENTIAL THERAPIES TARGETING PCSCs

It is broadly accepted that development of anti-cancer drugs to target determinant pathways and proteins of PCSCs will improve chemotherapeutic outcomes[15]. Eradication of these CSCs should be able to stop tumour progression and reduce future tumour insurgences[26,32]. Potential strategies to target PCSCs are discussed in the next section.

Non-cancer related drugs

Some non-cancer related drugs that show anticancer effects against different human CSCs could also represent an option in PCSCs (Table 1). They act through different mechanisms of action including the inhibition of some important PCSCs pathways (Figure 1).

Antibiotics are among the molecules that exhibit extraordinarily diverse biological activities. For example, salinomycin, an antibacterial and coccidiostat ionophore drug, interferes with the activity of KRAS-4B, Wnt, and EMT pathways reducing the viability of breast CSCs[33]. Interestingly, it has also been demonstrated that salinomycin blocks tumour growth and the metastatic spread of PDAC in a genetically engineered mouse model[34]. In addition, the FDA-approved antibiotic azithromycin, which binds to the 50S subunit of the bacterial ribosome, inhibits tumoursphere formation in PDAC and other cancers[35]. Also, the antibiotic tigecycline, developed in response to the antibiotic resistance of some bacteria, reduces the sphere formation of CSCs in pancreatic, breast, lung, and prostate cancers[29]. In particular, it eliminates the therapy-resistant chronic myeloid leukaemia CSCs[36], and a phase I clinical trial demonstrated the safety of its intravenous infusions in patients with acute myeloid

Table 1 Non-cancer related drugs and their potential effects on pancreatic cancer stem cells

Drug	Function	Relative pathway/process	Ref.
Salinomycin	Anti-bacterial antibiotic	Wnt, EMT	[33]
Azithromycin		Mitochondria	[35]
Nigericin		EMT	[38]
Tigecycline		OXPHOS	[29]
Chloroquine	Anti-malaria	OXPHOS	[39]
Atovaquone		OXPHOS	[36,40]
Aprepitant	Anti-emetic	Wnt	[41]
Ketamine	Anti-depressant	Wnt	[42,43]
Aspirin	Anti-pyretic Anti-inflammatory	ALDH1, NF-κB	[44,45]
Metformin	Anti-diabetic	mTOR, PI3K/Akt	[46-48]
Disulfiram	Anti-alcoholism	NF-κB	[49-53]
Atorvastatin	Anti-cholesterol	Mevalonate	[7,54]

Wnt: Wingless-type MMTV integration site family; EMT: Epithelial mesenchymal transition; OXPHOS: Oxidative phosphorylation; ALDH1: Aldehyde dehydrogenase 1; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; mTOR: Mammalian target of rapamycin; PI3K: Phosphatidylinositol 3-kinase; Akt: Protein kinase B.

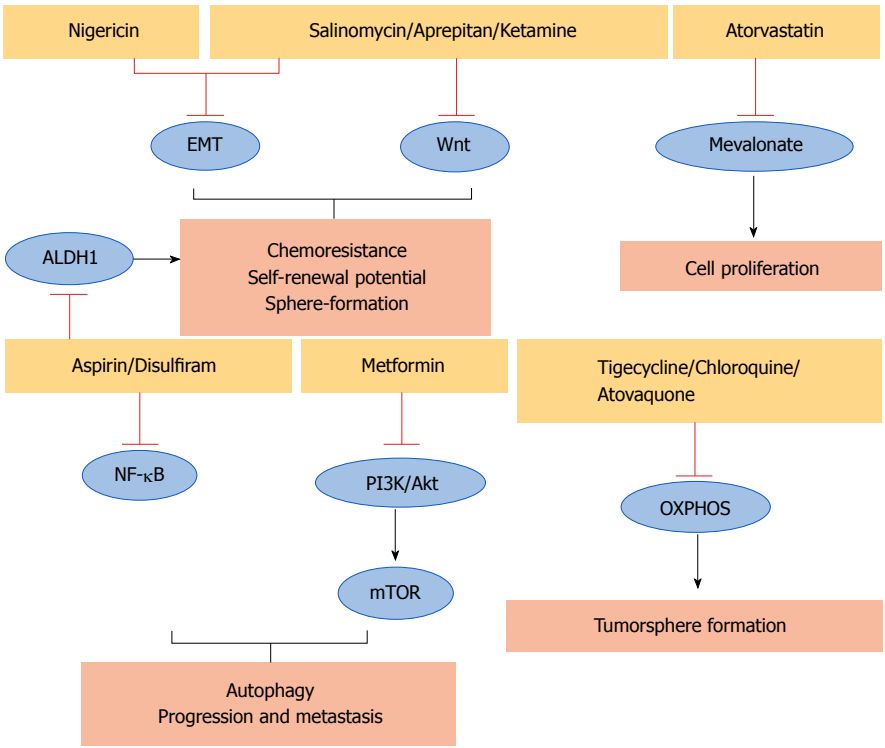

Figure 1 Mechanism of action of different non-cancer related drugs against pancreatic cancer stem cells. EMT: Epithelial mesenchymal transition; ALDH1: Aldehyde dehydrogenase 1; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; mTOR: Mammalian target of rapamycin; PI3K: Phosphatidylinositol 3-kinase; Akt: Protein kinase B; OXPHOS: Oxidative phosphorylation.

leukaemia[37], supporting its transfer to clinical use. Moreover, it has been demonstrated that the antibiotic nigericin increases E-cadherin expression and inhibits the EMT process of CSCs leading to a reduction of invasion and metastasis of colorectal cancer[38]. This observation suggests that it should be further investigated to determine whether if it is also effective for targeting PCSCs.

Some anti-malarial agents may have the potential to target PCSCs. For example, it has been demonstrated that chloroquine has significant effects on PCSCs by inhibiting CXCR4 and Hedgehog pathways[39]. The same can also be said for another anti-malarial compound atovaquone, which acts as a potent and selective OXPHOS inhibitor, inhibiting the sphere-formation of CSCs in breast cancer[40].

Also showing promise for targeting PCSCs is aprepitant, an FDA-approved antiemetic drug that inhibits Wnt signalling, sphere formation, growth, and stemness of CSCs in colon cancer[41]. Ketamine, a drug used as an anaesthetic and depression, reduces CSCs traits and tumour growth in a colorectal cancer model. In

particular, it acts by decreasing Wnt activity[42]. Notably, ketamine reportedly inhibits the proliferation of PDAC cells[43].

Salicylic acid, also known as aspirin, is another non-cancer related drug that may be a candidate for eliminating PCSCs in the successful treatment of PDAC. Indeed, aspirin, commonly used as an antipyretic and anti-inflammatory drug, counteracts PCSCs features such as ALDH1 activity, NF-κB signalling, self-renewal potential, and gemcitabine resistance[44]. A phase Ⅲ trial confirmed the beneficial effect of aspirin as an adjuvant treatment to prevent disease recurrence and contribute to survival after primary therapy in breast, colorectal, gastro-oesophageal, and prostate tumours[45].

Metformin, a dimethylbiguanide used as an anti-diabetic drug, is also able to counteract the features of PCSCs. It inhibits the mTOR and PI3K/Akt pathways, reducing the expression of PCSCs markers in pancreatic tissue, as well as the size and number of tumour spheres. Moreover, *in vivo* experiments demonstrated that metformin prevents progression and metastasis in PDAC[46]. Unfortunately, a phase Ⅱ trial showed that metformin does not improve the outcome in patients with advanced metastatic PDAC treated with standard therapy[47,48]. These findings suggest that future research should include studies of more potent biguanides.

Another non-cancer related drug is disulfiram, a drug widely used to control alcoholism, which is involved in the inhibition of NF-κB, ERK and proteasome pathways in PDAC. It has been demonstrated that disulfiram in combination with chemotherapy or chemoradiation, is able to target PCSCs[49-52]. Notably, a phase IIb trial demonstrated that the addition of disulfiram to chemotherapy prolonged survival in patients with newly diagnosed non-small cell lung cancer[53].

Moreover, we have recently demonstrated[7] that atorvastatin, a drug used to lower blood cholesterol, reduces the viability of PCSCs. Accordingly, the anti-cancer effect of cholesterol-reducing agents has been demonstrated against other CSCs. To date, in a clinical setting statin intake was significantly associated with longer recurrence-free survival in hepatocellular carcinoma patients with hepatectomy[54].

Taken together, these findings indicate that repurposing established compounds to target PCSCs could represent a good strategy for combating PDAC. It is also economically advantageous and assures rapid translation into clinical because these compounds often are already approved by the FDA and show minor side effects compared to traditional chemotherapeutic drugs[29].

Compounds focused on deregulated pathways and proteins

In the last ten years, many agents that target specific deranged pathways of pancreatic tumour cells have shown promise in preclinical studies. Accordingly, potential therapies targeting PCSCs could be developed based on their deregulated pathways and/or proteins (Table 2). As stated above, multiple signalling pathways are known to be important for stemness, including the Wnt/β-catenin, SHH, Notch, and mTOR pathways. Some compounds that inhibit the Wnt signalling pathway have been reported in the previous section on non-cancer related drugs (*i.e*., salinomycin, aprepitant, and ketamine). It has been demonstrated that crocetinic acid (a carotenoid obtained from saffron) is able to target PCSCs by inhibiting the expression of both SHH and smoothened proteins, which play a key role in the SHH pathways[55]. SHH and smoothened proteins lead to the activation of the Gli transcription factor and target genes involved in stem cell maintenance. In particular, crocetinic acid decreases the number and size of the spheroids in a dose-dependent manner and supresses the expression of DclK1, a PCSCs surface marker[55]. Another natural compound that inhibits the SHH pathways is sanguinarine (an isoquinoline alkaloid derived from *Sanguinaria canadensis*). It has been recently reported to be an effective agent for the inhibition of PCSCs[56]. It inhibits the self-renewal capacity of PCSCs, as well as their migration, invasion, and EMT by suppressing the SHH pathway. Recently, PCSCs have been efficiently eliminated by targeting the SHH pathway using the Gli inhibitor GANT61 in combination with rapamycin (an mTOR inhibitor)[57].

Another deregulated PCSCs pathway that can be targeted is Notch signalling. Its inhibition by γ-secretase inhibitor (RO4929097) as well as by Hes1 shRNA reduces the formation of tumour-spheres and the proportion of PCSCs[58]. Notch signalling can reportedly be inhibited by using quinomycin A (an antibiotic and also classifiable as a non-cancer related drug). Quinomycin A suppresses PCSCs by reducing Notch 1-4 receptors and by decreasing the expression of their ligands (Jagged1, Jagged2, DLL1, DLL3, and DLL4) of the downstream protein Hes1 and the γ-secretase complex[59]. Quinomycin A also decreases the expression of DclK1, CD44, CD24, and EPCAM, retarding the tumour-sphere formation of PCSCs[59]. Clinical trials published several decades ago and not related to pancreatic cancer indicated a modest activity of quinomycin A against some tumours.

Inhibition of mTOR signalling has also been proposed as a novel strategy for targeting CSCs. In particular, it has been shown that greater suppression of PCSCs is obtained by combining gemcitabine with the mTOR inhibitor rapamycin[60] or c-Met/RON inhibitor with the mTOR inhibitor AZD8055[61].

Changes in the expression of PCSC proteins may represent a good starting point to investigate potential therapeutic targets. Recently, we indicated that fatty acid synthase (FASN) might represent a means of eradicating PCSCs[7]. Treatment with cerulenin, a specific FASN inhibitor, led to a reduction of Panc1 CSCs viability and decreased the formation of spheroids. Accordingly, it has been demonstrated that FASN plays a pivotal

Table 2 Deregulated pathways and proteins to target pancreatic cancer stem cells

Deregulated pathways	Compound or strategy	Ref.
Hedgehog	Crocetinic acid	[55]
	Sanguinarine	[56]
	GANT61	[57]
Notch	RO4929097, shRNA	[58]
	Quinomycin A	[59,97,98]
mTOR	Rapamycin	[60]
	AZD8055	[61]
Deregulated proteins	**Compound or strategy**	
FASN	Cerulenin	[7]
AnxA1	siRNA	[64]
MARCKS	MANS peptide	[66]
Galectin-3	Polysaccharide RN1	[70]
PKM2	Lapachol	[72]
	Diallyl disulphide	[73]
ERRγ	GSK5182	[82,83]

shRNA: Short hairpin RNA; siRNA: Small interfering RNA; FASN: Fatty acid synthase; mTOR: Mammalian target of rapamycin; AnxA1: Annexin A1; MARCKS: Myristoylated alanine-rich C-kinase substrate; PKM2: Pyruvate kinase isozyme M2.

role in the maintenance of stemness in other CSCs[62]. Among the potential PCSCs targets we identified annexin A1 (AnxA1)[7], which is an important player in the development and progression of different types of cancer, including pancreatic cancer, and plays a role in the maintenance of stemness and drug resistance in some CSCs[63]. Recent studies have shown that knockdown of AnxA1 decreases cell invasion and metastatic potential in several types of cancer, including PDAC[64].

Another potential therapeutic target that is both overexpressed and oversecreted by Panc1 CSCs and that should be investigated to reduce the viability of PCSCs is myristoylated alanine-rich C-kinase substrate (MARCKS)[7,27], a protein involved in cell motility, cell shape, cell cycle regulation, secretion, and transmembrane transport[65]. It has been demonstrated that a peptide (MANS peptide) that inhibits the function of MARCKS reduces lung cancer metastasis[66].

Another protein overexpressed and oversecreted in Panc1 CSCs is galectin-3 (Gal3)[7,27], which activates RAS signalling[67]. Many studies reported that Gal3 is implicated in cancer stemness, in particular by activation of Notch signalling[68], and that it may therefore represent a good therapeutic target[69]. Accordingly, it has been shown that down-regulation of Gal3 by an allosteric inhibitor, *i.e.*, the polysaccharide RN1 (purified from the flower of *Panax notoginseng*), increases metastatic cancer cell apoptosis and decreases pancreatic cancer cell growth[70,71].

Another potential target of PCSCs we identified was pyruvate kinase isozyme M1/M2 (PKM1/PKM2)[7]. Although PKM2 is a key mediator of glycolysis in cancer cells, research focused on exploiting metabolic pathways for cancer therapy is still scarce. To date, anti-tumour effects have been demonstrated in melanoma cells following treatment with lapachol (a specific PKM2 inhibitor)[72], and inhibition of stemness has been reported in breast CSCs treated with diallyl disulphide which targets PKM2 (and also CD44 and AMPK signalling)[73]. Recently a new series of small molecule PKM2 inhibitors able to inhibit the growth of tumour cells has been synthesised[74]. It could be worthwhile to evaluate their efficacy also on PCSCs.

Another protein involved in metabolism that is overexpressed and oversecreted by Panc1 CSCs is lactate dehydrogenase A (LHDA)[7,27]. LDHA is an important supporter of glucose metabolism in cancer cells. It generates adequate extracellular lactate to provide a favourable microenvironment for CSCs growth and invasion[75]. Although inhibition of LDHA activity has been proposed as an approach to cancer therapy[76], a limited number of LDHA inhibitors are reported in the literature[77]. However, LDHA is transcriptionally regulated by the oncogenic transcription factor FoxM1[78], and some FoxM1 inhibitors (such as thiostrepton, troglitazone, and the FDI-6 molecule) have been reported that could indirectly lead to a reduction of LDHA[79].

Among the upstream regulators of deranged PCSCs proteins, we found there is the oestrogen-related receptor gamma (ERRγ)[7], which promotes metabolic reprogramming in CSCs, pluripotency, OXPHOS, and the glycolysis pathway[80]. A novel strategy for targeting PCSCs could be represented by the inverse agonists of ERRγ, which decrease OXPHOS and mitochondrial activity and promote apoptosis[81]. Accordingly, it has been demonstrated that GSK5182 (an inverse agonist of ERRγ) determines the up-regulation of p21 and p27, promotes G1 phase arrest, and leads to ROS accumulation and pluripotency inhibition in iPS cells[82-84].

Nanoparticles for improved drug delivery

Surgery, chemotherapy, and radiotherapy are the most common anti-cancer therapeutic approaches; however, the non-specific targeting of cancer cells has made these approaches often non-effective with the consequence that higher doses of drugs need to be administered to reach the tumour region[85]. In order to

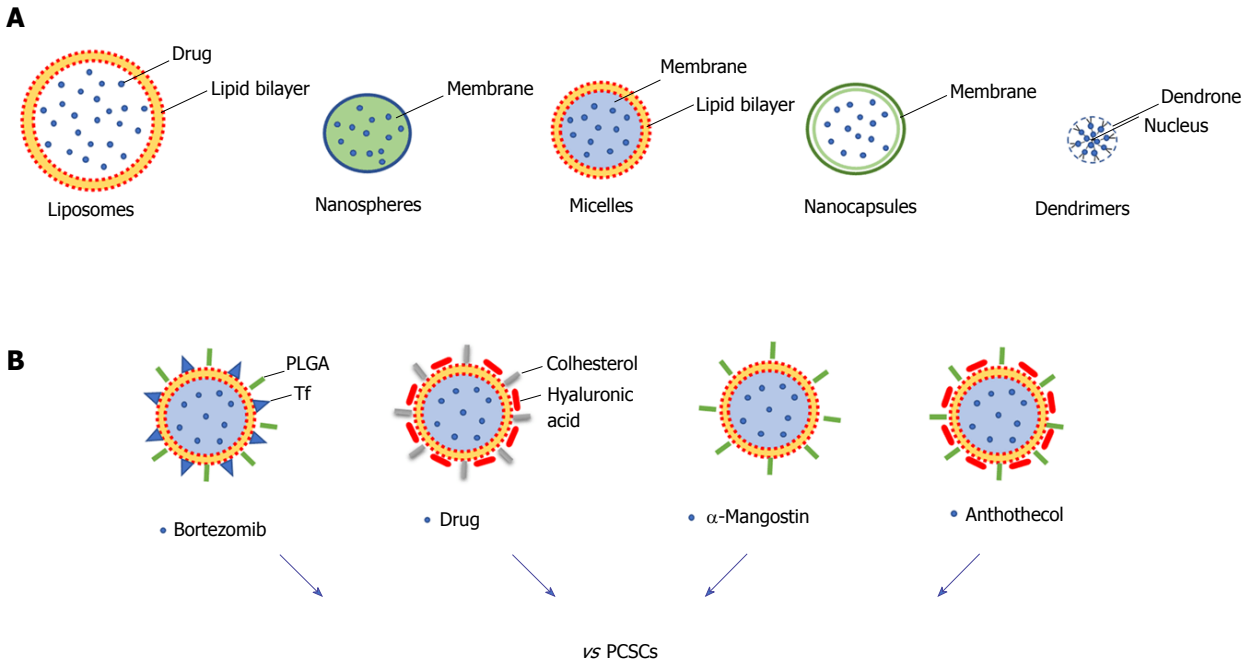

Figure 2 The types of nanoparticles. A: Different types of nanoparticles for targeted drug delivery; B: Specific nanoparticles for targeting pancreatic cancer stem cells. PCSCs: Pancreatic cancer stem cells.

improve the delivery of the drug, nanoparticles (NPs) have been developed to specifically and effectively target CSCs, reducing cytotoxicity and increasing the efficacy of treatments[86]. The different types of NPs include polymeric, magnetic, gold, and mesoporous silica NPs, and they provide a wide range of applications such as cancer therapy, tumour destruction through heating (hyperthermia), and drug/gene delivery[87,88]. In particular, for targeted drug delivery NPs comprise materials such as liposomes (100-400 nm), nanospheres (1-100 nm), micelles (10-100 nm), nanocapsules (10-1000 nm) and dendrimers (3-20 nm) (Figure 2A). These nanocarriers enhance the solubility and formulation of hydrophobic or water-insoluble drugs and control the drug delivery at the cancer tissue.

Some NPs have been developed to target pancreatic cancer, and liposomal formulations have gained regulatory approval[89]. The first clinical trial of NPs conducted in PDAC patients was done using a PEGylated colloidal gold-rhTNF nanomedicin, termed CYT-6091, which demonstrated that NPs greatly reduce the toxicity of chemotherapeutics and may target tumours[88]. In particular, some NPs have been developed to specifically target PCSCs (Figure 2B). PDAC is characterised by dense stroma with a high amount of hyaluronic acid (HA), which reduces drug delivery and interacts with CD44 surface marker regulating the invasion of PDAC cells. HA-based nanogel-drug conjugates with enhanced anticancer activity have been designed for the targeting of CD44-positive and drug-resistant tumours. These conjugates are based on membranotropic cholesteryl-HA (CHA) with various encapsulated drugs, such as the non-cancer related drug salinomycin, etoposide (a chemotherapeutic agent), or curcumin (a natural compound), and all have higher cytotoxicity in CD44-expressing drug-resistant PDAC cells compared to free drugs and to non-modified HA-drug conjugates[90]. Recently, HA-modified poly (dl-lactic-co-glycolic acid)-poly (ethylene glycol) (HA-PLGA-PEG) NPs have been developed for targeted delivery of TTQ (thio-tetrazolyl analogue of a clinical candidate, IC87114) to CD44 over-expressing cancer cells. *In vitro* results showed that cellular uptake led to higher cytotoxicity and enhanced intracellular accumulation of these NPs in high expressing CD44 MiaPaCa2 cells[91].

Natural product-based compounds can be an attractive strategy for the treatment of pancreatic cancer and could be integrated with NP approaches. For some of these, an inhibiting action against PCSCs has already been demonstrated (for example resveratrol, quercetin, and green tea catechins, and curcumin)[92], and for this reason they would deserve to be analysed as nanoparticle formulations. Among these natural compounds there are withaferin A (a major component of *Withania somnifora*) and carnosol (found in *Rosmarinus officinalis*, *Salvia carnosa*, and *Origanum vulgare*). They have suppressive effects on the proliferation, migration, and activation of c-Met in PCSCs[93]. A recent study investigated the role of α-mangostin (derived from the plant mangosteen) encapsulated NPs (Mang-NPs) in the inhibition of pancreatic carcinogenesis by targeting CSCs in human and transgenic mice. The data obtained indicated that Mang-NPs suppress PCSCs features (*i.e.*, EMT, cell proliferation, cell cycle, pluripotency, self-renewal, and apoptosis) and also target CSCs in mice[94]. A similar approach has been implemented for the investigation of the efficacy of anthothecol (an antimalarial compound) encapsulated by PLGA NPs (antho-NPs) against PCSCs.

Interestingly, it has been demonstrated that antho-NPs specifically inhibit PCSCs growth by modulating the SHH pathway[95].

Although significant progress has been made in the development of NPs, they are far from optimal. Indeed, there are already problems regarding the low drug loading capacity of some NPs. Liposomes are sometimes affected by drug diffusion through the liposome bilayer, and micellar drugs exhibit *in vivo* instability[90]. For these reasons, polymeric and nanogel drug conjugates, characterized by controlled drug release and higher drug loading capacity, provide a better strategy. Other challenges that must be addressed in the future for clinical use of NPs concern inefficient delivery, inherent toxicity, off-target effects, unfavourable biological distribution, and lack of clearance from the systemic circulation[96]. In conclusion, even if further research is needed for the development of efficient NPs, it is possible to speculate that the targeted delivery system for anti-cancer agents will be translated into clinical practice. It is tempting to imagine that in the near future modified NPs might serve as promising nanocarriers for site-specific drug delivery by targeting PCSCs and that protocol might be further improved for *in vivo* applications.

CONCLUSION

In conclusion, although further studies are needed, the new developments in targeting PCSCs are expected to have high impact in the treatment of PDAC in coming years. Nevertheless, some questions still need further investigation. While PCSCs represent an intriguing target for therapy, their complete characterisation is still needed. The identification of proteomic profiles and in particular of the deregulated pathways and proteins of PCSCs is fundamental to increasing our knowledge about pancreatic cancer and to identify new therapeutic approaches to eradicate PDAC stem cells that result in recurrence of the disease. Thus, enhanced biological knowledge of PCSCs, combined with the development of nanoparticle technology, promises to be key for the development of new effective treatments of pancreatic cancer.

REFERENCES

1 **Gallmeier E**, Gress TM. [Pancreatic ductal adenocarcinoma]. *Internist* (Berl) 2018; **59**: 805-822 [PMID: 29980819 DOI: 10.1007/s00108-018-0460-z]

2 **Luchini C**, Capelli P, Scarpa A. Pancreatic Ductal Adenocarcinoma and Its Variants. *Surg Pathol Clin* 2016; **9**: 547-560 [PMID: 27926359 DOI: 10.1016/j.path.2016.05.003]

3 **Waters AM**, Der CJ. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harb Perspect Med* 2018; **8**: pii: a031435 [PMID: 29229669 DOI: 10.1101/cshperspect.a031435]

4 **Houg DS**, Bijlsma MF. The hepatic pre-metastatic niche in pancreatic ductal adenocarcinoma. *Mol Cancer* 2018; **17**: 95 [PMID: 29903049 DOI: 10.1186/s12943-018-0842-9]

5 **Kleeff J**, Korc M, Apte M, La Vecchia C, Johnson CD, Biankin AV, Neale RE, Tempero M, Tuveson DA, Hruban RH, Neoptolemos JP. Pancreatic cancer. *Nat Rev Dis Primers* 2016; **2**: 16022 [PMID: 27158978 DOI: 10.1038/nrdp.2016.22]

6 **Guseva LN**. [Nitritometric determination of allacyl]. *Farmatsiia* 1969; **18**: 43-45 [PMID: 5793409 DOI: 10.1186/s13045-017-0551-7]

7 **Brandi J**, Dando I, Pozza ED, Biondani G, Jenkins R, Elliott V, Park K, Fanelli G, Zolla L, Costello E, Scarpa A, Cecconi D, Palmieri M. Proteomic analysis of pancreatic cancer stem cells: Functional role of fatty acid synthesis and mevalonate pathways. *J Proteomics* 2017; **150**: 310-322 [PMID: 27746256 DOI: 10.1016/j.jprot.2016.10.002]

8 **Ellenrieder V**, König A, Seufferlein T. Current Standard and Future Perspectives in First- and Second-Line Treatment of Metastatic Pancreatic Adenocarcinoma. *Digestion* 2016; **94**: 44-49 [PMID: 27438590 DOI: 10.1159/000447739]

9 **Liang C**, Shi S, Meng Q, Liang D, Ji S, Zhang B, Qin Y, Xu J, Ni Q, Yu X. Complex roles of the stroma in the intrinsic resistance to gemcitabine in pancreatic cancer: where we are and where we are going. *Exp Mol Med* 2017; **49**: e406 [PMID: 29611542 DOI: 10.1038/emm.2017.255]

10 **Amrutkar M**, Gladhaug IP. Pancreatic Cancer Chemoresistance to Gemcitabine. *Cancers* (Basel) 2017; **9**: pii: E157 [PMID: 29144412 DOI: 10.3390/cancers9110157]

11 **Al Haddad AH**, Adrian TE. Challenges and future directions in therapeutics for pancreatic ductal adenocarcinoma. *Expert Opin Investig Drugs* 2014; **23**: 1499-1515 [PMID: 25078674 DOI: 10.1517/13543784.2014.933206]

12 **Valle S**, Martin-Hijano L, Alcalá S, Alonso-Nocelo M, Sainz B Jr. The Ever-Evolving Concept of the Cancer Stem Cell in Pancreatic Cancer. *Cancers* (Basel) 2018; **10**: pii: E33 [PMID: 29373514 DOI: 10.3390/cancers10020033]

13 **Santamaria S**, Delgado M, Kremer L, Garcia-Sanz JA. Will a mAb-Based Immunotherapy Directed against Cancer Stem Cells Be Feasible? *Front Immunol* 2017; **8**: 1509 [PMID: 29170667 DOI: 10.3389/fimmu.2017.01509]

14 **Plaks V**, Kong N, Werb Z. The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell* 2015; **16**: 225-238 [PMID: 25748930 DOI: 10.1016/j.stem.2015.02.015]

15 **Dawood S**, Austin L, Cristofanilli M. Cancer stem cells: implications for cancer therapy. *Oncology* (Williston Park) 2014; **28**: 1101-1107, 1110 [PMID: 25510809]

16 **Dubarry JJ**, Quinton A, Bancons J. [Use of colopten in intestinal pathology]. *Bord Med* 1971; **4**: 561-564 passim [PMID: 5552673 DOI: 10.3389/fimmu.2017.00939]

17 **Liu P**, Wang Z, Brown S, Kannappan V, Tawari PE, Jiang W, Irache JM, Tang JZ, Armesilla AL, Darling JL, Tang X, Wang W. Liposome encapsulated Disulfiram inhibits NFκB pathway and targets breast cancer stem cells in vitro and in vivo. *Oncotarget* 2014; **5**: 7471-7485 [PMID: 25277186 DOI: 10.18632/oncotarget.2166]

18 **Lei Y**, Zhang D, Yu J, Dong H, Zhang J, Yang S. Targeting autophagy in cancer stem cells as an anticancer therapy. *Cancer Lett* 2017; **393**: 33-39 [PMID: 28216370 DOI: 10.1016/j.canlet.2017.02.012]

19 **Cojoc M**, Mäbert K, Muders MH, Dubrovska A. A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. *Semin Cancer Biol* 2015; **31**: 16-27 [PMID: 24956577 DOI: 10.1016/j.semcancer.2014.06.004]

20 **Li C**, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. *Cancer Res* 2007; **67**: 1030-1037 [PMID: 17283135 DOI: 10.1158/0008-5472.CAN-06-2030]

21 **Dalla Pozza E**, Dando I, Biondani G, Brandi J, Costanzo C, Zoratti E, Fassan M, Boschi F, Melisi D, Cecconi D, Scupoli MT, Scarpa A, Palmieri M. Pancreatic ductal adenocarcinoma cell lines display a plastic ability to bi-directionally convert into cancer stem cells. *Int J Oncol* 2015; **46**: 1099-1108 [PMID: 25502497 DOI: 10.3892/ijo.2014.2796]

22 **Ishiwata T**, Matsuda Y, Yoshimura H, Sasaki N, Ishiwata S, Ishikawa N, Takubo K, Arai T, Aida J. Pancreatic cancer stem

cells: features and detection methods. *Pathol Oncol Res* 2018; **24**: 797-805 [PMID: 29948612 DOI: 10.1007/s12253-018-0420-x]
23 **Rao CV**, Mohammed A. New insights into pancreatic cancer stem cells. *World J Stem Cells* 2015; **7**: 547-555 [PMID: 25914762 DOI: 10.4252/wjsc.v7.i3.547]
24 **Chalquest RR**. Preveterinary requirements and admission to American veterinary colleges: important changes. *J Am Vet Med Assoc* 1986; **189**: 27-29 [PMID: 3733496 DOI: 10.1002/0471141755.ph1425s61]
25 **Biondani G**, Zeeberg K, Greco MR, Cannone S, Dando I, Dalla Pozza E, Mastrodonato M, Forciniti S, Casavola V, Palmieri M, Reshkin SJ, Cardone RA. Extracellular matrix composition modulates PDAC parenchymal and stem cell plasticity and behavior through the secretome. *FEBS J* 2018; **285**: 2104-2124 [PMID: 29660229 DOI: 10.1111/febs.14471]
26 **Subramaniam D**, Kaushik G, Dandawate P, Anant S. Targeting Cancer Stem Cells for Chemoprevention of Pancreatic Cancer. *Curr Med Chem* 2018; **25**: 2585-2594 [PMID: 28137215 DOI: 10.2174/0929867324666170127095832]
27 **Brandi J**, Dalla Pozza E, Dando I, Biondani G, Robotti E, Jenkins R, Elliott V, Park K, Marengo E, Costello E, Scarpa A, Palmieri M, Cecconi D. Secretome protein signature of human pancreatic cancer stem-like cells. *J Proteomics* 2016; **136**: 1-12 [PMID: 26850699 DOI: 10.1016/j.jprot.2016.01.017]
28 **Ercan G**, Karlitepe A, Ozpolat B. Pancreatic Cancer Stem Cells and Therapeutic Approaches. *Anticancer Res* 2017; **37**: 2761-2775 [PMID: 28551612 DOI: 10.21873/anticanres.11628]
29 **Renz BW**, D'Haese JG, Werner J, Westphalen CB, Ilmer M. Repurposing Established Compounds to Target Pancreatic Cancer Stem Cells (CSCs). *Med Sci* (Basel) 2017; **5**: pii: E14 [PMID: 29099030 DOI: 10.3390/medsci5020014]
30 **Meidhof S**, Brabletz S, Lehmann W, Preca BT, Mock K, Ruh M, Schüler J, Berthold M, Weber A, Burk U, Lübbert M, Puhr M, Culig Z, Wellner U, Keck T, Bronsert P, Küsters S, Hopt UT, Stemmler MP, Brabletz T. ZEB1-associated drug resistance in cancer cells is reversed by the class I HDAC inhibitor mocetinostat. *EMBO Mol Med* 2015; **7**: 831-847 [PMID: 25872941 DOI: 10.15252/emmm.201404396]
31 **Zheng X**, Carstens JL, Kim J, Scheible M, Kaye J, Sugimoto H, Wu CC, LeBleu VS, Kalluri R. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature* 2015; **527**: 525-530 [PMID: 26560028 DOI: 10.1038/nature16064]
32 **Zhan HX**, Xu JW, Wu D, Zhang TP, Hu SY. Pancreatic cancer stem cells: new insight into a stubborn disease. *Cancer Lett* 2015; **357**: 429-437 [PMID: 25499079 DOI: 10.1016/j.canlet.2014.12.004]
33 **Najumudeen AK**, Jaiswal A, Lectez B, Oetken-Lindholm C, Guzmán C, Siljamäki E, Posada IM, Lacey E, Aittokallio T, Abankwa D. Cancer stem cell drugs target K-ras signaling in a stemness context. *Oncogene* 2016; **35**: 5248-5262 [PMID: 26973241 DOI: 10.1038/onc.2016.59]
34 **Schenk M**, Aykut B, Teske C, Giese NA, Weitz J, Welsch T. Salinomycin inhibits growth of pancreatic cancer and cancer cell migration by disruption of actin stress fiber integrity. *Cancer Lett* 2015; **358**: 161-169 [PMID: 25529011 DOI: 10.1016/j.canlet.2014.12.037]
35 **Lamb R**, Ozsvari B, Lisanti CL, Tanowitz HB, Howell A, Martinez-Outschoorn UE, Sotgia F, Lisanti MP. Antibiotics that target mitochondria effectively eradicate cancer stem cells, across multiple tumor types: treating cancer like an infectious disease. *Oncotarget* 2015; **6**: 4569-4584 [PMID: 25625193 DOI: 10.18632/oncotarget.3174]
36 **Skrtić M**, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, Hurren R, Jitkova Y, Gronda M, Maclean N, Lai CK, Eberhard Y, Bartoszko J, Spagnuolo P, Rutledge AC, Datti A, Ketela T, Moffat J, Robinson BH, Cameron JH, Wrana J, Eaves CJ, Minden MD, Wang JC, Dick JE, Humphries K, Nislow C, Giaever G, Schimmer AD. Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell* 2011; **20**: 674-688 [PMID: 22094260 DOI: 10.1016/j.ccr.2011.10.015]
37 **Reed GA**, Schiller GJ, Kambhampati S, Tallman MS, Douer D, Minden MD, Yee KW, Gupta V, Brandwein J, Jitkova Y, Gronda M, Hurren R, Shamas-Din A, Schuh AC, Schimmer AD. A Phase 1 study of intravenous infusions of tigecycline in patients with acute myeloid leukemia. *Cancer Med* 2016; **5**: 3031-3040 [PMID: 27734609 DOI: 10.1002/cam4.845]
38 **Zhou HM**, Dong TT, Wang LL, Feng B, Zhao HC, Fan XK, Zheng MH. Suppression of colorectal cancer metastasis by nigericin through inhibition of epithelial-mesenchymal transition. *World J Gastroenterol* 2012; **18**: 2640-2648 [PMID: 22690072 DOI: 10.3748/wjg.v18.i21.2640]
39 **Balic A**, Sørensen MD, Trabulo SM, Sainz B Jr, Cioffi M, Vieira CR, Miranda-Lorenzo I, Hidalgo M, Kleeff J, Erkan M, Heeschen C. Chloroquine targets pancreatic cancer stem cells via inhibition of CXCR4 and hedgehog signaling. *Mol Cancer Ther* 2014; **13**: 1758-1771 [PMID: 24785258 DOI: 10.1158/1535-7163.MCT-13-0948]
40 **Fiorillo M**, Lamb R, Tanowitz HB, Mutti L, Krstic-Demonacos M, Cappello AR, Martinez-Outschoorn UE, Sotgia F, Lisanti MP. Repurposing atovaquone: targeting mitochondrial complex III and OXPHOS to eradicate cancer stem cells. *Oncotarget* 2016; **7**: 34084-34099 [PMID: 27136895 DOI: 10.18632/oncotarget.9122]
41 **Garnier A**, Vykoukal J, Hubertus J, Alt E, von Schweinitz D, Kappler R, Berger M, Ilmer M. Targeting the neurokinin-1 receptor inhibits growth of human colon cancer cells. *Int J Oncol* 2015; **47**: 151-160 [PMID: 25998227 DOI: 10.3892/ijo.2015.3016]
42 **Blaj C**, Bringmann A, Schmidt EM, Urbischek M, Lamprecht S, Fröhlich T, Arnold GJ, Krebs S, Blum H, Hermeking H, Jung A, Kirchner T, Horst D. ADNP Is a Therapeutically Inducible Repressor of WNT Signaling in Colorectal Cancer. *Clin Cancer Res* 2017; **23**: 2769-2780 [PMID: 27903678 DOI: 10.1158/1078-0432.CCR-16-1604]
43 **Malsy M**, Gebhardt K, Gruber M, Wiese C, Graf B, Bundscherer A. Effects of ketamine, s-ketamine, and MK 801 on proliferation, apoptosis, and necrosis in pancreatic cancer cells. *BMC Anesthesiol* 2015; **15**: 111 [PMID: 26219286 DOI: 10.1186/s12871-015-0076-y]
44 **Zhang Y**, Liu L, Fan P, Bauer N, Gladkich J, Ryschich E, Bazhin AV, Giese NA, Strobel O, Hackert T, Hinz U, Gross W, Fortunato F, Herr I. Aspirin counteracts cancer stem cell features, desmoplasia and gemcitabine resistance in pancreatic cancer. *Oncotarget* 2015; **6**: 9999-10015 [PMID: 25846752 DOI: 10.18632/oncotarget.3171]
45 **Coyle C**, Cafferty FH, Rowley S, MacKenzie M, Berkman L, Gupta S, Pramesh CS, Gilbert D, Kynaston H, Cameron D, Wilson RH, Ring A, Langley RE; Add-Aspirin investigators. ADD-ASPIRIN: A phase III, double-blind, placebo controlled, randomised trial assessing the effects of aspirin on disease recurrence and survival after primary therapy in common non-metastatic solid tumours. *Contemp Clin Trials* 2016; **51**: 56-64 [PMID: 27777129 DOI: 10.1016/j.cct.2016.10.004]
46 **Mohammed A**, Janakiram NB, Brewer M, Ritchie RL, Marya A, Lightfoot S, Steele VE, Rao CV. Antidiabetic Drug Metformin Prevents Progression of Pancreatic Cancer by Targeting in Part Cancer Stem Cells and mTOR Signaling. *Transl Oncol* 2013; **6**: 649-659 [PMID: 24466367 DOI: 10.1593/tlo.13556]
47 **Kordes S**, Pollak MN, Zwinderman AH, Mathôt RA, Weterman MJ, Beeker A, Punt CJ, Richel DJ, Wilmink JW. Metformin in patients with advanced pancreatic cancer: a double-blind, randomised, placebo-controlled phase 2 trial. *Lancet Oncol* 2015; **16**: 839-847 [PMID: 26067687 DOI: 10.1016/S1470-2045(15)00027-3]
48 **Reni M**, Dugnani E, Cereda S, Belli C, Balzano G, Nicoletti R, Liberati D, Pasquale V, Scavini M, Maggiora P, Sordi V, Lampasona V, Ceraulo D, Di Terlizzi G, Doglioni C, Falconi M, Piemonti L. (Ir)relevance of Metformin Treatment in Patients with Metastatic Pancreatic Cancer: An Open-Label, Randomized Phase II Trial. *Clin Cancer Res* 2016; **22**: 1076-1085 [PMID: 26459175 DOI: 10.1158/1078-0432.CCR-15-1722]
49 **Owunari GU**, Minakiri SI. Disulfiram and copper gluconate in cancer chemotherapy; a review of the literature. *Cancer Res* 2014; **2**: 88-92 [DOI: 10.11648/j.crj.20140205.12]
50 **Kim SK**, Kim H, Lee DH, Kim TS, Kim T, Chung C, Koh GY,

Kim H, Lim DS. Reversing the intractable nature of pancreatic cancer by selectively targeting ALDH-high, therapy-resistant cancer cells. *PLoS One* 2013; **8**: e78130 [PMID: 24194908 DOI: 10.1371/journal.pone.0078130]

51 **Han D**, Wu G, Chang C, Zhu F, Xiao Y, Li Q, Zhang T, Zhang L. Disulfiram inhibits TGF-β-induced epithelial-mesenchymal transition and stem-like features in breast cancer via ERK/NF-κB/Snail pathway. *Oncotarget* 2015; **6**: 40907-40919 [PMID: 26517513 DOI: 10.18632/oncotarget.5723]

52 **Cong J**, Wang Y, Zhang X, Zhang N, Liu L, Soukup K, Michelakos T, Hong T, DeLeo A, Cai L, Sabbatino F, Ferrone S, Lee H, Levina V, Fuchs B, Tanabe K, Lillemoe K, Ferrone C, Wang X. A novel chemoradiation targeting stem and nonstem pancreatic cancer cells by repurposing disulfiram. *Cancer Lett* 2017; **409**: 9-19 [PMID: 28864067 DOI: 10.1016/j.canlet.2017.08.028]

53 **Nechushtan H**, Hamamreh Y, Nidal S, Gotfried M, Baron A, Shalev YI, Nisman B, Peretz T, Peylan-Ramu N. A phase IIb trial assessing the addition of disulfiram to chemotherapy for the treatment of metastatic non-small cell lung cancer. *Oncologist* 2015; **20**: 366-367 [PMID: 25777347 DOI: 10.1634/theoncologist.2014-0424]

54 **Higashi T**, Hayashi H, Kitano Y, Yamamura K, Kaida T, Arima K, Taki K, Nakagawa S, Okabe H, Nitta H, Imai K, Hashimoto D, Chikamoto A, Beppu T, Baba H. Statin attenuates cell proliferative ability via TAZ (WWTR1) in hepatocellular carcinoma. *Med Oncol* 2016; **33**: 123 [PMID: 27734263 DOI: 10.1007/s12032-016-0845-6]

55 **Rangarajan P**, Subramaniam D, Paul S, Kwatra D, Palaniyandi K, Islam S, Harihar S, Ramalingam S, Gutheil W, Putty S, Pradhan R, Padhye S, Welch DR, Anant S, Dhar A. Crocetinic acid inhibits hedgehog signaling to inhibit pancreatic cancer stem cells. *Oncotarget* 2015; **6**: 27661-27673 [PMID: 26317547 DOI: 10.18632/oncotarget.4871]

56 **Ma Y**, Yu W, Shrivastava A, Alemi F, Lankachandra K, Srivastava RK, Shankar S. Sanguinarine inhibits pancreatic cancer stem cell characteristics by inducing oxidative stress and suppressing sonic hedgehog-Gli-Nanog pathway. *Carcinogenesis* 2017; **38**: 1047-1056 [PMID: 28968696 DOI: 10.1093/carcin/bgx070]

57 **Miyazaki Y**, Matsubara S, Ding Q, Tsukasa K, Yoshimitsu M, Kosai K, Takao S. Efficient elimination of pancreatic cancer stem cells by hedgehog/GLI inhibitor GANT61 in combination with mTOR inhibition. *Mol Cancer* 2016; **15**: 49 [PMID: 27349387 DOI: 10.1186/s12943-016-0534-2]

58 **Abel EV**, Kim EJ, Wu J, Hynes M, Bednar F, Proctor E, Wang L, Dziubinski ML, Simeone DM. The Notch pathway is important in maintaining the cancer stem cell population in pancreatic cancer. *PLoS One* 2014; **9**: e91983 [PMID: 24647545 DOI: 10.1371/journal.pone.0091983]

59 **Ponnurangam S**, Dandawate PR, Dhar A, Tawfik OW, Parab RR, Mishra PD, Ranadive P, Sharma R, Mahajan G, Umar S, Weir SJ, Sugumar A, Jensen RA, Padhye SB, Balakrishnan A, Anant S, Subramaniam D. Quinomycin A targets Notch signaling pathway in pancreatic cancer stem cells. *Oncotarget* 2016; **7**: 3217-3232 [PMID: 26673007 DOI: 10.18632/oncotarget.6560]

60 **Matsubara S**, Ding Q, Miyazaki Y, Kuwahata T, Tsukasa K, Takao S. mTOR plays critical roles in pancreatic cancer stem cells through specific and stemness-related functions. *Sci Rep* 2013; **3**: 3230 [PMID: 24231729 DOI: 10.1038/srep03230]

61 **Zeng JY**, Sharma S, Zhou YQ, Yao HP, Hu X, Zhang R, Wang MH. Synergistic activities of MET/RON inhibitor BMS-777607 and mTOR inhibitor AZD8055 to polyploid cells derived from pancreatic cancer and cancer stem cells. *Mol Cancer Ther* 2014; **13**: 37-48 [PMID: 24233399 DOI: 10.1158/1535-7163.MCT-13-0242]

62 **Yasumoto Y**, Miyazaki H, Vaidyan LK, Kagawa Y, Ebrahimi M, Yamamoto Y, Ogata M, Katsuyama Y, Sadahiro H, Suzuki M, Owada Y. Inhibition of Fatty Acid Synthase Decreases Expression of Stemness Markers in Glioma Stem Cells. *PLoS One* 2016; **11**: e0147717 [PMID: 26808816 DOI: 10.1371/journal.pone.0147717]

63 **Bizzarro V**, Belvedere R, Milone MR, Pucci B, Lombardi R, Bruzzese F, Popolo A, Parente L, Budillon A, Petrella A. Annexin A1 is involved in the acquisition and maintenance of a stem cell-like/aggressive phenotype in prostate cancer cells with acquired resistance to zoledronic acid. *Oncotarget* 2015; **6**: 25076-25092 [PMID: 26312765 DOI: 10.18632/oncotarget.4725]

64 **Fang Y**, Guan X, Cai T, Long J, Wang H, Xie X, Zhang Y. Knockdown of ANXA1 suppresses the biological behavior of human NSCLC cells in vitro. *Mol Med Rep* 2016; **13**: 3858-3866 [PMID: 27035116 DOI: 10.3892/mmr.2016.5022]

65 **Fong LWR**, Yang DC, Chen CH. Myristoylated alanine-rich C kinase substrate (MARCKS): a multirole signaling protein in cancers. *Cancer Metastasis Rev* 2017; **36**: 737-747 [PMID: 29039083 DOI: 10.1007/s10555-017-9709-6]

66 **Chen CH**, Thai P, Yoneda K, Adler KB, Yang PC, Wu R. A peptide that inhibits function of Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) reduces lung cancer metastasis. *Oncogene* 2014; **33**: 3696-3706 [PMID: 23955080 DOI: 10.1038/onc.2013.336]

67 **Song S**, Ji B, Ramachandran V, Wang H, Hafley M, Logsdon C, Bresalier RS. Overexpressed galectin-3 in pancreatic cancer induces cell proliferation and invasion by binding Ras and activating Ras signaling. *PLoS One* 2012; **7**: e42699 [PMID: 22900040 DOI: 10.1371/journal.pone.0042699]

68 **Kang HG**, Kim DH, Kim SJ, Cho Y, Jung J, Jang W, Chun KH. Galectin-3 supports stemness in ovarian cancer stem cells by activation of the Notch1 intracellular domain. *Oncotarget* 2016; **7**: 68229-68241 [PMID: 27626163 DOI: 10.18632/oncotarget.11920]

69 **Nangia-Makker P**, Hogan V, Raz A. Galectin-3 and cancer stemness. *Glycobiology* 2018; **28**: 172-181 [PMID: 29315388 DOI: 10.1093/glycob/cwy001]

70 **Zhang L**, Wang P, Qin Y, Cong Q, Shao C, Du Z, Ni X, Li P, Ding K. RN1, a novel galectin-3 inhibitor, inhibits pancreatic cancer cell growth in vitro and in vivo via blocking galectin-3 associated signaling pathways. *Oncogene* 2017; **36**: 1297-1308 [PMID: 27617577 DOI: 10.1038/onc.2016.306]

71 **Glinsky VV**, Kiriakova G, Glinskii OV, Mossine VV, Mawhinney TP, Turk JR, Glinskii AB, Huxley VH, Price JE, Glinsky GV. Synthetic galectin-3 inhibitor increases metastatic cancer cell sensitivity to taxol-induced apoptosis in vitro and in vivo. *Neoplasia* 2009; **11**: 901-909 [PMID: 19724684 DOI: 10.1593/neo.09594]

72 **Shankar Babu M**, Mahanta S, Lakhter AJ, Hato T, Paul S, Naidu SR. Lapachol inhibits glycolysis in cancer cells by targeting pyruvate kinase M2. *PLoS One* 2018; **13**: e0191419 [PMID: 29394289 DOI: 10.1371/journal.pone.0191419]

73 **Xie X**, Huang X, Tang H, Ye F, Yang L, Guo X, Tian Z, Xie X, Peng C, Xie X. Diallyl Disulfide Inhibits Breast Cancer Stem Cell Progression and Glucose Metabolism by Targeting CD44/PKM2/AMPK Signaling. *Curr Cancer Drug Targets* 2018; **18**: 592-599 [PMID: 29110616 DOI: 10.2174/1568009617666171024165657]

74 **Ning X**, Qi H, Li R, Jin Y, McNutt MA, Yin Y. Synthesis and antitumor activity of novel 2, 3-didithiocarbamate substituted naphthoquinones as inhibitors of pyruvate kinase M2 isoform. *J Enzyme Inhib Med Chem* 2018; **33**: 126-129 [PMID: 29185365 DOI: 10.1080/14756366.2017.1404591]

75 **Talaiezadeh A**, Shahriari A, Tabandeh MR, Fathizadeh P, Mansouri S. Kinetic characterization of lactate dehydrogenase in normal and malignant human breast tissues. *Cancer Cell Int* 2015; **15**: 19 [PMID: 25705126 DOI: 10.1186/s12935-015-0171-7]

76 **Fiume L**, Manerba M, Vettraino M, Di Stefano G. Inhibition of lactate dehydrogenase activity as an approach to cancer therapy. *Future Med Chem* 2014; **6**: 429-445 [PMID: 24635523 DOI: 10.4155/fmc.13.206]

77 **Altamimi AS**, Alafeefy AM, Balode A, Vozny I, Pustenko A, El Shikh ME, Alasmary FAS, Abdel-Gawad SA, Žalubovskis R. Symmetric molecules with 1,4-triazole moieties as potent inhibitors of tumour-associated lactate dehydrogenase-A. *J Enzyme Inhib Med Chem* 2018; **33**: 147-150 [PMID: 29199484 DOI: 10.1080/14756366.2017.1404593]

78 **Cui J**, Shi M, Xie D, Wei D, Jia Z, Zheng S, Gao Y, Huang S, Xie K. FOXM1 promotes the warburg effect and pancreatic cancer progression via transactivation of LDHA expression. *Clin Cancer Res* 2014; **20**: 2595-2606 [PMID: 24634381 DOI: 10.1158/1078-0432.CCR-13-2407]

79 **Tabatabaei-Dakhili SA**, Aguayo-Ortiz R, Domínguez L, Velázquez-Martínez CA. Untying the knot of transcription factor druggability: Molecular modeling study of FOXM1 inhibitors. *J Mol Graph Model* 2018; **80**: 197-210 [PMID: 29414039 DOI: 10.1016/j.jmgm.2018.01.009]

80 **Kida YS**, Kawamura T, Wei Z, Sogo T, Jacinto S, Shigeno A, Kushige H, Yoshihara E, Liddle C, Ecker JR, Yu RT, Atkins AR, Downes M, Evans RM. ERRs Mediate a Metabolic Switch Required for Somatic Cell Reprogramming to Pluripotency. *Cell Stem Cell* 2015; **16**: 547-555 [PMID: 25865501 DOI: 10.1016/j.stem.2015.03.001]

81 **Yu DD**, Huss JM, Li H, Forman BM. Identification of novel inverse agonists of estrogen-related receptors ERRγ and ERRβ. *Bioorg Med Chem* 2017; **25**: 1585-1599 [PMID: 28189393 DOI: 10.1016/j.bmc.2017.01.019]

82 **Kim JH**, Choi YK, Byun JK, Kim MK, Kang YN, Kim SH, Lee S, Jang BK, Park KG. Estrogen-related receptor γ is upregulated in liver cancer and its inhibition suppresses liver cancer cell proliferation via induction of p21 and p27. *Exp Mol Med* 2016; **48**: e213 [PMID: 26940882 DOI: 10.1038/emm.2015.115]

83 **Singh TD**, Jeong SY, Lee SW, Ha JH, Lee IK, Kim SH, Kim J, Cho SJ, Ahn BC, Lee J, Jeon YH. Inverse Agonist of Estrogen-Related Receptor γ Enhances Sodium Iodide Symporter Function Through Mitogen-Activated Protein Kinase Signaling in Anaplastic Thyroid Cancer Cells. *J Nucl Med* 2015; **56**: 1690-1696 [PMID: 26338896 DOI: 10.2967/jnumed.115.160366]

84 **Mathieu J**, Zhou W, Xing Y, Sperber H, Ferreccio A, Agoston Z, Kuppusamy KT, Moon RT, Ruohola-Baker H. Hypoxia-inducible factors have distinct and stage-specific roles during reprogramming of human cells to pluripotency. *Cell Stem Cell* 2014; **14**: 592-605 [PMID: 24656769 DOI: 10.1016/j.stem.2014.02.012]

85 **Bahrami B**, Hojjat-Farsangi M, Mohammadi H, Anvari E, Ghalamfarsa G, Yousefi M, Jadidi-Niaragh F. Nanoparticles and targeted drug delivery in cancer therapy. *Immunol Lett* 2017; **190**: 64-83 [PMID: 28760499 DOI: 10.1016/j.imlet.2017.07.015]

86 **Golchin A**, Hosseinzadeh S, Roshangar L. The role of nanomaterials in cell delivery systems. *Med Mol Morphol* 2018; **51**: 1-12 [PMID: 29170827 DOI: 10.1007/s00795-017-0173-8]

87 **Aftab S**, Shah A, Nadhman A, Kurbanoglu S, Aysıl Ozkan S, Dionysiou DD, Shukla SS, Aminabhavi TM. Nanomedicine: An effective tool in cancer therapy. *Int J Pharm* 2018; **540**: 132-149 [PMID: 29427746 DOI: 10.1016/j.ijpharm.2018.02.007]

88 **Yang F**, Jin C, Subedi S, Lee CL, Wang Q, Jiang Y, Li J, Di Y, Fu D. Emerging inorganic nanomaterials for pancreatic cancer diagnosis and treatment. *Cancer Treat Rev* 2012; **38**: 566-579 [PMID: 22655679 DOI: 10.1016/j.ctrv.2012.02.003]

89 **Yang F**, Jin C, Jiang Y, Li J, Di Y, Ni Q, Fu D. Liposome based delivery systems in pancreatic cancer treatment: from bench to bedside. *Cancer Treat Rev* 2011; **37**: 633-642 [PMID: 21330062 DOI: 10.1016/j.ctrv.2011.01.006]

90 **Wei X**, Senanayake TH, Warren G, Vinogradov SV. Hyaluronic acid-based nanogel-drug conjugates with enhanced anticancer activity designed for the targeting of CD44-positive and drug-resistant tumors. *Bioconjug Chem* 2013; **24**: 658-668 [PMID: 23547842 DOI: 10.1021/bc300632w]

91 **Saneja A**, Nayak D, Srinivas M, Kumar A, Khare V, Katoch A, Goswami A, Vishwakarma RA, Sawant SD, Gupta PN. Development and mechanistic insight into enhanced cytotoxic potential of hyaluronic acid conjugated nanoparticles in CD44 overexpressing cancer cells. *Eur J Pharm Sci* 2017; **97**: 79-91 [PMID: 27989859 DOI: 10.1016/j.ejps.2016.10.028]

92 **Gupta S**, Pramanik D. Phytochemicals and cancer stem cells: A pancreatic cancer overview. *Current Chemical Biology* 2016; **10**: 10 [DOI: 10.2174/2212796810666160419152309]

93 **Aliebrahimi S**, Kouhsari SM, Arab SS, Shadboorestan A, Ostad SN. Phytochemicals, withaferin A and carnosol, overcome pancreatic cancer stem cells as c-Met inhibitors. *Biomed Pharmacother* 2018; **106**: 1527-1536 [PMID: 30119228 DOI: 10.1016/j.biopha.2018.07.055]

94 **Verma RK**, Yu W, Shrivastava A, Shankar S, Srivastava RK. α-Mangostin-encapsulated PLGA nanoparticles inhibit pancreatic carcinogenesis by targeting cancer stem cells in human, and transgenic (Kras(G12D), and Kras(G12D)/tp53R270H) mice. *Sci Rep* 2016; **6**: 32743 [PMID: 27624879 DOI: 10.1038/srep32743]

95 **Verma RK**, Yu W, Singh SP, Shankar S, Srivastava RK. Anthothecol-encapsulated PLGA nanoparticles inhibit pancreatic cancer stem cell growth by modulating sonic hedgehog pathway. *Nanomedicine* 2015; **11**: 2061-2070 [PMID: 26199979 DOI: 10.1016/j.nano.2015.07.001]

96 **Li J**, Liu F, Gupta S, Li C. Interventional Nanotheranostics of Pancreatic Ductal Adenocarcinoma. *Theranostics* 2016; **6**: 1393-1402 [PMID: 27375787 DOI: 10.7150/thno.15122]

97 **Wadler S**, Tenteromano L, Cazenave L, Sparano JA, Greenwald ES, Rozenblit A, Kaleya R, Wiernik PH. Phase II trial of echinomycin in patients with advanced or recurrent colorectal cancer. *Cancer Chemother Pharmacol* 1994; **34**: 266-269 [PMID: 8004762 DOI: 10.1007/s002800050139]

98 **Muss HB**, Blessing JA, DuBeshter B. Echinomycin in recurrent and metastatic endometrial carcinoma. A phase II trial of the Gynecologic Oncology Group. *Am J Clin Oncol* 1993; **16**: 492-493 [PMID: 8256763 DOI: 10.1097/00000421-199312000-00006]

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MINIREVIEWS

Cancer stem cell impact on clinical oncology

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Abstract

Cancer is a widespread worldwide chronic disease. In most cases, the high mortality rate from cancer correlates with a lack of clear symptoms, which results in late diagnosis for patients, and consequently, advanced tumor disease with poor probabilities for cure, since many patients will show chemo- and radio-resistance. Several mechanisms have been studied to explain chemo- and radio-resistance to anti-tumor therapies, including cell signaling pathways, anti-apoptotic mechanisms, stemness, metabolism, and cellular phenotypes. Interestingly, the presence of cancer stem cells (CSCs), which are a subset of cells within the tumors, has been related to therapy resistance. In this review, we focus on evaluating the presence of CSCs in different tumors such as breast cancer, gastric cancer, lung cancer, and hematological neoplasias, highlighting studies where CSCs were identified in patient samples. It is evident that there has been a great drive to identify the cell surface phenotypes of CSCs so that they can be used as a tool for anti-tumor therapy treatment design. We also review the potential effect of nanoparticles, drugs, natural compounds, aldehyde dehydrogenase inhibitors, cell signaling inhibitors, and antibodies to treat CSCs from specific tumors. Taken together, we present an overview of the role of CSCs in tumorigenesis and how research is advancing to target these highly tumorigenic cells to improve oncology patient outcomes.

Key words: Cancer; Targeted therapy; Clinical outcome; Drug resistance; Cancer stem cells

Core tip: Tumor heterogeneity can explain the presence of cells that display high tumorigenic capacity along with chemo- and radio-resistance properties. These cells, identified as cancer stem cells (CSCs), are partially responsible for recurrence and tumor progression. Most tumors follow the CSC model, which indicates the existence of a subset of highly tumorigenic cells. This has been shown to be the case for several patients with several types of tumors. In this review, we focus on the phenotypes used for the study and identification of CSCs from human samples, as well as promising strategies to target CSCs.

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INTRODUCTION

Cancer stem cells (CSCs) comprise a cell population within a tumor that, among other factors, is responsible for cancer initiation, propagation, metastasis and recurrence. It is known that solid tumors are composed of heterogeneous cell populations[1-3] with different phenotypic characteristics at different stages of development, with variable abilities to proliferate. However, only the CSC population is clonogenic *in vitro* and *in vivo*, suggesting that these cells are the only ones with the highest tumorigenic potential[4,5].

The existence of a subset of cancer cells that possesses an extensive proliferative capacity was reported in leukemia and multiple myeloma in the 1970s[6,7]. In both cancer types, only a cell population derived from a tumor was able to grow in clonogenic assays, where they formed spherical colonies, and induce tumors in mice that recapitulated the original tumor. At that time, the most reliable criterion for CSC identification was the capacity of these cells to produce colonies[6].

The first CSCs were isolated from acute myeloid leukemia (AML) by transplantation into severe combined immune-deficient (SCID) mice. They were identified as $CD34^+CD38^-$ cells and named AML-initiating cells because of their ability to establish human leukemia in SCID mice. Since the identified $CD34^+CD38^-$ cells were less differentiated than colony-forming cells, a hierarchy or heterogeneity in AML was proposed[1]. Later, in 1997, the model was reproduced in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) mice, where $CD34^+CD38^-$ CSCs were capable of differentiating into leukemic blasts *in vivo*, supporting the existence of a hierarchy in leukemia[8].

Some years later, enriched CSC populations were obtained from human brain tumors[9], using cells with a $CD133^+$ phenotype that showed a higher capacity for proliferation, self-renewal, and differentiation. $CD133^+$ cells were xenotransplanted into NOD/SCID mice and formed tumors that, when serially transplanted, recapitulated the original human tumor[10,11]. Since then, CSCs from various solid tumors have been reported[5].

In recent years, several research groups have focused on the identification and isolation of these cells. Besides leukemia and multiple myeloma, CSCs from solid tumors have been identified and isolated through the use of surface and functional markers[12-15], their growing capacity as spheroids *in vitro*[16,17], the evaluation of CSC clonogenic capacity[18,19] and their *in vivo* tumorigenic capacity in xenotransplant experiments[16,17,20,21].

Due to the reported participation of CSCs in chemo- and radio-resistance[22-24], an increasing interest in implementing strategies against CSCs in patients to improve their clinical outcome has grown in recent years because conventional therapies are effective in controlling tumor growth at the beginning, but over time, relapse is a main problem due to remaining CSCs[22,25,26].

CSC GENERALITIES

A CSC is defined as a cell within a tumor that is able to produce an identical cell with the same properties to give rise heterogeneous differentiated progeny, and has the ability to modulate differentiation and self-renewal (homeostatic control). These CSCs possess the ability to propagate themselves, as well as recapitulate a tumor[2,3,27].

A major characteristic of CSCs relies on their ability to regulate stemness pathways such as Wnt/β-catenin, Sonic hedgehog (Shh), transforming growth factor beta (TGF-β), *etc*[28]. These pathways are dysregulated in CSCs, and targeting them has been proposed as a strategy to increase the effectiveness of cancer therapies.

The CSC model postulates that solid tumors and leukemia are hierarchically organized, with CSCs at the apex of this hierarchy, driving tumor growth, relapse, metastasis and drug resistance[5,29]. Cell heterogeneity is responsible for varying cell morphology, different proliferative index, genetic changes and therapeutic response[30]. For a successful therapy, all CSCs should be specifically eliminated to avoid relapse.

Typically, CSCs are defined as a small or a rare cell population[2,31] that forms tumors after being xenotransplanted into immunodeficient mice. However, recent reports have suggested that the percentage of CSCs within a tumor can vary from 0.02% to 25% depending on the tumor type, where higher CSC proportions are found in undifferentiated tumors[31-34]. Typically, higher CSC frequencies have been found in mouse models, leukemias and lymphomas, while lower frequencies are frequently found in solid tumors[35]. Based on this information, it has been suggested that not all cancers follow the CSC model[27]. Instead, a dynamic or plastic CSC model has been proposed, where CSCs and non-CSCs could alternate between two phenotypic states[36].

In this dynamic model, both cell types show varying levels of tumor-forming capacity, drug response and the ability to give rise to differentiated cells[29,35]. CSCs and non-CSCs can still be easily distinguished through surface and functional markers, but mainly by their self-renewal capacity.

It is very important to note that the CSC model is widely reported in several cancer types (Figure 1), although there are a few publications about cancers that do not follow a CSC model or a dynamic CSC model, specifically in lymphoma mice models[37] and melanoma[32], where the tumors are homogeneous. In 2007, Strasser and his group inoculated 10 to 10^5 pre-B/B lymphoma cells into recipient mice. All of the animals developed lymphoma within 35 d, regardless of the number of inoculated cells, differing only in tumor growth rate[37].

Although CSCs are able to self-renew and differentiate, they do not necessarily originate from the malignant transformation of stem cells[33]. The cell of origin refers only to the cell type that received the first genetic or epigenetic hit, which confers the ability for self-renewal or tumor growth[35]. Examples of these cells are: normal stem cells, restricted progenitor cells and more differentiated cells. All of them could have acquired or maintained self-renewal capacity, and some of them can even undergo epithelial to mesenchymal transition (EMT), giving rise to metastatic CSCs[36].

In conclusion, the variable phenotype of the CSC population in patients and tumor types proposed in the CSC dynamic model constitutes the main challenge for the possible use of anti-CSC therapy.

CSC CHARACTERISTICS WITH CLINICAL RELEVANCE

The CSC population possesses several characteristics that can be useful for cancer therapy development, primarily focusing on the elimination of these cells.

Usually, a distinctive profile of surface and functional markers characterizes the CSC population, and their identification and purification usually begins with the description of such markers[3,29]. Moreover, there is an increasing interest in identifying the role of each marker in CSCs, as well as targeting CSC-specific pathways, which could increase the radio- and chemo-sensitivity of CSCs.

To date, several CSC markers from distinct tumor types have been proposed and validated through different experimental models (Table 1 and Figure 1). Some of these markers are discussed below.

Surface markers

Nowadays, there are CSC markers that are widely used to identify several tumor types. Such markers have been reported in CSC-enrichment culture models from cell lines or primary cultures derived from patient samples and serial xenotransplantation of putative CSCs in mouse models, which must be able to recapitulate the original heterogeneous populations and be directly validated in human tumor samples. It is important to note that the use of a single marker to define a CSC population is not recommended. For this purpose, a phenotypic profile that combines various markers should be established, as well as carrying out self-renewal assays (Figure 1)[2,25].

CD133, also known as prominin-1, is a transmembrane cell surface glycoprotein traditionally used as a hematopoietic stem cell marker that is effective for detection of non-stem cells from various tumor and tissue samples. The Dirks laboratory used the CSC marker CD133 for brain CSC identification. The purified $CD133^+$ population from primary human brain tumors samples showed higher proliferation and self-renewal capacity in neurosphere formation assays than $CD133^-$ cells[10]. Moreover, the inoculation of only a few $CD133^+$ cells was sufficient to produce a tumor, which was then successfully transplanted[11]. In 2013, the Pelicci laboratory reported that CD133 was found in an interconvertible state in glioblastoma patient-derived neurospheres and that the use of short hairpin RNA (shRNA) against CD133 diminished their self-renewal and tumorigenicity potential[18]. Interestingly, some studies have proposed that CD133 could maintain CSC properties through the Wnt/β-catenin signaling pathway[38].

CD133 has also been tested in colorectal cancer cell lines and tumor tissue samples[39,40] through the use of various techniques, including flow cytometry and serial xenotransplantation in mice[41]. Additionally, $CD133^+$ CSCs have been reported in many other solid cancer models, including endometrial cancer[42], lung cancer[43], small cell lung cancer[44], laryngeal cancer[45,46], liver cancer[47], colorectal cancer[48], and gastric cancer[49].

CD133 has been found in samples that represent higher stage tumors and are predictors of poor prognosis. For this reason, CD133 is considered a promising therapeutic target. This year, a phase Ⅰ trial for testing the efficacy of CD133-directed CAR-T cells showed that $CD133^+$ cells were successfully eliminated after CART-133 infusion[50].

CD44 is a multifunctional glycoprotein involved in cell adhesion, signaling, proliferation, migration, hematopoiesis, and lymphocyte activation[51]. It functions as a receptor for hyaluronan and other extracellular matrix components[52]. CD44 is widely used as a CSC marker, especially for tumors of epithelial origin, and it is used alone or in combination with CD24 for the identification of breast CSCs[5]. CD24 is a small surface protein that is found in many tumor types. However, reports from cancer cell lines show that there is a substantial variation in CD24 expression even among the same tumor types[53].

Though $CD24^-$ cells are commonly associated with CSC phenotypes, there are some cases in which $CD24^+$ has been found to be a marker for cell populations with CSC features. For example, in nasopharyngeal carcinoma (NPC) cell lines[54] and in HPV-16 SiHa cervical cancer

Table 1 Cancer stem cells markers in solid tumors

Cancer type	Phenotype	Model	References
Prostate cancer	$CD44^+$	PCa cell line and tumor xenograft in mice	[58]
Breast cancer	$CD44^+$ $CD24^{-/low}$	Patient-derived tumor xenograft in mice	[5]
Cervical cancer	$CD44^+$ $CD24^+$	SiHa cell line	[55]
Gastric cancer	$CD44^+$ $CD24^+$	AGS cell line and patient tissue samples	[56]
Nasopharyngeal carcinoma	$CD24^-$	NPC cell lnes, mice	[54]
Gastric adenocarcinoma	$CD44^+$ $CD133^+$	Patient tissue samples	[51]
Oral squamous cell carcinoma	$CD44^+$ ALDH1	Metastatic lymph nodes	[153]
Breast cancer	CD44v	Clinical samples	[154]
Prostate cancer	CD133	Primary prostate cancer cell lines	[155]
Endometrial cancer	CD133	Human endometrial cell lines	[42]
Liver cancer	CD133	Huh-7 cells and tumor xenograft in mice	[47]
Prostate cancer	CD133	Primary human prostate cancer cell lines	[155]
Cervical cancer	CD49f	SiHa and HeLa cell lines	[156]
Non-small cell lung cancer	CD49f	Patient-derived sphere-forming assays	[157]
Gastric cancer	CD49f	Gastric tumor tissues and tumor xenograft in mice	[75]
Colon cancer	CD49f	HT29 and Caco2 cell lines, clinical samples	[77]
Cervical cancer	ALDH	SiHa and HeLa cell lines, mice model	[85]
Colon cancer	ALDH1A3	HT29 cell line	[158]
Colon cancer	ALDH1A1	HT29 cell line and tumor xenograft in mice	[159]
Breast cancer	ALDH	Breast cancer tumor tissues	[160]

CSCs: Cancer stem cells; ALDH: Aldehyde dehydrogenase; NPC: Nasopharyngeal carcinoma.

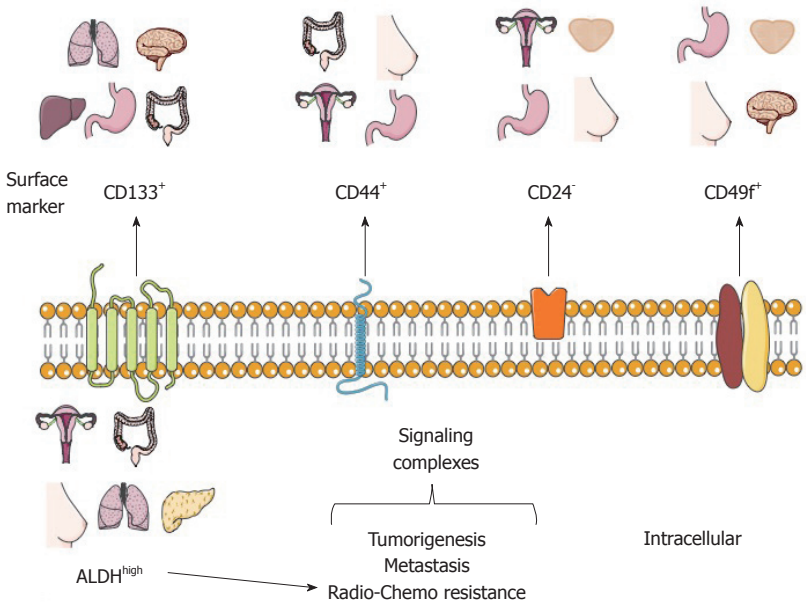

Figure 1 Schematic representation of common cancer stem cell markers. CD133, CD44, CD24 and CD49f are common phenotype markers used for the identification of cancer stem cells (CSCs) and their isolation from tissue samples from cancer patients, such as the stomach, lung, liver, ovary, breast, prostate and colon carcinoma. In addition, the metabolic and functional marker aldehyde dehydrogenase (ALDH) is represented in CSCs derived from ovarian carcinoma, colon carcinoma, breast, lung and liver cancer. The CSC markers shown have a specific and relevant function in the high tumorigenic capacity of CSCs, metastasis, and resistance to radio- and chemotherapy.

cells, isolated $CD44^+CD24^+$ cells were radioresistant and more tumorigenic than those negative for the same markers[55]. The same $CD44^+CD24^+$ phenotype was used to identify gastric CSCs[56].

A known classic publication demonstrated that only a small population isolated from breast tumors, defined as $CD44^+CD24^{-/low}$, has the capacity to sustain tumor growth in NOD/SCID mice and generate heterogeneous cell populations as the original breast tumor[5]. Later, in human prostate cancer samples, CSCs characterized through immunofluorescence with the $CD44^+/\beta_2\beta_1{}^{hi}/CD133^+$ phenotype were identified and characterized[57]. The next year, $CD44^+$ prostate cancer cell populations were obtained[58]. Also, CD44 and CD133 expression was evaluated in gastric adenocarcinoma tumors by immunohistochemistry, and it was found that both markers could be correlated with clinical and pathological parameters[51].

Although CD44 is widely reported as a CSC marker, it is very important to note that it is a ubiquitously expressed molecule derived from a gene with 18 exons. When all variable exons are spliced out, the standard form (CD44s) is expressed, and when alternative splicing occurs, variant forms (CD44v) are expressed[59]. In spite of this, there are only a few reports in which CD44 isoforms are considered when evaluating CSCs. In 2005, Mackenzie and his group demonstrated the existence of two CSC populations, both expressing $CD44^{high}$ (and $CD44^{+}$), derived from head and neck cutaneous squamous cell carcinoma. One was associated with EMT properties and the other one possessed an epithelial phenotype[60]. They demonstrated that the $CD44^{high}$ cells that undergo EMT preferably expressed the CD44s isoform; while the epithelial $CD44^{high}$ cells expressed the CD44v isoform. Using RNAseq, another group later confirmed these results. The CD44v6 isoform was identified as the predominant isoform in a prostate cancer epithelial cell line[61].

A very important contribution from the Mackenzie laboratory is that they demonstrated that the use of enzymes (for example, trypsin or collagenase) for cell extraction from tissues caused destruction of cell surface CD44v isoforms, leaving only the CD44s isoform[62]. Moreover, CD44-specific antibodies are not able to distinguish between all isoforms. Specifically, in breast cancer, CD44v was found to be associated with better prognosis while CD44s was related to poor prognosis[63]. As a consequence, CD44 is the most frequently found CSC marker[64,65]. Other examples are found in colorectal cancer, in which CD44 was found together with CD133[66,67], head and neck squamous cell carcinoma[68,69], ovarian CSCs[70], and gastric cancer using the specific isoform CD44v8-10[71].

CD49f or integrin α6, is a transmembrane glycoprotein that functions as the receptor for the extracellular matrix protein laminin[72,73]. CD49f is widely distributed in stem cells and in the brain[73]; because of its role in tumor cell proliferation, survival, self-renewal and tumor growth, it has been proposed that it could be used as a CSC marker[73].

In sphere colony forming cell culture using prostate cancer cells, CD49f was shown to be a better marker than CD133 and CD44[74]. In gastric cancer, $CD49^{high}$ cells displayed CSC characteristics, including resistance to doxorubicin, 5-fluorouracil and doxifluridine[75].This has also been reported in breast[76] and colon cancer[77]. Besides the examples mentioned above, there are other surface markers that have been proposed as CSC markers, such as CXCR4 and LGR5, among others.

Functional markers

Another strategy for CSC identification and purification is the use of functional or intracellular markers (Figure 1), which are considered to be more stable than surface markers. The principal functional CSC marker is aldehyde dehydrogenase or ALDH, part of an enzyme superfamily encoded by 19 genes that metabolize endogenous and exogenous aldehydes. It is present in practically all organisms, and its levels and isozymes vary depending on tissue and organ[78].

For ALDH identification, specific ALDH antibodies are available; nonetheless, we suggest that the most appropriate way for ALDH identification is the measurement of its activity using the commercial ALDH fluorescent substrate ALDEFLUOR® kit assay by Stem Cells Technologies, Inc. (Vancouver, BC, Canada). Cells that display high ALDH activity, (named $ALDH^{high}$ or $ALDH^{+}$ or $ALDH^{br}$), can be identified and isolated using flow cytometry[79]. Several works have shown that high ALDH activity is often associated with CSCs derived from solid tumor types[80]. These cells are generally characterized by a higher proliferation potential, colony-forming capacity, self-renewal, *in vivo* tumorigenic capacity, metastasis, and drug resistance. For instance, $ALDH^{high}$ CSCs have been identified in colon cancer[81,82], lung cancer[83], cervical cancer[14,84,85], breast cancer[86], pancreatic cancer[87,88], and melanoma[89,90], to mention some examples.

As for surface markers, ALDH is often reported in combination with other cell markers to increase the accuracy of CSC validation. In some cases, high ALDH activity is found together with high expression of markers like CD133. Some cases have been identified in ovarian cancer[91,92], invasive ductal breast carcinoma tumors[93], and lung cancer[94]. The combination $ALDH^{+}$/$CD44^{+}$ has been evaluated in various tumors such as breast cancer[95] and lung cancer[96].

CSCs AND THERAPY RESISTANCE

Several cancers acquire drug resistance during or after treatment, which is the case for cancers that possess cells that are more resistant than the rest of the tumor. Generally, resistant cells have proteins that remove drugs from cells[97]. One of the most studied mechanisms of drug resistance in CSCs is their ability to actively expel therapeutic drugs *via* transport proteins. Such proteins are a family known as ATP-binding cassette transporters. These proteins use ATP-dependent drug efflux pumps for drug elimination, mostly into the extracellular space, and they have been found to be overexpressed in CSCs using side population assays[41,98-100].

Additionally, high ALDH activity is directly related to a higher resistance to several drugs, for example, cyclophosphamide, temozolomide, irinotecan, paclitaxel, and doxorubicin[101-103]. Resistance conferred by ALDH has been observed in numerous cell lines and patient samples[97,104]. A well known case is the resistance to cyclophosphamide, where ALDH irreversibly oxidizes aldophosphamide, an active metabolite of cyclophosphamide, into an inert compound[105]. In breast cancer, the inhibition of ALDH activity in $ALDH^{high}$ $CD44^{+}$ cells leads to a reduction in chemoresistance to doxorubicin and paclitaxel[106]. This information suggests that the

inhibition of ALDH activity leads to cell sensitization to chemotherapeutics[99].

Besides higher resistance to conventional cancer treatments, evidence shows that highly metastatic tumors correlate with a higher percentage of CSCs[28].

CSCs IN PATIENTS: PHENOTYPE AND TYPE OF STUDIES

Most publications about the identification of CSCs have been performed in cell lines. However, in this section, we will discuss the cases in which CSCs were identified in patient samples.

CD133 was analyzed in a meta-analysis of 32 studies of non-small cell lung cancer, and a higher CD133 expression was associated with poor tumor differentiation and lymph node metastasis[107].

Gastric CSCs have been identified in tumor tissues and peripheral blood using the $CD44^+CD54^+$ phenotype[108]. Nevertheless, in another study, $CD133^+/CD44^+$ cells sorted from 44 patients who underwent gastrostomy failed to produce tumors in mice and did not show any CSC properties[109].

The presence of ALDH has been analyzed in normal mammary and breast cancer tissues[110]. The activity of ALDH1A3 is associated with metastasis in patient breast cancer samples by microarray analysis[86]. In another analysis of formalin-fixed paraffin-embedded tissue samples from primary stage Ⅳ breast cancer, ALDH and CD44/CD24 expression was correlated with response to endocrine therapy and clinical outcome but was not statistically significant[111].

CSC approaching therapy

Despite the broad variety of CSC publications in the last years, the discovery of effective therapies has remained elusive. However, some advances have been made in the field that could be getting us closer to direct CSC elimination. A brief outline of some of these strategies is showed in Figure 2.

Targeting deregulated pathways in CSCs aims at developing effective strategies against CSCs. In adult pancreas, the Hedgehog (Hh) signaling pathway is dormant, but it is upregulated in pancreatic ductal adenocarcinoma, specifically in $CD44^+/CD24^+/ESA^+$CSCs. In a phase Ⅰ study, 68 patients were treated with GDC-0449 or Vismodegib, a Hh pathway antagonist[112], alone or in combination with gemcitabine. GDC-0449 inhibited Hh signaling, but there was no correlation with survival or other parameters[113]. Other drugs that show promising results in inhibiting this pathway are PF-044449913[114] and thiostrepon, which attenuates $CD44^+/CD24^-$ triple-negative breast CSCs[115].

In addition, γ–secretase inhibitors target the Notch pathway and possess a stronger anti-neoplastic activity when combined with chemotherapeutic agents[116]. Nevertheless, adverse effects have been reported, as patients developed cutaneous rash in phase Ⅰ clinical trials[117,118].

Several drugs that aim to inhibit the Wnt/β-catenin signaling pathway are being developed. One such drug is Celecoxib, a non-steroidal anti-inflammatory drug that inhibits β-catenin signaling by cyclo-oxygenase (commonly known as COX)-dependent and COX-independent mechanisms[116]. This drug downregulates CD133 expression in colon cancer cells by inhibiting Wnt signaling[119] and intestinal cancer growth[120]. The Wnt inhibitor LGK-974 inhibits porcupine, an O-acyltransferase required for Wnt secretion. In liver cancer cells, LGK-974 blocks secretion of the Wnt3A protein, and as a consequence, cells become more sensitive to radiation[121]. A recent study showed that LGK-974 downregulates ALDH1A3 and reduces chemoresistance in glioblastoma cells[122].

Curcumin is an antioxidant derived from turmeric whose anti-cancer effect is well documented. Referring specifically to CSCs, curcumin has shown the potential to regulate the CSC self-renewal pathways, as well as specific microRNAs[123]. In $CD133^+$ lung CSCs, curcumin suppresses the activation of Wnt/β-catenin and Shh pathways, as well as other CSC traits[124]. It has been demonstrated that in bladder cancer, curcumin suppress the Shh pathway[125] and in laryngeal carcinoma treatment, curcumin enhances the effectiveness of cisplatin, reducing $CD133^+$ cells *in vitro*[46]. Additionally, a combination of curcumin and FOLFOX chemotherapy inhibits colorectal CSCs in *ex vivo* models[126].

An interesting strategy is to target CSCs using nanoparticles to reduce side effects on surrounding normal cells. In 2015, construction of glucose-coated gold nanoparticles (Glu-GNPs) that used glucose to facilitate GNP entry into leukemic stem cells overexpressing CD44 (TH1-P) was reported. Leukemic cells were cultured for one hour in the absence of glucose for better Glu-GNP uptake, and then X-ray irradiation tests were performed. Results showed that Glu-GNPs enhanced cell death compared to either irradiation or GNPs alone[127]. Formulated mangostin-encapsulated poly(lactic-co-glycolic acid) nanoparticles (Mang-NPs) successfully downregulated the known stemness genes c-Myc, Nanog and Oct4, two CSC markers, CD24 and CD133, and the Shh pathway[128]. Salinomycin and paclitaxel nanoparticles are also being used to eliminate breast cancer cells including CD44 breast CSCs[129].

Interestingly, CSCs have a strict dependence on mitochondrial biogenesis. Five classes of FDA-approved antibiotics that inhibit mitochondrial biogenesis were used on eight different cancer cell lines, and the results suggested that the observed therapeutic effects were infection-independent[130]. Clinical trials using doxycycline showed positive results in cancer patients[131]. Another drug that has been shown to specifically eliminate CSCs is metformin, and its effects are enhanced when it is used in combination with doxorubicin[132]. Moreover, it has been observed that metformin reduces metastasis by targeting both EMT

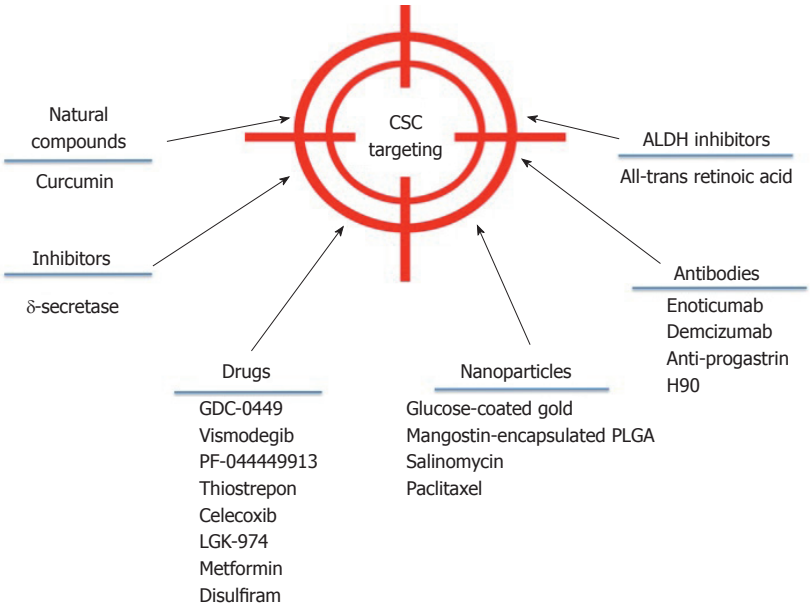

Figure 2 Drugs that may target cancer stem cells. Promising therapeutics to treat cancer patients. The flowchart highlights the new and more promising cancer therapies that can be directed toward cancer stem cells to eliminate them. CSC: Cancer stem cell.

and CSCs[133]. In the ovarian cancer cell line SKOV3, low doses of metformin diminished $CD44^+CD117^+$ CSCs in xenograft tissue and enhanced the effect of cisplatin[134]. In esophageal cancer, metformin reduced the number of ALDH+ cells, tumor growth *in vivo*[135], and in pancreatic cancer, it increased radiation sensitivity[136].

Using antibodies is another strategy to block CSC signaling pathways and reduce tumor activity in different models. For instance, the anti-DLL4 (Enoticumab) antibody that targets the dominant Notch ligand DLL4 has shown anti-tumor activity, especially in VEGF-resistant tumors in human phase Ⅰ studies[137]. Furthermore, another anti-DLL4 antibody (Demcizumab) is effective in decreasing tumor size but produces hypertension[138]. In colon cancer patients, increased progastrin levels in the blood have been observed, which is a tumor-promoting peptide that participates in colon CSC self-renewal and is also a direct target gene of β–catenin/Tcf4. Based on this information, specific anti-progastrin antibodies have been developed and tested in colon cancer cell lines and in mice. The antibodies, alone or in combination with chemotherapy, decreased self-renewal, migration and invasion. Moreover, they mitigated Wnt-driven intestinal neoplasia and induced tumor cell differentiation *in vivo*[139]. H90 is a mouse IgG1 mAb against human CD44 that directly targets CSCs to induce differentiation and proliferation in AML xenograft mouse models[140]. Additionally, anti-CD44s-specific antibodies are effective in eliminating pancreatic stem cells[141]. For more extensive information about antibodies against CSCs, we recommend reference[142].

ALDH is an important CSC marker that is overexpressed in several cancers. Specific ALDH inhibitors are effective in modulating cell growth, apoptosis and differentiation. Additionally, increased chemo- and radio-sensitivity is usually observed. All-trans retinoic acid (commonly known as ATRA) is a first generation systemic retinoid that promotes cell differentiation[143,144] and has been used in clinical trials[145]. ATRA has also been tested in breast cancer cells[106,146,147] and in gastric cancer, where it inhibited tumor growth[148], and in head and neck cancer, where it suppressed Wnt/β-catenin signaling[149]. In a phase Ⅰ/Ⅱ trial, advanced breast cancer patients did not show a significant improvement when treated with ATRA and tamoxifen compared with tamoxifen alone[150].

Disulfiram is a drug used for treating alcoholism, and it shows anti-cancer activity *in vitro* and *in vivo*, further potentiating the chemotherapeutic response. Its effectiveness has been demonstrated on paclitaxel-resistant triple-negative breast cancer cells[151], in non-small cell lung cancer cells[152], and glioblastoma.

CONCLUSION

CSCs are potential cancer therapy targets due to their tumorigenic capabilities, such as chemo- and radio-resistance, phenomena involved in tumor relapse in patients. Several efforts have been made to continue to identify the CSCs in several tumors to better understand the mechanisms related to tumor resistance in oncologic patients. It is known that de-regulated cell signaling pathways are partially responsible for maintaining CSC stemness. Consequently, Wnt, Notch and Hh signaling pathways have been studied to develop an efficient anti-CSC therapy. However, innovative anti-cancer treatments need to be developed to improve the lifespan and quality of life of cancer patients. Finally, we suggest that there cannot be a generalized CSC phenotype to design and promote new drugs, antibodies, nanoparticles, and cellular treatments to treat oncological patients. Taken together, we suggest

the full characterization of phenotypes and capabilities of CSCs in patients, a cellular component responsible for tumor progression, tumor relapse and metastasis.

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REFERENCES

1 **Lapidot T**, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645-648 [PMID: 7509044 DOI: 10.1038/367645a0]

2 **Clarke MF**, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* 2006; **66**: 9339-9344 [PMID: 16990346 DOI: 10.1158/0008-5472.CAN-06-3126]

3 **Dalerba P**, Cho RW, Clarke MF. Cancer stem cells: models and concepts. *Annu Rev Med* 2007; **58**: 267-284 [PMID: 17002552 DOI: 10.1146/annurev.med.58.062105.204854]

4 **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105-111 [PMID: 11689955 DOI: 10.1038/35102167]

5 **Al-Hajj M**, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; **100**: 3983-3988 [PMID: 12629218 DOI: 10.1073/pnas.0530291100]

6 **Park CH**, Bergsagel DE, McCulloch EA. Mouse myeloma tumor stem cells: a primary cell culture assay. *J Natl Cancer Inst* 1971; **46**: 411-422 [PMID: 5115909]

7 **Hamburger AW**, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977; **197**: 461-463 [PMID: 560061 DOI: 10.1126/science.560061]

8 **Bonnet D**, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730-737 [PMID: 9212098 DOI: 10.1038/nm0797-730]

9 **Hemmati HD**, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, Kornblum HI. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci USA* 2003; **100**: 15178-15183 [PMID: 14645703 DOI: 10.1073/pnas.2036535100]

10 **Singh SK**, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003; **63**: 5821-5828 [PMID: 14522905]

11 **Singh SK**, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. Identification of human brain tumour initiating cells. *Nature* 2004; **432**: 396-401 [PMID: 15549107 DOI: 10.1038/nature03128]

12 **Ginestier C**, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007; **1**: 555-567 [PMID: 18371393 DOI: 10.1016/j.stem.2007.08.014]

13 **Greve B**, Kelsch R, Spaniol K, Eich HT, Götte M. Flow cytometry in cancer stem cell analysis and separation. *Cytometry A* 2012; **81**: 284-293 [PMID: 22311742 DOI: 10.1002/cyto.a.22022]

14 **Rao QX**, Yao TT, Zhang BZ, Lin RC, Chen ZL, Zhou H, Wang LJ, Lu HW, Chen Q, Di N, Lin ZQ. Expression and functional role of ALDH1 in cervical carcinoma cells. *Asian Pac J Cancer Prev* 2012; **13**: 1325-1331 [PMID: 22799327 DOI: 10.7314/APJCP.2012.13.4.1325]

15 **Shackleton M**. Normal stem cells and cancer stem cells: similar and different. *Semin Cancer Biol* 2010; **20**: 85-92 [PMID: 20435143 DOI: 10.1016/j.semcancer.2010.04.002]

16 **Yu SC**, Ping YF, Yi L, Zhou ZH, Chen JH, Yao XH, Gao L, Wang JM, Bian XW. Isolation and characterization of cancer stem cells from a human glioblastoma cell line U87. *Cancer Lett* 2008; **265**: 124-134 [PMID: 18343028 DOI: 10.1016/j.canlet.2008.02.010]

17 **Qiang L**, Yang Y, Ma YJ, Chen FH, Zhang LB, Liu W, Qi Q, Lu N, Tao L, Wang XT, You QD, Guo QL. Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. *Cancer Lett* 2009; **279**: 13-21 [PMID: 19232461 DOI: 10.1016/j.canlet.2009.01.016]

18 **Brescia P**, Ortensi B, Fornasari L, Levi D, Broggi G, Pelicci G. CD133 is essential for glioblastoma stem cell maintenance. *Stem Cells* 2013; **31**: 857-869 [PMID: 23307586 DOI: 10.1002/stem.1317]

19 **Wang L**, Guo H, Lin C, Yang L, Wang X. Enrichment and characterization of cancer stem-like cells from a cervical cancer cell line. *Mol Med Rep* 2014; **9**: 2117-2123 [PMID: 24676900 DOI: 10.3892/mmr.2014.2063]

20 **Fillmore CM**, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res* 2008; **10**: R25 [PMID: 18366788 DOI: 10.1186/bcr1982]

21 **Bertolini G**, Roz L, Perego P, Tortoreto M, Fontanella E, Gatti L, Pratesi G, Fabbri A, Andriani F, Tinelli S, Roz E, Caserini R, Lo Vullo S, Camerini T, Mariani L, Delia D, Calabrò E, Pastorino U, Sozzi G. Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci USA* 2009; **106**: 16281-16286 [PMID: 19805294 DOI: 10.1073/pnas.0905653106]

22 **Li X**, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlick A, Zhang X, Chamness GC, Wong H, Rosen J, Chang JC. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst* 2008; **100**: 672-679 [PMID: 18445819 DOI: 10.1093/jnci/djn123]

23 **Liao J**, Qian F, Tchabo N, Mhawech-Fauceglia P, Beck A, Qian Z, Wang X, Huss WJ, Lele SB, Morrison CD, Odunsi K. Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism. *PLoS One* 2014; **9**: e84941 [PMID: 24409314 DOI: 10.1371/journal.pone.0084941]

24 **Bao S**, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006; **444**: 756-760 [PMID: 17051156 DOI: 10.1038/nature05236]

25 **Clarke MF**. A self-renewal assay for cancer stem cells. *Cancer Chemother Pharmacol* 2005; **56** Suppl 1: 64-68 [PMID: 16273355 DOI: 10.1007/s00280-005-0097-1]

26 **Eyler CE**, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol* 2008; **26**: 2839-2845 [PMID: 18539962 DOI: 10.1200/JCO.2007.15.1829]

27 **Gupta PB**, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? *Nat Med* 2009; **15**: 1010-1012 [PMID: 19734877 DOI: 10.1038/nm0909-1010]

28 **Ajani JA**, Song S, Hochster HS, Steinberg IB. Cancer stem cells: the promise and the potential. *Semin Oncol* 2015; **42** Suppl 1: S3-S17 [PMID: 25839664 DOI: 10.1053/j.seminoncol.2015.01.001]

29 **Vlashi E**, Pajonk F. Cancer stem cells, cancer cell plasticity and radiation therapy. *Semin Cancer Biol* 2015; **31**: 28-35 [PMID: 25025713 DOI: 10.1016/j.semcancer.2014.07.001]

30 **Visvader JE**. Cells of origin in cancer. *Nature* 2011; **469**: 314-322 [PMID: 21248838 DOI: 10.1038/nature09781]

31 **Ishizawa K**, Rasheed ZA, Karisch R, Wang Q, Kowalski J, Susky E, Pereira K, Karamboulas C, Moghal N, Rajeshkumar NV, Hidalgo M, Tsao M, Ailles L, Waddell TK, Maitra A, Neel BG, Matsui W. Tumor-initiating cells are rare in many human tumors.

Cell Stem Cell 2010; **7**: 279-282 [PMID: 20804964 DOI: 10.1016/j.stem.2010.08.009]
32 **Quintana E**, Shackleton M, Foster HR, Fullen DR, Sabel MS, Johnson TM, Morrison SJ. Phenotypic heterogeneity among tumorigenic melanoma cells from patients that is reversible and not hierarchically organized. *Cancer Cell* 2010; **18**: 510-523 [PMID: 21075313 DOI: 10.1016/j.ccr.2010.10.012]
33 **Visvader JE**, Lindeman GJ. Cancer stem cells: current status and evolving complexities. *Cell Stem Cell* 2012; **10**: 717-728 [PMID: 22704512 DOI: 10.1016/j.stem.2012.05.007]
34 **Eppert K**, Takenaka K, Lechman ER, Waldron L, Nilsson B, van Galen P, Metzeler KH, Poeppl A, Ling V, Beyene J, Canty AJ, Danska JS, Bohlander SK, Buske C, Minden MD, Golub TR, Jurisica I, Ebert BL, Dick JE. Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med* 2011; **17**: 1086-1093 [PMID: 21873988 DOI: 10.1038/nm.2415]
35 **Visvader JE**, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008; **8**: 755-768 [PMID: 18784658 DOI: 10.1038/nrc2499]
36 **Khan IN**, Al-Karim S, Bora RS, Chaudhary AG, Saini KS. Cancer stem cells: a challenging paradigm for designing targeted drug therapies. *Drug Discov Today* 2015; **20**: 1205-1216 [PMID: 26143148 DOI: 10.1016/j.drudis.2015.06.013]
37 **Kelly PN**, Dakic A, Adams JM, Nutt SL, Strasser A. Tumor growth need not be driven by rare cancer stem cells. *Science* 2007; **317**: 337 [PMID: 17641192 DOI: 10.1126/science.1142596]
38 **Alvarado-Ortiz E**, Sarabia-Sanchez MA, Garcia-Carranca A. Molecular mechanisms underlying the functions of cellular markers associated with the phenotype of Cancer Stem Cells. *Curr Stem Cell Res Ther* 2018 [PMID: 30147013]
39 **Ren F**, Sheng WQ, Du X. CD133: a cancer stem cells marker, is used in colorectal cancers. *World J Gastroenterol* 2013; **19**: 2603-2611 [PMID: 23674867 DOI: 10.3748/wjg.v19.i17.2603]
40 **Lin L**, Fuchs J, Li C, Olson V, Bekaii-Saab T, Lin J. STAT3 signaling pathway is necessary for cell survival and tumorsphere forming capacity in $ALDH^+/CD133^+$ stem cell-like human colon cancer cells. *Biochem Biophys Res Commun* 2011; **416**: 246-251 [PMID: 22074823 DOI: 10.1016/j.bbrc.2011.10.112]
41 **Catalano V**, Di Franco S, Iovino F, Dieli F, Stassi G, Todaro M. CD133 as a target for colon cancer. *Expert Opin Ther Targets* 2012; **16**: 259-267 [PMID: 22385077 DOI: 10.1517/14728222.2012.667404]
42 **Nakamura M**, Zhang X, Mizumoto Y, Maida Y, Bono Y, Takakura M, Kyo S. Molecular characterization of CD133+ cancer stem-like cells in endometrial cancer. *Int J Oncol* 2014; **44**: 669-677 [PMID: 24366104 DOI: 10.3892/ijo.2013.2230]
43 **Wang S**, Xu ZY, Wang LF, Su W. CD133+ cancer stem cells in lung cancer. *Front Biosci* (Landmark Ed) 2013; **18**: 447-453 [PMID: 23276935 DOI: 10.2741/4113]
44 **Sarvi S**, Mackinnon AC, Avlonitis N, Bradley M, Rintoul RC, Rassl DM, Wang W, Forbes SJ, Gregory CD, Sethi T. CD133+ cancer stem-like cells in small cell lung cancer are highly tumorigenic and chemoresistant but sensitive to a novel neuropeptide antagonist. *Cancer Res* 2014; **74**: 1554-1565 [PMID: 24436149 DOI: 10.1158/0008-5472.CAN-13-1541]
45 **Wu CP**, Du HD, Gong HL, Li DW, Tao L, Tian J, Zhou L. Hypoxia promotes stem-like properties of laryngeal cancer cell lines by increasing the CD133+ stem cell fraction. *Int J Oncol* 2014; **44**: 1652-1660 [PMID: 24573690 DOI: 10.3892/ijo.2014.2307]
46 **Zhang H**, Yu T, Wen L, Wang H, Fei D, Jin C. Curcumin enhances the effectiveness of cisplatin by suppressing CD133+ cancer stem cells in laryngeal carcinoma treatment. *Exp Ther Med* 2013; **6**: 1317-1321 [PMID: 24223665 DOI: 10.3892/etm.2013.1297]
47 **Piao LS**, Hur W, Kim TK, Hong SW, Kim SW, Choi JE, Sung PS, Song MJ, Lee BC, Hwang D, Yoon SK. CD133+ liver cancer stem cells modulate radioresistance in human hepatocellular carcinoma. *Cancer Lett* 2012; **315**: 129-137 [PMID: 22079466 DOI: 10.1016/j.canlet.2011.10.012]
48 **Abbasian M**, Mousavi E, Arab-Bafrani Z, Sahebkar A. The most reliable surface marker for the identification of colorectal cancer stem-like cells: A systematic review and meta-analysis. *J Cell Physiol* 2018; [PMID: 30317669 DOI: 10.1002/jcp.27619]
49 **Lee HH**, Seo KJ, An CH, Kim JS, Jeon HM. CD133 expression is correlated with chemoresistance and early recurrence of gastric cancer. *J Surg Oncol* 2012; **106**: 999-1004 [PMID: 22674531 DOI: 10.1002/jso.23178]
50 **Wang Y**, Chen M, Wu Z, Tong C, Dai H, Guo Y, Liu Y, Huang J, Lv H, Luo C, Feng KC, Yang QM, Li XL, Han W. CD133-directed CAR T cells for advanced metastasis malignancies: A phase I trial. *Oncoimmunology* 2018; **7**: e1440169 [PMID: 29900044 DOI: 10.1080/2162402X.2018.1440169]
51 **Nosrati A**, Naghshvar F, Khanari S. Cancer Stem Cell Markers CD44, CD133 in Primary Gastric Adenocarcinoma. *Int J Mol Cell Med* 2014; **3**: 279-286 [PMID: 25635255]
52 **Yan Y**, Zuo X, Wei D. Concise Review: Emerging Role of CD44 in Cancer Stem Cells: A Promising Biomarker and Therapeutic Target. *Stem Cells Transl Med* 2015; **4**: 1033-1043 [PMID: 26136504 DOI: 10.5966/sctm.2015-0048]
53 **Jaggupilli A**, Elkord E. Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity. *Clin Dev Immunol* 2012; **2012**: 708036 [PMID: 22693526 DOI: 10.1155/2012/708036]
54 **Yang CH**, Wang HL, Lin YS, Kumar KP, Lin HC, Chang CJ, Lu CC, Huang TT, Martel J, Ojcius DM, Chang YS, Young JD, Lai HC. Identification of CD24 as a cancer stem cell marker in human nasopharyngeal carcinoma. *PLoS One* 2014; **9**: e99412 [PMID: 24955581 DOI: 10.1371/journal.pone.0099412]
55 **Liu H**, Wang YJ, Bian L, Fang ZH, Zhang QY, Cheng JX. CD44+/CD24+ cervical cancer cells resist radiotherapy and exhibit properties of cancer stem cells. *Eur Rev Med Pharmacol Sci* 2016; **20**: 1745-1754 [PMID: 27212166]
56 **Zhang C**, Li C, He F, Cai Y, Yang H. Identification of CD44+CD24+ gastric cancer stem cells. *J Cancer Res Clin Oncol* 2011; **137**: 1679-1686 [PMID: 21882047 DOI: 10.1007/s00432-011-1038-5]
57 **Collins AT**, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; **65**: 10946-10951 [PMID: 16322242 DOI: 10.1158/0008-5472.CAN-05-2018]
58 **Patrawala L**, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, Reilly JG, Chandra D, Zhou J, Claypool K, Coghlan L, Tang DG. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* 2006; **25**: 1696-1708 [PMID: 16449977 DOI: 10.1038/sj.onc.1209327]
59 **Prochazka L**, Tesarik R, Turanek J. Regulation of alternative splicing of CD44 in cancer. *Cell Signal* 2014; **26**: 2234-2239 [PMID: 25025570 DOI: 10.1016/j.cellsig.2014.07.011]
60 **Biddle A**, Liang X, Gammon L, Fazil B, Harper LJ, Emich H, Costea DE, Mackenzie IC. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. *Cancer Res* 2011; **71**: 5317-5326 [PMID: 21685475 DOI: 10.1158/0008-5472.CAN-11-1059]
61 **Hernandez JR**, Kim JJ, Verdone JE, Liu X, Torga G, Pienta KJ, Mooney SM. Alternative CD44 splicing identifies epithelial prostate cancer cells from the mesenchymal counterparts. *Med Oncol* 2015; **32**: 159 [PMID: 25850653 DOI: 10.1007/s12032-015-0593-z]
62 **Biddle A**, Gammon L, Fazil B, Mackenzie IC. CD44 staining of cancer stem-like cells is influenced by down-regulation of CD44 variant isoforms and up-regulation of the standard CD44 isoform in the population of cells that have undergone epithelial-to-mesenchymal transition. *PLoS One* 2013; **8**: e57314 [PMID: 23437366 DOI: 10.1371/journal.pone.0057314]
63 **Inoue K**, Fry EA. Aberrant Splicing of Estrogen Receptor, HER2, and CD44 Genes in Breast Cancer. *Genet Epigenet* 2015; **7**: 19-32 [PMID: 26692764 DOI: 10.4137/GEG.S35500]
64 **Zöller M**. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer* 2011; **11**: 254-267 [PMID: 21390059 DOI: 10.1038/nrc3023]
65 **Lobo NA**, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells. *Annu Rev Cell Dev Biol* 2007; **23**: 675-699 [PMID:

17645413 DOI: 10.1146/annurev.cellbio.22.010305.104154]
66 **Zhang L**, Zheng W, Wang Y, Wang Y, Huang H. Human bone marrow mesenchymal stem cells support the derivation and propagation of human induced pluripotent stem cells in culture. *Cell Reprogram* 2013; **15**: 216-223 [PMID: 23713432 DOI: 10.1089/cell.2012.0064]
67 **Jing F**, Kim HJ, Kim CH, Kim YJ, Lee JH, Kim HR. Colon cancer stem cell markers CD44 and CD133 in patients with colorectal cancer and synchronous hepatic metastases. *Int J Oncol* 2015; **46**: 1582-1588 [PMID: 25625240 DOI: 10.3892/ijo.2015.2844]
68 **Joshua B**, Kaplan MJ, Doweck I, Pai R, Weissman IL, Prince ME, Ailles LE. Frequency of cells expressing CD44, a head and neck cancer stem cell marker: correlation with tumor aggressiveness. *Head Neck* 2012; **34**: 42-49 [PMID: 21322081 DOI: 10.1002/hed.21699]
69 **Faber A**, Barth C, Hörmann K, Kassner S, Schultz JD, Sommer U, Stern-Straeter J, Thorn C, Goessler UR. CD44 as a stem cell marker in head and neck squamous cell carcinoma. *Oncol Rep* 2011; **26**: 321-326 [PMID: 21617876 DOI: 10.3892/or.2011.1322]
70 **Meng E**, Long B, Sullivan P, McClellan S, Finan MA, Reed E, Shevde L, Rocconi RP. CD44+/CD24- ovarian cancer cells demonstrate cancer stem cell properties and correlate to survival. *Clin Exp Metastasis* 2012; **29**: 939-948 [PMID: 22610780 DOI: 10.1007/s10585-012-9482-4]
71 **Lau WM**, Teng E, Chong HS, Lopez KA, Tay AY, Salto-Tellez M, Shabbir A, So JB, Chan SL. CD44v8-10 is a cancer-specific marker for gastric cancer stem cells. *Cancer Res* 2014; **74**: 2630-2641 [PMID: 24618343 DOI: 10.1158/0008-5472.CAN-13-2309]
72 **Watt FM**. Role of integrins in regulating epidermal adhesion, growth and differentiation. *EMBO J* 2002; **21**: 3919-3926 [PMID: 12145193 DOI: 10.1093/emboj/cdf399]
73 **Lathia JD**, Gallagher J, Heddleston JM, Wang J, Eyler CE, Macswords J, Wu Q, Vasanji A, McLendon RE, Hjelmeland AB, Rich JN. Integrin alpha 6 regulates glioblastoma stem cells. *Cell Stem Cell* 2010; **6**: 421-432 [PMID: 20452317 DOI: 10.1016/j.stem.2010.02.018]
74 **Yamamoto H**, Masters JR, Dasgupta P, Chandra A, Popert R, Freeman A, Ahmed A. CD49f is an efficient marker of monolayer- and spheroid colony-forming cells of the benign and malignant human prostate. *PLoS One* 2012; **7**: e46979 [PMID: 23071686 DOI: 10.1371/journal.pone.0046979]
75 **Fukamachi H**, Seol HS, Shimada S, Funasaka C, Baba K, Kim JH, Park YS, Kim MJ, Kato K, Inokuchi M, Kawachi H, Yook JH, Eishi Y, Kojima K, Kim WH, Jang SJ, Yuasa Y. CD49f(high) cells retain sphere-forming and tumor-initiating activities in human gastric tumors. *PLoS One* 2013; **8**: e72438 [PMID: 24015244 DOI: 10.1371/journal.pone.0072438]
76 **Ye F**, Zhong X, Qiu Y, Yang L, Wei B, Zhang Z, Bu H. CD49f Can Act as a Biomarker for Local or Distant Recurrence in Breast Cancer. *J Breast Cancer* 2017; **20**: 142-149 [PMID: 28690650 DOI: 10.4048/jbc.2017.20.2.142]
77 **Haraguchi N**, Ishii H, Mimori K, Ohta K, Uemura M, Nishimura J, Hata T, Takemasa I, Mizushima T, Yamamoto H, Doki Y, Mori M. CD49f-positive cell population efficiently enriches colon cancer-initiating cells. *Int J Oncol* 2013; **43**: 425-430 [PMID: 23708747 DOI: 10.3892/ijo.2013.1955]
78 **Sládek NE**. Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. *J Biochem Mol Toxicol* 2003; **17**: 7-23 [PMID: 12616643 DOI: 10.1002/jbt.10057]
79 **Storms RW**, Trujillo AP, Springer JB, Shah L, Colvin OM, Ludeman SM, Smith C. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci USA* 1999; **96**: 9118-9123 [PMID: 10430905 DOI: 10.1073/pnas.96.16.9118]
80 **Toledo-Guzmán ME**, Ibañez Hernández M, Gomez-Gallegos AA, Ortiz-Sánchez E. ALDH as a Stem Cell marker in solid tumors. *Curr Stem Cell Res Ther* 2018 [PMID: 30095061 DOI: 10.2174/1574888X13666180810120012]
81 **Huang EH**, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS, Boman BM. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer Res* 2009; **69**: 3382-3389 [PMID: 19336570 DOI: 10.1158/0008-5472.CAN-08-4418]
82 **Shenoy A**, Butterworth E, Huang EH. ALDH as a marker for enriching tumorigenic human colonic stem cells. *Methods Mol Biol* 2012; **916**: 373-385 [PMID: 22914954 DOI: 10.1007/978-1-61779-980-8_27]
83 **Jiang F**, Qiu Q, Khanna A, Todd NW, Deepak J, Xing L, Wang H, Liu Z, Su Y, Stass SA, Katz RL. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res* 2009; **7**: 330-338 [PMID: 19276181 DOI: 10.1158/1541-7786.MCR-08-0393]
84 **Liu SY**, Zheng PS. High aldehyde dehydrogenase activity identifies cancer stem cells in human cervical cancer. *Oncotarget* 2013; **4**: 2462-2475 [PMID: 24318570 DOI: 10.18632/oncotarget.1578]
85 **Ortiz-Sánchez E**, Santiago-López L, Cruz-Domínguez VB, Toledo-Guzmán ME, Hernández-Cueto D, Muñiz-Hernández S, Garrido E, Cantú De León D, García-Carrancá A. Characterization of cervical cancer stem cell-like cells: phenotyping, stemness, and human papilloma virus co-receptor expression. *Oncotarget* 2016; **7**: 31943-31954 [PMID: 27008711 DOI: 10.18632/oncotarget.8218]
86 **Marcato P**, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, Helyer L, Pan L, Leidal A, Gujar S, Giacomantonio CA, Lee PW. Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. *Stem Cells* 2011; **29**: 32-45 [PMID: 21280157 DOI: 10.1002/stem.563]
87 **Kim SK**, Kim H, Lee DH, Kim TS, Kim T, Chung C, Koh GY, Kim H, Lim DS. Reversing the intractable nature of pancreatic cancer by selectively targeting ALDH-high, therapy-resistant cancer cells. *PLoS One* 2013; **8**: e78130 [PMID: 24194908 DOI: 10.1371/journal.pone.0078130]
88 **Hoshino Y**, Nishida J, Katsuno Y, Koinuma D, Aoki T, Kokudo N, Miyazono K, Ehata S. Smad4 Decreases the Population of Pancreatic Cancer-Initiating Cells through Transcriptional Repression of ALDH1A1. *Am J Pathol* 2015; **185**: 1457-1470 [PMID: 25769430 DOI: 10.1016/j.ajpath.2015.01.011]
89 **Luo Y**, Nguyen N, Fujita M. Isolation of human melanoma stem cells using ALDH as a marker. *Curr Protoc Stem Cell Biol* 2013; **26**: Unit 3.8. [PMID: 24510792 DOI: 10.1002/9780470151808.sc0308s26]
90 **Luo Y**, Dallaglio K, Chen Y, Robinson WA, Robinson SE, McCarter MD, Wang J, Gonzalez R, Thompson DC, Norris DA, Roop DR, Vasiliou V, Fujita M. ALDH1A isozymes are markers of human melanoma stem cells and potential therapeutic targets. *Stem Cells* 2012; **30**: 2100-2113 [PMID: 22887839 DOI: 10.1002/stem.1193]
91 **Kryczek I**, Liu S, Roh M, Vatan L, Szeliga W, Wei S, Banerjee M, Mao Y, Kotarski J, Wicha MS, Liu R, Zou W. Expression of aldehyde dehydrogenase and CD133 defines ovarian cancer stem cells. *Int J Cancer* 2012; **130**: 29-39 [PMID: 21480217 DOI: 10.1002/ijc.25967]
92 **Silva IA**, Bai S, McLean K, Yang K, Griffith K, Thomas D, Ginestier C, Johnston C, Kueck A, Reynolds RK, Wicha MS, Buckanovich RJ. Aldehyde dehydrogenase in combination with CD133 defines angiogenic ovarian cancer stem cells that portend poor patient survival. *Cancer Res* 2011; **71**: 3991-4001 [PMID: 21498635 DOI: 10.1158/0008-5472.CAN-10-3175]
93 **Mansour SF**, Atwa MM. Clinicopathological Significance of CD133 and ALDH1 Cancer Stem Cell Marker Expression in Invasive Ductal Breast Carcinoma. *Asian Pac J Cancer Prev* 2015; **16**: 7491-7496 [PMID: 26625750 DOI: 10.7314/APJCP.2015.16.17.7491]
94 **Roudi R**, Korourian A, Shariftabrizi A, Madjd Z. Differential Expression of Cancer Stem Cell Markers ALDH1 and CD133 in Various Lung Cancer Subtypes. *Cancer Invest* 2015; **33**: 294-302 [PMID: 26046383 DOI: 10.3109/07357907.2015.1034869]
95 **Qiu Y**, Pu T, Guo P, Wei B, Zhang Z, Zhang H, Zhong X, Zheng H, Chen L, Bu H, Ye F. ALDH(+)/CD44(+) cells in breast cancer are associated with worse prognosis and poor clinical outcome. *Exp*

Mol Pathol 2016; **100**: 145-150 [PMID: 26687806 DOI: 10.1016/j.yexmp.2015.11.032]
96 **Liu J**, Xiao Z, Wong SK, Tin VP, Ho KY, Wang J, Sham MH, Wong MP. Lung cancer tumorigenicity and drug resistance are maintained through ALDH(hi)CD44(hi) tumor initiating cells. *Oncotarget* 2013; **4**: 1698-1711 [PMID: 24091605 DOI: 10.18632/oncotarget.1246]
97 **Januchowski R**, Wojtowicz K, Zabel M. The role of aldehyde dehydrogenase (ALDH) in cancer drug resistance. *Biomed Pharmacother* 2013; **67**: 669-680 [PMID: 23721823 DOI: 10.1016/j.biopha.2013.04.005]
98 **Leonard GD**, Fojo T, Bates SE. The role of ABC transporters in clinical practice. *Oncologist* 2003; **8**: 411-424 [PMID: 14530494 DOI: 10.1634/theoncologist.8-5-411]
99 **Abdullah LN**, Chow EK. Mechanisms of chemoresistance in cancer stem cells. *Clin Transl Med* 2013; **2**: 3 [PMID: 23369605 DOI: 10.1186/2001-1326-2-3]
100 **Eyre R**, Harvey I, Stemke-Hale K, Lennard TW, Tyson-Capper A, Meeson AP. Reversing paclitaxel resistance in ovarian cancer cells via inhibition of the ABCB1 expressing side population. *Tumour Biol* 2014; **35**: 9879-9892 [PMID: 24993095 DOI: 10.1007/s13277-014-2277-2]
101 **Zhao J**. Cancer stem cells and chemoresistance: The smartest survives the raid. *Pharmacol Ther* 2016; **160**: 145-158 [PMID: 26899500 DOI: 10.1016/j.pharmthera.2016.02.008]
102 **Pearce DJ**, Taussig D, Simpson C, Allen K, Rohatiner AZ, Lister TA, Bonnet D. Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. *Stem Cells* 2005; **23**: 752-760 [PMID: 15917471 DOI: 10.1634/stemcells.2004-0292]
103 **Tanei T**, Morimoto K, Shimazu K, Kim SJ, Tanji Y, Taguchi T, Tamaki Y, Noguchi S. Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. *Clin Cancer Res* 2009; **15**: 4234-4241 [PMID: 19509181 DOI: 10.1158/1078-0432.CCR-08-1479]
104 **Sreerama L**, Sladek NE. Cellular levels of class 1 and class 3 aldehyde dehydrogenases and certain other drug-metabolizing enzymes in human breast malignancies. *Clin Cancer Res* 1997; **3**: 1901-1914 [PMID: 9815579]
105 **Vasiliou V**, Pappa A, Estey T. Role of human aldehyde dehydrogenases in endobiotic and xenobiotic metabolism. *Drug Metab Rev* 2004; **36**: 279-299 [PMID: 15237855 DOI: 10.1081/DMR-120034001]
106 **Croker AK**, Allan AL. Inhibition of aldehyde dehydrogenase (ALDH) activity reduces chemotherapy and radiation resistance of stem-like ALDHhiCD44$^+$ human breast cancer cells. *Breast Cancer Res Treat* 2012; **133**: 75-87 [PMID: 21818590 DOI: 10.1007/s10549-011-1692-y]
107 **Chen E**, Zeng Z, Bai B, Zhu J, Song Z. The prognostic value of CSCs biomarker CD133 in NSCLC: a meta-analysis. *Oncotarget* 2016; **7**: 56526-56539 [PMID: 27489355 DOI: 10.18632/oncotarget.10964]
108 **Chen T**, Yang K, Yu J, Meng W, Yuan D, Bi F, Liu F, Liu J, Dai B, Chen X, Wang F, Zeng F, Xu H, Hu J, Mo X. Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients. *Cell Res* 2012; **22**: 248-258 [PMID: 21727908 DOI: 10.1038/cr.2011.109]
109 **Rocco A**, Liguori E, Pirozzi G, Tirino V, Compare D, Franco R, Tatangelo F, Palaia R, D'Armiento FP, Pollastrone G, Affuso A, Bottazzi EC, Masone S, Persico G, Nardone G. CD133 and CD44 cell surface markers do not identify cancer stem cells in primary human gastric tumors. *J Cell Physiol* 2012; **227**: 2686-2693 [PMID: 21898409 DOI: 10.1002/jcp.23013]
110 **Singer CF**, Zabkova P, Rappaport C, Muhr D, Pfeiler G, Gschwantler-Kaulich D, Fink-Retter A, Staudigl C, Walter I, Hudelist G, Spiess AC, Kubista E. Presence of intratumoral stem cells in breast cancer patients with or without BRCA germline mutations. *Curr Cancer Drug Targets* 2012; **12**: 44-50 [PMID: 22111833 DOI: 10.2174/156800912798888938]
111 **Hashimoto K**, Shimizu C, Tsuda H, Saji S, Osaki A, Shigekawa T, Aogi K. Immunohistochemical detection of breast cancer stem cells in hormone receptor-positive breast cancer and their role in response to endocrine therapy and clinical outcome. *Oncology* 2012; **82**: 168-174 [PMID: 22433454 DOI: 10.1159/000336078]
112 **Singh BN**, Fu J, Srivastava RK, Shankar S. Hedgehog signaling antagonist GDC-0449 (Vismodegib) inhibits pancreatic cancer stem cell characteristics: molecular mechanisms. *PLoS One* 2011; **6**: e27306 [PMID: 22087285 DOI: 10.1371/journal.pone.0027306]
113 **Kim EJ**, Sahai V, Abel EV, Griffith KA, Greenson JK, Takebe N, Khan GN, Blau JL, Craig R, Balis UG, Zalupski MM, Simeone DM. Pilot clinical trial of hedgehog pathway inhibitor GDC-0449 (vismodegib) in combination with gemcitabine in patients with metastatic pancreatic adenocarcinoma. *Clin Cancer Res* 2014; **20**: 5937-5945 [PMID: 25278454 DOI: 10.1158/1078-0432.CCR-14-1269]
114 **Sadarangani A**, Pineda G, Lennon KM, Chun HJ, Shih A, Schairer AE, Court AC, Goff DJ, Prashad SL, Geron I, Wall R, McPherson JD, Moore RA, Pu M, Bao L, Jackson-Fisher A, Munchhof M, VanArsdale T, Reya T, Morris SR, Minden MD, Messer K, Mikkola HK, Marra MA, Hudson TJ, Jamieson CH. GLI2 inhibition abrogates human leukemia stem cell dormancy. *J Transl Med* 2015; **13**: 98 [PMID: 25889765 DOI: 10.1186/s12967-015-0453-9]
115 **Yang N**, Zhou TC, Lei XX, Wang C, Yan M, Wang ZF, Liu W, Wang J, Ming KH, Wang BC, Xu BL, Liu Q. Inhibition of Sonic Hedgehog Signaling Pathway by Thiazole Antibiotic Thiostrepton Attenuates the CD44+/CD24-Stem-Like Population and Sphere-Forming Capacity in Triple-Negative Breast Cancer. *Cell Physiol Biochem* 2016; **38**: 1157-1170 [PMID: 26963129 DOI: 10.1159/000443066]
116 **Takebe N**, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nat Rev Clin Oncol* 2015; **12**: 445-464 [PMID: 25850553 DOI: 10.1038/nrclinonc.2015.61]
117 **Krop I**, Demuth T, Guthrie T, Wen PY, Mason WP, Chinnaiyan P, Butowski N, Groves MD, Kesari S, Freedman SJ, Blackman S, Watters J, Loboda A, Podtelezhnikov A, Lunceford J, Chen C, Giannotti M, Hing J, Beckman R, Lorusso P. Phase I pharmacologic and pharmacodynamic study of the gamma secretase (Notch) inhibitor MK-0752 in adult patients with advanced solid tumors. *J Clin Oncol* 2012; **30**: 2307-2313 [PMID: 22547604 DOI: 10.1200/JCO.2011.39.1540]
118 **Messersmith WA**, Shapiro GI, Cleary JM, Jimeno A, Dasari A, Huang B, Shaik MN, Cesari R, Zheng X, Reynolds JM, English PA, McLachlan KR, Kern KA, LoRusso PM. A Phase I, dose-finding study in patients with advanced solid malignancies of the oral γ-secretase inhibitor PF-03084014. *Clin Cancer Res* 2015; **21**: 60-67 [PMID: 25231399 DOI: 10.1158/1078-0432.CCR-14-0607]
119 **Deng Y**, Su Q, Mo J, Fu X, Zhang Y, Lin EH. Celecoxib downregulates CD133 expression through inhibition of the Wnt signaling pathway in colon cancer cells. *Cancer Invest* 2013; **31**: 97-102 [PMID: 23245395 DOI: 10.3109/07357907.2012.754458]
120 **Egashira I**, Takahashi-Yanaga F, Nishida R, Arioka M, Igawa K, Tomooka K, Nakatsu Y, Tsuzuki T, Nakabeppu Y, Kitazono T, Sasaguri T. Celecoxib and 2,5-dimethylcelecoxib inhibit intestinal cancer growth by suppressing the Wnt/β-catenin signaling pathway. *Cancer Sci* 2017; **108**: 108-115 [PMID: 27761963 DOI: 10.1111/cas.13106]
121 **Tian D**, Shi Y, Chen D, Liu Q, Fan F. The Wnt inhibitor LGK-974 enhances radiosensitivity of HepG2 cells by modulating Nrf2 signaling. *Int J Oncol* 2017; **51**: 545-554 [PMID: 28627706 DOI: 10.3892/ijo.2017.4042]
122 **Suwala AK**, Koch K, Rios DH, Aretz P, Uhlmann C, Ogorek I, Felsberg J, Reifenberger G, Köhrer K, Deenen R, Steiger HJ, Kahlert UD, Maciaczyk J. Inhibition of Wnt/beta-catenin signaling downregulates expression of aldehyde dehydrogenase isoform 3A1 (ALDH3A1) to reduce resistance against temozolomide in glioblastoma in vitro. *Oncotarget* 2018; **9**: 22703-22716 [PMID: 29854309 DOI: 10.18632/oncotarget.25210]

123 **Li Y**, Zhang T. Targeting cancer stem cells by curcumin and clinical applications. *Cancer Lett* 2014; **346**: 197-205 [PMID: 24463298 DOI: 10.1016/j.canlet.2014.01.012]

124 **Zhu JY**, Yang X, Chen Y, Jiang Y, Wang SJ, Li Y, Wang XQ, Meng Y, Zhu MM, Ma X, Huang C, Wu R, Xie CF, Li XT, Geng SS, Wu JS, Zhong CY, Han HY. Curcumin Suppresses Lung Cancer Stem Cells via Inhibiting Wnt/β-catenin and Sonic Hedgehog Pathways. *Phytother Res* 2017; **31**: 680-688 [PMID: 28198062 DOI: 10.1002/ptr.5791]

125 **Wang D**, Kong X, Li Y, Qian W, Ma J, Wang D, Yu D, Zhong C. Curcumin inhibits bladder cancer stem cells by suppressing Sonic Hedgehog pathway. *Biochem Biophys Res Commun* 2017; **493**: 521-527 [PMID: 28870814 DOI: 10.1016/j.bbrc.2017.08.158]

126 **James MI**, Iwuji C, Irving G, Karmokar A, Higgins JA, Griffin-Teal N, Thomas A, Greaves P, Cai H, Patel SR, Morgan B, Dennison A, Metcalfe M, Garcea G, Lloyd DM, Berry DP, Steward WP, Howells LM, Brown K. Curcumin inhibits cancer stem cell phenotypes in ex vivo models of colorectal liver metastases, and is clinically safe and tolerable in combination with FOLFOX chemotherapy. *Cancer Lett* 2015; **364**: 135-141 [PMID: 25979230 DOI: 10.1016/j.canlet.2015.05.005]

127 **Hu C**, Niestroj M, Yuan D, Chang S, Chen J. Treating cancer stem cells and cancer metastasis using glucose-coated gold nanoparticles. *Int J Nanomedicine* 2015; **10**: 2065-2077 [PMID: 25844037 DOI: 10.2147/IJN.S72144]

128 **Verma RK**, Yu W, Shrivastava A, Shankar S, Srivastava RK. α-Mangostin-encapsulated PLGA nanoparticles inhibit pancreatic carcinogenesis by targeting cancer stem cells in human, and transgenic (Kras(G12D), and Kras(G12D)/tp53R270H) mice. *Sci Rep* 2016; **6**: 32743 [PMID: 27624879 DOI: 10.1038/srep32743]

129 **Muntimadugu E**, Kumar R, Saladi S, Rafeeqi TA, Khan W. CD44 targeted chemotherapy for co-eradication of breast cancer stem cells and cancer cells using polymeric nanoparticles of salinomycin and paclitaxel. *Colloids Surf B Biointerfaces* 2016; **143**: 532-546 [PMID: 27045981 DOI: 10.1016/j.colsurfb.2016.03.075]

130 **Lamb R**, Ozsvari B, Lisanti CL, Tanowitz HB, Howell A, Martinez-Outschoorn UE, Sotgia F, Lisanti MP. Antibiotics that target mitochondria effectively eradicate cancer stem cells, across multiple tumor types: treating cancer like an infectious disease. *Oncotarget* 2015; **6**: 4569-4584 [PMID: 25625193 DOI: 10.18632/oncotarget.3174]

131 **Lamb R**, Fiorillo M, Chadwick A, Ozsvari B, Reeves KJ, Smith DL, Clarke RB, Howell SJ, Cappello AR, Martinez-Outschoorn UE, Peiris-Pagès M, Sotgia F, Lisanti MP. Doxycycline down-regulates DNA-PK and radiosensitizes tumor initiating cells: Implications for more effective radiation therapy. *Oncotarget* 2015; **6**: 14005-14025 [PMID: 26087309 DOI: 10.18632/oncotarget.4159]

132 **Hirsch HA**, Iliopoulos D, Tsichlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res* 2009; **69**: 7507-7511 [PMID: 19752085 DOI: 10.1158/0008-5472.CAN-09-2994]

133 **Rattan R**, Ali Fehmi R, Munkarah A. Metformin: an emerging new therapeutic option for targeting cancer stem cells and metastasis. *J Oncol* 2012; **2012**: 928127 [PMID: 22701483 DOI: 10.1155/2012/928127]

134 **Zhang R**, Zhang P, Wang H, Hou D, Li W, Xiao G, Li C. Inhibitory effects of metformin at low concentration on epithelial-mesenchymal transition of CD44(+)CD117(+) ovarian cancer stem cells. *Stem Cell Res Ther* 2015; **6**: 262 [PMID: 26718286 DOI: 10.1186/s13287-015-0249-0]

135 **Honjo S**, Ajani JA, Scott AW, Chen Q, Skinner HD, Stroehlein J, Johnson RL, Song S. Metformin sensitizes chemotherapy by targeting cancer stem cells and the mTOR pathway in esophageal cancer. *Int J Oncol* 2014; **45**: 567-574 [PMID: 24859412 DOI: 10.3892/ijo.2014.2450]

136 **Fasih A**, Elbaz HA, Hüttemann M, Konski AA, Zielske SP. Radiosensitization of pancreatic cancer cells by metformin through the AMPK pathway. *Radiat Res* 2014; **182**: 50-59 [PMID: 24909911 DOI: 10.1667/RR13568.1]

137 **Chiorean EG**, LoRusso P, Strother RM, Diamond JR, Younger A, Messersmith WA, Adriaens L, Liu L, Kao RJ, DiCioccio AT, Kostic A, Leek R, Harris A, Jimeno A. A Phase I First-in-Human Study of Enoticumab (REGN421), a Fully Human Delta-like Ligand 4 (Dll4) Monoclonal Antibody in Patients with Advanced Solid Tumors. *Clin Cancer Res* 2015; **21**: 2695-2703 [PMID: 25724527 DOI: 10.1158/1078-0432.CCR-14-2797]

138 **Smith DC**, Eisenberg PD, Manikhas G, Chugh R, Gubens MA, Stagg RJ, Kapoun AM, Xu L, Dupont J, Sikic B. A phase I dose escalation and expansion study of the anticancer stem cell agent demcizumab (anti-DLL4) in patients with previously treated solid tumors. *Clin Cancer Res* 2014; **20**: 6295-6303 [PMID: 25324140 DOI: 10.1158/1078-0432.CCR-14-1373]

139 **Prieur A**, Cappellini M, Habif G, Lefranc MP, Mazard T, Morency E, Pascussi JM, Flacelière M, Cahuzac N, Vire B, Dubuc B, Durochat A, Liaud P, Ollier J, Pfeiffer C, Poupeau S, Saywell V, Planque C, Assenat E, Bibeau F, Bourgaux JF, Pujol P, Sézeur A, Ychou M, Joubert D. Targeting the Wnt Pathway and Cancer Stem Cells with Anti-progastrin Humanized Antibodies as a Potential Treatment for K-RAS-Mutated Colorectal Cancer. *Clin Cancer Res* 2017; **23**: 5267-5280 [PMID: 28600477 DOI: 10.1158/1078-0432.CCR-17-0533]

140 **Jin L**, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med* 2006; **12**: 1167-1174 [PMID: 16998484 DOI: 10.1038/nm1483]

141 **Li L**, Hao X, Qin J, Tang W, He F, Smith A, Zhang M, Simeone DM, Qiao XT, Chen ZN, Lawrence TS, Xu L. Antibody against CD44s inhibits pancreatic tumor initiation and postradiation recurrence in mice. *Gastroenterology* 2014; **146**: 1108-1118 [PMID: 24397969 DOI: 10.1053/j.gastro.2013.12.035]

142 **Naujokat C**. Monoclonal antibodies against human cancer stem cells. *Immunotherapy* 2014; **6**: 290-308 [PMID: 24762074 DOI: 10.2217/imt.14.4]

143 **Pérez-Alea M**, McGrail K, Sánchez-Redondo S, Ferrer B, Fournet G, Cortés J, Muñoz E, Hernandez-Losa J, Tenbaum S, Martin G, Costello R, Ceylan I, Garcia-Patos V, Recio JA. ALDH1A3 is epigenetically regulated during melanocyte transformation and is a target for melanoma treatment. *Oncogene* 2017; **36**: 5695-5708 [PMID: 28581514 DOI: 10.1038/onc.2017.160]

144 **Gudas LJ**, Wagner JA. Retinoids regulate stem cell differentiation. *J Cell Physiol* 2011; **226**: 322-330 [PMID: 20836077 DOI: 10.1002/jcp.22417]

145 **Petrie K**, Zelent A, Waxman S. Differentiation therapy of acute myeloid leukemia: past, present and future. *Curr Opin Hematol* 2009; **16**: 84-91 [PMID: 19468269 DOI: 10.1097/MOH.0b013e3283257aee]

146 **Ginestier C**, Wicinski J, Cervera N, Monville F, Finetti P, Bertucci F, Wicha MS, Birnbaum D, Charafe-Jauffret E. Retinoid signaling regulates breast cancer stem cell differentiation. *Cell Cycle* 2009; **8**: 3297-3302 [PMID: 19806016 DOI: 10.4161/cc.8.20.9761]

147 **Yan Y**, Li Z, Xu X, Chen C, Wei W, Fan M, Chen X, Li JJ, Wang Y, Huang J. All-trans retinoic acids induce differentiation and sensitize a radioresistant breast cancer cells to chemotherapy. *BMC Complement Altern Med* 2016; **16**: 113 [PMID: 27036550 DOI: 10.1186/s12906-016-1088-y]

148 **Nguyen PH**, Giraud J, Staedel C, Chambonnier L, Dubus P, Chevret E, Bœuf H, Gauthereau X, Rousseau B, Fevre M, Soubeyran I, Belleannée G, Evrard S, Collet D, Mégraud F, Varon C. All-trans retinoic acid targets gastric cancer stem cells and inhibits patient-derived gastric carcinoma tumor growth. *Oncogene* 2016; **35**: 5619-5628 [PMID: 27157616 DOI: 10.1038/onc.2016.87]

149 **Lim YC**, Kang HJ, Kim YS, Choi EC. All-trans-retinoic acid inhibits growth of head and neck cancer stem cells by suppression of Wnt/β-catenin pathway. *Eur J Cancer* 2012; **48**: 3310-3318 [PMID: 22640830 DOI: 10.1016/j.ejca.2012.04.013]

150 **Budd GT**, Adamson PC, Gupta M, Homayoun P, Sandstrom SK, Murphy RF, McLain D, Tuason L, Peereboom D, Bukowski RM, Ganapathi R. Phase I/II trial of all-trans retinoic acid and tamoxifen

in patients with advanced breast cancer. *Clin Cancer Res* 1998; **4**: 635-642 [PMID: 9533531]

151 **Liu P**, Kumar IS, Brown S, Kannappan V, Tawari PE, Tang JZ, Jiang W, Armesilla AL, Darling JL, Wang W. Disulfiram targets cancer stem-like cells and reverses resistance and cross-resistance in acquired paclitaxel-resistant triple-negative breast cancer cells. *Br J Cancer* 2013; **109**: 1876-1885 [PMID: 24008666 DOI: 10.1038/bjc.2013.534]

152 **Duan L**, Shen H, Zhao G, Yang R, Cai X, Zhang L, Jin C, Huang Y. Inhibitory effect of Disulfiram/copper complex on non-small cell lung cancer cells. *Biochem Biophys Res Commun* 2014; **446**: 1010-1016 [PMID: 24657266 DOI: 10.1016/j.bbrc.2014.03.047]

153 **Ortiz RC**, Lopes NM, Amôr NG, Ponce JB, Schmerling CK, Lara VS, Moyses RA, Rodini CO. CD44 and ALDH1 immunoexpression as prognostic indicators of invasion and metastasis in oral squamous cell carcinoma. *J Oral Pathol Med* 2018; **47**: 740-747 [PMID: 29791975 DOI: 10.1111/jop.12734]

154 **Hu J**, Li G, Zhang P, Zhuang X, Hu G. A CD44v+ subpopulation of breast cancer stem-like cells with enhanced lung metastasis capacity. *Cell Death Dis* 2017; **8**: e2679 [PMID: 28300837 DOI: 10.1038/cddis.2017.72]

155 **Kanwal R**, Shukla S, Walker E, Gupta S. Acquisition of tumorigenic potential and therapeutic resistance in CD133+ subpopulation of prostate cancer cells exhibiting stem-cell like characteristics. *Cancer Lett* 2018; **430**: 25-33 [PMID: 29775627 DOI: 10.1016/j.canlet.2018.05.014]

156 **Bigoni-Ordóñez GD**, Ortiz-Sánchez E, Rosendo-Chalma P, Valencia-González HA, Aceves C, García-Carrancá A. Molecular iodine inhibits the expression of stemness markers on cancer stem-like cells of established cell lines derived from cervical cancer. *BMC Cancer* 2018; **18**: 928 [PMID: 30257666 DOI: 10.1186/s12885-018-4824-5]

157 **Zhang Y**, Xu W, Guo H, Zhang Y, He Y, Lee SH, Song X, Li X, Guo Y, Zhao Y, Ding C, Ning F, Ma Y, Lei QY, Hu X, Li S, Guo W. NOTCH1 Signaling Regulates Self-Renewal and Platinum Chemoresistance of Cancer Stem-like Cells in Human Non-Small Cell Lung Cancer. *Cancer Res* 2017; **77**: 3082-3091 [PMID: 28416482 DOI: 10.1158/0008-5472.CAN-16-1633]

158 **Durinikova E**, Kozovska Z, Poturnajova M, Plava J, Cierna Z, Babelova A, Bohovic R, Schmidtova S, Tomas M, Kucerova L, Matuskova M. ALDH1A3 upregulation and spontaneous metastasis formation is associated with acquired chemoresistance in colorectal cancer cells. *BMC Cancer* 2018; **18**: 848 [PMID: 30143021 DOI: 10.1186/s12885-018-4758-y]

159 **Kozovska Z**, Patsalias A, Bajzik V, Durinikova E, Demkova L, Jargasova S, Smolkova B, Plava J, Kucerova L, Matuskova M. ALDH1A inhibition sensitizes colon cancer cells to chemotherapy. *BMC Cancer* 2018; **18**: 656 [PMID: 29902974 DOI: 10.1186/s12885-018-4572-6]

160 **Pal D**, Kolluru V, Chandrasekaran B, Baby BV, Aman M, Suman S, Sirimulla S, Sanders MA, Alatassi H, Ankem MK, Damodaran C. Targeting aberrant expression of Notch-1 in ALDH+ cancer stem cells in breast cancer. *Mol Carcinog* 2017; **56**: 1127-1136 [PMID: 27753148 DOI: 10.1002/mc.22579]

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ORIGINAL ARTICLE

Basic Study

Functional and molecular mechanism of intracellular pH regulation in human inducible pluripotent stem cells

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Abstract

AIM

To establish a functional and molecular model of the intracellular pH (pH_i) regulatory mechanism in human induced pluripotent stem cells (hiPSCs).

METHODS

hiPSCs (HPS0077) were kindly provided by Dr. Dai from the Tri-Service General Hospital (IRB No. B-106-09). Changes in the pH_i were detected either by microspectrofluorimetry or by a multimode reader with a pH-sensitive fluorescent probe, BCECF, and the fluorescent ratio was calibrated by the high K^+/nigericin method. NH_4Cl and Na-acetate prepulse techniques were used to induce rapid intracellular acidosis and alkalization, respectively. The buffering power (β) was calculated from the ΔpH_i induced by perfusing different concentrations of $(NH_4)_2SO_4$. Western blot techniques and immunocytochemistry staining were used to detect the protein expression of pH_i regulators and pluripotency markers.

RESULTS

In this study, our results indicated that (1) the steady-state pH_i value was found to be 7.5 ± 0.01 (n = 20) and 7.68 ± 0.01 (n =20) in HEPES and 5% CO_2/HCO_3^--buffered systems, respectively, which were much greater than that in normal adult cells (7.2); (2) in a CO_2/HCO_3^--buffered system, the values of total intracellular buffering power (β) can be described by the following equation: β_{tot} = 107.79 $(pH_i)^2$ - 1522.2 (pH_i) + 5396.9 (correlation coefficient R^2 = 0.85), in the estimated pH_i range of 7.1-8.0; (3) the Na^+/H^+ exchanger (NHE) and the Na^+/HCO_3^- cotransporter (NBC) were found to be functionally activated for acid extrusion for pH_i values less than 7.5 and 7.68, respectively; (4) V-ATPase and some other unknown Na^+-independent acid extruder(s) could only be functionally detected for pH_i values less than 7.1; (5) the Cl^-/ OH^- exchanger (CHE) and the Cl^-/HCO_3^- anion exchanger (AE) were found to be responsible for the weakening of intracellular proton loading; (6) besides the CHE and the AE, a Cl^--independent acid loading mechanism was functionally identified; and (7) in hiPSCs, a strong positive correlation was observed between the loss of pluripotency and the weakening of the intracellular acid extrusion mechanism, which included a decrease in the steady-state pH_i value and diminished the functional activity and protein expression of the NHE and the NBC.

CONCLUSION

For the first time, we established a functional and molecular model of a pH_i regulatory mechanism and demonstrated its strong positive correlation with hiPSC pluripotency.

Key words: Microspectrofluorimetry; Human induced pluripotent stem cells; Na^+/H^+ exchanger; Na^+/HCO_3^- cotransporter; Cl^-/OH^- exchanger; Cl^-/HCO_3^- exchanger; V-ATPase; Intracellular buffering power; Intracellular pH; BCECF

Core tip: For the first time, we established a model of the intracellular pH (pH_i) regulation mechanism in human induced pluripotent stem cells (hiPSCs). The steady-state pH_i value of hiPSCs was 7.50-7.68, which greater than that of normal adult cells. The Na^+-H^+ exchanger, the Na^+-HCO_3^- cotransporter and vacuolar-ATPase were the main acid extruders, while the Cl^--HCO_3^- anion exchanger and the Cl^--OH^- exchanger were the main acid loaders. Moreover, the pH_i and acid-extruding mechanism were decreased during the loss of pluripotency in hiPSCs. pH_i regulators represent an attractive target for differentiation efficiency or culture quality.

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INTRODUCTION

The homeostasis of intracellular pH (pH_i) affects many cellular functions, including cell proliferation, apoptosis, differentiation and epigenetic characteristics[1-7]. The pH_i in mammalian cells is maintained within an optimal narrow range through the combined operation of transmembrane transporters and the intracellular buffering capacity. Thus far, pH_i control in mammalian cells has been divided into the following categories: (1) intracellular buffering; (2) acid extrusion systems; (3) acid loading systems; and (4) monocarboxylate-H^+ transport[7-11]. Intracellular buffering power (β) minimizes immediate changes in pH_i, either in an acidic or alkaline direction. The total intracellular buffering power (β_{tot}) has two components as follows: the intrinsic buffering power

of the cell (β_i) caused by physicochemical buffers, such as weak acid/base moieties of cytoplasmic proteins, and the buffering capacity caused by intracellular CO_2/HCO_3^- (β_{CO2})[10]. Furthermore, different ion transporters are involved in the active pH_i regulatory mechanism. Acid-equivalent extruders, the Na^+-H^+ exchanger (NHE), the Na^+-HCO_3^- cotransporter (NBC) and vacuolar-ATPase (V-ATPase) are the main active acid extruders that are activated against intracellular acidification[7-9,12,13]. In contrast, the acid-equivalent loaders, such as the Cl^--HCO_3^- anion exchanger (AE) and the Cl^--OH^- exchanger (CHE), are activated to prevent intracellular alkalization[12,14,15]. In addition to acid extruders and acid loaders, there is also an H^+-monocarboxylate transporter (MCT), which is very important for all mammalian cells because the metabolism and transport of lactate is essential for metabolism and function under physiological or pathological conditions, such as in tumors or hypoxic conditions. The MCT has been demonstrated to play a role either as an acid extruder or an acid loader, depending on the concentration gradient of monocarboxylates, such as lactate acid and pyruvate, between the intracellular and extracellular environments[16,17]. The MCT carrier is stereoselective for L-lactate over D-lactate and has a stoichiometry of 1 H^+ with 1 lactate$^-$ anion[18,19].

Recently, the dysregulation of pH_i has been found to be a commonly adaptive feature in different types of cancer cells[20]. In normally differentiated adult cells, the pH_i and extracellular pH (pH_e) are generally approximately 7.2 and 7.4, respectively[8,9,13,21]. However, a reversed pH gradient of $pH_i \geqslant 7.2$ and $pH_e \leqslant 7.1$ has been demonstrated in cancer cells. This reversed pH gradient is caused the overexpression and increased set-point of the acid extrusion mechanism[12,20-23]. This dysregulated pH_i feature further promotes tumor progression, invasion and metastasis[21,24-26]. Indeed, metabolic changes have been reported to be a substantial hallmark of cancer cells[27]. Both in the absence or presence of oxygen, cancer cells tend to shift their metabolism from aerobic phosphorylation to aerobic glycolysis, which is known as the Warburg effect. However, the glycolytic byproducts lactate and H^+ increase during aerobic glycolysis. Therefore, intracellular acid extruders, such as NHE and MCT, are activated to maintain pH_i homeostasis. The overactivation and/or overexpression of the acid extrusion mechanism results in an increased pH_i that further promotes proliferation and prevents apoptosis in cancer cells[24,26,28,29]. Furthermore, accompanying extracellular acidification causes restructuring of the extracellular matrix and further promotes malicious metastasis and invasion[26,30,31].

Human induced pluripotent stem cells (hiPSCs), which are reprogrammed from somatic cells by expressing pluripotent transcription factors, are defined by their ability for self-renewal and differentiation into the three germ layers[32]. Pluripotent stem cells (PSCs) shared many similar properties with cancer cells, such as increased glycolysis, proliferation and adaptation to hypoxia[33-35]. Therefore, it has been proposed that the pH_i regulatory mechanism in hiPSCs is not typical compared to that in most adult cells. Indeed, a few studies have indicated that changes in pH_i affect the fate of stem cell differentiation. Decreased pH_i, either by a deficiency or the inhibition of NHE1, has been found to disturb retinoic acid-induced neuronal differentiation in mouse embryonal carcinoma cells. A similar phenomenon has been claimed to contribute to osteogenesis in human umbilical cord-derived mesenchymal stem cells[1,36]. Furthermore, overexpressed NHE1 has been shown to increase cardiomyocyte differentiation in mouse embryonic stem cells (mESCs)[6]. A recent study has reported that a decreased pH_i by knocking out or inhibiting NHE obstructed drosophila follicle stem cell differentiation and delayed the loss of pluripotency during spontaneous differentiation induced by the removal of LIF/2i[6]. Therefore, an elevated pH_i is considered necessary for PSCs to differentiate. Furthermore, another study has shown that acidic culture medium, caused by the accumulation of lactic acid from glycolysis, promotes pluripotency in both mESCs and hESCs through several mechanisms. However, studies that have optimized the culture environment showed that although acidic culture medium (pH < 7.0) promotes the retention of OCT-4 and pluripotency, it also causes significant growth arrest and an apoptotic effect in mESCs[37]. Notably, although decreasing pH_i has been shown to retain pluripotency during differentiation, the resting pH_i level in the pluripotent state is maintained at pH_i about 7.4 and is greater than that in differentiated adult cells[6]. Therefore, these recent results implicate that PSCs might share a cancer-like pH_i regulatory mechanism and consequently create a reversed pH gradient to promote pluripotent properties. However, there is a lack of reports on the correlation between the pH_i regulatory mechanism and pluripotency in hiPSCs.

Because of the importance of pH_i regulation in hiPSCs, the aims of this study are to further investigate the underlying mechanisms of pH_i regulation in hiPSCs. To determine transporter-mediated membrane fluxes of acid equivalents from measurements of pH_i, an accurate knowledge of intracellular buffering power is essential. Therefore, the first aim of this study is to estimate β_i and β_{CO2}, and the second aim is to characterize the active pH_i regulators in hiPSCs to provide the molecular and functional targets of pH_i regulators for future applications in clinics. Finally, the correlation between the pH_i regulatory mechanism and hiPSC pluripotency was examined in this study.

MATERIALS AND METHODS

Cell culture

The hiPSCs (HPS0077) were a kind gift from Dr. N.Z. Dai (TSGH-IRB No: 100-05-251) from the Tri-Service General Hospital, Taipei, Taiwan. In this study, vitronectin was used to support the growth and adhesion of HPS0077 cells. To prepare the vitronectin-coated culture

plate, 100 μL vitronectin (500 μg/mL) was directly added and mixed into cold DPBS. The vitronectin-DPBS solution was then added into the culture plate at a final concentration of 0.5 μg/cm^2 and incubated at room temperature for at least 2 h. This vitronectin-coated culture plate could be used immediately or stored at 4 ℃ for later use within 2 wk. To maintain pluripotency, HPS0077 cells were continuously cultured with mTeSR1 or mTeSR-E8 medium. When the cell colonies were grown to a sufficient size, Accutase was added to the cells at 37 ℃ for 3 min to suspend the cells. The cell suspension was centrifuged at 1000 rpm for 3 min, and the collected cell pellet was resuspended in fresh medium. The vitronectin solution was aspirated, and the cells were seeded in a suitable ratio with mTeSR1 or mTeSR-E8 medium containing 10 μmol/L Y-27632. The Y-27632-containing culture medium was replaced with Y-27632-free medium after 24 h, and the medium was subsequently changed every day. To induce the loss of pluripotency, the mTeSR1 or mTeSR-E8 medium was replaced by mTeSR-E6 medium for 1 to 4 d, and the medium was changed once every two days.

Immunocytochemistry staining and immunoblotting

For immunocytochemistry staining, a pluripotent stem cell 4-marker immunocytochemistry kit (Invitrogen), including primary antibodies against OCT4, SSEA4, SOX2 and TRA-1-60, was used to evaluate the pluripotency. Briefly, the experimental procedure was performed according to the manufacturer's instructions. For immunoblotting, whole cell lysates were prepared using RIPA lysis buffer containing 1% protease, 1% phosphatase, and 0.1% Triton X. The supernatant was collected after centrifugation at 12000 rpm for 30 min at 4 ℃. A total of 40 μg of total protein per sample was subjected to 10% SDS-PAGE and transferred to a PVDF membrane and subsequently blocked for 1 h with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST). The membranes were then incubated overnight with primary antibodies of different pH_i regulators and an internal control at 4 ℃. Then, the membranes were washed three times in TBST to remove the unbound primary antibodies and the secondary antibody was then added and incubated for 60 min at room temperature. The membranes were washed three times in TBST, and chemiluminescence was detected using a Clarity™ Western ECL substrate.

Measurement of intracellular pH

The measurement of the pH_i has been described in detail in our previous reports[12]. Briefly, to measure the change in pH_i, HPS0077 hiPSCs were analyzed by microspectrofluorimetry with a pH-sensitive fluorescent dye, BCECF-AM. When cell colonies (on a 24 mm round coverslip) were grown to a sufficient size, cells were then incubated with BCECF-AM (diluted to 6.25 μg/mL with standard HEPES solution) for 1 h at room temperature. Then, the coverslip containing the cells was moved to an inverted fluorescence microscope and excited with light at wavelengths of 490 and 440 nm. The change in the BCECF emission ratio of the 530 nm wavelength emission at a 490 and 440 nm excitation (490/440) was detected and indicated the change in pH_i. A high potassium/nigericin calibration method was used to convert the emission ratio to the pH_i value.

When the pH_i was measured using a Synergy 2 Multi-Mode Reader, the cells were seeded on 24-well culture plates. The solution was replaced with a pipette instead of a perfusion system (including a peristaltic pump and suction). The experimental procedure is similar to microspectrofluorimetry, and the details are described in our previous study[23].

Weak acid/base prepulse technique

NH_4Cl and Na-acetate prepulse techniques were used to induce intracellular acidification and alkalization, respectively, and the subsequent recovery from induced acidification and alkalization represent the activity of the acid extruder(s) and acid loader(s), respectively[12]. Taking NH_4Cl prepulse as an example, it can be described by 4 phases, as shown in Figure 1A. Cells were first perfused with 20 mmol/L NH_4Cl for 5 min, which caused an initial rapid alkalization. This mechanism is simply caused by the small molecular weight and nonpolar $[NH_3]_e$ easily crossing the cell membrane and acquiring hydrogen in the cytosol to produce NH_4^+ (phase 1: rapid alkalization, $NH_3 + H^+ \rightarrow NH_4^+$). Then, the pH_i slowly recovered and stabilized through the activation of acid loaders, such as AE and CHE (phase 2: slow recovery). The removal of NH_4Cl caused rapid intracellular acidification because $[NH_3]_i$ rapidly effluxed and further produced hydrogen from $[NH_4^+]_i$ in the cytosol (phase 3: rapid acidification, $NH_4^+ \rightarrow NH_3 + H^+$). The subsequent pH_i recovery following NH_4Cl-induced intracellular acidification is due to the activation of acid extruders, such as NHE and NBC, and this recovery slope represents the function of acid extruders (phase 4: pH_i recovery). To accurately quantify the H^+ flux through pH_i regulators, all pH_i recovery rate data was converted to the J_H (pH_i recovery rate multiplied by buffering power)[10].

Measurement of intracellular buffering power to derive the net influx or the net efflux

After the loading of BCECF-AM, cells were sequentially perfused with Na^+/Cl^--free HEPES or 5% CO_2/HCO_3^--buffered solution (the details of the composition of the solutions are listed in the *Solution* section below) containing different concentrations of $(NH_4)_2SO_4$ (40, 20, 10, 5, 2.5 and 0 mmol/L). Perfusion with $(NH_4)_2SO_4$ induced an initial intracellular alkalization, and the subsequent removal of $(NH_4)_2SO_4$ or decrease in $(NH_4)_2SO_4$ concentration caused acidification. The buffering power is defined as the ability to resist the change in pH_i induced by the impact of hydrogen, *i.e.*, $(NH_4)_2SO_4$. Therefore, if the buffering power is stronger, the change in pH_i will be smaller. The buffering power can be

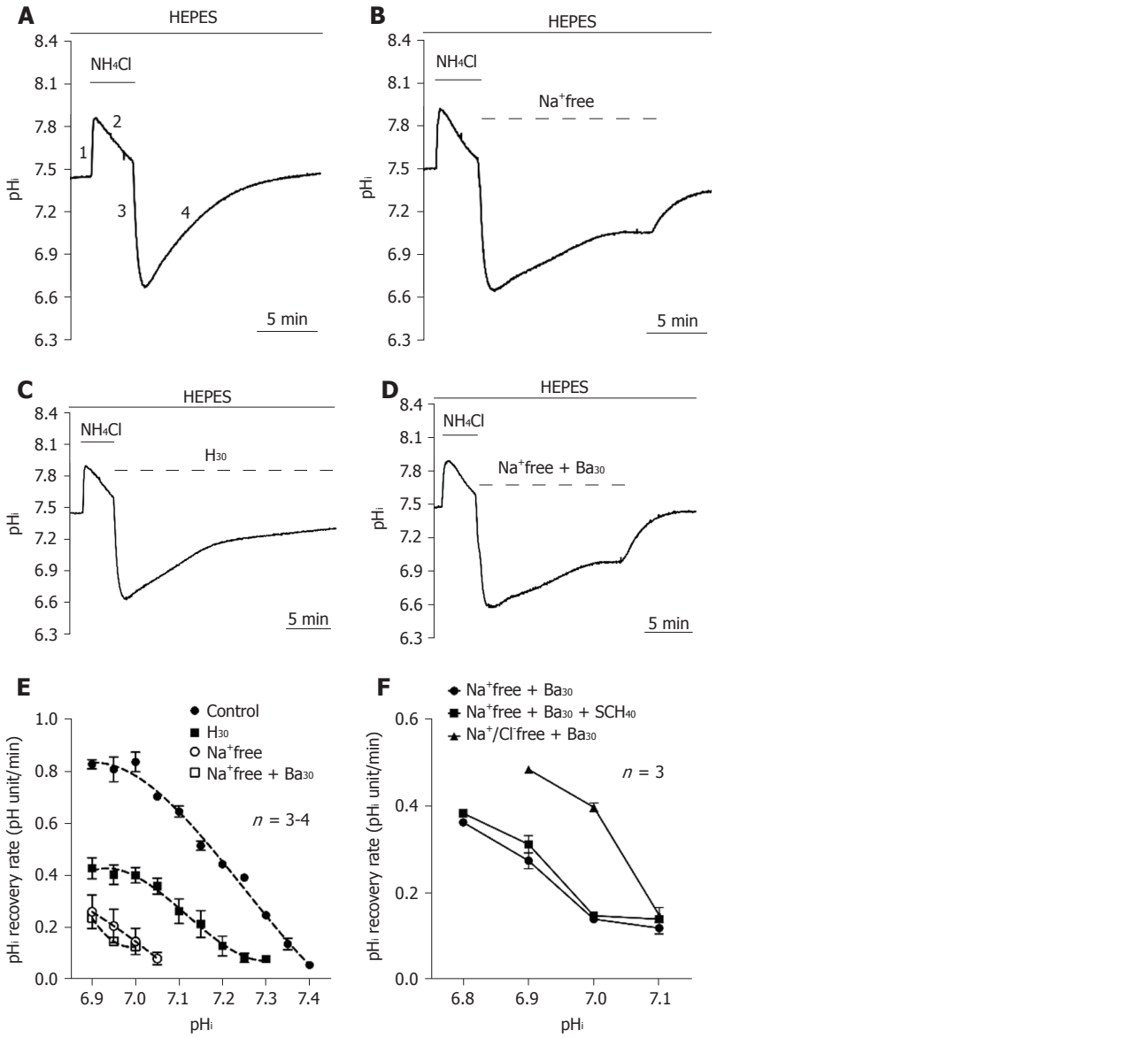

Figure 1 Functional characterization of acid extruders in the HEPES-buffered system. A-D: The top bar shows the buffer system used in perfusion experiments. The application of NH_4Cl and different conditions were respectively shown with the solid and dotted lines above the trace. The trace shown in A showed a typical pH_i recovery slope after NH_4Cl prepulse-induced intracellular acidosis in HEPES-buffered solution as a control. The traces shown in B-D showed the effect of the removal of extracellular Na^+ (Na^+-free), addition of 30 μmol/L HOE 694 (H_{30}) and Na^+-free + 30 μmol/L bafilomycin A1 (Ba_{30}) on the pH_i recovery slope. E: The curve of the pH_i recovery rates for Na^+-free, H_{30} and Na^+-free with Ba_{30} were collected from 3-6 similar experiments shown in A-D. F: After pre-treatment with NH_4Cl for 5 min, HPS0077 cells were treated with Na^+-free + Ba_{30}, Na^+-free + Ba_{30} + 40 μmol/L SCH-28080 (SCH_{40}) and Na^+/Cl^--free + Na^+-free + Ba_{30} in HEPES-buffered solution, and the change in pH_i was detected by a multimode reader. Error bars represent the mean ± SE.

defined by the following equation[38]:

$$\beta(mM) = [H^+]_i/\Delta pH_i, \quad (e.1)$$

Where $[H^+]_i$ is the change in the concentration of intracellular protons, and ΔpH_i is the resulting change in pH_i.

For experiments with the NH_4Cl prepulse technique, the application of $(NH_4)_2SO_4$ externally induces intracellular alkalization. This is due to the rapid diffusion of NH_3 into the cell and its subsequent hydrogenation to form NH_4^+. Upon the removal of extracellular $(NH_4)_2SO_4$, NH_4^+ exits the cell as uncharged NH_3, leaving behind an equal concentration of H^+ and causing intracellular acidification. If $[H^+]_i$ is assumed to equal the intracellular concentration of NH_4^+ at the moment of their removal from the external solution, then equation 1 can be expressed as follows:

$$\beta(mM) = [NH_4^+]_i/\Delta pH_i. \quad (e.2)$$

According to the Henderson-Hasselbalch equation, the relationship between internal and external NH_4^+ concentration is as follows:

$$pH_o - pH_i = \log([NH_4^+]_i / [NH_4^+]_o). \quad (e.3)$$

Equation 3 can then be rearranged as follows:

$$[NH_4^+]_i = [NH_4^+]_o \times 10^{(pHo-pHi)}. \quad (e.4)$$

In the extracellular solution, $pH_o = pKa + \log([NH_3]_o/[NH_4^+]_o)$ (Henderson-Hasselbalch equation). Therefore, this equation can be rearranged as follows:

$$[NH_4^+]_o = C/(10^{(pHo-pK)} + 1), \quad (e.5)$$

where C is the total extracellular concentration of

NH_4^+ and pK is the dissociation constant of $(NH_4)_2SO_4$. Combining equations 4 and 5, we can derive $[NH_4^+]_i$ at a given pH_i as follows:

$$[NH_4^+]_i = [C/(10^{(pHo-pK)} + 1)] \times 10^{(pHo-pHi)}. \quad (e.6)$$

In an open system, the theoretical β_{CO2} can be calculated as follows:

$$\beta_{CO2} = 2.3 \times [HCO_3^-]_i. \quad (e.7)$$

Similar to the calculation procedures outlined above for NH_4^+, $[HCO_3^-]_i$ can then be calculated as follows:

$$[HCO_3^-]_i = [C/(10^{(pK-pHo)} + 1)] \times 10^{(pHi-pHo)}. \quad (e.8)$$

Solutions and chemicals

Nigericin calibration solution was composed of 140 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.01 mmol/L nigericin and 10 mmol/L buffer (MES, HEPES or CAPSO), and the pH was adjusted to 5.5, 6.5, 7.0, 7.5, 8.5 or 9.5 with 6 mol/L NaOH. The buffers used in the calibration solution were in accordance with the pKa of the buffers and the pH of the solution (MES was used for pH = 5.5 and 6.5; HEPES was used for pH = 7.0, 7.5 and 8.5; and CAPSO was used for pH = 9.5).

Standard HEPES-buffered solution was composed of 140 mmol/L NaCl, 4.5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 11 mmol/L glucose, and 20 mmol/L HEPES. Standard bicarbonate-buffered Tyrode's solution (equilibrated with 5% CO_2/22 mmol/L HCO_3^-) was the same as above, except that the NaCl concentration was reduced to 117 mmol/L, and 22 mmol/L $NaHCO_3$ was added instead of HEPES (pH 7.40 at 37 ℃).

Ion-substituted solutions: For Na^+-free HEPES-buffered Tyrode's solution, NaCl was replaced with 140 mmol/L N-methyl-D-glucamine (NMDG). For Cl^--free CO_2/HCO_3^--buffered Tyrode's solution contained 117 mmol/L sodium gluconate, 4.5 mmol/L potassium gluconate, 12 mmol/L calcium gluconate, 22 mmol/L $NaHCO_3$, 1 mmol/L $MgSO_4$, and 11 mmol/L glucose. The Na^+/Cl^--free solution (for the buffering power experiment) was composed of 140 mmol/L NMDG, 4.5 mmol/L K-gluconate, 1 mmol/L Mg-gluconate, 2.5 mmol/L Ca-gluconate, 11 mmol/L glucose and 20 mmol/L HEPES (for 5% CO_2/HCO_3^--free system) or bubbled with 5% CO_2 (for 5% CO_2/HCO_3^- system). The pH was adjusted to 7.4 with 6 mol/L NaOH, HCl or H_2SO_4 at 37 ℃ for all solutions. NH_4Cl, Na-acetate and $(NH_4)_2SO_4$ were directly added as solids to the buffered solutions before use. HOE 694 (HOE, a NHE1 specific inhibitor), S0859 (an NBC-specific inhibitor), bafilomycin A1 (Ba, a V-type ATPase-specific inhibitor) and SCH-28080 (SCH, a KHE-specific inhibitor) were added as stocks to solutions shortly before use. All drugs mentioned above were obtained from Sigma-Aldrich.

Statistical analysis

The data were expressed as the mean ± SE of *n* preparations. The statistical significance was analyzed using one-way or two-way ANOVA followed by Tukey's or Dunnett's multiple comparisons with GraphPad Prism 6 software, respectively. A *P*-value less than 0.05 were regarded as statistically significant.

RESULTS

In situ calibration of BCECF and the detection of hiPSC pluripotency markers

To monitor the change in pH_i, an *in situ* calibration was conducted in hiPSCs. A high potassium/nigericin calibration method was used to convert the emission ratio to the pH_i value. Briefly, BCECF-loaded cells were perfused with six different nigericin calibration solutions with different pH levels (5.5-9.5) (the details of the composition of the six nigericin calibration solutions are listed above in the solutions section) that caused the pH_i to equal the pH_e, as shown in Figure 2A. The calibration equation was obtained from ten similar experiments and a nonlinear BCECF fluorescence-pH_i curve of function, as shown below and in Figure 2B. The following equation was used to convert the fluorescence ratio into pH_i:

$$pH_i = pK_a + \log[(R_{max} - R)/(R - R_{min})] + \log(F_{440min}/F_{440max})$$

Where R is the ratio of the 530 nm fluorescence emission at 490 nm and 440 nm excitation (490/400), and F is the fluorescence value at 490 nm and 440 nm excitation. The maximum and minimum ratios (R_{max} and R_{min}) of 530/490 and 530/440 (Em/Ex) were obtained from perfusion with pH 9.5 and 5.5 calibration solutions, respectively.

Because the HPS0077 cell line was used as a representative example of hiPSCs in this study, we first examined whether pluripotency markers, such as OCT4, SOX2, SSEA-4 and TRA-1-60, are present in HPS0077 cells. As shown in Figure 2C, the four pluripotency markers were clearly identified by immunofluorescence staining and labeling. Our results support the hypothesis that the HPS0077 cell line possesses the characteristics of hiPSCs and is suitable as the subject for this study.

Functional characterization of acid extruders in HEPES buffered system

To investigate whether there is an acid extrusion mechanism in the cultured hiPSCs, the cells were first perfused in HEPES-buffered solution (CO_2/HCO_3^--free). As shown in Figure 1A, a pH_i recovery slope following NH_4Cl prepulse-induced intracellular acidification was a typical trace for the control (n = 3). Either removal of the extracellular Na^+ (n = 3) or application of 30 μmol/L HOE 694 (H_{30}, n = 4) significantly inhibited the pH_i recovery rate, as shown in Figures 1B and 1C, respectively, which demonstrates the presence of Na^+-dependent acid extruder(s) and NHE1 in HPS0077 cells.

However, besides Na^+-dependent acid extruders, there is another acid extrusion mechanism responsible for the remaining acid extrusion in HEPES solution. Therefore, to further investigate whether the remai-

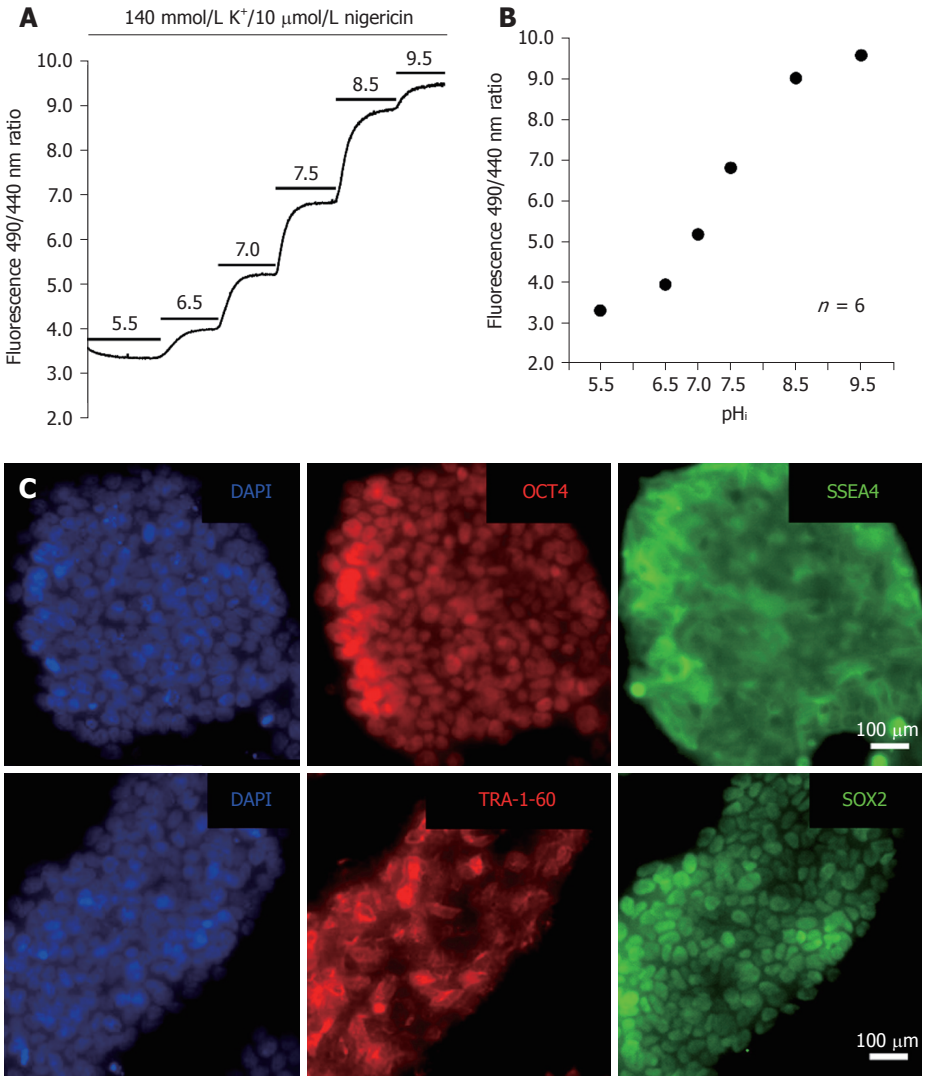

Figure 2 Calibration of the BCECF fluorescence ratio and pluripotency characterization. A: The trace showed the protocol of BCECF fluorescence ratio (510 nm emission at 490 nm and 440 nm excitations) calibration in HPS0077 cells. The top bars represent the application of different conditions; B: The plots of pH_i *vs* the BCECF fluorescence ratio were collected from 6 similar experiments shown in A; C: Immunofluorescence analysis showed the expression of pluripotency markers, OCT4, SSEA4, TRA-1-60 and SOX2, in HPS0077 cells.

ning Na^+-independent pH_i recovery (*i.e.*, could not be inhibited by Na^+-free solution) is caused by the vacuolar-type ATPase (V-ATPase), HPS0077 cells were perfused with an Na^+-free solution pulse with 30 μmol/L bafilomycin A1 (Ba_{30}; V-ATPase-specific inhibitor, n = 3), as shown in Figure 1D. However, either no significant inhibition or slight inhibition of pH_i recovery was observed between the Na^+-free solution group and the Na^+-free solution + Ba_{30} group (Figures 1B and 1D, respectively). These results suggest that V-ATPase does not play a role in acid extrusion to the cytosol in hiPSCs. Experimental data similar to those shown in Figures 1A-D were summarized and plotted as a function of the pH_i recovery rate *vs* pH_i in Figure 1E. As shown in Figure 1E, in HEPES solution (*i.e.*, when HCO_3^--dependent acid extruder(s) were not activated), the acid extrusion mechanism was mainly attributed to NHE1 (the difference between the trace of the Na^+-free group and the trace of the H_{30} group), apart from other Na^+-dependent acid extruder(s) (the difference between the trace of H_{30} and the trace of Na^+-free). Moreover, the other Na^+-independent acid extruder(s) were activated when the pH_i was less than 7.1 ± 0.01 (see the trace of Na^+-free + Ba_{30}).

To further examine whether the Na^+-independent acid extruders shown in Figure 1E are KHE or Cl^--dependent acid extruder(s), HPS0077 cells were either performed by adding 40 μmol/L SCH-28080 (SCH_{40}, a KHE-specific inhibitor) or removing $[Cl^-]_o$. The change in pH_i in this series of experiments was detected using a Synergy 2 Multi-Mode Reader with BCECF-AM dye. The data for this series of experiments were summarized and plotted as a function of the pH_i recovery rate *vs* pH_i in Figure 1F. As shown in Figure 1F, the pH_i recovery rate between the trace before and after adding SCH_{40} (n = 3, solid circles and squares, respectively) was not significantly different. Moreover, the removal of $[Cl^-]$ (n = 3, solid triangles) surprisingly caused a dramatic

increase in the pH_i recovery rate instead of inhibition. This phenomenon is most likely caused by the inhibition of the activity of the Cl^--dependent acid loader. In summary, these results provide clear pharmacological evidence that the NHE is mainly responsible for acid extrusion and functionally coexists with other Na^+-dependent and -independent acid extrusion mechanisms in HPS0077 cells. Moreover, the Na^+-independent acid extrusion mechanism is neither a KHE nor a Cl^--dependent acid extruder(s).

Functional characterization of acid extruders in a 5% CO_2/HCO_3^--buffered system

To quantify the $[H_i]^+$ flux through pH_i regulators in 5% CO_2/HCO_3^--buffered conditions, we first quantified intracellular buffering (β). The experimental details are shown in the materials and methods section, and we found that β increased as pH_i increased at pH_i = 7.0 to 8.0 (n = 35, data not shown). The equation can be expressed as $\beta = 107.79\ (pH_i)^2 - 1522.2\ (pH_i) + 5396.9$ (correlation coefficient $R^2 = 0.85$). The obtained β can be used to calculate the $[H_i]^+$ flux through pH_i regulators by the following equation: $J_H = \beta \times pH_i$ recovery rate (pH_i value/minutes). To further investigate whether the NBC is functionally involved in the 5% CO_2/HCO_3^- condition, we used a protocol similar to the previously mentioned experiments except for the replacement of HEPES-buffered solution with 5% CO_2/HCO_3^--buffered solution. The pH_i recovery slope following NH_4Cl prepulse-induced intracellular acidification in 5% CO_2/HCO_3^--buffered solution was a typical trace for the control (n = 7), as shown in Figure 3A. As shown in Figures 3B-E, the pH_i recovery rate was significantly inhibited under four different conditions as follows: removal of $[Na^+]$ (n = 3, addition of H_{30} (n = 4), addition of 90 μmol/L S0859 (S_{90}; an inhibitor of NBC, n = 3), and addition of H_{30} and S_{90} (H_{30} + S_{90}, n = 3). Experimental data similar to those shown in Figures 3A-E were summarized and plotted as a function of J_H *vs* pH_i in Figure 3F. As shown in Figure 3F, a similar pH_i recovery rate between Na^+-free and H_{30} + S_{90} conditions indicated that the NHE1 and the NBC were both involved in the Na^+-dependent acid extrusion mechanism in the 5% CO_2/HCO_3^- condition in HPS0077 cells.

Notably, the acid extrusion mechanism in the 5% CO_2/HCO_3^- condition was regulated mainly by the NBC in the pH_i range of 7.50-7.68 because the pH_i recovery rate could be completely inhibited by S_{90} (Figure 3D). Moreover, the addition of S_{90} did not affect pH_i recovery when the pH_i was less than 6.9 ± 0.01 (n = 3, see the trace of S_{90}), which indicated that the NBC was not responsible for acid extrusion in the relatively acidic cytoplasm (Figure 3D). In summary, NHE1, NBC and Na^+-independent acid extruder(s) were mainly functionally activated in the pH_i ranges of < 7.5, 6.9-7.68 and < 7.1, respectively.

Functional characterization of acid loaders

The homeostasis of pH_i is coregulated by both acid extruders and acid loaders. The CHE and the AE are two known acid loaders in mammalian cells. Unlike the NHE and the NBC, the acid loading mechanism depends on $[Cl^-]_o$ and further exchange of $[OH^-]_i$ or $[HCO_3^-]_i$ out of the cytoplasm to neutralize intracellular alkalization. To estimate the function of acid loaders, an Na-acetate prepulse was used to induce intracellular alkalization in this study. The subsequent pH_i recovery slope was expressed as the acid loading activity of acid loaders. Figures 4A and 4C show the typical pH_i recovery slope following the Na-acetate prepulse either in HEPES or HCO_3^--buffered solution, respectively (n = 3). Removal of $[Cl^-]_o$ in the 5% CO_2/HCO_3^--buffered solution completely inhibited the pH_i recovery (n = 3), as shown in Figure 4D, which indicated that the acid loading mechanism is completely Cl^--independent in HPS0077 cells. However, interestingly, a rapid acid loading phenomenon was observed before the total inhibition at pH_i = 7.9 ± 0.01 (n = 3) in HEPES solution, as shown in Figure 4B. These results indicated that CO_2 or HCO_3^- may inhibit this unknown Cl^--dependent acid loader(s), but the characterization requires further studies. Due to the lack of specific inhibitors of the CHE and the AE, according to previous studies on the acid loading mechanism in mammalian cells conducted by Leem *et al*[39], we speculate that the Cl^--independent acid loading mechanism is mainly attributed to the CHE and the AE[39]. Notably, as shown in Figure 4E, the pH_i recovery rate is nearly identical between the 5% CO_2/HCO_3^- system (solid circles) and the HEPES system (solid squares), which indicates that the CHE plays a more important role than the AE in the acid loading mechanism in HPS0077 cells.

Decrease in pH_i during the loss of pluripotency: molecular and functional evidence

Our previously mentioned results showed that the acid extruders NHE and NBC mainly functionally coexist in hiPSCs.

We further investigated the dynamic changes in pH_i during the loss of pluripotency in hiPSCs. In the pluripotent state, the resting pH_i observed from the pH_i completely recovered after NH_4Cl prepulse-induced intracellular acidification was found to be 7.5 ± 0.01 (n = 20) and 7.68 ± 0.01 (n = 20) in HEPES and 5% CO_2/HCO_3^- conditions, respectively, as shown in Figures 5A and 5B. Moreover, in 5% CO_2/HCO_3^--buffered solution, as expected, the resting pH_i shifted to 7.46 ± 0.02 (n = 5) and 7.66 ± 0.02 (n = 5) after adding S0859 (S_{90}) and HOE694 (H_{30}), respectively (Figure 5B, the data were collected from the data shown in Figures 3C and 3D). Notably, there was no significant difference between the resting pH_i in HEPES and in the 5% CO_2/HCO_3^- plus S_{90} conditions, which indicates that the set-point of NHE activation is pH_i = 7.5. In the 5% CO_2/HCO_3^- condition, the resting pH_i showed no significant difference between the untreated and H_{30}-treated conditions, which indicates that the pH_i is regulated by the NBC instead of the NHE in the pH_i range of 7.50-7.68.

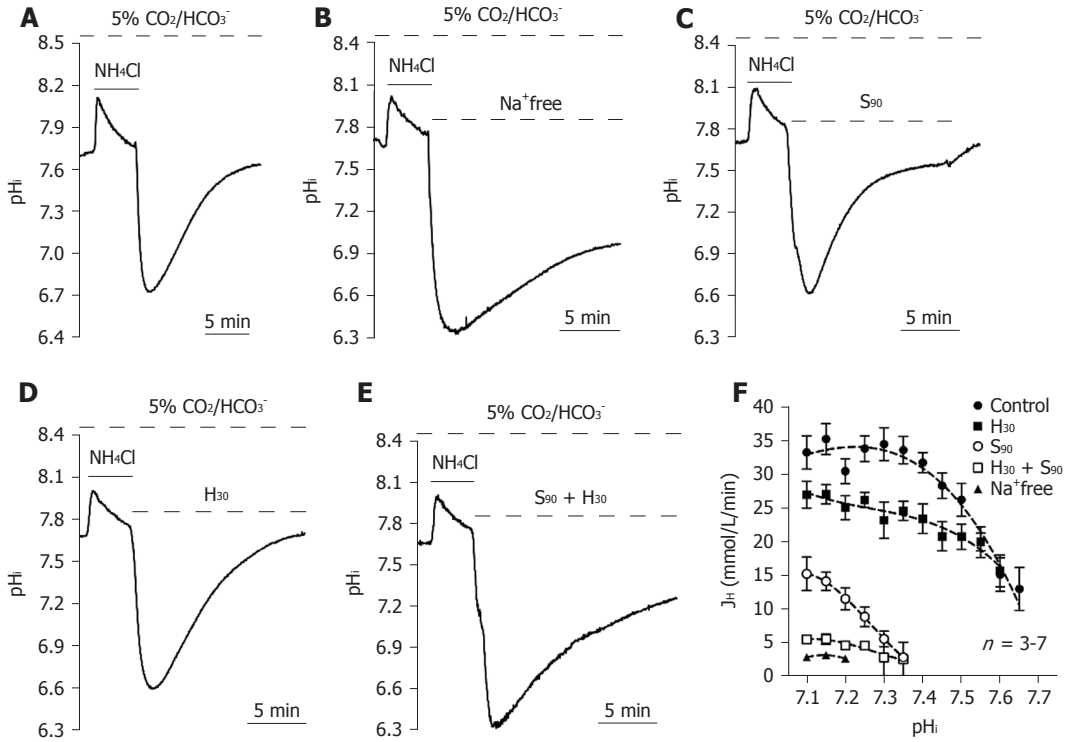

Figure 3 Functional characterization of acid extruders in the 5% CO_2/HCO_3^--buffered system. A-E: The trace shown in A showed a typical pH_i recovery slope after NH_4Cl prepulse-induced intracellular acidosis in HEPES-buffered solution as a control. The traces shown in B-E showed the effect of the removal of extracellular Na^+ (Na^+-free), addition of 90 μmol/L S0859 (S_{90}), addition of 30 μmol/L HOE 694 (H_{30}) and addition of S_{90} + H_{30} on the pH_i recovery slope; F: The curve of the pH_i recovery rates after the addition of Na^+-free, S_{90}, H_{30} and S_{90} + H_{30} were collected from 2-10 similar experiments shown in A-E. Error bars represent the mean ± SE.

To further induce the loss of pluripotency, HPS0077 cells were first transferred from mTeSR1 media (designed for maintaining long-term pluripotency) to mTeSR-E8 medium (containing fibroblast growth factor 2, FGF2, and transforming growth factor β1, TGFβ1) and then subsequently replaced with mTeSR-E6 medium (without FGF2 and TGFβ1) for 1 to 4 days (E6-1d to 4d) to induce the loss of pluripotency. Notably, the expression of the pluripotency marker OCT4 was significantly decreased after culture in mTeSR-E6 medium, as shown in Figure 5C. We also found that the expression of NHE1, NHE3, V-ATPase, NBCe1 and NBCe2 decreased during the loss of pluripotency, while the expression of NBCn1 did not decrease, as shown in Figure 5C.

To further investigate the role of the NHE and the NBC on the loss of pluripotency, we detected the pH_i recovery rate following NH_4Cl prepulse-induced intracellular acidification. The pH_i recovery traces in different culture mediums, *i.e.*, E8, E6-1d, E6-2d, E6-3d and E6-4d in HEPES and 5% CO_2/HCO_3^--buffered solution are shown in Figures 6A and 6D, respectively. The graphs in Figure 6B show the pH_i recovery rate in E6-1d to E6-4d normalized from the E8 condition (% of E8) in HEPES, estimated at pH_i = 6.9 and 7.2, respectively, and averaged for 3 experiments similar to that shown in Figure 6A. The NHE is mainly responsible for acid extrusion in the HEPES condition. When the pH_i recovery rate was measured at pH_i = 6.9, E6-1d showed no significant change, while E6-2d, E6-3d and E6-4d significantly decreased by 76.3%, 60.6% and 51.7%, respectively (n = 3). When the pH_i recovery rate was measured at pH_i = 7.2, the pH_i recovery rates of E6-1d, E6-2d, E6-3d and E6-4d significantly decreased by 82.7%, 67.4%, 47.6% and 16.3%, respectively (n = 3). The max/min charts in Figure 6C show the resting pH_i in E8, E6-1d, E6-2d, E6-3d and E6-4d, respectively, averaged from similar experiments as shown in Figure 6A (n = 5-20). The resting pH_i decreased from 7.5 to 7.49, 7.4, 7.28 and 7.21 in E6-1d, E6-2d, E6-3d and E6-4d, respectively (n = 5 to 20).

The graphs shown in Figure 6E show the pH_i recovery rate in E6-1d to E6-4d normalized to E8 (control) in 5% CO_2/HCO_3-buffered solution, which was estimated at pH_i = 6.9, 7.2 and 7.5, respectively, and averaged for 3 experiments similar to that shown in Figure 6D. As shown in Figure 6E, in the 5% CO_2/HCO_3^- condition (*i.e.*, where the NHE and the NBC were both involved in the acid extrusion mechanism), the pH_i recovery rate measured at pH_i = 6.9 and 7.2 showed no significant difference between E8 and E6-1d, but it was significantly decreased by 85.2% when measured at pH_i = 7.5. The pH_i recovery rate for E6-2d, E6-3d and E6-4d was significantly decreased by 88.7, 74.9 and 61%, respectively, when measured at pH_i = 6.9, decreased by 82%, 77.2% and 51.5%, respectively, when measured at pH_i = 7.2, and decreased by 53.4%, 44.8% and 22.3%, respectively, when measured at pH_i = 7.5 (n = 3). The max/min charts shown in Figure 6F show the resting pH_i in E8, E6-1d, E6-2d, E6-3d and E6-4d, averaged from similar experiments as those shown

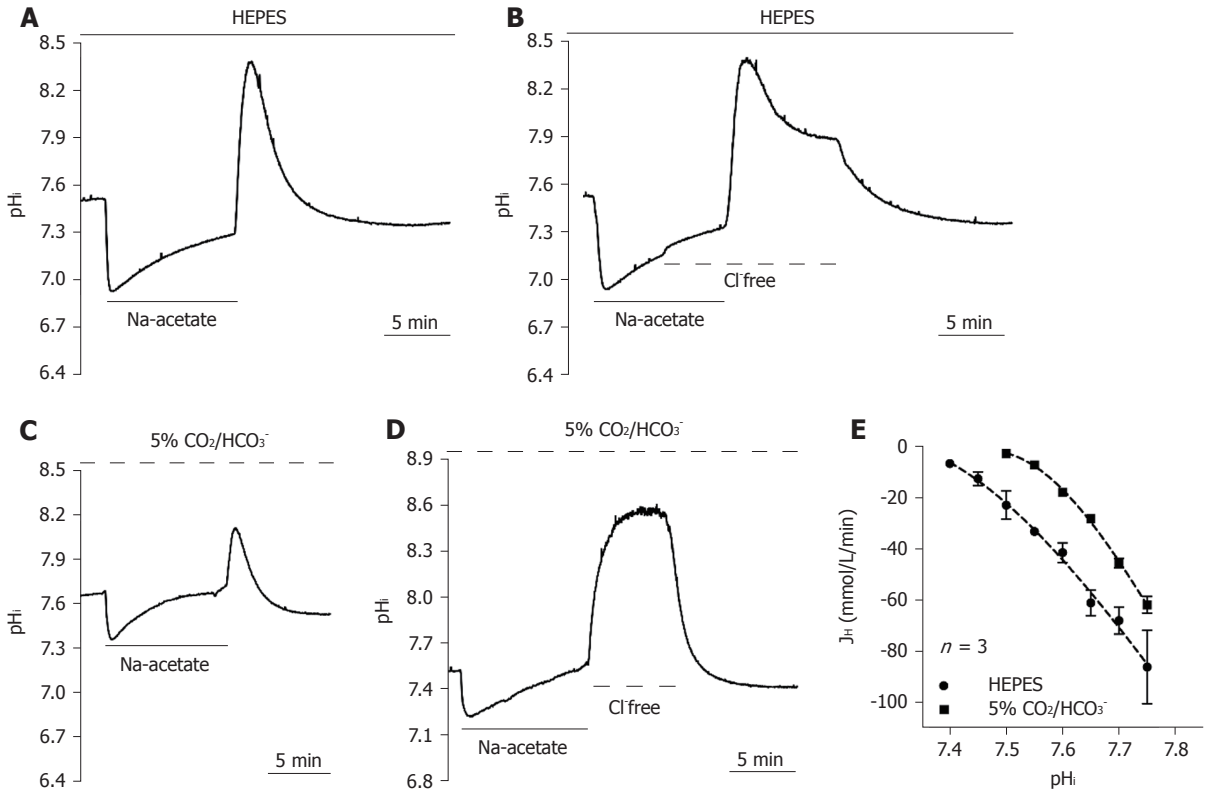

Figure 4 Functional characterization of the acid loader. A-D: The traces shown in A and C showed typical pH_i recovery slopes after Na-acetate prepulse-induced intracellular alkalization in HEPES and 5% CO_2/HCO_3^--buffered solution as a control. The traces shown in B and D showed the effect of the removal of extracellular Cl^- (Cl^--free) on the pH_i recovery slope in HEPES and 5% CO_2/HCO_3^--buffered solution; E: The curve of the pH_i recovery rates in HEPES and 5% CO_2/HCO_3^--buffered solution were collected from 3-4 similar experiments shown in A and C. Error bars represent the mean ± SE.

in Figure 6D (n = 5-20). We found that the resting pH_i decreased from 7.68 to 7.64, 7.61, 7.56 and 7.48 in E6-1d, E6-2d, E6-3d and E6-4d, respectively (n = 5, Figure 6F). In summary, our results provide clear evidence that the loss of hiPSC pluripotency decreased the activity and expression of acid extruders (NHE and NBC), further resulting in a decrease in the pH_i recovery rate and resting pH_i.

DISCUSSION

The functional and molecular evidence of active transmembrane acid extruders and acid loaders in hiPSCs

In this study, we have clearly demonstrated that transmembrane active pH_i regulators, such as NHE1, NBC, AE and CHE, functionally coexisted in hiPSCs (Figures 3 and 4). Moreover, we successfully quantified the net acid efflux of each functional acid transporter, as shown in Figures 3 and 7, by considering intracellular buffering. From Figure 3F, we can clearly observe that the active efflux was mainly dependent on the activity of the NBC in hiPSCs in the pH_i range less than 7.35 because the S_{90} group (*i.e.*, inhibiting NBC activity) substantially decreased the activity compared to other groups (inhibiting NHE1 or other Na-independent acid extruders). Moreover, the role of NHE1 on acid extrusion decreased as the pH_i increased (Figures 1, 3 and 7). Notably, the activity of NHE1 was nominally undetectable when the pH_i was greater than 7.50, as shown in Figures 1, 3 and 7.

Relevant molecular candidates for the NBC include at least five members of the slc4 family, including 2 electrogenic Na^+-HCO_3^- cotransporters (NBCe1/SLC4A4 and NBCe2/SLC4A5), 1 electroneutral Na^+-HCO_3^- cotransporter (NBCn1/SLC4A7) and 2 Na^+-dependent Cl^--HCO_3^- exchangers (NCBE/SLC4A10 and NDCBE/SLC4A8)[7,40,41]. In this study, we found that three isoforms of the NBC, NBCn1, NBCe1 and NBCe2, coexist in hiPSCs, which is similar to our previously reported results in cultured human renal artery smooth muscle cells[7]. However, the Aalkjaer group has demonstrated that the NBC is NBCn1, *i.e.*, it is electroneutral, in rat and mouse smooth muscle cells[42], which is similar to the results reported in guinea pig myocytes by the Vaughan-Jones group[10]. In other words, the coexistence of 3 types of NBCs in hiPSCs is different from the results in mouse and rat models (c.f. Aalkjaer's group) and guinea pig models (c.f. Vaughan-Jones's group, which is likely due to differences in species/organs.

Moreover, in contrast to the results reported in our previous studies in cardiovascular cells, we found that Na^+-independent acid extruder(s) and Cl^--independent acid loader(s) were substantially present for acid extrusion (pH_i < 7.1) and acid loading (pH_i > 7.9) in hiPSCs (Figures 1, 4 and 7). We further demonstrated

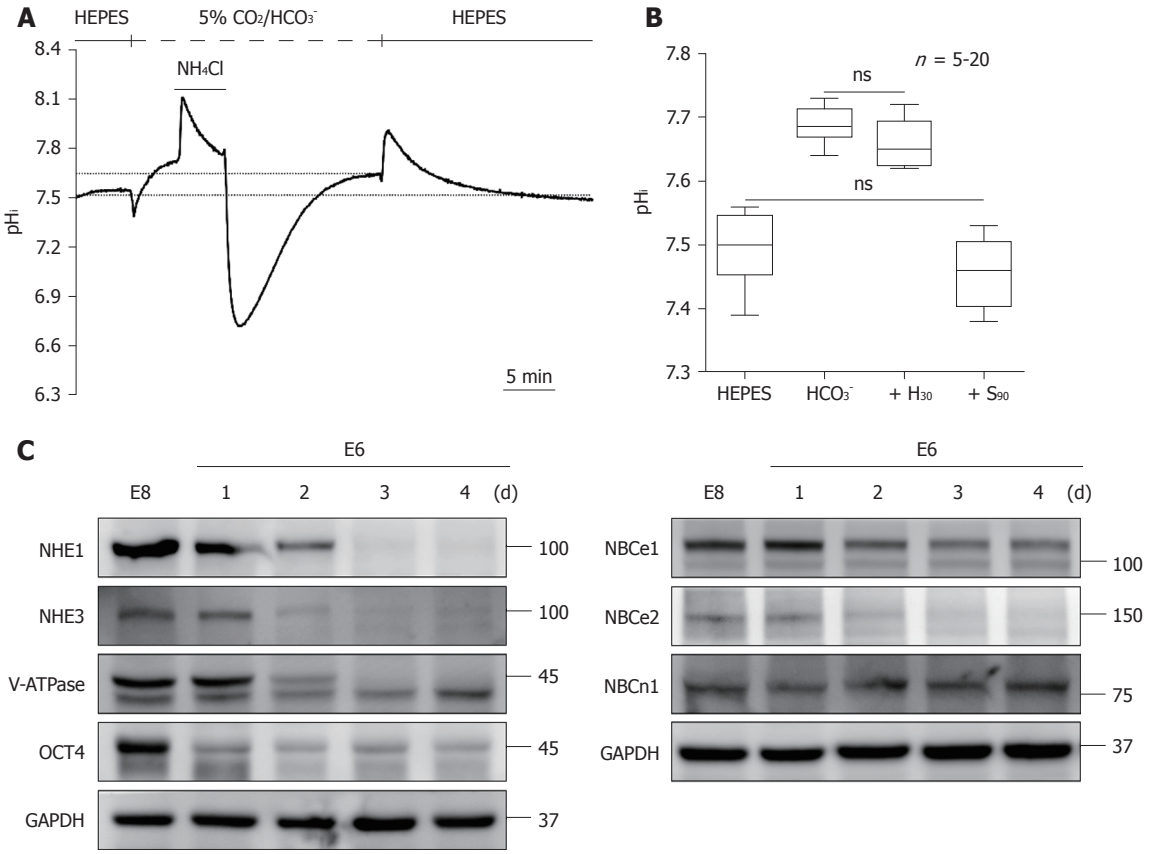

Figure 5 Steady-state pHi in HEPES and 5% CO_2/HCO_3^--buffered solution and the change in the expression of pHi regulators during the loss of pluripotency in human induced pluripotent stem cells. A: The resting pHi was a steady-state taken from the completely recovered pHi after intracellular acidification or alkalization. The dotted line indicates the value of the resting pHi; B: The max/min chart of the resting pHi in hiPSCs was collected from A (n = 20) and Figures 4C and D (n = 5). The means of the resting pHi in HEPES and 5% CO_2/HCO_3^--buffered solution were found to be 7.50 ± 0.01 and 7.68 ± 0.01, respectively. After treatment with H_{30} and S_{90} in 5% CO_2/HCO_3^--buffered solution, the resting pHi shifted to 7.66 ± 0.02 and 7.46 ± 0.02, respectively; C: Immunoblot analysis of the expression of NHE1, NHE3, V-ATPase, NBCe1, NBCe2, NBCn1 and OCT4 in hiPSCs in different culture media for different days (E8 and E6-1d to E6-4d). The histograms in B display the mean and the min to max values. hiPSCs: Human induced pluripotent stem cells; NHE: The Na^+/H^+ exchanger; NBC: The Na^+/HCO_3^- cotransporter; V-ATPase: Vacuolar-ATPase.

that the unknown Na^+-independent acid extruder(s) is not the V-ATPase, KHE[43] or Cl^--dependent acid extruder (localized on lysosome and gastric cell membranes)[44,45] (Figures 1D and 1F). Therefore, we hypothesize that this unknown Na^+-independent mechanism is most likely an ATP-dependent transporter instead of a concentration gradient-driven transporter. For example, ATP deficiency, induced by the addition of oligomycin, combined with the addition of bafilomycin A1 during the perfusion experiments would allow us to observe whether it inhibits Na^+/V-ATPase-independent acid extrusion in hiPSCs[46]. However, functional and molecular characterization requires further studies in the future.

In addition to being an acid extruder, NBCe1 has been reported to be responsible for the acid loading mechanism during the process of changing from the HEPES-buffered solution to the 5% CO_2/HCO_3^--buffered solution in mouse astrocytes[47]. However, in our findings, the addition of 50 μM S0859 still failed to inhibit the Cl^--independent acid extrusion mechanism in the HEPES-buffered condition (data not shown). This result suggested that the Cl^--independent acid extruder(s) was not NBCe1 in hiPSCs. Due to this unknown Cl^--independent acid extrusion mechanism being completely inhibited in the CO_2/HCO_3^--buffered system and the lack of related studies, future works should further characterize the possible existence of a CO_2-related pHi acid loading mechanism.

The implication of the existence of extra acid extrusion/loading mechanisms in hiPSCs

The existence of an unknown acid extrusion mechanism, *i.e.*, Na^+-independent acid extruder(s) (see Figure 3F) and acid loading mechanisms, *i.e.*, Cl^--independent acid loader(s), in hiPSCs might imply that the ability to resist the acid/base impact is very important for the pluripotency of hiPSCs[37,48,49]. It has been reported that hiPSCs share many cellular properties with cancer cells, such as increased cell proliferation and dependence on glycolysis for metabolism[24,26,50,51]. Many studies showed that a lower pHi decreased proliferation and energy production in either normal or cancer cells[15,26]. Indeed, in this study, we found that the acid extrusion mechanism was fully activated at an acidic pHi (< 7.2), including the NHE, the NBC

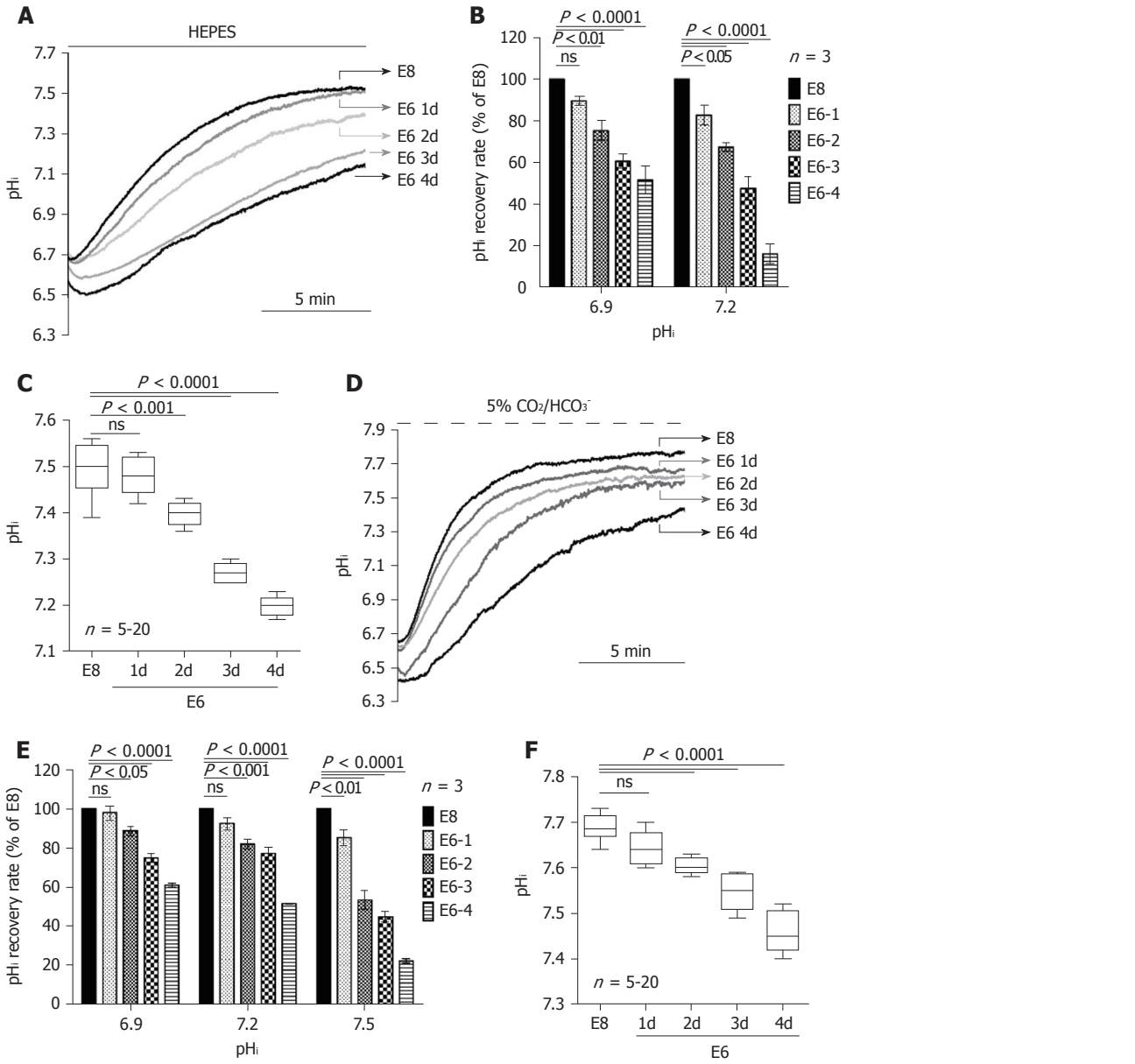

Figure 6 The change in the activity of the Na^+/H^+ exchanger and the Na^+/HCO_3^- cotransporter and the resting pH_i during the loss of pluripotency in human induced pluripotent stem cells. A: The traces showed the changes in pH_i recovery after NH_4Cl prepulse-induced intracellular acidification in E8 medium (containing fibroblast growth factor 2, FGF2, and transforming growth factor β1, TGFβ1) and E6 medium (without FGF2 and TGFβ1) for 1 to 4 d (E6-1d to E6-4d) in HEPES-buffered solution; B: The charts showed the pH_i recovery rate in E6-1d to -4d normalized to the rate in E8 (% of E8) in HEPES-buffered solution, which was estimated at pH_i = 6.9 and 7.2, respectively, and averaged for 3 experiments similar to that shown in A; C: The max/min plots showed the resting pH_i in E8, E6-1d, E6-2d, E6-3d and E6-4d media that were averaged from similar experiments shown in A (n = 5-20); D: The traces showed the changes in pH_i recovery after NH_4Cl prepulse-induced intracellular acidification in E8 and E6-1d to E6-4d media in 5% CO_2/HCO_3^--buffered solution; E: The graphs show the pH_i recovery rate in E6-1d to E6-4d normalized to the rate in E8 (control) in 5% CO_2/HCO_3^--buffered solution, which was estimated at pH_i = 6.9, 7.2 and 7.5, respectively, and averaged for 3 experiments similar to that shown in D; F: The max/min plots showed the resting pH_i in E8 E6-1d, E6-2d, E6-3d and E6-4d media, averaged from similar experiments shown in D (n = 5-20). Error bars represent the mean ± SE. The histograms in C and F show the mean and min to max values. NS: No significant difference; hiPSCs: Human induced pluripotent stem cells.

and an unknown Na^+-independent acid extruder(s), in hiPSCs. As expected, the resting pH_i in hiPSCs was found to be 7.5 and 7.68 in the HEPES and 5% CO_2/HCO_3^- conditions, respectively, and was relatively higher than that of normal differentiated adult cells (resting pH_i = 6.9-7.2), such as cardiovascular cells and tissues demonstrated in our previous studies[7,13,52,53]. In cancer, the reversal of the intracellular/extracellular pH (pH_i/pH_e) gradient (alkaline pH_i and acidic pH_e) is a common feature and further promotes carcinogenesis. The reason for the gradient reversal is that cancer cells overexpress and upregulate the set-point of acid extruders[24-26]. Therefore, it is likely that hPSCs may upregulate the acid extrusion mechanism to adapt to cancer-like cellular properties. Some studies showed that, in addition to hiPSC growth being inhibited by an acidic culture environment, the alkalization of culture medium significantly decreases the cell growth rate and

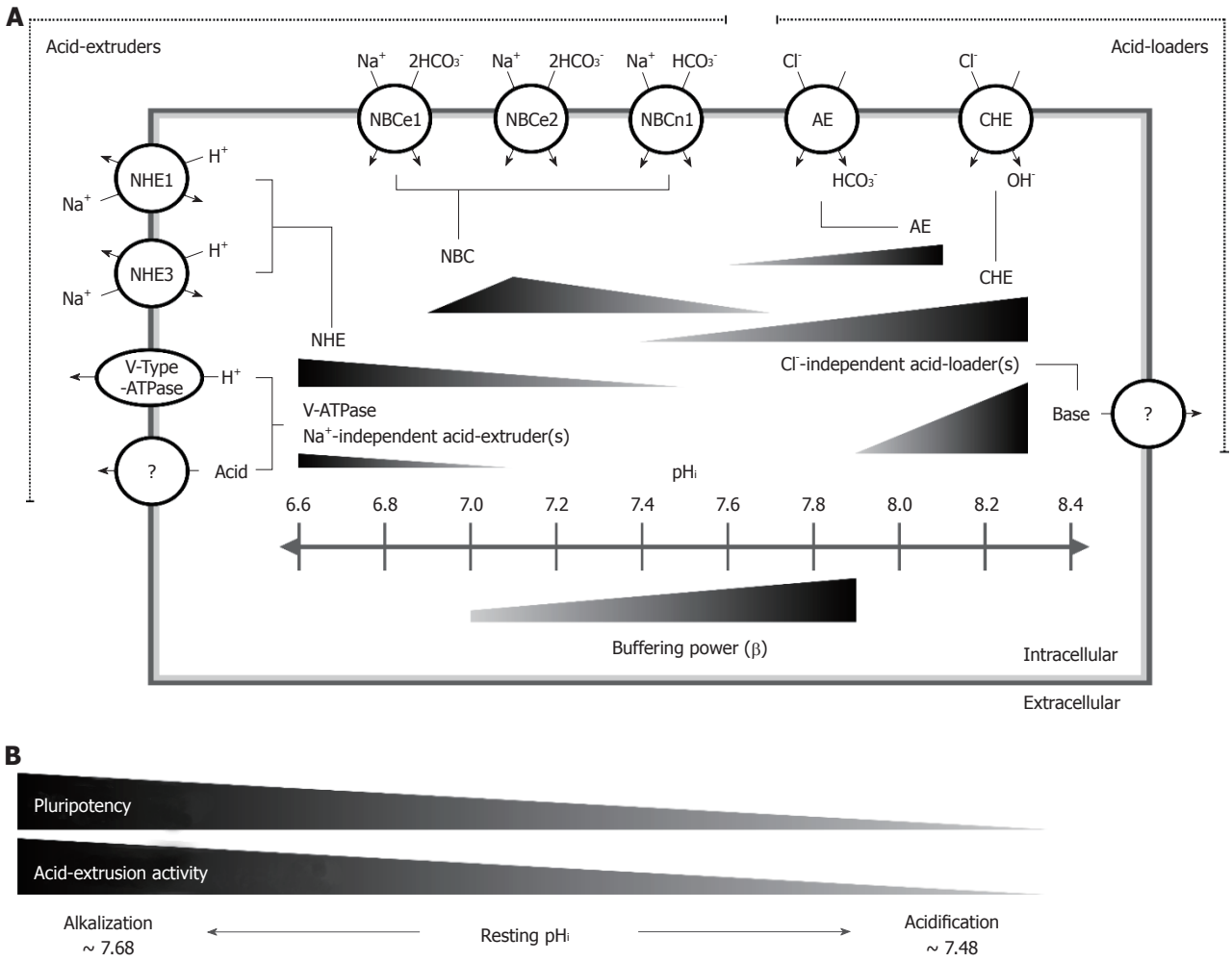

Figure 7 Kinetic model of the pHi regulatory mechanism in human induced pluripotent stem cells. A: A kinetic model illustrating the pH_i regulatory mechanism in HPS0077 cell, including acid extrusion, acid loading and passive buffering power. For the first time, we demonstrated that the active membrane pH_i regulators NHE1, NHE3, V-ATPase, NBCe1, NBCe2, NBCn1, AE and CHE functionally coexisted in hiPSCs, and in addition, unknown Na^+-independent acid extruder(s) and Cl^--independent acid loader(s) were also observed. The length of the triangle indicates the pH_i range of pH_i regulator activation, and the height indicates the magnitude of the pH_i regulatory activity. For example, the NHE, NBC, AE and CHE were activated at $pH_i \leqslant 7.5$, between 6.9 and 7.68, $\geqslant 7.4$ and between 7.6 and 8.1, respectively. The non-NHE acid extruders [V-ATPase, unknown Na^+-independent acid extruder(s)] and unknown Cl^--independent acid loader(s) were activated during extreme intracellular acidification, *i.e.*, $pH_i < 7.1$, and alkalization, *i.e.*, $pH_i > 7.9$, respectively. Moreover, the intracellular passive buffering capacity (β) increased as the pH_i shifted to the alkalization direction; B: In the process of the loss of pluripotency, the activity of the acid extrusion mechanism gradually decreased, including the participation of at least the NHE, the NBC and V-ATPase, and resulted in the resting pH_i shifting from 7.68 to 7.48. hiPSCs: Human induced pluripotent stem cells; NHE: The Na^+/H^+ exchanger; NBC: The Na^+/HCO_3^- cotransporter; V-ATPase: Vacuolar-ATPase; AE: Anion exchanger; CHE: Cl^--OH^- exchanger.

expression of pluripotency markers at a minimum pH_e = 7.8[37,48,49]. The proliferative ability and pluripotency in hPSCs are critical for development[54]. Therefore, the expression of additional unknown Na^--independent and Cl^--independent acid-regulating extruder(s) in hiPSCs implicates the function of resisting the potential impact of intracellular proton changes in hPSCs. However, further study on characterizing the mechanisms should be conducted in the future.

Decreases in acid extrusion activity during the loss of pluripotency in hiPSCs

A previous study showed that during the early spontaneous differentiation of mESCs, the resting pH_i significantly increased at 48 and 72 h and returned to baseline at 96 h, and this increase was dependent on the loss of NHE1 function[6]. However, in this study, the decrease in resting pH_i and the downregulation of the acid extrusion mechanism were demonstrated during the early loss of pluripotency in hiPSCs either in HEPES-buffered conditions or in 5% CO_2/HCO_3^--buffered conditions. These contradictory results may be due to the different pluripotent states between mESCs and hiPSCs, *i.e.*, naïve and primed pluripotency, respectively[54,55]. As expected, the cells in the preprimed (naïve) and primed states significantly increased the pH_i at 48 and 72 hours during early differentiation in mESCs. This result implies that increasing resting pH_i occurred during the naïve to primed pluripotency states[6]. Subsequently, the resting pH_i returned to baseline at 72-96 h, which may indicate that the primed state is further differentiated. Furthermore, to adapt to the intracellular acidification caused by increased glycolysis, *i.e.*, the Warburg effect, the acid extrusion

mechanism is upregulated and further alkalizes the resting pH_i in cancer cells[24,26]. During the processes of PSC development, metabolism has been found to rely on different metabolic pathways, *i.e.*, oxidative phosphorylation (OXPHOS), glycolysis and OXPHOS in naive, primed and early differentiation states, respectively[51,56,57]. This switch between OXPHOS and glycolysis supports the dynamic changes in the resting pH_i observed during the loss of pluripotency in mESCs and the decrease in the resting pH_i and acid extrusion in hiPSCs demonstrated in this study.

The possible underlying mechanism for the observed decrease in the acid extrusion mechanism during the process of the loss of pluripotency in hiPSCs may be due to the crosstalk between the PI3K/AKT and MEK/ERK signaling pathways, which plays a curial role in pluripotency[58]. To maintain pluripotency in hPSCs, FGF2 has been added to the culture medium to activate PI3K/AKT signaling[58,59]. The activation of PI3K/AKT signaling further promotes the relative gene expression of pluripotency markers and inhibits differentiation by suppressing MEK/ERK signaling[58]. Therefore, the removal of FGF2 decreases the ratio of AKT activity to ERK and further causes cell differentiation[58,60]. ERK is a well-known activator of NHE1[61,62], but we did not find that removal of FGF2 (in E6 medium) resulted in an increase of the NHE1-dependent acid extrusion rate in this study. Although AKT has been shown to inhibit NHE1 activity in cardiovascular cells[63], AKT is stimulated by insulin and growth factors and further activates NHE1 in cancer cells and fibroblasts[64,65]. Therefore, this study implicates that the removal of FGF2 causes the loss of AKT activity and thus decreases the acid extrusion rate in hiPSCs.

In conclusion, for the first time, we established a functional pH_i regulatory model in hiPSCs, as shown in Figure 7. In this model, we demonstrated that the steady-state pH_i value is approximately 7.50-7.68 in hiPSCs. Additionally, we showed that at least four types of acid extruders [NHE, NBC, V-ATPase and Na^+-independent acid extruder(s)] and three types of acid loaders [CHE, AE and Cl^--independent acid loader(s)] coexist and are responsible for the pH_i regulatory mechanism, and each is activated in different pH_i ranges in hiPSCs. Moreover, the activity of the acid extrusion mechanism decreased by changing both the expression and activity of acid extruders during the process of the loss of pluripotency in hiPSCs.

ARTICLE HIGHLIGHTS

Research background

Homeostasis of intracellular pH_i (pH_i) affects many cellular functions, such as cell proliferation and differentiation. However, the knowledge of pH_i regulation mechanism in human pluripotent stem cells still unknown.

Research motivation

The changes of acid-base kinetic were observed during the loss of pluripotency in mouse embryonic stem cells. Moreover, the balance of intracellular and extracellular pH significantly affected the reprogramming efficiency and culture quality of human induced pluripotent stem cells (hiPSCs).

Research objectives

We aimed to establish the pH_i regulation mechanism model and investigate the relationship of pH_i regulation and pluripotency in hiPSCs.

Research methods

In the pluripotent state and during the loss of pluripotency in hiPSCs, we observed the activity of pH_i regulation mechanism by acutely induced intracellular acidification and alkalization in the physiological buffered solution.

Research results

In hiPSCs, the Na^+-H^+ exchanger (NHE), the Na^+-HCO_3^- cotransporter (NBC) and vacuolar-ATPase (V-ATPase) were the main active acid extruders that were activated against intracellular acidification. In contrast, the acid-equivalent loaders, such as the Cl^--HCO_3^- anion exchanger (AE) and the Cl^--OH^- exchanger (CHE), were activated to prevent intracellular alkalization. In addition to the classic pH_i regulators NHE, NBC, V-ATPase, AE and CHE, we also demonstrated the functional existence of unknown acid-extruder(s) and –loader(s) in hiPSCs. Moreover, the pH_i and acid-extruding mechanism were decreased during the loss of pluripotency in hiPSCs.

Research conclusions

For the first time, we established a model of the pH_i regulation mechanism in hiPSCs. The higher resting pH_i and acid-extruding mechanism might be the specific feature to adaptive the cancer-like cellular function and pluripotency in hiPSCs.

Research perspectives

In summary, we characterized the pH_i regulation mechanism and its functional/expressional roles in maintenance of pluripotency of hiPSCs. We proposed that targeting either pH_i regulators or pH environments of culture medium could be an effective way to modify the pluripotency state of hiPSCs, which may contribute the differentiation efficiency or culture quality.

REFERENCES

1 **Gao W**, Zhang H, Chang G, Xie Z, Wang H, Ma L, Han Z, Li Q, Pang T. Decreased intracellular pH induced by cariporide differentially contributes to human umbilical cord-derived mesenchymal stem cells differentiation. *Cell Physiol Biochem* 2014; **33**: 185-194 [PMID: 24481225 DOI: 10.1159/000356661]

2 **Li X**, Karki P, Lei L, Wang H, Fliegel L. Na+/H+ exchanger isoform 1 facilitates cardiomyocyte embryonic stem cell differentiation. *Am J Physiol Heart Circ Physiol* 2009; **296**: H159-H170 [PMID: 19011045 DOI: 10.1152/ajpheart.00375.2008]

3 **McBrian MA**, Behbahan IS, Ferrari R, Su T, Huang TW, Li K, Hong CS, Christofk HR, Vogelauer M, Seligson DB, Kurdistani SK. Histone acetylation regulates intracellular pH. *Mol Cell* 2013; **49**: 310-321 [PMID: 23201122 DOI: 10.1016/j.molcel.2012.10.025]

4 **Park HJ**, Lyons JC, Ohtsubo T, Song CW. Acidic environment causes apoptosis by increasing caspase activity. *Br J Cancer* 1999; **80**: 1892-1897 [PMID: 10471036 DOI: 10.1038/sj.bjc.6690617]

5 **Pouysségur J**, Franchi A, L'Allemain G, Paris S. Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts. *FEBS Lett* 1985; **190**: 115-119 [PMID: 4043390 DOI: 10.1016/0014-5793(85)80439-7]

6 **Ulmschneider B**, Grillo-Hill BK, Benitez M, Azimova DR, Barber DL, Nystul TG. Increased intracellular pH is necessary for adult epithelial and embryonic stem cell differentiation. *J Cell Biol* 2016; **215**: 345-355 [PMID: 27821494 DOI: 10.1083/jcb.201606042]

7 **Loh SH**, Lee CY, Tsai YT, Shih SJ, Chen LW, Cheng TH, Chang CY, Tsai CS. Intracellular Acid-extruding regulators and the effect of lipopolysaccharide in cultured human renal artery smooth muscle cells. *PLoS One* 2014; **9**: e90273 [PMID: 24587308 DOI: 10.1371/journal.pone.0090273]

8 **Loh SH**, Chen WH, Chiang CH, Tsai CS, Lee GC, Jin JS, Cheng TH, Chen JJ. Intracellular pH regulatory mechanism in human atrial myocardium: functional evidence for Na(+)/H(+) exchanger and Na(+)/HCO(3)(-) symporter. *J Biomed Sci* 2002; **9**: 198-205 [PMID: 12065894 DOI: 10.1159/000059420]

9 **Loh SH**, Jin JS, Tsai CS, Chao CM, Chiung CS, Chen WH, Lin CI, Chuang CC, Wei J. Functional evidence for intracellular acid extruders in human ventricular myocardium. *Jpn J Physiol* 2002; **52**: 277-284 [PMID: 12230804 DOI: 10.2170/jjphysiol.52.277]

10 **Lagadic-Gossmann D**, Buckler KJ, Vaughan-Jones RD. Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *J Physiol* 1992; **458**: 361-384 [PMID: 1302269 DOI: 10.1113/jphysiol.1992.sp019422]

11 **Amos BJ**, Pocock G, Richards CD. On the role of bicarbonate as a hydrogen ion buffer in rat CNS neurones. *Exp Physiol* 1996; **81**: 623-632 [PMID: 8853270 DOI: 10.1113/expphysiol.1996.sp003963]

12 **Chen GS**, Lee SP, Huang SF, Chao SC, Chang CY, Wu GJ, Li CH, Loh SH. Functional and molecular characterization of transmembrane intracellular pH regulators in human dental pulp stem cells. *Arch Oral Biol* 2018; **90**: 19-26 [PMID: 29524788 DOI: 10.1016/j.archoralbio.2018.02.018]

13 **Lee CY**, Tsai YT, Chang CY, Chang YY, Cheng TH, Tsai CS, Loh SH. Functional characterization of intracellular pH regulators responsible for acid extrusion in human radial artery smooth muscle cells. *Chin J Physiol* 2014; **57**: 238-248 [PMID: 25241983 DOI: 10.4077/CJP.2014.BAD269]

14 **Vaughan-Jones RD**, Spitzer KW, Swietach P. Intracellular pH regulation in heart. *J Mol Cell Cardiol* 2009; **46**: 318-331 [PMID: 19041875 DOI: 10.1016/j.yjmcc.2008.10.024]

15 **Casey JR**, Grinstein S, Orlowski J. Sensors and regulators of intracellular pH. *Nat Rev Mol Cell Biol* 2010; **11**: 50-61 [PMID: 19997129 DOI: 10.1038/nrm2820]

16 **de Hemptinne A**, Marrannes R, Vanheel B. Influence of organic acids on intracellular pH. *Am J Physiol* 1983; **245**: C178-C183 [PMID: 6614155 DOI: 10.1152/ajpcell.1983.245.3.C178]

17 **Trosper TL**, Philipson KD. Functional characteristics of the cardiac sarcolemmal monocarboxylate transporter. *J Membr Biol* 1989; **112**: 15-23 [PMID: 2593136 DOI: 10.1007/BF01871160]

18 **Deuticke B**, Beyer E, Forst B. Discrimination of three parallel pathways of lactate transport in the human erythrocyte membrane by inhibitors and kinetic properties. *Biochim Biophys Acta* 1982; **684**: 96-110 [PMID: 7055558 DOI: 10.1016/0005-2736(82)90053-0]

19 **Wang X**, Poole RC, Halestrap AP, Levi AJ. Characterization of the inhibition by stilbene disulphonates and phloretin of lactate and pyruvate transport into rat and guinea-pig cardiac myocytes suggests the presence of two kinetically distinct carriers in heart cells. *Biochem J* 1993; **290**(Pt 1): 249-258 [PMID: 8439293 DOI: 10.1042/bj2900249]

20 **Reshetnyak YK**. Imaging Tumor Acidity: pH-Low Insertion Peptide Probe for Optoacoustic Tomography. *Clin Cancer Res* 2015; **21**: 4502-4504 [PMID: 26224874 DOI: 10.1158/1078-0432.CCR-15-1502]

21 **Damaghi M**, Wojtkowiak JW, Gillies RJ. pH sensing and regulation in cancer. *Front Physiol* 2013; **4**: 370 [PMID: 24381558 DOI: 10.3389/fphys.2013.00370]

22 **Swietach P**, Vaughan-Jones RD, Harris AL, Hulikova A. The chemistry, physiology and pathology of pH in cancer. *Philos Trans R Soc Lond B Biol Sci* 2014; **369**: 20130099 [PMID: 24493747 DOI: 10.1098/rstb.2013.0099]

23 **Lee SP**, Chao SC, Huang SF, Chen YL, Tsai YT, Loh SH. Expressional and Functional Characterization of Intracellular pH Regulators and Effects of Ethanol in Human Oral Epidermoid Carcinoma Cells. *Cell Physiol Biochem* 2018; **47**: 2056-2068 [PMID: 29975935 DOI: 10.1159/000491473]

24 **Webb BA**, Chimenti M, Jacobson MP, Barber DL. Dysregulated pH: a perfect storm for cancer progression. *Nat Rev Cancer* 2011; **11**: 671-677 [PMID: 21833026 DOI: 10.1038/nrc3110]

25 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

26 **Parks SK**, Chiche J, Pouysségur J. Disrupting proton dynamics and energy metabolism for cancer therapy. *Nat Rev Cancer* 2013; **13**: 611-623 [PMID: 23969692 DOI: 10.1038/nrc3579]

27 **Moses C**, Garcia-Bloj B, Harvey AR, Blancafort P. Hallmarks of cancer: The CRISPR generation. *Eur J Cancer* 2018; **93**: 10-18 [PMID: 29433054 DOI: 10.1016/j.ejca.2018.01.002]

28 **Marchiq I**, Pouysségur J. Hypoxia, cancer metabolism and the therapeutic benefit of targeting lactate/H(+) symporters. *J Mol Med* (Berl) 2016; **94**: 155-171 [PMID: 26099350 DOI: 10.1007/s00109-015-1307-x]

29 **Lee ZW**, Teo XY, Song ZJ, Nin DS, Novera W, Choo BA, Dymock BW, Moore PK, Huang RY, Deng LW. Intracellular Hyper-Acidification Potentiated by Hydrogen Sulfide Mediates Invasive and Therapy Resistant Cancer Cell Death. *Front Pharmacol* 2017; **8**: 763 [PMID: 29163155 DOI: 10.3389/fphar.2017.00763]

30 **Amith SR**, Fliegel L. Regulation of the Na+/H+ Exchanger (NHE1) in Breast Cancer Metastasis. *Cancer Res* 2013; **73**: 1259-1264 [PMID: 23393197 DOI: 10.1158/0008-5472.CAN-12-4031]

31 **Lucien F**, Brochu-Gaudreau K, Arsenault D, Harper K, Dubois CM. Hypoxia-induced invadopodia formation involves activation of NHE-1 by the p90 ribosomal S6 kinase (p90RSK). *PLoS One* 2011; **6**: e28851 [PMID: 22216126 DOI: 10.1371/journal.pone.0028851]

32 **Takahashi K**, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408 DOI: 10.1016/j.cell.2007.11.019]

33 **Gu W**, Gaeta X, Sahakyan A, Chan AB, Hong CS, Kim R, Braas D, Plath K, Lowry WE, Christofk HR. Glycolytic Metabolism Plays a Functional Role in Regulating Human Pluripotent Stem Cell State. *Cell Stem Cell* 2016; **19**: 476-490 [PMID: 27618217 DOI: 10.1016/j.stem.2016.08.008]

34 **Yoshida Y**, Takahashi K, Okita K, Ichisaka T, Yamanaka S. Hypoxia enhances the generation of induced pluripotent stem cells. *Cell Stem Cell* 2009; **5**: 237-241 [PMID: 19716359 DOI: 10.1016/j.stem.2009.08.001]

35 **Zhang J**, Nuebel E, Daley GQ, Koehler CM, Teitell MA. Metabolic regulation in pluripotent stem cells during reprogramming and self-renewal. *Cell Stem Cell* 2012; **11**: 589-595 [PMID: 23122286 DOI: 10.1016/j.stem.2012.10.005]

36 **Wang H**, Singh D, Fliegel L. The Na+/H+ antiporter potentiates growth and retinoic acid-induced differentiation of P19 embryonal carcinoma cells. *J Biol Chem* 1997; **272**: 26545-26549 [PMID: 9334233 DOI: 10.1074/jbc.272.42.26545]

37 **Chaudhry MA**, Bowen BD, Piret JM. Culture ph and osmolality influence proliferation and embryoid body yields of murine embryonic stem cells. *Biochem Eng J* 2009; **45**: 126-135 [DOI: 10.1016/j.bej.2009.03.005]

38 **Zaniboni M**, Swietach P, Rossini A, Yamamoto T, Spitzer KW, Vaughan-Jones RD. Intracellular proton mobility and buffering power in cardiac ventricular myocytes from rat, rabbit, and guinea pig. *Am J Physiol Heart Circ Physiol* 2003; **285**: H1236-H1246 [PMID: 12750065 DOI: 10.1152/ajpheart.00277.2003]

39 **Leem CH**, Lagadic-Gossmann D, Vaughan-Jones RD. Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. *J Physiol* 1999; **517** (Pt 1): 159-180 [PMID: 10226157 DOI: 10.1111/j.1469-7793.1999.0159z.x]

40 **Boedtkjer E**, Aalkjaer C. Acid-base transporters modulate cell migration, growth and proliferation: Implications for structure development and remodeling of resistance arteries? *Trends Cardiovasc Med* 2013; **23**: 59-65 [PMID: 23266155 DOI: 10.1016/j.tcm.2012.09.001]

41 **Romero MF**, Chen AP, Parker MD, Boron WF. The SLC4 family of bicarbonate (HCO_3^-) transporters. *Mol Aspects Med* 2013; **34**: 159-182 [PMID: 23506864 DOI: 10.1016/j.mam.2012.10.008]

42 **Boedtkjer E**, Aalkjaer C. Intracellular pH in the resistance vasculature: regulation and functional implications. *J Vasc Res* 2012; **49**: 479-496 [PMID: 22907294 DOI: 10.1159/000341235]

43 **da Costa-Pessoa JM**, Damasceno RS, Machado UF, Beloto-

Silva O, Oliveira-Souza M. High glucose concentration stimulates NHE-1 activity in distal nephron cells: the role of the Mek/Erk1/2/p90RSK and p38MAPK signaling pathways. *Cell Physiol Biochem* 2014; **33**: 333-343 [PMID: 24557342 DOI: 10.1159/000356673]

44 **Li X**, Wang T, Zhao Z, Weinman SA. The ClC-3 chloride channel promotes acidification of lysosomes in CHO-K1 and Huh-7 cells. *Am J Physiol Cell Physiol* 2002; **282**: C1483-C1491 [PMID: 11997263 DOI: 10.1152/ajpcell.00504.2001]

45 **Takahashi Y**, Fujii T, Fujita K, Shimizu T, Higuchi T, Tabuchi Y, Sakamoto H, Naito I, Manabe K, Uchida S, Sasaki S, Ikari A, Tsukada K, Sakai H. Functional coupling of chloride-proton exchanger ClC-5 to gastric H+,K+-ATPase. *Biol Open* 2014; **3**: 12-21 [PMID: 24429108 DOI: 10.1242/bio.20136205]

46 **Dascalu A**, Nevo Z, Korenstein R. The control of intracellular pH in cultured avian chondrocytes. *J Physiol* 1993; **461**: 583-599 [PMID: 8394427 DOI: 10.1113/jphysiol.1993.sp019530]

47 **Theparambil SM**, Naoshin Z, Thyssen A, Deitmer JW. Reversed electrogenic sodium bicarbonate cotransporter 1 is the major acid loader during recovery from cytosolic alkalosis in mouse cortical astrocytes. *J Physiol* 2015; **593**: 3533-3547 [PMID: 25990710 DOI: 10.1113/JP270086]

48 **Kim N**, Minami N, Yamada M, Imai H. Immobilized pH in culture reveals an optimal condition for somatic cell reprogramming and differentiation of pluripotent stem cells. *Reprod Med Biol* 2016; **16**: 58-66 [PMID: 29259452 DOI: 10.1002/rmb2.12011]

49 **Gupta P**, Hourigan K, Jadhav S, Bellare J, Verma P. Effect of lactate and ph on mouse pluripotent stem cells: Importance of media analysis. *Biochem Eng J* 2017; **118**: 25-33 [DOI: 10.1016/j.bej.2016.11.005]

50 **Liu A**, Yu X, Liu S. Pluripotency transcription factors and cancer stem cells: small genes make a big difference. *Chin J Cancer* 2013; **32**: 483-487 [PMID: 23419197 DOI: 10.5732/cjc.012.10282]

51 **Varum S**, Rodrigues AS, Moura MB, Momcilovic O, Easley CA 4th, Ramalho-Santos J, Van Houten B, Schatten G. Energy metabolism in human pluripotent stem cells and their differentiated counterparts. *PLoS One* 2011; **6**: e20914 [PMID: 21698063 DOI: 10.1371/journal.pone.0020914]

52 **Tsai CS**, Loh SH, Jin JS, Hong GJ, Lin HT, Chiung CS, Chang CY. Effects of alcohol on intracellular pH regulators and electromechanical parameters in human myocardium. *Alcohol Clin Exp Res* 2005; **29**: 1787-1795 [PMID: 16269908 DOI: 10.1097/01.alc.0000183512.31705.74]

53 **Tsai YT**, Lee CY, Hsu CC, Chang CY, Hsueh MK, Huang EY, Tsai CS, Loh SH. Effects of urotensin II on intracellular pH regulation in cultured human internal mammary artery smooth muscle cells. *Peptides* 2014; **56**: 173-182 [PMID: 24768794 DOI: 10.1016/j.peptides.2014.04.011]

54 **Weinberger L**, Ayyash M, Novershtern N, Hanna JH. Dynamic stem cell states: naive to primed pluripotency in rodents and humans. *Nat Rev Mol Cell Biol* 2016; **17**: 155-169 [PMID: 26860365 DOI: 10.1038/nrm.2015.28]

55 **Altshuler A**, Verbuk M, Bhattacharya S, Abramovich I, Haklai R, Hanna JH, Kloog Y, Gottlieb E, Shalom-Feuerstein R. RAS Regulates the Transition from Naive to Primed Pluripotent Stem Cells. *Stem Cell Reports* 2018; **10**: 1088-1101 [PMID: 29456180 DOI: 10.1016/j.stemcr.2018.01.004]

56 **Folmes CD**, Dzeja PP, Nelson TJ, Terzic A. Metabolic plasticity in stem cell homeostasis and differentiation. *Cell Stem Cell* 2012; **11**: 596-606 [PMID: 23122287 DOI: 10.1016/j.stem.2012.10.002]

57 **Ryall JG**, Cliff T, Dalton S, Sartorelli V. Metabolic Reprogramming of Stem Cell Epigenetics. *Cell Stem Cell* 2015; **17**: 651-662 [PMID: 26637942 DOI: 10.1016/j.stem.2015.11.012]

58 **Singh AM**, Reynolds D, Cliff T, Ohtsuka S, Mattheyses AL, Sun Y, Menendez L, Kulik M, Dalton S. Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation. *Cell Stem Cell* 2012; **10**: 312-326 [PMID: 22385658 DOI: 10.1016/j.stem.2012.01.014]

59 **Chen G**, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA. Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 2011; **8**: 424-429 [PMID: 21478862 DOI: 10.1038/nmeth.1593]

60 **Cho YM**, Kwon S, Pak YK, Seol HW, Choi YM, Park DJ, Park KS, Lee HK. Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochem Biophys Res Commun* 2006; **348**: 1472-1478 [PMID: 16920071 DOI: 10.1016/j.bbrc.2006.08.020]

61 **Malo ME**, Li L, Fliegel L. Mitogen-activated protein kinase-dependent activation of the Na+/H+ exchanger is mediated through phosphorylation of amino acids Ser770 and Ser771. *J Biol Chem* 2007; **282**: 6292-6299 [PMID: 17209041 DOI: 10.1074/jbc.M611073200]

62 **Javadov S**, Baetz D, Rajapurohitam V, Zeidan A, Kirshenbaum LA, Karmazyn M. Antihypertrophic effect of Na+/H+ exchanger isoform 1 inhibition is mediated by reduced mitogen-activated protein kinase activation secondary to improved mitochondrial integrity and decreased generation of mitochondrial-derived reactive oxygen species. *J Pharmacol Exp Ther* 2006; **317**: 1036-1043 [PMID: 16513848 DOI: 10.1124/jpet.105.100107]

63 **Snabaitis AK**, Cuello F, Avkiran M. Protein kinase B/Akt phosphorylates and inhibits the cardiac Na+/H+ exchanger NHE1. *Circ Res* 2008; **103**: 881-890 [PMID: 18757828 DOI: 10.1161/CIRCRESAHA.108.175877]

64 **Meima ME**, Webb BA, Witkowska HE, Barber DL. The sodium-hydrogen exchanger NHE1 is an Akt substrate necessary for actin filament reorganization by growth factors. *J Biol Chem* 2009; **284**: 26666-26675 [PMID: 19622752 DOI: 10.1074/jbc.M109.019448]

65 **Clement DL**, Mally S, Stock C, Lethan M, Satir P, Schwab A, Pedersen SF, Christensen ST. PDGFRα signaling in the primary cilium regulates NHE1-dependent fibroblast migration via coordinated differential activity of MEK1/2-ERK1/2-p90RSK and AKT signaling pathways. *J Cell Sci* 2013; **126**: 953-965 [PMID: 23264740 DOI: 10.1242/jcs.116426]

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ORIGINAL ARTICLE

Basic Study

Platelet-rich plasma enhances adipose-derived stem cell-mediated angiogenesis in a mouse ischemic hindlimb model

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Abstract

AIM

To evaluate the angiogenic effect of platelet-rich plasma (PRP)-preconditioned adipose-derived stem cells (ADSCs) both in vitro and in a mouse ischemic hindlimb model.

METHODS

ADSCs were divided based on culture medium: 2.5% PRP, 5% PRP, 7.5% PRP, and 10% PRP. Cell proliferation rate was analyzed using the MTS assay. The gene expression of CD31, vascular endothelial growth factor, hypoxia-inducible factors, and endothelial cell nitric oxide synthase was analyzed using reverse transcription polymerase chain reaction. Cell markers and structural changes were assessed through immunofluorescence staining and the tube formation assay. Subsequently, we studied the *in vivo* angiogenic capabilities of ADSCs

by a mouse ischemic hindlimb model.

RESULTS

The proliferation rate of ADSCs was higher in the 2.5%, 5%, and 7.5% PRP groups. The expression of hypoxia-inducible factor, CD31, vascular endothelial growth factor, and endothelial cell nitric oxide synthase in the 5% and 7.5% PRP groups increased. The 5%, 7.5%, and 10% PRP groups showed higher abilities to promote both CD31 and vascular endothelial growth factor production and tubular structure formation in ADSCs. According to laser Doppler perfusion scan, the perfusion ratios of ischemic limb to normal limb were significantly higher in 5% PRP, 7.5% PRP, and human umbilical vein endothelial cells groups compared with the negative control and fetal bovine serum (FBS) groups (0.88 ± 0.08, 0.85 ± 0.07 and 0.81 ± 0.06 for 5%, 7.5% PRP and human umbilical vein endothelial cells compared with 0.42 ± 0.17 and 0.54 ± 0.14 for the negative control and FBS, $P < 0.01$).

CONCLUSION

PRP-preconditioned ADSCs presented endothelial cell characteristics *in vitro* and significantly improved neovascularization in ischemic hindlimbs. The optimal angiogenic effect occurred in 5% PRP- and 7.5% PRP-preconditioned ADSCs.

Key words: Platelet-rich plasma; Adipose-derived stem cells; Mesenchymal stem cell; Angiogenesis; Endothelial differentiation; Mouse ischemic hindlimb model

Core tip: We reported the *in vitro* angiogenic effect of platelet-rich plasma (PRP) treated adipose-derived stem cells (ADSCs) and the neovascularization ability of these cells in animal models. This is significant because we demonstrated that ADSCs presented endothelial cell characteristics after PRP treatment. We were the first to observe that treatment with PRP-preconditioned ADSCs significantly enhanced circulation in mouse ischemic hindlimbs models. Our result further showed that 5% and 7.5% PRP exerted the optimal effect on promoting angiogenesis of ADSCs and improving perfusion. We developed a stem cell-based, safe, and efficient way to promote peripheral circulation in animal model.

Chen CF, Liao HT. Platelet-rich plasma enhances adipose-derived stem cell-mediated angiogenesis in a mouse ischemic hindlimb model. *World J Stem Cells* 2018; 10(12): 212-227
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INTRODUCTION

Peripheral artery disease (PAD) is caused by peripheral artery obstruction, which may lead to ischemic changes in the extremities. Due to insufficient blood flow in the musculature, patients may present with symptoms such as pain, claudication, or even tissue necrosis. Smoking, diabetes mellitus, hypercholesterolemia, hypertension, and renal insufficiency have all been reported to have high correlations with PAD. The pathological features of PAD include lumen obstruction caused by atherosclerotic plaques and destruction of vessel walls. Research has shown that elderly individuals and patients with diabetes mellitus are prone to these vasculature problems[1]. The current trend of the increasing populations of elder people and patients with diabetes mellitus is accompanied with the increased prevalence of PAD.

Current therapies for PAD are primarily aimed at relieving the discomfort and slowing the progress of the disease. In advanced PAD, revascularization surgery is indicated for large to medium-sized peripheral arteries with obstructions. However, ideal treatment for small arteries with obstructions has not been established. Therefore, treatments for obstructive lesions in small vessels are urgently required. Therapeutic angiogenesis provides a novel strategy for managing PAD; this strategy induces new vessel development in ischemic tissue, which can improve local perfusion.

Angiogenesis comprises many steps. Establishing stable and functional vascular networks is complicated. During ischemia, the damaged tissue releases growth factors to attract endothelial progenitor cells (EPCs). These cells proliferate, migrate, and form tubular structures, and finally achieve angiogenesis[2]. Microscopically, many growth factors are involved in angiogenesis. The angiogenic switch is initiated by hypoxia. Hypoxia-inducible factors (HIFs) are transcription factors that respond to hypoxia, and they play crucial roles in maintaining hemostasis during low oxygen conditions. During hypoxia, HIFs bind to targets, including the vascular endothelial growth factor (VEGF) gene, subsequently increasing the expression of downstream factors including transforming growth factor alpha and platelet-derived growth factor. Angiogenesis promotes endothelial cell proliferation and migration[3]. Endothelial cell nitric oxide synthase (eNOS), which is secreted by endothelial cells, exerts synergistic effects on neovascularization by increasing vessel wall permeability and promoting endothelial cell migration. CD31 also plays a crucial role in angiogenesis. It is a cell–cell adhesion molecule located on the endothelial cell membrane. Without CD31 stimulation, endothelial cells cannot form tubular structures. Through the synergistic effects of the mentioned factors, endothelial cells form new vessels at the ischemic site and subsequently establish a stable and functional perfusion system. Therefore, in research, these factors are commonly used as angiogenic markers for evaluating endothelial cell differentiation.

A previous study proved that mesenchymal stem cells (MSCs) can be used to form EPCs and promote

angiogenesis, and MSCs are thus useful for vascular tissue engineering[4]. However, limited stem cell numbers circulate in the blood, which poses a major problem to the clinical application of these cells[5]. Although human adult stem cells can be obtained from many accessible sources, such as the bone marrow, teeth, and skeletal muscle, isolating human adult stem cells from the aforementioned tissue is difficult due to limited cell numbers and high donor site morbidities[6]. Recently, researchers have focused their attention on fat tissue-derived MSCs, adipose-derived stem cells (ADSCs). ADSCs were discovered in 2002 by researchers at University of California at Los Angeles; they have become a popular therapeutic strategy in current stem cell research. Abundant ADSCs can be retrieved from autologous fat tissue, and no controversy and ethical concerns are associated with these cells. In contrast to bone marrow-, teeth-, or skeletal muscle-derived stem cells, ADSCs are much easier to obtain. ADSCs can be collected through liposuction, which is a commonly performed cosmetic procedure[7,8]. Furthermore, after appropriate induction, ADSCs exhibit endothelial cell properties. All these characteristics render ADSCs more suitable for clinical use than other types of stem cells.

Fetal bovine serum (FBS) is widely used in research settings for the *in vitro* culture of ADSCs. However, culturing cells for therapeutic purposes in patients is associated with zoonotic disease transmission and xeno-immunization concerns. Alternative culture medium for ADSCs should be human-derived and should meet the criteria proposed by the International Society of Cellular Therapy (ISCT) and International Fat Applied Technology Society (IFATS)[9,10]. Because human serum is a natural reservoir of growth factors, and it has already been proved to be effective endothelial lineage differentiation media, several researchers have applied human serum products to culture ADSCs and induce angiogenesis *in vitro*. Recently, autologous conditioned serum, namely platelet-rich plasma (PRP), has shown great potential. PRP is an autologous reservoir of growth factors and cytokines. In summary, PRP has great potential to replace animal serum as culture medium.

To date, limited data are available on the effects of PRP on ADSCs. Our study evaluated the angiogenic potential of PRP-preconditioned ADSCs. In addition, ADSCs' biological characteristics and their capability to induce angiogenesis both *in vitro* and *in vivo* were evaluated.

MATERIALS AND METHODS

All experimental procedures were performed as per hospital regulations and medical ethics standards.

Preparation of PRP

Concentrated human PRP (UltraGRO™) was purchased from AventaCell BioMedical Co., Ltd. (http://www.atcbiomed.com, United States). Blood donors were tested by the supplying company as per the United States regulations for the preparation of blood components. These donors were negative for mycoplasma, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, human T-lymphotropic virus type 1 [determined by polymerase chain reaction (PCR) and serologic testing], anti-*Trypanosoma cruzi* antibody, and syphilis (determined by serologic testing).

Preparation of human ADSCs from human fat

Human raw lipoaspirates were derived from our patients undergoing selective suction-assisted lipectomy and were isolated following the procedure described by Zuk *et al*[11] with modifications. After harvesting, the lipoaspirates were washed extensively to remove blood cells, and the lipoaspirates, which were obtained under local anesthetics, were digested with 0.075% collagenase in a 37 ℃ water bath for 30 min. Subsequently, the cell pellet was collected through centrifugation and was incubated overnight in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS at 37 ℃ in 5% CO_2.

Flow cytometry analysis

Flow cytometry analysis was performed to characterize the phenotypes of ADSCs. Cells were cultured in medium containing different concentrations of PRP (2.5%, 5%, 7.5%, and 10% PRP) or 10% FBS (control group). At least 1×10^6 cells per well were incubated with fluorescence-labeled monoclonal antibodies against human CD34 (BD Biosciences, San Jose, CA, United States), CD45 (BD Biosciences), CD73 (R&D Systems), CD90 (BD Biosciences), and CD105 (R&D Systems). After washing, the labeled cells were analyzed through flow cytometry using BD FACSCalibur™ and the BD CellQuest™ Pro.

Cell proliferation assay

To determine the optimal concentration of PRP for ADSC proliferation, ADSCs were cultured in medium containing different concentrations of PRP (2.5%, 5%, 7.5%, and 10% PRP) or 10% FBS (control group). At least 5×10^3 ADSCs were seeded per well and were incubated at 37 ℃ in 5% CO_2. Subsequently, the proliferation of ADSCs in each group was determined using the CellTiter 96 AQueous One Solution Reagent (Promega Co., Madison, WI, United States), which contains a novel tetrazolium salt (MTS). The tetrazolium salt MTS is reduced by living cells into a colored formazan product. The quantity of the formazan product is directly proportional to the number of viable cells. In our experiment, the colorimetric measurement of the formazan dye was performed at a wavelength of 490 nm on an enzyme-linked immuno sorbent assay plate reader (Molecular Devices, Sunnyvale, CA, United States). Cell numbers were determined using a calibration curve plotting the

number of ADSCs *vs* the absorbance values on days 0, 3, 5, and 7.

Reverse transcription-PCR

Mature and functional endothelial markers were evaluated through reverse transcription-PCR (RT-PCR). ADSCs were incubated in culture medium containing different concentrations of PRP (2.5%, 5%, 7.5%, and 10% PRP); 10% FBS was used as the control group. After culturing for 14 d, RNA was isolated using a GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, United States) in accordance with the manufacturer's protocols. The expression of angiogenic-related genes, namely CD31 and CD34, was analyzed. The constitutively expressed gene encoding β-actin was used as an internal control in RT-PCR to normalize the amounts of mRNA in each sample.

Immunofluorescence staining

ADSCs were cultured in medium containing different concentrations of PRP (2.5%, 5%, 7.5%, and 10% PRP) or 10% FBS (control group). Approximately 1 × 10^4 cells were incubated at 37 ℃ in 5% CO_2. On days 7 and 14, to identify the endothelial differentiation of ADSCs, immunofluorescence staining was performed using the following antibodies: rabbit polyclonal antibody (1:200) against human CD31 (1:50) and rabbit polyclonal antibody against VEGF. Antibodies with fluorescent labels were used. The immunostaining intensity was observed through confocal microscopy and was then quantified using Image J. The corrected total cellular fluorescence ratio was calculated using the following formula: corrected total cellular fluorescence = integrated density (area of selected cell × mean fluorescence of background readings).

Tube formation assay

Matrigel was thawed and suspended in 96-well plates and solidified at 37 ℃ for 30 min. ADSCs were cultured in medium containing different concentrations of PRP (2.5%, 5%, 7.5%, and 10% PRP) or 10% FBS (control group) for 14 d. Cells were then seeded at a density of approximately 1 × 10^5 cells per well and were labeled using Live-Dead Cell Staining Kits. The tubular structure was examined under a confocal microscope. Endothelial differentiation was assessed by determining the average length, total tube length, branch numbers, and total branch points through Image J.

Animal care protocol and nude mouse ischemic hindlimb model

Animal care and experiments were approved by the Local Ethics Committee for Animal Research Studies at Chang Gung Memorial Hospital. All nude mice were kept in laboratory conditions (controlled temperature 23 ℃ and humidity 50%) with free access to water and food for 4 wk prior to experimentation. ADSCs were preconditioned by culturing in medium containing 5% PRP, 7.5% PRP, and 10% FBS. Additionally, human umbilical vein endothelial cells (HUVECs) were used as a positive control. Mice administered with local injections of phosphate buffer solution (PBS) only (no ADSCs) were used as negative controls. General anesthesia was done by mixture of Zoletil and Xylazine 0.1 cc intraperitoneal injection before all experiments. The proximal portion of the right femoral artery of 8-wk-old nude mice was ligated using an electrosurgical pencil. About 3 × 10^6 preconditioned ADSCs and HUVECs were directly injected over the medial thigh muscle and lateral thigh muscle groups. Blood flow in both the ischemic hindlimb and normal hindlimb was measured using a laser Doppler blood flow meter on days 0, 1, 4, 7, 11, 14, and 18.

Immunohistochemistry study

Nude mice were sacrificed on day 18. All mice were euthanized by carbon dioxide overdose for tissue collection. The muscle of the ischemic hindlimb was fixed in paraformaldehyde and sectioned into slices. To identify endothelial cells, immunohistochemical analysis was performed using rabbit polyclonal antibody against mouse CD31 antibody. The sections were then treated with diaminobenzidine and were stained using hematoxylin and eosin stain. Capillary density was determined by measuring the capillary numbers/mm^2 under a microscope.

Statistical analysis

All the data are reported as mean ± standard deviation. Statistical analyses among the multiple group data are carried out using a one-way analysis of variance test to determine the significant differences. Turkey's *post-hoc* test is used to determine the difference between any two groups with $P < 0.05$ considered statistically significant.

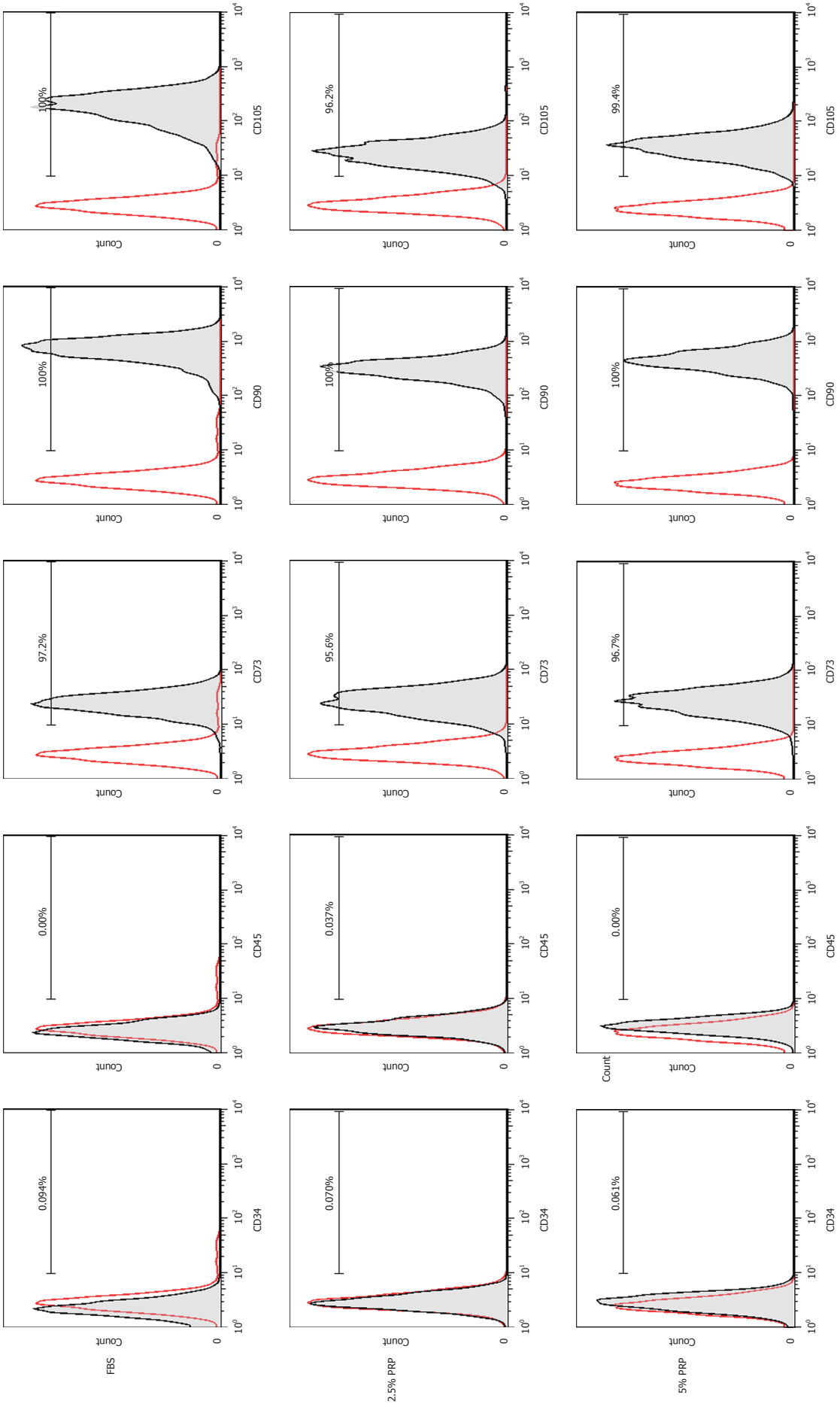
RESULTS

Characterization of ADSCs

Flow cytometry was performed to characterize the phenotypes of ADSCs after their culture in medium containing PRP (Figure 1). Most of PRP- preconditioned ADSCs (98%-99%) were positive for endoglin receptor (CD105), the surface enzyme ecto-59-nucleotidase (CD73), and extracellular matrix protein (CD90). However, they were negative for markers of hematopoietic lineage (CD34) and the leukocyte common antigen (CD45). The cells presented cell markers specific to MSCs and these phenotypes meet the criteria proposed by the ISCT and IFATS for ADSCs.

Cell proliferation assay

The cell proliferation rate is presented as the growth ratio (%), which was calculated using the following formula: ADSC numbers on day 3, 5, or 7/ADSC numbers on day 0. The cell proliferation rate was increased from


FBS
2.5% PRP
5% PRP
CD34
CD45
CD73
CD90
CD105
Count
0.094%
0.00%
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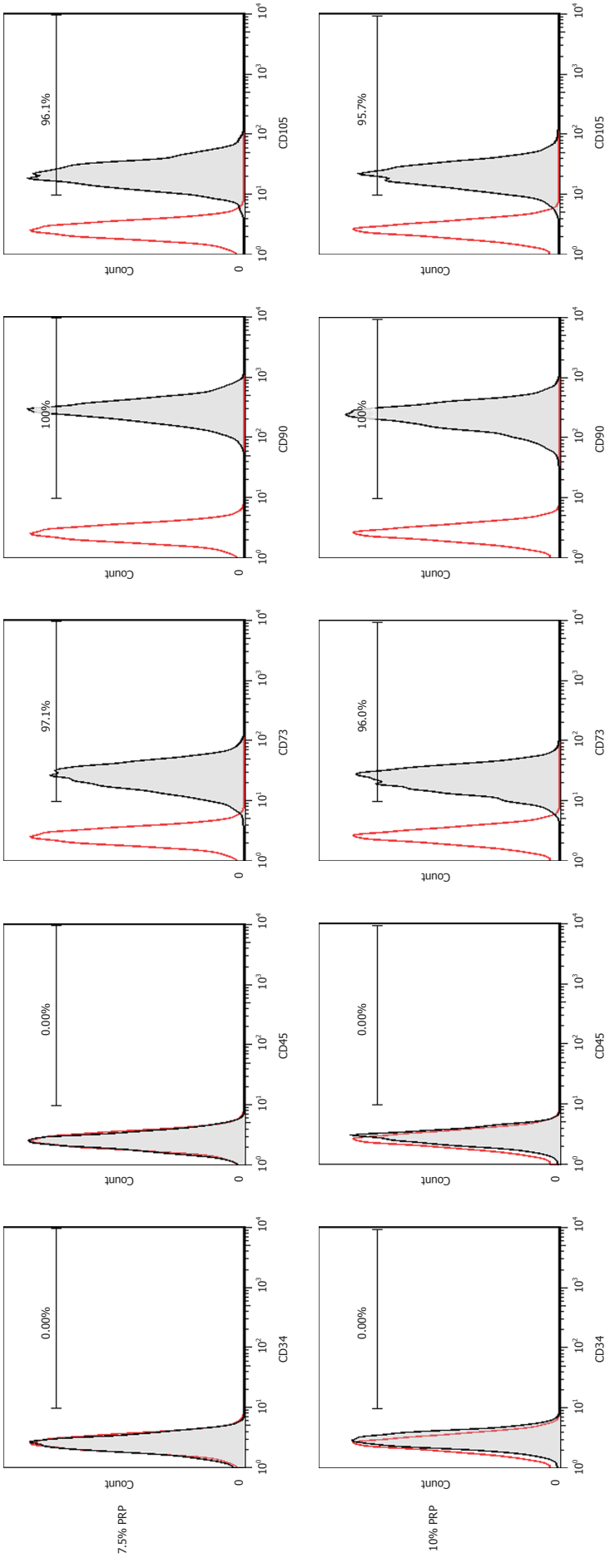

Figure 1 Flow cytometry of platelet-rich plasma preconditioned adipose-derived stem cells. Most of the platelet-rich plasma preconditioned cells (98%-99%) were positive for CD105, CD73 and CD90, but negative for hematopoietic lineage markers including CD34 and CD45. The cells presented cell markers specific to mesenchymal stem cells. PRP: Platelet-rich plasma; FBS: Fetal bovine serum.

day 3 in PRP groups compared with the FBS group. At the endpoint (day 7), the proliferation rate of ADSCs was significantly higher in the 2.5%, 5%, and 7.5% PRP groups (25.348 ± 2.572, 31.778 ± 2.523, 33.400 ± 5.428 for 2.5% PRP, 5% PRP, 7.5% PRP, respectively) than in the 10% FBS (control group) and 10% PRP groups (15.483 ± 3.071 and 14.168 ± 2.650 for 10% FBS and 10% PRP; $P < 0.01$). The results suggested that 2.5%, 5%, and 7.5% PRP showed a higher ability to increase ADSC proliferation compared with FBS (Figure 2).

RT-PCR

Angiogenic-related gene expression was analyzed by RT-PCR. Relative quantification of RT-PCR was done by calculating the difference of the dCt value between the target groups and the FBS group. Compared with the FBS group, the expression of HIF mRNA was significantly increased in the 5% and 7.5% PRP groups ($P < 0.01$; Figure 3A). The mRNA expression of endothelial cell markers, including CD31 and VEGF, was markedly higher in the 5%, 7.5%, and 10% PRP groups than in the 2.5% PRP and FBS groups ($P < 0.01$; Figure 3B and C). The eNOS mRNA expression level was also increased in the 5%, 7.5%, and 10% PRP groups ($P < 0.01$; Figure 3D). RT-PCR analysis revealed that the expression levels of *HIF*, *CD31*, *VEGF*, and *eNOS* genes were significantly higher in the 5% PRP and 7.5% PRP groups ($P < 0.01$). In RT-PCR, the expression of angiogenic-related genes was higher in the 5% and 7.5% PRP groups.

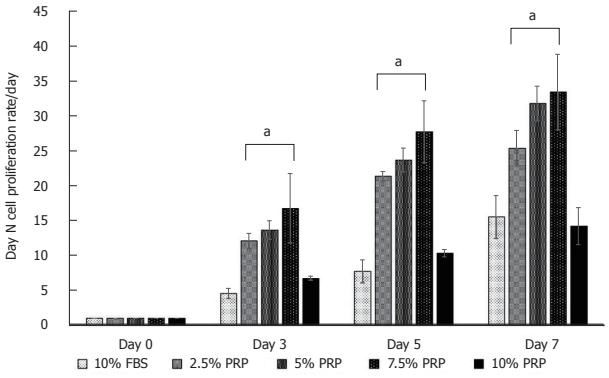

Figure 2 Cell proliferation assay. At the endpoint (day 7), the proliferation rate of ADSCs was significantly higher in the 2.5%, 5%, and 7.5% PRP groups (25.348 ± 2.572, 31.778 ± 2.523, 33.400 ± 5.428 for 2.5% PRP, 5% PRP, 7.5% PRP, respectively) than in the 10% FBS (control group) and 10% PRP groups (15.483 ± 3.071 and 14.168 ± 2.650 for 10% FBS and 10% PRP; $P < 0.01$). The results suggested that 2.5%, 5%, and 7.5% PRP showed a higher ability to increase ADSC proliferation compared with FBS. Data are expressed as mean ± standard deviation. $^{a}P < 0.01$ *vs* 10% FBS group. ADSC: Adipose-derived stem cell; PRP: Platelet-rich plasma; FBS: Fetal bovine serum.

Immunofluorescence staining for angiogenic protein expression

The protein production level was analyzed through immunofluorescence staining. After culturing for 14 d, CD31 expression was significantly higher in all PRP groups compared with the FBS group ($P < 0.01$; Figure 4A and C). According to the immunofluorescence staining results, the early elevated production of VEGF was noted from day 7 in the 5% and 7.5% PRP groups. On day 14, 5%, 7.5%, and 10% PRP groups showed considerable increases in VEGF production (Figure 4B and D). VEGF and CD31, which are produced by endothelial cells, are key proteins in angiogenesis. VEGF and CD31 production increased after ADSCs were cultured in medium containing PRP. The 5%, 7.5%, and 10% PRP groups showed a higher ability to promote both CD31 and VEGF production by ADSCs.

Morphological changes in ADSCs

EPCs form tubes, connecting to each other, and are then arranged in clusters. Thus, morphological change is also a crucial parameter for evaluating the level of endothelial differentiation. Microscopy revealed the tubular structure of ADSCs in all PRP groups (Figure 5A). We quantified tube formation by determining the average length, tube numbers, and branch points (Figure 5B). Overall performance was evaluated based on the total tube length, which was calculated using the following formula: average length × tube numbers. Although the tubular structure was observed through microscopy, morphological changes in ADSCs cultured in 2.5% PRP were nonsignificant compared with those in the control group ($P > 0.05$). The result showed that ADSCs in the 5%, 7.5%, and 10% PRP groups showed more tube formation, more cell–cell interconnections (evaluated by branch points), and longer tubes ($P < 0.01$).

PRP-preconditioned ADSCs improved revascularization in the ischemic mouse hindlimb

In our *in vitro* studies, 5% PRP and 7.5% PRP had higher abilities to promote the endothelial differentiation of ADSCs. We further designed an *in vivo* study to evaluate the angiogenic potential of these two groups. Blood vessel ligation was performed on the right hindlimb of nude mice. ADSCs from the PBS, FBS, 5% PRP, and 7.5% PRP groups and from HUVECs were applied to the wound immediately after surgery. Blood perfusion was measured immediately after surgery and on postoperative day 18 by using the laser Doppler blood flow meter. In representative images, red and blue indicated areas with normal blood perfusion and ischemia, respectively (Figure 6A). Image J was used to quantify laser Doppler images, and the result was expressed as the perfusion ratio (%), which was calculated using the following formula: blood flow in operated hindlimb/blood flow in non-operated hindlimb. Data revealed improved blood flow in the ischemic hindlimb after PRP treatment (Figure 6B). The revascularization rate remained low in the PBS and 10% FBS groups, with the ratios of 0.42 ± 0.16 and 0.54 ± 0.14, respectively, on day 18. The PRP and HUVEC groups showed significantly higher ratios on day 18 (0.88 ± 0.08, 0.85 ± 0.07, 0.81 ± 0.06 for 5% PRP, 7.5% PRP, and HUVECs, respectively) than the PBS group (0.42 ± 0.17; $P < 0.01$). No significant difference was observed in the perfusion ratios of the PRP and HUVEC groups ($P > 0.05$). Although serial laser Doppler images showed some natural recovery of hindlimb blood flow in control groups, administering PRP-preconditioned ADSCs to the ischemic site significantly increased tissue perfusion.

Histological staining of muscle sections

CD31 is a key glycoprotein expressed at endothelial cell intercellular junctions, and it is often used as an angiogenesis marker in research. In the present study, CD31 was used to demonstrate the presence of endothelial cells in histological tissue sections. Capillary density was assessed through quantification of CD31-positive capillaries, and the density was determined by measuring capillary numbers/mm^2. Consistent with the findings obtained from laser Doppler image analysis, the capillary densities of the PRP groups were significantly higher than those of the control and FBS groups, as shown in Figure 7A and quantified in Figure 7B (26.95 ± 9.21, 30.22 ± 4.80, 13.03 ± 4.46, and 14.22 ± 4.14 for 5% PRP, 7.5% PRP, 10% FBS, and the control group, respectively). The 5% PRP and HUVEC groups had similar angiogenesis effects, and the 7.5% PRP group had the optimal result among all groups. The capillary density significantly increased in the 7.5% PRP group, even when compared with the positive control HUVEC group (30.22 ± 4.80 and 20.03 ± 5.67 for 7.5% PRP and HUVEC, respectively; $P < 0.05$). Overall, these data demonstrated that 5% PRP and 7.5% PRP-preconditioned ADSCs significantly enhanced

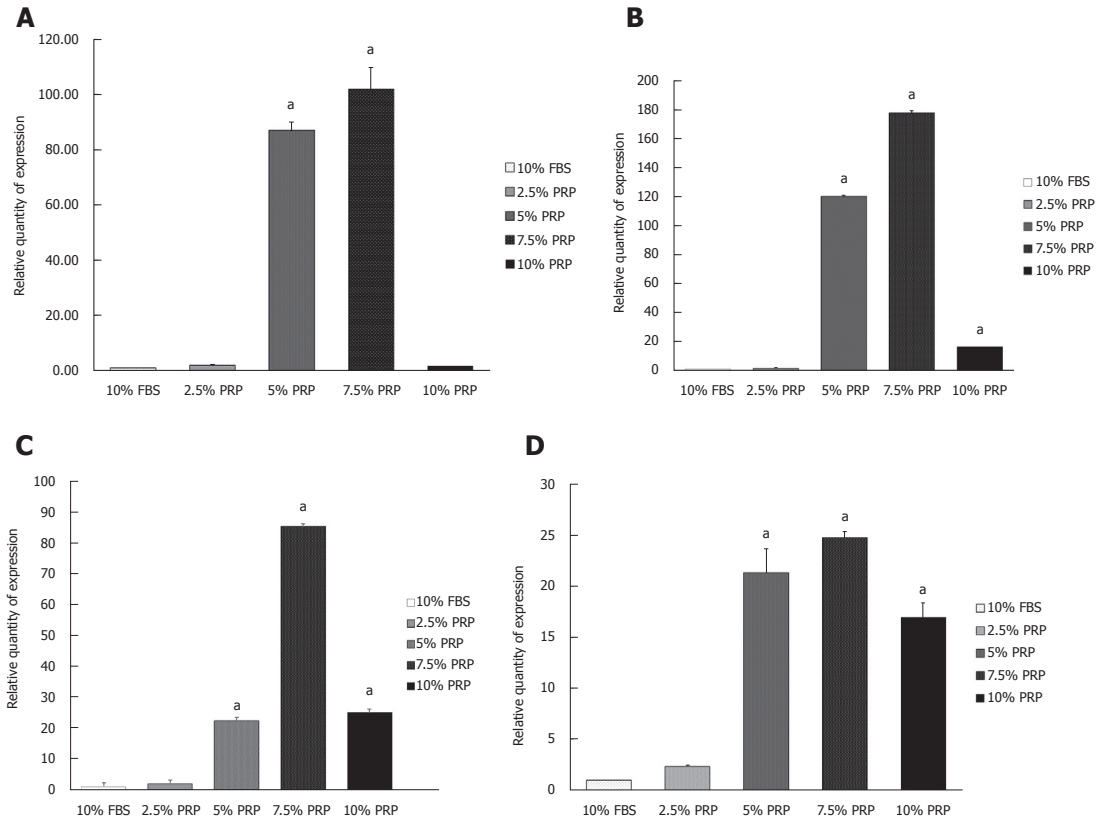

Figure 3 Analysis of gene expression of adipose-derived stem cell by reverse transcription polymerase chain reaction. A: The expression of hypoxia-inducible factor mRNA was significantly increased in the 5% and 7.5% PRP groups compared with the FBS group; B: The expression of CD31 mRNA was significantly higher in the 5%, 7.5%, and 10% PRP groups compared with the FBS group; C: The expression of vascular endothelial growth factor was markedly higher in the 5% and 7.5% PRP groups compared with the FBS group; D: The expression of endothelial cell nitric oxide synthase was significantly increased in the 5% and 7.5% PRP groups compared with the FBS group. Data are expressed as means ± standard deviation. $^{a}P < 0.01$ *vs* 10% FBS group. ADSC: Adipose-derived stem cell; PRP: Platelet-rich plasma; FBS: Fetal bovine serum.

physiologic neovascularization in ischemic tissue.

DISCUSSION

Our results showed that ADSCs cultured in medium containing PRP exhibited the properties of endothelial cells in terms of gene expression, angiogenic-related protein production, and morphological changes (*i.e.*, tubular structure formation). The ischemic hindlimb model showed significantly improved blood perfusion after local injection with PRP- preconditioned ADSCs. Histochemical analysis of CD31 in muscle sections also provided the same result. Notably, compared with FBS, PRP exhibited a higher ability to promote the endothelial differentiation of ADSCs. The 5% PRP and 7.5% PRP groups had the optimal result among all groups. These findings suggest that 5% PRP and 7.5% PRP are suitable substitutes for FBS when culturing ADSCs and can achieve angiogenesis and improve perfusion in ischemic tissue.

Flow cytometry

Cell surface markers such as CD105, CD73, and CD90 are associated with the stemness of MSCs. Any change in cell surface markers indicates that stem cells may have committed to other linages. In our study, PRP was added to medium to culture ADSCs. However, some concerns still exist that PRP might change the stem cell characteristics of MSCs. Li *et al*[12] found that after culturing in PRP, MSCs maintained their stem cell marker expression as well as multilineage differentiation capacity. Moreover, another study found that the percentage of surface markers remained the same in ADSCs after culturing in PRP[13]. We showed similar findings in flow cytometry. Our ADSCs presented cell markers specific to stromal stem cells after PRP treatment.

Cell proliferation

PRP is a human blood derivative that is rich in growth factors. A previous paper reported that PRP could promote proliferation of ADSCs[14]. The ability of PRP to accelerate cell proliferation of stem cells may come from α-granules, which have many growth factors in a physiological ratio, such as platelet-derived growth factor, transforming growth factor-β, VEGF, epidermal growth factor, insulin-like growth factor, *etc.*[15]. These growth factors all play crucial roles for enhancing cell proliferation. However, only a few studies report the effects of different PRP concentrations on ADSC

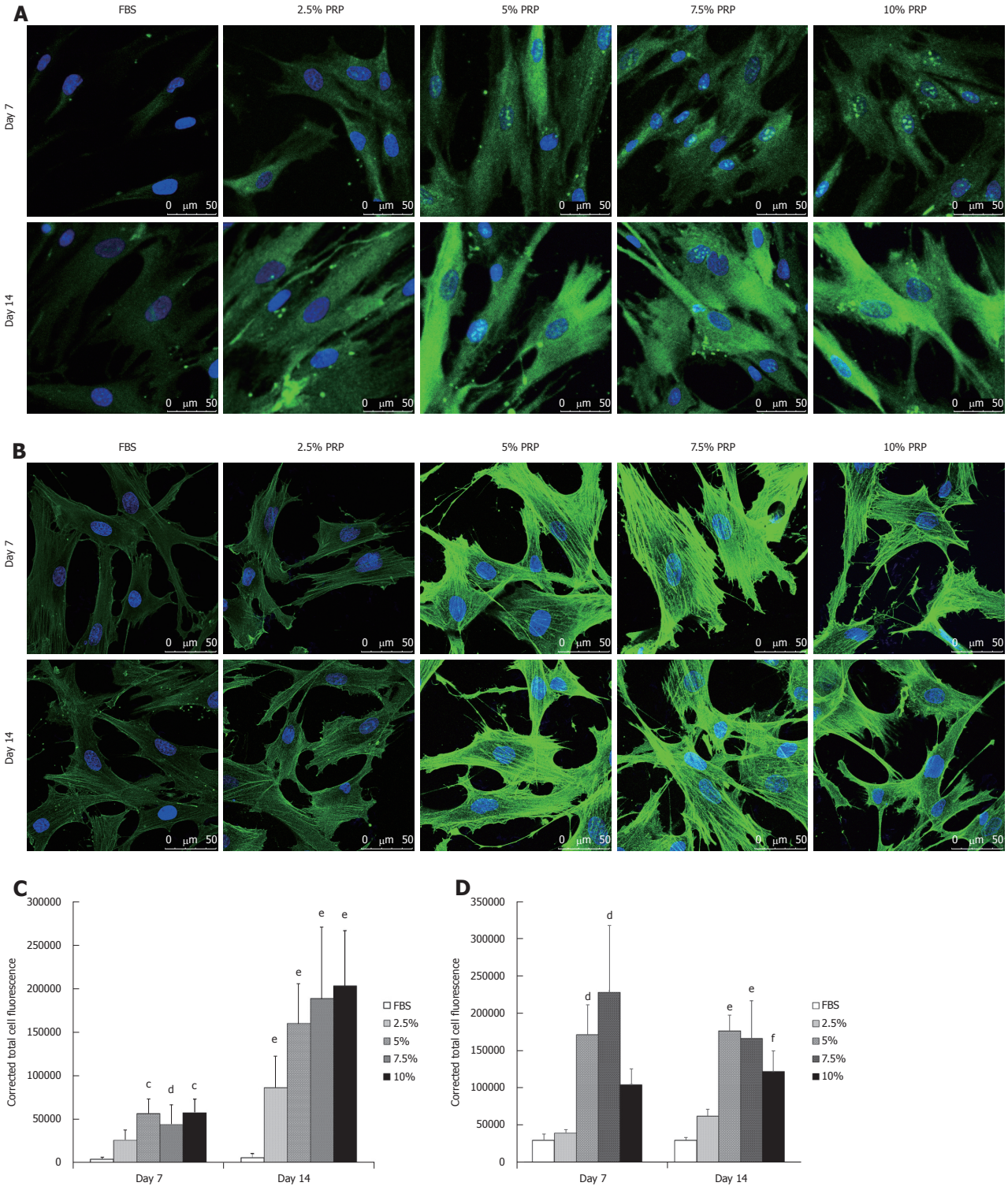

Figure 4 Immunofluorescence staining of CD31 and vascular endothelial growth factor. A: After culturing for 14 d, CD31 expression (in green) was higher in all PRP groups; B: After culturing for 14 d, VEGF expression (in green) was higher in all PRP groups; C: The immunostaining intensity was observed through confocal microscopy and was then quantified using Image J. The corrected total cellular fluorescence ratio was calculated using the following formula: corrected total cellular fluorescence = integrated density (area of selected cell × mean fluorescence of background readings). After culturing for 14 d, CD31 expression (in green) was significantly higher in all PRP groups than in the FBS group. D: The early elevated production of VEGF was noted from day 7 in the 5% and 7.5% PRP groups. On day 14, 5%, 7.5%, and 10% PRP groups showed considerable increases in VEGF production. Data are expressed as means ± standard deviation. $^{c}P < 0.01$ *vs* 10% FBS group at day 7; $^{d}P < 0.05$ *vs* 10% FBS group at day 7; $^{e}P < 0.01$ *vs* 10% FBS group at day 14; $^{f}P < 0.05$ *vs* 10% FBS group at day 14. PRP: Platelet-rich plasma; FBS: Fetal bovine serum; VEGF: Vascular endothelial growth factor.

proliferation. Early studies found that PRP had a dose dependent effect on proliferation of ADSCs[16-18]. However, later studies had opposite opinions. One study demonstrated that a PRP concentration of 10% to 20%

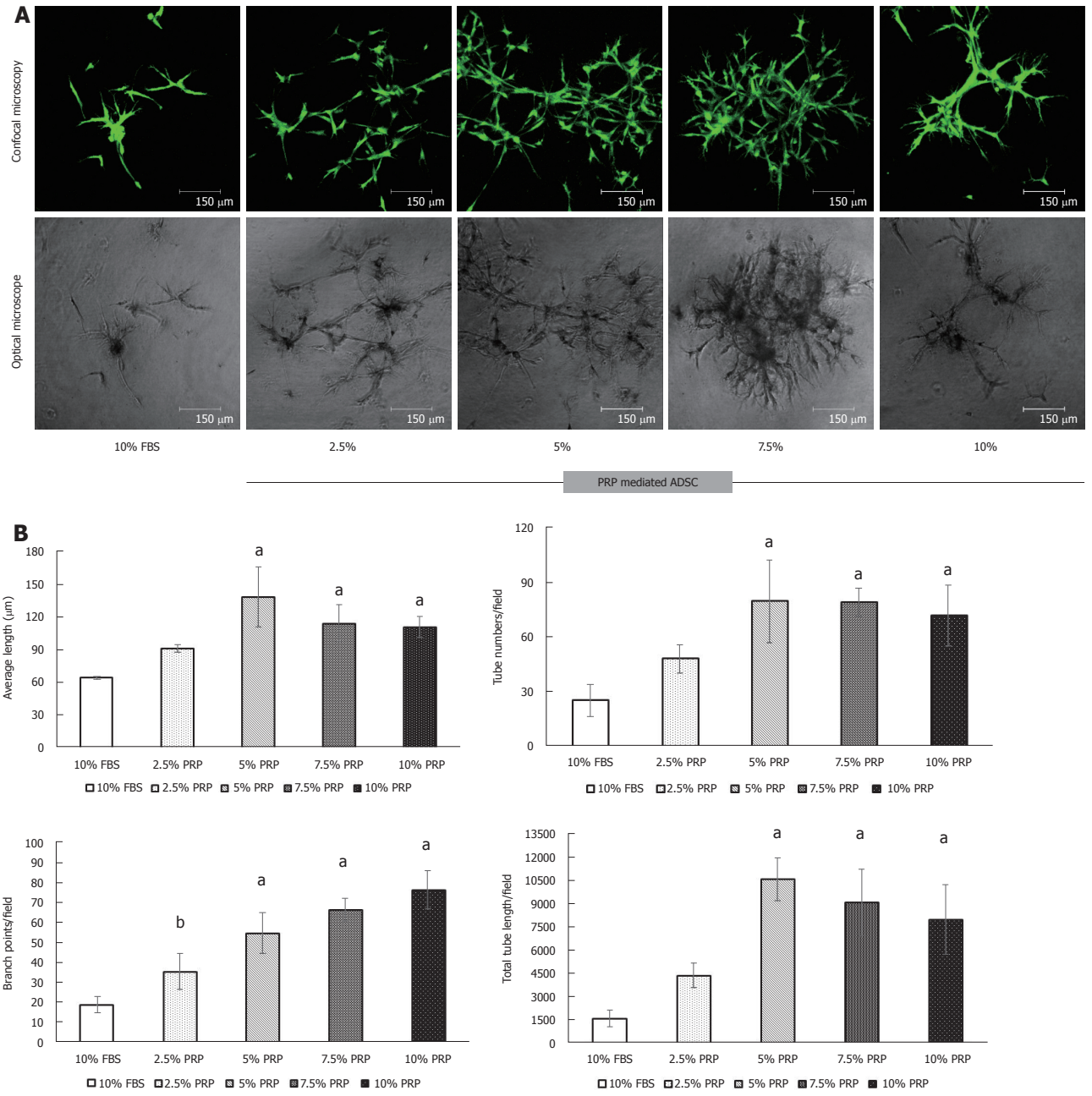

Figure 5 Tube formation assay. A: Tubular structure was noted in all PRP-treated ADSC groups; B: Tube formation was quantified by average length, tube numbers, and branch points. Overall performance was evaluated based on the total tube length, which was calculated using the following formula: average length × tube numbers. The result showed that ADSCs in the 5%, 7.5%, and 10% PRP groups showed more tube formation, more cell–cell interconnections (evaluated by branch points), and longer tubes. Data are expressed as means ± standard deviation. $^{a}P < 0.01$ *vs* 10% fetal bovine serum group. PRP: Platelet-rich plasma; FBS: Fetal bovine serum; ADSC: Adipose-derived stem cell.

had the highest impact on cell proliferation. Proliferation rates declined with higher PRP concentration[19]. In another study, ADSCs were cultured in medium with PRP concentrations from 1% to 30%. The result showed that ADSCs were better grown in 5% and 10% PRP[20]. Another paper had a similar result that 10% PRP in the culture medium markedly promoted cell proliferation while a higher concentration (30%) of PRP had less effect on cell proliferation[21]. It is possible that an inhibitory effect is exerted if the concentration of growth factors or cytokines is too high. In our study, we noted that PRP significantly increased ADSC proliferation compared with 10% FBS based medium. However, the PRP concentration was not directly proportional to the proliferation rate. It is notable that 2.5%, 5%, and 7.5% PRP had better results compared with others. Kakudo *et al*[22] also observed a similar finding. The best dose of PRP for cell proliferation of ADSC may varies from study to study due to different ADSC and PRP preparation methods. It is difficult to compare results between studies due to the lack of standardized protocols for ADSC and PRP collection.

Platelets contain abundant α-granules. These α-granules play important roles in regulating secretory and

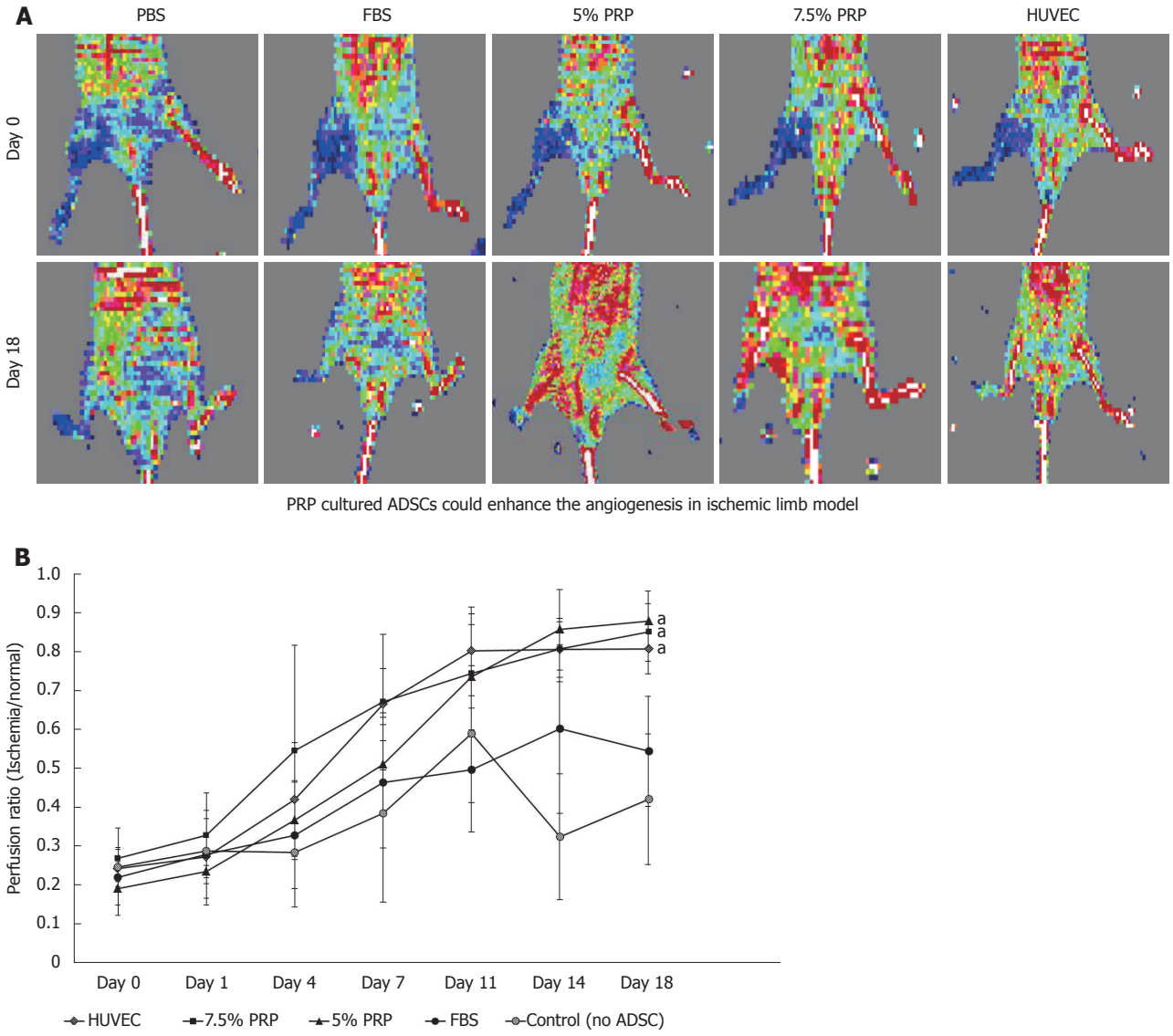

Figure 6 Ischemic hindlimb model. A: Red and blue indicate areas with normal blood perfusion and ischemia, respectively. Although serial laser Doppler images showed some natural recovery of hindlimb blood flow in control groups, administering PRP-preconditioned ADSCs to the ischemic site increased tissue perfusion; B: Quantification was done by perfusion ratio (%): blood flow in operated hindlimb/blood flow in non-operated hindlimb. The revascularization rate remained low in the phosphate buffer solution and 10% FBS groups, with the ratios of 0.42 ± 0.16 and 0.54 ± 0.14, respectively, on day 18. The PRP and HUVEC groups showed significantly higher ratios on day 18 (0.88 ± 0.08, 0.85 ± 0.07, 0.81 ± 0.06 for 5% PRP, 7.5% PRP, and HUVECs, respectively) than the phosphate buffer solution group (0.42 ± 0.17). Data are expressed as means ± standard deviation. $^{a}P < 0.01$ *vs* 10% FBS group. PRP: Platelet-rich plasma; FBS: Fetal bovine serum; ADSC: Adipose-derived stem cell; HUVEC: Human umbilical vein endothelial cell; PBS: Phosphate buffer solution.

angiogenesis pathways. However, α-granules not only release a variety of growth factors, but also contain regulatory proteins like the thrombospondin family. Thrombospondin-1 inhibits adhesion, proliferation, and tube formation of endothelial cells in culture, and it has been found to block neovascularization. Thrombospondin-2 also inhibits the migration and proliferation of endothelial cells. One study correlated large amounts of thrombospondin-1 detected in PRP with high concentrations of PRP could significantly decrease cell proliferation[23]. Whether the thrombospondin family is the most critical regulatory factor compromising angiogenic ability of PRP during high concentration needs further investigation.

RT-PCR and immunofluorescence staining

In this study, markers including HIF, VEGF, CD31, and eNOS were used to evaluate ADSC-mediated angiogenesis. Gene expression was also confirmed through immunofluorescence staining. ADSCs cultured in PRP gained an endothelial phenotype, as demonstrated by the high expression of CD31 and VEGF. In a previous study, HIF was upregulated in ischemic tissues in response to low oxygen status[24]. Another study showed that through HIF-1 production, downstream genes were activated to achieve angiogenesis, cell proliferation, and cell survival[25]. ADSCs produced more HIF under low oxygen conditions[26]. The present study is the first to report that not only oxygen deprivation but also PRP

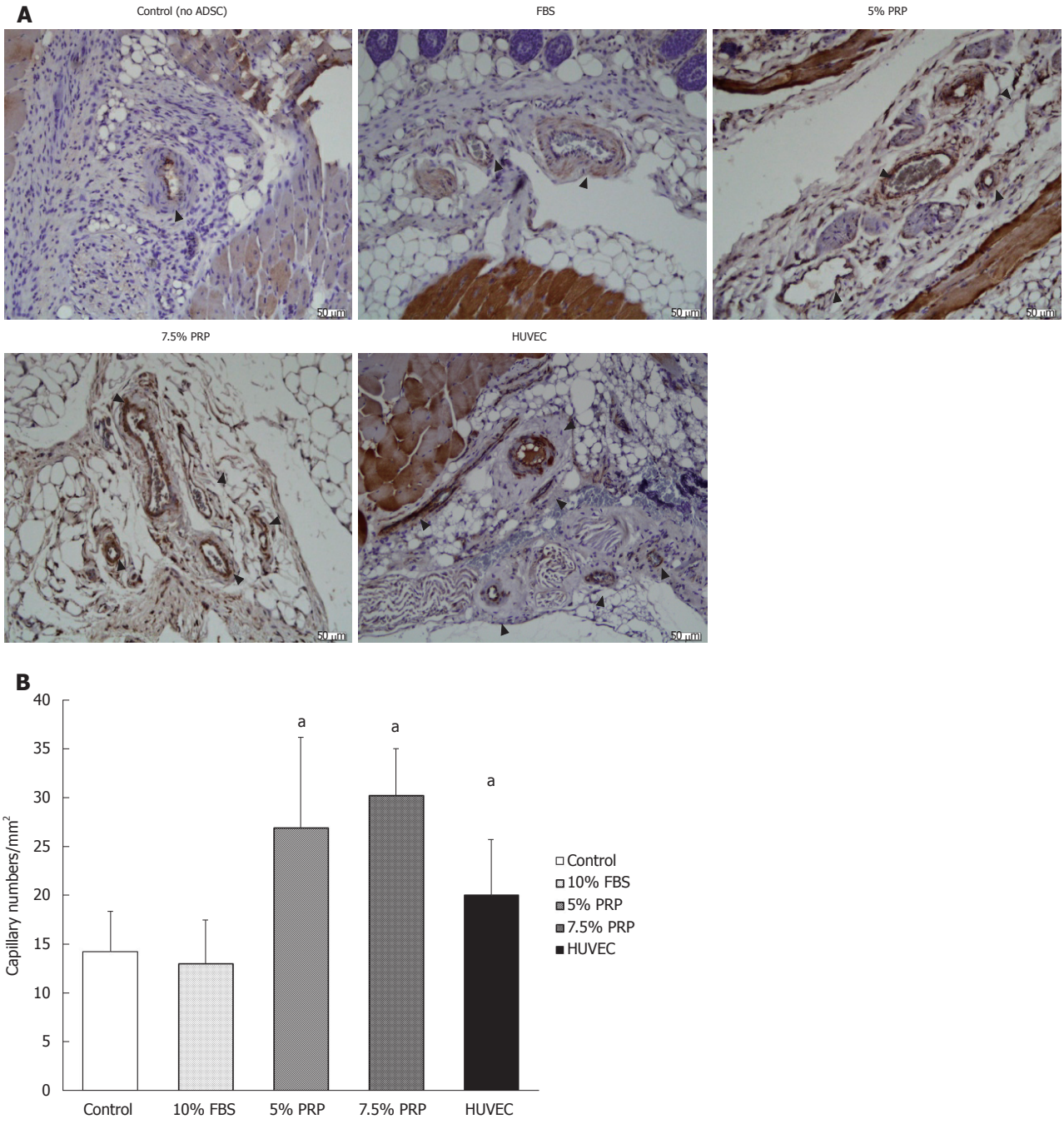

Figure 7 Histological staining of muscle sections. A: The capillary densities of the PRP groups were higher than those of the control and FBS groups; B: We used CD31 stained sections to identify capillary numbers under microscopy. Capillary density was determined by measuring capillary numbers/mm^2. The 5% PRP, 7.5% PRP, and human umbilical vein endothelial cell groups had better results than the FBS group. Data are expressed as means ± standard deviation. $^aP < 0.01$ *vs* 10% FBS group. PRP: Platelet-rich plasma; FBS: Fetal bovine serum; ADSC: Adipose-derived stem cell; HUVEC: Human umbilical vein endothelial cell.

treatment can assist ADSCs to produce more HIF, further activating angiogenesis.

HIF-1α is a subunit of HIF-1, and it can activate several angiogenic genes and their receptors under hypoxic status. VEGF is upregulated by HIF-1α, and it is the principal stimulatory factor in angiogenesis[27,28]. VEGF binds to VEGF receptor and activates the mitogen-activated protein kinase/extracellular signal–regulated kinase pathway to achieve cell proliferation. VEGF also activates the phosphatidylinositide 3-kinase/protein kinase B pathway to increase eNOS production[29]. eNOS then increases vessel wall permeability and facilitates the chemotactic migration of EPCs toward VEGF. VEGF also attracts cells including EPCs, mural cells, and hematopoietic stem cells to the ischemic site. These stem cells produce capillary plexuses and eventually form mature vessels. All these processes together cause the development of new vessels for vascular supply in ischemic limbs[30]. PRP is rich in VEGF and has been proven to enhance angiogenesis both *in vitro* and *in*

vivo. ADSCs were also able to increase the VEGF level in the nude mouse model established through local injection, enabling vessel growth in ischemic tissue[31]. We hypothesized that local VEGF concentration could be further elevated through both work from PRP and ADSC. In our study, we proved that HIF and the downstream angiogenic-related genes VEGF and eNOS were upregulated in the PRP groups. High expression of the endothelial cell surface marker CD31 in the PRP groups was also strong evidence that PRP treatment induced the endothelial differentiation of ADSCs.

Tube formation assay

A variety of stem cells had been adopted to cell therapy. Regardless of their tissue origins, MSCs from different origins can express similar endothelial-relevant functions *in vitro*[32]. ADSCs are a kind of MSC, with the capacity to become various cells including adipocytes, chondrocytes, and osteocytes. ADSCs also retained the ability to differentiate toward an endothelial lineage. The elder age, cardiovascular disease, obesity, or tobacco use of donors does not alter the isolation. Besides endothelial cell related molecular expression, functional characteristics were evaluated by the tube formation assay. After proper differentiation, ADSCs formed tubular structures upon plating on Matrigel, indicating that ADSCs were able to differentiate toward endothelial cells and participate in angiogenesis. Our finding was consistent to previous studies that demonstrated that ADSCs participate in angiogenesis.

Single growth factor VEGF or boosting VEGF expression by curcumin had been applied in a tubular formation assay to promote capillary structure formation of stem cells[33,34]. However, stimulation of neovascularization involves complex steps and the result is influenced by multiple growth factors. Single growth factor VEGF use may have limitations to establish stable blood vessels. It is notable that PRP is a natural growth factor reservoir and had been proved to accelerate proliferation and stimulate capillary tube formation of endothelial cells[35]. Compared with FBS, PRP better supported the formation of lumen-like structures and the alignment of multicentric junctions of endothelial cells[36]. One study had found that formation of capillary like structures of HUVECs became maximal at 5% PRP[37]. Another study demonstrated that capillary-like structures were more prevalent in certain concentrations of PRP (0.5%, 1% and 3% PRP) compared with either the control group (0% PRP) or higher concentrations (5% and 10% PRP) in a co-culture system of HUVECs and human dermal fibroblast cells[38]. These two studies demonstrated PRP induced capillary–like structures of HUVECs in a bell-shaped dose-response curve. A previous study has proven that ADSCs differentiated into endothelial cells and produced capillary-like structures as HUVECs did *in vitro*[7]. However, no studies worked on the dose-dependent effect of PRP on ADSCs for formation of capillary-like structures. In our study, we found PRP significantly induced morphologic change of ADSCs. Further investigation indicated that tubular formation was proportional to PRP concentration between 2.5% to 7.5% PRP level, suggesting that proper ratio of angiogenic factors in PRP is important for formation of functional blood vessels by ADSCs.

From our results, the higher concentration of PRP lowered the proliferation rate of ADSCs and further reduced endothelial differentiation. Pro-angiogenic and anti-angiogenic molecules released by activation of plasma thrombin receptors during tissue injury gives an explanation to above phenomenon. As mentioned above, α-granules in the platelets contain regulatory proteins like the thrombospondin family. These proteins regulate angiogenesis by inhibiting MSC proliferation, tubular formation, and migration. Besides, the pro-angiogenic VEGF in α-granules and the anti-angiogenic endostatin in plasma were released upon platelet activation[39]. Balance between local VEGF and endostatin concentration accounts for the net biological effect on injured tissue[40]. High levels of anti-angiogenic molecules present in plasma should be considered when using high concentrations of PRP in tissue engineering.

Nude mouse ischemic hindlimb model

Increasing research attention has been paid to PAD due to the increased disease prevalence. Various treatment strategies have been developed to induce new vessel formation and restore tissue perfusion through the use of exogenous molecular and cellular agents. The ischemic hindlimb model of mice has been widely used for the *in vivo* investigation of cell therapy.

Although preclinical results of single-dose growth factor use in ischemic animal models were promising, the therapy did not have long-lasting clinical effects due to the short half-life of growth factors[41]. Maintaining a stable level of growth factors is essential. Early studies have demonstrated that compared with the single-dose administration of VEGF, a drug delivery system that enabled the sustained release of growth factors significantly increased tissue blood flow, number of arterioles, and vascular density in the rabbit ischemic hindlimb model[42,43]. Gelatin hydrogels, polylactic-co-glycolic acid, and alginate are commonly used to make such granules. However, degradation time varies among different granules, and complete degradation may not be achieved. The residual foreign body is associated with a higher risk of infection and safety concerns, limiting the clinical application of granules. Gaining a comprehensive understanding of EPCs has enabled various stem cells to be utilized for endothelial differentiation and ischemic tissue repair. The focus has moved toward stem cell-based therapeutic angiogenesis, and ADSCs are promising stem cell types in therapeutic angiogenesis.

Many studies have demonstrated the angiogenic potential of ADSCs not only *in vitro* but also *in vivo*. In mouse ischemic hindlimb models, increased circu-

lating endothelial cells were detected after local ADSC injection. The ischemic limbs recovered from muscle injury, and muscle sections exhibited increased vascular density[44,45]. Studies have found that neovascularization in ischemic tissue is attributed not only to the endothelial differentiation of ADSCs but also to their paracrine effects. Conditioned media obtained from ADSCs contained multiple angiogenic cytokines, including HIF, VEGF, fibroblast growth factor, and hepatocyte growth factor[46]. Through these growth factors, ADSCs augmented surrounding cell remodeling, reduced endothelial cell atrophy, and stimulated angiogenesis. However, the survival rates of ADSCs transplanted in the animal ischemic hindlimb model were variable. Multiple treatments have been developed to increase the survival and modify the angiogenic potential of ADSCs for cell therapy.

Under hypoxic conditions, the survival rate of ADSCs and the revascularization of animal ischemic hindlimbs are improved with the upregulation of HIF-1α and VEGF as the underlying mechanism[47,48]. Although researchers have reported that growth factors may have positive effects on the proliferation and endothelial differentiation of ADSCs, PRP treated ADSCs have never been applied in animal models. This study is the first to apply PRP, which is a natural reservoir of growth factors, to culture ADSCs and to evaluate cell angiogenic potential *in vivo*. Our hypothesis was that synergistic effects of multiple growth factors in PRP would increase the survival rate and endothelial differentiation of ADSCs. Application of these preconditioned ADSCs to ischemic tissue may achieve higher revascularization. PRP-preconditioned ADSCs had a higher ability to promote neovascularization *in vivo* compared with ADSCs preconditioned in common culture medium (*i.e.*, FBS).

In the present study, we determined the effect of PRP concentration on cell angiogenic potential. HUVECs, which are known to have a strong ability to promote angiogenesis in animal ischemic hindlimb models, providing nearly 80% blood perfusion recovery (compared with the normal hindlimb) in our mouse experiment. However, 5% and 7.5% PRP-preconditioned ADSCs provided even more favorable results in the animal model. In the ischemic hindlimb, 5% and 7.5% PRP-preconditioned ADSCs achieved perfusion rates as high as 85% and 88%, respectively. The result of muscle histological sections staining was consistent with those obtained from laser Doppler image analysis.

Conclusion

In conclusion, our study provided a new therapeutic strategy for PAD. Both ADSCs and PRP offer many advantages, including abundant resources, efficient preparation, and safety. Moreover, the clinical application of ADSCs and PRP is associated with lower risks of xeno-immune responses and zoonotic disease transmission. Previous studies reported that PRP had limited effects on the recovery of blood perfusion and wound healing, which was mostly likely due to the rapid degradation of PRP *in vivo*. Therefore, instead of direct injection into tissue, ADSCs were preconditioned with PRP before cell implantation, which improved the angiogenic potential of cells and thus increased neovascularization in ischemic limbs. The balance between local angiogenic and proangiogenic factors accounts for the net biological effect on injured tissue. We further observed that 5% and 7.5% PRP provided the optimal effect on enhancing the angiogenic potential of ADSCs. Based on this finding, we believe that PRP treated ADSCs may be clinically applied for treating ischemic tissues and promoting wound healing in the future. Further research should focus on increasing the rate of ADSC differentiation into mature endothelial cells and finding key regulators for three-dimensional tubular structure formation in these cells. The optimal goal is to use ADSCs to form stable and functional vessels for patients with PAD.

ARTICLE HIGHLIGHTS

Research background

Peripheral artery disease (PAD) is caused by peripheral artery obstruction, which may lead to ischemic changes in the extremities. In advanced PAD, revascularization surgery is indicated for large to medium-sized peripheral arteries with obstructions. However, ideal treatment for small arteries with obstructions has not been established until now.

Research motivation

Therapeutic angiogenesis provides a novel strategy for managing PAD. Mesenchymal stem cells can be used to promote tissue angiogenesis. Among all mesenchymal stem cells, adipose-derived stem cells (ADSCs) are plentiful, easy to retrieve with less donor site morbidity, and free from ethical concerns, making it a good candidate for therapeutic angiogenesis. Fetal bovine serum (FBS) is widely used in research settings for culturing ADSCs. However, culturing cells for therapeutic purposes in patients is associated with zoonotic disease transmission and xeno-immunization concerns. Platelet-rich plasma (PRP) is an autologous reservoir of growth factors and cytokines, which have great potential to replace animal serum as culture medium.

Research objectives

To date, limited data are available on the effects of PRP on ADSCs. Our study evaluated the angiogenic potential of PRP-preconditioned ADSCs. In addition, ADSCs' biological characteristics and their capability to induce angiogenesis both *in vitro* and *in vivo* were evaluated.

Research methods

ADSCs were divided based on culture medium: 2.5% PRP, 5% PRP, 7.5% PRP, 10% PRP, or FBS as control. *In vitro*, we studied the cell proliferation rate, endothelial cell specific genes expression and cell morphology change. *In vivo*, we studied the angiogenic capability of ADSCs by mouse ischemic hindlimb mode.

Research results

The proliferation rate of ADSCs was higher in the 2.5%, 5%, and 7.5% PRP groups. The expression of hypoxia-inducible factor, CD31, vascular endothelial growth factor, and endothelial cell nitric oxide synthase increased in the 5% and 7.5% PRP groups. The 5%, 7.5%, and 10% PRP groups showed higher abilities to promote both CD31 and vascular endothelial growth factor production and tubular structure formation in ADSCs. According to laser Doppler perfusion scan, the perfusion ratios of ischemic limb to normal limb were significantly higher in the 5% PRP, 7.5% PRP and human umbilical vein endothelial cell

groups compared with the negative control and FBS groups.

Research conclusions

Our results showed that PRP-preconditioned ADSCs had a better ability to present endothelial cell characteristics *in vitro*. After PRP treatment, ADSCs significantly improved blood perfusion in ischemic hindlimbs. Furthermore, 5% PRP- and 7.5% PRP-preconditioned ADSCs exert the optimal angiogenic effect both *in intro* and *in vivo*.

Research perspectives

We were the first study to observe the angiogenic effect of PRP-preconditioned ADSCs on ischemic hindlimb models. We were also the first to discuss the correlation between PRP concentration and angiogenesis of ADSCs. Based on our results, we believe that PRP and ADSCs could be clinically applied for treating ischemic tissues and promoting wound healing in the future. Further research should focus on increasing the rate of ADSC differentiation into mature endothelial cells and finding key regulators for three-dimensional tubular structure formation in these cells. The optimal goal is to use ADSCs to form stable and functional vessels for patients with PAD.

REFERENCES

1 **Zhou R**, Zhu L, Fu S, Qian Y, Wang D, Wang C. Small Diameter Blood Vessels Bioengineered From Human Adipose-derived Stem Cells. *Sci Rep* 2016; **6**: 35422 [PMID: 27739487 DOI: 10.1038/srep35422]

2 **Adair TH**, Montani JP. Angiogenesis. San Rafael: Morgan & Claypool Life Sciences, 2010 [PMID: 21452444]

3 **Ziello JE**, Jovin IS, Huang Y. Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. *Yale J Biol Med* 2007; **80**: 51-60 [PMID: 18160990]

4 **Fujita Y**, Kawamoto A. Stem cell-based peripheral vascular regeneration. *Adv Drug Deliv Rev* 2017; **120**: 25-40 [PMID: 28912015 DOI: 10.1016/j.addr.2017.09.001]

5 **Nakagami H**, Maeda K, Morishita R, Iguchi S, Nishikawa T, Takami Y, Kikuchi Y, Saito Y, Tamai K, Ogihara T, Kaneda Y. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler Thromb Vasc Biol* 2005; **25**: 2542-2547 [PMID: 16224047 DOI: 10.1161/01.ATV.0000190701.92007.6d]

6 **Zhao L**, Johnson T, Liu D. Therapeutic angiogenesis of adipose-derived stem cells for ischemic diseases. *Stem Cell Res Ther* 2017; **8**: 125 [PMID: 28583178 DOI: 10.1186/s13287-017-0578-2]

7 **Cao Y**, Sun Z, Liao L, Meng Y, Han Q, Zhao RC. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. *Biochem Biophys Res Commun* 2005; **332**: 370-379 [PMID: 15896706 DOI: 10.1016/j.bbrc.2005.04.135]

8 **Planat-Benard V**, Silvestre JS, Cousin B, André M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Pénicaud L, Casteilla L. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation* 2004; **109**: 656-663 [PMID: 14734516 DOI: 10.1161/01.CIR.0000114522.38265.61]

9 **Bourin P**, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013; **15**: 641-648 [PMID: 23570660 DOI: 10.1016/j.jcyt.2013.02.006]

10 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]

11 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13**: 4279-4295 [PMID: 12475952 DOI: 10.1091/mbc.e02-02-0105]

12 **Li H**, Usas A, Poddar M, Chen CW, Thompson S, Ahani B, Cummins J, Lavasani M, Huard J. Platelet-rich plasma promotes the proliferation of human muscle derived progenitor cells and maintains their stemness. *PLoS One* 2013; **8**: e64923 [PMID: 23762264 DOI: 10.1371/journal.pone.0064923]

13 **Cervelli V**, Scioli MG, Gentile P, Doldo E, Bonanno E, Spagnoli LG, Orlandi A. Platelet-rich plasma greatly potentiates insulin-induced adipogenic differentiation of human adipose-derived stem cells through a serine/threonine kinase Akt-dependent mechanism and promotes clinical fat graft maintenance. *Stem Cells Transl Med* 2012; **1**: 206-220 [PMID: 23197780 DOI: 10.5966/sctm.2011-0052]

14 **Shen J**, Gao Q, Zhang Y, He Y. Autologous platelet-rich plasma promotes proliferation and chondrogenic differentiation of adipose-derived stem cells. *Mol Med Rep* 2015; **11**: 1298-1303 [PMID: 25373459 DOI: 10.3892/mmr.2014.2875]

15 **Fréchette JP**, Martineau I, Gagnon G. Platelet-rich plasmas: growth factor content and roles in wound healing. *J Dent Res* 2005; **84**: 434-439 [PMID: 15840779 DOI: 10.1177/154405910508400507]

16 **Tavakolinejad S**, Khosravi M, Mashkani B, Ebrahimzadeh Bideskan A, Sanjar Mossavi N, Parizadeh MR, Hamidi Alamdari D. The effect of human platelet-rich plasma on adipose-derived stem cell proliferation and osteogenic differentiation. *Iran Biomed J* 2014; **18**: 151-157 [PMID: 24842141]

17 **Van Pham P**, Bui KH, Ngo DQ, Vu NB, Truong NH, Phan NL, Le DM, Duong TD, Nguyen TD, Le VT, Phan NK. Activated platelet-rich plasma improves adipose-derived stem cell transplantation efficiency in injured articular cartilage. *Stem Cell Res Ther* 2013; **4**: 91 [PMID: 23915433 DOI: 10.1186/scrt277]

18 **Xiong BJ**, Tan QW, Chen YJ, Zhang Y, Zhang D, Tang SL, Zhang S, Lv Q. The Effects of Platelet-Rich Plasma and Adipose-Derived Stem Cells on Neovascularization and Fat Graft Survival. *Aesthetic Plast Surg* 2018; **42**: 1-8 [PMID: 29302732 DOI: 10.1007/s00266-017-1062-1]

19 **Felthaus O**, Prantl L, Skaff-Schwarze M, Klein S, Anker A, Ranieri M, Kuehlmann B. Effects of different concentrations of Platelet-rich Plasma and Platelet-Poor Plasma on vitality and differentiation of autologous Adipose tissue-derived stem cells. *Clin Hemorheol Microcirc* 2017; **66**: 47-55 [PMID: 28269759 DOI: 10.3233/CH-160203]

20 **Amable PR**, Teixeira MV, Carias RB, Granjeiro JM, Borojevic R. Mesenchymal stromal cell proliferation, gene expression and protein production in human platelet-rich plasma-supplemented media. *PLoS One* 2014; **9**: e104662 [PMID: 25115920 DOI: 10.1371/journal.pone.0104662]

21 **Cho HS**, Song IH, Park SY, Sung MC, Ahn MW, Song KE. Individual variation in growth factor concentrations in platelet-rich plasma and its influence on human mesenchymal stem cells. *Korean J Lab Med* 2011; **31**: 212-218 [PMID: 21779198 DOI: 10.3343/kjlm.2011.31.3.212]

22 **Kakudo N**, Minakata T, Mitsui T, Kushida S, Notodihardjo FZ, Kusumoto K. Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. *Plast Reconstr Surg* 2008; **122**: 1352-1360 [PMID: 18971718 DOI: 10.1097/PRS.0b013e3181882046]

23 **Hsu CW**, Yuan K, Tseng CC. The negative effect of platelet-rich plasma on the growth of human cells is associated with secreted thrombospondin-1. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009; **107**: 185-192 [PMID: 18805712 DOI: 10.1016/j.tripleo.2008.07.016]

24 **Krock BL**, Skuli N, Simon MC. Hypoxia-induced angiogenesis: good and evil. *Genes Cancer* 2011; **2**: 1117-1133 [PMID: 22866203 DOI: 10.1177/1947601911423654]

25 **Ke Q**, Costa M. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* 2006; **70**: 1469-1480 [PMID: 16887934 DOI: 10.1124/

mol.106.027029]
26 **Andreeva ER**, Lobanova MV, Udartseva OO, Buravkova LB. Response of Adipose Tissue-Derived Stromal Cells in Tissue-Related O2 Microenvironment to Short-Term Hypoxic Stress. *Cells Tissues Organs* 2015; **200**: 307-315 [PMID: 26407140 DOI: 10.1159/000438921]
27 **Zimna A**, Kurpisz M. Hypoxia-Inducible Factor-1 in Physiological and Pathophysiological Angiogenesis: Applications and Therapies. *Biomed Res Int* 2015; **2015**: 549412 [PMID: 26146622 DOI: 10.1155/2015/549412]
28 **Kakudo N**, Morimoto N, Ogawa T, Taketani S, Kusumoto K. Hypoxia Enhances Proliferation of Human Adipose-Derived Stem Cells via HIF-1α Activation. *PLoS One* 2015; **10**: e0139890 [PMID: 26465938 DOI: 10.1371/journal.pone.0139890]
29 **Hoeben A**, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev* 2004; **56**: 549-580 [PMID: 15602010 DOI: 10.1124/pr.56.4.3]
30 **Bir SC**, Esaki J, Marui A, Yamahara K, Tsubota H, Ikeda T, Sakata R. Angiogenic properties of sustained release platelet-rich plasma: characterization in-vitro and in the ischemic hind limb of the mouse. *J Vasc Surg* 2009; **50**: 870-879.e2 [PMID: 19679427 DOI: 10.1016/j.jvs.2009.06.016]
31 **Ii M**, Horii M, Yokoyama A, Shoji T, Mifune Y, Kawamoto A, Asahi M, Asahara T. Synergistic effect of adipose-derived stem cell therapy and bone marrow progenitor recruitment in ischemic heart. *Lab Invest* 2011; **91**: 539-552 [PMID: 21135814 DOI: 10.1038/labinvest.2010.191]
32 **Du WJ**, Chi Y, Yang ZX, Li ZJ, Cui JJ, Song BQ, Li X, Yang SG, Han ZB, Han ZC. Heterogeneity of proangiogenic features in mesenchymal stem cells derived from bone marrow, adipose tissue, umbilical cord, and placenta. *Stem Cell Res Ther* 2016; **7**: 163 [PMID: 27832825 DOI: 10.1186/s13287-016-0418-9]
33 **Trojahn Kølle SF**, Oliveri RS, Glovinski PV, Kirchhoff M, Mathiasen AB, Elberg JJ, Andersen PS, Drzewiecki KT, Fischer-Nielsen A. Pooled human platelet lysate versus fetal bovine serum-investigating the proliferation rate, chromosome stability and angiogenic potential of human adipose tissue-derived stem cells intended for clinical use. *Cytotherapy* 2013; **15**: 1086-1097 [PMID: 23602579 DOI: 10.1016/j.jcyt.2013.01.217]
34 **You J**, Sun J, Ma T, Yang Z, Wang X, Zhang Z, Li J, Wang L, Ii M, Yang J, Shen Z. Curcumin induces therapeutic angiogenesis in a diabetic mouse hindlimb ischemia model via modulating the function of endothelial progenitor cells. *Stem Cell Res Ther* 2017; **8**: 182 [PMID: 28774328 DOI: 10.1186/s13287-017-0636-9]
35 **Bir SC**, Esaki J, Marui A, Sakaguchi H, Kevil CG, Ikeda T, Komeda M, Tabata Y, Sakata R. Therapeutic treatment with sustained-release platelet-rich plasma restores blood perfusion by augmenting ischemia-induced angiogenesis and arteriogenesis in diabetic mice. *J Vasc Res* 2011; **48**: 195-205 [PMID: 21099226 DOI: 10.1159/000318779]
36 **Li X**, Hou J, Wu B, Chen T, Luo A. Effects of platelet-rich plasma and cell coculture on angiogenesis in human dental pulp stem cells and endothelial progenitor cells. *J Endod* 2014; **40**: 1810-1814 [PMID: 25175848 DOI: 10.1016/j.joen.2014.07.022]
37 **Kakudo N**, Morimoto N, Kushida S, Ogawa T, Kusumoto K. Platelet-rich plasma releasate promotes angiogenesis in vitro and in vivo. *Med Mol Morphol* 2014; **47**: 83-89 [PMID: 23604952 DOI: 10.1007/s00795-013-0045-9]
38 **Kakudo N**, Morimoto N, Ogawa T, Hihara M, Notodihardjo PV, Matsui M, Tabata Y, Kusumoto K. Angiogenic effect of platelet-rich plasma combined with gelatin hydrogel granules injected into murine subcutis. *J Tissue Eng Regen Med* 2017; **11**: 1941-1948 [PMID: 26489691 DOI: 10.1002/term.2091]
39 **Etulain J**, Mena HA, Meiss RP, Frechtel G, Gutt S, Negrotto S, Schattner M. An optimised protocol for platelet-rich plasma preparation to improve its angiogenic and regenerative properties. *Sci Rep* 2018; **8**: 1513 [PMID: 29367608 DOI: 10.1038/s41598-018-19419-6]
40 **Etulain J**, Mena HA, Negrotto S, Schattner M. Stimulation of PAR-1 or PAR-4 promotes similar pattern of VEGF and endostatin release and pro-angiogenic responses mediated by human platelets. *Platelets* 2015; **26**: 799-804 [PMID: 26082997 DOI: 10.3109/09537104.2015.1051953]
41 **Eppler SM**, Combs DL, Henry TD, Lopez JJ, Ellis SG, Yi JH, Annex BH, McCluskey ER, Zioncheck TF. A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. *Clin Pharmacol Ther* 2002; **72**: 20-32 [PMID: 12152001 DOI: 10.1067/mcp.2002.126179]
42 **Xie J**, Wang H, Wang Y, Ren F, Yi W, Zhao K, Li Z, Zhao Q, Liu Z, Wu H, Gu C, Yi D. Induction of angiogenesis by controlled delivery of vascular endothelial growth factor using nanoparticles. *Cardiovasc Ther* 2013; **31**: e12-e18 [PMID: 22954162 DOI: 10.1111/j.1755-5922.2012.00317.x]
43 **Doi K**, Ikeda T, Marui A, Kushibiki T, Arai Y, Hirose K, Soga Y, Iwakura A, Ueyama K, Yamahara K, Itoh H, Nishimura K, Tabata Y, Komeda M. Enhanced angiogenesis by gelatin hydrogels incorporating basic fibroblast growth factor in rabbit model of hind limb ischemia. *Heart Vessels* 2007; **22**: 104-108 [PMID: 17390205 DOI: 10.1007/s00380-006-0934-0]
44 **Moon MH**, Kim SY, Kim YJ, Kim SJ, Lee JB, Bae YC, Sung SM, Jung JS. Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. *Cell Physiol Biochem*2006; **17**: 279-290 [PMID: 16791003 DOI: 10.1159/000094140]
45 **Kondo K**, Shintani S, Shibata R, Murakami H, Murakami R, Imaizumi M, Kitagawa Y, Murohara T. Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis. *Arterioscler Thromb Vasc Biol* 2009; **29**: 61-66 [PMID: 18974384 DOI: 10.1161/ATVBAHA.108.166496]
46 **Kamihata H**, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, Masaki H, Mori Y, Iba O, Tateishi E, Kosaki A, Shintani S, Murohara T, Imaizumi T, Iwasaka T. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation* 2001; **104**: 1046-1052 [PMID: 11524400 DOI: 10.1161/hc3501.093817]
47 **Yu WY**, Sun W, Yu DJ, Zhao TL, Wu LJ, Zhuang HR. Adipose-derived stem cells improve neovascularization in ischemic flaps in diabetic mellitus through HIF-1α/VEGF pathway. *Eur Rev Med Pharmacol Sci* 2018; **22**: 10-16 [PMID: 29364466 DOI: 10.26355/eurrev_201801_14094]
48 **Rehman J**, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004; **109**: 1292-1298 [PMID: 14993122 DOI: 10.1161/01.CIR.0000121425.42966.F1]

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