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REVIEW

Translational products of adipose tissue-derived mesenchymal stem cells: Bench to bedside applications

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Abstract

With developments in the field of tissue engineering and regenerative medicine, the use of biological products for the treatment of various disorders has come into the limelight among researchers and clinicians. Among all the available biological tissues, research and exploration of adipose tissue have become more robust. Adipose tissue engineering aims to develop by-products and their substitutes for their regenerative and immunomodulatory potential. The use of biodegradable scaffolds along with adipose tissue products has a major role in cellular growth, proliferation, and differentiation. Adipose tissue, apart from being the powerhouse of energy storage, also functions as the largest endocrine organ, with the release of various adipokines. The progenitor cells among the heterogeneous population in the adipose tissue are of paramount importance as they determine the capacity of regeneration of these tissues. The results of adipose-derived stem-cell assisted fat grafting to provide numerous growth factors and adipokines that improve vasculogenesis, fat graft integration, and survival within the recipient tissue and promote the regeneration of tissue are promising. Adipose tissue gives rise to various by-products upon processing. This article highlights the

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significance and the usage of various adipose tissue by-products, their individual characteristics, and their clinical applications.

Key Words: Adipose tissue; Stem cells; Fat graft; Clinical applications; Mesenchymal stem cells

Core Tip: Promising evidence supports clinical application of derivatives of adipose-derived stem cells enriched with numerous growth factors and adipokines for improved vasculogenesis, graft integration, and survival within the recipient tissue. Analysis of its differential characteristics and practical applications became a necessity. In this review, we highlight the significance and usage of various adipose tissue by-products, their individual characteristics, and their clinical applications along with the evidence supporting its use.

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INTRODUCTION

With developments in the field of tissue engineering and regenerative medicine, the use of biological products for the treatment of various disorders has come into the limelight among researchers and clinicians. Among all the biological products, keen interest has been shown in adipose tissue and its by-products for translation from bench to clinical applications, with due consideration to its various unique properties. Because of the heterogeneous cellular population of adipose tissue, adipose tissue-derived products possess a greater advantage of regeneration compared with bone marrow-derived products[1,2].

Traditionally adipose tissue or fat graft transfer was practiced for elective cosmetic procedures and plastic and reconstructive surgery[3]. Because of the regeneration potential possessed by adipose tissue, use has become widespread in cosmetic-dermatological procedures, facial rejuvenation, breast and buttocks augmentation, and genital aesthetics[4-6]. Cellular viability and survival at the transplanted site depends on various factors involving the recipient site, donor site, laboratory processing, and manipulation[7,8].

Adipose tissue engineering aims to develop by-products and their substitutes for regeneration and restoring function[9]. That further eliminates the need for organ transplants and mechanical device placement. The use of biodegradable scaffolds along with adipose tissue products has a major role in cellular growth, proliferation, and differentiation[10,11]. Once implanted at an appropriate site, scaffolds degrade, progenitor cells proliferate with the help of growth factors and cytokines to form new tissue. Preclinical studies have investigated the ability of biomolecules and biodegradable three-dimensional scaffolds to interact with adipose tissue products to promote the adipogenesis of stem cells in vitro[12-15]. In this review, we discuss the differential characteristics of adipose tissue derivatives and elucidate their applications in clinical scenarios.

ADIPOSE TISSUE BIOLOGY

An adipocyte is usually 50-150 mm in diameter, it dies from ischemia if it grows larger, and its life expectancy varies from few months to 10 years in humans[16]. Adipose tissue, a specialized connective tissue with lipid-rich adipocytes, it contains a heterogeneous population of cells but adipocytes represents only 20% or less of the cellular mixture[17]. Based on adipocyte morphology, the two types of adipose tissue are

white adipose tissue found in adults and brown adipose tissue found in newborns [18]. Adipose tissue, apart from being the powerhouse of energy storage, also functions as the largest endocrine organ, with the release of various adipokines. Adipose tissue-derived adipokines include leptin, adiponectin, apelin, chemerin, interleukin (IL)-6, 8, and 10; monocyte chemoattractant protein (MCP)-1, plasminogen activator inhibitor (PAI)-1, retinol binding protein (RBP)-4, tumor necrosis factor (TNF)-α, progranulin, complement C1q tumor necrosis factor-related protein (CTRP)-4, interferon (IFN)-γ, and interferon-γ-inducible protein (IP)-10, which are readily available sources to induce stem cells[19-22]. The adipokines work in a paracrine fashion when transplanted as a cellular therapeutic tool[23,24]. The components of adipose tissue are lipid-laden adipocytes, fibroblasts, neural and vascular progenitor cells, multipotent progenitor cells, pericytes, extracellular matrices, cytokines, growth factors, and immune cells such as CD4+ T cells, as shown in Figure 1. The progenitor cells in adipose tissue are of paramount importance, as they have the capacity for regeneration of these tissues. The progenitor cells differentiate into mesodermal, ectodermal, and endodermal lineages[25-27]. Tissue engineering experts focus on adipose tissue and its products for their plasticity, relative ease of harvest, and potential autologous usage. Stem cells and progenitor cells from freshly prepared SVFs constitute up to 3%, which represents 2500-fold more than the stem cells isolated from bone marrow source (0.002%)[28].

The literature on adipose tissue survival and regeneration depicts "cell survival theory" and "cell replacement theory"[29-32]. Various studies have proven the promising results of adipose-derived stem cell (ADSC)-assisted fat grafting, which provides numerous growth factors and adipokines for improved vasculogenesis, fat graft integration, and survival within the recipient tissue[8,33]. Adipocytes contain three zones namely, (1) the outer surviving layer (100–300 microns), (2) middle regenerating layer (600–1200 microns), and (3) inner necrotic layer, as shown in Figure 2[30-32,34].

Yoshimura *et al*[35] demonstrated the sequence of changes that happen after grafting or transplantation of adipose tissue. In preclinical studies, it is shown that all adipocytes undergo apoptosis in the initial few days of grafting. Activation of adipogenesis was by adipose-derived progenitor cells, which were augmented by the adipokines at 3 mo after grafting. By the end of 9 mo, lipid droplets were absorbed by macrophages. The final fat graft retention at the recipient site was determined by the rate of successful replacement of the adipocytes[35]. ADSCs possess various advantages over bone marrow-derived mesenchymal stem cells (BM-MSCs)[36-38]. Harvesting adipose tissue by liposuction is less painful than bone marrow aspiration [39,40]. The quantity of stem/stromal cells obtained from adipose tissue than that obtained from bone marrow[41]. In long-term culture, ADSCs are more genetically stable[42,43].

Characterization of ADSCs

Researchers demonstrated that ADSCs have a consistent phenotyp and reproductive capacity, based on cellular yield, cellular viability, adipocyte differentiation, and cell surface markers[44-46]. During initial culture, ADSCs are polygonal cells that adhere to the flask surface. They exhibit fibroblastic plastic morphology and expand in in vitro cultures. within 2 d of primary culture, 90% of cells become confluent when subcultured within 2 d of primary culture with a demonstrated ADSC yield of 87% and viability of 94%[47]. Luna *et al*[47] recovered 1×10^6 adipocytes, 1×10^6 ADSCs, 1×10^6 vascular endothelial cells, and 1×10^6 other cells from 1 g of adipose tissue.

During in vitro culture, the ADSCs immunophenotype changes from $CD34^+$, major histocompatibility complex (MHC) I and II molecules, $CD80^+$, $CD86^+$, $CD45^+$, $CD11a^+$, $CD14^+$, $CD117^+$, human lymphocyte antigen (HLA)-DR^+, NOG^+, undifferentiated embryonic cell transcription factor (UTF)1^+, $WNT6^+$, and $WNT8A^+$ to increased expression of $CD9^+$, $CD13^+$, $CD29^+$, $CD44^+$, $CD63^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD166^+$, bone mor-phogenetic protein receptor (BMPR)2^+, collagen type VI alpha 2 chain (COL6A2)$^+$, transforming growth factor (TGF)-β$R1^+$, and vascular endothelial growth factor (VEGF)-A^+[48-52]. The lack of expression of HLA class I and II molecules in coculture and serial passages, confers ADSCs the property of immunosuppression and make them suitable for allogenic transplantation[53-55].

MSCs from adipose tissue have strong positive expression of STRO-1^+, $CD29^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD166^+$ & $CD44^+$ and weak positive expression for $CD34^+$ and $CD45^+$[56-57]. MSCs possess enhanced angiogenesis associated with the increased expression of $CD105^+$ and $CD34^+$. Proliferation and differentiation of MSCs is enhanced by $CD9^+$, $CD29^+$, $CD44^+$, $CD49d^+$, and $CD106^+$[58-61]. Gronthos *et al*[62] demon-strated that adipose tissue-derived stroma-vascular fraction (SVF) support

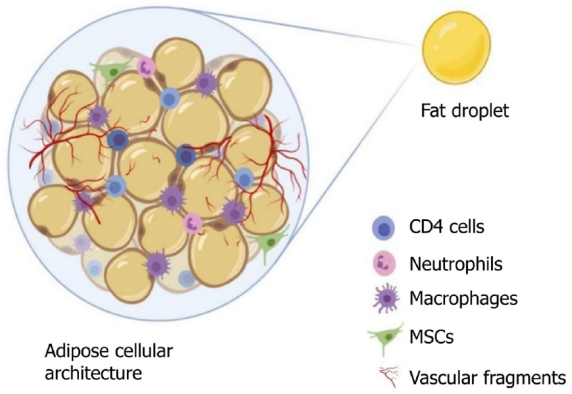

Figure 1 Organizational structure of adipose tissue. MSCs: Mesenchymal stem cells.

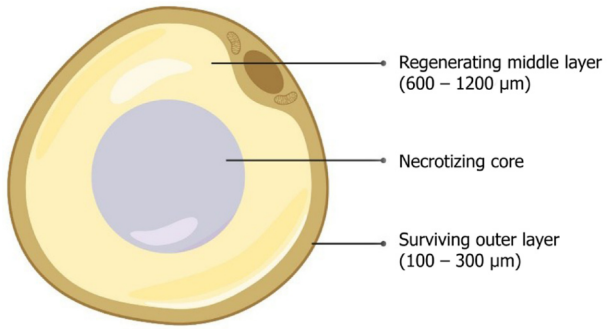

Figure 2 Zones of an adipocyte.

hematopoiesis in vitro. After about 8–12 cellular doublings in culture, ADSCs express $CD34^+$[63]. Various theories are available for the attribution of stem cell properties to pericytes. Szöke *et al*[64] stated that pericytes are present in both MSCs and ADSCs whereas Traktuev *et al*[65] and Crisan *et al*[66] stated that $CD34^+$ and $CD34^-$ pericytes are identical to adipose-derived stem cells.

Immunomodulation of ADSCs

The cellular components of ADSC induce and activate quiescent native MSCs to secrete biological micromolecules at the site of injury to establish local homeostasis by increasing the permeability of cells at the injury site, downregulating inflammatory processes, and recognition of host-tissue progenitor cells for final differentiation into the cells of interest in the injured tissue[67]. Therefore, ADSCs activate adaptive cellular responses and secrete IL-1Ra, IL-1β, PGE2, IDO, IL-4 & -10, and TGF-β that modulate and prime the native immune cells. The micromolecular interactions lead to a cascade of events responsible for immunotolerance of engraftment to a foreign site.

The immunotolerant/immunosuppressive/immunomodulatory activity of ADSCs is caused by the interplay of regulatory T cells, cytotoxic T cells, and B cells as shown in Figure 3. Immunomodulatory activity includes the inhibition of INF-γ and TNF-α production by effector T lymphocytic cells, upregulation of IL-4 and IL-10 by native immune cells, and inhibition of proliferation, migration, and differentiation of B cells that produce immunoglobulins[68]. The activity of natural killer cells is suppressed by the production of indolamine 2,3-dioxygenase by MSC-like cells[69,70]. Dendritic cells enhance the expression of IL-4 IL-10 and suppressed INF-γ and TNF-α[71,72]. The cascade of events directly suppresses tissue inhibitors of matrix metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs), resulting in the conversion of the proinflammatory environment to an anti-inflammatory environment. Adipose-derived MSCs secrete extracellular vesicles such as exosomes as a channel of communication with neighboring cells[73]. They act not only *via* direct cell-to-cell interaction but also *via* paracrine mechanisms[74] and are key mediators of signaling molecules such as TGF-β1[75], IL-10[76], PGE2[77], NO[78] and IDO[79,80]. The bioactive factors in-turn help to promote tissue regeneration and repair at the site of action.

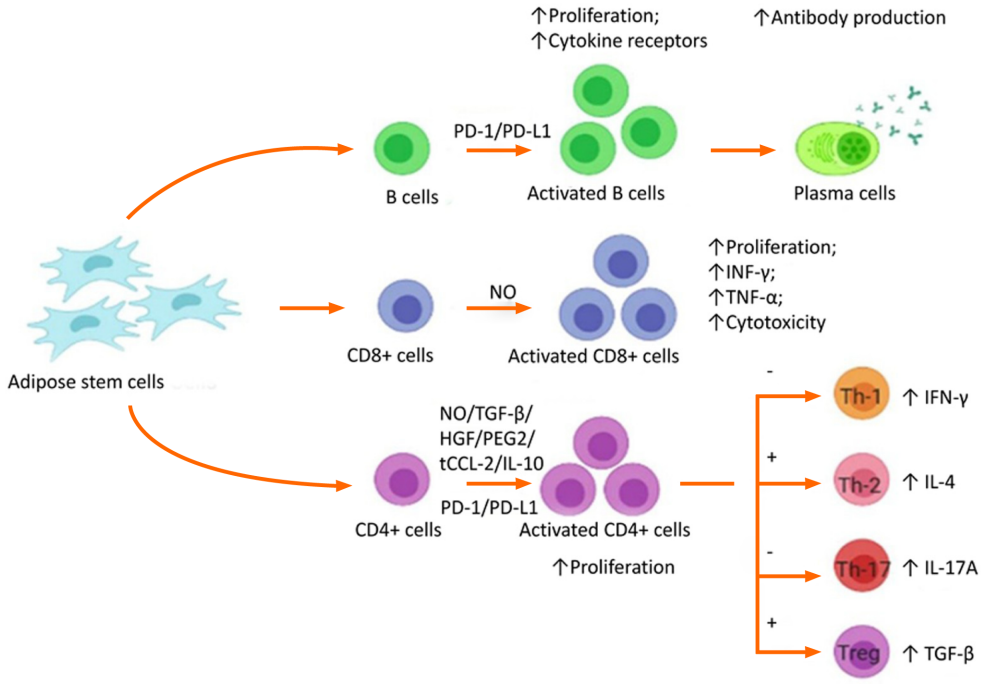

Figure 3 Immunomodulatory effects of mesenchymal stem cells *via* T and B lymphocyte system.

Derivatives and applications of adipose tissue

The various derivatives of adipose tissue that are of practical utility, with therapeutic potential are shown in Figure 4 and are discussed below:

Adipose stem cells: ADSCs are one of the forms of MSCs of adipose origin with an inherent property of self-renewal and multipotent differentiation[73,81]. Advantages of easy accessibility of the source, abundant availability, and fewer ethical concerns than embryonic stem cells, render ADSCs more suitable for use in regenerative medicine and tissue engineering. Tissue-engineered 3D scaffolds with ADSCs and biomolecules such as growth factors and extracellular matrix materials play a robust role in treating various disorders by cellular proliferation and differentiation[4]. Studies found that ADSCs have more grounded immunomodulatory impact than BM-MSCs[82-84]. Adipose tissue contains stem and progenitor cells in amounts of up to 3% of the uncultured SVF, which is 2500 times more than the stem cells obtained from bone marrow[85,86]. Depending on the donor and tissue harvesting site, lipoaspirates yield 1% to 10% stem cells, which is approximately 0.5×10^4 to 2×10^5 MSC/g adipose tissue[87]. ADSCs act in paracrine fashion by releasing adipocytokines, cytokines, and growth factors that form a secretome[88].

Following liposuction, adipose tissue is washed in phosphate-buffered saline containing 5% antibiotic, followed by tissue digestion by collagenase-1[89]. After the disintegration of adipose tissue, the resultant sample is transferred to tubes and centrifuged at 2000 rpm for 5 min to obtain a SVF that contains the ADSCs. The resultant cellular pellet is resuspended in 1 mL lysis buffer, recentrifuged at 2000 rpm for 5 min and the resulting cellular suspension is passed through a 70 μm cell strainer. The cellular mixture is transferred to lysine-coated culture plates and incubate at 37 °C with 5% CO_2. To obtain ADSCs, about 500 mg of the adipose tissue cell suspension is inoculated in the wells[87,90]. According to International Fat Applied Technology Society, uncultured ADSCs express $CD34^+$, $CD45^-$, $CD235a^-$ and $CD31^-$[63,91], whereas cultured ADSCs express $CD73^+$, $CD90^+$, $CD105^+$, $CD44^+$, $CD45^-$ and $CD31^-$[91,93].

The short- and long-term storage of ADSCs have been investigated. The cellular proliferative capacity of ADSCs decreases with the length of storage[43]. Hence, they must be supplemented with 10% human serum or platelet-rich plasma (PRP) in 0.9% saline at 4 °C for the first 2 h and not more than 4 h[1,94]. For long-term storage, ADSCs can be stored at −80 °C in liquid nitrogen for up to 6 mo[95,96]. Certain strategies can be followed to enhance and optimize the potential of the ADSC, such as culturing them in hypoxic conditions, which not only enhances the immunomodulatory effect but also reduces the risk of chromosomal aberration and tumorigenesis [97]. Further, the cells can be cryopreserved in the early passages for future usage

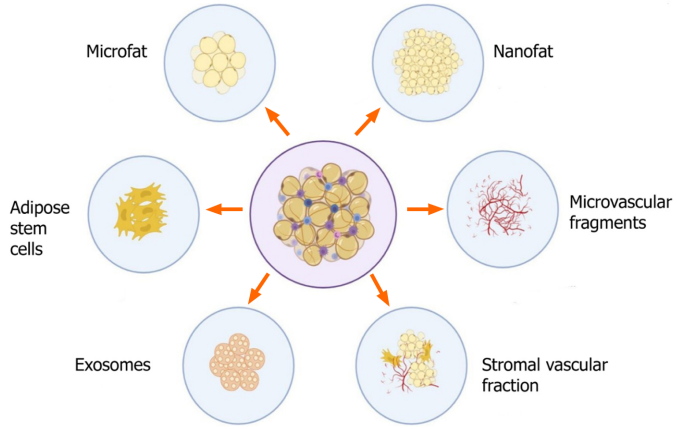

Figure 4 Derivatives of the adipose tissue complex.

thereby making them available for future use[98].

ADSCs are used in cardiac tissue engineering where they enhance regeneration of myocardial tissues, improve left ventricular ejection fraction, and reduce the scar volume in ventricular wall in rodent models[99]. ADSCs are seeded as bioscaffold in the healing of cutaneous ulcers and soft tissue injuries[100,101]. Several studies have proven the beneficial role of ADSCs in multiple sclerosis, diabetes mellitus, and rheumatoid arthritis. The anti-inflammatory and immunomodulatory effects of ASCs have been demonstrated in various preclinical models of autoimmune diseases[102]. ADSCs have regenerative potential for bone and cartilage healing in addition to electrical stimulation of cells and tissues[103]. Various researchers across the globe have been working on the therapeutic efficacy of ADSCs in osteoarthritis knees and hips[104-106]. Agostini *et al*[107] developed a protocol for ADSC application in osteoarthritis in terms of isolation, dose, frequency, analysis, and follow-up. There is no consensus on the use of cultured *vs* uncultured cells in the management of osteoarthritis[106,108]. ADSCs have been used in sports injuries of ligaments and tendons[109,110], but evidence to support the safety and efficacy is lacking.

The potency of the stem cells to differentiate into various lineages including nerve cells and nervous tissue makes them a good candidate for use in neurological disorders. Cultured and uncultured cells have been used in the treatment of various neurological diseases such as Alzheimer's disease, Parkinson's disease, intervertebral disc, amyotrophic lateral sclerosis, multiple system atrophy, post-polio residual paralysis and traumatic brain injury[111-114]. The cells enhanced neurovasculogenesis, counterfeit fibrosis, oxidative stress, anti-inflammation, and neuromodulation. In neurological diseases, the research toward the use of ADSCs is ongoing in animal models.

Microfat: Compared with nanofat, microfat appears deep yellow, with a fine granular structure and intact three-dimensional adipose tissue architecture[115]. Microfat is composed of mature adipocytes, SVF cells, pericytes, capillary fragments, and fibrous scaffolds are well preserved[116-118]. Hence, they are a natural recipient site for survival of grafts, and provide a niche for SVF cellular mixtures for tissue regeneration and rejuvenation. Yang *et al*[117] harvested microfat from adipose tissue with cannulas that had side holes of less than 1 mm. Examination of the harvests revealed intact fat lobular structures without need of any inter-syringe passages of emulsification procedures. Side holes of < 0.8 mm allowed the processed microfat to easily pass through the needles without any blockage. Such harvests, used for skin and facial rejuvenation through 27 gauge needles, revealed the preservation of micro-functional units of fat tissue that retained the regenerative properties of ADSCs[117]. Caggiati *et al*[119] demonstrated a higher yield of ADSCs from lipoaspirates harvested by barbed compared with blunt cannulas, but they found that barbed cannulas cut the fibrous septum and produced higher quantities of coarse fibers that increased the probability of molecular blockage in the needle. The cellular components in the microfat induced tissue regeneration through the recruitment of monocyte/macrophage differentiation at the recipient site[35,120]. The cellular yield in microfat (2.28 ± 1.90) × 10^5 cells/mL is higher than the yield in nanofat (4.12 ± 1.37) × 10^4 cells/mL, indicating that nanofat involves mechanical emulsification[117]. Microfat components integrate into the local environment because of the intact microintegrity of cellular transplant, with inherent

resistance to attack by host immune-mediated cells[121].

Microfat possess various advantages. (1) No mechanical emulsification or centrifugation is involved in preparation, other than the shearing forces during harvesting. (2) Intact vascular fragments with viable cellular components are present in the mixture. (3) There is less ischemic exposure time during adipose tissue harvest and while preparing the microfat graft. (4) Preservation of viable adipocytes that restore the degenerated and atrophied skin and subcutaneous tissues[117]. Compared with other adipose derivatives, microfat retains the three-dimensional architecture of the native tissue essential for the survival of mature adipocytes, thereby providing a natural niche for ADSC cells to ensure optimal tissue regeneration. Microfat is used in esthetic procedures. Because the three-dimensional architecture is retained in microfat, it is used as a lipofiller in breast reconstruction and facial and gluteal augmentation[122-125]. Microfat is also being tried in scar revision, burn injuries, lipodystrophies, rhinoplasty and wrinkles[3,40,117,126,127].

Nanofat: In 2013, Tonnard *et al*[128] developed nanofat, which is an ultrapurified adipose tissue-derived product that is devoid of mature adipocytes but contains CD34+ ADSCs, microvascular fragments, growth factors, biological peptides, and cytokines[93,94]. It is a liquefied, autologous injectable product with the property of biological integration with adjacent cells and tissues because of its homogenous consistency[129]. The size of nanofat components is approximately 400 to 600 μm [130]. Nanofat behaves much like adipose tissue-derived mesenchymal stromal cells. At the site of injury, stromal cells initiate a site-specific reparative response comprised of remodeling the extracellular matrix (ECM), enhanced and sustained angiogenesis, and immune system modulation. Because of the multi- and pluripotent nature of the cellular components in nanofat, it possesses the ability to differentiate into multiple lineages. Hence, nanofat can be used in preclinical and translational research in tissue engineering. Various preclinical and clinical studies have demonstrated antifibrotic, proangiogenic, neuroregenerative properties, and enhanced collagen deposition potential of adipose tissue-derived nanofat[131-134]. Apart from being an adipogenic derivative with ADSC, the proportion of ADSCs in nanofat is higher than that in microfat. The differences might be attributable to the method of preparation of the microfat, where the fibers and their accompanying capillaries that were the location of ADSCs are removed. In contrast, the ADSCs were mechanically separated from the native site and concentrated in nanofat, thereby making it more effective in terms of the number of ADSCs delivered to the target site. Sesé *et al*[135] estimated the total cellular load in mechanically prepared nanofat as 6.63 million cells/g of lipoaspirate whereas in enzymatically disintegrated SVF it was 0.68 million cells/g of lipoaspirate. The nucleated cellular count was 70% in nanofat and 7.3% in SVF. The cellular burden in nanofat contains predominantly the stromal cellular population[136].

Nanofat grafting enhances neoangiogenesis without producing any visible scars, and provides a favorable outcome in esthetic medicine for breast, buttocks, and genital augmentation, facial rejuvenation, and facial volume augmentation[137,138]. Nanofat injections retract the atrophic scars because of the presence of adipose tissue-derived stromal cells, and avoids the need for surgical procedures. Nanofat components can regenerate dermis and subcutaneous fatty tissues and enhance the dermo-epidermal junction[139]. They regenerate by laying down adipose tissue-derived ECM, collagen deposition, and neoangiogenesis. Klinger *et al*[140] reported that autologous fat grafting allowed the regeneration of skin that was soft, flexible, and matched the color of neighboring skin. This concept of skin rejuvenation can be extrapolated to scars present in joints, eyelids, the face, and mouth.

Nanofat grafting beneath and within the substance of the scar improves the quality, integrity, and texture of burn scars[141]. Improved skin texture, elasticity, skin moisture, facial rejuvenation, and anti-aging properties can be achieved by combining nanofat (autocrine and paracrine effects) with platelet-rich fibrin (PRF)[142]. In a preclinical trial, nanofat injection improved the thickness of the dermal layer and promoted angiogenesis in the photoaged skin of nude mice[143]. A wide range of improvements in wrinkles, discolorations, and burn scars have been seen with nanofat application[141]. Esthetically, nanofat grafting is used for the correction of dark circles [144,145], malar bags[3], hollow eyes[145], and blepharoplasty[146]. Because of fat atrophy in the aging process, nanofat has emerged as a plausible technique for facial rejuvenation[126,147,148]. Nanofat is also being increasingly used in primary rhinoplasty procedures[149]. Nanofat is being used to correct slight skin irregularities that do not require cartilage grafting[127,150]. Segreto *et al*[151] evaluated the role of a combination of nanofat grafting with autologous PRP in chronic nonhealing infected

wounds, where it enhanced the regeneration of soft tissue. The multi-differentiation potential of adipose tissue, which is a component in nanofat grafting, allows evaluation in avascular necrosis of femoral head, mild to moderate grades of osteoarthritis of the knees, tendinopathies, and nonunion of fractures. Preclinical and clinical studies of nanofat use have proven its regenerative capacity in various clinical settings.

Microvascular fragments: Microvascular fragments (MVFs), the byproduct of adipose tissue, range from 40–180 mm, and are composed of arteriolar, capillary, and vein segments[152-154]. MVFs release VEGF and basic fibroblast growth factor (bFGF) under culture conditions[155]. They are the richest source of proangiogenic factors that induce vasculogenesis in a paracrine fashion[154,156,157]. MVF contains Sca 1/VEGFR-2+ endothelial progenitor cells and MSCs expressing CD44+, CD73+, CD90+ and CD117+[158]. The components of MVF exhibit the morphology of intact lumen, endothelium, and perivascular stabilizing cells. MVF are the building blocks for therapeutic vasculogenesis[159].

It was initially speculated that the high vascularization potential of MVFs is mainly derived from stem cell populations. McDaniel *et al*[160] compared the regenerative properties of conventionally isolated adipose-derived stem cells and multipotent cells derived from an explant culture of microvascular fragments. They found that the latter source exhibited a higher proliferation rate, an increased expression of genes involved in differentiation, and an improved ability to form capillary-like structures. In line with the concept of the "stem cell niche," the findings indicated that compared with single-cell isolates, microvascular fragments including stem cell components provides a more physiological environment that maximizes the regenerative activity. Transplanted or injected MVFs rapidly integrate with native tissues to promote neoangiogenesis in the physiological environment[161]. The three phases of MVF-induced neoangiogenesis are (1) immature vascular segments with high proliferation capacity; (2) vascular remodeling with a high rate of apoptosis; and (3) vascular maturation with microvascular network organization[162,163]. The complex pattern of neoangiogenesis is associated with the upregulation of angiogenic genes.

Researchers have observed the regenerative potential in cartilage defects and skeletal muscle injury[164-167], myocardial infarction[152,163,168], partial- or complete-thickness skin defects[169,170] and diabetes mellitus[171]. MVF loaded scaffolds can reverse lymphatic network disorders by reducing edema formation and promoting vasculogenesis in the area of repair[154,172-174]. With the available, diverse potentials of MVF, the application of MVF in the clinical setting is plausible with the imperative question of technical and regulatory compliance from bench to bedside.

SVF: SVF is an ultra-byproduct of adipose tissue through various processing methods [175-177]. The development of biocellular regenerative medicine and cellular biology, has increased the use of SVF by regenerative surgeons and researchers. The components of SVF are MSCs, HSCs, T-regulatory cells, pericyte endothelial cells, mast cells, complex microvascular beds with fibroblasts, WBC, dendritic cells, intra-adventitial smooth muscular-like cells, and others, and extracellular matrix[44,177-179]. A sufficient number of SVF cells can be obtained without culture. Current restrictions on the use of cultured cells in humans cite the possibilities of contamination, tumorigenesis, unexpected cell differentiation, and limited cell sources. SVFs behave much like BM-MSCs. SVF mixtures contain 30% MSCs, 3% endothelial cells, and 14% endothelial precursor cells[180]. BM-MSCs contain 0.001% MSCs, 0.1% endothelial cells, and 2% endothelial precursor cells[181]. About 2% of isolated SVF express the hematopoietic markers $CD34^+$ and $CD\ 45^+$ and 7% express the MSC markers $CD105^+$, and $CD146^+$[92,182,183]. SVF cells express cell surface markers similar to those of BM-MSCs, such as $CD105^+/SH2^+$, $CD90^+$, $CD29^+$, $CD44^+$, $CD71^+$, and $SH3^+$, along with low expression of $CD31^+$, $CD45^+$, and $CD24^+$[184-186].

Various methods of isolation of SVF have been described in the literature[90,187-190]. SVF isolation needs fat obtained by liposuction and transportation to a cGMP certified laboratory for further processing[191-193]. The global researchers and clinicians widely use the enzymatic method for isolating and harvesting SVF by collagenase enzymes. After the addition of collagenase enzyme to the lipoaspirate, digestion and disintegration of adipose cells take place and result in the formation of an aqueous mixture including two phases, a floating adipocyte fraction and precipitated cellular components[194,195]. The separation can be enhanced by density gradient centrifugation and filtration[191]. The lower portion of the tube contains a yellowish red pellet which is the SVF mixture that can be used for in vitro expansion

[196]. Because of the ethical issues associated with SVF isolation by the enzymatic method, researchers opt for alternative methods for SVF isolation[197,198]. Researchers proposed mechanical agitation and disruption of adipose tissue, which releases a mixture of cellular components and stromal cells. The proportion of cells in the SVF obtained by mechanical disruption is lower that obtained by enzymatic degradation, but the regenerative potential of SVF mixtures obtained by mechanical disruption and enzymatic degradation are the same[198]. The anti-inflammatory effects of SVF result from the presence of higher amounts of IL-1 and 10 receptor antagonists, which are expressed by TNF-α and leptin released by monocytes and macrophages in the SVF mixture[199]. SVF mixtures provoke adaptive immunity by producing T-reg cells by suppressing the activation of dendritic cells[200,201].

Krawiec *et al*[202] demonstrated that SVF components seeded with biodegradable porous scaffolds *in vivo* for 8 wk resulted in the generation of tissue-engineered vascular grafts when populated with primary vascular components such as smooth muscle cells, endothelial cells, collagen, and elastin. ADSCs or SVF combined with PRP enhanced the regenerative potential in the form of neoangiogenesis, cellular proliferation, and differentiation[203]. The combination of SVF with PRP enhanced the stemness of MSCs, and the growth factors present in PRP induced locally available stem cells and prolonged the survival time and survival rates of cells present in the PRP[204]. Such combination treatments have been evaluated in preclinical and clinical trials of wound healing, osteoarthritis of the knees, bone and tendon regeneration, periodontal engineering, fat grafting procedures, and vascular diseases[203,205-208]. Osteochondral defects of the knees in goats were managed by the application of acellular type 1 and 3 collagen scaffolds along with SVF cells[209]. In rat models of acute kidney injury, the transplantation of autologous SVF cells induced antiapoptotic effects and the release of growth factors like VEGF and HGF to regenerate kidney cells [210]. In preclinical studies, the therapeutic effect, safety and functional outcome of autologous SVF uncultured or culture-expanded cells were observed in acute myocardial infarction, skin flap necrosis, and erectile dysfunction with cavernous nerve injury[211-213]. In human clinical studies, the use of SVF cells was extensively studied in conditions like breast reconstruction surgery, traumatic calvaria defects, types 1 and 2 diabetes mellitus, Crohn's disease with enterocutaneous fistula, and burn injuries[214-218]. Zhao *et al*[219] evaluated the efficacy of SVF in diabetic feet in terms of cellular survival, proliferation rate and differentiation and they determined the characteristics of transplanted cells. Various researchers demonstrated endobronchial administration of autologous SVF cells for treating idiopathic pulmonary fibrosis[220]. Autologous SVF is also being evaluated in COVID-19 individuals[221-223]. When cocultured with 5% or 20% PRP, the viability, motility, proliferation rate, and differentiation of ADSCs were increased[224]. In the case of fat grafting, clinicians are challenged by the fat graft rejection, with a reported resorption rate ranging from 25%-80%, which is mostly the result of apoptosis of mature adipocytes[225]. When the lipoaspirate was given along with SVF, a 35% greater graft retention was noted[194]. Further, more prominent microvasculature was noted compared with normal graft tissue, suggesting its clinical potential as a nourishing medium that enhanced the efficacy of the native therapy. Moreover, in situations such as osteoarthritis, an ultrafiltrate fraction of an adipose derivative such as SVF would be the ideal medium of choice to tap the benefits of ADSCs in cartilage regeneration[226].

ADSC exosomes: Exosomes are the cell-free regenerative tool in the field of tissue engineering. Exosomes are extracellular vehicles that are endosome-derived lipid bilayer spherical vesicles of 40 to 150 nm in size. They are found in all cell types and body fluids and are comprised of cytokines, proteins, lipids, DNA, and RNA from the parent cell. Exosomes are an integral part of both the diagnostic and therapeutic methods used in various diseases. ADSC exosomes (EXOs) differ from other MSC EXOs in proliferation and differentiation abilities and immunosuppressive pathways. Compared with ADSCs, ADSC-derived exosomes possess have a high biosafety profile with low immunogenicity[227]. They protect cargoes from degradation, have tissue and target specificity, good tissue permeability, intercellular signaling and communication, immune function, tissue homeostasis, and development of cell fate [227].

Ogawa *et al*[228] observed that mRNAs of adipose tissue-derived exosomes have a significant role in metabolic, immunological, and cellular responses. Various studies demonstrated that adipose tissue-derived exosomes possess the capability of regeneration of muscles and bones, promotion of wound healing, and enhancement of cellular proliferation, and neoangiogenesis. ADSCs-EXOs enhance the proliferation and migration of vascular endothelial cells and promote vasculogenesis. They retain

the fat graft volume by the stimulation of angiogenesis and regulating inflammatory responses[229,230]. ADSC EXOs were tested by Wang *et al*[231] for the promotion of wound healing in diabetic mice by enhancing angiogenesis, proliferation of fibroblasts, and collagen synthesis in the later stages. The evidence also supports the use of ADSC EXOs for treating diabetic foot patients[232]. To stabilize the ADSC EXOs concentration when used for local application, bioscaffolds like hydrogels or fibrin are augmented with exosomes to delay release at the therapeutic site[233,234]. ADSC EXOs are used for scarless cutaneous repair by retracting the size of scars, increasing the collagen 3 to collagen 1 ratio, and by regulating the migration, proliferation, differentiation, and gene expression of fibroblasts[235]. Kim *et al*[236] demonstrated that key cytokines and growth factors in ADSC EXOs facilitated tissue regeneration and repair through anti-oxidation, anti-wrinkle and skin-whitening activity. ADSC EXOs with multiple bioactive molecules for the management of aging warrants further extensive research. Zhao *et al*[237] demonstrated crosstalk that facilitated immune and metabolic homeostasis, providing a vital therapy for obesity and diabetes mellitus. ADSC EXOs combined with poly(lactide-co-glycolide) scaffolds improved the osteoinductive effects, MSC migration, and homing abilities in bone regeneration[238]. Chen *et al*[239] demonstrated that exosomes derived from miR-375-overexpressing ADSCs incorporated with hydrogel enhanced bone regeneration in a rat model of calvarial defect. Direct stem cell transplantation with ADSC EXOs primed by TNF-α, promoted the proliferation and differentiation of human osteoblasts promoted through the Wnt signaling pathway, which further widened the application of exosomes in bone regeneration[240]. Pers *et al*[241,242] reported that ADSC EXOs were a safe, effective, and inexpensive therapy for osteoarthritic knees. ADSC EXOs downregulated inflammation and oxidative stress in osteoarthritic knees[243]. Zhang *et al*[244] confirmed that intra-articular injection of ADSC EXOs inhibited cartilage and subchondral bone degradation and osteophyte formation and slowed the progression of the disease process in osteoarthritis. ADSC EXOs participated cellular communications and applications in plastic and cosmetic surgery. Although applications in clinical practice are lacking, ADSC EXOs have an increasing role in maximizing the therapeutic effectiveness for dermopathies and for tissue reconstruction[245]. There is a future for exosomes as a diagnostic tool for early identification of pathological processes involved in disease and as a therapeutic, targeted acellular conduit to optimize the pathological milieu at the site of interest.

Ethical concerns

An early therapy and drug development are dependent on translational phases, the best strategy is to standardize the process, and regularly acquire data to validate and certify the technologies being developed. The safety and efficacy of the developed therapy must be proven from various perspectives, including the donor, recipient, product, manufacturing, clinical application, and biovigilance. The procedures involved in making adipose-derived products involve the collection and preparation of fat tissue. The procedures require that the cells are obtained by safe methods without any adverse events. Clinicians who use the cells in their practice are obliged to abide by existing laws that control the use in clinical practice. The European Medicines Agency (EMA), United States Food and Drug Administration (FDA), and others consider adult human cells as biological products of two classes. The first involves cells processed with minimal manipulation techniques such as filtration, centrifugation, and mechanical disruption done in a single surgical window within a sterile operating environment. The second class of products undergoes significant manipulation such as expansion in cell culture, characterization, cultivation, and other manipulation techniques used outside of a single surgical window or the operating room. As per FDA and EMA regulations, adipose products are considered medicinal products that have to be harvested using validated procedures adhering to stringent regulatory protocols. The clinical quality attributes of therapeutic products ensure safety along with the maintenance of identity, purity, and potency as per the FDA Guidance for industry [Pharmaceutical development. Q8(R2). Current Step 4 version dated August 2009.Q8 (R2)]. In agreement with the reflection paper EMA/CAT/-600280/2010 Rev 1, 20 June 2014, it is presumed that for autologous use under a single therapeutic window with minimal manipulation in aseptic conditions, adipose-derived products would not require ethical committee underwriting for clinical use. However, there are no standardized methods or means to isolate ADSCs with predetermined critical quality attribute, making isolation of ADSC for targeted therapies difficult[246]. Hence, a modular system configured as a single-use kit that contained all the essential components in the exact proportions needed would be ideal and is recommended. Such kits would allow following a specific protocol to

standardize isolation of adipose derivatives with targeted action of ADSCs and comply with specific critical quality attributes.

Having discussed the practical constraints of the use of ADSCs for practical applications, the use of ADSC-derived exosomes as a therapeutic conduit is being explored [247]. Exosomes do not carry the risk of genetic instability and immune activation following their administration in the host environment. The differential advantage of using them *vs* their parent cell of origin include immune privilege in the host environment, which helps them to evade the native phagocytosis, their size in nanoscale enabling them to move in and out of cells with ease, their homing molecules on their surface enabling them to migrate to their site of interest[248]. Hence, with the above-conferred advantages in using ADSC-derived exosomes, they are the principal focus of research in advancing regenerative therapy in the future.

CONCLUSION

Of late, interest in adipose derivatives for regenerative therapies and constructive tissue engineering is on the rise. Having elaborated the biology, characteristics, immunology, and clinical applications of adipose-derived products, it is imperative that evidence to strengthen the clinical applicability of these therapeutic products is needed to warrant an official recommendation from regulatory authorities. Unraveling the utility of various adipose derivatives helps in the improvisation of the existing regenerative therapies and their associated biomedical applications.

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REVIEW

Unveiling the morphogenetic code: A new path at the intersection of physical energies and chemical signaling

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Abstract

In this editorial, we discuss the remarkable role of physical energies in the control of cell signaling networks and in the specification of the architectural plan of both somatic and stem cells. In particular, we focus on the biological relevance of bioelectricity in the pattern control that orchestrates both developmental and regenerative pathways. To this end, the narrative starts from the dawn of the first studies on animal electricity, reconsidering the pioneer work of Harold Saxton Burr in the light of the current achievements. We finally discuss the most recent evidence showing that bioelectric signaling is an essential component of the informational processes that control pattern specification during embryogenesis, regeneration, or even malignant transformation. We conclude that there is now mounting evidence for the existence of a Morphogenetic Code, and that deciphering this code may lead to unprecedented opportunities for the development of novel paradigms of cure in regenerative and precision medicine.

Key Words: Physical energies; Stem cells; Bioelectricity; Electromagnetic radiation; Mechanical forces; Morphogenetic code

Core Tip: The capability of biological systems to create dynamically evolving shapes, up to large-scale anatomy, raises a number of fundamental questions that are only partially addressed in terms of molecular signaling. Physical energies, including mechanical and electromagnetic waves, afford substantial control of somatic and stem cell fate under normal and pathological conditions. This editorial focuses on the remarkable role of bioelectricity in shape generation, and maintenance, up to growth

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regulatory patterning that lead to the specification of tissues/organs and of the whole individual. Implications of bioelectrical signaling in tissue regeneration and in the control of malignant transformation are also discussed.

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INTRODUCTION

There is increasing, compelling evidence that cellular dynamics and fate are fashioned by the capability to generate physical signaling, other than biochemical reactions[1]. Both somatic and stem cells produce mechanical and electric waves[2-5] to elaborate intra- and inter-cellular communications and orchestrate complex developmental pathways[6-9]. Compounding the complexity of this emerging picture, cells are also exploiting the ability of a selected number of molecules or molecular complexes to behave as chromophores, that is generating electromagnetic radiation in the form of light to orchestrate targeted signaling processes[1,10-13]. To this end, the list of molecules than can be actually deemed as chromophores includes a number of components in major ion channels involved in cytosolic calcium handling[14,15], essential players in redox signaling[16], such as multiple NAD(P)H oxidoreductases [17], as well as molecules involved in biochemical pathways leading to the formation of gasotransmitters, like nitric oxide (NO)[18,19].

Concerning the cellular interior, there is now evidence that microtubuli act as a major source for the generation of both mechanical waves and electromagnetic signaling[20-22]. The mechanical buckling of microtubuli, coupled with their inherent electric polarity, is a major determinant in the spreading of mechanoelectrical signaling across the cellular boundaries[22]. Microtubuli are themselves displaying chromophore characteristics[23], a trait that may further contribute long-range intercellular connectedness through electromagnetic radiation (light). Overall, microtubuli can be viewed as a sort of bioelectronic circuit, whose oscillatory patterns exhibit the features of both synchronization and swarming[24,25]. These mechanisms may play a remarkable role in a form of biomolecular recognition that transcends the lock-and-the-key scheme of interaction, being rather based upon the participation of molecules in the construction of signaling processes through a mechanism of resonance. Novel hypotheses are now being formulated, considering the resonant vibrational profiles associated with the helix-loop-helix structure shared by signaling peptides and transcription factors[26-29], and the possibility that microtubuli act as a viscoelastic matrix assembling these molecules into synchronous resonating clusters [25,30].

Taking into account these considerations, physical energies could be deployed to control cell behavior including the biology of stem cells. In this regard, we have provided evidence that the fate of stem cells, and their rescuing potential can be remarkably affected by electromagnetic fields[31-34], even reversing senescence in vitro[35,36], and mechanical forces[37]. Comprehensive review analyses focusing on the rescue of damaged tissues by the aid of physical energies, also including the use of shock waves and photobiomodulation, can be found elsewhere[1,38,39].

On the whole, a common feature arising from the inherent mechanical vibration of electrically polarized elements (*i.e.*, the cytoskeleton and virtually all the electrically charged subcellular components), and from the concerted activity of cellular ion channels, is the generation of endogenous bioelectric fields with radiation characteristics[1,40].

In this editorial, we focus on the role of bioelectricity as a constitutive element directing the development and assembly of shapes, from the subcellular/cellular level, up to large-scale anatomical patterning (Figure 1).

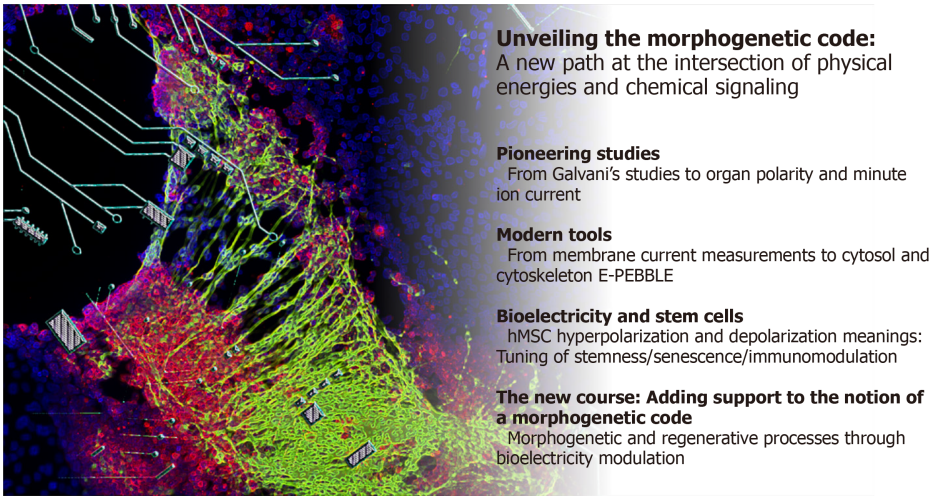

Figure 1 Graphical abstract: Symbolic representation of cellular bioelectronic circuitries in morphogenetic patterning. hMSC: human mesenchymal stem cell.

THE PIONEERING STUDIES: EVIDENCE FOR AN ELECTRO-DYNAMIC FIELD IN LIVING ORGANISMS

Bioelectricity is a term coined to identify the ability of electric fields endogenously generated in living cells to afford modulation of biological patterning from the cellular up to the tissue and organ level[40]. In all the cells and tissues, a part of electrically driven signaling originates from ion channels and related ion fluxes[40,41]. The differential distribution of resting potential across tissues represents an ancestral and conserved modality, highly integrated and connatural with chemical structures, in the establishment of cell signaling networks[42]. Bioelectricity plays a major role in the scaling up dynamics responsible for embryogenesis, and tissue regeneration, while altered non-coherent bioelectrical patterning appears to be involved in the onset of degenerative or malignant states[43].

The first studies on bioelectricity sink their roots in the beginnings of the 18th century. Seminal discoveries in this field emerged with the studies by Luigi Galvani, who showed the feasibility to achieve muscle twitching by touching the muscle with a deviating cut sciatic nerve in the absence of metal electricity, definitely providing evidence for animal electricity[44-46]. Through his experiments Galvani also unknowingly discovered the injury current and the injury potential[47].

Later in 1840's Emil du Bois-Reymond[48] provided crucial advancement in the bioelectricity, when he was able to show the existence of macroscopic levels of electrical activities in frog, fish and human bodies, while recording defined electric currents in living tissues and organisms by the aid of galvanometers made of insulated copper wires. He discovered the action potentials[48,49], and at the same time, he was able to demonstrate the existence of less fluctuating electricity at wound level, conclusively showing the injury current and potential[50].

Bioelectricity studies received a fundamental boost in the early 20th century, when the pioneering work of Harold Saxton Burr provided clear-cut evidence for the crucial role bioelectric fields play in the control of biological shapes, and embryonic development.

In his studies, Burr and Mauro[51] used a voltmeter, accurately dissecting voltage gradients in developing embryos[52], as well as at the level of malignant tissues[53]. He also extended his studies to plants, providing remarkable advancement in the understanding of the role exerted by bioelectricity in plant physiology and diversity [54,55].

Dr. Burr has shown that all living forms, of any species, generate and are embedded within electrodynamic fields, which can be quantitatively assessed and mapped under physiological, as well as diseased states[56-60]. He conceived that such Fields of Life or L-Fields constitute the fundamental blueprints of all kind of Life forms. Dr. Burr was also firmly convinced that the systematic assessment of L-Fields would have yielded unprecedented insights into the biophysical dynamics, even those associated with mental conditions, before symptoms of illness develop, paving the way for new strategies of Predictive and Precision Medicine.

In 1940's Dr. Langman, from the New York University and Bellevue Hospital Gynecological Service, performed a voltmeter-based assessment of the L-Field to screen more than 1000 women suffering from a variety of symptoms in the generative-urinary tract. The subjects who exhibited significant voltage gradient difference between the cervix and ventral abdominal wall were subjected to deeper analyses and laparotomy, as it was reported in 1949 in a paper published by Langman and Burr [53]. They found a hundred and two cases where there was a significant shift in the voltage gradient, suggesting malignancy. Surgical and biopsy confirmation was found in ninety-five of the one hundred and two cases[53,60].

These findings led Dr. Burr to perform additional studies, and found that changes in the L-Field not only allowed monitoring of tissue/nerve injury, but that precise L-Field signatures were associated with, or they may have even determined, the wound healing process[59].

Dr. Burr's studies discovered relevant implications and applications in the assessment of female menstrual cycle, also showing that the movement of ovulation can thoroughly be monitored electrically[58]. Somehow these studies, by providing remarkable information about the chronobiology of ovulation anticipated essential issues within, and also provided essential knowledge to a number of fields that would have been experiencing terrific development over the years, including modern gynecology, family planning, birth control and *in vitro* fertilization.

Revisiting Burr's work shows that he was a fantastic visionary pioneer of studies that only today are creating progressive evidence for the existence of a morphogenetic field and for the need to believe in this potential as an unprecedented chance to access a real comprehensive view on how biological systems acquire coherence[61]. Burr focused on their capability to create dynamically evolving shapes that, while sharing enormous similarities with the simplest microorganisms and our eukaryotic cells, nevertheless entail the evolutionary unfolding not only to complexity, like in multicellular organisms, but to the deeper meaning of biological forms and shapes. This includes the inherent susceptibility of biological forms and shapes to create further contexts and being then guided by those contexts to orchestrate the coherent morphologies and functions of the entire individual[61].

A fundamental merit of Burr was not only his pioneering work, but his ability to bring science at a subtle line where science itself should find the courage to accompany the scientist to the unrestrainable need of merging with other disciplines, like Arts, Philosophy and Religion, in the effort of accepting other view points to explore the mystery of Life and Universe.

Following Burr, applied electric fields were shown to interfere with the regeneration pattern in the planaria by Marsh and Beams[62], leading to head or tail formation at cut locations, and resulting in body polarity reversion.

Other remarkable contributions to the field of bioelectricity were brought about by Lionel Jaffe and Richard Nuccitelli, who afforded quantitative and non-invasive measurement of extracellular minute ion currents by their vibrating probe[63], further elucidating the role of bioelectric signaling in the control of developmental pattern[64-68]. Then, Borgens *et al*[69,70], Cone and Tongier[71], Cone and Cone[72], Stillwell *et al*[73], and McCaig *et al*[74] confirmed and extended Burr's findings in wound healing, limb regeneration, embryogenesis, and organ polarity.

These pioneering studies on bioelectricity led to seminal discoveries and set the basis for unprecedented ways of approaching fundamental issues in the unfolding of living organisms. Nevertheless, the narrative and implications set forward by bioelectricity studies have until very recently been left in the shadow, despite the inability of chemical and molecular approaches to answer fundamental questions of biology itself. As a result, the field of bioelectricity has been severely penalized in terms of biology education, popularity among scientists and funding opportunity.

Only recently, the need for more transdisciplinary and non-traditional approaches has emerged as an unavoidable path in Science, also favored by the novel developments and biological applications of Computer Science and AI. Additionally, these new synergies could benefit from the development of novel probes, allowing detection and 3D imaging of electric microcurrents in the form of fluorescent signals even at the single cell level.

MODERN TOOLS FOR STUDYING THE BIOELECTRIC SIGNALING IN LIVING CELLS AND TISSUES

Until quite recently, the assessment of intracellular electric fields was relying upon the

use of patch clamp, voltage clamp, or voltage dyes, allowing the measurement of electric pattern at the cellular membrane level. This site of investigation, although of remarkable relevance, only represents about 0.1% of the overall cellular volume. Moreover, with most of these membrane-associated tools cumbersome calibration approaches are needed. The recent development of nano-sized photonic voltmeter, initially referred to as E-PEBBLE (photonic explorer for biomedical use with biologically localized embedding), is now making affordable a 3D, and timely investigation of the electric patterning within the whole cellular volume[75-78]. These nanodevices can be calibrated extracellularly and can be subsequently targeted to the inside of any living cell or subcellular compartment, without further calibration. E-PEBBLEs embed Di-4-ANEPPS, a ratiometric, fast responding voltage-sensitive dye, whose fluorescence spectrum shifts in response to electric field changes[75,76]. The dye is encapsulated within a silane-capped mixed micelle, and it is therefore easily taken up by living cells. The possibility to address such shift ratiometrically, remarkably minimizes signal-to-noise ratio problems. These nanodevices exhibited an enhanced targeting efficiency when they are linked to multiple surface-conjugated targeting moieties. Interestingly, the use of these nanodevices provided evidence for the existence of intracellular electric fields that were not merely confined to the cellular membranes, but they were also ensuing within, and spreading throughout the cytosol [75-78]. These findings are consistent with the observations discussed above in the present article, showing that microtubules and microfilaments are electrically charged/polarized, and highly vibrating entities, behaving as electric power transmission lines for intra- and inter-cellular communication.

The chance for monitoring also non-membrane electric patterning inside living cells, has been challenging consolidated dogmas, holding promise for further dissecting cellular dynamics through the eyes of Physics. Worthy to note, the chance for combining E-PEBBLEs with confocal microscopy analysis is now opening the perspective to integrate subcellular chemical with physical signaling.

BIOELECTRICITY IN STEM CELL DYNAMICS: TUNING STEMNESS, SENESCENCE, PARACRINE AND DIFFERENTIATING PATHWAYS

The use of specific ratiometric fluorescent dyes, including $DiBAC_4$ and CC2-DMPE, has allowed a thorough analysis of ion channel-orchestrated bioelectric patterning in stem cell dynamics. In particular, membrane potential has been found to be essential in conducting the commitment of human mesenchymal stem cells (hMSCs) towards the osteogenic and adipogenic fates[79,80]. Data yielded with the voltage-sensitive dye $DiBAC_4$, revealed a characteristic trait of hyperpolarization in differentiating hMSCs, as compared with undifferentiated cells[79]. A causal role of hyperpolarization could be inferred by the observation that the differentiating process could be abrogated by disrupting the hyperpolarization progression by depolarizing hMSCs with two different strategies, including cell culturing in the presence of high [K^+], or ouabain, a specific inhibitor of Na^+/K^+ ATPase pump in the plasma membrane. Conversely, an upregulation in the expression of osteogenic markers was obtained when hMSCs were exposed to the KATP channel openers pinacidil and diazoxide, two compounds known for inducing hyperpolarization in different cell types. These findings strongly indicate that bioelectric fields play a major role in stem cell differentiation[79]. Bioelectric signaling not only is essential as a functional regulator of stem cell differentiation, but it also plays a relevant role in the maintenance of the differentiated state [81]. Depolarization of hMSC-derived osteoblasts and adipocytes resulted in the downregulation of bone and fat tissue markers, and therefore in phenotypic loss, even in the presence of specific differentiating factors for each commitment[81]. This observation suggests that bioelectric signaling might have overridden the molecular signaling in the maintenance of a differentiated state. The observed phenotypic suppression was not associated with an upregulation of stemness genes. Rather, the depolarized osteoblasts could be committed to competent adipocytes[81]. Thus, depolarization could be exploited to improve the transdifferentiation potential in hMSC-derived cells, without restoring stem-like signatures.

From a more general perspective, these results indicate that tuning of the bioelectric properties of stem cells may emerge as a novel approach to finely direct their therapeutic potential. Accordingly, the development of protocols to obtain electrically enriched hMSCs has provided the chance to isolate stem cells in which distinct electric states and ionic properties were associated with defined stemness and regenerative potential[82]. In these studies, hMSCs were sorted on the basis of their fluorescence

intensity for the trans-membrane potential indicator tetramethylrhodamine ethyl ester (TMRE). Subpopulations of electrically enriched hMSCs were found to differentially express genes involved in stemness, senescence, immunomodulation, and autophagy [82]. In particular, hMSCs with low levels of TMRE, accounting for a depolarized membrane potential, exhibited a reduced expression of senescence-associated markers, while overexpressing genes encoding autophagy and immunomodulatory players [82]. These findings indicate that hMSC sorting based upon cellular bioelectric properties would both allow stem cell enrichment for distinct features, and provide unprecedented strategies for selected cell therapy outcomes.

The relevance of bioelectricity in stem cell biology is further highlighted by the functional role of transmembrane potential (V_{mem}) in the regulation of stem cell proliferation and migration. In neurosphere-derived neural precursor cells (NPCs), IKIR and IKDR channels are involved in establishing a hyperpolarized resting V_{mem} of about -80 mV[83]. Depolarization through modulated IKIR enhanced NPC mitosis and neurosphere size[83]. Similarly, in both human and mouse embryonic stem (ES) cells, IKDR are expressed and exert a permissive role in proliferation, that can be antagonized by K^+ channel blockers[84].

The use of dyes of the DiBAC family also allowed to establish that bioelectricity is deeply involved in cell migration and communication. In fact, a combination of optical membrane-potential measurements with mechanical stimulation showed that the physical bridging provided by tunneling nanotubes (TNTs), embedding microtubules and to a lesser extent actin filaments, mainly acted as a form of electric connectedness [85,86]. Thus, neuronal migration and differentiation, as well as long-distance neuron-astrocyte communication, are supported by a form of bioelectronic circuitry through TNT-mediated depolarization. Consonant with these studies, modulation of potassium channels has been shown to control stem cell migration and invasiveness[87].

THE NEW COURSE: ADDING SUPPORT TO THE NOTION OF A MORPHOGENETIC CODE

While these findings point at the role of bioelectricity at cellular level, it is now becoming evident that the seminal intuitions and discoveries of Harold Saxton Burr and the studies of Scientists who continued to work in the groove traced by him, have laid the foundation to understand how bioelectric fields are a part of a morphogenetic code.

Cellular electric fields, electromagnetic and light radiation, as well as nanomechanical oscillations, are now emerging as vibrational signatures, imparting informational messages that contribute to the onset, unfolding and continuous remodeling of forms and of their inherent functions.

It is now clear that understanding the genetic, or protein level of cell signaling would only minimally help taking a glimpse on the most complex informational hierarchies underlying biological morphologies. Rather, considering biological systems as part of the vibrational nature of the Universe is now fostering more transdisciplinary efforts. The work of Scientists from multiple disciplines is gathering data showing that physical energies may act as a sort of software program driving the transition from nanoarchitectonics and supramolecular interactions to cellular shapes, and positioning, up to growth regulatory patterning that lead to the specification of tissues/organs and of the whole individual.

The observation that a wide-ranging spectrum of channelopathies, and consequent derangement in ion transport, are unequivocally linked to deep subversion of normal morphogenesis, leading to multi-organ crumbling and failure[88,89], should have been supported over time the notion that signaling through physical energies must had been regarded as a major determinant in the establishment of forms and functions. Nevertheless, discussing about forms, the "forma mentis" of Scientists has been slowly changing according to these evidence, and only recently a large body of compelling data is boosting molecular biologists to think in terms of biophysical signaling. The outstanding work performed by Michael Levin and his Coworkers is now providing clear-cut evidence for the intimate connection between molecular signaling, gene transcription and bioelectric signaling[90]. Levin's work is yielding fundamental data in showing that a continuous tuning of cellular patterning with the whole organism requirements underlies the establishment of shapes throughout embryogenesis[91-95] and the preservation of forms during the development of a hostile environment (*i.e.*, injury or tumorigenesis)[96-100]. Understanding the informational mechanisms that govern the establishment of complex anatomy has required a paradigm shift from the

observation of local cell communication to the attempt of approaching biological intelligence as a computational process that entails a network of maps encoded by physical energies. By this approach, Levin's group have shown the feasibility of modeling somatic computation *via* non-neural bioelectric networks, and that the spreading of multifaceted ion fluxes over space and time merges with molecular signaling in non-excitable cells[101,102]. This strategy has been further refined by the use of voltage sensitive dyes in combination with defined extracellular ionic solutions, an approach that has allowed investigating the consequences of resting membrane potential manipulation on cellular dynamics[78-80]. The development of targeted 4D imaging and related data analysis has also been part of studies that highlighted membrane potential as a tunable tool in the modulation of calcium-primed signaling in Xenopus embryogenesis[101,103-105]. Further contribution to the understanding of these dynamics, and to the deployment of control strategies of bioelectric patterning, came from the development of the BioElectric Tissue Simulation Engine (BETSE) by Pietak and Levin[106]. BETSE proved effective as a multiphysics simulator, and for a predictive spatio-temporal profiling of bioelectric patterns from the modeling of ion channel and gap junction activity. These approaches have been at the basis of a number of interrelated findings showing a crucial role of defined bioelectric patterns in: (1) The establishment of morphogenetic patterning during embryo development [107-110]; (2) The deployment of optogenetics in developmental biology, through the use of light to handle ion flux-dependent voltage and signals in embryo development [101]; (3) The physiological control of the large-scale mechanisms operating in tissue regeneration[111,112]; (4) Regenerative processes from the level of wound healing, up to the rescue of brain defects induced following animal model exposure to teratogenic or mutagenic agents[113]; and (5) The establishment of micro-environmental signals suitable for revealing, inducing or even counteracting cancer onset and progression[96, 97,114].

CONCLUSION

The journey for understanding how Physics may orchestrate molecular and cellular patterning up to contribute shapes and anatomical homeodynamics has started many years ago. Nevertheless, only recently we are facing the re-discovery of the potential for using physical energies to afford efficient modulation of cell signaling, tissue patterning and rescue.

Considering the diffusive properties of such physical stimuli, we may also envision a novel strategy of regenerative medicine relying upon the reprogramming of stem cells in situ, where they are resident in all tissues of the human body. This approach may hold promise for affording tissue regeneration without cell or tissue transplantation, but rather boosting our own self-healing potential. So far, the effects elicited at the stem cell level by endogenous electric patterning, or by mechanical vibration and electromagnetic radiation (including light), have been investigated mainly in mouse ES cells, and hMSCs. Nevertheless, besides hematopoietic stem cells, other tissue-resident multipotent elements, such as pericytes[115,116], and cells exhibiting pluripotency features in the adulthood, as the "multilineage-differentiating, stress-enduring" cells[117-119], may conceivably be targeted by mechanical and/or electromagnetic stimulation. Addressing this issue may disclose novel perspectives in regenerative and precision medicine, and should be the subject for future investigations.

We hope that our efforts will lead to a progressive awareness among the scientific community of the chance of using bioelectricity, electromagnetic radiation and nanomechanics to afford efficient re-setting in the epigenetics up to modulate tissue morphogenesis and regeneration, even offering chances to control oncogenesis and metastatic dissemination.

Progression within this context may be supported by the development of a transdisciplinary endeavor led by a novel generation of committed Scientists, and by the availability of targeted funding platforms.

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REVIEW

Alternative RNA splicing in stem cells and cancer stem cells: Importance of transcript-based expression analysis

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Abstract

Alternative ribonucleic acid (RNA) splicing can lead to the assembly of different protein isoforms with distinctive functions. The outcome of alternative splicing (AS) can result in a complete loss of function or the acquisition of new functions. There is a gap in knowledge of abnormal RNA splice variants promoting cancer stem cells (CSCs), and their prospective contribution in cancer progression. AS directly regulates the self-renewal features of stem cells (SCs) and stem-like cancer cells. Notably, octamer-binding transcription factor 4A spliced variant of octamer-binding transcription factor 4 contributes to maintaining stemness properties in both SCs and CSCs. The epithelial to mesenchymal transition pathway regulates the AS events in CSCs to maintain stemness. The alternative spliced variants of CSCs markers, including cluster of differentiation 44, aldehyde dehydrogenase, and doublecortin-like kinase, α6β1 integrin, have pivotal roles in increasing self-renewal properties and maintaining the pluripotency of CSCs. Various splicing analysis tools are considered in this study. LeafCutter software can be considered as the best tool for differential splicing analysis and identification of the type of splicing events. Additionally, LeafCutter can be used for efficient mapping splicing quantitative trait loci. Altogether, the accumulating evidence re-enforces

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the fact that gene and protein expression need to be investigated in parallel with alternative splice variants.

Key Words: Alternative splicing; Stem cell; Cancer stem cell; Transcriptome; Expression analysis

Core Tip: The alternative splicing machinery can produce various variants, associated with stemness characteristics of both stem cells (SCs) and cancer SCs. In this study, the role of spliced variants in SCs and stem-like cancer cells is reviewed. We highlight the importance of transcript-based expression concurrent with the gene and protein expression that leads to better understanding of self-renewal features of tumor cells.

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INTRODUCTION

Alternative ribonucleic acid (RNA) splicing is an emerging topic in molecular and clinical studies[1]. Alternative splicing (AS) is the key mechanism to generate a large number of messenger RNA (mRNA) transcripts from the relatively low number of human genes, which can lead to the assembly of different protein isoforms with distinct functions. This structural modification of gene transcripts and their encoded proteins is considered a vital process that increases diversity of protein functions in order to generate the complex cellular proteome[2,3].

Stem cells (SCs) are undifferentiated cells that are able to self-renewal, or differentiate into any types of differentiated cells. SCs can be found in both embryos and adult cells[4]. The self-renewal characteristics also can be found in cancer SCs (CSCs) within the tumor environment[5].

In this review, we discuss the significance of AS in determining the final fate of SCs. Also, the impact of AS events in promoting stemness features in CSCs is highlighted. To address all aspects related to AS, this review provides comprehensive detail regarding various types of AS tools.

MAIN TEXT

Alternative RNA splicing and its different types

The outcome of AS can result in a complete loss of function or the acquisition of new functions[2,3]. AS can also change the gene expression pattern in cancer cells. Exon-skipping (a form of alternative RNA splicing) in tumour suppressor genes can lead to truncated proteins, similar to classical nonsense mutations, resulting in cancer-specific AS in the absence of genomic mutations[6]. It has been demonstrated that nearly half of all active AS events are altered in ovarian and breast tumour cells compared to normal tissue[3].

There is ample evidence that AS coordinates significant changes in protein isoform expression and is the main cause of the functional diversity in proteins and proteome [7-10]. In humans, it is estimated that up to 94% of genes undergo AS, resulting in more than 100000 transcripts[7-9].

As presented in Figure 1, transcripts are products of precursor mRNAs (pre-mRNAs) splicing processes, where novel transcripts are discovered with increasing regularity and added to public databases, providing a valuable resource for analysis of AS[11]. During the transcription process, pre-mRNAs are produced. Then, through the RNA splicing process, the non-coding regions of pre-mRNAs (introns) are removed,

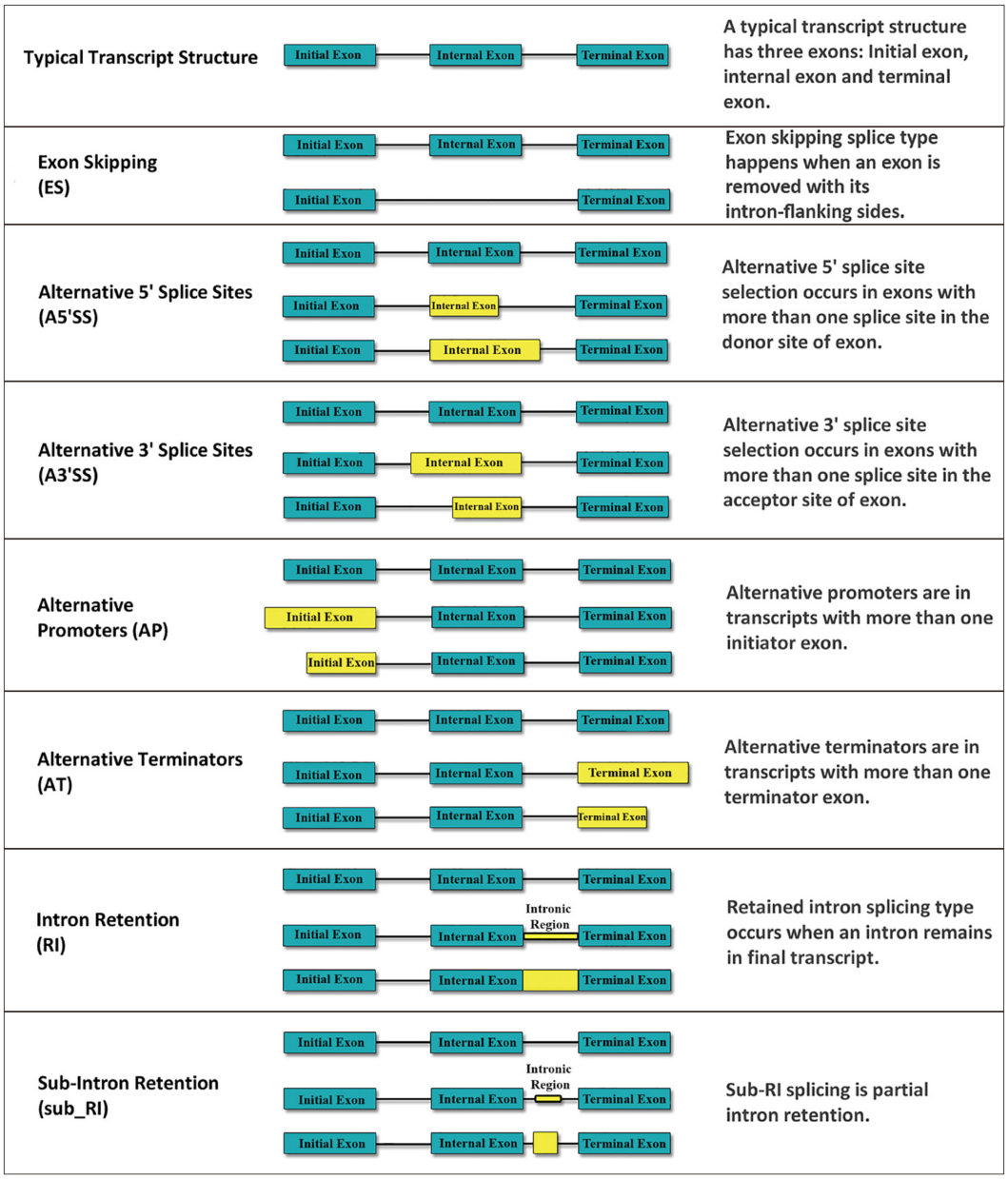

Figure 1 Different types of alternative RNA splicing events.

and coding regions (exons) are joined, and they produce mature mRNAs[12]. Also, it is well-established that the more complex process (AS) may increase the diversity of the mRNAs which are expressed from a single gene. During AS, different exons can be extended or skipped, or introns can be retained within spliced transcripts and produce different mRNAs[13]. The majority (approximately ninety percent) of human genes are alternatively spliced[14]. AS has been considered as a gene expression regulator and miss-splicing can contribute to risk for various human diseases, like cancer[14]. With the advent of high-throughput sequencing methods, analysis of human genomic and transcriptomic has been efficiently developed. Using bioinformatics tools, the sequenced transcripts can be aligned to the genomic reference sequences to find AS events[15,16]. Five standard forms of AS events have been identified including skipped exon (SE, also known as cassette exons), alternative 5′ splice site (A5SS), alternative 3′ splice site (A3SS), retained intron (RI) or intron retention (IR), mutually exclusive exons (MXE). Also, alternative first exons (AFE), and alternative last exons (ALE) are considered as less common AS events[17].

Exon skipping is the most common AS event, in which the exon as a whole is skipped from the mature mRNA transcript[18]. During A3SS and A5SS events, exons are flanked on one side by a constitutive splice site (fixated) and on the opposite side are flanked by two (or more) competing for alternative splice sites, leading to an alternate region (extension) that either is included within the transcript or is excluded

[17]. IR and MXE are the least abundant subtypes among all five major AS events. Normally, during pre-mRNA splicing, introns are fully spliced and excluded from the mature mRNA. However, during IR splicing, introns are retained in mature mRNAs. IR-spliced isoforms have different destinations, some of them may degrade by the nonsense-mediated decay (NMD) pathway, due to the existence of premature termination codons within introns. In some cases, further splicing is applied to the IR isoforms and helps them to escape NMD. NMD escaped-IR isoforms are translated into truncated proteins which have fewer or extra domains in their structure and increase the risk of diseases[19]. In the splicing of MXEs, exons are spliced in a coordinated manner. MXEs can leave the size of the spliced isoform products unchanged. In these isoforms, 1 out of 2 exons (or 1 group out of 2 exon groups) is retained, while the other one is spliced out[20].

There are more events that are not categorised among five standard forms including alternative AFE (alternative promoters) and ALE (alternative 3′ terminal exons). An AFE is the first exon of one splice variant of a gene, which is located downstream of some isoforms of this gene, or this exon is excluded from another isoform because it is located in the intronic region[21]. The AFE definition is also referred to as ALE in which the last exon (3′ terminal exon) may be retained or be excluded in different variants of a single gene[22].

The challenge of alternative RNA splicing analysis in cancer research

There is a gap in knowledge of abnormal estrogen-associated RNA splice variants in breast cancer, and their prospective contribution to breast cancer progression while breast cancer is mainly a disease in which the sex hormone estrogen stimulates uncontrolled growth. Furthermore, their relative abundance and their potential to use AS in cancer diagnosis and treatment has been neglected.

In addition to the novelty of the subject, the major reasons for this shortcoming are: (1) Heavy computation and high computer skills are required for AS detection and analysis; and (2) A high depth of sequencing is required for comprehensive profiling of splicing events. Recently published in Scientific Reports (2018)[11], we developed a Windows-based, user-friendly tool for identifying AS events without the need for advanced computer skills. Additionally, this tool operates as an online module, and employs the SpliceGraphs module without the need for additional resource updates. First, SpliceGraph generates data based on the frequency of active splice sites in the pre-mRNA. Then, the presented approach compares the query transcript exons to SpliceGraph exons in the online genome browser ENSEMBL.

Our team in the Dame Roma Mitchell Cancer Research Laboratories is exceptionally well positioned to unravel the complexity of AS in breast cancer, and thereby, utilise the aberrant alternative splice variants unique to breast cancer as an accurate diagnosis tool. Moreover, this proposed project has the capacity to employ alternative splice variants as a determinant of better outcomes of disease—a completely novel paradigm of disease prevention and diagnosis.

SCs and CSCs

There are various types of SCs. The highest differentiation potential which allows SCs to differentiate into any cell type of a whole organism is found in totipotent SCs, like a zygote. totipotent SCs can generate embryo and extra-embryonic structures in cells [23]. Pluripotent SCs (PSCs) are the other types of SCs that are not able to form extra-embryonic structures in cells, but they have the potential to differentiate into cells of three germ layers (endoderm, ectoderm, and mesoderm)[24]. Embryonic SCs (ESCs) and induced PSCs (iPSCs) are categorised in PSCs. ESCs are derived from the inner cell mass of a blastocyst (preimplantation embryos) and the indefinite self-renewal ability and plasticity are their vivid characteristics[25]. iPSCs are artificially derived from somatic cells, and their function and features are similar to PSCs. iPSCs have shown promising impacts on present and future regenerative medicine[26]. Another SC types are multipotent SCs which have limited differentiation abilities than PSCs and they only differentiate into a specific cell lineage. Haematopoietic SCs (HSCs) are multipotent SCs which can only differentiate into blood cells. Unipotent SCs have the least differentiation capabilities which they can only form one cell type, like dermatocytes[27].

Due to the similarities between cancer state and embryonic development, several studies have focused on the existence of CSCs within the tumor environment[5]. It has been well-established that tumor progression, anti-tumor drug resistance, and post-treatment tumor regeneration are driven by a special cancerous cell type, called CSCs. Generally, CSCs characterized by self-renewing, multipotent, and tumor-initiating properties. It is really difficult to detect CSCs within a tumor environment because

some CSCs that lack specific markers have tumor regeneration abilities[28]. To illustrate, cluster of differentiation (CD) 133 marker firstly was utilized to isolate CSCs from colon carcinoma, but some *CD133*$^-$ metastatic colon carcinoma cells were found which had self-renewal properties as well as *CD133*$^+$ CSCs[29]. Then, studies showed that CSCs could contain some specific subpopulation, like *CD133*$^-$ metastatic colon carcinoma cells, they are known as migrating SCs subpopulation[30]. So, in several solid tumors, the subpopulation of migrating and resident SCs can be found which they have been identified by various surface markers including *CD24*, *CD29*, *CD44*, *CD90*, *CD133*, aldehyde dehydrogenase 1 (*ALDH1*), and epithelial-specific antigen. These markers can be used as a potential target for developing anti-CSCs specific therapies[31].

AS in SCs

The differentiation of SCs and their progenitors relies on various molecular controls associated with the gene expression regulation, including chromatin modification, transcription factors, post-transcriptional regulation by AS, microRNAs (miRNAs), and post-translational modifications. Advanced technologies, as an illustration of high-throughput RNA sequencing (RNA-seq), have demonstrated the contribution of AS patterns in SCs maintenance and differentiation[32].

ESCs are pluripotent cells which able to self-renew, and they have the ability to differentiate into the endoderm, the ectoderm, and the mesoderm cells[33]. Hence, they are the best model for monitoring early embryonic development. Also, ESCs are the potential source for developing differentiated cells for therapeutic approaches in regenerative medicine. One of the first genome-wide studies on splicing patterns in the ESCs reported more than thousands of AS events in ESCs[32]. The results of one study revealed that human and mouse ESC-related AS events are mostly found in genes associated with the cytoskeleton (dystonin, adducin 3), plasma membrane (dynamin 2, integrin subunit alpha 6), and kinase activity (calcium/calmodulin dependent serine protein kinase, microtubule affinity regulating kinase 2, and mitogen-activated protein kinase kinase 7)[34].

Both RNA-seq and splicing microarrays studies reported AS events, particularly those associated with changing protein sequence[15]. Also, the gene expression alteration of the proteins that regarded as splicing regulators was detected during developmental stages, suggesting various AS events during the process of differentiation[16]. For example, increasing expression of splicing regulators like muscle blind-like (*MBNL1* and *MBNL2*) RNA binding proteins (*RBPs*) was shown during ESC differentiation which is highly associated with ESC-differential AS. The presence of the MBNL motif downstream and upstream of the flanking intronic sequences is associated with exon skipping and exon inclusion in ESC respectively[34]. It has been revealed that the AS events of genes that they contribute to ESC differentiation foster this process, like embryonic SC-specific event in forkhead box p1 (*FOXP1*), *FOXP1-ES*. The *FOXP1-ES* spliced isoform stimulates the expression of genes, including octamer-binding transcription factor 4 (*OCT4*), nanog homeobox (*NANOG*), nuclear receptor subfamily 5 group A member 2, and growth differentiation factor 3 which these transcription factors are required for pluripotency. Along with maintaining ESC pluripotency, *FOXP1-ES* showed effective involvement in the reprogramming of somatic cells to iPSCs[35,36]. The fibroblast growth factor 4 splice isoform (*FGF4si*) of *FGF4* is another example of transcription factor that play major roles in ESC fate determination and promotes differentiation at later stages of development[37].

One of the most important regulators of self-renewal in ESCs is POU domain proteins (POU class 5 homeobox 1, also known as *OCT4*) and its gene expression is stimulated by *FOXP1-ES*. *OCT4* has various isoforms including *OCT4A*, *OCT4B*, *OCT4B1*, *OCT4B2*, and *OCT4B4*. The expression of *OCT4A* only has been detected in ESCs and embryonal carcinoma cells (ECCs). The *OCT4B* detected in differentiated cells and *OCT4B1* had higher expression levels in ESCs and ECCs rather than non-pluripotent cells[38]. Also, it has been revealed that expression patterns of *OCT4B2* and *OCT4B4* are similar to *OCT4A* (they highly expressed in undifferentiated cells), and like *OCT4B1*, their expression is lower in differentiated cells[39,40].

AS events also have found as critical factors to control the hematopoiesis process which during this process the HSCs produce mature blood cells in the bone marrow. The importance of AS in hematopoiesis has been identified through the analysis of AS related to RUNX family transcription factor 1 (*RUNX1*) which is a critical transcription factor for this process. *RUNX1a* isoforms are generated from *RUNX1* by exon 6 AS and increase the capacity of the HSC pool[41]. Moreover, multiple isoforms of the other HSC-specific genes (homeobox A9, Meis homeobox 1, PR/SET domain 16, and HLF transcription factor-PAR bZIP family member) have been found using whole-

transcriptome splicing of murine HSCs[42]. Also, a bioinformatics analysis revealed that the IR in HSC-specific transcripts led to a decrease in the expression of genes involved in DNA binding and RNA processing. This process consequently promoted the NMD pathway in HSCs[43,44].

Along with ESCs and HSCs, there are neural SCs (NSCs) that are responsible for generating neurons and glia during embryonic development. The nervous system applies AS for cell differentiation, morphogenesis, and the formation of complex neuronal networks. The three major alternatively spliced isoforms of Quaking (*Qki*) have been found in NSCs. *Qki5* is one of these isoforms which regulates NSC functions [45]. Another example of AS involvement in the transition of SC to neuron cells is the existence of exon 10 in the mRNA of polypyrimidine tract binding protein 2 (PTBP2) protein[46]. Interestingly, it has been cleared that the downregulation levels of the other paralog of this protein, polypyrimidine tract binding protein 1 (PTBP1), inhibit the existence of exon 10 of *PTBP2*[47]. Hence, in neural cells, the neuron-specific microRNA miR-124 contributes to the downregulation of *PTBP1*[48]. On the other hand, there is another ubiquitous RNA-binding protein which suppresses PTBP1/PTBP2 levels by inhibiting the inclusion of exon 11/10 of *PTBP1/PTBP2* in myoblast cells[49,50].

AS and CSC

SCs in normal tissues are capable of renewing themselves, also, stem-like cells within tumors, which have been called CSCs have the ability of self-renewing to seed new tumors. Hence, they have also been termed "tumor-initiating cells"[51].

Due to gene expression regulation at the transcriptional level, the contribution of AS events has been clearly reported in tumor-related biological processes like proliferation, cell death, migration, and angiogenesis. AS changes transcriptome and proteome profile in human cells, therefore, its deregulation may greatly contribute to tumor plasticity[9]. Effectively, AS plays a regulatory role in maintaining the balance between pluripotency and differentiation of human ESCs during embryogenesis and tissue differentiation. Hence, defective AS machinery could mimic the oncogenic effects in non-tumorigenic cells. Results of an RNA-seq-based study on mammary cells revealed that AS events regulated by serine and arginine rich splicing factor 1 altered in human tumors. These defective AS events led to an enhancement of the proliferation and decreased apoptosis in MCF-10A cell cultures[52]. The involvement of defective AS events in controlling tumor heterogeneity suggesting that they could also lead to re-programming of stem pathways, triggering metastasis, and tumor progression[53].

Phenotypic conversions of cells between epithelial and mesenchymal states, known as epithelial-mesenchymal transition (EMT), is activated during metastasis and enhances the re-activation of stem pathways. Hence, EMT is associated with tumor aggressiveness and resistance of cancer cells to anti-tumor drugs[54]. Various types of regulators including cytokines and growth factors are dysregulated in cancer cells which mainly involved in promoting EMT. EMT influences the mRNA maturation of some splicing factor-like epithelial splicing regulatory protein (ESRP). This splicing factor regulates the Wnt signaling pathway through exon 4 skipping of T-cell factor 4 (*TCF4*). The activation of *TCF4* is prompted by nuclear localization of beta-catenin and they are major transcriptional mediators of the canonical Wnt signaling pathway[55]. It has been revealed that, during EMT, the transactivation of exon 4 carrying *TCF4* isoforms is reduced. On the contrary, the lack of exon 4 Led to surge Wnt signaling during EMT[56].

Moreover, ESRP-splicing factors can alter splicing of the Fibroblast growth factor receptor 2 (*FGFR2*)[57]. There are two different isoforms of *FGFR2* including IIIb and IIIc which have pivotal roles in ligand binding specificity. ESRPs mainly help to the production of *FGFR2-IIIb* by inhibiting exon IIIc. The *FGFR2-IIIc* levels increase in the absence of ESRPs[58]. Spliced transcripts of *FGFR2* were detected in primary tumors, and significantly enriched in metastases and tumor plasticity[59].

Also, the association between EMT and the emergence of CSCs has been identified [60]. Studies showed that EMT induces AS events in genes involved in stem-like cancer cells. NUMB endocytic adaptor protein (NUMB) is an endocytic adaptor protein that has various functions in cell polarity maintenance, cell migration, and EMT. The importance of *NUMB* AS has been reported in various cancers including breast[61,62], lung cancer[63], and hepatocellular carcinoma cells[64]. Four *NUMB* isoforms based on the AS of exon 3 and exon 9 have been identified in vertebrates[65]. Exon 9 mainly founds in SCs rather than differentiated cells, also enhanced expression of *NUMB* exon 9 has been clearly shown in various cancer types. Interestingly, MEK/ERK signaling pathway regulated *NUMB* exon 9 splicing[66]. In cancer cells,

EMT triggers *NUMB* exon skipping, which leads to enhance invasive properties, and abnormal AS events in *NUMB* may also affect the balance between stem-like and non-stem cancer cells[67].

One of the most important SC factors which contribute to embryogenesis and pluripotency of cancer stem-like cells is a member of *POU* family genes called *OCT4*. This SC factor plays a pivotal role in self-renewal and pluripotency properties in ESCs and ECCs[68]. AS leads to produce three main isoforms of the human *OCT4*, including *OCT4A*, *OCT4B*, and *OCT4B1*[38]. *OCT4A* contributes to maintaining stemness properties in ESCs and ECCs. On the contrary, *OCT4B* isoforms are not able to show these features[38,39].

Another gene that its spliced variants have been found in CSCs is Kirsten rat sarcoma viral oncogene homolog (*KRAS*). The occurrence of mutation and AS in this gene results in the production of transformed proteins that promote malignancies[69]. The spliced variants of *KRAS* are *KRAS4A* and *KRAS4B* which are known as minor and major isoforms respectively and in CSCs the splicing process of *KRAS* is regulated by the RNA binding motif protein 39 splicing complex. Hypoxic state in tumor environment activates the expression of *KRAS4A* in CSCs and there is a correlation between the expression of *KRAS4A* isoform and SC marker ALDH in cells with stem-like properties. *KRAS4A* spliced variant controls the metabolic requirements in SCs particularly the level of adenosine triphosphate and lactate. These metabolites increase in the absence of *KRAS4A*. Furthermore, Chen *et al*[70] reported that Indisulam, which is a novel sulfonamide compound with potential antineoplastic activity, is an inhibitor of *KRAS4A* splicing, but has no obvious effect on *KRAS4B*. Also, previously the involvement of *Kras4* isoforms in murine SCs during development was reported[71].

Erb-B2 receptor tyrosine kinase 2 (HER2) is a member of the epidermal growth factor receptor family of receptor tyrosine kinases and its overexpression is a common feature of invasive breast carcinomas. Cells that express HER2 receptors considered as HER2-positive breast cancer. The full-length HER2 is known as wild-type HER2 (wtHER2) which has about 1255 amino acids. While the altered form of HER2 has been identified with the absence of sixteen amino acids from the extracellular domain (deletion of exon 16). This *HER2* splice variant is known as *d16HER2* which highly enriched in the regulation of the breast CSCs (BCSCs) activity by its functional interaction with the notch receptor 1 family members[72]. Also, *d16HER2* spliced variants are involved in initiation and aggressiveness of tumors, CSC properties, EMT, and the trastuzumab susceptibility of HER2 positive BC cells compared with wtHER2 [73].

Another example of EMT-promoting splicing changes is occurred by two splicing factors including QKI and RNA-binding Fox proteins (RBFOX1/RBFOX2). QKI and RBFOX2 are responsible for exon skipping of cortactin transcript during EMT[74]. The roles of QKI and RBFOX1 in establishing the SC features in breast tumor cells and regulating the EMT have been declared by applying exon 30 splicing (exon skipping) in the actin-binding protein FLNB[75].

AS in the CSC marker

One of the most well-known cell surface adhesion receptors of CSCs is *CD44* which also famous as a CSC marker. CD44 is a non-kinase transmembrane glycoprotein comprised of 20 exons which AS leads to produce two different isoforms including the standard (*CD44s*) and variant (*CD44v*) isoforms[76]. Exons 1–5 and 16–20 are involved in *CD44s* isoform and make it the smallest isoform which is expressed by the mesenchymal cells. On the other hand, the middle nine exons (exons 6-15 of the genomic DNA) can be alternatively spliced and located between exons 1–5 domain and exons 16–20 region which form *CD44v* isoforms[77]. CD44 activates by binding to its main ligand, hyaluronic acid. Activated CD44 Leads to cell proliferation and metastasis[78]. *CD44s* isoforms predominantly express on hematopoietic and mesenchymal cells and the *CD44s* isoforms abundance in cancer cells induces stem-like features[79]. This protein is highly expressed in many cancer cells and its alternative spliced variants play a critical role in tumor progression.

Previously, it has been reported that switching from *CD44v* to *CD44s* isoform led to promoting EMT in cells. Cancer cells that undergo an EMT acquire SC-like properties, and *CD44* expression increases in these cells, while the expression levels of splicing factor *ESRP* reduced[54]. Splicing factor ESRP contributes to switching *CD44v* isoform into *CD44s* which is required for promoting EMT[80]. Various studies have been done to reveal the exact role of *CD44* isoforms in cancer cells. It has been reported that increasing tumor cell survival, invasiveness, and migration is associated with *CD44s* [81]. Ouhtit *et al*[82] showed the increased expression of *CD44* in metastatic breast tumors and they provided *in vivo* evidence for the role of the *CD44s* isoform in

promoting breast tumor invasion and metastasis to the liver. Moreover, Hiraga et *al* [83] declared that *CD44* can induce functional properties in CSCs and its *CD44s* variant contributes to the promotion of bone metastases. Besides *CD44s*, there is some evidence that proved the role of *CD44v* in enhancing CSC activities[84,85]. Also, protecting CSCs from reactive oxygen species-induced stress by interacting with a glutamate-cystine transporter that subsequently promotes tumor growth[86].

One of the driving factors that control EMT-associated splicing changes are ESRP-1 and -2 splicing factors. ESRP1 inhibits CSC traits by inhibiting the production of *CD44s*. Increased expression of *CD44s* upon EMT-induction leads to the invasive phenotype in cancer cells. Also, the results of bioinformatics analysis of the Cancer Genome Atlas program of breast cancer showed that there is a significant negative correlation between the ratio of the *CD44* isoforms and the *ESRP1* splicing factor[79]. Also, it has been proved that the expression levels of *ESRP1* decreased in triple-negative breast cancer (TNBC) compared with non-TNBC samples. Along with direct regulatory effects of ESR1 on *CD44s* isoform, this splicing factor also controls the CSCs functions by splicing α6β1 integrin. α6 integrin subunit (*CD49f*) is a well-known biomarker for BCSC and other CSCs and there are two splice variants for this subunit including α6Aβ1 and α6Bβ1 isoforms[87,88]. ESRP1 impedes CSC function by inducing the expression of the α6Aβ1 splice variant and repressing α6Bβ1 integrin isoform[58, 89].

Despite the above arguments, there are some controversies about the role of ESRP1 in promoting[90] or inhibiting[89] CSC properties. The results of a study which was conducted by Yae *et al*[90] seem contradictory. They reported that ESRP1 contributes to breast cancer metastasis through a mechanism in which these splicing factors stimulate the upregulation of the *CD44v* isoforms and results in lung metastasis of breast cancer cells accompanied by the expansion of stem-like cancer cells[90]. Also, Hu *et al*[91] revealed that *CD44v* is expressed on $CD24^{-}/CD44^{+}$ breast CSCs which enhances the risk of metastasis to the lung, rather than cells that expressing *CD44s*.

ALDH is a common CSC marker which catalyses the oxidation of aldehydes. There are 19 various types of *ALDH* in the human genome. Upregulation of *ALDHs* has found in SCs and CSCs[92]. However, *ALDH1* mainly considered as SCs and CSCs markers and contributes in self-renewal activity which has three isoenzymes *ALDH1A1*, *ALDH1A2*, and *ALDH1A3*. Among these isotypes, *ALDH1A1* is the most prominent SC marker in renal cell carcinoma (RCC) tumor, breast cancer[93], colon cancer[94], and is linked to tumorigenesis, mortality, and self-renewal activity[95].

Another marker of CSCs in the gastrointestinal tract is doublecortin-like kinase 1 (*DCLK1*) which mostly correlated with tumor initiation, EMT, and progression[96,97]. In RCC tumor, *DCLK1* alternative spliced variants (*DCLK1 ASVs*) overexpressed compared to control samples. *DCLK1*-long isoforms (Isoforms 2 and 4) are associated with RCC recurrence and they mainly co-expressed with renal tumor SC markers including *ALDH1A1*, C-X-C motif chemokine receptor 4, and *CD44*. It has been proved that a high level of *DCLK1* alternative transcript (Isoform 2) promotes the expression of RCC SC markers and increases self-renewal activity[98].

In summary, the AS machinery could regulate the self-renewal features of stem-like cancer cells. This process is performed by producing spliced variants of cancer cell markers. The most important spliced variants of CSCs markers are *CD44s*, *CD44v*, and α6Bβ1 integrin because they have found on the surface of various CSCs. Also, the splicing factor ESRP is responsible for the splicing changes of both *CD44* isoforms and α6Bβ1 integrin. The Table 1 represents the association of the alternative RNA splice variants and their activities in both SCs and CSCs[99-105].

AS analysis tools

Since the pivotal role of AS events has been cleared in increasing self-renewal properties and maintaining the pluripotency of CSCs, it is highly important to detect differential splicing using computational approaches. Sequencing methods have paved the way to survey AS. Initial studies by microarray profiling and EST-cDNA sequence data reported that about two-thirds of human multi-exon genes are alternatively spliced[106]. Then, high-throughput sequencing technologies provide a high depth of coverage and sensitivity to identify human AS. For the first time to survey the splicing complexity, the Genome Analyzer system of Illumina was used by Pan *et al*[7] Their results proved the effectiveness of the RNA-seq method to analyze AS events. However, it has been found that the RNA-seq method is not sufficient itself due to its short sequencing reads (approximately 100-150 bp), and needs a number of computational approaches to monitor differential splicing[107].

Table 1 Genes that undergo splicing events in stem cells and stem-like cancer cells

Type	Gene	Ensembl ID	Alternative RNA splice variants	Activities	Ref.
Non-cancerous stem cell	*IGF1*	ENSG00000017427	*IGF1Ec, IGF1 Ea*	*IGF-IEc* enhances proliferation and represses muscle progenitors differentiation, *IGF-IEa* activates anabolic pathways	[99]
	POU5F1 (OCT4)	ENSG00000204531	*OCT4A, OCT4B, OCT4B1*	Play roles in pluripotency and self-renewal of embryonic stem cells	[38]
	RBM4, RBM14, CoAA	ENSG00000173933	*CoAZ, ncCoAZ*	Influence co-transcriptional splicing	[100]
	DNMT3B	ENSG00000088305	*DNMT3B3, DNMT3B3Δ5*	*DNMT3B3Δ5* expressed in ESCs and functionally distinct from *DNMT3B3*	[101]
	VEGFA	ENSG00000112715	*VEGF120, VEGF164, VEGF188*	All promote MSC proliferation; some enhance paracrine signaling, osteogenic, or endothelial differentiation	[102]
	FGF4	ENSG00000075388	*FGF4, FGF4si*	*FGF4* is important to stem cell maintenance, while *FGFsi* antagonizes some of *FGF4*'s activity	[37]
	PKCd	ENSG00000163932	*PKCdI, PKCdII*	*PKCdI* is caspasecleavable and *PKCdII* is caspase in-cleavable	[103]
	POU2F2 (OCT2)	ENSG00000028277	*OCT2.2, OCT2.4*	*Oct2.2* is sufficient to induce neural differentiation in mouse ESCs, *Oct2.4* is able to block neural differentiation	[104]
	RMB14, CoAA	ENSG00000239306	*CoAA, CoAM*	*CoAA* is downregulated in favor of *CoAM* during early embryonic development	[105]
	RUNX1	ENSG00000159216	*RUNX1a*	Increases the capacity of the HSC pool	[41]
	Qki	ENSG00000112531	*Qki5*	Regulates neural stem cell function	[45]
	PTB	ENSG00000117569	*nPTB* (*PTBP2*) with exon 10	Transition of stem cell to neuron cells	[46]
	FOXP1	ENSG00000114861	*FOXP1-ES*	Promotes the maintenance of ESC pluripotency and contributes to efficient reprogramming of somatic cells into induced pluripotent stem cells	[35]
Cancer stem cell	*NUMB*	ENSG00000133961	*NUMB* exon skipping by EMT	Affect the balance between stem-like and non-stem cancer cells	[67]
	KRAS	ENSG00000133703	*KRAS4A*	Enriched in the cancer stem cell population and helps to modulate the metabolic requirements and stress responses associated with the cancer stem-progenitor cell transition	[70]
	HER2	ENSG00000141736	*D16her2*	Role in the regulation of the BC stem cells (BCSCs) activity through its functional interaction with the NOTCH family members	[72]
	CD44	ENSG00000026508	*CD44s*	*CD44s* isoforms in cancer cells induces stem-like features	[79]
	CD44	ENSG00000026508	*CD44v*	Promote CSC activities and protecting CSCs from ROS-induced stress	[86]
	α6β1 integrin *(ITGA5, VLA-6, CD49f/CD29)*	ENSG00000091409	α6Bβ1 integrin	Promotes the function of breast CSCs and tumor initiation	[89]
	FLNB	ENSG00000136068	FLNB exon 30 skipping	Associated with EMT gene signatures in basal-like breast cancer/ establishing the mesenchymal and stem-like cell state in breast cancers	[75]
	DCLK1	ENSG00000133083	DCLK1-long isoforms (Isoforms 2 and 4)	Promotes expression of RCC stem cell markers and increases self-renewal activity	[98]
	POU5F1 (OCT4)	ENSG00000204531	OCT4A	Promotes stemness properties in embryonal carcinoma cells	[38]

ALDH1	ENSG00000165092	*ALDH1A1*	Promotes tumorigenesis, mortality, and self-renewal activity in RCC	[98]

IGF-1: Insulin like growth factor 1; *POU5F1*: POU domain proteins; *OCT4*: Octamer-binding transcription factor 4; *RMB*: RNA binding motif protein; *DNMT3B*: DNA methyltransferase 3 beta; *VEGFA*: Vascular endothelial growth factor A; *FGF4*: Fibroblast growth factor 4; *PKCd*: Protein kinase C delta; *RUNX1*: RUNX family transcription factor 1; *Qki*: Isoforms of Quaking; *PTB*: Polypyrimidine tract binding; *FOXP1*: Forkhead box p1; *NUMB*: NUMB endocytic adaptor protein; *KRAS*: Kirsten rat sarcoma viral oncogene homolog; *HER*: Erb-B2 receptor tyrosine kinase; *CD*: Cluster of differentiation; *ITGA5*: Integrin subunit alpha 5; *FLNB*: Filamin B; *DCLK1*: Doublecortin-like kinase 1; *ALDH1*: Aldehyde dehydrogenase 1.

There are three approaches to perform differential splicing analysis, including exon-based, isoform-based, and event-based methods. Mainly, exon-based and event-based methods are categorized in one strategy called the count-based method (Table 2)[108-146].

Isoform-based method

Isoform-based methods are based on the reconstruction of full-length transcripts at the first step, then these methods estimate the relative isoform abundance across samples or conditions. Following that, statistical testing is used to identify the significant differences in the relative transcript abundances among various experimental conditions and samples. It is noticeable to say that the effectiveness of this method relies on accurate transcript quantification. Some of the isoform-based tools have been developed including cuffdiff2, HMMSplicer, PennDiff, rSeqDiff, DiffSplice, NSMAP, and MISO. The last two mentioned tools are also able to detect splicing events while it is not possible for others.

Cufflinks is an isoform-based pipeline which contains three programs including Cufflinks, Cuffmerge, and Cuffdiff. Cufflinks first applies transcript assembly by generating overlap graphs with fragments and quantifying the aligned reads. Transcript abundance of a transcript is then estimated in form of FPKM (fragments per kilobase per million mapped fragments). Then, using Cuffmerge, collected assemblies are merged to create a consensus reference. Cuffdiff2 finally performs different tests for detecting differentially expressed genes and differential isoform changes are calculated by applying a one-sided t-test[147].

HMMSplicer is a precise algorithm for analyzing canonical and non-canonical splice junctions in short-read datasets. HMMSplicer firstly divides each read in half, then seeds the halves to the reference genome and based on Hidden Markov Model, finds the exon boundary. At final points, a score is assigned to each junction, based on the alignment strength, number, and quality of bases supporting the splice junction. The true and false positives can be distinguished perfectly using this scoring algorithm and lead to find novel both canonical and non-canonical splice junctions[110]. PennDiff, as an accurate statistical method takes information regarding both gene structures and pre-estimated isoform relative abundances into consideration, then analyzes differential AS or transcription for RNA-seq data[111].

rSeqDiff is implemented as an R package for the detection of differential isoform expression from multiple RNA-Seq samples using the hierarchical likelihood ratio test. rSeqDiff can considering three cases for analysis, including genes with no differential expression, genes with differential expression without differential splicing, and genes with differential splicing[112].

DiffSplice is additionally another isoform-based method for the detection and visualization of differential transcription. The DiffSplice approach is not based on transcript or gene annotations, it overcomes the requirement for full transcript inference and quantification, which may be a challenge due to short read length. So, what makes DiffSplice distinct from other methods is that this tool uses a divide-and-conquer approach to seek out the difference between transcriptomes within the variety of AS modules (ASMs), where transcript isoforms separate. The abundance of various AS isoforms existing in each ASM is calculated for every sample and is compared across sample groups. A non-parametric statistical test is used for each ASM to demonstrate significant differential transcription with a controlled false discovery rate (FDR)[113].

NSMAP (non-negativity and sparsity constrained maximum a posteriori) model is provided to estimate the expression levels of isoforms using RNA-seq data. Like DiffSplice, NSMAP does not require annotation information. This tool drives the structures of isoforms and estimates the expression levels simultaneously[114].

MISO (mixture of isoforms) model with the help of bayesian inference estimates the expression level of alternatively spliced genes from RNA-seq data. Also, it is a probab-

Table 2 Alternative splicing analysis tools based on isoform-based method and count-based method (including the exon- and event-based approaches)

Tools	Human/ plants/ animals	Types of detecting splices	Type of tool	Year of publish	Ref.	Citation
Isoform-based method						
Cuffdiff2	All	-	Linux	2012	[109]	9550
HMMSplicer	All	-	Linux	2010	[110]	79
PennDiff	All	-	Linux	2018	[111]	9
rSeqDiff	All	-	R	2013	[112]	29
DiffSplice	All	-	Windows/Linux	2013	[113]	141
NSMAP	All	-	MatLab package	2011	[114]	46
MISO	All	SE / A3SS / A5SS / MXE / TandemUTR / RI / AF / AL	Linux	2010	[115]	1166
Exon-based method						
TopHaT	All	-	Linux	2009	[116]	11026
DEXSeq	All	-	R	2012	[117]	1091
edgeR	All	-	R	2010	[118]	19012
JunctionSeq	All	-	R	2016	[119]	81
limma	All	-	R	2015	[120]	11033
rSeqNP	All	-	R	2015	[121]	10
Event-based method						
MAJIQ	All	SE / A5SS / A3SS	Linux	2016	[122]	171
rMATS	All	SE / A5SS / A3SS / RI / MXE	Linux	2014	[123]	746
SUPPA	All	SE / A5SS / A3SS / RI / MXE / AF / AL	Linux	2015	[124]	134
SUPPA2	All	SE / A5SS / A3SS / RI / MXE / AF / AL	Linux	2018	[125]	-
ASGAL	All	SE / A5SS / A3SS / RI	Linux	2018	[126]	12
Astalavista version 3.0	All	SE / A5SS / A3SS / RI	Linux	2012	[127]	62
LeafCutter	All	SE / A5SS / A3SS	Linux	2018	[128]	200
SpliceGrapher	All	SE / A5SS / A3SS / RI	Linux	2012	[129]	132
KisSplice	All	SNPs, indels and AS events (SE / A5SS / A3SS / RI)	Linux	2012	[130]	-
Alt Event Finder	All	SE	Linux	2012	[131]	24
Matt	All	SE / A5SS / A3SS / RI	Linux	2018	[132]	14
ALEXA-seq	All	SE / A5SS / A3SS / RI	Linux	2010	[133]	340
Outrigger	All	SE / A5SS / A3SS / RI	Linux	2016	[134]	86
ASDT	All	SE / A5SS / A3SS / RI	Perl	2018	[135]	5
SplicePie	All	SE / RI	R	2015	[136]	17
VAST-TOOLS	All	SE / A5SS / A3SS / RI	R	2017	[137]	118
spliceR	All	SE / A5SS / A3SS / RI / AF/AL	R	2014	[138]	80
Pro-Splicer	Human	SE / A5SS / A3SS	Website (http://prosplicer.mbc.nctu.edu.tw/)	2003	[139]	50
ASPicDB	Human	SE / A5SS / A3SS / RI / AF/AL	Website (http://srv00.recas.ba.infn.it/ASPicDB/)	2006	[140]	33

H-DBAS	Human	SE / A5SS / A3SS / RI / AF/AL	Website (http://www.h-invitational.jp/h-dbas/)	2007	[141]	56
ASIP	Plant	SE / A5SS / A3SS / RI	Website (http://www.plantgdb.org/ASIP/)	2006	[142]	535
ASG	Human	SE / A5SS / A3SS / RI	Website (https://brcwebportal.cos.ncsu.edu/asg/)	2004	[143]	116
VastDB	vertebrate	SE / A5SS / A3SS / RI	Website (https://vastdb.crg.eu/wiki/Main_Page)	2017	[137]	118
Astalavista	Human/ Animals	SE / A5SS / A3SS / RI	Website (http://astalavista.sammeth.net/)	2007	[144]	205
SpliceDitector	Plant/ Human	SE / A5SS / A3SS / RI	Windows	2018	[11]	1
dSpliceType	All	SE / A5SS / A3SS / RI / MXE	Windows/ Linux	2015	[145]	4
AltAnalyze	All	SE / A5SS / A3SS / RI / MXE	Windows/ Linux	2010	[146]	253

SE: Skipped exon; A3SS: Alternative acceptor site; A5SS: Alternative donor site; RI: Retained intron; MXE: Mutually exclusive exons; AF/AL: Alternative first/last exons.

ilistic framework that takes RNA-seq data of single-end or paired-end to perform more accurate AS analysis at either the exon or isoform level. Interestingly, MISO provides confidence intervals for estimates of exon and isoform abundance, detects differential expression, and uses latent information to enhance accuracy. Also, this tool using GFF annotations can generate various types of AS events including SE, A3SS/A5SS, MXE, TandemUTR, RI, AFE, and ALE[115].

Count-based method

Count-based methods comprised of both exon-based and event-based models. In exon-based approaches read counts are assigned to different features, such as exons or junctions. The main difference between these two approaches is that exon-based methods are not able to provide the type of splicing event; they just estimate the differentially expressed exons/junctions between samples. Tools which have been developed based on the exon-based methods are TopHaT, DEXSeq, edgeR, JunctionSeq, limma, and rSeqNP. Except for TopHat, all exon-based tools are launched in the R environment.

TopHat is a software package that finds spliced junctions ab initio by large-scale mapping of RNA-seq reads. TopHat performs mapping the reads using Bowtie (http://bowtie-bio.sourceforge.net), an ultra-fast short-read mapping program to reference genome[116]. Also, TopHat is considered as a prior separately running tool for other software including Alt Event Finder, FineSplice, SplicingCompass, and NSMAP[148]. Firstly, the RNA-Seq data is processed by TopHat to find the splicing junctions. Then, NSMAP re-counts all the possible isoforms formed by the combinations of collected exons from TopHat, and identifies the expressed isoforms and their expression level estimations[114].

DEXSeq is another exon-based method which uses a linear model to detect differential splicing genes from RNA-seq data[117]. Identifying differential expression levels of genes, exons, or transcripts is also done by the edgeR package. Moreover, using a negative binomial generalized log-linear model, edgeR can be used to analyze the count data. Then, the expression levels are calculated by comparing the logFC of an exon to the logFC of the entire gene[118]. The JunctionSeq package works based on the statistical approach of DEXSeq to calculate the differential exon usage and exon junctions[119].

Limma package is a well-known R package for detecting differential gene expression also provides differential splicing using exon count data. Limma integrates various statistical principles to perform large-scale expression studies accurately. First, this package applies a linear model to calculate differential expression tests for the exon-level expression data. Then, the exon-level statistics are turned to gene-level statistics for detecting differential spliced genes[120].

rSeqNP like other exon-based methods is able to test differential expression and differential splicing of genes. This tool uses standard non-parametric tests based on ranks of expression values of genes and isoforms[121].

Event-based methods through calculating the percentage spliced in (PSI) values can generate splicing events including SE, A3SS/A5SS, MXE, RI, AFE, and ALE. There are various types of software in this category which some are available as python, R, Perl

packages, or others are accessible through online websites. Some of the most important tools are MAJIQ, rMATS, LeafCutter, SUPPA/SUPPA2, ASGAL, spliceR, SpliceDitector, *etc*.

MAJIQ (modeling alternative junction inclusion quantification) is able to find splice graphs and local splice variations (LSV) using RNA-seq data and transcriptome annotation file. An LSV can be determined as a split in a splice graph or from a single exon (reference exon). Single source LSV is related to splits from a reference exon to multiple 3′ splice sites in downstream exons, while, single target LSV is related to multiple 5′ splice sites spliced to an upstream reference exon. Both simple splicing events and complex transcript variations can be included in the LSVs. The MAJIQ builder and MAJIQ quantifier make up MAJIQ. The MAJIQ builder tries to detect known and novel LSVs and construct a splice graph for genes collected from RNA-seq data and transcriptome annotation. Following that, the MAIJQ quantifier applies Bayesian PSI modeling and bootstrapping to estimates PSI values for each quantified LSV. MAJIQ is mainly used with the Voila package which is a visualization tool that using the output of MAJIQ (builder and quantifier), creates interactive summary files with gene splice graphs, LSVs, and their quantification[122].

MATS (multivariate analysis of transcript splicing) is a Bayesian statistical framework for the identification of differential spliced genes obtained from RNA-Seq data. MATS applies multivariate uniform before estimating the correlation of exon splicing between samples, also it uses a Markov Chain Monte Carlo (MCMC) method along with a simulation-based adaptive sampling method to report the *P* value and FDR of differential AS[149]. rMATS is a developed version of the MATS method. rMATS uses a hierarchical framework to account for sampling uncertainty in individual replicates and variability among both paired and unpaired replicates between sample groups, also it can estimate the PSI of each event[123].

LeafCutter is an event-based method developed to analyze samples and population variation in intron splicing using short-read RNA-seq data. LeafCutter can detect differential splicing between sample groups, and used for mapping splicing quantitative trait loci (sQTLs). So, analyzing sQTLs by LeafCutter can help to identification of disease-associated variants. To compare LeafCutter and MAJIQ tools, MAJIQ is used to predict local splicing variation using split-reads and identification of complex splicing events, but MAJIQ does not scale properly more than thirty samples and has not been adapted to map sQTLs. The LeafCutter output of AS events is mainly focused on SEs, 5′ and 3′ alternative splice site usage, and additional complex events that can be summarized by differences in intron excision. Comparison of LeafCutter to other methods for differential splicing analysis revealed that the majority of the introns that LeafCutter reported as the most significant differentially spliced, shared a splice site with rMATS and MAJIQ. It can be considered that LeafCutter is able to detect the same differentially spliced events[123].

SUPPA is another event-based method which uses RNA-seq data to calculate PSI for differential spliced events[124]. SUPPA and its different version, SUPPA2, are able to consider AFE and ALE events coupled with other standard types of events. SUPPA2 has more advantages, including working with various replicates per condition and with different conditions. Also, SUPPA2 is able to cluster the differentially spliced events among various conditions to determine common splicing patterns and regulatory mechanisms[125].

Alternative Splicing Graph ALigner (ASGAL) is an event-based tool that uses RNA-Seq samples and a gene annotation to detect AS events. Firstly, ASGAL constructs the splicing graph from the gene annotations. Then, the RNA-reads alignment is done against the constructed splicing graph of the input gene. Finally, AS events are determined. The most prominent feature of ASGAL is that this tool can have higher accuracy to predict events. Because as compared with other tools which perform spliced alignment against a reference genome, ASGAL tries to detect novel splice sites based on a splicing graph[126].

SpliceR is an R package to detect AS events uses the full-length transcript output from RNA-seq data. For each event, spliceR annotates the genomic coordinates of the differentially spliced elements, to enhance downstream sequence analysis. Moreover, the possibility of the coding potential and NMD sensitivity of each transcript are determined by spliceR[138].

SpliceDetector is a windows-based user-friendly software for the identification of AS events directly from transcripts without any computer skill requirement or database download. Furthermore, to construct splicing graphs, data updating is not necessary because SpliceDetector uses the updated information deposited in the Ensembl database. To use SpliceDetector software, first, it takes human, plant, and model organisms transcript IDs as input. Then, it constructs a SpliceGraph based on all

of the exon coordinates of the related gene. Finally, AS events are provided as output [11].

The interaction between epigenetic and AS within SCs and CSCs

The critical role of AS in producing different mRNA variants, and even non-coding RNAs (ncRNAs), form one gene is well-established. AS can directly enhance transcriptomic and proteomic diversity. AS is regulated by RBPs which monitor the splicing machinery by binding to the pre-RNA. AS can be subjected to epigenetic regulation, like DNA methylation and histone modifications, and regulation by ncRNAs[150]. Long ncRNA (lncRNAs) are ncRNAs mainly longer than 200 nucleotides which have emerged as an essential regulator in various cellular processes[151]. Also, the critical roles of lncRNAs in adult SCs have been identified. Long intergenic non-protein coding RNA PNKY (*Pnky*) is a neural-specific lncRNA within the nucleus of NSCs. This lncRNA maintains the undifferentiating features of NSCs and neural differentiation enhanced by knocking down *Pnky*. Interestingly, *Pnky* could regulate AS through interacting with splicing regulator PTBP1[152,153].

MiRNA are small non-coding RNAs, about 23 nucleotides in length, play role in gene expression regulation by pairing with complementary sequences in protein-coding transcripts[154]. The microRNA-290 cluster maintains the pluripotency and stemness properties of ESCs by monitoring AS, also known as a master regulator of AS. The splicing factors *MBNL1/2* are targeted by one of the members of this cluster, miR-294, and the downregulation levels of *MBNL1/2* have been found in ESCs and during reprogramming of iPSCs. Also, it has been reported that the majority of AS events are regulated by miR-294 through *MBNL1/2* repression and this repression leads to up-regulation of other splicing factors in ESCs, so miR-294-dependent AS events are enhanced[155].

Also, the indirect involvement of some specific spliced variants in the miRNA expression regulation process has been demonstrated in CSCs. CSCs of human head and neck squamous cell carcinoma (HNSCC) are mostly characterized by *CD44v3* isoforms which generate through alternative mRNA splicing of *CD44*. Also, *ALDH1* markers along with the high expression of transcription factors *OCT4*, SRY-box transcription factor 2 (*SOX2*), and *NANOG* are the other features of HNSCCs. In CSCs, *Oct4-Sox2-Nanog* TFs are activated by binding the *CD44v3* to its ligand hyaluronan and these TFs have binding sites on miR-302 promoter. So, the interaction between hyaluronan-induced *CD44v3*-spliced variants and mentioned TFs plays a pivotal role in the downregulation of miR-302 target genes (like epigenetic regulators: Lysine-specific histone demethylase and DNA methyltransferase 1), and this process is critical for the acquisition of CSC features[156].

Transcript-based expression analysis adds value to understanding of transcriptome in SC research

Measuring the expression of alternatively spliced mRNAs, instead of overall gene expression, is considered as a new and more accurate approach for marker discovery in cancer research[157]. For example, many apoptotic regulatory genes, such as *BCL-x*, encode for alternatively spliced protein variants with opposing functions: One apoptotic and the other anti-apoptotic[158]. In recent years, there has been a discernible shift in cancer diagnosis and therapy due to the perception about the role of genes and their proteins. Cancer can occur irrespective of changes in expression of a gene or protein, but rather as a result of aberrant splice variants that are linked to cancer progression and/or drug resistance and is compensated by the decreased expression of other splice variants originating from that same gene.

Future of AS

AS contributes to a range of phenotypic traits of tumours as they progress and metastasis, and is a potential target for gene therapy[6,9]. With the advent of next-generation sequencing technology (NGS) and bioinformatics approaches, studying AS patterns has been performed in both cancerous and non-cancerous cells with increasing details. However, assembled transcripts obtained from NGS technology are not complete because this technology could sequence short reads. Hence, NGS platform may not be suitable enough for AS analysis. To overcome this limitation, the Pacific Bioscience (PacBio) platform has been developed based on the single-molecule real-time sequencing technology. PacBio technology provides full-length transcript sequencing without assembly. The PacBio full-length transcriptome data is the most accurate source to investigate AS[159]. AS analysis has been performed in various plant and mammalian species using PacBio platform[160-162].

Some cancer-specific differential spliced isoforms have been identified which can be used in cancer diagnosis and as potential targets for selective anti-tumor treatments in the future[163]. Currently, single-cell transcriptomic studies have paved the way for analyzing the gene-level expression and identification of isoforms originating from the same gene, to determine the distinct gene expression signatures within cells, especially tumor cells. Also, single-cell techniques have provided a powerful method for identifying CSCs[164]. There are some controversies regarding the AS pattern in single-cells. Some evidence reported that several spliced transcripts exist in single-cells. Faigenbloom *et al*[165] measured pairs of included and skipped isoforms obtained from spliced exons in single-cells. While, other studies demonstrated that single-cells express only one transcript variant or the dominance of a single transcript variant[166,167]. Further work in single-cell AS analysis is required to unravel the potential future of using AS events to develop personalized medicine for various diseases including cancer.

CONCLUSION

Unravelling AS opens a new avenue towards the establishment of new diagnostic and prognostic markers of cancer progression and metastasis as well as the development of a new generation of anticancer therapeutics: Treatments that inhibit specific splice variants, rather than targeting genes. Although the significant roles of alternatively spliced transcripts in promoting self-renewal properties of CSCs have been identified, more studies are needed to identify the whole CSCs-related splicing events to strengthen the therapeutic benefits of AS in the future.

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REVIEW

SOX transcription factors and glioma stem cells: Choosing between stemness and differentiation

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Abstract

Glioblastoma (GBM) is the most common, most aggressive and deadliest brain tumor. Recently, remarkable progress has been made towards understanding the cellular and molecular biology of gliomas. GBM tumor initiation, progression and relapse as well as resistance to treatments are associated with glioma stem cells (GSCs). GSCs exhibit a high proliferation rate and self-renewal capacity and the ability to differentiate into diverse cell types, generating a range of distinct cell types within the tumor, leading to cellular heterogeneity. GBM tumors may contain different subsets of GSCs, and some of them may adopt a quiescent state that protects them against chemotherapy and radiotherapy. GSCs enriched in recurrent gliomas acquire more aggressive and therapy-resistant properties, making them more malignant, able to rapidly spread. The impact of SOX transcription factors (TFs) on brain tumors has been extensively studied in the last decade. Almost all *SOX* genes are expressed in GBM, and their expression levels are associated with patient prognosis and survival. Numerous SOX TFs are involved in the maintenance of the stemness of GSCs or play a role in the initiation of GSC differentiation. The fine-tuning of *SOX* gene expression levels controls the balance between cell stemness and differentiation. Therefore, innovative therapies targeting SOX TFs are emerging as promising tools for combatting GBM. Combatting GBM has been a demanding and challenging goal for decades. The current therapeutic strategies have not yet provided a cure for GBM and have only resulted in a slight improvement in patient survival. Novel approaches will require the fine adjustment of multimodal therapeutic strategies

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that simultaneously target numerous hallmarks of cancer cells to win the battle against GBM.

Key Words: Glioblastoma; SOX transcription factors; Glioma stem cells; Stemness; Differentiation

Core Tip: Despite the remarkable progress that has been made in understanding the cellular and molecular biology of gliomas, current therapeutic strategies have not yet provided a significant benefit to patients or a cure. This review highlights the key functions of SOX transcriptional factors (TFs) in glioblastoma (GBM) and glioma stem cells (GSCs). SOX TFs influence stemness, self-renewal, proliferation, differentiation, viability, migration, invasion, apoptosis, therapy resistance, sphere-forming capacity and tumorigenicity. We emphasized that fine-tuning of the SOX expression level is required to control the balance between the stemness and differentiation of GSCs, making SOX TFs promising therapeutic targets for combatting GBM.

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INTRODUCTION

Cancer is among the leading causes of death worldwide, representing a substantial public health burden affecting welfare and life expectancy globally, with enormous impacts on individuals, families and health systems. The global burden of cancer continues to grow with its increasing incidence in the 21st century, mainly due to the growth and ageing of populations, adoption of unhealthy behaviors and exposure to unhealthy environments.

Cancers comprise a large group of diseases that arise from cells that escape the normal checkpoints of cell division and are capable of uncontrolled growth and proliferation. Deregulation of the precise molecular networks operating at the molecular and cellular levels that control cell proliferation, differentiation and cell death leads to the transformation of normal cells into cancer. Despite decades of intensive research, the underlying mechanisms that transform normal cells into cancer cells and enable cancer cells to spread and metastasize other sites in the body, leading to a fatal outcome, are still not completely understood.

The transformation of normal cells into cancer cells is a complex multistep process, and in recent decades, tremendous efforts have been made to understand the underlying mechanisms. In a highly influential article published more than 20 years ago, Hanahan and Weinberg[1] elucidated six essential biological capabilities of cancer cell, widely accepted as the six hallmarks of cancer. Most, if not all, cancers acquire this set of capabilities during the multistep development of human tumors. The six hallmarks of cancer (Figure 1) include cell sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis[1]. Together, these hallmarks constitute an organized framework for interpreting the remarkable diversity of neoplastic diseases[1]. It has been proposed that the genomic instability underlying these hallmarks generates genetic diversity, which contributes to multiple cancer hallmark functions[1].

In subsequent years, remarkable progress was made towards understanding the mechanisms underlying each of the six hallmarks. New technologies and significant advances in the understanding of the cellular and molecular biology of cancer cells provided better insight into the multiple events that allow cancer cells to acquire additional functional capabilities enabling them to survive, proliferate and disseminate. Thus, two additional enabling characteristics of cancer cells-genomic

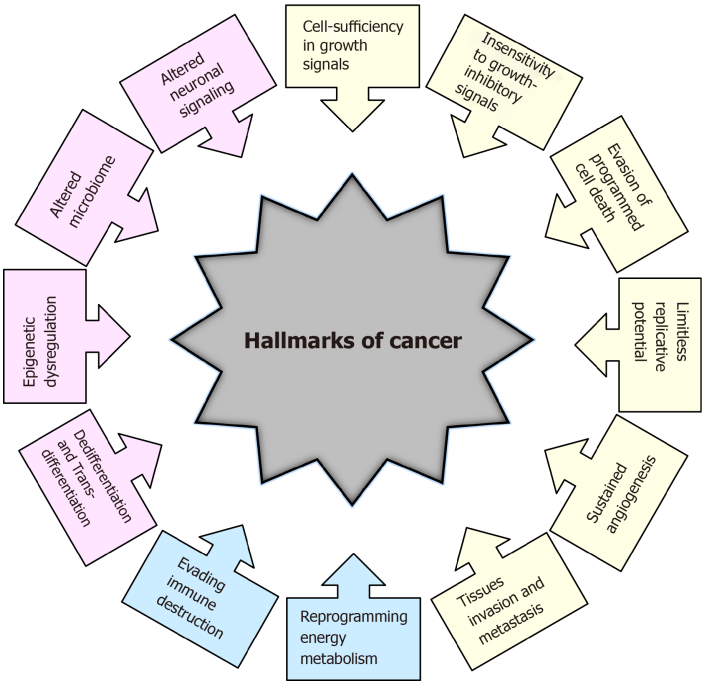

Figure 1 Historical overview of the hallmarks of cancer. Hallmarks of cancer proposed by Hanahan and Weinberg[1,2] in 2000 and 2011 are presented in yellow and blue colors, respectively. Additional hallmarks of cancer proposed by Senga and Grose[3] in 2021 are presented in pink.

instability and inflammation *via* innate immune cells, have been defined[2]. Genomic instability in cancer cells generates random mutations and acquired genetic diversity, endowing cancer cells with genetic alterations that drive tumor progression[3]. Inflammation that fights infections and heals wounds under physiological conditions can assist tumor progression in premalignant and malignant lesions[4]. Research on the intersections between inflammation and cancer pathogenesis has provided overwhelming evidence of the essential effects that immune cells, largely of the innate immune system, have on tumor-promoting neoplastic progression[4-6].

In an updated article published by Hanahan and Weinberg[2] in 2011, two novel hallmarks were proposed and added to the list of core hallmarks of cancer: reprogramming energy metabolism and evading immune destruction. The first includes the major reprogramming of cellular energy metabolism that is required to support uncontrolled cell proliferation. Since the immune system serves as an important barrier to tumor formation, the second hallmark implicates the ability of cancer cells to evade attack and elimination by immune cells[2].

It has become necessary for the hallmarks of cancer to be revised and upgraded as a result of the progression of cancer research and the accumulation of knowledge over the last decade. Four novel hallmarks of cancer were recently proposed, justified and incorporated into the mainstream hallmark conceptualization. These hallmarks include dedifferentiation and transdifferentiation, epigenetic dysregulation, altered microbiome and altered neuronal signaling (Figure 1)[7].

STEM CELLS: DEDIFFERENTIATION AND TRANSDIFFERENTIATION

Stem cells are defined as cells that have self-renewing capacity and the ability to differentiate into multiple cell types[8]. Stem cells are present in mammalian embryos at the blastocyst stage as well as in the tissues and organs of adults. While embryonic stem cells (ESCs) have the ability to differentiate into any cell type present in the adult body, adult stem cells are capable of generating and replacing terminally differentiated cells in specific tissues and are involved in the continual maintenance and repair of tissues and organs throughout life[9,10].

An unidirectional developmental model suggesting that pluripotent stem cells progressively lose their pluripotency as they differentiate along developmental pathways until they reach a terminally differentiated state was widely accepted for several decades[11]. It was believed that adult stem cells have the ability to generate only the differentiated cell phenotypes of the tissue in which they reside. At the

beginning of the 21st century, numerous studies challenged this strict hierarchy of stem cells and their unidirectional differentiation. The phenomenon of stem cell plasticity, or transdifferentiation, has emerged, suggesting that some adult stem cells have phenotypic potential that extends beyond the cell types of their resident tissue [12,13]. It was proposed that some stem cells under specific conditions might diverge from their predetermined pathway and generate cells of a different tissue by entering into a process of transdifferentiation, or that mature cells dedifferentiate into cells with a stem cell phenotype and eventually differentiate into cells of a different tissue[9].

In 1962, Gurdon[14] first proposed the hypothesis that the genome of every specialized cell of an adult organism has all the information required to develop into all different cell types. This hypothesis was proven in the seminal publication of Takahashi and Yamanaka[15], which demonstrated that adult differentiated cells could be reprogrammed into induced pluripotent stem cells that have the ability to differentiate into any of the endodermal, ectodermal and mesodermal cell lineages. This reprogramming is achieved by the overexpression of stem cell-associated genes, also known as stemness factors or Yamanaka factors, in the differentiated cells.

These findings validated the phenomenon of dedifferentiation and transdifferentiation, laying the groundwork for cancer stem cell (CSC) theory and the discovery of CSCs.

CSCs

The first indication of the existence of cancer cells with a stem cell phenotype came from the study of teratomas, which showed that undifferentiated cells preferably gave rise to nontumorigenic differentiated cells[16]. The CSC hypothesis was proposed in line with these data, suggesting that tumors comprise a mixture of malignant stem cells and their benign counterparts[17].

CSCs represent a small subpopulation of tumor cells with the capabilities of self-renewal, differentiation, and tumorigenicity when transplanted into an animal host [18]. CSCs and stem cells share similar properties, including self-renewal ability, unlimited growth potential, invasiveness and blockade of differentiation (reviewed in [19]), whereby indefinite self-renewal capability enables CSCs to initiate and maintain tumor growth.

CSCs were first identified in acute myeloid leukemia[20] and subsequently in a wide variety of tumor types, including melanoma, osteosarcoma, leukemia, breast, colorectal, brain, prostate, pancreatic, ovarian, liver and lung cancer[21-23].

Advances in whole-genome sequencing have revealed the remarkable genetic complexity of malignant tumors and the presence of subpopulations of cells with distinct genotypes and phenotypes[24]. The distinct genotypes of cancer cells within the tumor endow the cells with different biological features and phenotypes, providing the basis for intra- and inter-tumor heterogeneity[25]. The CSC model explains this phenotypic and functional heterogeneity among cancer cells[26-29].

It has been proposed that CSCs originate from either adult tissue-resident stem cells or from differentiated cells that have been reprogrammed to a pluripotent state by the process of dedifferentiation[30]. The CSC hypothesis proposes that many heterogenic cancers are organized in a hierarchical fashion based on the differentiation capacity of the cells comprising the tumor[31]. It has been suggested that this hierarchical order recapitulates the normal tissue hierarchy established by healthy stem cells. Thus, CSCs generate cellular heterogeneity by imposing a differentiation hierarchy by generating a range of distinct cell types present within the tumor[26]. However, the established hierarchy is not permanent, and under specific conditions, could be reversed as terminally differentiated cells become dedifferentiated and regain CSC properties[22, 27].

CSCs have been shown to exhibit high plasticity, resulting in changes in their phenotypic and functional appearance in response to chemo- and radiotherapeutics that cause alterations in the tumor microenvironment (TME)[30]. The negative effects of senescence can directly promote cancer stemness by increasing CSC plasticity, activating stemness pathways in non-CSCs, and promoting senescence escape and subsequent activation of a stemness pathway[30].

CSCs share a number of unique features that distinguish them from other tumor cells. CSCs are believed to be responsible for cancer initiation, progression, metastasis, recurrence and drug resistance[32]. Epithelial CSCs express many genes/pathways typically associated with normal stem cells (reviewed in[31]). In many types of tumors, some CSCs acquire epithelial-to-mesenchymal transition profiles through the upregu-

lation of the expression of specific genes driving metastasis. CSCs are suggested to be responsible for drug resistance and cancer relapse due to their ability to self-renew and differentiate into heterogeneous lineages of cancer cells[33]. Drug resistance has been linked to the ability of CSCs to become quiescent, upregulate the expression of enzymes such as aldehyde dehydrogenase, and upregulate the expression of anti-apoptotic proteins and multidrug resistance pumps that increase chemotherapeutic elimination from cells, resulting in low intracellular drug concentrations[31].

Accordingly, the majority of CSC features depend on the deregulation of signaling pathways that, in turn, rely on the altered activity of specific transcription factors (TFs).

SOX TFs AND CSCs

SOX (Sry-related HMG box) proteins constitute a large family of diverse and well-conserved TFs comprising at least 20 SOX family members in mammals[34]. They have been divided into eight distinct groups designated A-H based on their structure, expression profiles and homology (Table 1)[35]. The SOXB group is further subdivided into subgroup B1 comprising SOX1, SOX2 and SOX3 and subgroup B2 consisting of SOX14 and SOX21[36].

SOX proteins display properties of both classical TFs and architectural components of chromatin (reviewed in[37]). SOX TFs possess a 79 amino acid HMG domain that enables their specific DNA binding and additional domains involved in transcriptional regulation (reviewed in[37]). SOX TFs exert regulatory functions to activate or repress gene transcription through specific interactions with their partner factor(s) and by establishing contacts with the basic transcription machinery[38].

Several essential roles have been attributed to SOX TFs since their discovery. SOX TFs are a component of a regulatory network and, together with other TFs, signaling pathways, epigenetic modifiers and microRNAs, govern diverse cellular processes during development, such as the maintenance of stem cell pluripotency, cell proliferation, cell fate decisions, germ layer formation and the terminal differentiation of cells into tissues and organs (reviewed in[39,40]). However, the roles of SOX TFs are not limited to development as they also influence cell survival, regeneration and death, and control homeostasis in adult tissues[41,42].

Numerous studies have reported the roles of SOX TFs in the preservation of stem cell characteristics, playing a part of regulatory network required to establish ESCs and to maintain their pluripotent and proliferative state. SOX2, OCT4 (octamer-binding TF 4) and NANOG (named after the mythological Celtic land of the ever-young, "Tir nan Og")[43] comprise the core transcriptional circuit that orchestrates the maintenance of stem cell self-renewal and pluripotency[44].

Accumulating evidence has demonstrated that OCT4, SOX2 and NANOG are the core factors in a pluripotency gene network involved in the induction, maintenance and loss of pluripotency (reviewed in[45,46]). The state of pluripotency, displaying regulatory flexibility, is supported by a highly interconnected pluripotency gene regulatory network that functionally relies on a set of core pluripotency TFs. The state of pluripotency integrates external signals and exerts control over the decision between self-renewal and differentiation at the transcriptional, post-transcriptional and epigenetic levels[46]. Growing evidence shows that the overexpression of these core stemness-associated TFs occurs in various types of human cancers, and aberrant expression of these TFs is associated with tumor initiation, progression and therapy resistance (reviewed in[47]). Although core stemness-associated TFs play critical roles in maintaining pluripotency and self-renewal in both ESCs and CSCs, distinct mechanistic functions between them have been proposed[48].

Since the first reviews of the involvement of the *SOX* genes in cancer[49], the roles of particular members of this gene family in the development of multiple cancer types have been extensively studied. It has been shown that *SOX* genes may act as oncogenes, tumor suppressor genes, or both depending on the cellular context (reviewed in[50,51]). Oncogenic SOX TFs are overexpressed in multiple cancer types, exerting their oncogenic function *via* several mechanisms, including the promotion of proliferation, suppression of apoptosis, promotion of metastasis, and maintenance of CSCs[50,52-54].

SOX2 TF is one of the most studied SOX proteins, and during the last decade, aberrant SOX2 expression has been associated with various types of cancer (reviewed in[55,56]). In various tumor types, SOX2 has been linked to cancer stemness, and elevated SOX2 expression has also been associated with chemotherapy resistance[57],

Table 1 Classification of the human *SOX* genes[35]

SOXA	*SOXB*		*SOXC*	*SOXD*	*SOXE*	*SOXF*	*SOXG*	*SOXH*
	SOXB1	*SOXB2*						
SRY	*SOX1*	*SOX14*	*SOX4*	*SOX5*	*SOX8*	*SOX7*	*SOX15*	*SOX30*
	SOX2	*SOX21*	*SOX11*	*SOX6*	*SOX9*	*SOX17*		
	SOX3		*SOX12*	*SOX13*	*SOX10*	*SOX18*		

endothelial-to mesenchymal transition[58], promotion of clonogenicity, and *in vivo* tumorigenicity[57,59]. In addition, elevated SOX2 expression in glioblastoma (GBM) is associated with increased cell motility and tumor spreading, and its expression is detected amongst circulating CSC islets[60-62]. A summary of the various roles that SOX2 plays in cancer is presented in Figure 2.

The impact of SOX proteins on brain tumors has been extensively studied in the last few years, and significant contributions have been made to the understanding of the roles of SOX in the initiation, progression, dedifferentiation and spreading of glioma tumors.

GBM

GBM, classified as a grade IV glioma tumor, is the most common and malignant primary brain tumor[63]. GBM accounts for more than 60% of all brain tumors in adults and approximately 45% of all gliomas; the annual incidence of GBM is 3-4 cases per 100000 people[64-66]. The ratio of GBM occurrence is, to some extent, higher in men than in women (1.6:1)[63]. The median overall survival of patients with this type of tumor is approximately 12–15 months[67], with a median age at diagnosis of 65 years[68]. It has been demonstrated that the 5-year survival rate of GBM is 5.3%[69]. It is the most aggressive malignant brain tumor in adults, is among the most vascularized solid tumors (reviewed in[70,71]), consists of heterogeneous cell populations in different phases of differentiation[72], and recapitulates most of the hallmarks of cancer, including uncontrolled proliferation, resistance to apoptosis, dysregulation of the cell cycle and angiogenesis (reviewed in[73]).

GBMs are defined as primary, arising *de novo*, or secondary, developing from lower-grade glioma tumors (grade II or III astrocytoma) through malignant transformation [74]. Four clinically relevant subtypes of GBM have been identified based on molecular expression patterns: proneural, neural, classical and mesenchymal[75]. It has been demonstrated that various molecular subclasses can be detected within the same tumor[76]. It is reported in the literature that the proneural and neural GBM subtypes arise in or near the subventricular zone, while the mesenchymal and classical subtypes occur distal to the subventricular zone (reviewed in[77]). Furthermore, based on the gene expression profile, Park *et al*[78] identified three prognostic subtypes of GBM: mitotic (favorable), intermediate and invasive.

GBM persistently communicates with its TME, and the TME contributes to the tumorigenesis and progression of GBM (reviewed in[79,80]). The GBM TME is extremely heterogeneous, consisting of the extracellular matrix, tumor cells such as glioma stem cells (GSCs), and non-tumor cells, including endothelial cells, pericytes, microglia, immune cells, oligodendrocytes, neurons, astrocytes and myeloid-derived suppressor cells (reviewed in[81]). Extracellular vesicles containing soluble proteins, DNA, mRNA and noncoding RNAs enable communication between the GBM and the TME, and these vesicles contribute to angiogenesis, invasion, evasion of apoptosis and resistance to drugs (reviewed in[82,83]). Furthermore, it has been shown that GBM cells communicate by thin membrane channels (tunneling nanotubes), and the results obtained by Valdebenito *et al*[84] revealed that these nanotubes mediate the protection of GBM cells from temozolomide (TMZ) and ionizing radiation treatment.

The precise cell of origin of GBM is still a controversial issue. It has been reported in the literature that neural stem cells (NSCs) of the subventricular zone and oligodendrocyte precursor cells represent two major candidates for the GBM cell of origin (Figure 3) (reviewed in[85]). Furthermore, several studies proposed that GBM might arise from the malignant transformation of glial and astrocyte precursor cells as well as from the dedifferentiation of astrocytes and neurons (reviewed in[85-87]).

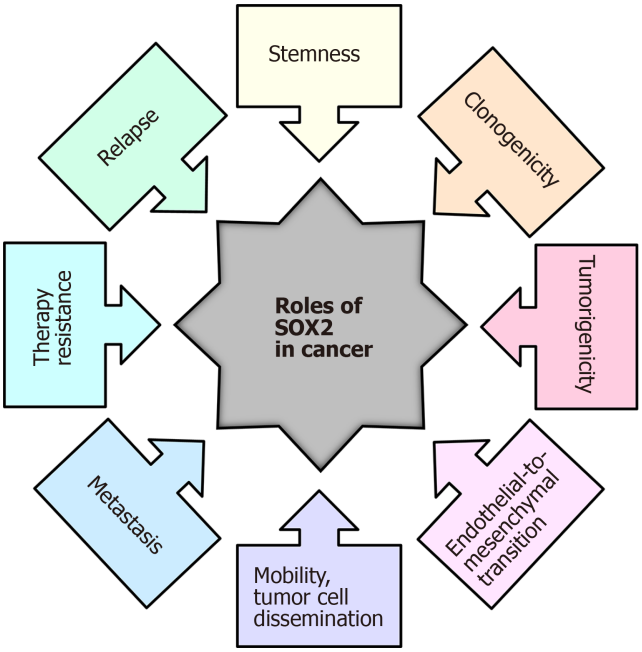

Figure 2 The roles of the SOX2 transcription factor in cancer. Modified from[223].

The current standard therapeutic treatment of GBM includes tumor resection by surgery, followed by radiotherapy and concomitant chemotherapy with TMZ[67]. However, GBM still remains incurable and usually recurs due to a high level of intertumoral and intratumoral heterogeneity demonstrated at the histological, cellular and molecular levels (reviewed in[88-90]), the presence of GSCs, infiltration into the healthy brain parenchyma, the high rate of migration from the tumor core and the ability to generate secondary tumors (reviewed in[91]).

GBM tumor initiation, progression, relapse, and resistance to treatments are associated with GSCs (reviewed in[92,93]), and these cells represent one of the main therapeutic targets.

ROLE OF *SOX* GENES IN GBM AND CORRELATIONS WITH PROGNOSIS AND SURVIVAL OF PATIENTS

Almost all members of the *SOX* gene family contribute to the malignant phenotype of GBM by regulating cell proliferation, migration, invasion, apoptosis, stemness, tumorigenicity or angiogenesis. The correlation of *SOX* gene expression levels with patient clinical outcomes has been documented (Table 2).

Various *SOX* genes exert oncogenic properties in GBM. *SOX1* regulates the self-renewal and proliferation of patient-derived cells, tumor initiation and progression [94]. The expression of *SOX1* in GBM is higher than that in the normal brain and low-grade glioma and correlates with shorter overall survival[94]. *SOX2* is one of the most studied *SOX* genes and is overexpressed in tumor samples from patients with GBM and in GBM cell lines[95-97]. *SOX2* gene exerts oncogenic activity in glioma cells[98]. High expression levels of SOX2, together with other stem cell markers, are observed in poorly differentiated GBM and are associated with its aggressiveness and poor prognosis[99-101]. It has been shown that recurrence of the tumor arises at the original site with a more invasive, aggressive phenotype and that is less sensitive to radiochemotherapy, and exhibits increased *SOX2* expression levels compared to corresponding observations in the primary tumors[102]. In contrast, there are data showing that *SOX2* expression is decreased in recurrent gliomas in comparison with its expression in primary gliomas and that patients with lower *SOX2* expression have a poor prognosis after receiving chemoradiotherapy[103]. Furthermore, we have shown that *SOX3* promotes the malignant behavior of GBM cells. *SOX3* affects GBM cell viability, proliferation, migration, invasion, and autophagy, and its high expression does not correlate with the overall survival of patients with GBM[104]. However, another study revealed that higher *SOX3* expression levels in glioma and glioma cell lines correlate with poor outcome[105]. *SOX9* exhibits oncogenic functions; its

Table 2 *SOX* gene activity in glioblastoma and correlation with the clinical outcome of glioblastoma patients

Ref.	Gene	Activity	Clinical outcome
[94]	*SOX1*	Oncogenic	Poor
[98-101]	*SOX2*	Oncogenic	Poor
[104,105]	*SOX3*	Possible oncogenic	No correlation / poor
[129-131]	*SOX4*	Oncogenic in glioma-initiating cells; tumor suppressor in GBM	Poor/good
[115,125]	*SOX5*	Tumor suppressor	Poor
[116,125]	*SOX6*	Tumor suppressor	Poor
[117,127]	*SOX7*	Tumor suppressor; glioma angiogenesis	Poor
[106-108]	*SOX9*	Oncogenic	Poor
[110,111]	*SOX10*	Possible oncogenic	Poor
[119]	*SOX11*	Tumor suppressor	Good

Glioblastoma: GBM.

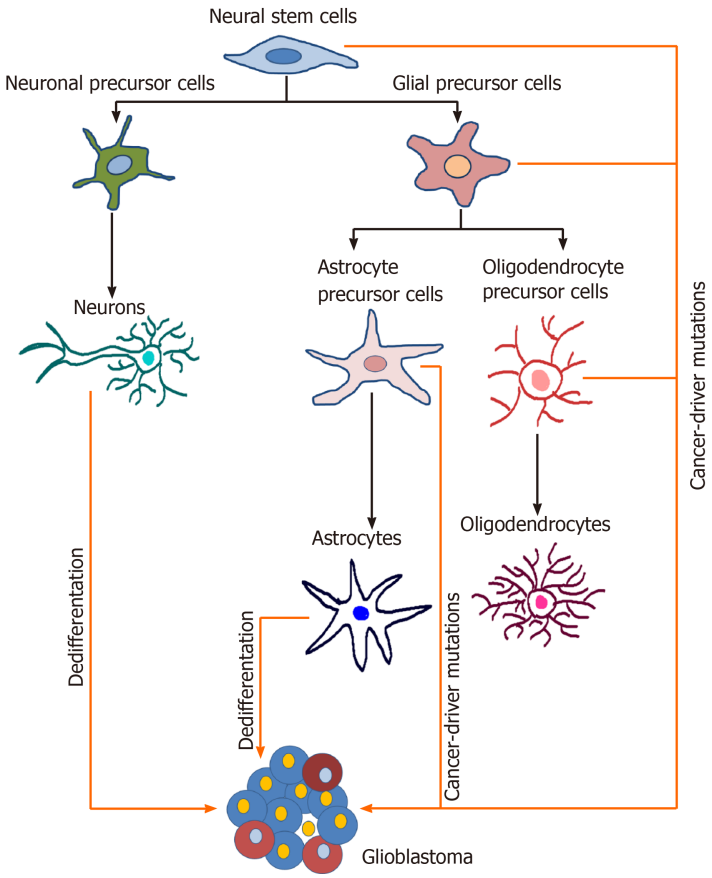

Figure 3 Potential cells of origin of glioblastoma. During the neural differentiation process (black arrows), neural stem cells differentiate into fate-restricted precursors that are capable of differentiating into neurons, astrocytes or oligodendrocytes. Glioblastoma cells can arise due to the accumulation of cancer driver mutations in different cell types or due to the dedifferentiation of mature cells (orange arrows). Modified from[224]. References are included in the main text.

silencing reduces proliferative capacity, arrests the cell cycle in the G2/M phase and increases glioma cell apoptosis[106]. The *SOX9* gene is also involved in the survival, proliferation and senescence of glioma cells[107]. Compared to the survival time of patients with lower *SOX9* expression, the survival of glioma patients with increased *SOX9* gene expression is shorter[106,108]. In addition, *SOX10* is expressed in gliomas [109] and, together with platelet-derived growth factor B, induces glioma development [110]. Moreover, high expression of *SOX10* correlates with reduced overall survival

[111].

Despite the well-established oncogenic activities of some *SOX* genes, others show tumor suppressive properties and are associated with good clinical outcomes. It was shown that *SOX5* expression was high in glioma samples and glioma cell lines compared to the *SOX5* expression in the normal adult brain and that the survival of patients with GBM with IgG antibodies against SOX5 in the sera was significantly prolonged compared to the survival of patients with no IgG antibodies[112]. Furthermore, *SOX5* overexpression inhibits clone formation of human glioma cells [113]. Schlierf *et al*[114] showed that the expression level of *SOX5* in gliomas was the same or lower than that in adult brain tissue. In contrast, Tian *et al*[115] revealed that high expression of *SOX5* correlates with poor prognosis in patients with GBM. These contradictory data probably arose as a consequence of GBM heterogeneity. Furthermore, it was shown that low *SOX6* expression in patients with GBM contributes to a better survival rate than that in patients who display high *SOX6* expression [116]. Functional analyses have shown that *SOX7* acts as a tumor suppressor. It has been demonstrated that *SOX7* represses glioma cell proliferation and migration *in vitro* and suppresses tumor formation *in vivo*[117]. It has been reported that *SOX11* is overexpressed in glioma samples[118]. Hide *et al*[119] showed that downregulation of *SOX11* expression is associated with a decrease in the survival of patients with GBM. Another study showed that SOX11, together with neurogenin 2, can reprogram glioma cells into neuron-like cells, inhibiting tumor growth and improving survival[120]. Recent data confirmed the tumor suppressor activity of the *SOX15* gene in glioma tumors. Specifically, *SOX15* overexpression inhibits proliferation and invasion, while its upregulation delays tumor formation *in vivo*[121]. Furthermore, it has been demonstrated that *SOX21* acts as a tumor suppressor[122] that inhibits *SOX2* expression, induces apoptosis in human glioma cells[123] and leads to the differentiation of glioma cells, thus inhibiting glioma progression *in vivo*[124]. Moreover, it has been shown that simultaneous ectopic expression of the *SOX5*, *SOX6* and *SOX21* genes in human primary GBM cells induces senescence and apoptosis, thus reducing their malignant potential[125]. Their downregulation increases the capacity of stem cells from the subventricular zone of the mouse brain to form glioma tumors upon encountering oncogenic stimuli[125].

Angiogenesis represents one of the hallmarks of GBM[126]. Upregulation of *SOX7* expression promotes tumor angiogenesis in a mouse model of high-grade glioma, and its high expression in GBM patients is associated with poor survival and early recurrence[127]. *SOX13* silencing reduced the tube formation abilities of U87 glioma-exposed endothelial cells[128]. Downregulation of SOX17 expression inhibited tumor angiogenesis in a mouse model of high-grade glioma, while no correlation between its expression level and the clinical outcome of patients with GBM was documented[127].

Some *SOX* genes display both oncogenic and tumor suppressive functions depending on the cellular context. For instance, *SOX4* exerts tumor suppressor activity in glioma cells by inducing GO/G1 cell cycle arrest and inhibiting growth[129]. In contrast, this gene is involved in maintaining the stemness of glioma-initiating cells [130]. There are also contradictory data regarding the correlation between *SOX4* expression and the clinical outcome in patients with GBM. Galatro *et al*[131] reported that patients with GBM with combined high expression of *ID4*, *SOX4* and *OCT4* exhibited lower survival. On the other hand, Zhang *et al*[129] demonstrated that *SOX4* expression is positively correlated with a good prognosis for patients with primary GBM.

Taken together, these reported findings demonstrate that almost all *SOX* genes are expressed in GBM and the expression levels of the majority of these genes are associated with the prognosis and survival of patients with GBM. *SOX*genes that exert oncogenic roles are usually associated with a poor prognosis and shorter survival, making them potential candidates as prognostic biomarkers and future therapeutic targets.

GSCs

GSCs were among the first CSCs identified in solid tumors[132]. GSCs are one of the major contributors to GBM heterogeneity; these cells exhibit a high proliferation rate and self-renewal capacity (reviewed in[71]), an ability to differentiate into diverse cell types, the ability to recapitulate a whole tumor (reviewed in[133]), enhanced invasive capacity, increased angiogenic potential[134] and an ability to initiate tumor growth and progression shortly after the surgical removal of the primary GBM tumor

(reviewed in[92]). GSCs usually express specific markers, such as CD133 (Prominin 1), CD44, NANOG, SOX2, OCT4, POU class 3 homeobox 2 (POU3F2), MYC proto-oncogene (c-Myc), Spalt Like TF 2 (SALL2) and KAT8 regulatory NSL complex subunit 2[135,136]. It has been reported that GBM cells are able to interconvert between GSC and non-GSC states[137]. Additionally, it has been found that a single GBM tumor contains different subsets of GSCs: proneural and mesenchymal[138].

Numerous studies have demonstrated the importance of signaling pathways in the maintenance of GSC properties, including the Hedgehog (HH)[139], Wnt/β-catenin [140], NOTCH[141], Epidermal growth factor receptor[142], Phosphatidylinositol-3-phosphate kinase/AKT/Mammalian target of rapamycin[143], Mitogen-activated protein kinase (MAPK)[144], Inhibitory kappa B kinase/Nuclear factor-kappa B (NF-κB)[145], Transforming growth factor-beta (TGF-β)/Small mothers against decapentaplegic[130] and Janus kinase/Signal transducers and activators of transcription (STAT) [146] pathways (Figure 4). It has been proposed that the regulation of the GSC phenotype results from a complex interplay between multiple signaling cascades[143, 147-149]. Accordingly, targeting several GSC-related signaling pathways at the same time seems to be necessary for the increased efficiency of anticancer treatments. A variety of small-molecule inhibitors of the HH, NOTCH and WNT/β-catenin signaling pathways have been identified/developed, along with inhibitors that target different pathways simultaneously, and the efficacy of some of these inhibitors have been demonstrated in preclinical and clinical studies[150,151].

GSCs are enriched in recurrent gliomas (reviewed in[152]), and they form more aggressive and diffuse tumors after intracranial transplantation in athymic nude mice than GSCs originating from the primary tumor of the same patient[153]. These factors make GSCs a primary therapeutic target. Cho *et al*[154] described five methods for targeting GSCs: development of GSC-specific chemotherapeutic agents, application of radiosensitizers, usage of immune cells capable of attacking GSCs, induction of GSC differentiation and gene therapy.

GSCs reside in the perivascular and perinecrotic GBM niches (reviewed in[79]). Perivascular niches contain normal and reactive astrocytes, pericytes, glioma-associated microglia/macrophages, myeloid cells, fibroblasts and normal NSCs in addition to GSCs and tumor cells (reviewed in[79]). GSCs residing in the perivascular niches divide slowly, are resistant to therapy and produce high levels of proangiogenic factors (reviewed in[155,156]). It has been reported that endothelial cells stimulate GSC self-renewal and tumorigenicity; on the other hand, GSCs may regulate and contribute to the tumor vasculature by producing cytokines and chemokines and by transdifferentiating into endothelial cells or pericytes (reviewed in[157]). Perinecrotic niches contain GSCs around necrotic foci induced by hypoxia (reviewed in[158]). Hypoxia induces signaling pathways that can impact GSC self-renewal, proliferation and invasion (reviewed in[158]). Cells expressing molecular markers of both hypoxia and GSCs (*e.g.*, SOX2, NANOG, CD133) are largely found in perinecrotic niches (reviewed in[134]).

SOX GENES AND MAINTENANCE OF GSCs PROPERTIES

It has been reported that *SOX* genes play important roles in the maintenance of GSC properties (Figure 5). Knockdown of *SOX1* expression in GSCs impairs their self-renewal, proliferation, viability and tumor-forming capacity, while *SOX1* overexpression promotes their malignant phenotype[94]. Among the SOX TF family, SOX2 is considered to be the most important player in the maintenance of GSC properties, including their tumorigenicity. Gangemi *et al*[159] demonstrated that silencing the *SOX2* gene in GSCs terminated cell proliferation, consequentially leading to the loss of tumorigenicity. The results obtained by Song *et al*[160] indicate that SOX2 plays a primary role in the regulation of CD133-positive GBM cell tumorigenicity. On the other hand, Cox *et al*[161] demonstrated that a 3-fold increase in *SOX*2 expression in GBM cells decreases their capacity to proliferate and generate spheres. These results suggest that the level of *SOX2* expression must be precisely regulated by tumor cells during gliomagenesis. Furthermore, it has been found that co-expression of SOX2, oligodendrocyte TF 2 (OLIG2) and zinc finger E-box binding homeobox 2 is involved in the maintenance of GSCs[162]. Downregulation of SOX2 expression impairs sphere-forming capacity, self-renewal, viability, proliferation, migratory and invasive capabilities of brain tumor stem cells derived from GBM[98]. Furthermore, knockdown of SOX3 expression in GSCs decreases the proliferation, migration and invasion capabilities of these cells and enhances their apoptosis[163], while knockdown of SOX4

Figure 4 Signaling pathways involved in the maintenance of glioma stem cell properties. The main signaling pathways involved in the maintenance of glioma stem cell properties include Hedgehog, Wnt/β-catenin, NOTCH, Epidermal growth factor receptor, Phosphatidylinositol-3-phosphate kinase/AKT/Mammalian target of rapamycin, Mitogen-activated protein kinase, Inhibitory kappa B kinase/Nuclear factor-kappa B, Transforming growth factor-beta/Small mothers against decapentaplegic and Janus kinase/Signal transducers and activators of transcription. References are included in the main text. MAPK: Mitogen-activated protein kinase; JAK/STAT: Janus kinase/Signal transducers and activators of transcription; EGFR: Epidermal growth factor receptor; PI3K/AKT/mTOR: Phosphatidylinositol-3-phosphate kinase/AKT/Mammalian target of rapamycin; IKK/NF-κB: Inhibitory kappa B kinase/Nuclear factor-kappa B; TGF-β/SMAD: Transforming growth factor-beta/Small mothers against decapentaplegic; GSCs: Glioma stem cells.

expression in glioma-initiating cells results in reduced sphere-forming and self-renewal capacities[130]. Silencing of *SOX9* leads to inhibition of sphere-forming capacity[164], while *SOX9* overexpression increases the sphere-forming capacity of GBM cells and induces the formation of larger tumors[165]. In glioma-initiating cells, inhibition of the midkine/anaplastic lymphoma kinase axis promotes SOX9 degradation, resulting in a decrease in the self-renewal and tumorigenic capacities of glioma-initiating cells[166]. Kurtsdotter *et al*[125] demonstrated that simultaneous forced expression of SOX5, SOX6 and SOX21 factors blocks the tumor-inducing capacity of human primary GBM cells.

THE ROLES OF *SOX* GENES IN STEMNESS AND DIFFERENTIATION OF GSCs

It has been reported that SOX TFs have important functions in both the maintenance of the stemness of GSCs and the dedifferentiation of glioma cells (Figure 6). The results obtained by Berezovsky *et al*[100] indicate that *SOX2* may play a central role in the regulation of dedifferentiation and acquisition of GSC properties. They found that knockdown of *SOX2* expression blocks the dedifferentiation of HF2303 GBM cells and reduces their tumorigenicity. Additionally, downregulation of *SOX2* expression in brain tumor stem cells derived from GBM led to an increase in the expression of differentiation markers[98]. Furthermore, it was shown that the TGF-β/SOX4/SOX2 pathway plays a crucial role in the maintenance of stemness and tumorigenicity in glioma-initiating cells[130]. It has been shown that the combination of SOX2, POU3F2, SALL2 and OLIG2, detected in the proneural subtype of GBMs, is able to reprogram differentiated tumor cells into GSCs[136].

In addition to the overwhelming evidence of the importance of the *SOX2* gene in the maintenance of the stem cell properties of GSCs, it was shown that the other two members of the SOXB1 subgroup also play important roles. Dedifferentiation of GBM cell lines is accompanied by an increase in *SOX1* expression. Additionally, *SOX1* expression was higher in patient-derived GSCs than in conventional glioma cell lines, and after the differentiation of these cells, the levels of SOX1 decreased dramatically [94]. Recently, we revealed that *SOX3* expression was higher in patient-derived GSCs, as well as in oncospheres derived from GBM cell lines, compared to *SOX3* expression

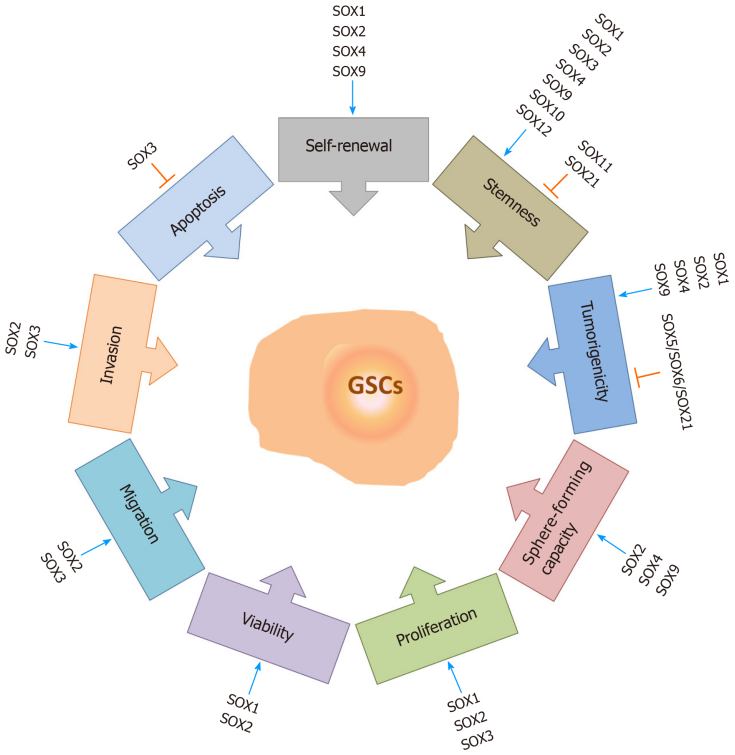

Figure 5 The roles of SOX transcription factors in glioma stem cells. SOX transcription factors exert numerous functions in glioma stem cells (GSCs). They influence GSC self-renewal, stemness, tumorigenicity, sphere-forming capacity, proliferation rate, viability, migration and invasion capacities, and apoptosis. Some of the members of the *SOX* gene family are involved in the resistance of GSCs to therapy. Blue arrows represent stimulation, and orange bars represent inhibition. References are included in the main text. GSCs: Glioma stem cells.

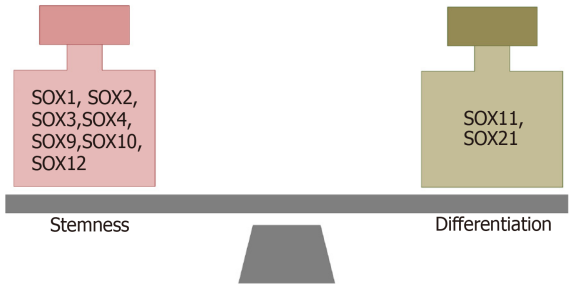

Figure 6 SOX transcription factors involved in the maintenance of stemness and differentiation of glioma stem cells. References are included in the main text.

in their differentiated counterparts, suggesting that *SOX3* expression is associated with the undifferentiated state of GBM cells[104].

Additionally, the roles of other *SOX* genes in the regulation of stemness and differentiation of GSCs have been described. It was found that the number of differentiated cells is increased after SOX4 knockdown in glioma-initiating cells[130]. On the other hand, the expression of *SOX9* is increased in oncospheres derived from GBM cell lines compared to its expression in their differentiated counterparts, and silencing of *SOX9* downregulated the expression of the stem cell markers CD133, NESTIN and SOX2 [164]. Overexpression of *SOX10* in LN229 GBM cells increased the expression levels of the stemness markers CD133, NESTIN, OCT4, NANOG and CD44[111]. On the other hand, *SOX11* overexpression in glioma-initiating cell-like cells and human glioma-initiating cells from malignant glioma blocked their tumorigenic ability by inducing neuronal differentiation[119]. The results obtained by Wu *et al*[167] indicate that SOX12 regulates the stemness of GBM cells and that SOX2, CD133, and OCT4 act as downstream effectors of SOX12 in these cells. Additionally, the results obtained by Caglayan *et al*[124] suggest that *SOX21* overexpression decreases the stem-like cell properties of glioma cells and initiates their differentiation *in vivo*.

Together, these reported findings indicate that SOX TFs play important roles in GBM and GSCs. Since GSCs are the main drivers of GBM progression and therapy resistance, further studies are needed to comprehensively delineate the roles of SOX TFs in these cells.

SOX GENES AND CHEMO-AND RADIORESISTANCE OF GSCs

GBM inevitably recurs following the initial therapy as a result of intratumoral heterogeneity and resistance to chemo- and radiotherapy, which are considered to be promoted by GSCs (reviewed in[133,156]). Conventional chemo- and radiotherapies affect the proliferative cells within the tumor, while GSCs successfully evade their effects through multiple intrinsic GSC features and adaptive mechanisms (Figure 7). These mechanisms include enhanced expression of the DNA mismatch repair gene *MGMT* (O^6-methylguanine-DNA methyltransferase)[168], high expression of drug efflux transporters[168], activation of the antiapoptotic pathway[168], increased replication stress leading to a constitutively active DNA damage response[169,170] and aberrant activation of multiple signaling pathways, such as Notch, HH, Wnt/β-catenin and NF-κB[171-174].

One of the essential features of cancer cells that confers resistance to therapy is their plasticity, *i.e.*, the capability of going through phenotypic changes in response to different environmental signals without genomic alteration. It has been shown that extrinsic factors such as radiation[175] or TNF-α (tumor necrosis factor α) through activation of the NF-κB pathway[171] promote proneural to mesenchymal shift of GSCs leading to a radioresistant phenotype[171]. Segerman *et al*[176] demonstrated that GBMs contain a heterogeneous population of glioma-initiating cells that exhibit variable levels of resistance to radiation and drugs[176]. The observed variations in therapy responses are the result of the slow shifts between the proneural- and mesenchymal-like cell states regulated by the changes in DNA methylation of the cis-regulatory regions of mesenchymal master regulator genes.

Another important mechanism underlying GBM resistance to therapy is the ability of differentiated cancer cells to undergo dedifferentiation into cancer stem-like cells. Auffinger *et al*[177] showed that long-term exposure of glioma cells to clinically relevant doses of TMZ caused the phenotypic switch of non-GSCs towards GSCs that exhibited expression of pluripotency- and stemness-related markers CD133, SOX2, OCT4, and Nestin, a highly infiltrative phenotype and increased chemoresistance. Analyses of the long-term effects of sub-toxic doses of radiation on patient-derived differentiated GBM cells and the phenotypic and molecular changes in GBM towards stemness *in vitro* were demonstrated, including the expression of stem cell markers SOX2, Nestin and OLIG2, as well as increased tumorigenicity *in vivo*[178].

Quiescent, slow-cycling CSCs play a critical role in tumor recurrence, as they escape the effects of chemo- and radiotherapy that mostly eradicate proliferative cells in the tumor bulk. TMZ treatment in a transgenic mouse model of glioma demonstrated that recurrent tumors derived from dormant glioma cells exhibited stem cell properties [179]. Qazi *et al*[180] revealed that the combined chemoradiotherapy of primary human GBMs resulted in acquired resistance of brain tumor stem cells, which was accompanied by increased expression of stemness-associated factors SOX2 and BMI1 and an enhanced self-renewal capacity of primary GBM cells.

The roles of the *SOX2* and *SOX9* genes in the chemo- and radioresistance of GSCs have been well documented among the members of the SOX TF family (Figure 8). High expression of *SOX2* in GBM patient-derived CD133-positive GSCs was shown to play a key role in promoting the self-renewal of GSCs, thereby contributing to their drug resistance[160]. Yang *et al*[181] showed that the miR-145/OCT4/SOX2 axis plays an important role in the chemoradioresistance of CD133-positive GSCs. Inhibition of stemness factors SOX2 and OCT4 by miR-145 was followed by an increased sensitivity of patient-derived CD133-positive GSCs to TMZ and radiation. The reduced chemoradioresistance of GSCs was also accompanied by a decrease in the expression of a few drug resistance (*MDR1* - multidrug resistance protein 1 and *ABCG2*-ABC subfamily G member 2) and antiapoptotic genes (*Bcl-2* - B-cell lymphoma 2 and *Bcl-xL* - B-cell lymphoma-extra large)[181]. Overexpression of *ID-4* (inhibitor of differentiation 4) induced the dedifferentiation of human glioma cells into glioma stem-like cells and downregulated the expression of *SOX2*-repressing miR-9*, leading to enhanced *SOX2* expression[182]. SOX2, as a direct transcriptional regulator, upregulates the expression of ABCC3 and ABCC6 transporters, conferring chemoresistance on ID4-induced glioma stem-like cells and patient-derived GSCs[182]. Furthermore, it was revealed

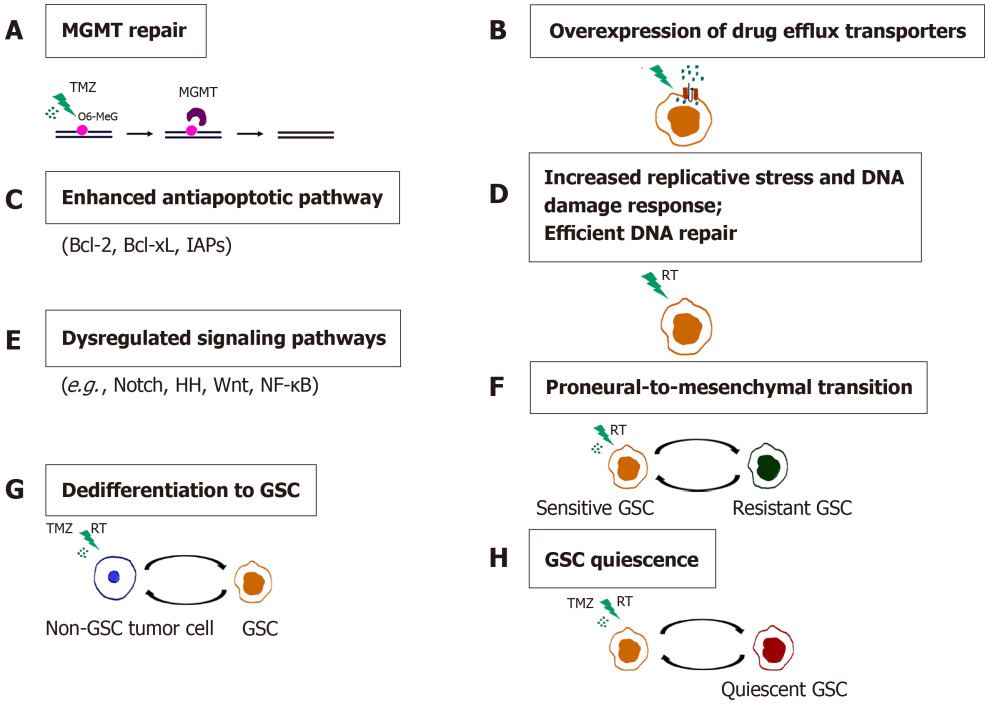

Figure 7 Mechanisms of chemo- and radioresistance of glioma stem cells. A: O^6-methylguanine-DNA methyltransferase (MGMT) repair; B: Overexpression of drug efflux transporters; C: Enhanced antiapoptotic pathway; D: Increased replicative stress and DNA damage response. Efficient DNA repair; E: Dysregulated signaling pathways; F: Proneural-to-mesenchymal transition; G: Dedifferentiation to GSC; H: Glioma stem cells (GSC) quiescence. Resistance of GSCs to temozolomide is achieved through increased expression of MGMT, drug efflux transporters and antiapoptotic proteins. Replicative stress, constitutively active DNA damage response and efficient DNA repair confer radioresistance to GSCs. Aberrantly activated signaling pathways, the ability of non-GSC tumor cells to dedifferentiate, proneural-to-mesenchymal transition and the capacity of GSCs to become quiescent contribute to the chemo- and radioresistance of glioblastoma. References are included in the main text. RT: radiation; O6-MeG: O-6-methylguanine; GSC: Glioma stem cell; TMZ: Temozolomide.

that *SOX2* is a direct transcriptional target of Forkhead box M1 (FOXM1) and that the FOXM1/SOX2 axis promotes the stemness and radioresistance of GBM[183].

A study by Garros-Regulez *et al*[165] demonstrated the correlation between high levels of SOX2 and SOX9 expression and resistance of GSCs to TMZ. The SOX2/SOX9 axis acts downstream of the mTOR signaling pathway and contributes to the observed chemoresistance. The authors proposed pharmacological inhibition of SOX2 by using specific inhibitors of the Sonic Hedgehog (SHH) and mTOR signaling pathways as a promising therapeutic approach to overcome TMZ resistance in a group of GBMs that display high expression levels of SOX2 and SOX9[165]. Wang *et al*[164] uncovered the *PDK1* (pyruvate dehydrogenase kinase 1) gene as a direct target of SOX9 that was shown to play a role in the regulation of GSC self-renewal and resistance to TMZ. The silencing of *SOX9* and inhibition of *PDK1* both reduced the activity of the AKT pathway and sensitized GSCs to TMZ treatment *in vivo*, highlighting the SOX9/PDK1 axis as a new promising target for more efficient GBM therapy. Most recently, Sabelström *et al*[184] demonstrated the critical role of the ERK1/2/miR-124/SOX9 axis in triggering the differentiation of stem-like GBM cells towards a neuronal phenotype. Inhibition of ERK1/2 activation by using a MEK inhibitor caused the enhanced expression of miR-124 and a reduction in the expression of its target gene, *SOX9*, consequently leading to decreased tumorigenicity and radioresistance in patient-derived GBM xenografts. These results imply that the induction of neuronal differentiation of GSCs represents a promising therapeutic approach in anti-GBM therapy.

CONCLUSION

Combatting GBM has been a demanding and challenging goal for decades. Heterogeneity of cell types within the tumor, the contribution of patient-to-patient variability to the growth, the response to treatment driven by the genomics of each tumor and the transition between proliferative and non-proliferative phases make GBM extremely difficult to treat (reviewed in[185]). GBM is one of the deadliest cancers, and GBM studies face many challenges, some of which are unique to brain tumors (blood-brain

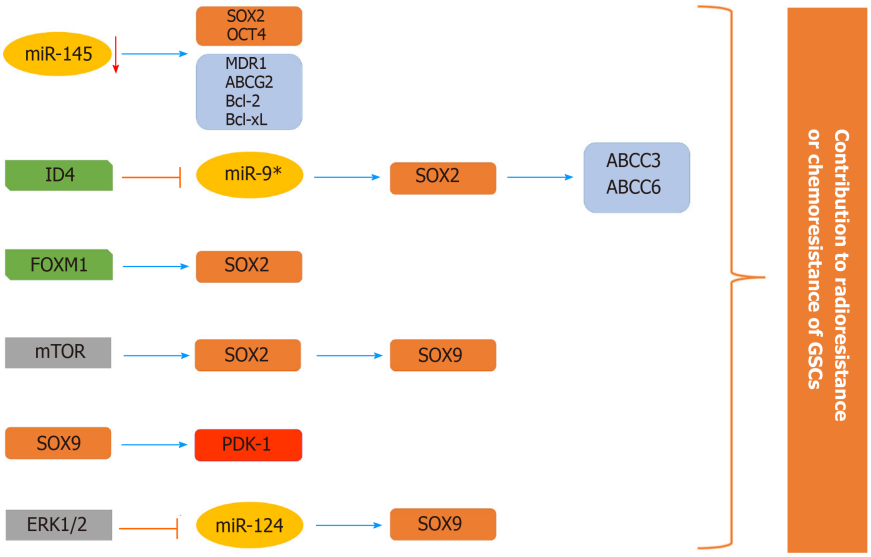

Figure 8 Schematic illustration of the involvement of SOX2 and SOX9 in the chemoresistance and radioresistance of glioma stem cells. The expression of stemness-related transcription factor SOX2 is elevated in glioma stem cells (GSCs) through various mechanisms (decreased expression of miR-145 and miR-9* that directly target *SOX2*, direct transcriptional activation by Forkhead box M1 and through mammalian target of rapamycin signaling) contributing to chemo- and radioresistance of GSCs. SOX2 directly activates the expression of ABCC3 and ABCC6 transporters. mTOR signaling affects the SOX2/SOX9 axis, contributing to chemoresistance. The ERK1/2/miR-124/SOX9 axis and direct targeting of *PDK1* (pyruvate dehydrogenase kinase 1) by SOX9 have a role in resistance to radiation and temozolomide, respectively. References are included in the main text. GSCs: Glioma stem cells; FOXM1: Forkhead box M1; mTOR: Mammalian target of rapamycin; PDK1: Pyruvate dehydrogenase kinase 1.

barrier and immunosuppressive environment) and others that are shared with other tumors (tumor heterogeneity at the cellular and molecular levels, plasticity, and the presence of CSCs). One of the most essential hallmarks of GBM is tumor heterogeneity, which has been documented at the histological, cellular and molecular levels. Both intertumor heterogeneity (comprising distinct genetic alterations between different individual tumors) and intratumor heterogeneity (comprising diversity within individual tumors) have been identified in GBM (reviewed in[90]). The heterogeneity of GBM has been defined at multiple molecular levels using-omics technologies. Heterogeneity documented at the genomic and transcriptomic levels enables the molecular classification of histopathologically indistinguishable tumors into different subtypes. An advanced understanding of intratumor heterogeneity has been achieved by single-cell sequencing. Patel *et al*[186] performed the first single-cell RNA-seq and confirmed that GBM subtype classifiers are variably expressed across individual cells within a single tumor, while a recent study revealed that malignant cells in GBM exist in four main cellular states that are reminiscent of the canonical neurodevelopmental cell types[187]. Another level of heterogeneity is linked to treatment-induced plasticity and temporal heterogeneity. By sequencing exomes of initial low-grade gliomas and recurrent tumors from the same patients, Johnson *et al*[188] revealed that at least half of the mutations in the initial tumor were undetected at recurrence in 43% of cases. Recently, a database of initial tumor and tumor recurrence samples from patients assembled by the Glass consortium revealed that 35 out of 222 patients exhibited treatment-related hypermutation at recurrence, while 70% of the cohort had an increased mutational burden after recurrence compared with the mutational burden of their initial tumor[189]. Taken together, these findings indicate that GBM heterogeneity provides additional layers of complexity that must be taken into consideration in the development of more effective therapies aimed at improving survival in GBM patients.

Remarkable progress has been made towards understanding the cellular and molecular biology of gliomas in the last decade. Advanced technologies have provided novel insights into multiple events that allow GSCs to acquire functional capabilities, enabling them to survive, proliferate and disseminate.

CSCs are considered to be, at least in part, responsible for drug resistance and cancer relapse due to their ability to self-renew and differentiate into heterogeneous lineages of cancer cells. Accordingly, the eradication of CSCs has become one of the major goals in cancer therapies. However, CSCs are very often therapy-resistant, representing the main obstacle to their eradication. Therapy resistance of CSCs is

mediated by many different mechanisms, including the acquisition of dormancy, decreased apoptosis, increased DNA repair and drug efflux capacity, interaction with their supporting microenvironment within the CSC niche and hypoxic stability (reviewed in[190]). GBM tumors may contain different subsets of GSCs[138]. GSCs may adopt a quiescent state that protects them from chemotherapy and radiotherapy (reviewed in[191]), and these treatments might increase the population of GSCs over time (reviewed in[192]). GSCs successfully evade the effects of conventional chemo- and radiotherapies through multiple intrinsic GSC features and adaptive mechanisms (Figure 7). GSCs enriched in recurrent gliomas acquire more aggressive and therapy-resistant properties than GSCs originating from primary tumors that become more malignant and rapidly spread, leading to therapeutic failures[152,153,185].

All these properties make GSCs a promising but very complex and challenging therapeutic target for GBM treatment. A number of approaches for targeting CSCs have been developed, including inhibition of drug efflux ABC transporters, targeting signaling pathways, the microenvironment and cell surface markers of CSCs, differentiation therapy, immunotherapy and application of radiosensitizers (reviewed in[133,193-196]).

Dedifferentiation of tumor cells into a stem cell-like state is considered an additional source of cellular heterogeneity, pointing to tumor cell plasticity as an important driver of GBM heterogeneity[90]. Gimple *et al*[137] proposed novel advanced therapies that are aimed at targeting tumor cell plasticity conferred by dedifferentiation (reviewed in[7,137]). One of the proposed approaches focuses on blocking dedifferentiation by applying a combination of therapies to prevent early resistance to therapeutics endowed by dedifferentiation and acquired lineage plasticity. Differentiation therapy is a novel approach that aims to target dedifferentiation by enhancing the conversion of dedifferentiated tumor cells towards permanently differentiated cells that are more sensitive to chemotherapy. The final proposed approach is focused on the differentiation of CSCs into harmless cell lineages that lack tumorigenic potency and metastatic potential by applying TFs or small molecules.

Significant conceptual and mechanistic similarities between cellular transformation in cancers and cellular reprogramming and dedifferentiation driven by various stem cell-associated pathways have been revealed[197]. The master regulators of stemness operate through transcriptional control of various stem cell-associated pathways during cellular reprogramming.

Since cell renewal is an ability that cancer cells must acquire to survive therapeutic treatment and reconstitute the tumor, master regulators of stemness have attracted significant attention in the treatment of GBM. SOX TFs are master regulators of stemness with known essential roles in GBM. Accordingly, SOX proteins and SOX2, in particular, have emerged as one of the major therapeutic targets for GBM. Overwhelming evidence of the importance of the *SOX2* gene in the maintenance of the stem cell properties of GSCs has been revealed. Numerous studies have demonstrated that glioma cells rely on SOX2 to maintain their tumorigenic activity, with GSCs displaying high levels of SOX2 (reviewed in[198]). SOX2 overexpression has been found in approximately 90% of human biopsies in GBM patient samples with enrichment in undifferentiated GSC populations. Downregulation of SOX2 in GSCs impairs cell proliferation and the ability of the cells to form tumors *in vivo*.

It has also been shown that numerous SOX TFs are involved in the maintenance of the stemness of GSCs (SOX1, SOX2, SOX3, SOX4, SOX9, SOX10, SOX12) or play a role in the initiation of GSC differentiation (SOX11 and SOX21). Accordingly, the fine-tuning of *SOX* gene expression levels is associated with the control of the balance between stemness and differentiation.

As outlined in this review, SOX TFs play multiple roles in GSCs. They influence GSC stemness, self-renewal, proliferation, differentiation, viability, migration, invasion, apoptosis, therapy resistance, sphere-forming capacity, and tumorigenicity (Figure 5). Therefore, innovative therapies aimed at targeting SOX TFs, and SOX2 in particular, are emerging as promising tools to combat GBM. These therapeutic approaches include the pharmacological inhibition of signaling pathways involving *SOX* genes[165,199,200], application of natural and synthetic compounds that control the expression of these genes[201-204], inhibition of SOX-DNA binding[205], inhibition of ubiquitylation and degradation of the SOX2 transcriptional repressor[206], immunotherapy[207,208], epigenetic silencing by miRNAs[181], blocking SOX function by peptide aptamers[209] and direct transcriptional repression of *SOX* gene expression [210] (Figure 9).

Modulation of *SOX* gene expression in GSCs represents an attractive approach to combat GBM. *SOX* gene expression in GSCs might be targeted by applying different approaches. One of the approaches to modulate *SOX* gene expression is treatment

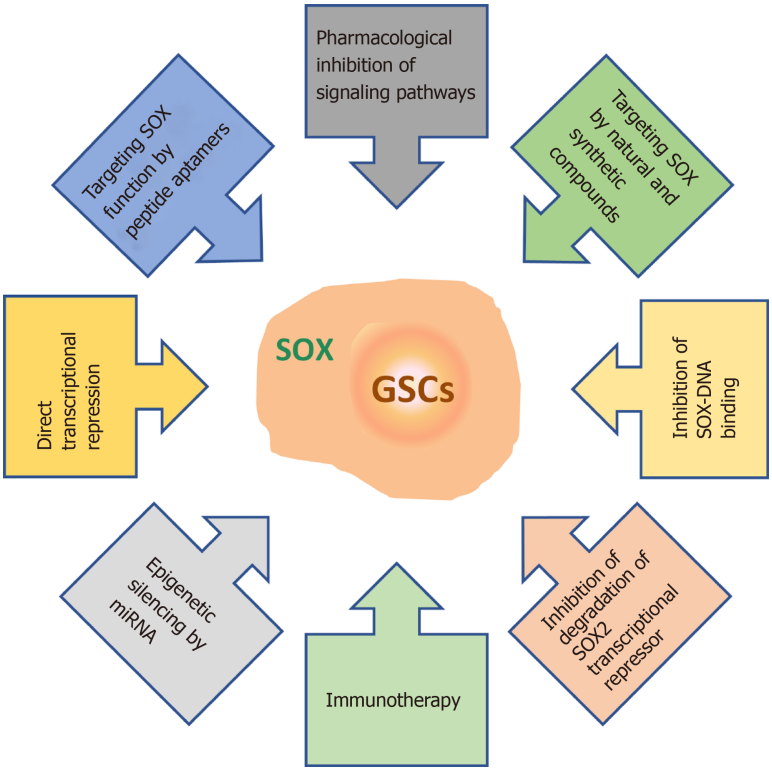

Figure 9 Overview of the therapeutic strategies for targeting *SOX* in glioma stem cells. References are included in the main text. GSCs: Glioma stem cells.

with natural plant products. Studies on natural plant products have revealed that they can act as effective antitumor agents[211]. Forty-nine per cent of the small molecules approved for tumor treatment between 1940 and 2014 were natural plant products or their derivatives[211]. Some of these products have anti-GBM potential alone or in combination with other chemotherapeutics[211-214]. Our results revealed that extracts from *Anthrisci cerefolii* and *Ononis spinosa* L. plants have anti-GBM activity[215,216]. Furthermore, it has been reported that nuciferine obtained from the leaves of *N. nucifera* Gaertn. inhibited GBM progression *via* the SOX2/AKT/STAT3/Slug pathway [201]. On the other hand, it was found that curcumin, which inhibits the proliferation of glioma cells and induces apoptosis and autophagy, simultaneously induces glioma cells to become stem-like and enhances the expression of SOX2, SOX4 and OCT4[203]. In addition to natural plant products, antibiotics such as salinomycin and actinomycin D reduce SOX2 expression in GSCs, indicating that drug repurposing may provide additional options for GBM treatment[202,204].

The expression of *SOX* genes in GSCs could be targeted by pharmacological inhibitors of signaling pathways that regulate GSC properties. Rapamycin, an inhibitor of mTOR complex 1, and cyclopamine, an inhibitor of the SHH pathway, reduced the expression of SOX2 and SOX9 in GBM cell lines[165]. Additionally, *SOX* genes could be targeted by engineered artificial TFs that selectively modulate *SOX* gene expression. Stolzenburg *et al*[210] developed zinc finger-based TFs that reduced *SOX2* expression, leading to reduced proliferation and colony formation in breast cancer cells.

Another approach is targeting SOX TFs in GSCs by the delivery of vectors that express miRNAs that were shown to specifically downregulate the expression of SOX proteins in GSCs. For example, the delivery of miRNA-145 to GBM patient-derived CSCs *in vitro* and *in vivo* resulted in the downregulation of the expression of stemness factors SOX2 and OCT4, suppression of tumorigenicity, increased sensitivity to TMZ and radiation and enhanced survival rate of xenotransplant mice when applied in combination with radiotherapy and TMZ[181].

GSCs could also be combated by vaccine approaches. It was demonstrated that vaccination of immunocompetent mice with Sox2 peptides significantly delayed tumor development[207]. Furthermore, Ueda *et al*[208] found that the SOX6 peptide induces SOX6 peptide-specific cytotoxic T lymphocytes, which are able to lyse GSCs derived from GBM.

In the last decade, immunotherapy that engages naturally occurring or genetically engineered oncolytic viruses has provided an additional strategy, especially for the treatment of cancers with poor prognosis, such as GBM. Although oncolytic viruses can infect both normal and cancer cells, they can only replicate in cancer cells[217]. Preclinical and clinical trials have pointed to the capability of oncolytic viruses to stimulate antitumor immune responses by recruiting T cells (reviewed in[218,219]). It has been reported that the Zika virus exerts oncolytic activity against GSCs. Compared to Zika virus infection of differentiated GBM cells, the Zika virus preferentially infects and selectively kills GSCs and stem-like cells in a SOX2-dependent manner[220]. It has also been reported that an immunotherapeutic approach in gliomas that uses a vesicular stomatitis virus expressing HIF-2a, SOX10 and c-Myc together with checkpoint inhibitors anti-PD1 and anti-CTLA-4 enhances the antitumor response [221].

The current therapeutic strategies for GBM have not yet provided a cure and have only resulted in a slight improvement in patient survival. Considering that GBM therapies result in poor outcomes and that GBM treatment is one of the most expensive cancer treatment *per capita* in the United States[222], there is a critical need for novel and more effective therapeutic options. Novel approaches to GBM treatment will require the fine-tuning of multimodal therapeutic strategies that simultaneously target numerous hallmarks of cancer cells to win the battle against GBM, one of the deadliest cancers.

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REVIEW

Retina stem cells, hopes and obstacles

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Abstract

Retinal degeneration is a major contributor to visual dysfunction worldwide. Although it comprises several eye diseases, loss of retinal pigment epithelial (RPE) and photoreceptor cells are the major contributors to their pathogenesis. Early therapies included diverse treatments, such as provision of anti-vascular endothelial growth factor and many survival and trophic factors that, in some cases, slow down the progression of the degeneration, but do not effectively prevent it. The finding of stem cells (SC) in the eye has led to the proposal of cell replacement strategies for retina degeneration. Therapies using different types of SC, such as retinal progenitor cells (RPCs), embryonic SC, pluripotent SCs (PSCs), induced PSCs (iPSCs), and mesenchymal stromal cells, capable of self-renewal and of differentiating into multiple cell types, have gained ample support. Numerous preclinical studies have assessed transplantation of SC in animal models, with encouraging results. The aim of this work is to revise the different preclinical and clinical approaches, analyzing the SC type used, their efficacy, safety, cell attachment and integration, absence of tumor formation and immunorejection, in order to establish which were the most relevant and successful. In addition, we examine the questions and concerns still open in the field. The data demonstrate the existence of two main approaches, aimed at replacing either RPE cells or photoreceptors. Emerging evidence suggests that RPCs and iPSC are the best candidates, presenting no ethical concerns and a low risk of immunorejection. Clinical trials have already supported the safety and efficacy of SC treatments. Serious concerns are pending, such as the risk of tumor formation, lack of attachment or integration of transplanted cells into host retinas, immunorejection, cell death, and also ethical. However, the amazing progress in

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the field in the last few years makes it possible to envisage safe and effective treatments to restore vision loss in a near future.

Key Words: Retina regeneration; Stem cells; Retina stem cell transplantation; Cancer stem cells; Photoreceptor replacement

Core Tip: Retinal degeneration is a major cause of visual loss worldwide, having no cure or suitable treatments. Transplantation of stem cells into the eye to replace lost cells is being evaluated as a new therapeutic strategy. Establishing the most suitable source of stem cells and ethical and safety concerns still require to be solved. Preclinical studies and ongoing clinical trials support stem cells as a promising therapeutic approach to restore vision loss.

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INTRODUCTION

Stem cells (SC) are rather undifferentiated cells present in most tissues of nearly all multicellular organisms, from humans[1-3], to plants[4-6], having the amazing capacity to either activate self-renewal or differentiate into specific cell types[7]. Hence, they are potentially capable of regenerating whole tissues or organs subjected to ablations or damages. This capacity varies between species. It is particularly remarkable in Platyhelminthes, such as the planarian worms, which when beheaded can regenerate their entire bodies in just a few days[8-10]. Many cold-blooded species retain a considerable regenerative capacity; some teleosts, like zebrafish, can regenerate their limbs, spinal cord, retina, heart and brain. By contrary, regenerative capacity in mammals is quite restricted[11,12].

SC have attracted great attention due to their potential for regeneration and knowledge about them has increased enormously in the last few years. Most SC have a rather simple morphology, resembling undifferentiated cells, and essentially, they have the same organelles and molecular machinery present in most eukaryotic cells. However, embryonic pluripotent SCs (PSCs) are capable during development of an extraordinary feat, to give rise to nearly all the cell types present in the body. In addition, after ablations or injuries to a given tissue, they activate a complex response leading to their reentry into the cell cycle and, eventually, to the activation of a differentiation pathway, acquiring the morphology and functions of the cells of the damaged tissue, thus being able to replace lost cells.

Based on their capacity for differentiation, SC can be classified either as unipotent, multipotent, pluripotent or totipotent. Whereas unipotent SC, such as epidermal or muscle SC, are able to generate only one type of cells upon differentiation[13,14], multipotent SC can generate a very limited amount of cell types, belonging to a closely related cell family[14], as usually is the case of adult SC. Müller glial SC in the retina belong to this group, as they potentially differentiate into just one or two retinal neuronal cell types and Müller glial cells (MGCs). PSCs, such as embryonic SC (ESCs) in the inner cell mass of the blastula, may differentiate into nearly all the cell types, while totipotent SC, like the zygote and the first few cells derived from it during zygote segmentation, have the highest capacity for differentiation and can give rise to all the cell types of an organism (Figure 1).

Based on their source, there are two main types of SC, ESC and adult (mature) SC. ESCs are included in a family of Stem/progenitor cells found in 3-5 d old embryos, which includes retinal progenitor cells (RPCs) and mesenchymal stromal cells. ESCs are pluripotent cells that can give rise to cells of the three embryonic germ layers: ectoderm, endoderm or mesoderm, and can generate all tissues in the body.

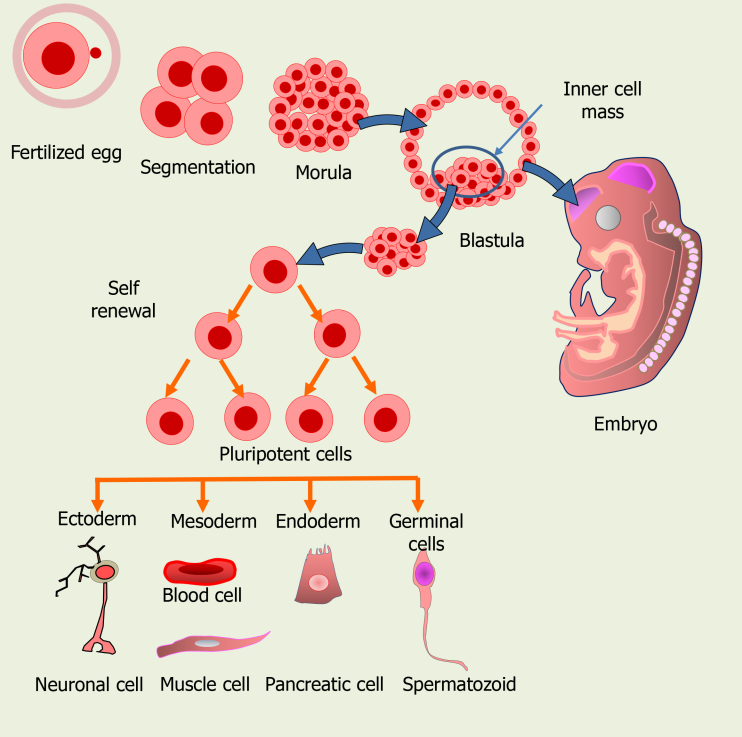

Figure 1 Origin of the different cell types from embryonic stem cells (ESCs) in the developing embryo. ESCs from the inner cell mass of a preimplantation embryo can give rise to all the cell types and to an entire organism.

A major breakthrough in SC research was achieved less than a decade ago; the introduction of a few SC specific genes, such as *Myc*, *Sox2*, *Oct3/4* and *Klf4*, induced somatic cells to acquire the morphology, characteristics, and markers of SC, thus being defined as induced PSCs (iPSCs). Their transplantation in nude mice generates tumors exhibiting tissues from the three germ layers[15], suggesting they may be used for cell replacement therapies.

Mature SC are present in small amounts in adult, differentiated tissues; these multipotent cells can give rise to a few types of specialized cells[2]. Mature SC are also found in the umbilical cord and placenta after birth. They also include hematopoietic SC (HSC) from bone marrow (BM), peripheral blood or umbilical cord blood, and are commonly used for transplantation, one of their advantages being that they rarely generate unwanted cell types.

The most relevant features of SC are their ability for self-renewal and for giving rise to multiple cell types. A very active SC self-renewal occurs during the first stages of development, both in the morula and in the inner mass of the blastula to generate the new cells required for the developing organism (Figure 1). Later, SC can activate in damaged tissues, proliferating and then differentiating to replace lost cells. In addition to the "ready to use" pool of undifferentiated SC, some differentiated cells may eventually undergo a dedifferentiation process, reentering the cell cycle and becoming "active" SC. To regain the proliferating capacity, they repress genes required for cell differentiation, activating those required for a proliferative, undifferentiated state[16].

Noteworthy, SC are similar to cancer SC in their ability to self-renew and generate large populations of more differentiated descendants; they also share the phenotypic plasticity that allows them to enter or exit the cell cycle, an ability closely associated with the stemness properties and invasiveness of cancer cells[17]. These morphological and functional similarities compromise current therapeutic efforts, as SC transplantation very frequently leads to deregulation of the mitotic cell cycle and tumor formation[18,19].

The ability of SC to repair damaged tissues and regenerate lost cells has encouraged scientists and clinicians to explore their use to treat or cure different diseases. However, many issues have still to be unraveled before using SC safely in humans. The aim of this review is to analyze the characteristics and potential advantages of using SC for treating retina neurodegenerative diseases, and the risks that remain to be addressed before using these cells in the clinic.

RETINAL DEGENERATIONS AND TREATMENTS

Contrary to early beliefs, nerve tissues in many animals, including mammals, have SC able to give rise to new neurons[20-23], sparking the hope for regenerative approaches to neurodegenerative diseases. However, at least presently, it seems nearly impossible to regenerate the brain as a whole. Humans have about 10^{11} neurons with different morphologies and multiple functions[24], which added to the complex interactions among the different neuronal types, and between neurons and other cell types (*i.e.*, glial cells), imposes tremendous limitations for brain regeneration.

Retinal degeneration (RD) represents one of the most common causes of irreversible vision impairment leading to blindness worldwide. It comprises several eye diseases, including age-related macular degeneration (AMD), diabetic retinopathy, Stargardt's disease and retinitis pigmentosa (RP). Among them, AMD is the leading cause of severe vision loss in people over 60 years old, with a global prevalence of 8.7 %, and it currently affects about 200 million persons in western world countries[25]. In spite of the numerous differences between these diseases, dysfunctions of RPE and photoreceptors (PHRs) are the major contributors to their pathogenesis[26].

The retina is part of the central nervous system (CNS); fortunately, its structure is simpler than that of the brain, making its regeneration more feasible. The retina is responsible for receiving light and transforming it into electrical signals that travel to the brain, where visual images are generated. It is a thin tissue of easy access, located in the back of the eye, with an ordered layered organization and a limited number of cells. These features make it possible to use SC to develop strategies for alleviating or, eventually curing, retinal neurodegenerative diseases.

Moreover, and in contrast to other organs of the body, the eye is a relatively immune privileged organ, which supports the likelihood of retina transplantation[27-29]. The ocular immune privilege, which is intended to limit local immune and inflammatory responses, can also contribute to avoid the rejection of grafts placed in the anterior chamber of the eye and protect the eye from inflammatory insults[30,31]. However, this protection is not absolute and this must be taken into account in cell replacement strategies. Treatments using iPSCs may activate immune rejection, as they can upregulate genes that induce a T cell response, and thus lead to the rejection of transplanted cells. Since the molecular mechanisms involved in these processes are incompletely understood[32], many studies resort to immunosuppression to reduce the risk of rejection[33]. Increasing our knowledge of the molecular processes that inhibit immune rejection of transplants would contribute to elaborate new strategies to facilitate the acceptance of tissue allografts during retinal transplantation.

SC therapy and neural regeneration

The initial efforts to treat RD were oriented to halt degenerative processes of the retina. The proposed therapies included a variety of medical compounds and treatments, including several neurotrophic factors, aimed at controlling oxidative stress and cell death[34,35] or anti-vascular endothelial growth factor agents, to prevent the formation of leaky blood vessels, mainly used to treat wet AMD[36]. However, these strategies pose diverse difficulties. In wet AMD, treatments to avoid or minimize choroidal neovascularization, very frequently cause complications, such as uveitis and vitreous hemorrhages, which compromise their effectiveness. On the other hand, the clinical efficacy of the neuroprotective strategies has not been firmly established. Furthermore, surgical interventions like laser therapies may not prevent the progression of the disease in patients with AMD or other RD and can cause inflammatory-related damages[37-39].

The finding of SC in the retina has provided additional tools to develop strategies for repairing the damaged retinas. Therapies using different types of SC, such as stem/progenitor cells, RPCs, PSCs, ESCs, iPSCs, and mesenchymal SCs (MSCs), capable of self-renewal and of differentiating into multiple cell types, have now gained ample consensus. Numerous preclinical studies using SC in several animal models of RD have been performed and their results are encouraging. Emerging evidence suggests that RPCs are among the best candidates for RD treatment; they do not present ethical concerns and they have a relatively low risk of tumorigenesis and immunorejection[40]. Moreover, increasing evidence suggests that the combination of SC therapies with the provision of survival molecules might provide the best strategy to treat RD.

Regeneration following retina damages might require the recapitulation of developmental specification/differentiation programs. Following PHR damage, zebrafish retina evidences an increase in the expression of several developmental competence factors, required for generating ganglion, amacrine, and PHR cells[41]. Retinal injury

might turn on cell specification programs in neuronal progenitor cells, which recapitulate the temporal expression sequence occurring during retina development and hence provide precursors suitable for replacing lost cells. A critical question is, which are the features that characterize and distinguish SC from other cells present in the organism? Cumulative evidence indicates that their extraordinary capacities depend, in part, on complex interactions between cell surface proteins and a variety of external and internal signals that activate signaling pathways to regulate pluripotency. These external signals include LIF/STAT3, Wnt/β-catenin, FGF/ERK, TGF/SMAD, bone morphogenetic protein (BMPs), Sonic Hedgehog, and the Wnts and Notch proteins[42].

The internal regulatory system comprises many transcription factors, such as Oct4, Sox2 and Nanog, which interact with specific target genes that regulate self-renewal and pluripotency[43,44] (Figure 2). *Oct4* (also known as *Oct3* o *Pou5f1*) controls the Sox2 transcription factor, involved not only in self-renewal of SC, but also in embryo development. Sox2 maintains SC in an undifferentiated state, after concluding embryo development[45,46], being important also for regulating proliferation and differentiation of neuronal SC progenitors[47,48]. In addition, both Sox2 and Oct4 interact upstream with the promoter of Nanog, another transcription factor, activating different genes that inhibit differentiation[49,50] (Figure 2). Nanog is involved in the self-renewal of ESC and is critical for maintaining pluripotent cells in an undifferentiated state[51]. Interestingly, genetic deletion of Nanog in ESCs does not abolish pluripotency, although it reduces cell self-renewal activity. As a general rule, Nanog prevents SC differentiation, so it is considered a guardian of pluripotency[50].

In addition, epigenetical regulation of global changes in genetic expression control SC rate of mitosis, along with other morphological and physiological changes. Acetylation or methylation of H3 and H4 histones regulate the distribution of active and inactive forms of chromatin, and consequently genetic transcription in mouse pluripotent cells. Noteworthy, mouse ESCs have poised (bivalent) domains containing both active and inactive forms of chromatin; hence, poised chromatin has histone modifications associated with both gene activation and repression[52]. Most bivalent domains are associated with the so called, highly conserved noncoding elements, found in clusters around different genes, including many transcription factors implicated in the regulation of cell differentiation during development[53]. Some of these transcription factors are members of the Sox, Fox, Pax, Irx and Pou families. Bivalent domains in SC are thought to maintain stemness by equilibrating the expression of relevant genes involved in differentiation, whereas signals released during development give way to an irreversible differentiation process[54].

Sox2 binds to bivalently marked promoters of poised proneural genes in neural progenitor cells and a subset of other genes, to maintain the bivalent chromatin state, and prevent excessive polycomb repressive complex 2 activities. It decreases the trimethylation of H3 on lysine 27 (H3K27me3) through histone methyl transferase activity. H3K27me3 often interacts in bivalent domains with H3K4me3, another epigenetic modification to H3, which plays a significant role in SC fate determination and early embryo development. Thus, *Sox2* maintains a permissive epigenetic state, enabling proper activation of the neuronal differentiation program under suitable neurogenic cues[55]. Therefore, *Sox2* plays an essential role in preserving pluripotency of SC and its interplay with *Oct4* and *Nanog* generates a network that preserves the pluripotent state of SC.

MicroRNAs (miRNAs) are also major regulators of self-renewal and differentiation, modulating the expression of genes involved in cell cycle progression and pluripotential state. Several miRNAs have been proposed to target transcripts that, directly or indirectly, coordinate cell cycle progression in embryonic, somatic and cancer SC. Among these miRNAs, miRNA (MiR)-290-295, miR-302, miR-17-92, miR-106-b25 and miR-106a-363, together with members of the let-7 family, regulate ESC cell cycle, mostly by facilitating the G1/S transition[56,57].

MiRNAs also participate in regulating and promoting SC differentiation (Figure 3). miR-145 is important during hESCs differentiation, having the pluripotency factors Oct4, Sox2, and Klf4 as direct targets (Figure 3). Overexpression of miR-145 Leads to repression of the 3' untranslated regions of *Oct4, Sox2, and Klf4*, thus inhibiting self-renewal and promoting differentiation. Interestingly, Oct4 binds to miR-145 promoter and inhibits its transcription in hESCs, acting as a negative loop[58].

MiR-145 promotes neuronal differentiation and regulates neural SC by repressing the expression of sex determining region *Y-box2*, and *Sox2* (Figure 2), along with that of Lin28, a well-characterized RNA binding protein and a pluripotency promoter, which suppresses the biogenesis of let-7 miRNA. MiR-145 also upregulates let-7a and let-7b during neurogenesis[59]. In turn, the let-7 family inhibits proliferation by

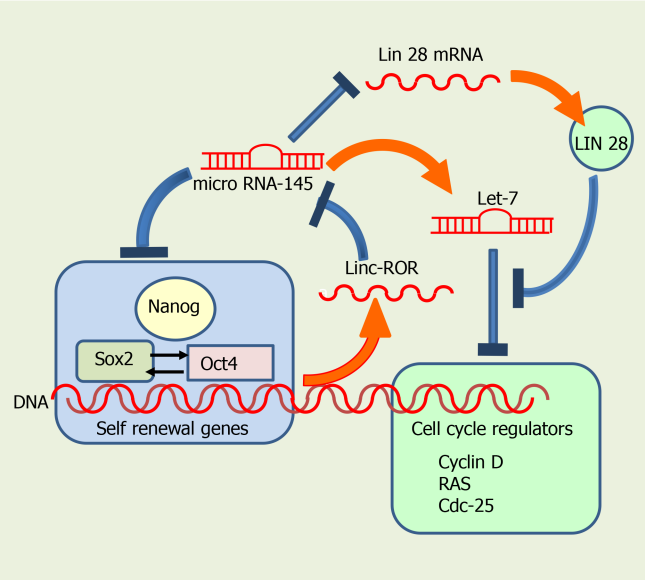

Figure 2 Regulatory control of self-renewal and differentiation by transcription factors and microRNAs. Oct4, Sox2 and Nanog prevent microRNA-145 from blocking the self-renewal of SC. Conversely, microRNA-145 promotes cell differentiation by inhibiting expression of Oct4, Sox2 and Nanog. Let-7, another microRNA, promotes differentiation by interfering with cell cycle regulators, and is regulated in turn by microRNA-145 and Lin 28.

interfering with cell cycle regulators such as RAS, Cyclin D, and CDC25. Pos-transcriptional regulation of let-7 by Lin28 appears to be required for normal development. Moreover, let-7 might have a central role in the regulation of 'stemness', by repressing self-renewal and promoting differentiation, not only during normal development, but also in cancer cells[60,61].

Exogenous LIN28 expression has been shown to suppress Let-7 activity, thus reverting inhibition of cell proliferation in human neural progenitor cells[61-65] (Figure 2). Moreover, Sox2 is required for preserving the expression of physiological levels of Lin28 in the developing neural tube[61]. Sox2 binds to a promoter region of LIN28, and promotes acetylation, by interacting with the histone acetyltransferase complex[61]. Collectively, these data imply that miRNAs and several transcription factors regulate self-renewal in SC, interacting very precisely to decide whether they continue in the cell cycle or start their differentiation.

Wang *et al*[66] established an additional mechanism for regulation of pluripotency, involving a large intergenic noncoding RNA (lincRNA), the linc-regulator of reprogramming, or linc-ROR, which belongs to a larger group of non-coding RNA (ncRNA). While the vast majority of the mammalian genomic DNA is transcribed, the largest fraction are ncRNAs, as only a small fraction are protein-coding genes. In addition to the well-known transfer RNAs, ribosomal RNAs, and miRNAs, these ncRNAs include long ncRNAs (lncRNAs), which are longer than 200 nucleotides.

LincRNAs are transcribed from both strands of DNA in intergenic regions[67], and have both exons and introns. LncRNAs are shorter than lincRNAs, and they both have single-stranded sequences, able to form secondary structures[67]. LincRNA transcripts are generally found in the mammalian nucleus, while lncRNA transcripts are usually in the cytoplasmic region. LincRNAs regulate the transcription of neighboring genes by increasing or repressing transcriptional activation, and are believed to be involved in several pathologies. In contrast, the exact functions of lncRNAs are not fully established. Linc-ROR was the first identified linc-RNA; it promotes reprogramming of differentiated cells into iPSCs and maintains ESCs[68]. Noteworthy, linc-ROR interacts with several miRNAs and has been reported to be controlled by *Oct4*, *Sox2* and *Nanog* in iPSCs. The presence of binding sites for these pluripotency transcription factors in linc-ROR indicates they regulate its expression, and hence that of human ESC (hESC)[68]. In turn, linc-ROR has been shown to maintain hESC self-renewal by functioning as a "sponge", trapping miR-145 and preventing miRNA-mediated suppression of the pluripotency factors Oct4, Nanog, and Sox2[66] (Figure 2).

Linc-ROR has also been proposed to modulate the reprogramming of human iPSCs [69]. LINC-ROR may also be oncogenic, having as a target the EF-hand calcium binding protein tescalcin, an oncogene significantly upregulated in ocular melanoma cell lines and animal models[70].

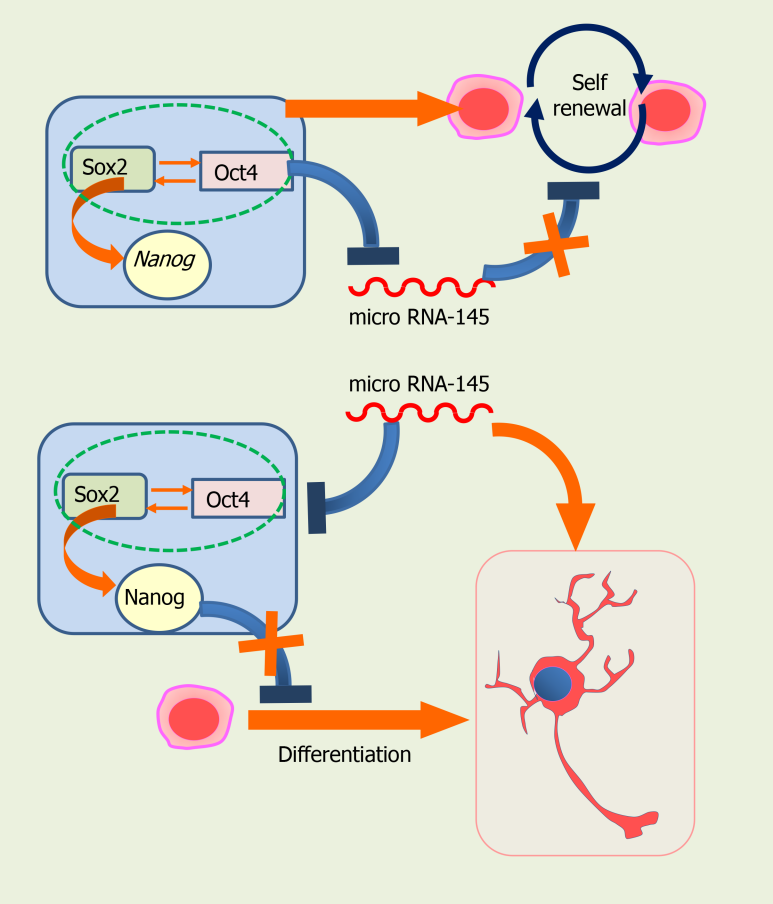

Figure 3 microRNA-145 and transcription factors Sox2, Oct4 and Nanog regulate cell renewal and differentiation. Sox2, Oct4 and Nanog interact with microRNA-145 to regulate cell cycle and differentiation of stem cells.

Most of the above described regulatory signals and mechanisms are operative in the retina. Our knowledge on the mechanisms regulating SC function in this tissue has enormously increased during the last two decades. Growth factors and signaling pathways, such as fibroblast growth factor (FGF2), epidermal growth factor (EGF), insulin growth factor (IGF), ciliary neurotrophic factor (CNTF), Wnt/β-catenin, Notch–Delta, and others, regulate the regenerative response in the retina, supporting and maintaining the status of endogenous SC by activating proliferation and reprogramming cells to replace injured or dead retinal neurons. Some inhibitory extracellular matrix or cell adhesion molecules, along with Bmp4 and other signaling pathways, often have the opposite effect[71]. Other systemic factors such as hormones, growth factors, cells of the immune system and blood, are regulators of the regenerative behavior of endogenous retinal SC[72].

Substantial evidence underscores the role of trophic factors in regulating SC activity. Early work evidenced the relevance of trophic factors in activating SC after injuries; several trophic factors and other molecules, including FGF2, EGF, SC factor, erythropoietin, and brain derived trophic factor (BDNF) increase adult neurogenesis by stimulating generation of new neuronal cells or improving their survival[73]. Trophic factors usually contribute to improve the milieu in which SC proliferate; provision of an enriched environment to neural stem/progenitor cells promotes neurogenesis in the brain subventricular zone after inducing a cortical stroke[74]. Furthermore, SC appear to contribute to enrich their own environment by releasing trophic and survival molecules. MSC secrete many survival factors such as EGF, IGF-1, FGF2, BMP-7, TGF-b1, and interleukin-6 (IL-6), among other factors, which protect injured cells[75-77]. These factors might regulate SC proliferation, migration, differentiation, and interactions after injuries. Interestingly, enriched environment has a neuroprotective effect in the retina in diverse animal models of pathological situations, such as glaucoma and ischemia reperfusion, preserving or increasing BDNF levels[78, 79]; whether it also impacts on SC regulation is a pending question that demands further research.

Retina SC have profiles similar to those of RPCs at early stages of eye development. They respond to many well-known intracellular factors, including the transcription factors Pax6, Chx, Rx, Six, Sox, Prox, Pitx, and others[80-82]. Their regenerative potential is epigenetically regulated[83-85], and they are also influenced by trophic and survival factors. MGCs have been proposed as retina SC and our research group has established that trophic factors as glial-derived neurotrophic factor (GDNF), FGF2, insulin and IGF-1 increase their proliferation and expression of SC markers such as Pax6 and nestin. This implies that the intrinsic, but dormant proliferative capacity of these cells can be regulated by trophic factors and other environmental molecular cues [86] (Figure 4).

Interestingly, dental pulp SC, which originate from the neural crest, are able to differentiate into neurons when supplemented with EGF, FGF2, and retinoic acid[87]. When transplanted intravitreally, they have been shown to secrete significant amounts of nerve growth factor, BDNF, neurotrophin-3, and GDNF, promoting neuroprotection and axon regeneration in retinal ganglion cells after axon injury[88-90]. Further research is required to identify the trophic factors required for preserving multipotentiality and proliferation in retina SC.

Transcription factors and miRNAs have been shown to control the formation of new retinal neurons derived from endogenous SC. Particularly, miRNAs are involved in controlling the ability of MGCs in non-mammalian and mammalian vertebrates to generate new RPCs[91]. In zebrafish, which, as mentioned before, effectively regenerates the retina after injury[92], miR-216a regulates reprogramming in MGCs, maintaining them in a quiescent state in undamaged retina. miR-216a suppression is necessary and sufficient for MGC dedifferentiation and proliferation, having the disruptor of telomeric silencing-1-like (Dot1 L) as a target; this miR-216a/Dot1 L regulatory axis mediates the initiation of retina regeneration through the Wnt/β-catenin pathway[93]. In addition, miR-9 has been recently identified in zebrafish MGC as a critical factor controlling retinal NSCs proliferation and fate[94], its depletion increasing the number of neural progenitors and neurons. miR-9 has different targets, including lin-28, which is necessary to promote the proliferation of retinal NSCs after injury[95], TLX and ONECUT mRNAs, which promote NSCs differentiation into neurons in both zebrafish and humans[94]. miR-9 might act as a negative regulator of the Sox2-Ascl1a/Atoh7-Lin-28 pathway to prevent MGC proliferation and as an activator of the TLX-ONECUT pathway for reprogramming endogenous MGC into functional retinal neurons[96]. Furthermore, miR-9 is involved in regulating mouse RSC differentiation through repression of polypyrimidine tract-binding protein 1 (PTBP1) expression, which is a repressor for polypyrimidine tract-binding protein 2 (PTBP2), during neuronal differentiation. Both proteins are highly expressed in the fetal stage and show lower transcript levels in the mature brain, retaining PTBT1 expression in glial cells and that of PTBP2 mostly in neurons[97]. miR-9 promotes the differentiation of neuronal cells from mouse RSCs, reducing the expression of PTBP1 and consequently increasing the expression of PTBP2[98]. Moreover, over-expression of miR-25 and miR-124, or let-7 antagonism induces the expression of proneural transcription factor Ascl1, a crucial regulator in retinal regeneration[99], and promotes the conversion of mature MGC into a neuronal/RPC phenotype[100].

In contrast to miRNA, most biological functions of lncRNAs are still poorly understood. They are believed to control key biological processes, including cell proliferation, apoptosis, differentiation, oxidative stress and inflammation, and have been shown to regulate SC maintenance and neural SCs proliferation[101,102]. In the eye, lncRNAs also appear to regulate SC maintenance, cell lineage commitment, and cellular phenotype differentiation[103] and have been involved in several ocular pathological conditions, such as glaucoma, proliferative vitreoretinopathy, diabetic retinopathy, and ocular tumors[104-107]. Interestingly, a retina-specific lncRNA, Vax2os, is involved in cell cycle progression in PHR progenitor cells during development of the mammalian retina[108,109]. Other lncRNAs, such as RNCR2[110], MIAT[111], and Gomafu have been found in the developing retina[112]. MIAT plays a critical role in regulating mammalian retinal cell specification and is involved in several diseases leading to visual impairment as well[113]. Expression levels of the lncRNA MALAT1 are significantly upregulated in diabetic retinas and it has been suggested to regulate retinal neurodegeneration in several rodent models[114]. MALAT1 expression is upregulated in cultured MGCs and retinal ganglion cells following stress, while its suppression decreases reactive gliosis, suggesting that MALAT1 dysregulation leads to neurodegenerative processes[115].

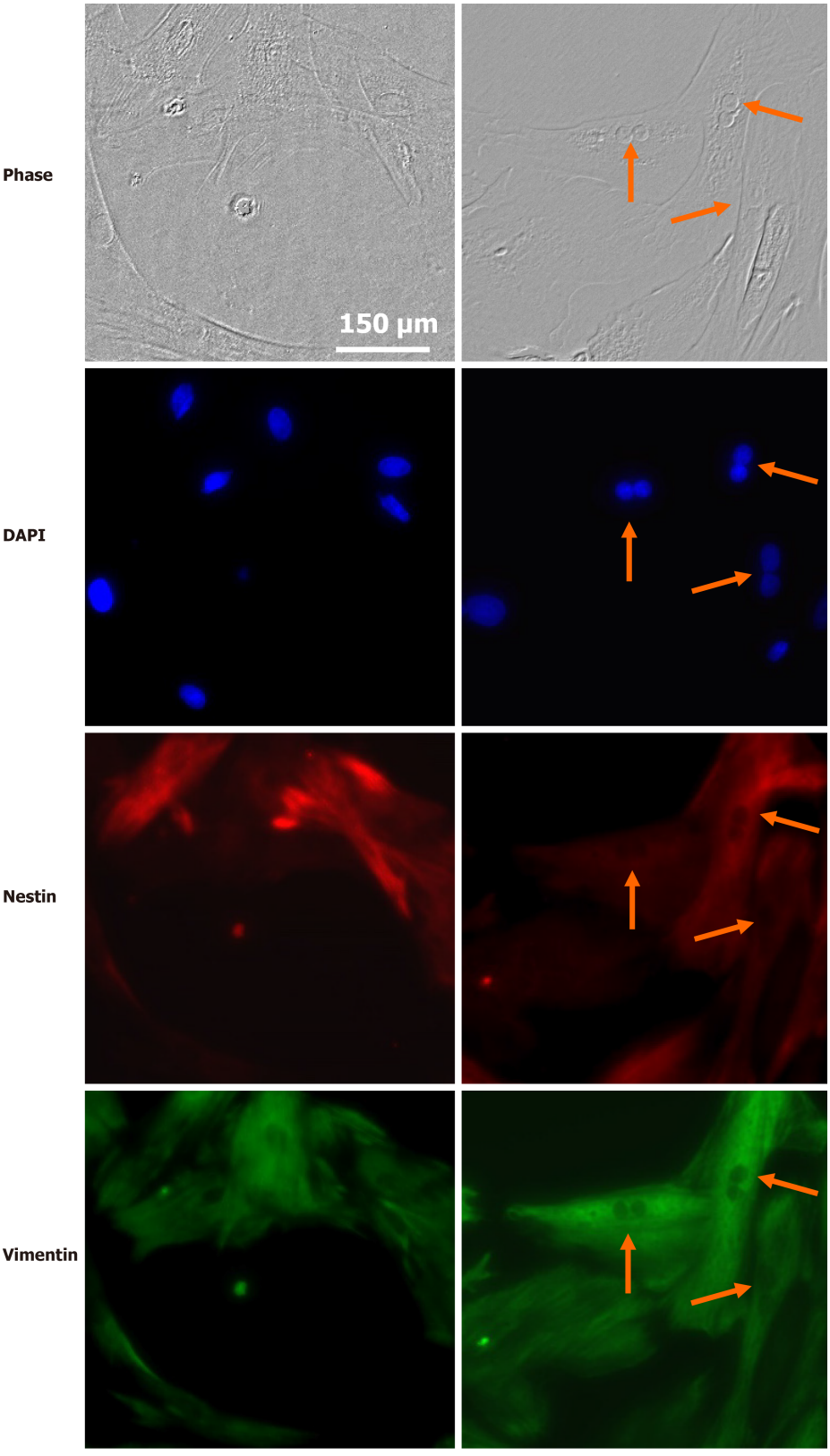

Figure 4 Stem cell properties of Müller glial cells. Photomicrographs of Müller glial cell cultures from rat retina in interphase (left) or mitosis (self-renewal) (right), showing nuclei labeled with the DNA probe DAPI, expression of the stem cell marker nestin (red) and of vimentin (green). Arrows indicate mitotic anaphases. The bar indicates 150 μm.

SC IN RD: CURRENT APPROACHES

An advantage of SC therapies in the eye is that they may provide a tool for achieving cellular regeneration in diseases leading to the loss of particular cell types, as retinal ganglion cells are to be replaced in glaucoma, and PHRs or RPE cells in AMD or RP.

Noteworthy, the recovery of a particular cell type might also have a trophic role, restoring the provision of trophic factors released by that cell type. As stated above, the fact that the vitreous cavity is a relatively immune-privileged site also contributes to the feasibility of SC transplantation.

These advantages have prompted researchers to establish the most suitable sources of SC and define which protocols to apply for each particular situation. Two main approaches can be described; one of them takes into account the cell type contributing to the degeneration; thus, many studies focus in RPE cells, when their damage plays a pivotal role in the pathogenesis of the disease, such as AMD; a second approach aims to directly replace the retinal neurons affected in each disease.

In the first approach, the efforts have been directed mainly to transplanting SC-derived RPE cells into the eye, either as a monolayer or as a cell suspension. Numerous protocols have been assayed, with different advantages and disadvantages; establishing the most suitable therapeutic strategy demands a careful analysis of all of them. A successful treatment would allow the replacement of the damaged RPE cells, thus preventing the progression of diseases such as AMD. A potential disadvantage is that in these diseases the initial degeneration of RPE cells is usually followed by the degeneration of neurons, mainly PHRs. Since most of the SC used are unipotent and will only generate new RPE cells, this approach has the limitation that it can only be useful at early stages of the disease, as no replacement of lost PHRs or other neuronal cells will be achieved when used at advanced stages.

Using SC to replace retina neurons faces several obstacles. The first is the difficulty to achieve their differentiation into the required neuronal cell type. Even if this is surpassed, the integration of the newly generated cells into the host retina confronts numerous critical complications such as their engrafting onto Bruch's membrane, achievement of their adequate polarization, reestablishing an adequate circuitry, and finally avoiding immune rejection[116,117].

PHR death is the cause of most RDs, excluding glaucoma. Hence, a second approach to treat most RDs is to directly replace PHRs. With this aim, several strategies have been evaluated. Early work showed that transplants of embryonic murine retinas into the anterior chamber of adult eyes survive and develop, resulting in the differentiation of both neurons and MGC, with few cases of graft rejection[118,119]. Subsequent strategies involve the reactivation of the dormant potential for regeneration of endogenous populations of cells within the retina to generate new PHRs or also to attempt retinal repair by transplantation of healthy PHRs into the vitreous or the subretinal space[120,121].

Therapeutic efficacy of different retina SC

Since the expectation of recovering visual function by using SC emerged, there has been an active search to establish the most adequate SC sources. As occurs with other tissues in the body, the retina has several cell types that display properties of SC. This is evident in fish and amphibians during development and regeneration. During embryogenesis, most of the retina originates from the ciliary marginal zone, a ring of cells found at the periphery of the retina. In contrast, different sources might provide new neurons during regenerative responses. In the amphibian retina of urodeles, RPE cells play a critical role during retina regeneration; these cells dedifferentiate into retinal progenitors that have to recapitulate the normal development of the retina, first proliferating, and then differentiating into the different retinal cell types[122]. In zebrafish, MGCs are recruited following damage of the retina, and transiently de-differentiate, express SC markers, and re-enter the cell cycle, to generate retinal progenitors that migrate to replace the lost neurons[92].

Consequently, cumulative evidence has shown that the ciliary margin zone, the RPE cells, the iris, and MGCs are the major sources of SC in the eye[71,120,123-125]. Some of these cells are represented by stem and low-differentiated cells and others by latent, differentiated progenitors[71,120,123-125]. In spite of their different origin, all of them share the essential requisites of SC, namely, the capacity for unlimited self-renewal and the ability to differentiate into different cell lineages. These traits turn them into ideal sources for recovering the different cell types lost in diverse retina degenerative diseases, thus repairing the retina and restoring vision. The feasibility of reprogramming resident, non-neuronal cell types into neurons *in vivo*[126] has led to include them as well among the SC types apt to contribute to neuronal regeneration in the retina.

We will now describe briefly the most explored candidates for cell replacement in the retina and the research, including clinical trials, that support their use.

RPCs are among the most promising SC type for treating RDs (Table 1). All cells in the retina derive from them, making these cells, obtained from human fetal retinas, an attractive source of SC for retina repairing. The evolutionary conserved, temporal organization of the genesis of each cell type, and even of particular subtypes, during development of the retina is well established[127]. RPCs proliferate actively, are generally multipotent and have been shown to produce a specific repertoire of cell types at defined developmental stages, suggesting they exhibit intrinsic changes in their state of competence along development[128]. Noteworthy, RPCs are very heterogeneous regarding their gene expression[129]. Although intrinsic cues play a central role in defining cell fate, this fate is not strictly determined and the context, i.e., regulatory and transcription factors, miRNAs, active/inactive signaling pathways, influences the cell response to a certain perturbation[127]. In addition, extrinsic cues may contribute to modulating the temporal progression of cell fate acquisition; soluble factors released by ganglion or amacrine cells can limit the generation of the respective cell type[129,130], implying their involvement in feedback inhibition.

SC-derived RPCs (SC-RPCs) obtained from embryos are a possible source of cells for retina cell replacement strategies[131]. These multipotent cells display markers indicative of retinal SC fate, such as *Pax6*, *Vsx2*, *Lhx2*, *Six3*, *Rax*, and can differentiate into MGCs and the six types of retinal neurons[132-136]. Establishing the strategies required for achieving the differentiation of SC-RPCs into the multiple cell types that constitute the retina and drawing a more precise roadmap of the cues involved is crucial for their successful use in regeneration schemes.

Initially, the feasibility of expanding these cells in order to achieve a sufficient amount for transplantation, while simultaneously preserving their multipotency was a limiting factor for using RPCs isolated from the fetal neural retina. Promising *in vitro* and *in vivo* studies have shown that SC isolated from embryonic or fetal retina can be expanded *in vitro*[12], under particular conditions, such as low-oxygen culture conditions[137], paving the way for their use.

The ability of human or mouse fetal RPCs to repair retinal damages has been analyzed in multiple sub-retinal transplantation studies, which have evidenced they can rescue PHRs, preserving rhodopsin expression and visual function[18,138-141].

Human RPCs are able to differentiate into specific retinal cell types, including PHRs, preserving vision in rats[132,137,142-144]. RPCs isolated from retinas of postnatal day 1 mice, expanded in culture and grafted in the degenerating retina of RD or rho$^{-/-}$ mice differentiate and express PHR markers; in rho$^{-/-}$ mice, RPCs integrate in different retina layers, increasing outer nuclear layer thickness and improving light-mediated behavior[12]. Transplantation of RPCs in the subretinal space has been proved feasible; mice retinal progenitors obtained from postnatal day 1 retinas and injected into the subretinal space of different adult mouse models of retina degeneration can integrate, differentiate as PHRs and improve visual function[132,145]. Transplantation of human RPCs into Royal College of Surgeons (RCS) rats, an animal model in which a mutation in RPE cells leads to PHR degeneration, preserves the outer nuclear layer thickness and cell count and prevents visual loss[138]. Preclinical studies have also used RPCs obtained from human fetal retinas, between 14 wk and 20 wk of gestation, a time point at which PHR progenitors are exiting the cell cycle and initiating their differentiation[146]. Delivery of human RPCs by intravitreal transplantation has also been proved effective; injecting human RPCs, obtained from retinas of 16 wk to 18 wk' gestational age, in the vitreal cavity of RCS rats has no adverse effects, and preserves PHR cell nuclei in the outer nuclear layer and visual function for 8 wk, decreasing afterwards[40]. However, the low efficiency of integration of RPCs in these studies, usually restricted to areas near the injection site, with few cells achieving differentiation are significant drawbacks still to be overcome.

Fetal RPCs have been used to repair atrophied retina areas in patients with RP or AMD[147-149] (Table 1). Intravitreal and subretinal delivery of allogeneic RPCs for RP treatment are still under evaluation in clinical trials. A sole intravitreal injection of human RPCs has been associated to an improvement in visual acuity in treated *vs* non-treated eyes, after a 12-month follow-up in phase I/IIa RP patients[151] and a phase IIa trial has been initiated[152] to evaluate safety.

Although RPCs emerge as a promising source of SC to achieve PHRs and retina regeneration, further knowledge is necessary to improve the efficiency of their integration, and control their differentiation into the required cell type, the extent of their reparative effect and, when allogeneic conditions are used, the magnitude of potential immune-derived damage.

PSCs are another potential source of cells to treat RDs. These cells were initially restricted to ESCs deriving from the inner cell mass of preimplantation embryos, and can be indefinitely maintained in the pluripotent state (Figure 1). This pluripotent state

Table 1 Stem cells in retinal diseases

Retinal disease	Type of SC used	Type of intervention	Clinical trial status	Results: Advantages and disadvantages	Ethical concerns
RP, AMD	Fetal RPCs[33,147-150]	Subretinal or intravitreal transplantation	Yes[40,151]	Rescue of PHRs and low risk of tumorigenesis. Able to differentiate into MGCs and retinal neurons; improvement of retinal sensitivity. Adverse effects: limited amount of cells and low tissue integration. Promising therapeutic treatment.	Little
RP, AMD, most retinal degenerations	PSCs and iPSCs	Reprogramed to iPSC[40]	Yes[40,200,293]	Potential replacement of damaged retinal cells. Low immunogenicity. Adverse effects: risk of teratoma development[40,50,156,160-163]. Genomic instability in iPSC. Further research still needed.	Little or none
AMD, retinal degenerations	RPE	Transplantation of RPE sheets from human fetal eyes in AMD patients	Yes[204,283]	Substantial rescue of PHRs. Visual improvement[147,148]. Little or no evidences of draft rejection[33,203,204,281,285]. Adverse effects: unwanted cell aggregation; lack of attachment; cases of anoikis[162,163].	Concerns about using human tissues
AMD, stargardt disease	Human ESC-RPE	Injection of differentiated ESC-RPE cells in subretinal space[238]	Yes phase 1/2a trial[175,176]	Cells form monolayers and display typical RPE features[171-173]. Improvement of visual acuity. No tumorigenicity or rejection after 4 years[187]. Cell sheets preserve RPE characteristics better than cell suspension[175-178]. Absence of serious adverse effects[131,188]. Effectiveness still uncertain.	Concerns about using human tissues
AMD	iPSC-RPE	Autologous transplantation into an AMD patient of cell-sheets of RPE, differentiated from iPSC obtained from the skin's patient[195]	Yes[195,199,200]	Preservation of main RPE features. Long term survival of transplanted iPSC-RPE cells[196,199,200]. Visual acuity stable for 4 years[197,198]. Presence of mutations in iPSCs[199]. Little immune rejection[202]. Adverse effects: Risk of tumorigenesis. Still pending to establish the adequate iPSC-RPE cells and the effectiveness of transplantation.	Important concerns about safety
AMD, stargardt macular distrophy	human fetal SC	Transplantation of human fetal RPE cells into subretinal space	Yes[176,203]	Improvement of visual parameters.No immunosuppression; No restoring of retinal morphology; no expression of retinal markers[169,208]. Adverse effects: little graft rejection in patients with AMD.Further research still required to provide effective and safe treatments.	Concerns about using aborted human fetuses
Retinal degeneration	MGCs	Not established	No	Express most SC markers; potentially capable of retinal regeneration after reprogramming. Obstacles: gliosis, low regenerative potential.	Not determined
Retinal degeneration, retinal injuries and uveitis	Bone marrow (MSC) and hematopoietic SC	Intravitreal injection (in mouse models of RP)	No	Promote regeneration of different retinal cells[238,246-248]. Safety not determined.	Not determined

The use of different stem cells for treating retinal degenerations, such as retinal pigment or AMD, entails many advantages but also disadvantages and ethical concerns. SC: Stem cells; RP: Retinal pigment; AMD: Age-related macular degeneration; PHRs: Photoreceptors; MGCs: Müller glial cells; PSCs: Pluripotent stem cells; iPSCs: Induced pluripotent stem cells; RPE: Retinal pigment epithelial; ESC: Embryonic stem cells; MSC: Mesenchymal stem cells.

is preserved by a complex and coordinated gene network, along with several signaling pathways activated by environmental cues that start in blastocysts (in the blastula stage) and persist until gastrulation; at this time point, levels of *Oct4* and *Nanog* decrease, and pluripotency can no longer be preserved[152-155]. However, a major step forward for SC-based therapies came from the *in vitro* technology developed by Takahashi and Yamanaka[15], and Takahashi *et al*[156], that allowed to obtain PSCs by inducing dedifferentiation of adult somatic cells through their reprogramming to a pluripotent state, thus generating iPSCs. As ESCs, iPSCs cells can be expanded indefinitely, preserving an undifferentiated state and, eventually, can differentiate into cells of the three germ layers: ectoderm, mesoderm and endoderm[157].

Numerous *in vitro* studies have now evaluated the capacity of IPSCs for retina cell replacement. Addition of low molecular-mass compounds to iPSC cultures leads to their differentiation into retinal progenitors, RPE cells and PHRs[158]. In a recent work, Fligor *et al*[159] demonstrated that three-dimensional retinal organoids derived from human iPSCs can recapitulate retina differentiation and are useful models to investigate guidance of developing neurites toward their targets.

The ability of human iPSCs to differentiate into a wide range of cell types turns them into a suitable and attractive alternative to ESCs, avoiding the ethical issues associated to the later. Hence, human iPSCs appear as likely candidates to be successfully used in the near future, providing better models for studying, treating, and eventually curing retinal degenerative diseases. Nonetheless, a crucial problem precluding their use is that they have been shown to give rise to teratomas when injected into immune-deficient mice[160,156,161-163].

Further research on the mechanisms underlying self-renewal properties, and safety of human PSCs are still needed to solve the pending questions associated with their therapeutic possibilities[164] (Table 1).

Ciliary-derived cells and RPE cells have also been reported as a potential source of progenitor cells that can be mobilized to the injured retina[165]. RPE cells form a monolayer between the PHRs and the choroidal vasculature. They transport ions, water and metabolic end products from the subretinal space to the blood, they represent the only blood supply for the outer retina and have the critical function of constantly clearing shed PHR outer segments by phagocytosis. Their interactions with the Bruch's membrane and the choriocapillaris constitute a barrier that regulates exchange of substances between the neural retina and the circulation. To perform these functions adequately, RPE cells must maintain a polarized structure, which is crucial for the homeostasis of the outer retina, the disruption of which leads to degenerative retinopathies[166].

Transplantation of SC-derived RPE cells has been in the spotlight for over 10 years as a cell replacement strategy, particularly for patients with macular disorders, in which the early loss of RPE cells leads to the subsequent death of PHRs. A huge amount of information has been accumulated regarding their efficacy and safety, and clinical trials are already on course.

RPE cells were among the first candidates evaluated for subretinal transplantation since they can be easily obtained from patients, for autologous replacement therapies, or even from corpses, and can be maintained *in vitro* for long periods. Autologous RPE cells have been used in patients with RP or AMD, transplanting patches of RPE cells into the damaged areas of the retina[147-149]. A weakness to the therapeutic possibilities of adult RPE cells is that the normal functions of RPE cells are not fully reestablished after these procedures, and they may retain some aging features[117].

Transplantation of RPE cells has many advantages, appearing as a suitable strategy to treat inherited diseases, such as AMD. The possibility of differentiating RPE cells from hESC (hESC-RPE) and from iPSC (iPSC-RPE) has paved the way for their use, since it provides a potentially unlimited source for the replacement of affected or dead RPE cells[167]. hESC-RPE cells can be obtained by culturing ESC colonies, in which cells spontaneously differentiate into RPE cells after removal of FGF. Sheets of RPE cells can be obtained from cultures of either ESCs, or iPSCs, and they can form confluent monolayers, reproducing many of the functions of RPE cells[168-170]. They not only form monolayers, but also display the typical RPE microvilli and pigment-containing melanosome granules, and express RPE markers, such as Na^+K^+ATPase, Pax6, and RPE65, together with proteins associated with tight junctions and involved in retinol cycling[163-171]. hESC-RPE cells have also been shown to express and release pigment epithelial derived factor from their apical surface[174]. Following their transplantation into RCS dystrophic rats, hESC-RPE cells survive in the subretinal space, expressing low levels of RPE65 and downregulating the cell cycle and Pax6 expression, while maintaining expression of other markers[172].

The efficacy of transplanting these cells as either a cell suspension or as sheets is a matter of extensive analysis. ESC-derived RPE cell suspensions were safely used in a phase I/IIa trial for treating AMD and Stargardt disease patients[175,176] (Table 1). However, the effectiveness of this strategy is still uncertain as these suspension are unable to form the typical RPE monolayers and to survive long periods of time[177, 178]. Animal studies evidence the feasibility of subretinal transplantation of a hESC-RPE monolayer re-grown in a biocompatible membrane, which shows a normal implantation[179,180]. Current evidence suggests that cell sheets, rather than cell suspensions, might be more effective for preserving morphology, polarization, survival and physiology of RPE cells[177].

Different materials have been used to support RPE monolayers, with or without artificial scaffolds, as these materials may influence inflammation, adequate insertion and interaction of RPE cells with other cell types in the retina[181]. These patches allow the formation of tight junctions, required to acquire a fully polarized morphology, which, as stated above, is critical for RPE cells functions and for their interaction with PHR segments[182]. However, the survival of these patches after transplantation is a significant problem to deal with (Table 1).

The transplantation site is also still subject of considerable research. The subretinal space, a frequent transplantation site, is a relatively immune privileged site, and RPE cells located in this space have immunosuppressive functions. However, transplanted RPE grafts can be eventually rejected, as they can be attacked by immune cells due to their immunogenicity[181,183] (Table 1). Initial studies to evaluate the efficacy and safety of RPE cells for retina regeneration therapies were performed in animals, and most of them involved immunosuppression. Transplantation of RPE sheets prepared from human fetal eyes in RCS rat eyes indicates a substantial rescue of PHRs in the area of the RPE patches, with no evidence of draft rejection[33]. Later work evidenced that subretinal transplantation of hESC-RPE in RCS rat eyes leads to PHR rescue and improvement in visual performance; donor hESC-RPE localized adjacent to the RPE layer show no uncontrolled proliferation or evidence of tumor formation[171]. Similarly, long-term functional rescue is observed after subretinal injection of ESC-RPE in animal models of AMD and Stargardt disease, diseases in which RPE degeneration leads to PHR loss and visual deficiency; the retinas preserve PHR integrity and function, without evidence of teratomas or pathological changes[184].

Some problems still remain. Cell transplantations of RPE in the retina frequently causes unwanted cell aggregation or lack of attachment onto the Bruch´s membrane [185], with RPE cells tending to form aggregates (rosettes) instead of functional monolayers[172] or undergoing anoikis when dissociated from their usual extracellular matrix[186] (Table 1).

Further studies are required to solve these pending issues; nevertheless, cumulative information support ESC-RPE as a potentially useful and inexhaustible source of SC for treating retina degeneration.

The first clinical study evaluating the feasibility of transplantation of hESC-RPE cells to human patients was reported by Schwartz and colleagues in 2012[175]. These researchers injected differentiated hESC-RPE cells in the subretinal space of patients having macular degeneration. The cells integrated in the host RPE layer, forming monolayers, and improved visual acuity in over half of the patients, with no visible hyperproliferation, tumorigenicity or rejection-related inflammation even after 4 years [187]. A similar study evidenced an improvement in visual acuity and the absence of serious adverse effects[188]. In a Phase I trial, hESC-RPE cells on a coated, synthetic basement membrane transplanted in two patients with severe exudative AMD successfully survived for a year and increased visual acuity[189]. Therefore, retinal implants of ESC-RPE cells appear as a promising therapeutic tool to treat retinal diseases (Table 1).

iPSC-RPE are also a promising source of RPE cells for transplantation as they provide a virtually unlimited number of RPE cells, in a non-invasive manner (Table 1). iPSC-RPE cells have the same genetic background, and display morphological and functional characteristics of mature RPE cells, which they retain after their transplantation in the rodent retina[190-193]. iPSC-RPE cells have been established from mouse, monkey and human, using different methods to obtain the iPSCs and then induce their differentiation into RPE cells[194-196]. These cells preserve key RPE features; Carr *et al*[191] showed that following their transplantation into the RCS dystrophic rat, iPSC-RPE phagocyte PHR material, both *in vitro* and *in vivo*.

Several studies have reported that human iPSC-RPE cells also exhibit native RPE features, such as gene expression and cellular functions, and have immunosuppressive properties, suppressing T-cell activation *in vitro*[181].

Ensuring safety and efficacy are major concerns for their clinical applications. Tumorigenesis is a major risk, due to the reprogramming methods used and their possible contamination with iPSCs, which might contribute to formation of teratomas [181].

The potential use of iPSC-RPE cells has been tested in fewer clinical trials than ESC-RPE cells. The first human pilot trial was conducted by Mandai *et al*[197] and generated great public attention. iPSCs were induced from the patient´s skin cells by introducing reprogramming factors and then differentiated into RPE cells; an autologous RPE sheet was then prepared and transplanted into a patient with neovascular AMD[197]. The graft remained stable, with no signs of rejection or increased proliferation, and the patient´s visual acuity remained constant for four years[197,198]. A further trial was suspended due to the presence of mutations in iPSCs from another subject[199]. Takahashi´s group is currently evaluating the use of allogeneic iPSC-RPE cell grafts, which would be faster to prepare and easier to control genomic stability; their use requires to take into account the different factors leading to a higher risk of immune rejection.

In an early clinical study, Sugita *et al*[200] used iPSC-RPE cells derived from major histocompatibility complex (MHC) homozygous donors; after being transplanted into patients with exudative AMD having a matched MHC, the graft cells remained stable for one year, showing no abnormal growth. Although the patients experienced many moderate adverse events, such as corneal damages, retinal edema, elevated intraocular pressure, endophthalmitis, and mild immune rejection in the eye, this trial demonstrates long term survival and safety of transplanted iPSC-RPE cells[196,200,201]. Ongoing clinical studies in five patients with neovascular AMD have safety as their main concern and report the survival of the transplanted grafts and only one case of immune rejection[202]. Once the safety of their use is established, further clinical trials will be required to determine which iPSC-RPE cells are the most adequate and whether their transplantation is effective to improve visual function. Of note, treatments with iPSC-derived RPE cells are limited to early stages of RDs; they may be ineffective in patients with an irreversible PHR loss.

Fetal RPE cells provide another source of RPE cells for transplantation. In an early work, Algvere *et al*[203] transplanted human fetal RPE cells into the subretinal space in AMD patients, with no immunosuppression; although patients with exudative lesions showed graft rejection, patients with geographic atrophy of dry AMD presented little evidence of rejection (Table 1). Even when alerting about the risk of rejection, they concluded that it is feasible to transplant human RPE into the submacular space of nonexudative AMD patients without adversely affecting visual function. Further clinical trials show that subretinal human RPE allografts transplanted into eyes of AMD patients, without immunosuppression, exhibit high rejection rates after 2 years, probably through a disruption in the blood retinal barrier; on the other hand, small extrafoveal transplants remain essentially unchanged for over 2 years in non-exudative AMD without immunosuppression[204]. Interestingly, transplants of fetal retinal tissues as intact sheets, or even as aggregates, in the sub retinal space in rats have been shown to survive, adhered to the host RPE sheets[116,205,206]. In these cases, the occurrence of a pre-existing RPE sheet in the transplanted recipient provides paracrine trophic support for the grafted tissue, preventing cell death[205,206].

Human fetal neural SC are another possible source of SC. These cells, obtained from aborted fetuses, were identified by the expression of the cell-surface marker CD133, and cultured under conditions that induce rosette formation. After injection into the subretinal space in RCS rats, these cells survived away from the injection site and improved visual parameters, even when they neither restored retinal morphology, nor expressed retinal markers[207,208] (Table 1).

Taken as a whole, the above findings imply that transplantation of fetal cells is a promising approach, still requiring further research to provide an effective and safe treatment.

MGCs emerge as a further promising source of SC for retina regeneration. MGCs are the principal glial cells in the retina and play crucial roles in the preservation of retinal structure and function[209]. They provide the main trophic and metabolic support for retinal neurons, playing a major role in the preservation of homeostasis, the regulation of nerve signal transduction, and the formation of synaptic structures in the retina. Emerging evidences suggest that MGCs are dormant stem-like cells present throughout the retina that serve as a source of progenitor cells to regenerate retinal neurons after injury[209,210]. In teleost fish, MGCs are a major source of progenitors for retina regeneration after injury. In the damaged zebrafish retina, the activation of a reprogramming process in MGCs leads to their de-differentiation to generate neuronal progenitor cells, which proliferate and finally differentiate into all the cell types

forming the retina[92]. This remarkable regeneration capacity is much diminished in vertebrates; although vertebrate MGCs have been established as retina SC, they have a very limited capacity to achieve retina regeneration upon damage. In spite of this limitation, MGCs would provide an intrinsic source of SC, in contrast to ESCs, iPSCs, or embryonic fetal RPCs, for regenerative purposes. A further complication is that injuries to the mammalian retina turn on a reactive process in MGCs, termed "gliosis", through which MGCs initially orchestrate a neuroprotective response and then, if the injury persists, turn on a pro-inflammatory response that further impairs neuronal function and tissue repair (Figure 5). This gliotic process is common to other glial SC in the CNS; following injuries to nerve tissues, astrocyte-like cells with SC properties activate a reactive gliotic response that interferes with neurogenesis, turning gliosis into a considerable obstacle for regenerative processes[211] (Table 1).

During this gliotic response MGCs up-regulate the expression of intermediate filaments and recruit macrophages. Reactive gliosis following transplantation also occurs in response to many other donor cell types, including neuronal cells, iPSCs, and PHR precursors, when transplanted either into the vitreous or in the subretinal space. This suggests gliosis is independent of the type and origin of transplanted SC. Intravitreally transplanted cells secrete CNTF and IL-6, among other factors, that activate the JAK2/STAT3 cascade, and STAT3 mediates glial fibrillary acidic protein (GFAP) upregulation (Figure 5). Noteworthy, pharmacological inhibition of STAT3 in BM MSC reduces GFAP expression and improves their retinal engraftment[212]. Moreover, activation of JAK/STAT signaling cascades is required for increasing proliferation of MGC-derived progenitors in NMDA-injured chicken retinas[213].

In spite of eliciting gliotic responses and scar formation in damaged retinas, that prevent neurite elongation and cell migration, MGCs still retain regenerative capabilities. Several studies have shown that a reduced amount of vertebrate MGC de-differentiates and re-enters the cell cycle, after different injuries, and eventually differentiate as retinal neurons[214].

MGCs have been shown to replace some or all retinal cell types in various species [215]. In response to damage, or when exposed to a combination of insulin and FGF2, MGCs in the chick retina can de-differentiate into proliferating progenitor cells, re-enter the cell cycle and express neuronal transcription factors such as CASH-1, PAX6 and CHX10[210,216]. However, their neurogenic competence is limited and they can only generate a few amacrine and bipolar cells[216-218].

In the mouse retina, neurotoxic injury activates proliferation and the expression of progenitor markers in MGCs[219], which can then differentiate into specific neuronal types[220,221]. Our work has shown that oxidative stress induces the de-differentiation and increases the proliferation of rat cultured MGCs[222]. Moreover, in mixed neuro-glial cultures, MGCs preserve the proliferative potential and SC characteristics of retina progenitor cells, even after successive reseedings, and also stimulate their differentiation as PHRs, increasing opsin expression and markers of PHR function, such as glutamate uptake and light-dependent cGMP degradation[223]. Interestingly, MGCs from the *rd1* mice retina fail to preserve their proliferative capacity and the expression of SC markers, such as Sox2 and Nestin, in mixed *rd1* neuron-glial cultures. Nestin expression is recovered when *rd1* MGCs are co-cultured with wild type neurons and, conversely, it decreases in wild type MGCs co-cultured with *rd1* neurons; this suggests that an active crosstalk between MGCs and PHRs is essential for the preservation of the regenerative potential of MGCs[224].

A recent work shows that culturing of human surgical retinal explants obtained from the equatorial retina reveals spontaneously migrating cells that express ESC markers, as Pax6, Sox2, Nestin and also MGC markers, such as GFAP and glutamine synthetase. This implies that following injury, this area of the retina might provide a source of RPCs, since it generates cells that possess the potential for regeneration, with markers consistent with Müller cell lineage[225].

As a whole, these findings imply that although with a restricted capacity, MGCs have the potential for neuronal regeneration. Understanding the mechanisms that limit this regenerative capacity and how to unleash it would allow reprogramming of MGCs as a source of progenitors for retina regeneration. Furthermore, given the undesired long-term effects of reactive gliosis, a better comprehension of the mechanisms of gliosis is essential before considering the use of MGCs for transplantation therapies.

A recent promising strategy has been successfully applied to unleash SC features in MGCs, through the transfer of cytoplasmic materials between transplanted and recipient cells. This transfer, by membrane fusion, exosome delivery or other methods of intercellular trafficking has been shown as an efficient tool for reprogramming cells [226,228]. Endogenous hematopoietic stem and progenitor cells transplanted into

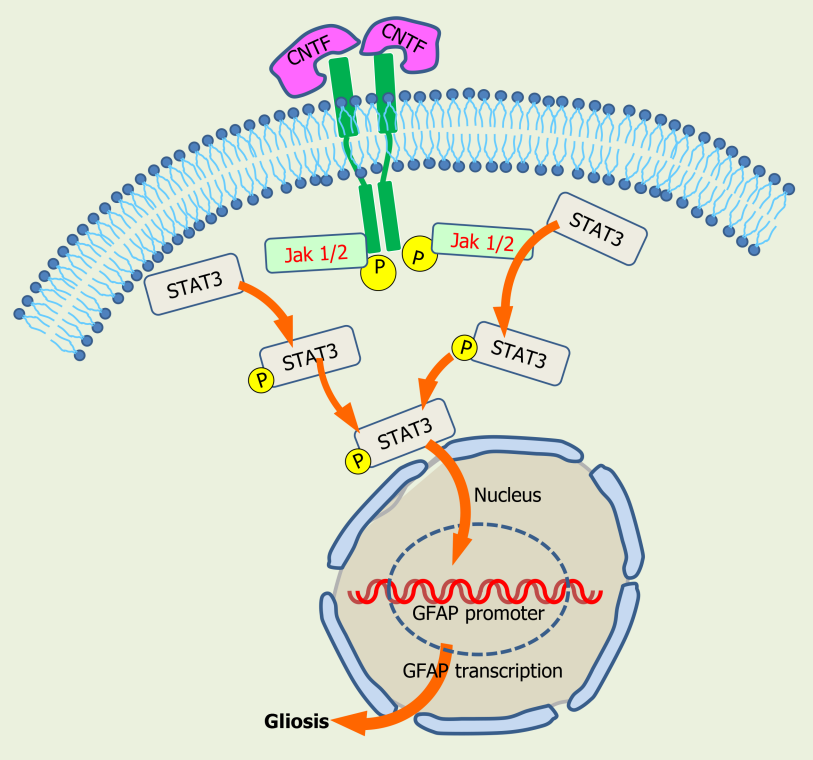

Figure 5 Activation of gliosis by the JAK2/STAT3 signaling cascade. Jak/Stat signaling cascade is activated by ciliary neurotrophic factor and other factors, generating phosphorylated STAT3 intermediates, which turn on glial fibrillary acidic protein promoter gene to induce gliosis. CNTF: Cliary neurotrophic factor; GFAP: Glial fibrillary acidic protein.

retinas of genetic and drug-induced mouse models of retina degeneration efficiently fuse with retinal MGCs *in vivo*, reprogramming them back to a neural progenitor-like state, to finally differentiate into PHRs, improving the electrophysiological response and the regeneration of the retina[228]. This supports transfer of intracellular material from SC as a new tool for turning on regenerative programs in MGCs.

Although this transfer appears as a promising strategy, understanding the molecular mechanisms involved is crucial for improving effectiveness of MGCs reprogramming. Extensive research has been devoted to uncover the diverse signaling pathways and the genetic network leading to MGCs reprogramming[229]. In zebrafish, this reprogramming involves the activation of different injury-induced genes that regulate neurogenic competence; among them, the proneural transcription factor ascl1a emerges as a crucial regulator in retinal regeneration[99]. Overexpressing *Ascl1* and modifying MGCs epigenome, with a histone deacetylase inhibitor, promotes the generation of inner retina neurons from MGCs in adult mice after retinal damage [230]; this implies *Ascl1* activation and epigenetic regulation are required for MGCs reprogramming. In contrast, the Hippo pathway repression of the transcription cofactor YAP blocks the ability of MGCs to adopt a proliferative, progenitor-like identity[231]. Exciting new data uncovers the genetic networks that control the regenerative capacity of MGCs in zebrafish and mice[232]. Retina injury triggers a reactive state in MGCs in zebrafish and mouse; however, while most zebrafish MGCs then adopt a progenitor fate, a dedicated gene regulatory network, which includes upregulation of nuclear factors I (NFI) factors, restore reactive mice MGCs to quiescence. Deletion of NFI factors in mice MGCs allows the generation of MGC-derived inner retina neurons, implying these factors are crucial to suppress neurogenesis from MGCs[232]. Although many questions remain to be answered, these findings suggest MGC reprogramming is a promising tool to unleash the SC potential of MGCs, which would contribute to human retina regeneration (Table 1).

As regardless of their source, most SC have the capacity to differentiate into nearly all of the cell types occurring in the human body, SC types like those from umbilical or placental tissues, have been evaluated in animal studies and in clinical trials for their therapeutic use in the eye. Umbilical tissues contain adult MSC, and other non-ocular cell types, such as placental cells and bone-marrow derived MSCs are also available [117,233-235]. An early study compared the efficacy of these three human-derived types of SC injected at early stages of RD in the subretinal space in RCS rats. Cells

obtained from human umbilical cords were the most effective, rescuing larger areas of PHRs and preserving visual function, with no sign of tumor formation[236]. These cells were found to rescue phagocytic dysfunction in RCS-derived RPE cells in culture, by releasing trophic factors such as BDNF, GDNF, hepatocyte growth factor, and bridge molecules that bind to PHR outer segments and facilitate RPE phagocytosis [237] (Table 1). Adipose, BM and umbilical MSCs have been shown to secrete multifunctional exosomes with low risk of toxicity and immunological rejection[238]. In a clinical trial evaluating the safety and tolerability of the subretinal injection of human umbilical cord tissue-derived cells in patients with visual impairment due to geographic atrophy[239], patients showed a variable and consistent increase in visual acuity after 12 months, with no rejection or tumor formation[240]. Umbilical cord cells might thus provide a potential source of SC, able to contribute to PHR survival and function.

The use of BM SC has also gained relevance, given the evidences that these cells can rescue degenerating and ischemic retina[238]. Studies injecting BM SC subretinally in mouse models of RP show improvements in visual parameters and in the structure and function of RPE cells and PHRs[241,242] (Table 1).

BM SCs comprise MSC and HSC. MSC are easily accessible primary cells, with various biological functions and properties such as multi-lineage differentiation, anti-inflammation, immune suppression, and neuroprotection. They express specific cell surface markers including CD105, CD73, CD44, CD90, CD166, CD146 CD54, and CD49. HSC are capable of self-renewal, and can be identified by cell surface markers, like CD34+ in humans[243]. They have been used for transplantation treatments in retinal diseases, as in mice models of diabetic retinopathy or in ischemia-reperfusion injury[244].

BM SCs have generated great expectations for treating several RDs, including retinal injury, and autoimmune uveitis. Their intravenous injection reduces laser-induced damage in the retina, by inhibiting apoptosis and inflammatory responses, even when they do not migrate to the injured retina[245]. These cells release many soluble factors and exosomes; interestingly, exosome administration prevents the potential risks caused by MSC transplantation, mainly allogeneic and xenogeneic immunological rejection, and malignant transformation. MSCs can also be incorporated intravitreally into the damaged retinas, releasing molecules that activate the cell cycle, thus promoting regeneration of different retinal cells[238,246-248]. Although promising, the safety of these treatments remains to be established.

ESCs are pluripotent cells, with the ability to differentiate into any cell type in the body; hence, in addition to giving rise to ESC-RPE cells they represent an attractive source for replacement of retinal neurons. ESCs have been cultured with Wnt and nodal antagonists, involved in patterning the embryo and in the maintenance of pluripotency and carcinogenesis[249], and then with activin to induce retinal fate, thus generating cells with a PHR phenotype, expressing Rhodopsin and recoverin[250]. Using a combination of noggin, Dickkopf (dkk1), an inhibitor of Wnt signaling pathway, and IGF-1, Lamba *et al*[251] generated retinal progenitors from human ESCs, which integrate into degenerating retinas, increasing PHR differentiation.

ESCs emerge as a promising source of cells for retinal replacement. Nevertheless, extensive research is still necessary to identify the signals that promote retinal fate, allowing ESC differentiation into particular neuronal types, and to establish whether neurons derived from ESCs can be functionally integrated in human host retinas.

Moreover, taking advantage of the ability of ESCs for differentiating into multiple cell types is challenged by ethical questions, since human embryos have to be used as donors to obtain them. In contrast, as discussed for RPE cells, iPSCs represent a source to generate ESC-like cells that do not require a human embryonic cell donor, posing no ethical objections. However, transforming donor cells into iPSCs still has the potential risk of developing tumors or cancer cells[116,252] (Table 1).

iPSCs are similar to human ESCs in their morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. The successful reprogramming of adult somatic cells (*i.e.*, skin, or blood cells) into iPSCs by introducing the so called Yamanaka´s factors (Oct3/4, Sox2, Klf4 and c-Myc)[15], allows the generation of essentially all kind of tissues, including human dermal fibroblasts[15,156,253-255], as these iPSCs can differentiate into cell types from the three germinal layers *in vitro*[256]. One of the main difficulties of this approach is the requirement for *c-Myc*, a proto-oncogene capable of transforming iPSCs into cancer cells; to avoid it, a combination of *Oct4, Sox2, Nanog* and *Lin28* has been used[257]. Human iPSCs have been shown to generate several types of neurons and even brain organoids, showing both excitatory and inhibitory synapses and exhibiting functional synaptic activity[258] and demonstrating an extraordinary

capacity to emulate human fetal synapses[259]. An amazing finding was that cultured human iPSCs release intrinsic cues that allow them to recapitulate the main steps of retinal development, leading to the formation of three-dimensional retinal tissue exhibiting rather differentiated, photosensitive PHRs. Thus, iPSCs cells have proved to be very effective in developing organoids of three-dimensional tissues, containing mini optic vesicles, with characteristics similar to those of tissues and organs developed *in vivo*[18,260,261]. These organoids are useful to investigate the pathophysiology of various diseases and to evaluate therapeutic strategies[262].

While this approach is very promising, several safety concerns should be dealt with before using iPSCs for treating degenerative retina diseases. A troublesome finding is the report that iPSCs retain an epigenetic memory, since the pluripotent cells obtained by these reprogramming methods preserve residual DNA methylation signatures, characteristic of their original donor cell types[263]. Alteration of the genome due to *in vitro* manipulation, leading to oncogene mutations, is another major concern[18,262,264]. Due to the reprogramming process and their active cell division, iPSCs can accumulate mutations with a high risk of developing cancer cells[265]. This is a shared feature of many SC, which harbor malignant SC in their niches, able to maintain an active self-renewal while generating differentiated cells[266]. Although embryonic and adult iPSCs have the capacity of preventing the accumulation of genetic damages and avoid their propagation to daughter cells, this capacity is hampered by mutations occurred during their life span. Many critical functions of SC, like self-renewal, survival, proliferation, and differentiation are regulated by Jak/STAT kinase, phosphatidylinositol 3-kinase/phosphatase, NF-κB, and other signaling pathways, the dysregulation of which could lead to cancer development[267].

Cancer SC are particularly tolerant to DNA damages and fail to undergo senescence or regulated cell death. In spite of the accumulation of genetic lesions, they remain proliferating, contributing to form tumors and resisting chemo- and radio-therapy [266]. Since the risk of different cancer types correlates strongly with the amount of mitotic cycles of the normal self-renewing cells, SC are believed to be particularly prone to generating cancer cells and tumors[268], a feature that threatens their use in regenerative medicine.

The versatility of iPSC-derived organoids described above and their similarity to specific tissues and organs turn them into effective tools to evaluate the progression of multiple diseases and the effectiveness of new drugs. This has become apparent during the coronavirus disease 2019 (COVID-19) pandemic. This disease is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), identified in Wuhan, China, in December 2019. The respiratory system is the primary infected organ, but several other organs, including the CNS, the eyes and the retina may also be infected[269,270].

The angiotensin-converting enzyme 2 (ACE2) is the main host cell receptor for the entry of SARS-CoV-2. The eye expresses ACE2, and this expression is present in its inner part, including the retina[271-273]. Furthermore, SARS-CoV-2 is detected in post mortem retinas of COVID-19 patients[274]. As human iPSC-derived retinal organoids have been used to investigate disease progression, and for drug testing[275], the onset of the COVID-19 pandemic led researchers to use iPSC-derived organoids to investigate both SARS-CoV-2 pathogenesis and the effectiveness of antiviral drugs [276]. iPSC-derived human neuronal progenitor cells, neurospheres and brain organoids are permissive to SARS-CoV-2[277], suggesting SARS-CoV-2 can productively infect the brain and might thus be involved in the neurological symptoms observed in the disease. As ACE2 is expressed in human iPSC-derived retinal organoids and monolayer cultures derived from their dissociation and both platforms can be infected by a GFP-expressing lentivirus with a SARS-CoV-2 spike (S) protein[278], these platforms appear as suitable models to investigate SARS-CoV-2 pathogenesis and evaluate drug efficacy.

In addition, to using SC for cell replacement strategies, the feasibility of transplanting PHR cells into the damaged retinas has been a subject of extensive study. Using these cells presents particular advantages and difficulties. Their position as a layer in the retina makes their transplantation feasible; however, since PHRs do not divide, how to reach a critical mass of replacement cells, sufficient to lead to vision improvement is a pending, imperative question that remains to be answered[116,169].

On the other hand, regeneration of PHRs faces less complications that of brain neurons. The short single axons of PHRs would allow an easier reestablishment of the appropriate contacts and connections, when compared to the complex neuronal circuitries found in other regions of the CNS. Axonal growth would be further facilitated since the myelin components that bind to the Nogo 66 receptor, inhibiting this growth in the brain, are relatively absent in the retina[116,279].

Early work evidenced that when PHR sheets or dissociated cells are transplanted into the subretinal space of *rd* (*C3H rd/rd*) mice, the transplanted PHRs survive for one month, developing outer segment-like processes, and synaptic terminals[141,280]. Transplantation of PHRs as a cell suspension in *rd1* (C3H/HeNHsd *rd1*) mice regenerates a functional outer nuclear layer[281,282]. Even if no host rod cells are left in the degenerating retina, the transplanted PHRs reestablish the outer nuclear layer, preserve their appropriate polarization, and adequately reconnect their axons with the host neurons.

In an early clinical trial, subretinal transplantation of a sheet of PHR cells obtained from corpses to two advanced RP patients, without immunosuppression, showed no evidence of rejection. Although no improvement in visual acuity was observed, this trial demonstrated that PHRs can be harvested from human cadaveric eyes and safely transplanted to patients with RP[283]. Subretinal transplantation of human fetal retinal micro-aggregates in patients with RP and neovascular AMD evidences an apparent high tolerance for graft tissue, even when no positive effect on visual function has been observed. Interestingly, the transplantation of retinal micro-aggregate suspensions or retinal sheets from human fetuses in patients with RP and wet AMD leads to a transient improvement of light sensitivity in about 30% of the patients[284]. Similarly, subretinal transplantation of fetal retinal sheets in RP and AMD patients improves their visual acuity, supporting the efficacy of these therapies[281,285,286].

Other clinical trials are currently taking place. Many variables occurring during PHR transplants still require to be defined before successfully using these transplants in RD patients.

Regulation of SC death is a further critical problem that most regenerative processes still face. Thus, the initial exacerbation of the cell cycle in ESC, required for replenishing the cell loss occurring during degeneration, leads to a progressive accumulation of DNA damages, to which ESCs are very sensitive[287-289], and leads them to trigger apoptosis even after low damage doses[290]. The reason for this sensitivity remains unclear, as, in contrast, adult SC evidence a variable sensitivity to damage. Deregulated proliferation of SC increases the risk of mutations associated with cancer development. Thus, ESCs have to choose between cell death resistance, which may lead to the accumulation of mutations and cancer, or a high sensitivity to DNA damage, which may cause SC depletion, and regeneration failure[290], due to the activation of cell death response to preserve genetic stability[291].

In addition, PHR death is intrinsically generated in several retina degenerative diseases, such as RP, due to genetic causes. These causes will persist, even when allogenic therapies with human iPSCs allow a successful transplantation. Therefore, the combination of cell replacement therapies with new strategies aimed at inhibiting cell death will be essential to prevent the death of the newly generated PHRs.

Finally, it is important to mention the risks regarding the inadequate use of SC therapies. Attracted by the big economic benefits, a growing "stem cell" industry is developing[292]. The excitement regarding the therapeutic possibilities of SC and their potential capacity for regenerating most damaged tissues has led to the emergence of many unauthorized "Stem cell clinics" around the world, proposing treatments that lack carefully tested protocols, thus exposing desperate patients to high risks. Due to the relatively easy anatomic access and physiological monitoring of the retina, SC therapies for many ocular conditions are already being offered for improving or curing eye degenerative diseases without providing solid scientific data. Usually the efficacy of the treatments is exaggerated, and the potentially serious health risks of the untested "stem cell therapies" underestimated. It is important to remark that, up to now, only few SC therapies have been approved by the United States Food and Drug Administration[117]. Our knowledge on SC therapies develops much faster than the regulations supervising the ethical and legal concerns related to their clinical use. Hence, governmental offices around the world have neither approved nor disapproved numerous SC treatments offered in multiple websites. In spite of the warnings regarding the risks of undergoing non-approved stem-cell based, ocular treatments, such as that of the American Academy of Ophthalmology (2016), many vulnerable patients throughout the world have been deceived into believing that they are under safe, legal, which instead have repeatedly resulted in severe vision loss (American Academy of Ophthalmology, 2016. Intraocular Stem Cell Therapy).

CONCLUSION

Degeneration of RPE and PHR are the main pivotal causes of RD, a major cause of

blindness around the world. The finding of SC in the retina, potentially able to restore the lost cells and repair this degeneration, has opened a new era toward more effective treatments or eventually cures for these diseases. Most SC types like the iPSCs, RPE, and RPCs, are potentially able to replenish the cell loss. Up to now, RPE and RPCs, derived from multiple sources, emerge as the best candidates for treating RD and several "proof of concept" studies support their use. However, none of the proposed SC fulfill yet all the requirements to successfully restore vision and significant problems remain to be addressed before using them safely in the clinic. The list of difficulties is significantly large; while some SC do not attach adequately to their substrata, others are immune-rejected, only survive for short periods of time, fail to integrate into the host retina or do not improve visual function[116]. How to avoid the risk of tumor development, and to address ethical issues are questions still unanswered and a careful balance of benefits and potential harms is required to choose an effective strategy for transplanting SC in the retina.

Additional pending difficulties are how to control the migration of the new cells in the host retina, their differentiation into the required cell types and their reestablishment of synaptic connectivity. Since our knowledge regarding the mechanisms underlying these processes are in their infancy, clinicians strongly rely in the capacity of the tissues surrounding the implanted grafts to provide the cues for the successful integration and function of the newly generated cells.

In spite of these enormous obstacles, outstanding progress has been made around the world to uncover the molecular mechanisms and understand the pathways that control SC potential. This progress allows us to hope that, in a near future these obstacles will be overcome, thus paving the road toward the treatment or eventual cure of RD by using SC.

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REVIEW

Considerations for the clinical use of stem cells in genitourinary regenerative medicine

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Abstract

The genitourinary tract can be affected by several pathologies which require repair or replacement to recover biological functions. Current therapeutic strategies are challenged by a growing shortage of adequate tissues. Therefore, new options must be considered for the treatment of patients, with the use of stem cells (SCs) being attractive. Two different strategies can be derived from stem cell use: Cell therapy and tissue therapy, mainly through tissue engineering. The recent advances using these approaches are described in this review, with a focus on stromal/mesenchymal cells found in adipose tissue. Indeed, the accessibility, high yield at harvest as well as anti-fibrotic, immunomodulatory and proangiogenic properties make adipose-derived stromal/SCs promising alternatives to the therapies currently offered to patients. Finally, an innovative technique allowing tissue reconstruction without exogenous material, the self-assembly approach, will be presented. Despite advances, more studies are needed to translate such approaches from the bench to clinics in urology. For the 21st century, cell and tissue therapies based on SCs are certainly the future of genitourinary regenerative medicine.

Key Words: Genitourinary tract; Cell therapy; Tissue engineering; Stem cells

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Core Tip: Considering the lack of adequate tissue to perform repair or replacement of organ/tissue for urologic patients, new strategies must be developed, and stem cell-based therapies and tissue engineering approaches seem promising therapeutic alternatives. A complete overview of stem cells used in urology will be presented with a focus on adipose-derived stem cells which have particularly drawn the attention of researchers. Finally, an innovative technique allowing tissue reconstruction without exogenous material, the self-assembly approach, will be presented.

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INTRODUCTION

From the kidneys to the extremity of the urethra, urological tissues can be affected by several pathologies. These pathologies can mainly be divided in two groups: Congenital and acquired anomalies. Due to the severity of the anomaly or its recurrence, patients may require surgical reconstruction to restore the genitourinary tract's normal function. The repair and reconstruction of these damaged/abnormal tissues are still a challenge nowadays. Using transplantation of autologous tissues to restore the urogenital function remains the gold-standard[1]. However, this technique is limited by the characteristics of the donor, the secondary donor site injuries, and the adequacy of the function of the grafted tissue[2]. Indeed, it is easy to conceive that the intestinal bowel, used for ureteral reconstruction, cannot provide the required impermeability function[3] since the leading role of the intestinal bowel is to absorb nutrients, while the role of the ureters is the opposite, acting as a barrier to protect other tissues from urine. On the other hand, allogeneic transplantation is limited by the risk of tissue rejection and the availability of these tissues. The demand for transplantable tissues is increasing with the ageing of the population and the increasing incidence of several anomalies (*e.g.*, hypospadias), while the offer remains low. Due to the lack of available tissues for these genitourinary reconstructions, efforts are invested in thinking outside the box. In the last decades, the emergence of regenerative medicine has been recognized as a promising avenue for meeting these clinical needs. Regenerative medicine allows to regenerate or replace human cells, tissues or organs, to restore a normal function[4]. This highly collaborative scientific field brings together many disciplines such as electrical, mechanical and tissue engineering, biochemistry, biophysics, cellular and molecular biology.

Regenerative medicine strategies can rely on two distinct approaches to restore the tissue functions: (1) Cell therapy: Injection of autologous or allogeneic cells or their secretome/conditioned medium to allow the regeneration of the tissues; and (2) Tissue therapy: Implantation of a synthetic or natural biomaterial, seeded or not with cells and eventually including growth factors, to improve and guide the repair process. It appears that the presence of cells in the biomaterials before grafting, including stem cells (SCs), is particularly important to the success of the implantation[5].

This review will shortly present the genitourinary tract anatomy to better understand its pathologies and why current therapies need improvement. Second, we will provide an overview of the different sources of SCs available for genitourinary regenerative medicine. The description of works in this field using SCs will be done with a particular focus on mesenchymal stem/stromal cells isolated from adipose tissue (AT) since data is now available from an increasing number of studies. Finally, an innovative technique allowing tissue reconstruction without the use of exogenous material, the self-assembly approach, will be presented.

ANATOMY, PATHOLOGIES AND CURRENT TREATMENTS

Kidneys

The kidney is one of the most challenging organs of the genitourinary system (Figure 1) to repair/reconstruct in regenerative medicine as its structure and roles are complex. Kidneys can be affected by numerous malfunctions. Chronic kidney disease (CKD) is one of the top causes of death and affects 9%-14% of the United States adult population[6,7]. CKD is defined as a glomerular filtration rate inferior to 60 mL/min *per* 1.73 m² or presence of kidney damage markers for at least three months[8]. It is mainly caused by diabetes, glomerulonephritis, pyelonephritis or hypertension, with a variation of its prevalence by ethnicity[8]. Congenital autosomal dominant polycystic kidney disease can also be a cause, with a prevalence of 1/400 to 1/1000 birth[9]. The quality of life of the patients and socioeconomic status are reduced as CKD progresses.

Because nephrogenesis is limited to embryonic development, the intrinsic capacity to self-repair of adult kidneys is limited, causing a permanent loss of nephrons, which can lead to end-stage kidney disease[10,11]. Treatments at this stage are renal replacement or hemodialysis, but the mortality and morbidity remain high[12]. Non-drug related therapies for CKD rely mainly on lifestyle changes: Enhancing hydration and maintaining a diet rich in vegetables and fruits, smoking cessation, exercising, limiting alcohol intake, maintaining body mass index within the normal range, and limiting sodium and protein intake[13]. Treatments to control hypertension and hyperglycemia can also be administered to the patients[13].

Bladder

Bladder characteristics can be observed in Figure 1. Hormonal and metabolic factors contribute to the integrity and function of this organ. Its disturbance induces a dysfunction, which can lead from simple pain to even the need for the ablation of the bladder.

Regenerative medicine for bladder reconstruction or regeneration is necessary following various pathological conditions, such as cancer, trauma, congenital malformation, overactive bladder, interstitial cystitis, infectious cystitis (*e.g.*, unresolved *E. Coli*-induced cystitis), ketamine-induced cystitis, inflammation, stress incontinence or voiding dysfunction. A loss of storage capacity or bladder compliance induces a frequent need to urinate. Chronic urinary tract infections, incontinence, and renal calculi, which can extend to renal failure, can affect these patients[14]. Despite side effects, bladder augmentation using bowel segments or "enterocystoplasty" often remains the proposed treatment to patients presenting these pathologies. This surgery is considered the "gold-standard" treatment[15]. However, short- and long-term complications often present since the leading role of the intestinal tissue is to absorb nutrients, whereas the role of the bladder is to protect against urine toxicity[16]. Thus, malignancies, metabolic complications such as reabsorption of acid, electrolyte disturbance and mucus retention, bladder calculi, bladder perforation, upper tract deterioration and chronic infections are commonly affecting patients that underwent enterocystoplasty[17,18].

Ureters and urethra

Ureters and urethra are the conduits of the urinary tract (Figure 1). These structures can be affected by both congenital and/or acquired anomalies. Due to their location, the most common causes of ureteral injuries are iatrogenic in 80% of the cases, while 20% are due to external trauma[19]. Ureteral injury occurs most commonly during gynecologic surgeries, with an estimation of 52% to 82%[19]. Depending on the pathology stage and evolution, different operations may be performed, such as endoscopic surgery, partial ablation by segmental resection or total ablation of the injured ureter with or without radical nephroureterectomy. End-to-end anastomosis is applied for patients presenting short-length ureteral deficit due to the successful outcomes of this technique[20]. For the long-segment reconstruction, the use of autologous bowel is the gold-standard[3]. However, this kind of tissue presents many potential complications, including metabolic imbalance, malabsorption of vitamins, cholelithiasis, nephrolithiasis and infections[21]. It is not unusual that multiple surgical procedures are needed over time due to stenosis or strictures at the repair site, which may require nephrectomy[3].

On the other extremity of the urinary tract, the urethra is prone to congenital anomalies. With 1/250 newborn boys affected, hypospadias represents 73.3% of congenital penile anomalies[22,23] and its prevalence is increasing[24]. This anomaly is due to a defect of the tubularization and the fusion of the urethral plate. Hypospadias

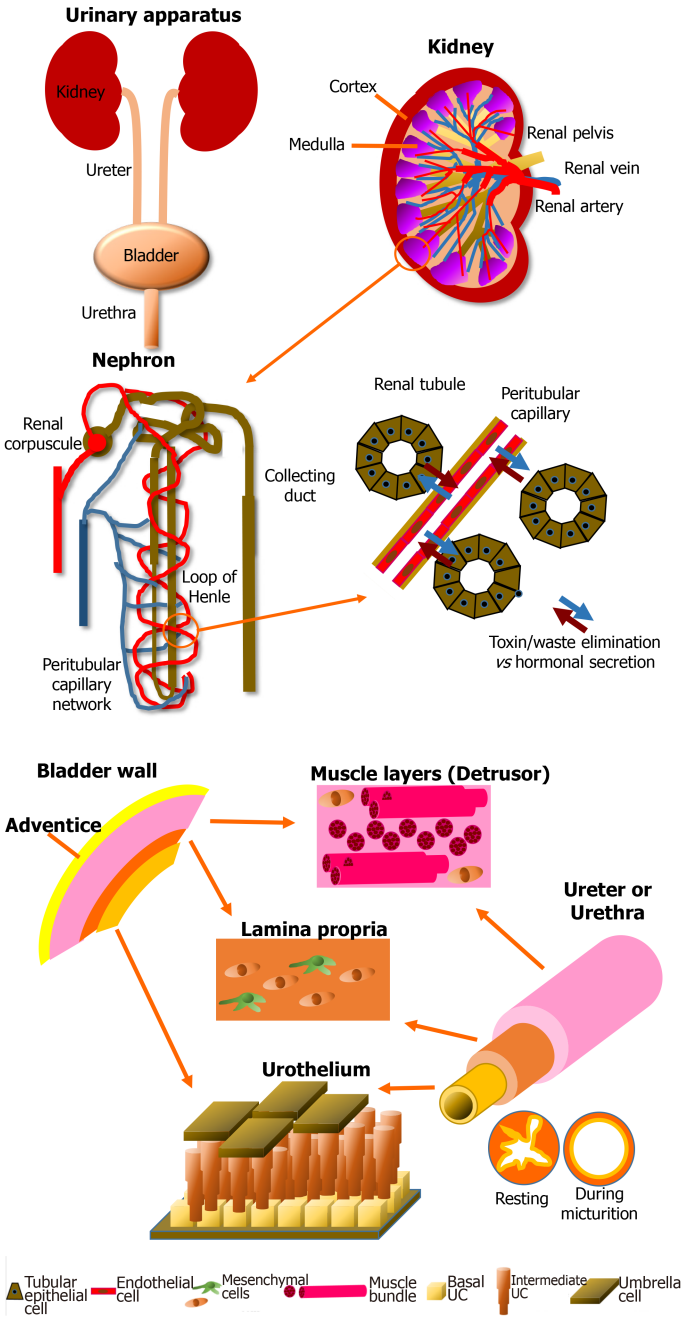

Figure 1 Schematic of the anatomy of the urinary apparatus. Kidney: Kidneys are the blood filtration unit of the body, extracting toxins and metabolic waste to produce the urine purifying 180 L of blood every day. The nephron is the element which allows exchange between blood and urine through the renal tubules and the capillaries. Another role of the kidney is the regulation of the water balance when the body is sweating or in the presence of abundant/inadequate hydration by reabsorption of components during blood filtration. Kidneys also produce hormones essential for blood pressure regulation, such as renin, which will cause, *via* angiotensin II, stimulation of the secretion of aldosterone. Finally, 90% of the erythropoietin is secreted by this organ. This glycoprotein stimulates the proliferation and differentiation of erythrocyte precursors. Pathologies can impair these functions and endanger the patient's life. Bladder: The central role of the bladder is to receive and dynamically store the urine produced by the kidneys through structural and regulatory mechanisms. The urine is brought to the bladder *via* the ureters. The bladder is also a complex organ composed of various layers. It is described from its outer surface to the lumen with the adventice (layer of fatty tissue), the detrusor muscle, the lamina propria (a connective tissue including a muscularis mucosae) and the epithelium, which is named the urothelium. The latter can be divided in three layers: basal, intermediate and superficial (umbrella). The impermeability is due to a thin asymmetric unitary membrane of uroplakin, expressed at the apical surface of mature urothelial cells called umbrella cells. Ureter and urethra: Ureters and urethra share a common primary role: to transport urine, allowing its excretion outside of the body. Anatomic organization of these organs are histologically related to the bladder due to their similar protective role against urine through the urothelial cell layers. From the inside to the outside, these two organs are composed of the transitional urothelial cell layer, a submucosa comprising fibroblasts and extracellular matrix, especially type I and III collagens, a muscular cell layer of smooth muscle cells, and an adventitia surrounding the organ. During the micturition, the star-shaped configuration of the urethra allows to compensate the increase in pressure. The muscular layers also play a critical role in the maintenance of the tubular shape.

is characterized by the inadequate position of the urethral meatus under the glans penis. This meatus can be localized on the ventral face of the penis in a position more or less close to the normal opening. The opposite urethral anomaly, epispadias, is less

frequent with 1/10000 boys affected[25] and is characterized by a malposition of the urethral meatus from the dorsal side of the penis to the pubic symphysis. On the other side, acquired anomalies appears after birth, due to an external event (*e.g.*, accident or a trauma). The most commonly acquired urethral anomaly is urethral stenosis (called stricture for anterior urethra), with an incidence of 0.6%, leading to more than 5000 hospital visits *per* year in the United States[26]. Stenosis is characterized by a urethral lumen becoming impeded by surrounding fibrotic tissues leading to a narrowing or even a total obstruction of the conduit. Stenosis is most commonly caused by an injury/trauma (*e.g.*, motorcycle or bicycle accident), but it can also be induced by infection, surgical complications, *lichen sclerosus* or cancer. Depending on the severity of the pathology, few tissues can be used to repair or replace the penile anomalies. Skin grafts, *tunica vaginalis*[27,28], lingual or buccal mucosa have all been evaluated [29-36], but it appears that oral mucosa remains the gold standard[37]. However, these approaches are associated with many complications such as re-stenosis, numbness, submucosal scars, dry mouth, difficulty to open the mouth (contracture), neurosensory defects, lesions, discomfort, pain, and risk of infection. In addition, considering that a limited amount of tissue can be harvested, this is problematic for longer urethral anomalies in need of correction[38-43].

Problems related to current treatments

The paucity of native tissues available for repair, the anatomical characteristics, and the side effects such as morbidity at the donor site are significant limitations of the current treatments for severe urologic cases[3]. Moreover, long-term complications are commonly found such as fibrosis, malignancies, metabolic troubles, stenoses and fistulas[44]. To improve the quality of life of urologic patients, new and innovative therapeutic strategies need to be developed to be more efficient with less debilitating side effects. Regenerative medicine strategies using SC transplantation or their use in tissue engineering have been proposed as solutions.

REGENERATIVE MEDICINE STRATEGIES USING SCs

Stem cell sources for urologic regenerative medicine: A concise overview

SCs can be described as clonogenic self-renewing cells with the potential to differentiate into one or more cell types. These cells are maintained in an undifferentiated state in very specific microenvironment called niches[45,46]. They are classified according to their differentiation potency. Pluripotent SCs [or embryonic SCs (ESC)] are derived from the inner cell mass of the embryo blastocyst and can produce cells of the three germ layers. The use of ESCs is controversial due to ethical issues but also to their high capacity to create teratomas after injection. Interestingly, adult SCs can be harvested from many tissues (Figure 2), such as the brain, bone marrow (BM), peripheral blood, blood vessels, skeletal muscle, skin, cornea, retina, tooth dental pulp, digestive system, liver and, pancreas[47]. They can be divided in two main categories. The first one, composed of multipotent SCs able to differentiate into many cell types, include, for example, neuronal SCs (derived from the ectoderm), mesenchymal SCs (MSCs) and hematopoietic SCs (both derived from the mesoderm). The other category contains the unipotent SCs, which have a reduced range of differentiation, such as epidermal SCs (of ectodermal origin), muscle SCs (of mesodermal origin) or urothelial SCs (of endodermal origin).

Since the discovery of adherent SCs in BM by Friedenstein *et al*[48] 50 years ago[48], the number of MSC family members has increased considerably. These cells can now be extracted from various tissues such as bone marrow (BM-MSC), AT (adipose-derived SC or ASC), synovium (SMSC), dental pulp (DP-MSC), muscle, peripheral blood, yellow ligament, menstrual blood, endometrium, maternal milk, amniotic fluid, placenta (PDSC), umbilical cord blood (UCB-MSC), and umbilical cord Wharton's jelly [49,50] (Figure 2). MSCs are heterogeneous populations, which can be important in regard of their differentiation efficiency in the context of animal grafting[49].

Many types of SCs have been studied (Table 1), but due to greater accessibility[51], postnatal SCs used in urology are mainly MSCs, especially but not exclusively BM-MSCs and ASCs. Indeed, through their secretome and multilineage potential of differentiation, MSCs are the basic building blocks used in regenerative medicine, including in the field of urology. For example, MSCs can differentiate into different cell types presenting features of various lineages, such as endothelial cells, epithelial cells (including urothelial cells), myoblasts, smooth muscle cells (SMCs), fibroblasts or neurogenic cells[52]. Because MSCs also have a significant cell proliferation and differ-

Table 1 *In vivo* studies for regenerative medicine of urologic tissues (excluding studies using adipose tissue-derived stem cells) (words used in the PubMed research engine (National Library of Medicine): "Urology" "regeneration" "reconstruction" "stem cells")

Stem cell type	Organ treated	Pathology	Animal model	Ref.
AFSC	Bladder	OAB	Rat	[203,204]
AFSC	Bladder	DUA	Rat	[205]
AFSC	Bladder	PD	Rat	[206]
AFSC	Bladder	Stroke	Rat	[203]
BM-MSC	Bladder	OAB	Rat	[207,208]
BM-MSC	Bladder	pBOO	Rat	[209]
BM-MSC	Bladder	IC/BPS	Rat	[210]
BM-MSC	Bladder	PD	Rat	[206,211]
BM-MSC	Bladder	SCI	Rat	[212-214]
BM-MSC	Bladder	Partial cystectomy	Rat	[215]
BM-MSC	Kidney	Renal regeneration	Mouse	[216]
BM-MSC	Kidney	CKD	Rat	[69,70]
BM-MSC	Kidney	AKI	Rat	[7,71]
BM-MSC	Kidney	AKI	Human	[83]
BM-MSC	Bladder	Partial cystectomy	Rat	[90]
BM-MSC	Urinary sphincter	SUI	Rat	[91]
BM-MSC	Bladder	Hemicystectomy	Dog	[97]
BM-MSC	Bladder	Augmentation	Rat	[98]
BM-MSC	Penis	ED	Rat	[217,218]
BM-MSC	Urethra	Urethroplasty	Rabbit	[103]
DP-SC	Bladder	IC/BPS	Rat	[219]
ESC	Kidney	Reconstruction	Rat/mouse	[88]
ESC-MGE	Bladder	SCI	Mouse	[220]
ESC-MSC	Bladder	IC/BPS	Rat	[221-223]
MDC	Bladder	DUA	Rat	[224,225]
MDC	Bladder	DUA	Mouse	[225]
NPC	Bladder	SCI	Rat	[226-228]
OM-SC	Bladder	SCI	Rat	[229]
Sk-MSC	Bladder	DUA	Rat	[230]
Sk-MSC	Urinary sphincter	SUI	Monkey	[231]
Sk-MSC	Urinary sphincter	SUI	Human	[92-94]
Sk-MSC	Urethra	Urethral defect	Rat	[102]
UCB-MSC	Bladder	OAB	Rat	[232]
UCB-MSC	Bladder	IC/BPS	Rat	[233-235]
UCB-MSC	Bladder	Cerebral Ischemia	Rat	[232]
USC	Bladder	IC/BPS	Rat	[236]
USC	Urethra	Urethral defect	Rabbit	[237]
USC	Penile Cavernous body	ED	Rat	[238]
USC	Kidney	AKI	Rat	[84]
USC	Bladder	Augmentation	Rat	[101]

AKI: Acute kidney injury; ASCs: Adipose-derived stromal/stem cells; BM-MSC: Bone marrow-mesenchymal stem cells; UCB-MSC: Umbilical cord blood-mesenchymal stem cells; USC: Urine derived stem cells; DPSC: Dental pulp stem cells; OAB: Overactive bladder; DUA: Detrusor underactivity; PD: Peyronie's disease; pBOO: Partial bladder outlet obstruction; IC/PBS: Interstitial cystitis/bladder pain syndrome; SCI: Spinal cord injury; SUI: Stress urinary incontinence.

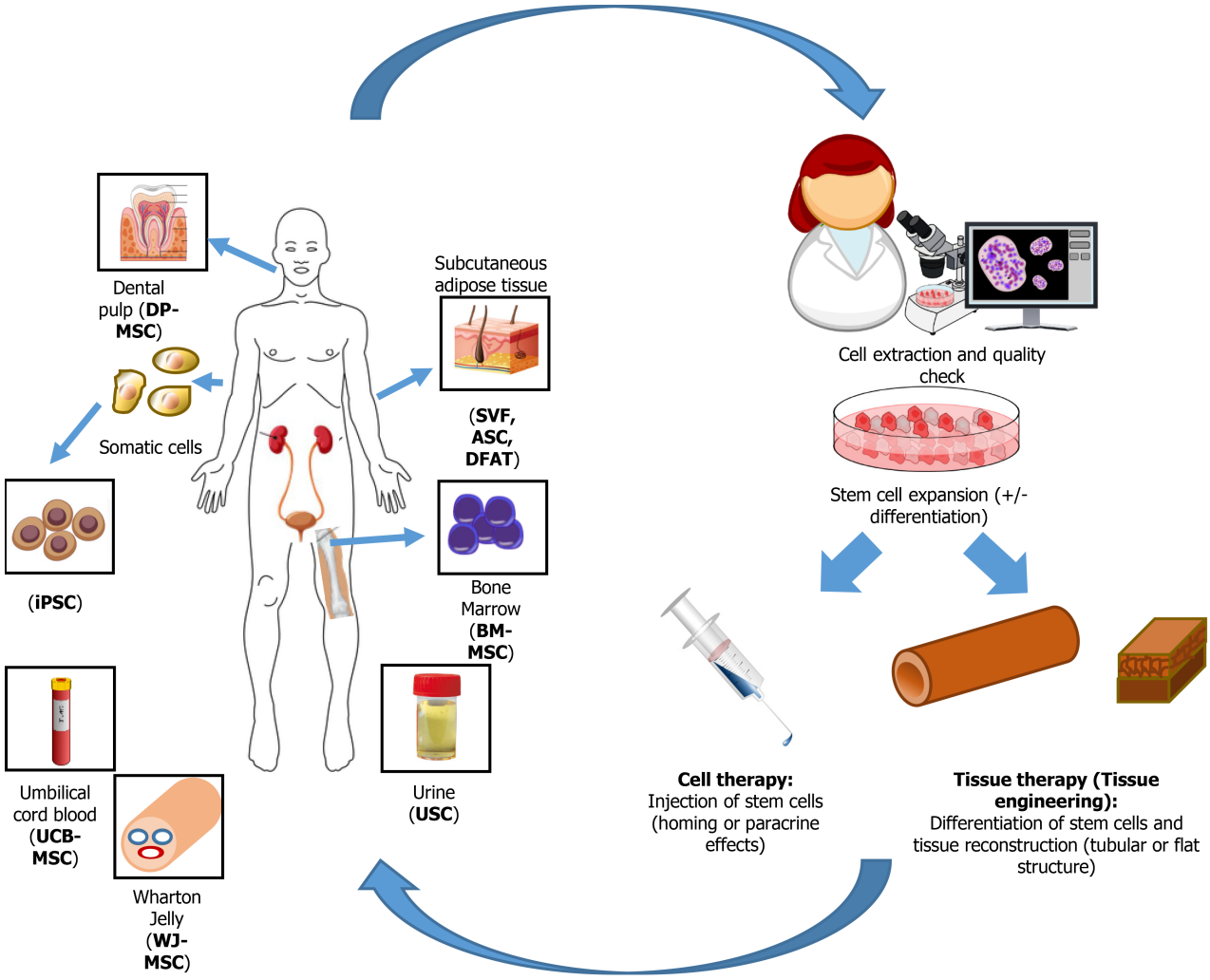

Figure 2 Source of stem cells used for genitourinary regenerative medicine. After extraction from various tissues, stem cells are expanded and eventually differentiated, depending on their origin, before to be reinjected to the patients or used to reconstruct genitourinary engineered tissues. DP-MSC: Dental pulp-mesenchymal stem cells; BM-MSC: Bone marrow-mesenchymal stem cells; iPSC: Induced pluripotent stem cells; SVF: Stromal vascular fraction; ASC: Adipose-derived stromal/stem cells; DFAT: Dedifferentiated fat; UCB-MSC: Umbilical cord blood-mesenchymal stem cells; WJ-MSC: Wharton Jelly-mesenchymal stem cells.

entiation potential, only one sample containing multipotent SCs could theoretically allow the reconstruction of an entire urological organ. For example, human dental pulp SCs (DP-SCs) are also of interest in the urological field. Indeed, Song *et al*[53] successfully differentiated DP-SCs into bladder SMCs using a conditioned medium. Studies have shown that somatic SCs can be obtained from testes throughout the male lifetime[54]. Other reports have shown that even endometrial SCs (EnSCs) could be differentiated into SMCs and urothelial cells for bladder engineering[55,56]. The less invasive way to obtain these cells is by collecting menstrual blood, but an endometrial biopsy can also be performed. Harvesting EnSCs does not necessitate anesthetic procedures compared to the protocols used for ASCs or BM-MSCs[57].

Of note, in 2008, Zhang *et al*[58] showed for the first time that SCs present in urine, urine derived SCs (USCs) could differentiate into urothelial, SMCs, endothelial and interstitial cells, allowing a non-invasive collection of SCs for genitourinary tissue engineering[58]. This promising method generates approximately 2 to 7 progenitor cells for 100 mL of urine, which can be extensively expanded in culture. Subsequently, the differentiation of these cells toward the meso-, endo- and ectodermal lineages have been shown by using appropriate induction media, further increasing their interest

[59].

Finally, a new source of SCs was created by human hands in 2006, the induced pluripotent SCs (iPSCs). After collecting somatic cells, such as fibroblasts or white blood cells, the latter are transduced using viral vectors to express transcription factors associated with pluripotency[60]. These reprogrammed cells can then be redifferentiated *in vitro* towards the cell type needed: *e.g.*, into MSCs[61]. Because from one blood sample many differentiated cells can be obtained, but also because cells from patients suffering from various pathologies cannot be cultivated *in vitro*[62], iPSCs represent a promising SC source. However, their production appears much more technically challenging than for the previously described sources.

In the next sections, key examples of the use of SCs for genitourinary regenerative medicine will be presented before focusing on the most recent work using postnatal MSCs isolated from AT.

Regenerative medicine for kidney

The kidney function relies on complex vascular structures, and this organ is an ecosystem where 24 cell types synergize over the vascular, interstitial, glomerular and tubular compartments[63]. Reconstructing/regenerating this organ using regenerative medicine is a true challenge. Especially, the microvasculature of glomeruli and the complex tissue structure must be preserved. Even if progress has been made to support the renal function, the following section will only focus on studies using SCs as therapeutics. Two main strategies can be distinguished: (1) The repair and regeneration of the kidney using cell therapy; and (2) The reconstruction of the entire organ for transplant using tissue engineering.

Cell therapy for the kidney: BM-MSCs have been the most studied postnatal mesenchymal cell type, undoubtedly due to the demonstration of their safety in clinical trials (Table 1)[64]. Three hypotheses have been established concerning the effects of MSCs of various sources: (1) The regenerative potential of tissues is improved by the paracrine secretion of bioactive factors; (2) The regeneration of tissues is allowed by the differentiation of MSCs into resident cells to repopulate the tissue; or (3) The fusion of MSCs with resident cells[63]. Nevertheless, very few studies indicated that MSCs could fuse with resident cells[65] or transdifferentiate[66]. However, as low detectable differentiation of BM-MSCs into kidney epithelial cells to repopulate the tissue has been reported, a paracrine mechanism of action of MSCs to regenerate the kidney is suspected[67,68]. Beneficial effects were shown after exogenous administration of BM-MSCs in various models of acute and CKD. Direct injection of BM-MSCs in the kidney reduced or prevented renal dysfunction and renovascular hypertension in a CKD rat model[69,70]. Furthermore, in acute kidney injury (AKI) models and following BM-MSC injection, cellular proliferation was increased while the tubular dysfunction was reduced, including decreased apoptotic and necrotic cell death[71,72]. Supporting the paracrine hypothesis, studies have shown that most of the BM-MSC's effects can be obtained using conditioned medium only[71,73,74]. Indeed, the immunomodulatory properties of MSCs are well-known, with an induction of anti-inflammatory factors such as interleukin (IL)-10 and a decrease in inflammatory factors (IL-1 and -6)[71,75,76]. On the other hand, the secretome of the MSCs is also characterized by growth factors such as hepatocyte growth factor (HGF), insulin-like growth factor-1, transforming growth factor (TGF)-α and -β, fibroblast growth factor (FGF) and vascular endothelial growth factors (VEGF), which can be enhanced by the preconditioning of the MSCs (*e.g.*, hypoxic conditioning)[51,77,78]. MSCs can release these factors, also known to be produced by renal cells during kidney injury[63], either in a free-state or contained within exosomes, vesicles of 20-100 nm diameter originating from endosomes, or microvesicles, vesicles of 50-1000 nm originating from the plasma membrane[51]. These extracellular vesicles contain nucleic acids such as mRNAs and microRNAs, cytoplasmic and membrane proteins, and even bioactive lipids, all contributing to cell-cell communication and signal transmission[79].

Depending on the culture conditions (*e.g.*, 3D microspheres), BM-MSCs can modulate their secretome, overproducing factors found in low quantity when cells are grown as monolayers, such as antiapoptotic and anticancer factors including tumor necrosis factor (TNF)-stimulated gene 6 protein (TSG-6) and IL-24[80,81]. The mechanism of action of the paracrine effects of MSCs to regenerate the kidney can be advantageous for clinical use compared to transplantation. Indeed, the large-scale culture of MSCs and harvesting of their secretory products, or the production of a recombinant mixture of proteins, can be done in good manufacturing practice (GMP) facilities and presents lower risks than direct injection of SCs[63]. However, these cell-

free therapies' therapeutic efficacy has been limited by the low stability and retention of these components[82]. Indeed, cells are continuously producing factors, whereas cell-free therapies would need repeated injections. Using new technologies as supramolecular nanofibers peptides could be an alternative to improving kidney repair, enhancing, and prolonging bioavailability of these factors[82]. A clinical study on 66 patients with postoperative AKI showed that intra-aortic infusion of allogenic BM-MSCs led to a worse prognosis in the postoperative period[83]. Further studies are therefore needed to expand the use of global MSCs in clinical routine.

Interestingly, Sun *et al*[84] showed that USCs used as a therapy in a rat AKI model significantly improved the renal function and histological damage[84]. Furthermore, it inhibited the inflammation and apoptosis processes in the kidney while promoting tubular epithelial proliferation.

Tissue engineering for kidney reconstruction: Whole organ production has also been evaluated using tissue engineering. First, acellular structures have been produced and studied from porcine, human and rat kidneys[85]. The main advantage is the preservation of the architecture of the decellularized matrix. This architecture has been shown to impact cell morphology and differentiation[86]. Fully differentiated epithelial and endothelial renal cells can be seeded on the decellularized scaffold as done by Song *et al*[85] before perfusion in a whole-organ bioreactor[85]. When perfused through their intrinsic bed, the resulting substitute produced rudimentary urine *in vitro*. When grafted *in vivo*, grafts were perfused by the recipient's circulation and produced urine through the ureteral conduit[85]. However, only a negligible excretion of urea and creatinine was measured. Similar results have been found by other teams[87]. In another study, rat kidneys were decellularized and seeded with pluripotent murine ESCs[88]. After the proliferation of the primitive precursor cells within the glomerular, vascular and tubular structures, cells were reported to express epithelial cell's differentiation markers[88]. Despite promising preclinical outcomes obtained with animal models, such as the reduced acute and chronic kidney injuries [64,89], clinical trials remain in the early phases, mainly investigating the safety and some efficacy of allogenic MSC infusion[64,89].

Regenerative medicine for bladder

The bladder is a highly elastic hollow organ surrounded by three muscular layers forming the *Detrusor*. These muscular cells are essential to maintain a fully functional bladder. Moreover, neural and vascular networks are required for a healthy, competent bladder and self-control of the micturition. As for the kidneys, the bladder architecture must be preserved to ensure that efficacy is preserved.

Cell therapy for the bladder: Several studies have described phenotypic and physiologic similarities between adult MSCs and bladder SMCs. Sharma *et al*[90] indicated that unstimulated BM-MSCs have a similar contractile protein profile as the bladder SMCs[90]. Furthermore, no significant difference of increased magnitude of intracellular Ca^{2+} release has been found between both groups when stimulated. Another study using adult nude rats to evaluate bladder augmentation found that the BM-MSCs seeded on the poly 1,8 octanediol-cocitrate scaffold maintained a high level of protein expression of smooth muscle markers, significantly increasing the muscle-to-collagen ratios at ten weeks post augmentation[90]. Therefore, it has been of interest to evaluate the impact of MSC injection for patients affected by stress urinary incontinence (SUI). In an injured rat model, using serial vaginal dilatation, Dissaranan *et al*[91] showed that the leak point pressure (LPP) was significantly improved in animals receiving an injection of BM-MSCs compared to the controls[91]. These results suggest that factors secreted by MSCs, in general, can have therapeutic effects when injected. Local injection of autologous muscle-derived SCs has been evaluated in clinical trials and successfully improved patients' quality of life affected by SUI[92-94]. Since the main effects of MSCs have been attributed to their secretome, it will be interesting to evaluate the secretome-only injection as treatment or as prophylaxis for patients with SUI. Indeed, even without engraftment, the only use of MSC injection could result in the release of soluble factors and enhanced quality of life of patients [64]. Therefore, MSCs and their secretome could offer a safe and effective treatment for bladder dysfunction, targeting the pathophysiology instead of only targeting symptoms. However, the current state of knowledge needs more data in the long term to ensure a safe large scale and efficient use of these cells. Indeed, more studies are needed to determine the method of delivery (systemic or local injection), the dose and the most secure and efficient cell origin (ASCs, BM-MSCs, *etc*.).

Tissue engineering for bladder reconstruction: Reconstruction of a whole bladder to replace the affected one has been investigated by many teams. Tissue engineering for bladder reconstruction is based on the use of natural, synthetic or hybrid scaffolds. Decellularized scaffolds such as bladder acellular matrix (BAM) and small intestinal submucosa (SIS) have been studied for bladder reconstruction[2,95]. However, issues have been encountered. Indeed, the maximum distance allowing tissue regeneration by native cells using an acellular graft has been evaluated to 0.5 cm[5]. It is, therefore, necessary to seed cells on the scaffold before its implantation[5]. Nevertheless, autologous cells could not be used in patients with cancer[96] or benign end-stage bladder diseases[62]. In these cases, the use and differentiation of SCs could represent an alternative.

BM-MSCs have been evaluated as an alternative cell source. Zhang *et al*[97] used BM-MSCs on an SIS scaffold for bladder reconstruction in a hemicystectomy canine model[97]. They showed that BM-MSCs had a similar cell proliferation, histological appearance, and contractile phenotype as primary cultured bladder SMCs. Using an amniotic membrane, BM-MSC contributed to regenerate the detrusor and urothelium in a rat model of bladder augmentation[98]. However, proper urinary bladder function could not be achieved, and further studies are required.

Urine-derived SCs can also be considered an alternative source for bladder reconstruction as only a fresh -collected less than 24 h before- urine sample is necessary. To this end, studies showed that USCs could be differentiated in SMCs with contractile function comparable to native SMCs[99]. In another study, USCs have been differentiated in urothelial and SMCs that were then seeded on a bacterial cellulose scaffold[100]. They showed that a multilayered urothelium could be obtained with the colonization of the cells into the matrix, holding promises for urinary reconstruction using USCs. Lee *et al*[101] directly seeded undifferentiated USCs on a surface modified composite scaffold (polycaprolactone/pluronic bladder submucosa matrix) in a rat model to improve bladder capacity[101]. They showed a significant functional improvement of the bladder compliance compared to the control group and an increased regeneration of SMC tissues with a well-differentiated multilayered urothelium.

Regenerative medicine for the ureters and urethra

The primary function of ureters and the urethra are a strictly waterproof barrier preventing the toxic urine diffusion beneath the epithelium. The reconstruction of these structures must preserve this function otherwise fibrosis induction will occur. As these tubular organs are surrounded by a muscular layer allowing to compensate the increase of pressure during the micturition, elasticity and mechanical resistance have to be maintained in the reconstructed substitutes.

Cell therapy for ureters and urethra: Human skeletal muscle derived SCs were applied on the damaged urethral site in a rat model and improved penile functional recovery[102]. Indeed, six weeks after transplantation, a higher functional recovery was found in the transplanted group than controls (70.2% *vs* 39.1%). The authors indicated that transplanted human cells differentiated into skeletal muscle fibers, nerve-related Schwann cells, perineuriums, and vascular pericytes while active paracrine angiogenic cytokines were also detected.

Tissue engineering for ureter and urethra reconstruction: SCs have also been used for urethra and ureteral reconstruction. Yudintceva *et al*[103] seeded allogenic BM-MSCs on a bilayer poly-D,L-lactide/poly-ε-caprolactone scaffold for urethra reconstruction in a rabbit model in comparison with conventional urethroplasty, performed using an autologous buccal mucosa graft[103]. After a follow-up of 12 wk, the absence of complications, reduced fibrosis and inflammatory cell infiltration were observed in the experimental group compared to the group for which buccal mucosa was grafted.

In other studies, SCs have been first differentiated before being seeded on scaffolds. Urothelial and SMCs derived from USCs have been compared to native cells when seeded on a SIS scaffold[104]. The authors indicated that USCs expressed urothelial cell markers or SMC markers according to the differentiation protocol. Furthermore, the resulting tissues were similar to those formed when urothelial cells and SMCs derived from native ureters were used.

The optimization of the culture medium/media used to differentiate SCs is a challenge and must be different for each kind of SCs because their engagement status is different: ESCs is different from definitive endoderm SCs or urothelial cell progenitors. As an example of this kind of works, murine ESCs have been successfully differentiated into urothelial cells using chemically defined conditions. Such protocols

could facilitate the generation of epithelial cells for *in vitro* tissue production[105].

TOWARDS CLINICAL APPLICATIONS

AT as a source of therapeutic cells

Beyond its metabolic and endocrine functions, white AT represents an important source of multipotent cells for regenerative medicine[106,107], with subcutaneous depots being most abundant and accessible in humans through lipoaspiration procedures. ASCs originate from the cultivation of cells extracted from the stromal vascular fraction (SVF) of AT. This classical way of extracting multipotent human ASCs has been mastered by Zuk *et al*[108,109] and reported in 2001 and 2002[108,109]. SVF corresponds to the fresh cellular pellet obtained after AT collagenase digestion and centrifugation[110] (Figure 3). SVF is heterogeneous, being composed of fibroblasts, endothelial cells, SMCs, macrophages, monocytes, preadipocytes and mesenchymal stromal/SCs[111,112]. This cell heterogeneity plays an important role conferring the SVF several important therapeutic characteristics such as pro-angiogenic, anti-inflammatory and immunomodulating properties[113-115]. However, it is also associated with a high variability of the SVF's therapeutic potential among individuals, making it more complicated to standardize SVF-based treatments[116]. The use of SVF as treatment of urogenital disorders has been widely investigated in recent years in particular for preclinical studies for erectile disfunction and for Peyronie's disease[117-120]. Moreover, a small number of early clinical studies using injected SVF for urogenital system related pathologies, like urinary incontinence, erectile disfunction, and Peyronie's disease, have been performed in the last years[121-123]. Since treatments based on freshly extracted SVF cells have been recently reviewed[115,124], the following sections will focus on the use of cultured mesenchymal cells obtained from AT [ASCs and dedifferentiated (DFAT) cells] for regenerative medicine applied to the urologic system.

There are two approaches for obtaining stromal/SCs from AT (Figure 3). ASCs represent the more common source as they are obtained following culture of the fresh SVF. ASCs are described as relatively homogenous adherent cultures of cells possessing key functional properties: Important multilineage differentiation potential and relevant secretome-related therapeutic potential[109,125,126]. Since their discovery, cell therapies based on ASCs have been developed for many pathological conditions including wound healing, cardiovascular diseases, bone fractures and many other health problems, including urological-related pathologies[115,127-131]. The second and most recent method of obtaining progenitor cells from AT depots is by inducing the dedifferentiation of floating mature adipocytes obtained following collagenase digestion, leading to cell populations commonly named DFAT cells (Figure 3). In fact, as soon as 1986, Sugihara *et al*[132] developed a ceiling culture method that induced the dedifferentiation of mature rat adipocytes into fibroblast-like cells[132]. Later in 2004, Yagi *et al*[133] showed that isolated fibroblast-like cell populations obtained following the dedifferentiation of mature murine adipocytes exhibited long term viability in culture and adipogenic potential upon induction[133]. The seminal study by Matsumoto *et al*[134] in 2008 showed that human cells originating from dedifferentiated adipocytes extracted from subcutaneous depots exhibited critical features associated with ASCs and BM-MSCs, including cell surface markers such as CD13, CD29, CD44, CD90 and CD105, and multilineage differentiation potential towards adipogenesis, osteogenesis, chondrogenesis[134]. More recent studies also indicate that DFAT cells could acquire myogenic and neural-like cells phenotype[135,136]. Importantly, as part of the secretome of these dedifferentiated cells, many growth factors with proangiogenic action have been quantified such as the VEGF-A, HGF, FGF and Angiopoietin-1 (Ang-1)[137]. DFAT cells, has been more widely studied in recent years for different fields of regenerative medicine. Some preclinical studies have shown DFAT cardiomyocyte differentiation and pro-angiogenic potential in myocardial infarction and nerve regeneration using rat models [136,138,139]. Despite the recent characterization of DFAT cells, some preclinical studies have already shown promising therapeutic potential for these cells. The following section details the recent studies that used ASCs and DFAT cells for the treatment of urogenital diseases.

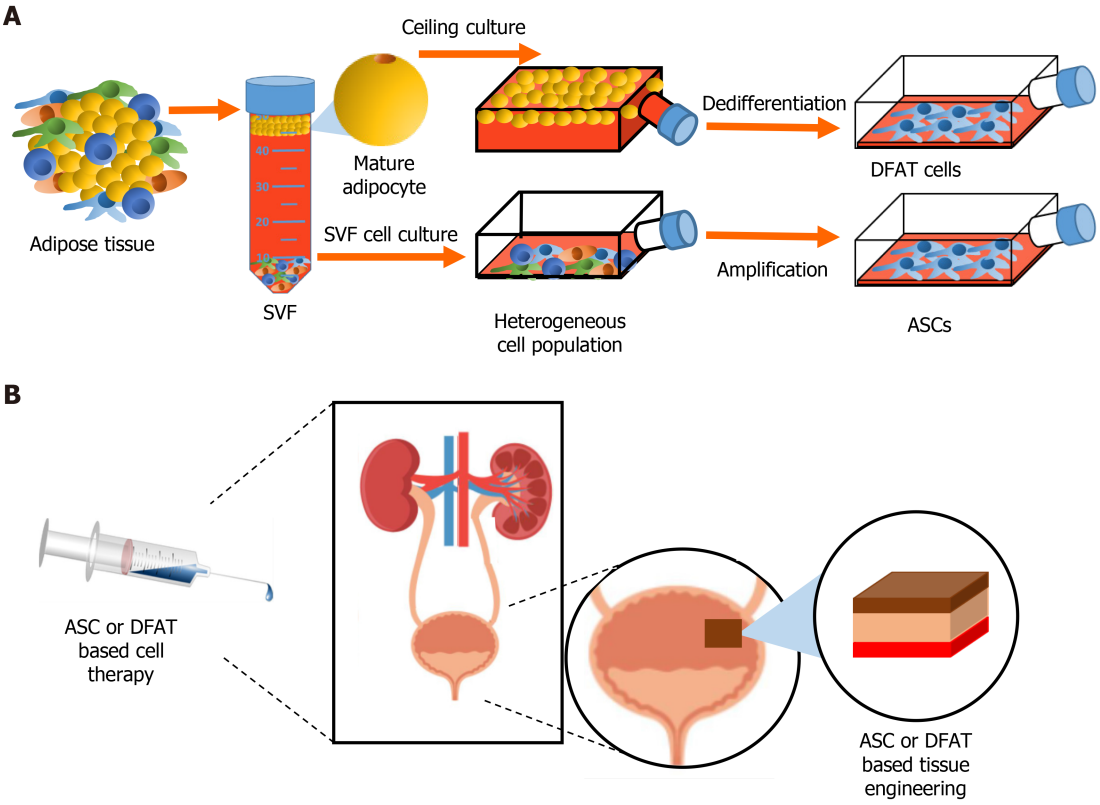

Figure 3 Stem cells from adipose tissue in regenerative medicine. A: Extraction of the stromal vascular fraction from adipose tissue and obtention of adipose-derived stromal/stem cells (ASCs) after cell culture. Ceiling culture of mature adipocytes and resulting populations of dedifferentiated fat (DFAT) cells; B: Potential uses of ASCs and/or DFAT cell-based therapies in genitourinary regenerative medicine. SVF: Stromal vascular fraction; ASC: Adipose-derived stromal/stem cells; DFAT: Dedifferentiated fat.

ASC-based therapies and tissue engineering approaches for treating urogenital-related diseases/pathologies

In the last five years (2016-2021), different ASC-based therapeutic approaches have been developed to treat genitourinary-related pathologies. Recent cell therapies based on ASCs injections for treating kidney, ureter, bladder and urethral related pathologies in different animal models are presented (Table 2), as well as the latest tissue engineering efforts using ASCs to reconstruct 3D urologic tissues (Table 3).

ASC-based therapies in renal diseases: Various ASC-based therapies have been developed in the last five years to treat different aspects of CKD due to their therapeutic properties. Fibrosis is a late manifestation of CKD that is often irreversible. Therefore, numerous teams are focusing their efforts on using ASCs for the treatment of Renal Interstitial Fibrosis (RIF) (Table 2). Among recent studies, Song *et al*[140] described the anti-inflammatory effects of rat ASCs in RIF rat models, where lower gene expression of TNF-α and IL-1 were observed in treated rats compared to control [140]. Moreover, ASCs also mediated partial inhibition the TGF-β1 signaling axis, therefore significantly suppressing the epithelial-mesenchymal transition (EMT) of tubular epithelial cells involved in fibrosis[140]. Rivera-Valdes *et al*[141] also showed the potential of human ASCs in reducing RIF in rats[141]. Treated groups showed significantly reduced gene expression of *COL1A1* and *ACTA2*, leading to 89% less collagen deposition and to a 40% reduction of fibrosis as assessed by a morphometric analysis of microphotographs of Masson trichrome and Sirius red stained tissues when compared to control groups[141].

Fibrosis typically results from chronic inflammation. The anti-inflammatory effects of MSCs have long been known as an important tool for treating fibrotic tissue phenotypes[142]. Recent research suggests that production of glial-derived neurotrophic factor (GDNF) by human ASCs can provide renoprotective effects in treated RIF mouse models[143]. Indeed, Wang *et al*[143] recently showed that compared to wild-type human ASC-based therapy, ASCs genetically modified to overexpress GDNF (GDNF-ASCs) enhanced tissue repair by increasing macrophage transition from an M1 inflammatory phenotype to an M2 reparative phenotype in mice

Table 2 Adipose-derived stromal/stem cells-based preclinical studies for treating urogenital related diseases/pathologies (2016-2021)

Year	Organ/tissue	Disease/method	Animal model	Type of therapy (cell type/host anatomic site)	Cell/molecule concentration	Outcomes	Ref.
2021	Kidney	Renal interstitial fibrosis/unilateral urethral obstruction	Nu/nu mice 6–8 week-old males, *n* = 40	Injection of genetically modified SC human GDNF-ASCs and non-modified ASCs/intravenous	5×10^5 cells in 150 µL of saline	Improvement of vascular rarefaction/Renal protection against microvascular injuries/Oxidative stress reduction	Li *et al*[144], 2021
2020		Kidney injury/ischemia-reperfusion	Wistar rats 100-200 g males, *n* = 28	Injection of SC rat ASCs/tail vein	2×10^6 cells in 1 mL of PBS	Reduction of total tissue damage and urine mineral concentration/ASC anti-inflammatory effects	Changizi-Ashtiyani *et al*[153], 2020
2020		Kidney injury/ischemia-reperfusion	SD rats 8–12 week-old males, *n* = N/S	Injection of epididymal rat ASCs/left kidney	2×10^6 cells in 100 µL of decellularized kidney ECMH	Epithelial differentiation of post transplanted ASCs/accelerated repair of renal tubular injury *via* ASC pro-angiogenic molecules	Zhou *et al*[152], 2020
2020		Sepsis-induced AKI/cecal ligation and puncture	C57/BL6 mice 6–8 week-old males, *n* = 140	Injection of SC human ASCs-derived exosomes/tail vein injection	100 µg of exosomes in 200 µL of vehicle solution	Exosome protective functions against AKI/apoptosis and inflammation reduction *via* Sirtuin-1 pathway regulation	Gao *et al*[149], 2020
2019		Renal interstitial fibrosis/unilateral ureteral obstruction	Nude mice, *n* = 12	Injection of SC human GDNF-ASCs/tail vein	5×10^5 cells in 150 µL of vehicle solution	Macrophage transition from inflammatory (M1) to reparative (M2) phenotype/reduction of renal fibrosis and inflammation	Wang *et al*[143], 2019
2019		Diabetic nephropathy/induced diabetes	C57BL/KsJ db/db mice 8 week-old males, *n* = 20	Injection of SC murine ASCs-derived exosomes/tail vein	N/S	Attenuation of spontaneous diabetes and nephropathy by reduced proteins levels in the urine of treated mice	Jin *et al*[148], 2019
2017		Renal interstitial fibrosis/unilateral ureteral obstruction	Wistar rats 6 week-old males, *n* = 45	Injection of epididymal rat ASCs/tail vein	5×10^6 cells in 1 mL of vehicle solution	Significantly reduced EMT and inflammatory response *via* TGF-β1 signaling pathway inhibition in treated rats	Song *et al*[140], 2017
2017		Chronic kidney injury/adenine intoxication	Wistar rats 250 g males, *n* = 12	Injection of SC human ASCs/tail vein	2×10^6 cells in vehicle solution	Reduction of kidney fibrosis/improved creatine and urea in serum/significantly lower expression of profibrogenic genes in treated rats	Rivera-Valdes *et al*[141], 2017
2017		Acute kidney injury/ischemia-reperfusion	SD rats 220-250 g males, *n* = 32	Injection of perinephric human ASCs or SVF/intra-parenchymal	2×10^6 cells in 100 µL of PBS	SVF and ASCs equally improved renal injury by promoting cell proliferation and decreasing tubular injury and cell apoptosis	Zhou *et al*[146], 2017
2016		Acute kidney injury/ischemia-reperfusion	SD rats 250-300 g males, *n* = 72	Injection of rat ASCs/tail vein	1×10^6 cells in vehicle solution	Significantly lower kidney injury scores at days 1 and 3 post-treatment/not significant improvement at day 7 post-treatment	Sheashaa *et al*[145], 2016
2016		Acute kidney injury/IRI	SD rats 320-350 g males, *n* = 40	Injection of epididymal rat ASCs and ASCs-derived exosomes/intravenous	1.2×10^6 cells + 100 µg of ASCs-derived exosomes	Combined ASCs and exosomes confer higher kidney protection towards IRI than either one alone	Lin *et al*[147], 2016
2016		Chronic kidney disease/already present	Cats (various sex, age and breeds), *n* = 8	Injection of allogenic cryopreserved feline ASCs/cephalic vein	2×10^6 cells *per* kg in vehicle solution	No significant improvement of renal functions between treated and control groups/not adverse side effects noticed using allogenic ASCs	Quimby *et al*[150], 2016
2018	Urethra	Stress Urinary Incontinence/pudendal nerve	SD rats adult females, *n* = 48	Injection of exosomes derived from SC human ASCs/peripheral urethral	50 µg of exosomes in 50 µL of saline	Increased bladder capacity and leak point pressure/higher muscle fiber and nerve fiber regeneration	Ni *et al*[171], 2018

	transection					
2018	Stress Urinary Incontinence/pudendal nerve transaction	SD rats 6-7 week-old females, *n* = 144	Injection of inguinal rat ASCs/transurethral sphincter	1×10^6 cells in 400 μL of D-Hanks's solution	ASCs in vivo viability 60 d post-implantation/higher content of striated muscle in the urethra/higher values of leak point pressure	Cui *et al*[170], 2018
2018	Urethral stricture/N/S	SD rats, N/S	Injection of miR-21 modified SC human ASCs/ urethral wall	1×10^6 cells in 100 μL of saline	miR-27 cells increased epithelium and smooth muscle layer formation compared to normal ASCs/improve the epithelial wound healing microenvironment	Feng *et al*[168], 2018
2016	Urethral fibrosis/TGF-β1 induced model	SD rats 300 g males, *n* = 18	Injection of inguinal rat ASCs/urethra	2×10^5 cells in 50 μL of saline	Significantly decreased fibrosis evaluated by reduced collagen type I and III expression	Sangkum *et al* [167], 2016
2016	Urethral stricture/induced by TGF-β1 and surgical incision	SD rats 300-350 g males, *n* = 36	Injection of SC human ASCs/urethral wall	1×10^6 cells in 100 μL of PBS	Increased bladder capacity (50%)/wider urethral lumen/decreased expression of fibrosis-related genes	Castiglione *et al* [166], 2016

AKI: Acute kidney injury; ASCs: Adipose-derived stromal/stem cells; db/db: Spontaneous diabetes; ECMH: Extracellular matrix hydrogel; EMT: Epithelial mesenchymal transition; g: grams; GDNF: Glial-derived neurotrophic factor; IRI: ischemia-reperfusion injury; N/S: Not specified; PBS: Phosphate-buffered saline; SC: Subcutaneous; SD: Sprague-Dawley; SVF: Stromal vascular fraction; TGF-β1: Transforming growth factor-β1.

[143]. More recently, Li *et al*[144] showed in 2021 that interstitial fibrosis mice treated with GDNF-ASC exhibit reduced capillary rarefaction, significantly reducing oxidative stress, leading to renal protection to microvascular injuries (EMT inhibition *via* PI3K/AKT signaling pathway), and renal fibrosis in treated mice[144]. This suggests that GDNF upregulation could be a future candidate for improving CKD-related fibrosis.

In the last years, different teams have developed ASC-based treatments for Ischemia-Reperfusion-Injury (IRI) animal models of AKI. In 2016, Sheashaa *et al*[145] showed in a IRI rat model that rat ASC-based systemic therapy significantly reduced creatinine levels in serum by increasing creatinine clearance for seven days after treatment. In the first three days, lower injury scores were also observed in treated rats compared to controls[145]. However, this study stipulates that injury scores did not improve further after one week of therapy and creatinine levels were not followed beyond seven days of treatment.

A study by Zhou *et al*[146] showed that injection of human SVF and cultured ASCs significantly reduced proinflammatory and immunomodulatory cytokine production (TNF-α and IL-10) in treated IRI rats. In this study, treated animals showed increased densities of peritubular kidney capillaries, with similar improvements achieved by SVF and ASCs[146]. Using a different approach, Lin *et al*[147] tested a combination of rat ASCs and ASC-derived exosomes (Ex-ASC) in IRI rat models. Their results showed that rats treated with this combined therapy showed a significant improvement of AKI features compared to ASCs and exosome only treated groups[147]. Exosomes therapeutic mechanisms of action are still not well understood. However, recent studies involving a murine model of diabetic nephropathy suggest that miR-486, carried in murine ASC-derived exosomes, downregulated mTOR activation-mediated autophagy in podocytes of treated mice[148]. This translated into a decreased

Table 3 Preclinical studies of urogenital related pathologies/disease using adipose-derived stromal/stem cells in tissue engineering (2016-2020)

Year	Organ/tissue	Approach	Animal model	Substitute implantation (cell type and scaffold/host anatomic site)	Cell concentration *per* scaffold	Outcomes	Ref.
2020	Kidney	Diabetic nephropathy/unilateral nephrectomy	SDT fatty rats 5-week-old males, *n* = 21	SC rat ASCs three-layer sheets/renal capsule transplantation	1×10^6 cells in 35-mm culture dish/sheet	14-d survival of transplanted sheets/significantly lower urinary TNF-α levels/maintained renal tubular structure in treated rats	Takemura *et al* [155], 2020
2018		Kidney reconstruction	Wistar rats 6-8 week-old males	Inguinal rat ASCs seeded onto a rat decellularized kidney/no implantation	1×10^7 cells in 2 ml of culture medium *per* decellularized kidney	ASCs differentiated into endothelial and tubular cells after 5 d of culture/few cells attached to the scaffold after 10 d	Xue *et al*[154], 2018
2016	Ureter	Artificial ureter injury/surgical excision	New Zealand white rabbits3.5 kg females, *n* = 20	Smooth muscle like-cells from SC rabbit ASCs seeded onto ventral aorta/decellularized matrix/graft placed over ureter defect	N/S	Seeded ASCs showed urothelial and smooth muscle-like cells phenotype in the ureter substitute 8 wk after implantation	Zhao *et al*[165], 2016
2020	Bladder	Complete bladder removal/surgical excision	SD rats 300 g adult females, *n* = 9	SFP human ASCs seeded onto a decellularized rat bladder matrix/bladder transplantation	1×10^6 cells in 500 μL of cells suspension/bladder scaffold	Acquisition of a smooth muscle-like phenotype of seeded ASCs seeded/ASC paracrine effect increased vascularization and innervation	Moreno-Manzano *et al* [163], 2020
2020		Sub-totally resected urinary bladder/upper two-thirds bladder excision	Athymic rats 200 g adult females, *n* = 9	Smooth muscle-like cells from SC human ASCs seeded onto 3-layer PLGA sheet/bladder graft anastomosis	1×10^6 cells mixed with 500 μL of human plasma/scaffold	Complete bladder regeneration and functionality restoration/fusion of smooth muscle-like cells in the regenerated muscular layer	Salem *et al* [162], 2020
2019		Bladder injury/surgical incision(1 cm)	SD rats 6-week-old females, *n* = 48	Inguinal rat ASCs cells and PGA combined sheets/bladder patch anastomose	1×10^5 cells/cm^2 *per* sheet reconstruction	Patches promote urothelium, smooth muscle, neural and blood vessel regeneration/restored bladder function	Wang *et al* [151], 2019
2018		Bladder augmentation/cystotomy incision (1 cm)	SD rats 8 week-old males, *n* = 34	Inguinal rat ASCs seeded onto PCL-Chitosan scaffold/bladder substitute anastomose	15×10^7 cells/mL *per* scaffold	Higher smooth-muscle regeneration from ASCs/larger bladder capacity/increased angiogenesis	Zhou *et al*[161], 2018
2017		Bladder augmentation/surgical incision(1 cm)	SD rats 8 week-old females, *n* = 46	SC rat ASCs seeded onto an AM-SF scaffold/bladder substitute anastomose	10×10^7 cells/mL in 40 μL of saline *per* scaffold	Bladder capacity augmentation (30%)/relatively normal micturition pattern/ASC viability after 12 wk of implantation	Wang *et al* [157], 2017
2017		Bladder augmentation/surgical incision (1 cm)	SD rats 8 week-old males, *n* = 30	Inguinal rat ASCs seeded onto a BAMG-SF scaffold/bladder substitute anastomosis	50 μL of cell suspension at 1×10^8 cells/mL *per* scaffold	Higher bladder capacity (2.3-fold)/Enhanced VEGF angiogenic potential by ERK ½ phosphorylation	Xiao *et al*[160], 2017
2017		Augmentation cystoplasty/surgical incision (1 cm)	SD rats 8 week-old males, *n* = 30	Rat ASCs encapsulated in an ADA/GEL seeded onto a porcine BAMG/bladder substitute anastomosis	100 μL of encapsulated cells at a 1×10^6/mL concentration *per* scaffold	Morphological bladder restoration by enhanced scaffold degradation/enhanced VEGF-mediated angiogenesis and smooth muscle regeneration in treated rats	Xiao *et al*[210], 2017
2016		Bladder augmentation/surgical incision (1 cm)	SD rats immunocompetent 36 week-old males, *n* = 30	Inguinal rat ASCs seeded onto a porcine BAMG/Bladder substitute anastomosis	15×10^7/mL cell suspension *per* scaffold	Greater bladder capacity in experimental group/equal urothelial regeneration in the treated and non-treated groups at 4- and 14-wk post-implantation	Zhe *et al*[156], 2016
2016		Partial cystectomy/half upper	Beagle dogs 10-12 Kg	Human ASCs seeded onto a whole	1×10^5 cells *per* cm^2 of each	Complete urothelial coverage of seeded and	Hou *et al*[159],

		bladder transection	males, n = 12	porcine BAMG/scaffold grafted onto bladders' dome	scaffold	unseeded bladder after 6 mo/higher capillary density and smooth muscle organization in treated dogs' bladder	2016
2020	Urethra	Urethral injury/surgically induced	New Zealand white rabbits 9-week-old males, n = 24	SC rabbit ASCs seeded onto a human DAM scaffold/urethral graft	1×10^6 cells *per* scaffold	Higher number of urethras healed following seeding of ASCs onto DAM	Hariastawa *et al* [172], 2020
2020		Urethral injury/surgically induced (2 cm × 0.6 cm)	New Zealand white rabbits males, n = 15	Inguinal rabbit ASCs seeded in a nanofibrous scaffold/graft placed over urethral defect	1×10^7 cells *per* scaffold	Hypoxia preconditioning of ASCs increased urethral lumen diameter/preserved morphology/enhanced angiogenesis	Wan *et al*[173], 2020

ADA-GEL: Alginate dialdehyde-gelatin; AM-SF: Autologous myofibroblast-silk fibroin; ASCs: Adipose-derived stromal/stem cells; BAMG-SF: Bladder acellular matrix graft-silk fibroin; bFGF: Basic fibroblast growth factor; DAM: Dried amniotic membrane; g: gram; Kg: Kilogram; N/S: Not specified PCL: Polycaprolactone; PGA: Polyglycolic acid; PLGA: Poly(lactid-*co*-glycolic acid); SC: Subcutaneous; SD: Sprague-Dawley; SDT: Spontaneously diabetic Torii; SFP: Suprapatellar fat pad; TNF-α: Tumor necrosis factor-α; VEGF: Vascular endothelial growth factor.

podocyte autophagy disfunction-mediated injury, reducing tissue damage and improving renal function[148]. Findings in sepsis-derived AKI mice models also suggest that ASC-derived exosomes contribute to reduced renal damage by inhibiting apoptotic responses *via* Sirtuin-1 (SIRT1) pathway activation, reducing the inflammatory reaction[149]. However, similar to most studies available, rather short-term effects of ASC treatments have been described, and further investigations are needed to better understand and establish if long-term therapeutic outcomes can be achieved.

The study by Quimby *et al*[150] in 2016 evaluated longer term effects of ASCs in a CKD feline model by performing three allogenic injections of cat ASCs at two, four, and six weeks. Although this study highlighted the safety of repeated allogenic ASC injections, treated cats showed no improvement compared to controls (n = 4 *per* group) [150]. However, important parameters must be taken into consideration, such as the cat's sex, age, and breed, which were considerably different between the experimental groups. In addition, the cells used for each of the three injections were obtained from different cat donors. These conditions complicate the analyses and conclusions that can be drawn.

ASC's delivery vehicle is an essential component of the SCs therapies since it can act on cell viability and therapeutic potential by improving cell retention and reducing cell stress[151]. Embedding cells into an acellular matrix can have such protective effects. Indeed, the use of a decellularized matrix hydrogel (DMH) was shown to improve ASC local delivery and survival in a IRI rat model. After 30 d of ASC-DMH injection into the kidney, tubular epithelial-like cells differentiation and viability of ASCs were observed in the kidney of treated mice[152]. This suggests that the use of decellularized matrix may induced ASC differentiation into an epithelial-like phenotype, promotes growth factor secretion and improved ASCs long term viability in treated rats after IRI. In 2020, Changizi-Ashtiyani *et al*[153], showed the impact of rat ASCs injected before inducing IRI induced CKD. Results revealed the renoprotective effects of ASC-based therapy in a IRI rat model. Compared to non-treated animals, treated

rats showed 2.5 times lower urea levels in the blood, higher urine osmolarity, significantly reduced oxidative stress levels and higher protection of kidney tissue 48 h after treatment[153].

As mentioned before, organ transplantation is one of the most common therapeutic approaches for advanced CKD. Therefore, many researchers, in addition to developing ASC-related cell therapies, have also undertaken the development of tissue engineering approaches using ASCs as the primary therapeutic component. Selected studies are described in Table 3. For example, Xue *et al*[154] recently developed a reconstructed rat kidney model by seeding rat ASCs in a decellularized rat whole kidney. The authors concluded that ASCs underwent differentiation towards endothelial-like and tubular cells with high cellular adherence to the scaffold induced by the action of stromal cell-derived factor 1 (SDF-1) and the cell-scaffold interactions [154]. Therefore, even if *in vivo* implantation was not performed in this study, it suggests ASCs can be beneficial when used in recellularization approaches for future clinical applications[154]. Although complete kidney reconstruction seems like an ideal goal, research is still far from the development of an entire functional kidney to be used in clinical trials. However, different tissue engineering approaches could still contribute to kidney disease improvement. Takemura *et al*[155] recently showed that grafting reconstructed sheets produced from rat ASCs significantly lowered the secretion of proinflammatory molecules, reduced renal tubules atrophy, and contributed to the maintenance of typical renal tubular structures in diabetic nephropathy treated mice[155]. However, ASC survival was only followed and observed for 14 d after implantation of the cell sheets in treated mice. Additional follow-up studies showing are thus needed to understand better the therapeutic potential of ASCs reconstructed sheets.

ASC-based tissue engineering approaches targeting bladder disorders: Many studies aim to achieve bladder reconstruction as a clinical alternative for treating different bladder-related diseases. Either complete or partial bladder reconstruction with MSCs have been suggested as promising approaches using bladder augmentation different animal models[52]. Among those studies, selected research from last five years using ASC-based tissue engineering approaches are shown in Table 3.

Zhe *et al*[156] used a porcine BAM scaffold seeded with undifferentiated rat ASCs, using an incubation period in the peritoneal cavity in a rat bladder augmentation model. This study showed that 14 wk postoperatively, treated rats displayed significant signs of improved regeneration of bladder SMCs, nerve cells, as well as increased bladder capacity compared to the BAM only controls[156]. Of note, seeded ASCs were not detected in the graft after 14 wk post-implantation. Increasing ASCs long-term viability and maintenance still represent a challenge for bladder engineering.

BAM graft is commonly used as a scaffold for achieving bladder tissue reconstruction, but new scaffolds have been tested lately. For example, Wang *et al*[157] in 2017 showed a novel autologous myofibroblast (AM)-silk fibroin (SF) scaffold obtained by incubating pig BAM treated with a SF solution[157]. This scaffold was implanted subcutaneously in the back of female SD rats. Their results showed that using an AM-SF scaffold contributed to bladder augmentation in rats with ASC viability still detected 12 wk after implantation[157]. Similar results were also obtained by this team using a pig BAMG-SF for bladder reconstruction in rats[158].

A recent preclinical study in larger animals, namely a dog model (n = 13) evaluated the therapeutic properties of human ASCs for bladder reconstruction when seeded in a pig BAM scaffold[159]. This study showed significantly increased bladder volume and compliance, in addition to an increased bladder regeneration by smooth muscle differentiation and improved vascularization in treated dogs six months after implantation compared to BAM only controls[159]. Different biomaterials and seeding methods have been developed in recent years, such as encapsulation of rat ASCs in an alginate dialdehyde gelatin (ADA/Gel) hydrogel[160] or polycaprolactone/chitosan scaffolds [161] promoting bladder regeneration in rats with a contribution of ASCs cells to differentiation into smooth muscle-like cells in vivo, as shown in previous studies.

The more recent work of Salem *et al*[162] highlighted the potential of human ASCs when the latter were predifferentiated into SMCs and seeded in a triple layered poly(lactid-co-glycolic acid) (PLGA) sheet for bladder reconstruction[162]. This study showed that short-term results (two weeks) of the implanted grafts in athymic rats were similar among ASC-PLGA and PLGA only treated groups. However, in the follow-up analyses after 12 wk, only the ASC-PLGA treated group showed regeneration within the main bladder layers (mucosal, stratified urothelium, submucosal and muscular layer) with a significantly restoration of bladder functions

[162]. The study by Moreno-Manzano *et al*[163] showed similar results of bladder regeneration in a model of partial cystectomy in rats after treatment with a BAM seeded with undifferentiated human ASCs[163]. Taken together, these studies support a promising use of ASCs, warranting further research on their mechanisms of action, before future clinical trials are designed.

ASC-based cellular therapies and tissue engineering for ureteral and urethral diseases: The ureters and the urethra are fibromuscular tubes that can be affected by physiological defects such as ureteral and urethral fibrosis, urethral stricture (US), among others, that can compromise these organs' optimal function, justifying the need for new therapeutic strategies[164].

To our knowledge, cell therapy by ASC injection has not been performed yet for ureters. However, tissue reconstruction has been achieved (Table 3). For example, in 2016, Zhao *et al*[165] showed that rabbit ASCs seeded onto decellularized matrix obtained from rabbit ventral aorta and cultured *in vitro* for six weeks produced a well-structured ureter eight weeks post-transplantation in a rabbit[165]. This study suggested a five-step strategy for ureteral tissue engineering such as ASCs culture, urothelium and smooth muscle phenotype induction, sandwich co-culture of the vessel extracellular matrix scaffold and the differentiated cells followed by ureteral maturation within the omentum before transplantation[165].

For urethral therapies, ASCs have been used to treat US and fibrosis in the last years (Table 2). First, in 2016 Castiglione *et al*[166] showed that the injection of human ASCs directly in the urethral wall of stricture in rats resulted in an increased bladder capacity (50%) with a wider urethral lumen in addition to decreased expression of fibrosis-related genes compared to the non-treated control[166]. In the same year, Sangkum *et al*[167] showed in a rat model of urethral fibrosis that injection of rat ASCs directly onto the urethra of treated animals significantly decreased submucosal fibrosis and collagen type I and III protein production[167]. Taken together, these studies suggest that ASCs injection could represent a potential treatment for preventing scar formation in US disease. Recent studies aimed at increasing the therapeutic potential of ASCs. For example, Feng *et al*[168] used genetically modified miR-21 human ASCs for treating US in rat models[168]. This study showed that miR-21-ASCs significantly increased proangiogenic gene expression, such as hypoxia inducible factor-α, VEGF, bFGF, HGF-1, stem cell factor and SDF-1. Furthermore, miR-21-ASCs therapy also improved the epithelial wound healing microenvironment, smooth muscle layer formation and enhanced SC survival compared to normal ASC-treated rats[168]. This work thus suggests a new approach for enhancing urethral repair for future urethroplasty interventions.

Urinary incontinence is also a common medical condition and is related to a lowered basal LPP below 60 cm H_2O or a maximal urethral closure pressure below 20 cm H_2O[169]. Recent studies using ASC injections have been developed using a urinary incontinence rat model to better understand the therapeutic effects of ASCs. First in 2018, Cui *et al*[170] showed that injection of rat ASCs was associated with higher content of striated muscle in the urethra and higher values of LPP compared to non-treated rats[170]. ASCs survival was also detected 60 d post-implantation. Later that same year, Ni *et al*[171], using a different approach, studied the therapeutic potential of human ASC-derived exosomes[171]. Their study showed an enhanced proliferation of skeletal muscle and Schwann cell lines *in vitro* upon exosome exposure. When injected *in vivo*, a higher bladder capacity and LPP were observed, associated with enhanced muscle fibers and peripheral nerve fibers regeneration in the urethra of the exosome-treated rats, eight weeks after injection. These results suggest that local injection of exosomes derived from human ASCs can improve functional and histological recovery of the urethra of incontinent rats[171].

Finally, ASC-based tissue engineering approaches have also been developed for urethral reconstruction (Table 3). Hariastawa *et al*[172] study showed that rabbit ASCs seeded onto a human dried amniotic membrane (DAM) increased urethral healing in a surgically induced urethral injury rabbit[172]. This study showed that 28 d after implantation, rabbits treated with ASC-DAM exhibited less features of fibrosis with decreased fistula presence in the healed tissue compared to DAM only treated and untreated groups, suggesting this novel ASC-DAM seeded scaffold as a potential graft for urethral reconstruction. Also in 2020, Wan *et al*[173] tested a nanofibrous blend scaffold (PLLA/PCL/PLGA) seeded with rat ASCs preconditioned by hypoxia for urethral reconstruction in an induced urethral injury rabbit model[173].

This study showed that hypoxia preconditioning of ASCs, combined with the nanofibrous scaffold, led to larger urethral lumen diameter, preserved urethral morphology, and increased angiogenesis by enhanced VEGF secretion compared to

the normoxia ASCs treated group. This study showed that hypoxia preconditioning of ASCs mediated an upregulation of angiogenesis in comparison to the use of non-preconditioned ASCs seeded into the scaffold.

DFAT cells for treatment of urogenital diseases

An increasing number of studies have characterized DFAT cell properties to gain insights into the biology of this unique cell population[174]. Since their discovery, only a few preclinical studies have been performed using DFAT cells as therapeutics, with a handful having investigated their use for urogenital-related conditions (Table 4).

In 2009, Sakuma *et al*[175] reported a study based on the use of murine DFAT cells to promote smooth muscle-like differentiation in a mouse bladder injury model[175]. Their work showed a significant contribution of DFAT cells to bladder tissue regeneration, as assessed 30 d after cell transplantation. Treated mice had almost twice higher levels of α-smooth muscle actin (α-SMA) expressing cells in the injured areas compared to untreated controls, indicating favorable wound healing by reducing scar tissue with smooth muscle like cells[175]. Then in 2011, a study performed by Obinata *et al*[176] showed the contribution of undifferentiated rat DFAT cells for the treatment of urethral sphincter atrophy caused by vaginal distention[176]. The study showed two groups of Sprague-Dawley rats that underwent vaginal distention causing urethral injury with reduced LPP of the urethral sphincter. DFAT cell transplantation resulted in a significant improvement of LPP (DFAT group: 37.3 ± 6.4 *vs* control group: 21.7 ± 5.7 mmHg, $P < 0.01$). Immunohistochemistry quantification revealed that the striated muscle thickness as well as α-SMA positive areas were significantly increased in the DFAT injected group than in the control group[176]. Therefore, this study suggests that undifferentiated DFAT cells can differentiate *in vivo* after injection to help rebuild damaged smooth muscle tissue. DFAT cell therapy was then evaluated in 2015 for its potential to improve glomerulonephritis related disease in Wistar rats with immunological and non-immunological induced renal injury[177]. This study showed that systemic tail vein injection of rat DFAT cells generally led to high numbers of cells trapped in the lungs, DFAT cells significantly reduced proteinuria ($P < 0.01$), as well as interstitial fibrosis, in association with decreased expression of kidney-injury molecule 1 in the non-immunological renal injury model. However, DFAT cell-based therapy did not show significant renal improvement in the non-immunological induced renal injury model[177]. The authors suggested that DFAT cells trapped in the lungs might secrete anti-inflammatory and/or immunosuppressive substances that contribute to the renal injury healing process.

Finally, DFAT cells were used by Ikado *et al*[178] in 2016 in their study investigating vesicoureteral reflux in rats that underwent urethral clamping and placement of cystostomy followed by intravesical pressurization[178]. In this study, undifferentiated rat DFAT cells were transplanted in treated rats, resulting in significantly lower vesicoureteral reflux grade and reduced hydronephrosis leading to lower renal scaring during the healing process[178]. This work suggested that DFAT cells expression of TGF-β1 and tissue inhibitors of metalloproteinases, contributed to extracellular matrix production and stabilization in the scar tissue. Interesting advantages have been observed so far using DFAT cell-based therapy for treating urogenital-related pathologies. However, more extensive studies are needed to evaluate DFAT cell's mechanisms of action and long-term outcomes.

THE SELF-ASSEMBLY APPROACH FOR GENITOURINARY TISSUE ENGINEERING

As we have seen, tissue engineering strategies rely on scaffolds such as synthetic or natural biomaterials, including decellularized tissues[179]. Studies indicate that cellularized structures have better potential than acellular ones[44]. Furthermore, obtention of a better long-term outcome highly depends on preserving the pool of SCs [180,181]. As they naturally reside in privileged locations called niches[182], SC conservation depends on the biomaterial's capacity to recreate this unique SC-type specific structure. Twenty years ago, Dr. François A. Auger and his team at the LOEX research center developed a unique and innovative strategy used since then to reconstruct a wide variety of tissues, including urologic tissues. This technique is called the "self-assembly" approach using tissue engineering[183]. It relies on mesenchymal cells' ability to secrete and assemble their own ECM when cultivated long-term in the presence of serum and ascorbate, a cofactor of lysyl- and prolyl-hydroxylase, enzymes involved in the process of maturation of collagen fibers[184,185]. Because cells produce

Table 4 Preclinical studies for treatment of urogenital related diseases using dedifferentiated fat cells

Year	Disease/injury	Animal model	Type of therapy (cell type/injection site)	Cell concentration	Outcomes	Ref.
2016	VUR	SD rats 8 week-old females weighing 200 g, *n* = 10	Injection of undifferentiated rat DFAT cells/bilateral vesicoureteral junction	1 × 10^6 cells in 30 μL of saline	Significant amelioration of VUR in treated rats/nephroprotective effects in rats	Ikado *et al* [178], 2016
2015	Immunologically induced glomerulonephritis and adriamycin induced nephropathy	Wistar rats, males weighing 250 g, *n* = 64	Injection of undifferentiated rat DFAT cells/RA or TV	1 × 10^6 cells in 20 μL of saline	TV DFAT cell injection showed lower proteinuria and renal degeneration than direct cell implantation/DFAT immunosuppressive effects significantly reduced glomerulonephritis in treated rats	Maruyama *et al* [177], 2015
2011	Urethral sphincter injury by VD	SD rats 8 week-old females, *n* = 16	Injection of undifferentiated rat DFAT cells/paraurethral connective tissue at mid-urethra	1 × 10^6 cells in 20 μL of saline	Sphincter muscle regeneration by DFAT cell therapy/improvement of "lowered leak point"	Obinata *et al* [176], 2011
2009	Cryo-injured bladder wall (2 mm diameter)	C57BL/6 mice 8-9 week-old males, *n* = 10	Injection of smooth muscle-like cells differentiated from human DFAT cells/bladder wall	1 × 10^6 cells in 20 μL of Hanks' balanced solution	DFAT differentiation potential into smooth muscle-like cells/approximately 2-fold higher αSMA expressing cells in scar tissue 30 d post-injection in treated mice	Sakuma *et al* [175], 2009

αSMA: alpha smooth muscle actin; DFAT: Dedifferentiated fat; RA: Renal artery; SD: Sprague-Dawley; TV: Tail vein; VD: Vaginal distension; VUR: Vesicoureteral reflux.

their own microenvironment, the physicochemical conditions allowing SCs to be preserved are thus recreated. This technique was used in the clinic to produce reconstructed skin for patients with severe burns[186] and those suffering from chronic ulcers[187]. In this context, preservation of epithelial SCs was demonstrated for this organ undergoing monthly cell renewal[188].

The self-assembly approach has then been similarly applied to urologic tissues. Porcine, then human urethra substitutes have been produced[189], matured[190] then grafted subcutaneously into mice[191]. Bladder substitutes have also been reconstructed[192,193] and further matured using bioreactors[194]. The need for organ-specific stroma has been demonstrated in this model by obtaining a better epithelial differentiation after replacing skin fibroblasts with bladder mesenchymal cells[195]. In these substitutes, the urothelium presented characteristics close to native tissue as evidenced by functional tests (permeability of urea using Franz's diffusion cells).

Furthermore, electron microscopy indicated the maturation of uroplakin plaques and the presence of discoid and fusiform vesicles near the plasma membranes, and immunostaining of uroplakins, tight junction proteins, and various markers such as keratins. These reconstructed tissues were used as models to study several pathologies such as bladder cancer[196], ketamine-induced cystitis[197] or the effect of metallic stent use[198]. Soon after, the preservation of epithelial SCs (*i.e.* urothelial cell progenitors) was studied, not only during the expansion of cells before reconstruction but during the engineering phase itself, and showed significant improvement under hypoxic conditions[199].

The use of ASCs for urological tissue reconstruction was also evaluated to further improve the substitutes reconstructed by the self-assembly technique. This was performed with the final goal of increasing the potential of graft take through enhanced angiogenesis and limiting surgery-induced inflammation. In this study, different combinations of reconstructed stroma were evaluated[200]. The use of skin fibroblast and ASC sheets gave compelling results with improved mechanical properties, mainly required for surgical manipulation. These features need to be confirmed by performing *in vivo* experiments. Also, the combination of bladder mesenchymal cells and ASC remains to be evaluated. Self-assembly protocols based on ASCs also lead to the production of tubular structures[201], supporting their future use for urethra or ureter engineering using this approach. The self-assembly approach is thus very promising based on these results showing that reconstructed tissues feature structural and functional properties closely resembling those of native tissues and support long-term preservation of SC pool required for positive graft outcomes.

CONSIDERATIONS ON THE USE OF SC FROM VARIOUS ORIGINS

As detailed above, promising studies have been performed using SCs to provide therapeutic solutions to urologists, with the goal of reducing side effects for the patients. It is expected that improvements in the knowledge and use of SCs to treat kidney, bladder or ureters/urethra pathologies will continue to be increasingly evaluated and published soon. The risks associated with SC-based therapies, however, should not be minimized. Most preclinical studies have been performed at a laboratory scale, with few animals and SCs extracted and amplified under conditions that will have to be adjusted to cGMP guidelines to respect safety and regulatory issues. Translating the preclinical findings to routine use in urology, with thousands or even millions of patients over the years, will necessitate collaborative efforts in designing the most appropriate clinical trials based on reliable and trusted SC sources. Indeed, depending on their source and their degree of potency, SCs use can be associated to various degrees of risk. Researchers and clinicians must keep in mind that SCs can share properties associated with cancer cells, such as an enhanced ability to proliferate. This is best illustrated by the formation of teratomas after injection of ESCs or iPSCs into animals, for example, during tumorigenicity assays. It is essential to devise strict protocols and safety assays to ensure a tight regulation of SC fate after induction of differentiation towards the desired cell type and the elimination of residual pluripotent undifferentiated cells. The use of defined culture media and the engineering of a microenvironment (*e.g.*, ECM, cytokines) inducing the proper target cell differentiation will likely contribute to the success of increased cell safety and efficacy. However, in the near future, iPSCs could represent a great alternative as the potential of urothelial cells extracted from diseased bladders has been shown to be impacted[62].

When using postnatal SCs such as MSCs, their reduced multilineage potency is associated with an advantageous safety profile. However, many parameters must be taken into considerations including the choice of the culture media used for expansion, the number of cell passages required to obtain enough cells *per* patient, the method of implantation (systemic or local injection, tissue engineering strategies) that can all affect their therapeutic properties and secretory profile. Therefore, more regulated clinical trials need to be initiated, ideally as multicenter studies, to determine not only the safety but also the efficacy of a specific SC-based treatment for a specific indication. The notion that a single SC type, prepared and implanted is a specific fashion could treat a wide variety of unrelated diseases can lead to hasty conclusions on SCs potential and to unsafe clinical practices, such as stem cell tourism[202].

CONCLUSION

With the rapid progress of knowledge, technical breakthroughs, and reliable SC manufacturing, we can hope that the present century will be one of regenerative medicine and that SCs will play an essential role in the development of safe and efficient therapies available to a greater number of patients. There is no doubt that therapies using SCs necessitate close patient's follow-up with competent personnel. These new medications could allow prescription of treatment which better meet the patient's need with less side-effects.

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REVIEW

Age and genotype dependent erythropoietin protection in COVID-19

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Abstract

Erythropoietin (EPO) is the main mediator of erythropoiesis and an important tissue protective hormone that appears to mediate an ancestral neuroprotective innate immune response mechanism at an early age. When the young brain is threatened-prematurity, neonatal hyperbilirubinemia, malaria- EPO is hyper-secreted disproportionately to any concurrent anemic stimuli. Under eons of severe malarial selection pressure, neuroprotective EPO augmenting genetic determinants such as the various hemoglobinopathies, and the angiotensin converting enzyme (ACE) I/D polymorphism, have been positively selected. When malarial and other cerebral threats abate and the young child survives to adulthood, EPO subsides. Sustained high ACE and angiotensin II (Ang II) levels through the ACE D allele in adulthood may then become detrimental as witnessed by epidemiological studies. The ubiquitous renin angiotensin system (RAS) influences the α-klotho/fibroblast growth factor 23 (FGF23) circuitry, and both are interconnected with EPO. Here we propose that at a young age, EPO augmenting genetic determinants through ACE D allele elevated Ang II levels in some or HbE/beta thalassemia in others would increase EPO levels and shield against coronavirus disease 2019, akin to protection from malaria and dengue fever. Human evolution may use ACE2 as a "bait" for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) to gain cellular entry in order to trigger an ACE/ACE2 imbalance and stimulate EPO hypersecretion using tissue RAS, uncoupled from hemoglobin levels. In subjects without EPO augmenting genetic

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determinants at any age, ACE2 binding and internalization upon SARS-CoV-2 entry would trigger an ACE/ACE2 imbalance, and Ang II oversecretion leading to protective EPO stimulation. In children, low nasal ACE2 Levels would beneficially augment this imbalance, especially for those without protective genetic determinants. On the other hand, in predisposed adults with the ACE D allele, ACE/ACE2 imbalance, may lead to uncontrolled RAS overactivity and an Ang II induced proinflammatory state and immune dysregulation, with interleukin 6 (IL-6), plasminogen activator inhibitor, and FGF23 elevations. IL-6 induced EPO suppression, aggravated through co-morbidities such as hypertension, diabetes, obesity, and RAS pharmacological interventions may potentially lead to acute respiratory distress syndrome, cytokine storm and/or autoimmunity. HbE/beta thalassemia carriers would enjoy protection at any age as their EPO stimulation is uncoupled from the RAS system. The timely use of rhEPO, EPO analogs, acetylsalicylic acid, bioactive lipids, or FGF23 antagonists in genetically predisposed individuals may counteract those detrimental effects.

Key Words: Erythropoietin; Angiotensin converting enzyme; Angiotensin II; Hemoglobinopathy; Malaria; Coronavirus disease 2019; Fibroblast growth factor 23

Core Tip: Erythropoietin (EPO) appears to mediate an ancestral neuroprotective innate immune response mechanism mitigating tissue injury and pathogen invasion at an early age. Age-dependent but anemia-unrelated EPO elevation has been reported in conditions that threaten the young brain such as prematurity, incipient kernicterus, and malaria. Malaria protective genetic determinants such as the angiotensin converting enzyme (ACE) D allele and the thalassemias can raise EPO and extend their protection against coronavirus disease 2019 in an age-dependent manner but could turn detrimental in genetically predisposed adults. ACE2 could represent a "bait" for severe acute respiratory syndrome coronavirus-2 to induce ACE/ACE2 imbalance and angiotensin II engendered protective EPO increase at a young age irrespective of genetic predisposition.

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INTRODUCTION

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the cause of the coronavirus disease of 2019 pandemic (COVID-19) has to date (September 11, 2021) infected almost 225 million people worldwide, causing nearly 4.6 million deaths[1]. The COVID-19 pandemic continues to be a global threat despite increasing vaccinations[1]. We and others have recently proposed that the thalassemias and especially HbE, might confer resistance to and/or protection from SARS-CoV2 infection and severity[2,3]. Supporting this hypothesis, Littera *et al*[4] from Sardinia found none of their seriously ill COVID-19 patients were carriers of beta-thalassemia while a recent metanalysis reported a pooled incidence rate of COVID-19 in patients with beta thalassemia at 1.34 per 100000 personday, which is less than half of that observed in the general population (2.89)[5]. We hypothesized that host immune system modulations engendered by malarial selection pressure *via* thalassemia/HbE mutations might confer this protection akin to an antimalarial effect[2]. Another genetic variant significantly associated with mild malaria *vs* severe malaria is the D allele of angiotensin converting enzyme (ACE) I/D polymorphism, that codes for higher ACE levels and subsequently increased angiotensin II (Ang II) production *vs* the I allele[6-8]. We attempted, therefore, to trace a common denominator to explain

the emergence of those two genetic determinants forced by malarial evolutionary pressure. We posit here that the evolutionary selection of thalassemias and the ACE D allele as adaptive alleles for pathogen resistance is neither coincidental nor surprising. Both genetic determinants appear to elicit and sustain a phylogenetically preserved ancestral neuroprotective innate immune response mechanism against tissue injury or pathogen invasion mediated either *via* systemic or/and local increases in erythropoietin (EPO) production[9].

In the present review, we will attempt to explain how (1) Elevated EPO can account for COVID-19 protection in the young; (2) EPO augmenting genetic determinants can predispose for severe COVID-19 complications in adults, and (3) Endogenous and/or pharmacological EPO modulation may offer innovative approaches to treat and/or mitigate SARS-CoV-2 disease severity.

EPO'S TISSUE PROTECTIVE ACTIONS

EPO is an evolutionary conserved hormone, well known for almost a century as the main mediator of erythropoiesis but its widespread effects throughout the body might transcend its primary role[9]. EPO's principal physiologic stimulus for secretion is tissue hypoxia which upon detection by renal interstitial cells is subsequently secreted [9]. Apart from its two main sites of secretion, the kidney and liver, EPO is locally produced and released in a paracrine or autocrine fashion by cells of various tissues including the heart, lungs, testes, ovaries, enterocytes, breast gland and human milk, spleen, bone marrow macrophages, placenta, retina, astrocytes, and neurons[10,11]. EPO's erythropoietic effects are mediated *via* binding to an EPO receptor (EPOR) homodimer $(EPOR)_2$ on erythroid precursors[9]. Evidence supports the renin angiotensin system (RAS) system *via* Ang II and the EPO-fibroblast growth factor 23 (FGF23) signaling pathway as additional regulatory pathways, possibly involved in EPO's non-hematological functions[12,13]. EPO's two distinctive activities (erythropoiesis and tissue protection) appear to reside in different EPO domains and bind to two distinct receptors[14].

When pathogen invasion, tissue trauma or insult occurs, a defensive strategic ensemble is summoned, spearheaded by chemokines and inflammatory cytokines, to attract armies of immune cells that fend off, isolate, kill and remove pathogens and dead cells. This process needs to be controlled and must not be allowed to propagate. Thus, a tissue protective mechanism is required and seems to be provided by the presence of EPO *via* its binding to the tissue-protective receptor (TPR), a heteromeric complex between the EPOR and the β common receptor[9,14]. The TPR is typically not highly expressed but compartmentalized intracellularly and is up-regulated and exposed when insult, trauma, hypoxia, and inflammation invoke subsequent tissue protection[9]. It also has a much lower EPO affinity and needs as high as fivefold systemic EPO levels to be activated[9]. EPO's tissue protective, tissue regenerative, angiogenetic, anti-inflammatory, and anti-apoptotic effects have been documented *via* exogenous EPO administration in both vertebrates and invertebrates and in a variety of disease models[11,15,16] and correlates to the expression of the EPOR in those non-hematopoietic tissues[11]. EPO *via* EPOR expressed on various immune cells, can directly affect the way immune cells exert their immunoregulatory effects, and shift the function of the immune system towards suppression, swing the inflammatory response to immune tolerance, protect injured tissues from apoptosis, and promote wound healing[17]. EPO's immunoregulatory effects have been demonstrated in experimental autoimmune encephalomyelitis[18] and in Th17 cell–associated immune-mediated kidney diseases *via* EPO binding to T cell–expressed EPOR inhibiting Th17 cell induction[19]. Furthermore, EPO's beneficial pleiotropic effects on alveolar-capillary barrier integrity in acute lung injury/acute respiratory distress syndrome (ARDS) have been proposed to be potentially mediated through EPO's anti-inflammatory, anti-apoptotic, anti-oxidative, pro-angiogenic and cytoprotective actions[20,21]. Finally, EPO stimulates bone marrow endothelial progenitor cell mobilization possibly contributing to pulmonary endothelial repair through fusion with resident cells, paracrine effects, or combinations of both[20,21].

YOUNG AGE AND EPO AUGMENTING GENETIC DETERMINANTS: EVOLUTIONARY LESSONS ON HOW TO "SAVE THE CHILDREN"

As TPR has a much lower EPO affinity, local tissue concentrations need to be high to activate it[9].High endogenous EPO, dissonantly elevated from what is expected by a concurrent anemic stimulus and presumably to exert its non-erythropoietic tissue protective functions, has been reported in few studies[22-25]. In all these situations, an imminent tissue insult or pathogen invasion are present while young age (< 13 years) appears to be an important and independent determinant of EPO response unrelated to the circulating hemoglobin levels (Figure 1)[22-25]. Cord blood EPO levels are strongly correlated to cord blood bilirubin in pathological neonatal hyperbilirubinemia potentially shielding the newborn brain from an imminent kernicterus[23]. In extremely premature newborns, elevated endogenous EPO levels varied with circulating levels of inflammation-related proteins possibly mediating protective and repair mechanisms[24]. As a response to pathogen invasion, younger children at all degrees of severe malarial anemia (SMA), tends to have significantly higher EPO levels than expected from their degree of anemia, a phenomenon that declines with increasing age[25]. That the maximum EPO response in SMA occurred very early and at a time when cerebral malaria is uncommon reinforces the notion of an appropriate tissue protective role for EPO[25]. In that sense, the emergence of the two specific classes of malaria protective genetic determinants (the thalassemias and the ACE D allele) is congruent with the evolutionary objective of augmenting either systemic and/or local tissue EPO concentrations to mitigate tissue injury and/or pathogen invasion. The above SMA described age-related EPO pattern has also been reported in sickle cell, and HbE/β-thalassemic children without malaria[22,25]. The numerous mutations of the globin genes in thalassemias cause various degrees of anemia that are a potent and sustained stimulus for renal EPO secretion with elevated systemic EPO levels[22,25]. The ensuing ineffective erythropoiesis in thalassemias[25] avoids polycythemia and subsequent prothrombotic complications but ensures persistent and high enough EPO levels to engage the TPR in various tissues to protect against malaria and its feared cerebral complications[26]. The ACE D allele, also significantly associated with milder forms of malaria in areas of high malarial burden, is another sophisticated genetic selection[5,27-29]. Widespread RAS presence in every human organ and the presence of the ACE D allele ensure that adequate substrate, and enzyme levels (ACE) are abundant[30,31], to provide for systemically and/or locally elevated Ang II levels[7,8] sufficient for endocrine or paracrine effects on EPO secretion stimulation[12,32]. In addition, Ang II may exert immune system modulation [33] and/or direct anti plasmodium activity[34]. The subsequently increased local tissue EPO levels would thus bypass systemic EPO prothrombotic effects while possibly also conferring the demanded tissue protection[35] and mitigation against Plasmodium invasion[12,26,32]. Significantly higher age-related ACE activities in serum are found in newborns and premature infants as well as healthy children and teenagers than adults [36]. Furthermore, lower nasal ACE2 expression in children relative to adults has been reported (Figure 1)[37].

EPO IS AN ANCESTRAL NEUROPROTECTIVE MECHANISM PREVENTING LETHAL CEREBRAL INSULTS AT YOUNG AGE: IMPLICATIONS FOR COVID-19

The above findings and the presence of EPO-like signaling involved in neuroprotection in insects that lack hematopoiesis[38], reinforce the rational assumption that, in younger age groups, high EPO levels could mediate a phylogenetically preserved ancestral neuroprotective innate immune response mechanism preventing lethal cerebral damage from both non-communicable (kernicterus, prematurity)[23,24] and communicable insults (cerebral malaria) (Figure 1)[25,26]. Preliminary evidence suggests that children are indeed less likely to be symptomatic or develop severe symptoms when infected with SARS-CoV-2[39] but whether elevated EPO levels could account for the milder COVID-19 course is currently not known as EPO levels have not been reported in pediatric COVID-19 patients. It is however, known that EPO levels are significantly decreased in adult patients with critical COVID-19[40,41]. It is conceivable that evolution uses the ACE2 as a "bait" for SARS-CoV-2 to gain cellular entry in order to trigger an ACE/ACE2 imbalance[42-44] and stimulate EPO hypersecretion using RAS, uncoupled from hemoglobin levels. Low nasal ACE2 Levels

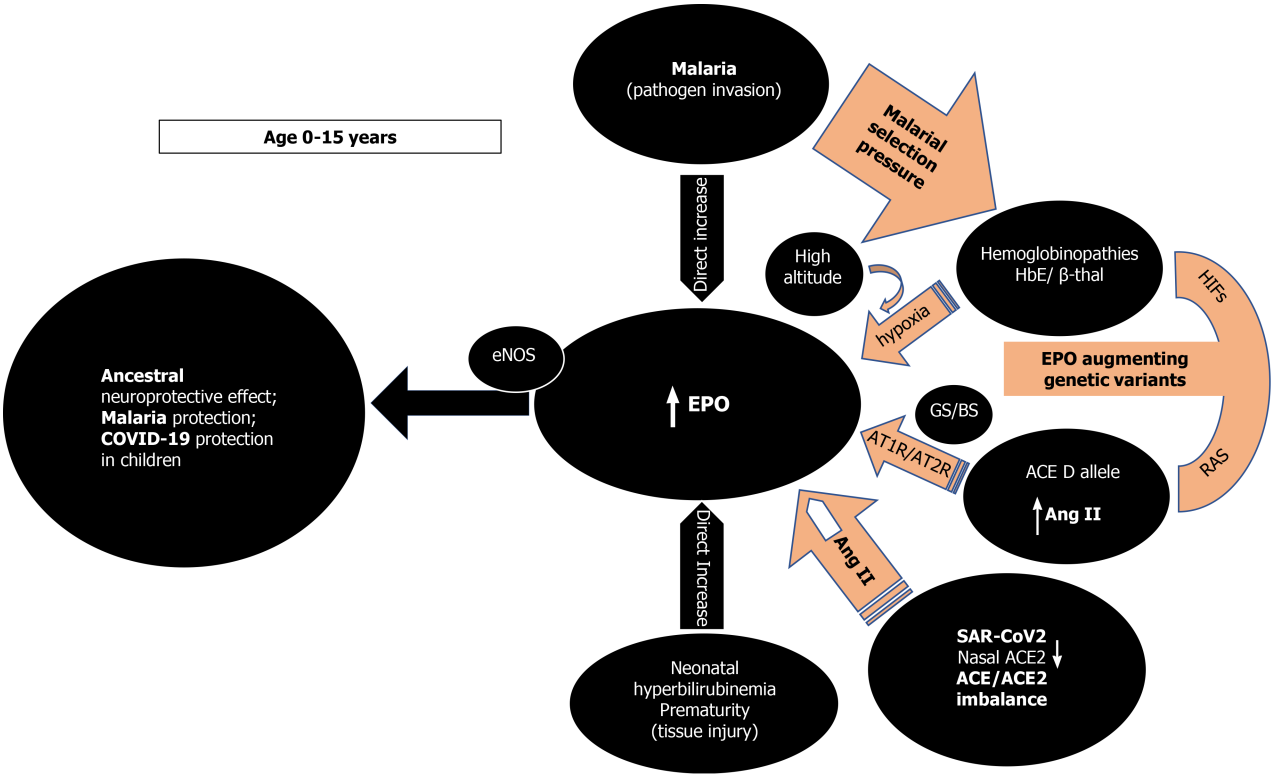

Figure 1 Age dependent erythropoietin secretion and effect of erythropoietin augmenting genetic determinants inducing ancestral neuroprotection, malaria protection, and possibly coronavirus disease 2019 protection in children. ACE: Angiotensin converting enzyme; ACE2: Angiotensin converting enzyme 2; EPO: Erythropoietin; Ang II: Angiotensin II; β-thal: Beta thalassemia; GS/BS: Gitelman syndrome/ Bartter Syndrome; HIFs: hypoxia inducible factors; SARS-COV-2: Severe acute respiratory syndrome coronavirus-2; COVID-19: Coronavirus disease 2019; RAS: Renin angiotensin system; AT1R: Ang II type 1 receptor; AT2R: Ang II type 2 receptor; eNOS: Endothelial nitric oxide synthase.

present in children[37] would beneficially intensify this imbalance, especially for those without protective genetic determinants[37]. Genetically predisposed children already enjoy protective EPO levels through sustained elevated Ang II levels, through the ACE D allele in some, the ACE2 T allele leading to lower ACE2 expression in females[6,45], or HbE/beta thalassemia in others, thus protecting against coronavirus disease 2019 (COVID-19), in similar ways seen in malaria and dengue fever[46] (Figure 1). EPO secretion augmenting genetic determinants alone or synergistically, might protect from or allow an asymptomatic and uncomplicated SARS-CoV2 infection leading to seropositivity and subsequent immunity[2]. In the 2nd Indian serosurvey, where only 3% of the seropositive individuals reported symptoms suggestive of COVID-19[47], the highest seropositivity rate was from the state of Odisha (formerly Orissa), where almost one quarter of the malaria burden of India is found[48]. Surreptitiously, in the same area, α-thalassemia, sickle cell and β-thalassemia alleles were found in 50.84%, 13.1% and 3.4% of subjects[49], respectively while in the same geographical region, the frequency of ACE D allele was significantly higher (57.9%) in mild malaria patients as compared to those in severe malaria patients[6].

It seems intuitive to assume that endogenously increased EPO levels represent an innate "survival mode" that indeed protects the young from tissue injury and pathogen invasion. Longitudinal studies show an overall decrease in EPO levels with increasing age, but the influence of the ACE D allele/DD genotype on EPO decline is not known. Sustained and chronically elevated EPO levels in young or middle-aged non-anemic adults could herald an evolving glucose intolerance or hypertension (HT) [50,51] and later in life establish unfavorable associations with cardiovascular events [52], kidney function decline[52], fracture risk[53], and mortality[52]. Most, if not all the above conditions share associations with the ACE D allele[54] and thus, elevated EPO levels in non-anemic individuals maybe a marker for the presence of the D allele and the elevated Ang II it subsequently encodes[7,8,55,56]. The malarial protection engendered by the EPO augmenting ACE D allele[6,26-28], and the ACE2 T allele[6,45], may thus represent an evolutionary trade off and come at the expense of creating a disadvantage in older age[52] including increased risk of infection, complications, and mortality in COVID-19[45,57-59]. The association of HT with higher risk of severe or

fatal COVID-19[60] and association of HT with the ACE D/ACE2 T alleles reported in several Indian populations[44,61,62] could explain the statistics observed in India during the current phase of the COVID-19 pandemic[1].

THE ACE D ALLELE / DD GENOTYPE AND EPO INTERPLAY: IMPLICATIONS FOR COVID-19

RAS and Ang II effects demonstrate impressive complexity (Figure 2)[30,31].

First, in severe acute respiratory syndrome (SARS) and COVID-19 most deaths occur due to ARDS[63]. The frequency of the ACE D allele was reported to be significantly higher in ARDS[64] but also in the hypoxemic group in Vietnamese patients with SARS related ARDS in the first SARS epidemic[65]. The association of the ACE D allele/DD genotype with increased mortality is now being increasingly reported in various ethnic groups in SARS-CoV-2 as well[59,66]. This association might reflect the effects the ACE D allele exerts *via* Ang II on interleukin 6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1) levels (Figure 2)[67,68]. Both IL-6 and PAI-1 Levels correlate with Ang II and are the highest in individuals with the ACE DD genotype[67-70]. IL-6 can inhibit EPO secretion in the kidney[71], is a prognosticator of COVID-19 disease severity, progress to severe disease and mortality[72,73]. Similarly, elevated PAI-1 is an independent risk factor for poor ARDS outcomes in COVID-19[74] and IL-6 induced significantly elevated PAI-1 Levels in critically ill COVID-19 patients [74,75]. This suggests that the ACE gene I/D polymorphism may play important roles in SARS-CoV-2 infection disease progression into ARDS, and dysregulated immune response[59].

Congruent to its primary evolutionary (neuroprotective) objective of enhanced EPO secretion when threatened by pathogen invasion, ACE D allele/DD-genotype elevated levels of Ang II, reduce ACE2 tissue expression and activity by stimulation of lysosomal degradation through an Ang II type 1 receptor (AT1R) dependent mechanism and thus, might mitigate entry of pathogens using the ACE2 receptor[76, 77]. The ACE2 malaria protective T allele could further reduce ACE2 expression and similarly mitigate pathogen entry[45]. ACE2 is ubiquitous and also present in type I and type II alveolar epithelial cells[78,79]. Loss of ACE2 expression with increasing age, in males, and type 2 diabetes (DM)[80], is known to precipitate severe acute lung failure[81]. Binding and internalization of ACE2 by SARS-CoV-1/2 involves the same AT1R dependent mechanism as Ang II[44], in reducing ACE2 cell surface expression [42,43]. A vicious circle of ACE/Ang II/ACE2 imbalance and persistently increased Ang II levels through continual RAS over-activation might lead to lung shut-down, in similar mechanistic ways as described in human H7N9[82] and H5N1[83]. Additionally, an aberrant T-cell-mediated immune response and cytokine storm could be further mediated by the excessively elevated and unopposed Ang II levels[63,84]. Clonally expanded tissue-resident memory-like Th17 cells have been reported in the bronchoalveolar lavage fluid from patients with severe COVID-19[85]. Th17 cells are under the influence of Ang II signaling[86] and their cell numbers were associated with disease severity and lung damage. Th17 cells demonstrate a potentially pathogenic profile of cytokine expression that may lead to immune-mediated inflammatory diseases[57,85,86]. Both EPO binding to T cell–expressed EPOR as well as AT1R block have been shown to inhibit Th17 cell induction[19,86].

Moreover, Ang II from a functional T-cell RAS plays a pivotal role in T-cell activation towards pro-inflammatory effects, proliferation, chemotaxis, cytokine production, and regulation of memory CD8+ T cell development[33,86]. All these Ang II effects could explain the adverse ACE D allele autoimmunity associations across several ethnicities and autoimmune conditions such as multiple sclerosis (MS)[86], systemic lupus erythematosus (SLE)[87,88], rheumatoid arthritis[89] and vitiligo along with higher IL-6 Levels[89-92]. In addition, Ang II induced pyroptosis, an inflammasome initiated lytic form of programmed cell death further contributes to the COVID-19 cytokine storm[93]. In COVID-19 and under the influence of the ACE D allele and the excessively increased Ang II levels[84], caspase-1 mediated pyroptotic inflammatory cell necrosis could lead to autoantigen exposure and stimulate multiple autoantibody production[94], thus leading to the development of a myriad of autoimmune conditions such as MS, SLE, antiphospholipid antibodies and syndrome, autoimmune hemolytic anemia, and thrombocytopenia, Guillain-Barré syndrome, vasculitis as well as a Kawasaki like syndrome with autoantibodies to ACE2 in children[95]. This pattern that is analogous to our findings in sarcoidosis where ACE D allele induced serum ACE increase and subsequent Ang II elevation can steer the

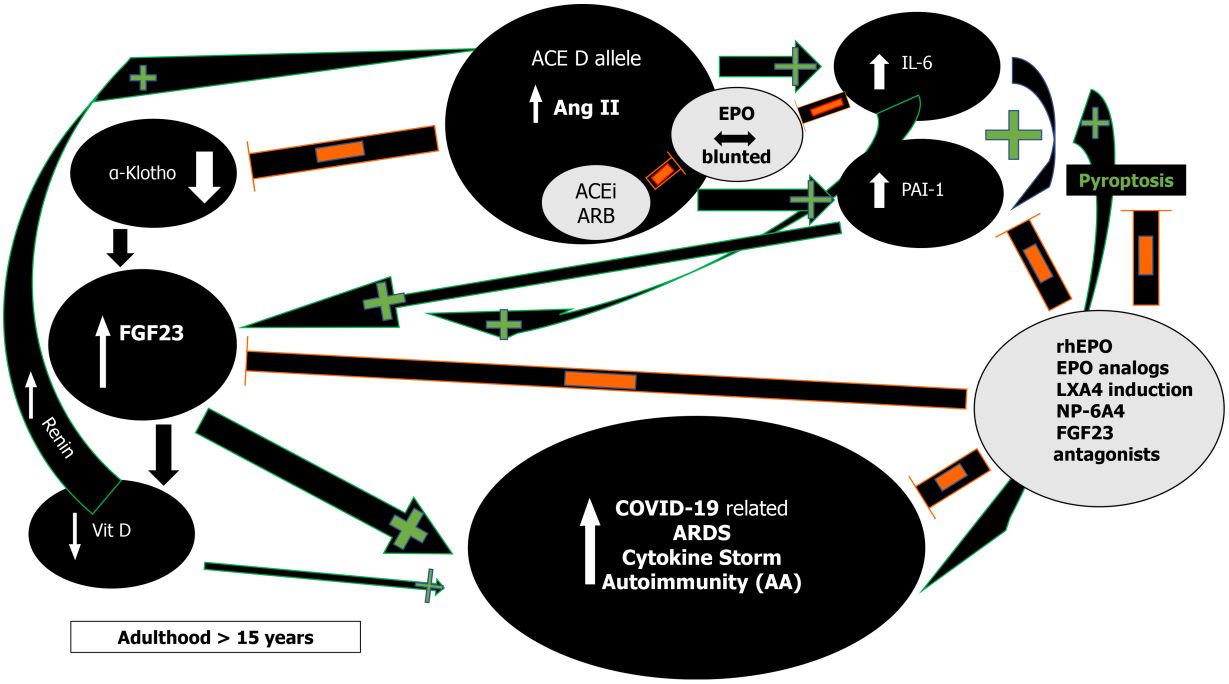

Figure 2 Proinflammatory effects of angiotensin converting enzyme D allele induced Angiotensin II *via* plasminogen activator inhibitor-1 and interleukin 6 induction and their effects on the α-Klotho/fibroblast growth factor 23 axis; inhibitory action of recombinant human erythropoietin/erythropoietin analogs/Lipoxin A4/fibroblast growth factor 23 antagonists. Orange minus sign denotes inhibition. Green plus sign denotes stimulation. ACE: Angiotensin converting enzyme; FGF23: Fibroblast growth factor 23; PAI-1: Plasminogen activator inhibitor-1; IL-6: Interleukin 6; rhEPO: Recombinant human erythropoietin; Vit D: Vitamin D; ARDS: Acute respiratory distress syndrome; AA: Autoantibodies; LXA4: Lipoxin A4; ACEi: Angiotensin converting enzyme inhibitors; ARB: Angiotensin receptor blockers; NP-6A4: AT2R peptide agonist.

immune system towards a protracted course with aberrant gastrointestinal immune reactivity and endocrine autoimmunity including polyglandular autoimmune syndromes[96-98]. Moreover, it has been reported that in acute sarcoidosis presenting with erythema nodosum and usually a benign and self-restricting course, the ACE DD genotype, significantly worsens prognosis[99]. Caspase-1 mediated pyroptosis and autoantigen exposure could lead to AT1R autoantibodies[94], shown to correlate significantly with IL-6[100], that can further mediate persistent proinflammatory Ang II effects by agonistic stimulation of AT1 receptors and increased AT1 receptor activity, even in the absence of the ACE D allele. Low-dose acetylsalicylic acid (ASA) [101] and increasing bioactive lipid (BAL) intake [arachidonic acid (20:4 n-6), eicosapentaenoic acid (20:5 n-3), and docosahexaenoic acid (22:6 n-3)] may result in the formation of increased amounts of endogenous Lipoxin A4 (LXA4) thus offering novel treatment options in the prevention and management of COVID-19 (Figure 2)[102]. Drug design research using LXA4 as a lead compound might result to innovative treatment modalities in autoimmune diseases[94].

Second, RAS influence on EPO levels likely represents an amalgam of complex, intercalated and interrelated set of signals involving multiple molecular mechanisms [12,32,103-106]. Endogenously elevated EPO levels due to hypoxia in high altitude[107, 108] or in human genetic models seem protective[109] while low EPO levels are associated with dismal COVID-19 prognosis (Figure 1)[41]. Epidemiological studies suggest that physiological adaptation in a hypoxic environment at high altitude may protect persons from the severe impact of acute infection caused by SARS-CoV-2[107, 108]. Reductions in cumulative incidence and mortality rates of COVID-19 with increasing altitude have been reported[107,108]. Possible explanations are related to reduced virulence and decreased SARS-CoV-2 pathogenicity at high altitude[107] along with physiological acclimatization to chronic hypoxia *via* increased EPO and genetically adapted high altitude native populations with lower ACE DD genotype frequency[108,110]. Recently, patients with fatal COVID-19 at 4150 meters above sea level displayed 2.5 times lower EPO levels compared to survivors but Ang II levels were not measured in that study[41].

Furthermore, studies in patients with inherited genetic defects in specific kidney transporters and ion channels such as Gitelman's and Bartter's Syndromes (GS/BS) showed a statistically significant absence of COVID-19 infection and COVID-19

symptoms (Figure 1)[109]. In GS/BS patients, the above-mentioned genetic defects result in defective salt reabsorption in the thick ascending limb of loop of Henle[109]. The resulting salt wasting, hypokalemia, and metabolic alkalosis with relatively low levels of serum chloride induce chronic RAS activation with elevated Ang II levels but due to AT1R signaling defects a hypertensive phenotype is not seen[111]. Instead, endogenously increased levels of aberrantly glycosylated ACE2[112] and Ang 1-7 counteract Ang II effects[109,112]. Intriguingly, GS/BS patients also demonstrate Ang II receptor type 2 dependent increase in EPO levels[103] and lack of Ang II induced increase of the PAI-1 gene and protein expression compared to healthy adults[113], both phenomena being possibly protective against COVID-19 at any age.

In critical and deceased COVID-19 patients, EPO levels have recently been reported to be significantly lower and not in accordance with the similarly low hemoglobin levels[40,41]. Moreover, elevated Ang II levels, strongly associated with viral load and lung injury have been reported in another study[84], and in avian influenza A virus H5N1 infected mice and H7N9 infected patients[82,83]. To date, no study has been reported in COVID-19 patients that has investigated the simultaneous measurement of Ang II and EPO and/or correlations to their ACE I/D polymorphism.

Renin and Ang II increase and RAS inhibitors inhibit EPO secretion in healthy volunteers[106]. Severe COVID-19 is also frequently associated with HT, DM, obesity, and metabolic syndrome[114], all resulting in RAS activation through various mechanisms[106]. Nevertheless, the expected Ang II induced EPO rise does not occur in critically ill COVID-19 patients even though the RAS augmenting ACE D allele may be overrepresented in both COVID-19 and associated risk diseases[58,59,61]. Marathias *et al*[106] recently elegantly reviewed RAS and Ang II influence on EPO secretion. Glucose and sodium reabsorption, hyperinsulinemia, the G-protein-coupled receptor 91, all induce RAS activation. The increased Ang II is expected to enhance EPO secretion through tubulointerstitial ischemia, direct upregulation of EPO transcription factors and bone marrow stimulation along with enabling erythropoiesis supportive iron metabolism[106]. On the other hand, glucose toxicity in the renal parenchyma in concurrent DM, obesity, and metabolic syndrome, induce damage on the renal EPO-producing cells and lower EPO secretion. Additionally, HT with widespread use of RAS inhibitors, diabetic hyporeninemic hypoaldosteronism, autonomic neuropathy, obesity or DM induced hypogonadism with low testosterone, chronic and acute inflammation through Ang II induced IL-6 increase[72], all inhibit renal EPO secretion (Figure 2)[71,106]. Finally, blunted EPO response has been documented in critically ill patients while a recent meta-analysis suggests that EPO therapy may decrease mortality[115].

Moreover, elevated Ang II reduces renal α-Klotho expression, interfering with FGF23 signaling and resulting in elevated FGF23 Levels (Figure 2)[116]. FGF23 will inhibit 1α-hydroxylase, leading to the lowering of 1,25-dihydroxyvitamin D3 production and cause or aggravate an incipient vitamin D deficiency, implicated in numerous adverse outcomes including morbidity and mortality in COVID-19[116,117]. All the ACE D allele associations as in HT, type 2 DM, kidney disease, and possibly mortality in COVID-19 could be explained by Ang II induced FGF23 elevations[84,116]. FGF23 serves as a proinflammatory paracrine factor, secreted mainly by M1 proinflammatory macrophages[118]. Powerful and dose-dependent associations have been demonstrated between elevated FGF23 Levels and higher risks for chronic kidney disease, left ventricular hypertrophy and congestive heart failure, autosomal dominant hypophosphatemic rickets, osteomalacia, vitamin D deficiency, fibrous dysplasia, aging, and mortality[119]. Unifying these mechanisms is the finding that both IL-6 and PAI-1 are significant regulators of FGF23 homeostasis[119-121]. Dexamethasone abolished IL-6 induced FGF23 increase[119,120] while PAI-1 inhibition substantially decreased FGF23 levels (Figure 2)[121]. rhEPO administration significantly decrease PAI-1 levels in multi-trauma patients[122] and led to the miraculous recovery of a critically ill elderly COVID-19 patient[123]. EPO's inhibitory effect on PAI-1 and subsequently FGF23 may well have contributed to the patient's recovery and further studies are planned to investigate the potentially favorable rhEPO effect in severe COVID-19[124-126]. Human data show that both endogenous and exogenous EPO influence FGF23 levels *via* alterations of the ratio of active to inactive FGF23 in favor of its inactive form, thus attenuating effects of bioactive intact FGF23 levels and explain EPO's protective effects[118,127]. At present, no study has been reported that investigated FGF23 levels in COVID-19.

THERAPEUTIC CONSIDERATIONS

Currently, therapeutic approaches are symptomatic and include empirical immunosuppressive and anti-inflammatory tactics (dexamethasone)[128], interferons [129], targeting of individual cytokines (IL-6: Tocilizumab/statins/heparin; PAI-1: Statins, and numerous target substances in development)[75,130-132] and correction of isolated laboratory abnormalities (*e.g.*, sodium disturbances)[133]. Prolonged use of these interventions may lead to serious adverse effects and reduction of host defenses with resurgence of opportunistic infections.

An Occam's razor therapeutic strategy guided by mendelian, and mechanistic evidence might be pursued. ACE I/D polymorphism genetic testing could be predictive and guide patient triage and treatment decision making as individuals with the DD genotype are predisposed to a more severe COVID-19 disease course[59]. Research evidence supports the notion that endogenously[109,112] and exogenously increased EPO levels[123] could break the vicious circle of persistent ACE D allele augmented Ang II stimulation on PAI-1, IL-6 and FGF23 by both synergistic and individual inhibition[21,122,123,127,134]. Whenever the administration of rhEPO is not possible due to contraindications or heightened prothrombotic risk, EPO derivatives can coax EPO's tissue-protective activity *via* its TPR for therapeutic use without the risks attributed to EPO's hematological actions[10,14,134]. Furthermore, EPO mediates reduction of auto-and alloantibody formation and used together with LXA4 inducing BALs and/or ASA could prevent recently reported AT1-AA induced collateral damage and autoimmune pathology[94,101,102,135,136]. Moreover, in hematologic patients, rhEPO treatment is associated with an enhanced antibody response to the influenza vaccine, similar to that of healthy subjects and it is conceivable that this effect could also be replicated in COVID-19 vaccinations, especially in immunocompromised patients[137]. Additional treatment modalities could employ a combination of autologous peripheral blood or umbilical cord-derived mesenchymal stromal cells and rhEPO/EPO derivatives that induce notable clinical improvement shortly after initiating treatment in a critically ill patient with severe ARDS[138,139].

Recently, NP-6A4, a novel AT2R peptide agonist with an FDA orphan drug designation for pediatric cardiomyopathy, increased expression of AT2R and cardioprotective EPO in a pre-clinical model with severe obesity and pre-diabetes (ZO rat), along with suppression of nineteen inflammatory cytokines including IL-6 without increasing expression levels of ACE2[140]. NP-6A4 appears as an ideal adjuvant drug candidate for EPO mediated tissue protection and mitigation of cytokine storm[140]. Finally, elucidating FGF23 Levels in COVID-19 could help prognosticate, prevent, and help treat potential future complications. The use of FGF23 antagonists such as the FGF23 antibody burosumab, could be employed to lower FGF23 Levels in FGF23-mediated disorders[141], including COVID-19. To date and to the authors' knowledge, such clinical trials do not exist.

CONCLUSION

Age dependent EPO secretion[22-25] and the contribution of EPO augmenting genetic determinants in children and adults as a disease modifier in malaria is established[6, 25-28]. In the present work, we posit that this EPO effect extends to and explains COVID-19 protection in children[39] and can provide new pathophysiological insights and therapeutic avenues in adults (Figure 1). Elevated protective EPO mRNA levels were recently reported being 2.6 times higher in nasopharyngeal swab samples of adult SARS-CoV-2 patients that were asymptomatic or showing mild COVID-19 clinical symptoms, as compared to a control group[142]. EPO induces endothelial nitric oxide (NO) synthase and increases NO production in endothelial cells[14]. Increased NO bioavailability is shown to inhibit fusion of the SARS-CoV spike protein to ACE2 and early production of viral RNA [143], potentially mediating EPO protection in SARS-CoV-2 too.

The intricate balance between the components of the RAS axis (peptides and peptidases) and its interactions with the EPO and α-Klotho/FGF23 axes are incompletely understood in the context of chronic stable and acute decompensated environments. Known and unknown genetic determinants and concurrent diseases with their pharmacological interventions further complicate the view. High Ang II and low EPO levels in COVID-19, have been reported and strongly associate with viral load[84], lung injury[84], and critical disease[40,41]. Ang II, excessively augmented in the presence of the ACE D allele[7,8], leads to reduction in ACE2[44], and increases

FGF23, PAI-1, and IL-6 levels[67-70,116], that along with increasing age, co-morbidities and concurrent pharmacological RAS interventions, all blunt EPO response[50,71,106] and potentially reduce EPO levels in critically ill COVID-19 adult patients (Figure 2) [40,41]. In adults with COVID-19, this proinflammatory constellation would promote progress to ARDS, and cytokine storm with pyroptotic inflammatory reactions, autoantigen exposure, autoantibody production and subsequent autoimmune disorders[95].

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MINIREVIEWS

Overview of nutritional approach in hematopoietic stem cell transplantation: COVID-19 update

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Abstract

The coronavirus disease 2019 (COVID-19) is caused by the newly discovered SARS-CoV-2. Hematopoietic stem cell transplantation (HSCT) is a high-risk procedure. The novelty of COVID-19 has created more uncertainty during all phases of HSCT. It is thought that HSCT patients taking immunosuppressive agents are more likely to contract COVID-19 than healthy individuals are. Appropriate care precautions should be taken with patients undergoing HSCT to minimize the risk of COVID-19, and appropriate treatment methods must be followed in patients infected with COVID-19. Malnutrition has become a significant problem in HSCT patients during the COVID-19 pandemic. The causes of malnutrition in HSCT patients are multifactorial. However, the most important reason is the decrease in energy and nutrient intake. The HSCT procedure can lead to many complications such as dysgeusia, mucositis, diarrhea, constipation, xerostomia and vomiting/nausea. Improving the nutritional status of HSCT patients by managing each of these special complications with an appropriate nutritional approach is essential for successful engraftment. This review aims to provide a comprehensive overview of the specific complications affecting the nutritional status of HSCT patients and their nutritional approach during the challenging COVID-19 pandemic.

Key Words: COVID-19; Hematopoietic stem cell transplantation; Nutrition; Nutritional approach

Core tip: Hematopoietic stem cell transplantation (HSCT) is a high-risk procedure due to the presence of initial hematological malignancies and the high risk of complications. The novelty of the coronavirus disease 2019 (COVID-19), lack of literature and

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lack of antiviral agents leads to more uncertainty and an increased risk in HSCT procedures. It is important to protect HSCT patients from COVID-19 infection. Malnutrition is another important problem in HSCT patients. A proper nutritional approach is important in all phases of the HSCT procedure. Therefore, professional intervention with multidisciplinary nutrition support teams is indispensable.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) caused by the novel SARS-CoV-2 first appeared in Wuhan, China in late December 2019. The first cases outside of China were initially reported in January 2020. Subsequently, the epidemic dispersed rapidly across the world. On March 11, 2020, the World Health Organization (WHO) declared COVID-19 a pandemic[1]. As of December 26, 2020, WHO has reported 78383527 confirmed cases and 1740390 deaths globally[2].

The COVID-19 pandemic is continuing to place an enormous burden on healthcare around the world. Currently, > 40000 cases receive hematopoietic stem cell transplantation (HSCT) every year worldwide. However, the ultimate impact of COVID-19 on the HSCT procedure is still unknown[3]. Immunosuppressed patients undergoing active HSCT or survivors of HSCT are considered to be a susceptible population on which COVID-19 infection can have devastating consequences[4]. HSCT recipients are susceptible to multiple infections due to the immunosuppressive therapy received for graft failure prevention. Hereby, these patients are immunosuppressed and may be vulnerable to a worse prognosis of COVID-19[5]. Although it is early to estimate the risk of COVID-19 and its severity in HSCT patients, it is likely to follow the course of other respiratory viruses[4].

HSCT involves administration of healthy HSCs in patients with dysfunctional or depleted bone marrow[6]. It is applied to treat cancers such as leukemia, lymphoma, multiple myeloma, bone-marrow-related diseases, as well as various immunological and genetic diseases[7]. There are three categories of HSCT: allogeneic, autologous and syngeneic transplantation. Autologous transplantation is a type of transplantation in which the donor and recipient are the same. It is performed using the hematopoietic stem cells of the patient themselves. Allogeneic transplantation is the receiving of cells from a living donor with or with no blood tie. The donor should be immunologically similar to the recipient for allogeneic transplantation[8]. Syngeneic transplantation is allogeneic stem cell transplantation performed by collecting stem cells from twin siblings[9].

All patients undergoing HSCT are at high risk of malnutrition in the pre- and postengraftment phases[10]. COVID-19 burden further increases the risk of malnutrition in HSCT patients. As a result, nutritional intervention in the pre-, peri- and postengraftment phases is crucial, as the pretransplant nutritional status affects post-transplant complications and the end results. If this process is not managed properly, response to treatment may be limited.

We aim to review some complications affecting the nutritional status of HSCT patients and the nutritional approach to be followed in the challenging COVID-19 pandemic.

INFECTION COMPLICATIONS IN HSCT

Infections are the most common and important cause of mortality and morbidity associated with HSCT[11]. Especially in allogeneic HSCT, factors including the presence of myeloablation, reconstruction of a new immune system and the use of immunosuppressive agents in addition to other complications such as graft versus host disease (GVHD) can predispose to infections[12]. The immune cell reconstitution subsets after HSCT takes at least 2–3 years. After HSCT, this process carries a high risk

of infection[11]. Three different phases are defined for the risk of infection after HSCT.

The main risk factors in the first phase, the pre-engraftment phase, are neutropenia and mucosal damage. Neutropenia continues for 5–7 d (reduced intensity) or 15–30 d (conditioning regimen), according to the type of conditioning regimen. Bacteremia/sepsis, pneumonia, oropharyngitis, sinusitis, proctitis and cellulitis are common in this phase[11]. In addition, the most common pathogens in this stage are streptococci, Gram-negative bacteria, *Candida* and *Aspergillus* species if neutropenia persists for an extended period. Neutrophil-count recovery reduces the risk of bacterial infection in autologous transplants, while the risk continues in allogeneic transplants[13].

Cellular and humoral immune deficiency is predominant in the early postengraftment phase[13]. There is an increased risk of infection caused by GVHD in addition to catheter-related infections. These infections can lead to life-threatening outcomes. During this phase, adenovirus, the BK virus, respiratory viruses, *Pneumocystis jirovecii*, *Candida* and *Aspergillus* species in addition to other fungi are also common[11]. Patients receiving immunosuppressive therapy for GVHD are highly susceptible to these pathogens[13].

In the late postengraftment phase, where regeneration of cellular and humoral immunity continues, varicella zoster virus infection is frequently seen. Pathogens are also seen in the early postengraftment phase[14]. *Aspergillus* species, *P. jirovecii*, respiratory syncytial and parainfluenza virus may cause severe infections associated with the respiratory system[13]. The most common infections in the three phases of HSCT are summarized in Figure 1.

Respiratory viruses such as influenza, respiratory syncytial virus, parainfluenza virus, human metapneumovirus, the rhinovirus and adenovirus are well defined in HSCT recipients. There are limited data on coronavirus infection in these patients[15]. The main recommendation for HSCT recipients is to avoid being exposed to this virus. In all three phases, methods of protection from infection including, vaccination and application of pharmacological approaches as recommended are important in terms of preventing or at least reducing infectious complications[16].

COVID-19 IN HSCT

HSCT is a high-risk procedure due to the presence of initial hematological malignancies and the high risk of complications, particularly those which are infectious. However, HSCT remains the only curative treatment for most malignancies[17]. The emergence of COVID-19 is a global crisis which HSCT patients have to face[18]. The novelty of COVID-19, lack of literature and limited antiviral agents have led to further uncertainty as well as increased risk in HSCT procedures[19].

HSCT patients with weak immune systems resulting from long-term and regular administration of immunosuppressive agents are more likely to get COVID-19 compared to healthy individuals[20]. The immunosuppressive therapy may exacerbate immune-related pneumonia or T-cell cytokine release in HSCT patients[21]. A published meta-analysis has shown that immunosuppressive therapy is associated with longer hospital stay, higher risk of bacterial infection and mortality rates in patients with COVID-19 pneumonia[22]. High-dose, but not low-dose, immunosuppressive therapy can potentially increase mortality rates in severe COVID-19 patients. Therefore, high-dose immunosuppressive therapy should be used with extreme caution concerning COVID-19[23]. It is also possible that the immune system cannot effectively produce antibodies as a result of malnutrition and lymphocyte dysfunction in HSCT patients[3]. HSCT patients differ in many ways from the general population in relation to COVID-19. Firstly, they are more vulnerable. Secondly, it makes pathogens harbor longer, making them more contagious. Thirdly, it is difficult to keep them in quarantine due to their frequent medical needs. Finally, the course of the infection does not usually follow the natural course of the disease, as is seen in immunosuppressed hosts[24].

In order to minimize the risk of COVID-19, HSCT patients must limit their contact with potentially infected individuals and follow national prevention guidelines such as hand hygiene, home isolation and social distancing. They may contact healthcare providers *via* the internet or phone to reduce the frequency of hospital visits and manage non-emergency health problems[18]. They need to refrain from traveling. If travel is absolutely necessary, it is advisable to travel by private transport rather than public transport, including the train, bus or plane[25].

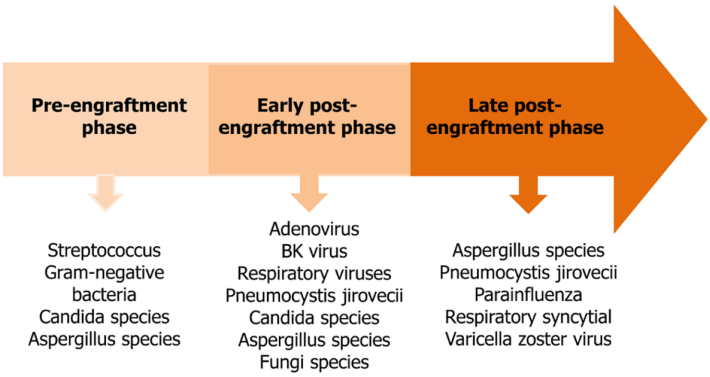

Figure 1 The most common infections in the three phases of hematopoietic stem cell transplantation.

HSCT is one of the most important treatment methods for many hematological malignancies. Unfortunately, HSCT can lead to many immediate and long-term issues such as donor rejection, conditioning-related toxicity, GVHD, recurrence of primary cancer and infections. Therefore, during the COVID-19 pandemic, the decision to continue with HSCT patients, depends on many factors such as the primary disease of the patient, the local burden of COVID-19, availability of alternative treatment, national guidelines, and the experience of the transplantation center[26]. A special assessment has been made for patients undergoing autologous or allogeneic HSCT and practical guidelines have been published by the Infectious Diseases Working Party on behalf of the European Society for Blood and Marrow Transplantation (EBMT)[25]. First of all, the presence of intensive care unit beds, ventilators and stem cell products should be investigated before starting the HSCT procedure. However, it has been suggested that nonemergency HSCT be postponed, especially for nonmalignant diseases. While it is clear that COVID-19 testing is mandatory for all patients before the conditioning regimen, the decision as to how long transplantation will be delayed if they become infected with COVID-19 is controversial[27]. The recommendations for the management of COVID-19 in HSCT patients are given in Table 1. However, the currently available recommendations are provisional and additional guidelines are expected to be published in the upcoming period[19].

If the HSCT patient has a positive reverse transcription polymerase chain reaction (RT-PCR) test result, or the chest computed tomography scan is suspicious of COVID-19, it is required that the patient is managed and treated according to the national COVID-19 guidelines. Supportive care is the mainstay of therapy. Immunosuppressive therapy should be continued for prophylaxis and treatment of GVHD[18]. The multidisciplinary team approach is required for the management of medical and nutritional therapy of these patients[4].

EFFECT OF HSCT ON NUTRITIONAL STATUS

Malnutrition is common in HSCT patients. The studies have shown that 10%–50% of all patients with hematological malignancies in the pre-HSCT period are already undernourished and often have a low body mass index (BMI)[28-30]. During and after the HSCT phase, many of the patients continue to lose weight and their nutritional status worsens[30,31].

The causes of involuntary weight loss and malnutrition during HSCT are multifactorial due to a number of factors such as underlying disease, nutritional status before transplantation and a conditioning regimen. HSCT patients often have decreased energy and nutrient intake. In addition, these patients have altered nitrogen balance, glucose tolerance, and energy needs along with antioxidant requirements. As a result of malabsorption, increased catabolism, changes in biochemical signs and the anorectic effects of cytokines, these patients may lose weight, particularly fat free mass [32]. Additionally, the adverse effects of treatments such as chemotherapy and radiotherapy are the main cause of malnutrition. The adverse effects of chemotherapy and radiotherapy include loss of appetite, dry mouth, oral mucositis, nausea, vomiting, changed sense of taste and smell as well as other gastrointestinal disturbances. Also, GVHD can cause nutritional issues such as dysphagia, nausea, vomiting, severe diarrhea, mucositis, anorexia or early satiety. These symptoms can

Table 1 The EBMTR recommendations for the management of COVID-2019 in HSCT patients[25]

No.	
1	All recipients are required to have tested negative for COVID-19 RT-PCR before starting conditioning regimen irrespective of respiratory symptoms
2	All recipients who are to be accepted for HSCT should be advised 14 d of home isolation before starting transplant conditioning. It should also be recommended to avoid unnecessary clinical visits to reduce the risk
3	For patients who have had close contact with a person diagnosed with COVID-19, performance of all transplant-related procedures should not be performed within at least 14 d and preferably 21 d from the last date of contact. The recipient is advised to be closely monitored for COVID-19 symptoms. COVID-19 RT-PCR negativity must be confirmed before any transplant procedure is undertaken
4	For confirmed COVID-19 patients with high-risk malignancies, HSCT should be delayed for a minimum of 14 d until the patient is asymptomatic and has at least 2 consecutive negative RT-PCR tests ≥ 24 h apart. In patients infected with COVID-19 with low-risk malignancies, HSCT must be delayed for a 3 mo

EBMT: European Society for Blood and Marrow Transplantation; RT-PCR: reverse transcription polymerase chain reaction; HSCT: hematopoietic stem cell transplantation.

negatively affect oral intake[31]. The American Society for Parenteral and Enteral Nutrition (ASPEN) clinical guidelines declared that all patients undergoing HSCT with myeloablative conditioning regimens are therefore at risk of malnutrition[33].

Malnutrition causes serious adverse outcomes in HSCT patients and results in higher susceptibility to bacterial and fungal infections, more days of fever[34], as well as higher mortality and lower overall survival rates[35]. A study examining 544 adult patients undergoing allogenic HSCT found that patients with BMI < 20 kg/m^2 had a higher mortality rate from infection and disease relapse[36]. All patients undergoing HSCT are required to undergo a comprehensive assessment of their nutritional status before the initiation of treatment. Also, their nutritional status should be monitored and re-evaluated throughout the entire HSCT process[7].

EFFECT OF COVID-19 ON NUTRITIONAL STATUS

The European Society for Clinical Nutrition and Metabolism (ESPEN) states that COVID-19 patients are at high risk of malnutrition[37]. This is explained by inflammatory syndrome, hypercatabolism and increased energy expenditure due to ventilation burden. If nutritional support is not initiated early, malnutrition can aggravate the end results of viral pulmonary damage, leading to rapid deterioration of respiratory muscle function. Hypermetabolism and physical inactivity can cause rapid muscle atrophy along with reduced food intake. Finally, gastrointestinal disturbances (diarrhea, nausea vomiting, or abdominal pain), anxiety, confinement, organizational problems and lack of staff can contribute to the limitation of food presentation and intake[38].

Muscle atrophy is also an important issue in COVID-19 patients. Recovery time is approximately 2 wk in mild patients and 3–6 wk in critical patients. Inactivity in this process results in rapid muscle atrophy as well as loss of muscle strength and function [39]. Even 10 d of bed rest in elderly healthy individuals causes a significant reduction in total fat free mass[40,41]. Malnutrition caused by COVID-19 induces loss of muscle mass along with the weakening of the immune system and the severity of COVID-19 may increase as a result[38]. Therefore, it is important to evaluate the nutritional status of these patients and to monitor them for extended periods especially in terms of fat free mass.

ASSESSMENT OF NUTRITIONAL STATUS IN HSCT

The immediate and serial nutritional status assessment of all pre-HSCT patients is required due to expected nutritional issues associated with the conditioning regimen [42]. In addition, nutritional status assessment with a validated tool is necessary during and after HSCT[43].

There is no gold standard in evaluating the nutritional status of HSCT patients. Anthropometric measurements (*e.g.* body weight, BMI, triceps skinfold thickness, fat free mass), biochemical parameters (albumin, prealbumin, transferrin, retinol binding protein), screening tools [Nutritional Risk Screening tool (NRS 2002), Mini Nutrition

Assessment (MNA), Tools (MUST) and Subjective Globe Assessment], in addition to functional tests (hand grip strength) can be used to assess their nutritional status[44]. It is recommended to use the combination of different screening tools along with clinical laboratory indicators for accurate and comprehensive nutritional status assessment [45].

There is no consensus on particular assessment methods to be used in different phases. This assessment should be made according to the protocol of each institution at all stages of HSCT. It is recommended that the nutritional status of patients is evaluated during phases of admission, the onset of a conditioning regimen, the day of stem cell infusion, the onset of immunosuppression, hospital discharge, 1–3 mo after HSCT, 6 mo after HSCT and 1 year after HSCT[43].

The ESPEN strongly recommends that the food intake of patients, the effect of symptoms, muscle mass, physical performance and the degree of systemic inflammation are evaluated following risk identification[46]. In outpatients, nutritional assessment should be performed within the first 15 d in patients with nutritional risk and within 30 d in those with no risk. Also, inpatients should be assessed up to 48 h after hospitalization and re-evaluated after 7 d. Weekly re-evaluation should be done until the patient is discharged[33,43].

During the pandemic, it has become clear that remote contact with patients is necessary to continue to provide adequate care to patients[47]. One of the WHO suggestions is to use telemedicine to strengthen the response of the health system to COVID-19. Telemedicine is a method of remote communication used to provide medical and clinical services to patients in different locations. The nutritional assessment can be made by telephone, teleconference or other digital channels whenever possible[39].

Nutritional screening and assessment

According to ASPEN, especially patients undergoing HSCT with the myeloablative conditioning regimen are at nutritional risk and the nutritional status of these patients should be screened[33]. In addition, ESPEN recommends screening for malnutrition during transplantation[48]. Although ESPEN and ASPEN recommend routine malnutrition screening for HSCT patients, there is no consensus on how to evaluate malnutrition in these patients[45]. The nutritional status of these patients can be evaluated using a validated screening tool, such as NRS-2002, MUST, the Malnutrition Screening Tool or the MNA-Short Form (MNA-SF)[43]. NRS-2002 is advised due to its easy applicability by ESPEN[48].

Apart from all the tools mentioned above, ESPEN recommends the use of a combination of two simple validated clinical tools (MUST and SARC-F) to remotely assess the nutritional risk and loss of muscle mass along with function. The acronym for the tool is R-MAPP, as for the Remote Malnutrition APP, and is being developed as an app. As a first step, MUST is required to be performed on all patients remotely. SARC-F is advised to be performed on elderly patients. It is also suggested to be applied on all patients with acute and chronic muscle-atrophy diseases. If the patient is classified with or at risk of malnutrition and/or SARC-F is predictive of sarcopenia along with poor outcomes, a nutritional care plan should be prescribed[39].

Anthropometric measurements

Anthropometric measurements are essential in determining underweight and overweight status as a tool of nutritional surveillance[43]. Body composition should be routinely evaluated in patients undergoing HSCT[43]. In practice, body weight and height, triceps skinfold thickness, arm circumference and arm muscle circumference are measured and BMI is calculated[49]. However, in clinical practice, it is not always possible to use these parameters when there is some limitation in movement, the presence of access, edema or immobility[43].

It is important to remember that splenomegaly may affect body weight in these patients. In addition, body weight may differ due to fluid retention or fat accumulation in patients receiving corticosteroid therapy[50]. Body weight should be corrected for excess fluids caused by pleural edema and ascites[43]. The muscle capacity of malnourished patients is significantly decreased. Hand dynamometer is an appropriate method used to evaluate the nutritional status of patients as a prognostic marker[51].

NUTRITIONAL GOALS IN HSCT

HSCT patients are a highly heterogeneous population in terms of nutritional goals. The different doses in conditioning regimens in addition to the presence of GVHD further alter the nutritional goals of patients[42].

Energy requirements

Determining energy requirements is important for individuals to maintain their energy balance and reach a healthy body weight. While calculating the energy requirements of patients, different conditions such as the diagnosis of the individual, the presence of other diseases, the purpose of the treatment, anticancer treatments, the presence of fever or infection or refeeding syndrome need to be considered[52].

The energy requirements of patients can be calculated with standard equations or indirect calorimetry. According to ASPEN, indirect calorimetry is the suggested method for determining the energy requirements of critically ill cancer patients. In cases where indirect calorimetry is not accessible, predictive equations such as Harris–Benedict, Scholfield and others can be used. Also, the EBMT states that energy requirements can be calculated according to the Harris–Benedict formula[7]. Another quick method is a simple formula that uses calories per current or adjusted body weight[43].

Due to hypercatabolic status, energy requirement is increased in HSCT patients. Although the energy expenditure by a HSCT recipient may vary according to whether it is autologous or allogeneic, there is agreement as to the energy requirements of the transplant recipient, which may increase up to 130%–150% in terms of the estimated basal energy expenditure corresponding to 30–50 kcal/kg/d[53], which corresponds to 30–50 kcal/kg/d[9]. Regarding the target energy intake after HSCT, 25–30 kcal/kg/d is usually recommended for patients with no severe malnutrition. In patients with malnutrition, 35–45 kcal/kg/d is suggested. However, it should be known that high energy intake in the postengraftment phase is associated with the risk of hyperglycemia[54].

Macronutrient requirements

Tissue damage may occur in HSCT patients as a result of several factors such as fever, infections, chemotherapy or radiotherapy. Adequate protein intake reduces muscle loss after cytoreductive therapy[43]. These patients need additional protein to repair and regenerate their tissues and to maintain a healthy immune system. The degree of malnutrition, the extent of the disease and the degree of stress are important in determining the protein requirement. The daily protein requirements are usually calculated using actual body weight rather than ideal body weight[52]. There is a consensus on protein requirements ranging from 1.4 to 1.5 g/kg/d and reaching up to 2.0 g/kg/d for both autologous and allogeneic HSCT patients[9,43].

After HSCT, glutamine therapy can minimize intestinal damage associated with a conditioning regimen. The effect of glutamine on glucose homeostasis may be beneficial for patients after allogeneic HSCT[55]. Oral glutamine may reduce mucositis and GVHD, while intravenous glutamine may reduce infections. Glutamine can have harmful effects, especially on critically ill patients with impaired renal function[56]. Although the mechanism of the harmful effects of glutamine in critically ill patients is unclear, nutritional supplements containing high doses of glutamine may cause amino acid overload in patients with renal dysfunction. Therefore, caution is advised when using glutamine in patients with impaired renal function after allogeneic HSCT. The well-designed studies in patients after allogeneic HSCT are needed to confirm possibly beneficial effects of glutamine[54].

Glucose intolerance may occur in HSCT patients due to the administration of cyclosporine and steroids, or sepsis. Although lipid metabolism abnormalities are less common in the early postengraftment phase, high cholesterol and triglyceride levels are observed in the late postengraftment phase. It should be kept in mind that hyperglycemia and hyperlipidemia in these patients increase the risk of comorbidity [43]. The optimum carbohydrate and fat requirements have not been determined by ESPEN or ASPEN in HSCT patients. Glucose intake in the early postengraftment phase should be ≤ 5 g/kg/d[54]. Lipids are recommended to be used to supplement energy requirements (20%–30%). Dietary lipids should contain long- and medium-chain triglycerides. In addition, *trans* fatty acids need to be removed from the diet of these patients. Saturated fatty acids are advised to be < 7%–10% of the total energy and unsaturated fatty acids should be 10%–15% of the total energy[43]. Omega 3 fatty acids play a role as an immunomodulatory factor. Theoretically, omega 3 fatty acids may

alleviate the cytokine storm and contribute to a reduction in the incidence of complications after HSCT[54].

Micronutrients requirements

There are few studies on the micronutrient requirements of HSCT patients[43]. Therefore, the micronutrients requirements of HSCT patients has not been established. It is known that vitamin C deficiency during the acute phase of HSCT is prominent and that this increases systemic inflammation[57]. Similarly, vitamin D deficiency before transplantation may increase the risk of GVHD and infection after transplantation[58]. Vitamin D can reduce the prevalence of chronic GVHD and mortality. After HSCT, zinc supplementation has been suggested as a potential treatment to help with immunosuppressive therapy, yet it has no adverse effects. However, this practice is not routine in transplant centers[43]. Moreover, it is important to remember that deficiency in antioxidants such as vitamin E and β-carotene may also be seen. It is considered safe to use a standard multivitamin along with a mineral supplement, not exceeding 100% of the dietary reference intake in the event that individuals have poor oral intake and side effects related to treatment[52].

Fluid requirements

Fluid requirements for HSCT patients are similar to those of healthy individuals. In other words, fluid requirements are reported as 1 mL/kcal or 35 mL/kg/body weight [43]. However, the presence of fever, ascites, edema, fistulas, excessive vomiting or diarrhea may cause changes in fluid balance[52]. Fluid requirement should be determined in these patients by considering these conditions[50].

NUTRITIONAL SUPPORT THERAPY IN HSCT

A proper nutritional approach is important in all phases of the HSCT procedure. Therefore, professional intervention by multidisciplinary nutrition support teams is indispensable[54]. Oral nutrition is indicated for HSCT patients with a functional gastrointestinal tract. Dietary counseling in addition to oral nutritional supplements are the first step for patients who are treated at all clinics and outpatient clinics with malnutrition or who are at risk of malnutrition. When oral intake is not sufficient to meet the nutritional requirements, enteral or parenteral nutrition may be indicated [42]. The nutritional support therapy recommendations in HSCT patients are given in Table 2.

NUTRITIONAL APPROACH IN SPECIAL COMPLICATIONS

The care plans for nutritional management are associated with a variety of complications, particularly gastrointestinal complications. Nausea and vomiting are most acute during cytoreduction therapy, yet mild symptoms continue for 3–6 wk. Mucositis peaks 10–14 d after transplantation and the associated pain along with swelling are the main deterrents of eating during the neutropenic phase. Cramping, abdominal pain and diarrhea due to mucosal crypt abnormalities, epithelial flattening, and cell degeneration in addition to increased intestinal permeability return to normal 1–2 wk after the onset of a conditioning regimen and 3–4 wk after transplantation[50]. In addition, since gastrointestinal symptoms such as diarrhea, nausea/vomiting and dysgeusia are among the well-known symptoms of COVID-19, nutritional treatment of these conditions has become more important in HSCT patients[59].

The recommendations cannot be generalized to all HSCT recipients[42]. The nutritional assessment is required to be conducted individually and an appropriate nutritional approach needs to be recommended by the nutritionist[43]. The nutritional approach in some specific complications are summarized in Table 3.

Mucositis

Mucositis is tissue damage caused by cancer therapy, especially high-dose chemotherapy and radiation therapy used in HSCT. It is a common adverse effect in patients undergoing HSCT. It causes inflammation and ulceration of the gas-trointestinal tract. There are two types of mucositis, oral and gastrointestinal. Oral mucositis characterized by the presence of erythematous along with ulcerative lesions in the oral cavity. Gastrointestinal mucositis affects the entire gastrointestinal system and can

Table 2 Nutritional support therapy recommendations in hematopoietic stem cell transplantation patients by phases[9]

	Oral/EN		PN (Digestive intolerance)	
	Severe malnutrition			
	No	Yes	No	Yes
Before transplantation	Normal protein: Low microbial diet	High protein EN	Minimal EN, normal protein PN	Minimal EN, high protein PN
Conditioning regimen (–7 to 0 d)[1]	High protein: Low microbial diet	High protein EN, supplements	Minimal EN, normal protein PN, glutamine	Minimal EN, high protein PN, glutamine
After Transplantation				
Phase I[2] (0–30 d)	Low microbial diet, EN	High protein EN, glutamine	Minimal EN, normal protein PN, glutamine	Minimal EN, high protein PN, glutamine
Phase II[3] (30–90 d)	Decrease EN, bland diet	Normal protein EN, supplements	EN 50% + PN 50%, normal protein	EN 50% + PN 50%, high protein
Phase III[4] (> 90 d)	Standard, bland diet	Standard diet, supplements	EN 100% or normal protein bland diet	EN 100% or high protein bland diet

In some sources, durations are given as follows:
[1]-10 to 0 d.
[2]0 to +21 d.
[3]+21 to +100 d.
[4]> 90 d. EN: Enteral Nutrition; PN: Parenteral Nutrition.

cause symptoms such as anorexia, nausea, vomiting and diarrhea[43]. Oral mucositis is a common complication of HSCT, with a high incidence of 75%–99%[42]. Lesions are painful and clinical management of oral mucositis is difficult[43].

There is no consensus on the prevention or treatment of mucositis. Regular dental check-ups are important in patients with mucositis. General care guidelines recommend daily oral care and mouthwashes in a nonirritating manner. While nonirritating liquid and soft foods are generally better tolerated in patients with oral or esophageal mucositis, they should avoid consumption of heavily flavored, acidic or spicy foods[52]. In patients with mild mucositis, it is important to change the consistency of the diet, even if oral intake is still possible. Gastrointestinal mucositis damages crypt cells and reduces the production of digestive enzymes. Also, temporary intolerance to lactose may occur. Patients with gastrointestinal mucositis may need a light dairy diet[43]. Patients with more severe mucositis should be fed safely through a tube. Parenteral nutrition may be indicated for patients who cannot be fed orally or enterally due to ileus, persistent vomiting or obstructive conditions[50].

Glutamine supplementation has long been used as part of gastrointestinal mucositis management. However, recent studies have shown conflicting results regarding its benefits[60]. There is no consensus on the benefit of glutamine in HSCT patients. Oral administration is indicated for mucositis and associated odynophagia. However, the exact results are unknown due to the different methodologies in the studies[43,46]. A meta-analysis found that glutamine reduces the severity and duration of mucositis as well as GVHD[61]. However, no benefit of glutamine on overall survival or reduction in infection rates has been identified in any randomized controlled trial[7]. Iyama *et al*[62] conducted a small retrospective study of patients who received an oral solution containing glutamine, fiber and oligosaccharides after allogenic HSCT. They found a statistically significant reduction in severe diarrhea, mucositis and weight loss. Glutamine administration is not routine in the treatment of mucositis due to these inconsistent results[44].

Dysgeusia

Dysgeusia is characterized by abnormal taste bud function and loss or impairment of the sense of taste[43]. The chemotherapy and total body irradiation reduce and destroy taste receptor cells. This results in dysgeusia or altered sense of taste, which affects food selection and contributes to poor oral intake[42]. Impairment in the bitter taste is first observed, followed by loss of sweet and salty tastes[43].

The dietary management of dysgeusia requires proper dietary counseling along with a thorough clinical and nutritional assessment[42]. In this case, flavors can be enhanced with the use of seasonings and spices. Citric foods can be consumed. Food

Table 3 Nutritional approach in specific complications

Special complications	Nutritional approach
Vomiting/nausea	Developing a diet plan which includes salty or sour foods, cold and clear liquids, small frequent meal portions, low-fat and low-fiber foods
	Starting parenteral nutrition or postpyloric enteral nutrition if symptoms are severe
Dysgeusia	Promotion of good oral hygiene
	Developing a diet plan which includes a variety of highly spicy, tasty foods (unless mucositis/esophagitis) to optimize oral intake
Xerostomia	Development of a diet plan which includes the use of highly-moist foods, saliva stimulants (*e.g.* citric acid drinks, sugarless sugar, sugar-free gum) to optimize oral intake
	Supplementation with additional oral or IV fluids as needed
	Making a saliva substitute
Mucositis	Developing a diet plan with varying texture/temperature foods and drinks in addition to providing adequate vitamin/mineral supplements to optimize oral intake
	Consideration of enteral or parenteral nutrition if oral intake is insufficient
Diarrhea	Discontinuing oral intake if stool exceeds approximately 2000 mL/d for several days
	Consideration of parenteral nutrition in prolonged lack of oral intake
	Intake of soluble fiber
	Maintaining a low lactose diet and prescribing lactase enzymes in the presence of lactose intolerance
	Developing a diet plan including low-fat, low-insoluble fiber
	Avoiding gastrointestinal irritants/stimulants and irritating disaccharides
Constipation	Development of a diet plan which increases insoluble fiber and fluid intake
	Promotion of physical activity
	Assessment of the need for pharmacological intervention
Anorexia	Eating smaller portions and more frequently
	Consumption of high-calorie snacks

may be served warm and cold instead of hot. Iron supplements and red meat may be reduced as the metallic taste in foods can worsen due to the use of platinum-based chemotherapeutic agents such as oxaliplatin, cisplatin and carboplatin[43]. As these patients have a specific sensitivity to meat, they can flavor them with fruit juice and salad dressings. They may use flavored herbs or spices such as basil or rosemary[63]. Patients who have problems due to metallic flavors can use plastic items instead of metal items[52]. Zinc supplements have been suggested as one of the treatment methods for dysgeusia, functioning in the regeneration process of injured taste buds [64].

Xerostomia

Xerostomia, commonly referred to as dry mouth syndrome, is the result of decreased saliva flow or absence of saliva causing mucosal thirst. Chemotherapy and other anticancer drugs administered during the conditioning regimen may cause xerostomia. When caused by damage to the salivary gland, it can be a lifelong problem. Chemotherapy also causes xerostomia when the salivary glands are exposed to radiation[65]. Patients with xerostomia face symptoms such as mucosal dryness, mouth discomfort and change in taste, chapped lips as well as dry nasal passages. These symptoms can make simple activities such as swallowing, talking and sleeping difficult as well as painful. If xerostomia is left untreated, it can significantly reduce the cellular composition and oral pH, which can increase caries[66].

One of the most important goals of xerostomia treatment is to promote the increase in natural saliva secretion. Patients can take frequent sips of fluids throughout the day to keep the oral cavity moist. If patients do not have open wounds, they may consume sour foods to stimulate saliva secretion. They can consume liquids sip by sip with successive bites during meals. Patients should avoid alcoholic or caffeinated beverages along with alcohol-containing mouthwash and need to maintain efficient oral hygiene.

Patients may use cold steam humidifiers while resting or sleeping[52]. Consumption of soft and room temperature foods is advisable. It is possible to moisten dry foods with broth, sauces, butter or milk. It is important to refrain from dry, packaged or solid foods, acidic or spicy foods, as well as sticky or sugary foods and drinks[66].

Dysphagia

Dysphagia is the change in the swallowing mechanism due to the toxicity of some antineoplastic drugs, and especially the radiotherapy used during the conditioning regimen[67]. The problems often occur in the oral cavity, pharynx, esophagus or esophagogastric passage due to dysphagia[43]. Dysphagia can result in symptoms such as food sticking in the throat or food/fluid entering the airway (aspiration), putting patients at risk of life-threatening pneumonia and negatively impacting their quality of life[67]. Also, it can cause nutrient deficiency, dehydration and even malnutrition[43]. Patients with dysphagia should be referred to a speech and language therapist. Also, it is recommended to include thickeners for liquids in the diet[43].

Mouth and throat pain

Mouth and throat pain are often caused by chemotherapy and radiotherapy. These patients must avoid salty, acidic, hard and dry foods. Consumed food is advised to be prepared soft, at room temperature and in small pieces[63]. Consumption of additional sauces, dressings or broths as well as liquid and juicy foods is also recommended. Individuals undergoing chemotherapy and radiotherapy need to avoid consumption of dry, coarse or rough foods, alcoholic beverages, citrus fruits, caffeine, tomatoes, vinegar and hot peppers[52].

Anorexia

Anorexia is defined as the loss of appetite and early satiety. It can be divided into two categories based on changes in voluntary or nonvoluntary eating control. It is defined as primary anorexia (or anorexia nervosa) if it occurs due to a change in body image perception leading to voluntary rejection of eating. It is defined as secondary anorexia (or disease-specific anorexia) if it occurs as a result of a higher and/or persistent inflammatory response secondary to chronic or acute diseases. Secondary anorexia is common in cancer patients and leads to limited food intake, worse disease outcomes, reduced life quality, and increased morbidity and mortality rates[68]. In HSCT patients, anorexia can be caused from the metabolic effects of cancer and the adverse effects of treatment and depression. It may also be associated with the effects of nausea and vomiting[43].

Patients with anorexia should eat smaller portions and more frequently. These patients also need to consume high-calorie snacks and increase their consumption of foods which they can tolerate better[43]. Energy and protein content of the patients' favorite foods can be increased[52]. In addition, snacks such as cheese, crackers, ice cream, peanut butter, fruit and yoghurt should be kept handy. As beverages create a feeling of satiety, very few drinks should be consumed with meals. Regular exercise can also lead to an increased appetite. Exercise can be done in consultation with a physician[63].

Nausea and vomiting

The etiology of nausea and vomiting in HSCT patients is multifactorial, including delayed gastric emptying, drugs such as opioids, mechanical bowel obstruction, vestibular dysfunction, increased intracranial pressure, metabolic problems and cortical effects such as anxiety and/or depression[69]. These disorders can lead to dehydration, electrolytic changes and prolonged hospital stays as well as nutrient deficiencies due to inadequate food intake. These symptoms may adversely affect the nutritional status of the patient[43].

Antiemetics are routinely prescribed to prevent and control symptoms in order to allow patients to continue to eat and take oral medications. Individuals with severe gastrointestinal symptoms may need nutritional support[42].

The use of behavioral approaches such as relaxation, distraction and relaxation training in addition to utilizing muscle relaxants may be beneficial[69]. Nutritional recommendations should be adjusted according to the diet tolerance of the patient to relieve nausea and vomiting. It is recommended to change foods which cause discomfort and avoid contact of the food by the patient during meal preparation as it may aggravate odor symptoms[43]. Pungent-smelling foods should be avoided[52]. Patients are advised to remain in a half-lying position (sitting or half-sitting, bed-head upright) while eating and to chew food thoroughly. They should avoid excessively

sweet or fatty foods and opt for cold foods. It is recommended to maintain adequate oral hygiene and to eat meals in places with good air circulation[43].

There is insufficient evidence in favor of or against the use of ginger, acupuncture/acupressure and other complementary or alternative therapies for prevention of nausea and vomiting in patients[69]. However, ginger is considered a medicinal herb that has an important role in preventing nausea (antiemetic properties) in patients undergoing chemotherapy. A total of 1.5 g of ginger powder/three doses per day has proven to be efficient in women undergoing chemotherapy for advanced breast cancer [70]. More clinical human studies should be done to establish the effectiveness of this herb. Therefore, there is not yet sufficient evidence to recommend the use of ginger in clinical practice[43].

Diarrhea

Diarrhea is common after high-dose cytoreductive therapy, oral antibiotics, intestinal infections such as cytomegalovirus enteritis or *Clostridium difficile* colitis, intestinal GVHD, lactose intolerance, gastric motility agents and magnesium salts[42,43]. Diarrhea can lead to dehydration by causing abnormal transport of water and electrolytes[43]. Therefore, if not managed well, it may lead to the depletion of fluid and electrolytes, malnutrition as well as hospitalization[52].

Patients with diarrhea should limit lactose, fiber-rich or naturally laxative food intake[71]. Due to the suppression of the immune system in HSCT patients, it is suggested that patients follow all hygiene recommendations during the processing and preparation of foods for these patients and that they minimize the risk of infectious complications in the gastrointestinal tract as much as possible[43]. Consumption of plenty of fluids, frequent and fewer meals, foods such as bananas, peaches, cooked potatoes in addition to cooked carrots with high sodium and potassium content is advised. It is necessary to avoid consumption of fatty foods, fried or raw vegetables and salads, nuts such as peanuts, walnuts, broccoli, dried beans, peas, pumpkin, corn, cauliflower, as well as fruits such as apricots, pears and plums [63]. It is crucial to avoid foods containing sugar and alcohol such as chewing gums (mannitol, xylitol, sorbitol, *etc.*). Increasing water-soluble fiber intake from sources such as applesauce, bananas, peaches, white rice and pasta is recommended[52].

Constipation

The use of opioid is known to cause constipation in HSCT patients[72]. Constipation is thought to be caused by opioids that can cause constipation by different mechanisms, such as increased fluid absorption and inhibition of gastrointestinal secretions. These mechanisms lead to decreased intestinal motility, prolonged contact between the intestinal contents and mucosa, greater absorption of water along with electrolytes, and thus dry stool formation[43].

Patients with constipation need to consume 2 L of fluid every day[52]. Their diet should contain modules of soluble and insoluble fiber. Consumption of cereals, brown rice, whole grain pasta, wheat bran, oat bran, whole grain foods such as flaxseeds and quinoa flakes, legumes (beans, lentils, peas, chickpeas and soybeans), properly washed fresh fruit, leafy vegetables and laxative juices (papaya, orange and prunes) is advised [43]. Daily life activities in addition to physical activities should be supported as much as possible[52].

Sinusoidal obstructive syndrome

Sinusoidal obstructive syndrome is a common and high-risk complication of HSCT. The syndrome is characterized by hyperbilirubinemia, jaundice, weight gain, ascites and painful hepatomegaly, which develops 10–20 d after initiation of cyclophosphamide-based cytoreductive therapy and later after other myeloablative regimens [73].

Its treatment is mainly supportive and contains sodium restriction, diuresis, renal replacement therapy, analgesia and therapeutic paracentesis[73]. Sodium and water balance need to be managed by reducing both oral and intravenous sodium in patients with sinusoidal obstructive syndrome. Continuous renal replacement therapy may be required in patients with impaired renal function. If hypertriglyceridemia (serum triglycerides > 350 mg/dL) develops, the intravenous lipid dose is required to be changed in order to provide 4%–8% of the total energy to prevent essential fatty acid deficiency. The nutritional support level of the patient should also be evaluated to minimize the risk of overfeeding and to ensure optimal carbohydrate metabolism as a lower dextrose load may be seen[42].

Renal complications

Despite the overall improvement in end results after HSCT, renal damage remains a common complication and contributes to procedural morbidity as well as mortality [74]. Acute renal failure is a known complication of HSCT, with a prevalence as high as 70% following HSCT. Renal failure ranges from prerenal failure to acute renal failure requiring continuous renal replacement therapy. Nutritional support goals during renal failure are to prevent malnutrition while minimizing uremic toxicity and other metabolic disorders. Nutritional intervention includes correcting fluid and electrolyte imbalances and maintaining adequate intravascular volume[42]. Patients with severe acute renal failure may require dialysis to achieve these goals, and the choice of a dialysis method often depends on the hemodynamic stability of the patient, the degree of volume overload and blood pressure[74].

Pulmonary complications

Pulmonary complications account for significant morbidity and mortality in HSCT recipients and are associated with infections, regimen-related toxicities along with chronic GVHD[75]. Nutritional intervention for patients who develop transplant-related pulmonary risk includes reducing total sodium intake (oral, intravenous, and medications) and limiting fluids using concentrated dextrose as well as amino acid solutions in addition to lipid emulsions[52]. It is also necessary to evaluate the nutritional support level of the patient to minimize the risk of overfeeding. Finally, for patients with excessive CO_2 production, it may be necessary to adjust the energy ratio from carbohydrate and fat. Pulmonary failure associated with chronic GVHD may increase metabolic requirements causing hypercaloric needs to prevent weight loss and muscle atrophy. Therefore, close nutritional monitoring is required[42].

GVHD

GVHD is one of the most challenging complications in hematological malignancies, especially after allogenic HSCT[76]. GVHD causes excessive diarrhea, abdominal pain, nausea, vomiting, gastrointestinal bleeding, dysphagia and malabsorption. These patients have a higher risk of malnutrition[7].

Standard treatment for GVHD consists of high-dose corticosteroids. In addition to increased appetite and weight gain, dietary adverse effects of corticosteroids include fluid and sodium retention, hyperglycemia, hypertriglyceridemia, hypercholesterolemia, muscle atrophy as well as bone demineralization. In addition, the relationship of high-dose corticosteroid intake with the risk of COVID-19 should not be ignored [76].

Energy requirements are higher in these patients, as energy loss occurs through diarrhea. The diets of these patients should be low in fiber and fat. Also, it is recommended that the diets contain no lactose. Complete bowel rest and total parenteral nutrition are indicated in severe GVHD grade IV and a stool volume > 1.5 L in 24 h[48]. These patients also have high protein requirements. Recommendations range from 1.2 to 2.5 g/kg/d. In the absence of severe renal failure it needs to be targeted at 1.5-2 g/kg/d. Such patients are often deficient in vitamins and trace elements. In addition, these micronutrients require regular measurement to assess their need for supplements[7,48].

Application of a gradual nutritional approach is required on these patients. The first phase consists of total bowel rest and the use of parenteral nutrition until diarrhea resolves. The second phase is the reintroduction of iso-osmotic, low-residue as well as lactose-free beverages to compensate for the loss of intestinal enzymes secondary to changes in the intestinal villi and mucosa. If these beverages are tolerated, the third stage involves the reintroduction of solid foods with low levels of lactose and fiber. In the fourth stage, dietary restrictions are gradually reduced as food intake is slowly resumed and tolerated. The fifth phase is the resumption of the normal diet of the individual[52].

Neutropenia and low bacterial diet/low microbial diet/neutropenic diet

Neutropenia is characterized by either a neutrophil count below 500/μL or below 1000/μL and expected to decrease to 500/μL within 2 d. Certain prophylactic preventions are taken to control the risk of neutropenia infection[43]. Neutropenia, which is associated with a high risk of infection, is a potential adverse effect of chemotherapy administered during myeloablative conditioning regimens. Due to the suppression of the immune system in this process, the risk of developing foodborne infections is high[46].

Although various studies have examined diet and infection risk, the protective benefit of a low microbial diet against infection has not been determined[42]. DeMille *et al*[77] examined the effects of a neutropenic diet on infection in 28 adult patients and reported that there was no significant difference in the rates of febrile admission or positive blood cultures between compliant and noncompliant patients. A study by Moody *et al*[78] compared the neutropenic diet with Food and Drug Administration-approved food safety guidelines in children undergoing aggressive chemotherapy regimens and observed that the infection rates were similar between the groups.

ASPEN recommends providing neutropenic patients with nutritional counseling on foods that may pose a risk of infection and safe food consumption[33]. ESPEN states that there are insufficient consistent data to suggest a low bacterial diet for patients > 30 d after allogeneic transplantation[46]. It is considered sufficient to use safe food processing methods, to avoid raw meat or dairy products and to avoid consumption of raw fruits or vegetables which have not been properly washed and/or peeled in neutropenic patients. Stricter neutropenic diets have shown no benefit on infection rates or survival, which argues against their routine use[35].

There is no standardized protocol in the neutropenia and differences between centers are high[7]. Some cancer centers continue to provide low microbial or low bacterial diets to individuals with low white blood cell counts. In general, autologous patients follow a neutropenic diet for the first 3 mo after transplantation, while allogeneic patients follow a neutropenic diet until all immunosuppression is ceased [42]. Follow-up is recommended for 3 mo after chemotherapy in autologous recipients, up to 1 year after transplant in allogeneic recipients, or longer in the event that they are currently taking immunosuppressants for GVHD[50]. In current recommendations, nutritional education includes dietary counseling on safe food processing and avoiding consumption of foods at risk of infection while patients are neutropenic or until immunosuppressive therapy is completed[52].

Safe cooking methods for HSCT patients include conventional cooking with gas and electric ovens, boiling and pressure-cooking as well as steaming. If conventional gas and electric ovens are used, they should always be preheated to allow fast cooking of food. The cooking time is recommended to be sufficient for the core temperature of the food to reach 70°C. If necessary, this can be checked using a probe on a binary food sample. It should be noted that the probe should not be used on food to be eaten by the patient. If the boiling method is used, it is advisable to put food into boiling water and bring it to the boiling point as soon as possible. If pressure cooking and steaming are preferred, either a home pressure cooker or a large-scale catering steamer can be used[50]. Hypochlorite solutions containing 200 ppm of active chlorine can be used to sterilize fruits and vegetables. After the food is washed, it can be rinsed with purified water[43]. Apart from these recommendations, food restrictions and food safety recommendations to be followed for immunosuppressed HSCT patients are given in Table 4.

After discharge from the hospital, patients will need to follow clean food precautions for 3–6 mo or until their white cell count reaches a level which provides adequate immunity. When clean food prevention is no longer required, patients are often advised to avoid high-risk foods such as unpasteurized milk and dairy products, soft cheeses, live yogurts and shellfish for one month after quitting the diet. Opportunistic infections, including foodborne illness, can occur during periods of immunosuppression[50].

CONCLUSION

HSCT is an intensive and usually long-term medical treatment that presents a wide range of nutritional challenges. It is known that HSCT patients are more vulnerable to infection and therefore to COVID-19 due to many factors such as disease burden and applied conditioning regimens. For this reason, it is essential to closely monitor these patients in terms of COVID-19 risk, to evaluate the procedures to be applied by considering all factors, and to make the final decision multidisciplinary. The current COVID-19 pandemic and issues in the health system as a result of the pandemic can make this complex procedure even more difficult. Therefore, these patients need special care and attention during this period.

Some of the most important problems in HSCT patients are nutrition-related complications and malnutrition that may occur as a result. These patients may face complications that affect nutritional status such as mucositis, dysphagia, xerostomia, GVHD and neutropenia. Each of these situations should be evaluated individually and

Table 4 Nutritional approach in immunosuppressed patients

Food restrictions	
1	Raw and undercooked meat, fish, shellfish, poultry, eggs, sauce
2	Unpasteurized or raw milk, cheese, yoghurt and other dairy products, soft cheeses, delicatessen cheeses, cheeses containing hot peppers or other uncooked vegetables, cheeses with molds
3	Foods containing raw or uncooked meat, tofu, pre-cooked cold meats, pickled fish, tempeh, raw eggs
4	Raw cereal products, unpackaged bread, cakes, pastries, cream cakes, dried fruits, nuts or coconut
5	Unwashed and unpeeled raw fruit, damaged fruit, berry fruits (strawberry, raspberry, blackberry), grapes (unless peeled), unroasted raw nuts, unpasteurized juice, dried fruit
6	Unwashed raw vegetables or herbs, delicatessen salad Herbs and spices should not be sprinkled on food after cooking
7	Well water (unless tested annually and found safe), cold brewed tea made with lukewarm or cold water, prepared and brewed unpasteurized fruit or vegetable juices
8	Uncooled, cream filled pastry products
9	Unwrapped or communally used butter, margarine, spreads or ghee, fresh salad dressings containing aged cheese or raw eggs, stored in refrigerated case
10	Raw or unpasteurized honey, herbal and non-traditional nutritional supplements, brewer's yeast if eaten without cooking
Food safety recommendations	
General	Particular care should be taken when handling, preparing and consuming food to avoid contracting a foodborne illness. 4 steps to follow in terms of food safety: (1) Cleaning: Washing hands, surfaces and products; (2) Separation: Avoiding cross-contamination; (3) Cooking: Cooking at proper temperature; and (4) Chilling: Refrigerate immediately
Shopping	Always check expiration dates. Avoid purchasing food close to its expiration date and never consume after the specified date. Do not buy damaged packaged food such as crushed boxes or ripped packages. Do not taste foods in supermarkets. Buy perishable foods first, then frozen perishables and finally refrigerated perishable products. Check for water on the floor by meat stands, freezers or refrigerators. Avoid hot places when carrying food
Storage	Store raw and cooked food separately. Check if your fridge and freezer are at the right temperature. Adjust refrigerator temperature to below 5C, freezer below 18C. Discard wastes after 3 d of refrigeration. Never leave perishable products out of the refrigerator for more than 2 h
Preparation	Wash hands thoroughly with hot water and soap before and after touching food. Use separate cutting boards to prepare beef, fish, chicken or vegetables. Prefer boards made of glass to wooden ones. Use only treated water to prepare food and clean the surface before you start preparation. Make sure canned food is clean. Wash cans before opening
Cooking	Avoid using a microwave for cooking; it can be used to heat food or defrost frozen food. Cook food thoroughly; pay special attention to larger portions and thicker cuts of meat. When warming pre-prepared food, check that the temperature inside the product is the same. Never reheat preheated food. Never re-freeze thawed frozen food
Eating out	It is safer to avoid eating and drinking outside while on a clean food diet. If you have to eat out, use sauces, condiments, salt and sugar only in disposable bags. Check that the toilets are clean and that there is soap to wash hands. Avoid iced drinks of unknown origin

an appropriate nutritional approach must be recommended by the nutritionist. There are limited numbers of observations regarding the nutritional approach in HSCT patients with COVID-19 and there is no specific guideline on this issue. However, appropriate nutritional intervention is recommended as an integral part of the approach to treating COVID-19 patients. The nutritional approach specific to HSCT patients should not be ignored during this nutritional approach.

To reduce the risk of harm to highly vulnerable groups of patients who await or have undergone a potentially life-saving HSCT procedure, the publication of new guidelines and new clinical studies along with international exchange of information in this area is necessary.

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MINIREVIEWS

Stem cell therapy and diabetic erectile dysfunction: A critical review

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Abstract

Erectile dysfunction (ED) has been identified as one of the most frequent chronic complications of diabetes mellitus (DM). The prevalence of ED is estimated to be about 67.4% in all DM cases worldwide. The pathophysiological process leading to ED involves endothelial, neurological, hormonal, and psychological factors. In DM, endothelial and neurological factors play a crucial role. Damages in the blood vessels and erectile tissue due to insulin resistance are the hallmark of ED in DM. The current treatments for ED include phosphodiesterase-5 inhibitors and penile prosthesis surgery. However, these treatments are limited in terms of just relieving the symptoms, but not resolving the cause of the problem. The use of stem cells for treating ED is currently being studied mostly in experimental animals. The stem cells used are derived from adipose tissue, bone, or human urine. Most of the studies observed an improvement in erectile quality in the experimental animals as well as an improvement in erectile tissue. However, research on stem cell therapy for ED in humans remains to be limited. Nevertheless, significant findings from studies using animal models indicate a potential use of stem cells in the treatment of ED.

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Core Tip: Erectile dysfunction (ED) is one of the most frequent complications of diabetes mellitus in males, which interferes with the patient's quality of life. Current available treatments, whilst improve the symptoms, are not able to repair damages of the affected tissues. Stem cells have the potential ability to renew and repair damaged endothelial cells and penile tissue in the diabetic ED. Recent studies have provided promising results on the use of stem cells to treat this condition. This article reviews current advances on this area of research.

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INTRODUCTION

Erectile dysfunction (ED) is defined as the persistent or recurrent inability to attain or maintain an adequate erection to reach satisfaction in sexual intercourse[1]. Although this condition does not directly coincide with serious health problems, it correlates with the decline in the quality of life[2]. Moreover, ED has been determined to be more common in men with diabetes mellitus (DM). Previous studies have shown that the prevalence of ED in DM is very high, *i.e.*, at 67.4%, among those with DM[3]. Thus, DM can be regarded as a major risk factor for acquiring sexual dysfunction in men, increasing the risk of ED in men with DM three times higher than that in those without diabetes[4].

MECHANISM OF DIABETIC ED

Erection is a condition when the penile organ becomes rigid and elevated as a result of the erectile tissue being filled with blood. It is modulated by several mechanisms involving endothelial cells and the autonomic nervous function. To better understand the pathogenesis of ED, we need to comprehend the physiological process of erection, as discussed briefly below.

Three key processes occur during erection: (1) The neurologically mediated arterial inflow; (2) The relaxation of smooth muscle within the corpora spongiosa to allow the blood to fill the penile vasculature; and (3) The obstruction of the veins to retain the blood within the penile vasculature[5].

In response to sexual stimuli, the brain transmits parasympathetic signals through the spinal cord. The signal then reaches the non-adrenergic non-cholinergic neuronal terminals, which will then induce the production of nitric oxide (NO) by neuronal NO synthase (nNOS). Likewise, the endothelium also produces NO *via* the activation of endothelial NO synthase (eNOS). eNOS and nNOS act as the endothelial and neuronal regulators, respectively. Then, the NO will be transported to the corpora spongiosa where it converts GTP to cyclic guanosine monophosphate (cGMP) with the help of the guanylate cyclase enzyme. The cGMP induces smooth muscle relaxation, allowing arterial flow to fill the cavernosal space. cGMP also potentiates protein kinase G activity that leads to free intracellular calcium being taken up by the endoplasmic reticulum, causing a decrease in calcium flux and an increase in potassium flux from the cells and in turn causing depolarization followed by the relaxation of vascular smooth muscle. When the cavernosal space congests with blood, the veins become compressed and occluded which traps the blood in the cavernosal space during tumescence[1,5,6].

Based on its mechanism, the pathological causes of diabetic-induced ED can be classified into several different categories, *i.e.*, endothelial, neuronal, and endocrinal causes and others, such as psychological and diabetic-related infections and multiple drug prescriptions. However, all of these dysfunctions can intertwine to form ED (Figure 1).

Endothelial factors

One of the most important factors that contribute to the development of diabetic ED is endothelial dysfunction. Endothelial dysfunction is primarily characterized by the loss of NO biological activity and/or biosynthesis at the cellular level, but it may also refer to the reduction in endothelial-dependent vasodilatory response of the smooth muscle cells[7]. The reduction in NO bioavailability is primarily caused by the reduced activity and/or low level of eNOS expression. One possible factor that affects the impairment of eNOS function in diabetic ED is the specific glycosylation process that incapacitates the activation of vascular endothelial growth factor (VEGF) signaling[8]. VEGF is proven to be a survival factor for endothelial cells[9] and also contributes to the upregulation of eNOS expression at the molecular level[10]. Despite its important role in modulating eNOS function, it seems that VEGF is not involved in the regulation of nNOS[11,12]. Meanwhile, the role of the RhoA-ROCK (Rho-associated protein kinase) complex in the development of ED has also been comprehensively studied in the past few years. ROCK, which is activated by RhoA, regulates myosin light chain phosphatase (MLCP) *via* phosphorylation. This leads to the deactivation of MLCP and subsequently promotes the contraction of the cavernosal body by lowering the levels of calcium in smooth muscles, facilitating the chronic-tonic contraction, thus maintaining the flaccid state of the penis[13]. ROCK has two isoforms: ROCK-1 and ROCK-2. Chiou *et al*[14] have shown that the induction of type 2 diabetes in rats resulted in the downregulation of eNOS, nNOS, and protein kinase G and the upregulation of the RhoA-ROCK pathway, particularly ROCK-1 isoform. Recently, studies have been conducted to assess the effects of ROCK inhibitors on diabetic ED. Treatment with ROCK inhibitors (SAR407899) has been found to induce penile erection through mechanisms independent of eNOS activity. Thus, this treatment can be targeted to cases of diabetic ED where eNOS activity is impaired[14].

The RhoA-ROCK pathway is also linked with the endothelin-1 (ET1)-induced vasoconstriction[15]. ET1 is a powerful vasoconstrictor, which is released from the penile vascular endothelium. In diabetic patients, the level of plasma ET1 is high[16], which may induce the constriction of the penile blood vessels. ET1 Level may also be elevated in response to the increase in oxidative stress in DM. Chronic hyperglycemia induces the release of free radicals (reactive oxidative species) due to the formation of advanced glycation end products (AGEs). This may contribute to the development of diabetic ED through oxidative cell damage and the suppression of NO action[17,18].

These processes will affect the elasticity of the blood vessels. This may be crucial in the development of ED since elastic blood vessels are important to enable better erections. Moreover, endothelial damage can cause plaque formation and subsequently the blockage of blood vessels. This blockage will result in the inability of the blood vessels to expand properly, thus leading to lower blood capacity and decreased speed of blood flow, resulting in reduced erectile ability.

Neuronal factors

The neuronal aspects of diabetic ED are less well understood due to lacking diagnostic tools available to support this etiology. Several studies have been conducted to assess nocturnal penile erection and whole sexual cycle *via* magnetic resonance imaging and positron emission tomography scan to characterize the disorder in the central aspects of erection[19]. Diabetic neuropathy is proven to be an important factor in the development of diabetic ED.

In DM, microangiopathy and nerve damage are caused by increased oxidative stress, accumulation of AGEs, impaired axonal transport, increased flux through the polyol pathway, altered protein kinase C activity, and poly(ADP-ribose) polymerase activity[20]. In a study comparing the significant microangiopathy and macroangiopathy factors in DM, the results indicated that microangiopathy factors, especially diabetic neuropathy, have a more significant impact than macroangiopathy factors in the development of diabetic ED[6].

Endocrine factors

Hypogonadotropic hypogonadism occurs in approximately 30%-40% of men with type 2 diabetes[21]. This condition reduces the production of testosterone, which has been identified to stimulate the synthesis, storage, and release of pro-erectogenic

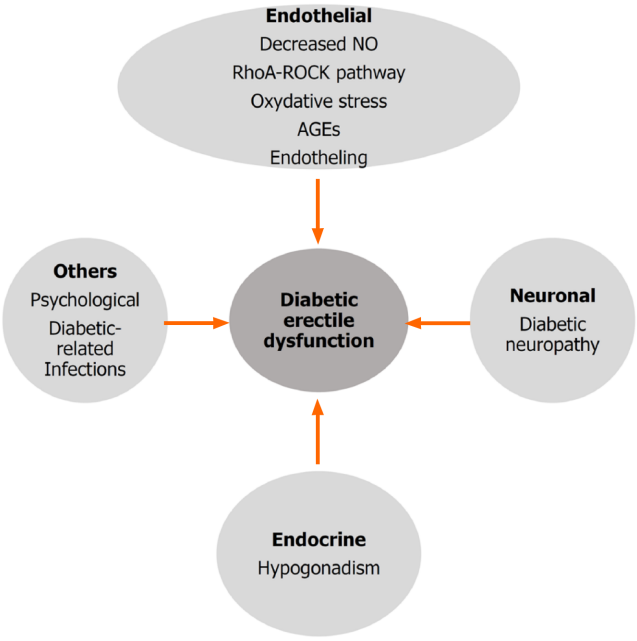

Figure 1 Factors that affect diabetic erectile dysfunction. Major factors that affect the development of diabetic erectile dysfunction include endothelial damage, neuronal dysfunction and endocrine abnormality. Other factors such as psychological factor and diabetic-related infections may also affect the development of diabetic erectile dysfunction. NO: Nitric oxide; AGEs: Advanced glycation end products.

neurotransmitters; modulate neuronal activity, receptor sensitivity, neurotransmitter liberation, and socio-sexual behavior (increasing libido); and positively influence the levels of dopamine, NO, oxytocin, *etc.*[1]. In a population-based cohort, the prevalence of hypogonadism in men with ED was 35% compared with 22.7% in men without ED [22]. A study in Japan conducted by Imai *et al*[23] showed that decreased testosterone has been associated with severe or moderate ED as opposed to men with mild or no ED and is a risk factor for the development of ED.

EVALUATION OF ED

Decreased erectile capacity is the most common symptom of patients with ED. The standard way to evaluate ED is by using the International Index of Erectile Function-5 (IIEF-5) or Erectile Hardness Score (EHS) criteria. IIEF-5 has five questions that can be used to evaluate a person's sexual life. This questionnaire has a sensitivity of 98% and a specificity of 88%[24].

Moreover, the EHS scoring system can also be used to evaluate the progression of ED following treatment as this scale is convenient to use. The EHS scale categorizes ED into five levels of erectile ability: (1) If the penis does not change; (2) If the penis is slightly enlarged without hardening; (3) If the penis is enlarged and hardens but cannot be used for sexual intercourse; (4) When the penis is enlarged, hardens, and can be used for sexual intercourse, but not maximally; and (5) If the penis is enlarged and hardens to the maximum[25].

In various studies using experimental animals, the evaluation of ED can be performed at the molecular and tissue level, starting from intracavernosal pressure measurement, assessment of angiogenesis process using certain markers, and analysis of erectile tissue profile including the blood vessel content and smooth muscle structure. Other molecular processes can also be used as parameters in the evaluation of ED.

TREATMENT APPROACHES OF DIABETIC ED

Lifestyle modifications

In an effort to regain the capability of erection in diabetic ED, many approaches in terms of lifestyle change have to be applied. Long-term control of glycemic levels is

important. Using HbA1c as an index of hyperglycemia, Cho *et al*[26] showed a significant connection between the severity of ED and the level of HbA1c. Physical activity has beneficial effects on the prevention and/or improvement of ED in several prospective studies[27]. With regard to consistent physical activity, weight loss in obese or overweight diabetic patients is strongly correlated with ED, as observed in a prospective study with a study duration of 5 years to 25 years showing that overweight and obese individuals displayed an increased probability in developing ED compared to individuals with normal weight[28]. Importantly, smoking[5] and alcohol appear to be risk factors for ED. In a recent study focusing on alcohol intake and erectile function, it was shown that moderate alcohol consumption conferred the highest protection of erectile function[29].

Pharmacological approach

Phosphodiesterase-5 (PDE5) inhibitors are the first-line therapy for treating ED. The mechanism of the PDE5 inhibitor drug is to inhibit the PDE5 enzyme in vascular smooth muscle cells, thus preventing cGMP degradation to GMP resulting in a sustained erection as the penile blood vessels will be kept dilated. Currently, there are four different types of PDE5 inhibitors available for ED treatment: sildenafil, vardenafil, tadalafil, and avanafil[5].

The most common adverse effects related to the treatment with PDE5 inhibitors are headache and flushing due to the vasodilatory effects of PDE5 inhibitors on the blood vessels. Abnormal vision is experienced by about 6% of individuals taking sildenafil, which could be attributed to the inhibitory action of the drug against PDE6, which is abundant in the retina[5]. On the other hand, back pain and muscle cramps are common adverse effects of tadalafil[5].

In the early 1980s, the first report of intracavernosal injection with papaverine was published and opened a new line of treatment of ED[30]. This second line of treatment is normally used for patients who demonstrated low response or low effectivity or those displaying severe side effects following PDE5 inhibitor treatment. Drugs that are available for ICI are alprostadil (10 mcg, 20 mcg, 40 mcg) and papaverine. ICI treatment induces vasodilation in the arterial smooth muscles, inducing blood flow and blood entrapment within the lacunar spaces of the penis. This treatment has shown better efficacy than oral pharmacological treatment. However, the dropout rate for ICI therapy remains relatively high, and it may be associated with priapism, ecchymoses, hematoma formation, and penile fibrosis[31]. The intraurethral application of alprostadil is also used for second line treatments of ED[30]. The most common side effects of this therapy are penile pain and urethral burning sensation.

Vacuum and surgical approach

Vacuum therapy uses negative pressure to distend the corporal sinusoids and to increase blood inflow to the penis[32]. However, studies that evaluate the use of vacuum constriction device (VCD) reported up to 30% patient dropout due to inadequate rigidity, penile pain, difficulty to ejaculate, and aesthetic causes[33].

The implantation of penile prosthesis as a surgical therapeutic approach for ED is used when the pharmacological (oral, injection, or trans-urethral) and mechanical (VCD) approaches do not achieve satisfactory effects or induce side effects that bothered the patient. The implantation of prosthesis provides a reliable and predictable erection and the highest satisfaction rate to both partners when compared with all of the treatments in ED[34].

All of the treatments available for diabetic ED are developed to achieve sexual satisfaction by improving the erection, but none of them have the capacity to repair the endothelial blood vessels in patients with diabetic ED. New therapeutic strategies to address the main problem in ED, for example, regenerative therapy, have not been widely explored, despite their potential to improve endothelial function. Regenerative therapy, such as stem cell-based therapy, has been identified to have the potential to address the root of the problem in diabetic ED. In the next part of this review, we discuss this therapeutic approach for the treatment for diabetic ED.

STEM CELLS AS A FUTURE THERAPY FOR DIABETIC ED

Ernst Haeckel first introduced stem cells (*stammzelle* in German) in 1868 to delineate unicellular organism from which all multicellular organisms originated[35]. Since then, many studies were conducted to identify different types of stem cells and their potency to treat various human diseases. In 2006, Yamanaka successfully developed

induced pluripotent stem cells (iPSCs), which were derived from adult somatic cell reprogramming, increasing the hope of using this type of stem cells as a therapeutic approach for many chronic diseases[35]. Stem cells are unique because they have several features such as the capability to self-renew, extensive proliferation capacity, the potential to differentiate to different cell types, the ability to minimize DNA damage through several DNA repair system, and the ability to maintain a very low metabolism to reduce reactive oxygen species level (quiescent stem cells)[35-37].

Classification of stem cells

Stem cells are undifferentiated cells which are able to differentiate to specialized cell types because of their ability to self-renew and to differentiate to one or more cell lineages. Stem cells can be classified based on their origin and potency[36,38]. To date, adipose-derived mesenchymal stem cell (ADSC) is considered as one of the most frequent cells used in the field of uro-andrology because it is abundant and easy to obtain[39-41]. Moreover, ADSCs have anti-inflammatory properties and an ability to repair vascular and nerve damage, which are the hallmarks in the development of diabetic ED[42].

Three fundamental aspects in stem cell therapy, including that for diabetic ED, need to be considered. These include (1) the origin of the stem cells, tissue biopsy, and cell harvesting, (2) *ex vivo* stem cell culture and clonal expansion through numerous distinct growth signals to produce specified cell type that we need, and (3) the delivery of these stem cells to the organ of interest[38,40].

Different routes of delivery are still being investigated to determine which one gives the best results. So far, several preclinical and clinical trials using various stem cell types have been conducted.

After delivery, two different mechanisms may occur in the tissues following stem cell implantation. First, the stem cells may differentiate to specific cells and hence, regenerate the damaged tissue. Second, stem cells may secrete various growth factors through the paracrine pathway[40,43], which can induce propagation and the differentiation of resident progenitor cells resulting in the repair of damaged tissue[43].

Current progress in stem cell therapy for diabetic ED

We have described that there are currently three main approaches to ED therapy which have been adopted worldwide, these are oral medication, intra-cavernosal injection and vacuum devices and penile prosthesis. However, all of these available options can only ameliorate the symptoms without restoring the damage of the penile tissue[39]. Stem cell therapy may address this problem.

The use of stem cells for the treatment of ED has been developed in several cases of ED post-prostatectomy. Several studies have reported many cases of cavernous nerve damage following prostatectomy[44], and the use of stem cells is now being investigated for the treatment of diabetic ED. It is estimated that ED occurs in approximately 67.4% of all cases of DM[3], illustrating that ED requires effective treatment to improve the quality of life of many patients with diabetes affected with ED.

Erection occurs as a result of the release of NO from sinusoidal endothelial cell walls. The vasodilation that occurs in these blood vessels in response to NO causes the compression of venules located between the trabecula and the tunica albuginea so that the blood becomes trapped within the blood vessels[43]. In simpler terms, it can be described that endothelial cells, cavernous smooth muscle cells, NO and nerve cells are key factors in erection, and DM damages these components. Stem cells, which have the ability to differentiate to specialised cells, may have the ability to correct these defects and restore proper erectile function[43]. This is the rationale for using stem cells as a therapy for ED in the future. However, the underlying mechanism still cannot be fully explained and needs further exploration.

Stem cell research in cases of ED has been pursued since the 2000s. However, most studies were limited to experimental animals. Of the 25 studies reviewed in this article, we found that 21 were conducted on animals (Table 1) and four on humans (Table 2). Of those experiments on animals, the stem cells used were isolated from adipose tissue, bone and human urine. Further, the type of stem cells used was autologous and allogenic, and the average number of stem cells injected per animal was 1×10^6 cells, which were delivered intra-cavernosally. However, the number of cells injected among these studies ranged from 2×10^6 to 5×10^5 cells per animal[46,47]. Moreover, in addition to stem cell therapy, several studies added growth factors, hormones and drugs, including insulin[48], magnetic iron oxide nanoparticles[49], microtissue[50], inducible NOS[47], myocardin[51], icariin[52], corin[53], exosomes from corpus cavernosum smooth muscle cells[54], fibroblast growth factor 2 (FGF2)[55], stromal cell-derived cell factor-1 (SDF-1)[56], VEGF[57] and hepatocyte growth factor[45].

Table 1 List of articles on stem cell therapy in animal models of diabetic erectile dysfunction

Ref.	Animal model (species, age, *n* number)	ED model	Evidence of previous DE	Type of stem cell	Source of stem cell	Characterization of stem cell	Number of cells injected and site of injection	Modification	Duration of follow-up	Parameter of therapy	Conclusion
Garcia *et al*[68]	10-wk-old Zucker (fa/fa) rats; *n* = 10 in each group (ADSCs and control)	Obese and type 2 diabetic rats with ED	Yes, CN electrostimulation	ADSCs; Autologous	Perigonadal adipose tissue	Not clear	1×10^6/IC	None	3 wk	ICP-MAP, nNOS, corporal body collagen, and corporal body endothelial cell	ADSCs improve ED
Liu *et al* [57]	10-wk-old male Sprague-Dawley rats; *n* = 12 in each group (lentivirus-VEGF, GFP ADSCs, VEGF/GFP ADSCs, control)	Type 1 diabetes induced by streptozotocin with ED	Yes, APO procedure	ADSCs; Allogenic	Bilateral groin	Yes, by flow cytometry	1×10^6/IC	VEGF, GFP	3 wk	ICP-MAP, VEGF, and e-NOS *in vivo* Endothelial, smooth muscle, and pericyte marker	Injection of ADSCs expressing VEGF displayed more efficiently and significantly raised ICP-MAP and increased endothelial markers
Ouyang *et al*[55]	Male Sprague-Dawley rats unspecific age; *n* = 10 in control group, *n* = 15 in each treatment group (USCs, lentivirus FGF2, USCs-FGF2)	High-fat diet following streptozotocin-induced diabetes	Yes, APO procedure	HUDSCs	Human urine	Yes, flow cytometry (CD24, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD146)	1×10^6/IC	FGF-2	4 wk	ICP-MAP, expression of endothelial markers (CD31, VEGF, and eNOS), smooth muscle markers (desmin and smoothelin), histological changes	USCs or USCs-FGF2 improved erectile function in type 2 diabetic rats
Li *et al*[69]	8-wk-old BALB/c mice; *n* = 8 in each group (control and treatment)	Streptozotocin-induced diabetes with ED	Not clear	BMSCs; Allogenic	Unspecified bone marrow	Yes, by flow cytometry. Identification with Sca-1 micromagnetic, identification of CD29, CD44, CD13, and CD34	Not mentioned	None	3 wk	ICP-MAP, penile histology	Flk-1 Sca-1 MSCs differentiate into skeletal and endothelial cells *in vivo* and *in vitro*
Liu *et al* [45]	Male Sprague-Dawley rats with no mentioned age; *n* = 10 in each group (negative control, ADSCs, ADSCs + hepatocyte growth factor)	Streptozotocin-induced diabetes with ED	Not clear	ADSCs	Not mentioned	Yes	2×10^6/IC	Hepatocyte growth factor	4 wk	ICP-MAP, smooth muscle, and endothelium (PECAM-1, SMA), apoptotic index	Significantly enhance the erectile function
Wang *et al*[70]	10-wk-old Male Sprague-Dawley rats; *n* = 15-16 in each group (control, normoxia AMCS, and hypoxia AMCS)	Streptozotocin-induced diabetic mouse	Yes, APO procedure	ADSCs; Allogenic	Inguinal adipose	Yes, fluorescent-activated cell sorting (CD90, CD29, CD34, CD45)	1×10^6/IC	Normoxia-hypoxia	4 wk	ICP-MAP, n-NOS, endothelial and smooth muscle histology	Hypoxia AMSCs improved ICP-MAP, nNOS. Hypoxia AMSCs condition effective to enhance theuraphetic effect of ED

Kovanecz *et al*[71]	7-mo-old Zucker (fa/fa) rats; *n* = 8 in each group (untreated, early diabetic treated with SC, early diabetic with high glucose treated with SC, late diabetic treated with SC, non-diabetic untreated)	Not mentioned specifically	Not clear	MSDCs; Allogenic	Hindlimb muscles	Not clear	1×10^6/IC	Early and late diabetes	8 wk	ICP-MAP, nNOS-eNOS, collagen ratio, calponin, inflammation marker	Stem cell decreased collagen and fat infiltration, upregulated nNOS-eNOS, and improved erectile function
Ryu *et al* [46]	12-wk-old C57BL/6J mice; *n* = 6 in each group (control, diabetic, diabetic with PBS, and diabetic with BMSCs)	Streptozotocin-induced diabetic mouse	Not clear	BMSCs; Allogenic	Tibiae and Femur	Yes, flow cytometry (CD3, CD44, CD45, CD103, CD105, CD117, MHC-1, and Sca-1)	3×10^5/IC	None	2 wk	e-NOS-nNOS, endothelial and smooth muscle content histology	BMSCs improved significant recovery of erectile tissue
Zhou *et al* [48]	8-wk-old male Sprague-Dawley; *n* = 5 in each group (negative control, ASCs + ad-luc-myocardin, ASCs + ad-luc, ED without treatment)	Streptozotocin-induced diabetic mouse	Not clear	ADSCs; Autologous	Paratesticular fat tissue	Not clear	1×10^6 ADSC/IC	Insulin and neutral protamine hagedorn	4 wk	ICP-MAP, AGEs, and RAGE; growth factors and cytokine in penis	ADSCs combined with insulin improved erectile function and pathological changes
Wang *et al*[52]	10-wk-old male Sprague-Dawley rats; *n* = 14-15 in each group (negative control, icariin, ADMSCs, ADMSCs + icariin)	Streptozotocin-induced diabetic mouse	Yes, APO procedure	ADSCs; Allogenic	Inguinal adipose	Yes, fluorescent-activated cell sorting (CD90, CD29, CD34, CD45)	1×10^6/IC	Icariin	4 wk	ICP-MAP, histology and immunohistology of penis tissue, intracellular ROS levels	Icariin-enhanced ADSCs in erectile function Icariin could protect ADSCs against oxidative stress
Zhou *et al* [50]	8-wk-old male Sprague-Dawley rats; (*n* = 8 in control, *n* = 20 in treated groups)	Streptozotocin-induced diabetic mouse	Yes, APO procedure	ADSCs; Allogenic	Paratesticular fat tissue	Not clear	1×10^6 ADSC and 1×10^4 ADSCs per MT/IC	Microtissues (MTs)	4 wk	ICP-MAP, nNOS, smooth and endothelial content histology	MTs improved histopathology and erectile function rather than traditional ADSC
Zhu *et al* [49]	10-wk-old male Sprague-Dawley rats; *n* = 8-10 in each group (non-diabetic controls, diabetic with PBS, ADSCs, ADSCs + Magnetic application	Streptozotocin-induced diabetic mouse	Not clear	ADSCs; Autologous	Paratesticular fat tissue	Yes, by flow cytometry (CD34, CD45, CD44)	1×10^6 ADSC	Magnetic iron oxide nanoparticle	4 wk	ICP-MAP, contents, smooth muscle (α-SMA), endothelium (von Willebrand factor), VEGF	ADSCs improved erectile function External magnetic field improved efficiency of labeled ADSC in the corpus cavernosum
Jeon *et al* [56]	8-wk-old male Sprague-Dawley rats; *n* = 12 in each group (control, DM ED, BM-MSC, SDF-1 (stromal	Streptozotocin-induced diabetic with ED	Not clear	BMSCs	Not mentioned	Not mentioned	1×10^6/IC	SDF-1	4 wk	ICP-MAP, nNOS-eNOS, FGF-VEGF *in vivo*	SDF-1 improved ED recovery and smooth muscle content, increased nNOS-eNOS, FGF-VEGF

	cell-derived factor-1)										
Chen *et al* [42]	Male Sprague-Dawley rats with unspecific age; *n* = 10 in each group (ADSC, BMSC, and control)	ED in type 2 rats with diabetes induced by high-fat and high-sugar diet and streptozotocin	Yes, by APO procedure	ADSCs and BMSCs; Allogenic	Medullary cavity of femur, tibia, and fibula	Yes, by flow cytometry Identification with CD34,CD45,CD73,CD90, and CD105	1×10^6/IC	None	2 wk	ICP-MAP, number of blood vessels, collagen	Stem cell improved ICP-MAP, increased the number of blood vessels, and reduced collagen content
Ouyang *et al*[72]	Male Sprague-Dawley rats with unspecific age; *n* = 8 in each group (control and treatment)	Streptozotocin-induced diabetes	Yes, APO procedure	HUDSCs	Human urine	Yes, flow cytometry and Western blot (CD63 and protein calnexin)	Not mentioned	None	4 wk	ICP-MAP, eNOA, phospho-eNOS, nNOS, and endothelial markers (CD31)	Human-derived stem cells improved ICP, eNOS, nNOS, and endothelial markers
Zhang *et al*[51]	Male Sprague-Dawley rats; *n* = 10 in each group (negative control, ASCs + ad-luc-myocardin, ASCs + ad-luc, ED without treatment)	Streptozotocin-induced diabetic mouse	Yes, APO procedure	ADSCs; Allogenic	Inguinal fat tissue	Yes, flow cytometry (CD29, CD90, CD34, and CD45)	1×10^6/IC	Ad-luc-myocardin modified	3 wk	ICP-MAP, collagen and smooth muscle histology: myocardin, collagen 1, cleaved caspase 3, α-SMA, and calponin	Myocardin enhanced therapeutic for ASCs for ED in diabetic mouse
Zhang *et al*[58]	CCECs culture, not specific rats; *n* = 15 each group (case and control)	CCECs treated with advanced glycation end products and streptozotocin-induced diabetic mouse	*In vivo* CCECs mimicking diabetic and APO procedure	HUDSCs	Human urine	Yes, by flow cytometry (CD24, CD31, CD34, CD44, CD45, CD73, CD90, CD105)	Not mentioned	None	4 wk	ICP-MAP, eNOS, p-NOS, VEGFRA, VEGFR2, authophagic flux	Urine stem cell improved cavernosal endothelium through upregulated authophagic activity
Zhang *et al*[47]	8-wk-old male Sprague-Dawley rats; *n* = 9 in each group (control group, DM with ED, ADSCs, ADSCs-EFGP, ADSCs + iNOS)	Streptozotocin-induced diabetic mouse	Yes, APO procedure	ADSCs; Allogenic	Inguinal fat tissues	Yes, by flow cytometry (CD2, CD31, CD49, CD90, CD106, CD34, CD45, CD73)	5×10^5/IC	EFGP and iNOS	2 wk	ICP-MAP, endothelial and smooth muscle content marker (NO, collagen-I, collagen IV, TGF-β, β- actin)	ADSCS-iNOS significantly reduced penile fibrosis
Song *et al* [54]	Male Sprague-Dawley rats; *n* = 7-8 in each group (BMSC-EXO, ADSC-EXO, CCSMC-EXO, control)	Streptozotocin-induced diabetic mouse	Yes, APO procedure	ADSCs and BMSCs; Allogenic	Inguinal adipose and rat femoral	Yes, flow cytometry (CD29, CD34, CD45, and CD90	100 μg of exosome/IC	Exosome CCSMC	4 wk	ICP-MAP, α-SMA collagen, TGF-β1, β-actin, e-NOS, and n-NOS, NO, and cGMP	CCSMC-EXOs improve erectile function, reducing collagen deposition and expression of eNOS-nNOS, NO, and cGMP
Wang *et al*[53]	Male Sprague-Dawley rats with unspecified age; no data on n number, four experimental groups in total (negative control, DM, DM +	Streptozotocin-induced diabetic mouse	Yes, but not mention what method	ADSCs; Allogenic	Not Mention	Yes, CD29, CD90, CD44, CD105, and von Willebrand factor	100 μL of exosome from ADSCs/IV	siCorin ADSC-EXO	2 wk	ICP-MAP, neurovascular function (ANP, BNP, nNOS, cGMP, β-actin) , inflammatory factor (IL6, IL1β), expression, delivery of corin	ADSC-EXO enhanced endothelial recovery and decreased inflammatory process

	ADSC-EXO, DM + siCorin-ADSC-EXO)

APO: Apomorphine; ADSCs: Adipose-derived stem cells; BMSCs: Bone marrow stem cells; CCECs: Cavernosal vascular endothelial cells; CCSMC: Corpus cavernosal smooth muscle cell; DM: Diabetes mellitus; ED: Erectile dysfunction; HUDSCs: Human urine-derived stem cells; IC: Intracavernosal; ICP-MAP: Intracavernosal pressure-mean arterial pressure; MSCs: Muscle-derived stem cells; PMSCs: Placental matrix stem cells.

Table 2 List of articles on stem cell therapy in humans with diabetic erectile dysfunction

Ref.	Study subject and n number	Type of ED	Evidence of previous DE	Type of stem cell	Source of stem cell	Stem cell characterization	Number of cells injected and site of injection	Modification	Duration of follow-up	Parameter of therapy	Conclusion
Bahk *et al* [61]	Impotent diabetic type 2 patients range from 57 yr to 87 yr; n = 7 in treated groups, n = 3 in controls.	Diabetic ED	Yes, by clinical diagnosis and other parameters	UCBSCs; Allogenic	Umbilical cord	Yes, by flow cytometry (CD13, CD14, CD34, CD31, CD45, CD44, CD49e, CD54, CD90, CD106, and HLA-DR)	1.5×10^7/IC	None	9 mo	IIEF-5, SEP, GAQ, erection diary, blood glucose diary	Umbilical cord blood stem cell had positive effects on ED
Levy *et al* [59]	Men aged 40-70 yr who had organic ED at least 6 mo; n = 8	Diabetic ED	Yes, by clinical diagnosis and other parameters	PM-MSCs; Allogenic	Placenta	Not clear	1 mL PM-MSCs /IC	None	6 wk, 3 mo, 6 mo	Peak systolic velocity, end-diastolic velocity, stretched penile length, penile width, IIEF-5	The treatment may be beneficial
Al demour *et al*[60]	Men aged 25-65 yr who had ED at least 6 mo; n = 4	Diabetic ED	Yes, by clinical diagnosis and other parameters	BMSCs; Autologous	Iliac crest	Yes, positive for CD90, CD105, CD73, and CD44	15×10^6/IC	None	2 yr	IIEF-5, EHS	Stem cell effective and improved erectile function
Protogerou *et al*[62]	Male with ED due to DM, hypertension, hypercholesterolemia, and Peyronie's disease; n = 5 in each group (control and ADSC treated)	Diabetic ED	Yes, by clinical diagnosis	ADSCs; Autologous	Not mentioned	Yes, alizarin red S and alcian blue staining, flow cytometry	2×10^5/IC	None	3 mo	IIEF-5, penile triplex	No difference in IIEF-5 between the case and control group, but significant between before and after treatment in both groups

ADSCs: Adipose-derived stem cells; BMSCs: Bone marrow stem cells; ED: Erectile dysfunction; GAQ: Global assessment questions; IIEF-5: International index of erectile function; PMSCs: Placental matrix stem cells; SEP: Sexual encounter profile; UCBSCs: Umbilical cord blood stem cells.

All of the studies have reported that stem cells provide improvements of the erectile function both functionally and structurally in various animal models. As eNOS and nNOS play a major role in penile erection, these two factors are used as parameters in stem cell studies. Nearly all studies have reported improved levels of these two

factors. This indicates that stem cells improve endothelial function at the molecular level.

Moreover, factors, such as VEGF, FGF, and von Willebrand factor, are believed to be vital in the improvement of ED in these studies. The increase of these factors suggests an induction of angiogenesis in the penile tissue. Furthermore, stem cells are also reported to reduce the fibrotic process in penile erectile tissue as well as modulate autophagy process[58]. Autophagy was reported to increase following stem cell treatment, which is believed to be a sign of tissue recovery and regeneration. All of these findings indicate that stem cells play a role in cell regeneration and recovery by increasing the levels of growth factors and suppressing inflammatory factors. Addition of specific factors may further improve tissue repair and erectile function compared to the administration of stem cells alone.

So far, only four studies have focused on stem cell therapy for ED in humans. Levy *et al*[59] have reported the administration of PM-SCs (placental matrix stem cells**)** in eight people with ED. After 6 mo following treatment, Levy *et al*[59] concluded that stem cell therapy improves erectile function. Another study by Al Demour *et al*[60] reported that the administration of bone marrow stem cells to people with ED resulted in the improvement of both the IIEF-5 and EHS scores; they further claimed that stem cell administration in the context of ED was well tolerated and safe for humans. However, these two studies have some weaknesses, for example, the studies did not include placebo control, were not randomized or blinded, and had a very small number of samples[60].

Bahk *et al*[61] conducted a study on seven diabetic patients aged 57-87 years and compared the effects of stem cell treatment with three patients who receive treatment for ED, such as PDE5 inhibitor alone. The stem cells used were umbilical cord-derived mesenchymal stem cells with a dose of 1.5×10^7 cells administered intracorporeally. This study was followed up for 9 mo, and at the end of the study, it was found that the stem cells produced a positive effect on ED as seen in the improvement of IIEF-5, SEP, GAQ, and erectile diary[61].

Protogerou *et al*[62] reported a different approach by using autologous adipose stem cells to treat ED in five people with DM. This group was compared with five other people who received platelet lysate. The number of stem cells given was 2×10^5 cells for 3 mo. At the end of the study, no difference in IIEF-5 was observed between the treatment and the control groups. However, a significant improvement was observed before and after treatment in both groups[62].

Possible mechanisms of stem cell therapy for diabetic ED

There are at least two possible mechanisms underlying the therapeutic effects of stem cell implantation for diabetic ED. First, stem cells may differentiate to specific cells and hence, regenerate the damaged tissue. In diabetic ED, the implanted stem cells may differentiate to myogenic cell precursor that subsequently in the presence of growth factors such as FGF and TGF-Beta can differentiate to myoblasts[40,63]. The myoblastic cells are known to have the capability to differentiate to vascular smooth muscle cells. The new smooth muscle cells may repair and improve damaged vascular tissues and hence improve the erectile function[64,65].

Second possible mechanism may involve the capability of stem cells to secrete beneficial paracrine factors. Stem cells are known to secrete angiogenic factors such as VEGF, basis fibroblast growth factor and SDF-1. These factors are potent inducers of angiogenesis and neovascularization[66,67]. Stem cells can also produce factors such as Wnt4 and Wnt7b, which are able to promote the differentiation of myoblasts to smooth muscle cells[63]. Together, the ability of stem cells to differentiate to smooth muscle cells and to produce beneficial paracrine factors that induce angiogenesis and neo-vascularization may be the underlying mechanisms of the therapeutic effects for diabetic ED.

CONCLUSION

Overall, based on the published data, it seems that stem cell therapy can become an alternative approach for the treatment of diabetic ED in the future. Stem cells can promote recovery and regeneration of the penile tissue following damage due to inflammation and free radicals. Studies reviewed in this paper, which are almost entirely conducted on experimental animals, certainly require further follow-up research. Information on stem cell research for ED treatment remains to be limited, but it can form the basis to develop further research in this area. In particular, larger

human studies with appropriate research designs are needed to provide more objective information on the possibility of translational application.

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MINIREVIEWS

Current knowledge on the multiform reconstitution of intestinal stem cell niche

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Abstract

The development of "mini-guts" organoid originates from the identification of Lgr5$^+$ intestinal stem cells (ISCs) and circumambient signalings within their specific niche at the crypt bottom. These *in vitro* self-renewing "mini-guts", also named enteroids or colonoids, undergo perpetual proliferation and regulated differentiation, which results in a high-performance, self-assembling and physiological organoid platform in diverse areas of intestinal research and therapy. The triumphant reconstitution of ISC niche *in vitro* also relies on Matrigel, a heterogeneous sarcoma extract. Despite the promising prospect of organoids research, their expanding applications are hampered by the canonical culture pattern, which reveals limitations such as inaccessible lumen, confine scale, batch to batch variation and low reproducibility. The tumor-origin of Matrigel also raises biosafety concerns in clinical treatment. However, the convergence of breakthroughs in cellular biology and bioengineering contribute to multiform reconstitution of the ISC niche. Herein, we review the recent advances in the microfabrication of intestinal organoids on hydrogel systems.

Key Words: Intestinal organoids; Reconstitution; Stem cell niche; Bioengineering; Hydrogel

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Core Tip: Organoid technique results in a high-performance, self-assembling and physiological platform in intestinal research and therapy. Despite the promising prospect of organoids research, their expanding applications are hampered by the canonical culture pattern, which reveals limitations such as inaccessible lumen, confine scale, batch to batch variation and low reproducibility. The tumor-origin of Matrigel also raises biosafety concerns in clinical treatment. The convergence of breakthroughs in cellular biology and bioengineering contribute to the development of biomaterial-based matrix or bioink for intestinal stem cells and incorporation of 3D printing and organ-on-a-chip technique, which may further advance organoid in future pathophysiological studies or functional tissue reconstitution.

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INTRODUCTION

In mammals, intestinal epithelium hosts diverse cell types at different stages of differentiation and possesses high self-renewal efficiency regulated by an sophisticated extracellular niche environment with a renewal cycle of 4-5 d, to ensure absorption of nutrients and defense against microorganisms[1,2]. As the inner layer of the intestinal wall, the intestinal epithelium and inferior lamina propria, which line the lumen, display a specific organization of crypt and villus structures to maximize the absorption surface especially in the small intestine[3].

Over the last 4 decades, efforts have been focused on identifying proliferative intestinal stem cells (ISCs) and reconstituting an *in vitro* model of the intestine[4]. Due to the complexity of the intestinal microenvironment and deep-seated residence of stem cell, the most available research tools are genetically variable cell lines derived from colorectal cancers, such as Caco-2 and HT-29[5,6]. However, Caco-2 cell-based culture lacks precise 3D architecture, interactions among different cell types and biochemical gradients, which are essential for the ISC niche[5,7]. Hence, stable culture and long-term expansion of ISCs *in vitro* was thought to be unattainable until 2005, when Wnt signaling was found to play a key role in maintaining the stemness and proliferative status of ISCs for the first time[8,9]. Subsequently, leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) was recognized as the marker gene of ISCs[4]. In addition, Sato *et al*[10] creatively applied Matrigel, which was extracted from Engelbreth-Holm-Swarm (EHS) sarcoma and resembles basal membrane in terms of its components, to provide biochemical support and construct a 3D niche environment, thus establishing the first generation of organoids. Conventional organoids cultured within Matrigel exhibit micron-sized 3D aggregates with projecting crypt-like buds and sealed-off lumen lined by epithelium[10-12]. ISCs interspersed in the crypt-like region are surrounded by Paneth cells, transit-amplifying cells (TA cells) and adhesion sites of matrices, which constitute the stem cell niche along with biochemical signaling gradients and mechanical cues[1,13].

In recent years, organoids have represented a biomimic platform for human development and physiological research and disease modeling including infectious, malignant, inherited and nutritional diseases, serving as an alternative to conventional animal models[14-16]. Even though stem cell research and organoid techniques have made remarkable breakthroughs, limitations have now surfaced such as low operability, uncertain biosecurity, poor plasticity and immunity deficiency[17,18]. Efforts have been made to develop alternative materials to Matrigel and enrich conventional organoid culture systems *via* 3D printing or microengineering[19,20].

This review focuses on integrant keys to reconstitute the stem cell niche microenvironment in organoid, the numerous candidate materials for culturing matrices and bioengineered models of the intestinal organoids.

NICHE CUES WITHIN THE MICROENVIRONMENT OF ISCS

Long term establishment of the novel intestinal organoid culture system depends on the preservation of self-renewal and self-organizing properties of the ISCs, which are regulated by various external environmental cues. So far, stemness-relevant biochemical signaling, niche cells, mechanical cues *etc.* have been identified as optimal niche cues for the reconstitution of ISCs *in vitro*.

Biochemical signaling within the ISC niche

Wnt/R-spondin signaling is the dominant regulator in the proliferation of ISCs[8]. Secreted mainly by Paneth cells and subepithelial fibroblasts, Wnt ligands bind to Frizzled and Lrp5/6 receptor complexes on ISCs and TA cells[21,22]. After binding, the complexes induce translocation of β-catenin into the nucleus to stimulate the expression of target genes that preserve the proliferating and undifferentiated status of stem cells, such as Axin2, Lgr5, Rnf43 and Znrf3[23]. Moreover, such activation is sustained and enhanced by the binding between R-spondin, a subepithelial fibroblast secreted protein, and its ligand Lgr5, which blocks negative feedback from the Rnf43 gene[23]. In human derived small enteroids or colonoids and mouse derived colonoids, exogenous Wnt and R-spondin are both entailed (Table 1). R-spondin alone is sufficient for mouse small ISC propagation[24-26].

Constituting the largest subdivision of the transforming growth factor-β (TGF-β) family, mesenchymal-derived bone morphogenetic proteins (BMPs) induce epithelial stem cell differentiation towards enterocytes and goblet cells by activating downstream phosphorylated SMAD1/5/8[24,27]. As BMP antagonists, Noggin, Gremlin1/2 and DMH-1 are involved in the maintenance of ISC numbers and proliferation status in long-term organoid culture, which play determinant roles on the avoidance of ISC exhaustion[28,29]. Therefore, exogenous Noggin is added as an essential cue to regulate ISCs in most organoid cultures[10].

Notch ligands such as Delta-like 1 (Dll1) and Dll4 are mainly expressed on Paneth cells interspersed among ISCs[30]. Once bound to Notch receptors at the surface of neighboring ISCs, the Notch intracellular domain (NICD) will be released and transported into the nucleus, where NICD undergoes enzymatic shedding by ADAM10 and γ-secretase to activate the transcription of Hes1/3/5. Hes1 in turn blocks Atoh-1 mediated differentiation toward the secretory lineage[31]. Knockout of Dll1 and Dll4 in intestinal epithelium could lead to complete exhaustion of ISCs and TA cells[32]. Dll1/Dll4-mediated Notch signaling is required for long-term homeostasis of intestinal epithelium. In the prometaphase of organoid culture, constant binding between Dll1/4+ Paneth cells and Notch+ ISCs contributes to the commencement of symmetry breaking and formation of proliferating buds, highlighting the pivotal role of Paneth cells in the ISC niche and organoid development[33,34].

Epidermal growth factor (EGF) is a canonical determinant that facilitates intestinal epithelial cells' self-renewal including ISCs and TA cells, and is mostly secreted by Paneth cells[35]. Activated EGF receptor (EGFR) initiates downstream RAS kinase and PI3K pathways, resulting in the translocation of ERK1/2 and boosting the mitotic signal[36]. As a significant factor in the organoid culture medium, EGF can be replaced by mesenchymal cell-derived insulin-like growth factor 1, which indicates the complementary effect of Paneth cells and the mesenchyme in constituting the ISC niche[37] (Figure 1).

Niche cells around ISCs

After being encapsulated into matrices, single Lgr5+ ISCs experience mitosis and end up with a cystiform sphere around day 3[24]. During the 4-cell stage and 16-cell stage, asynchronous cell division and alterations in extracellular matrix (ECM) density drives the variability in mechanosensor Yes-associated protein 1 (YAP1) subcellular localization, which is a cellular proliferating signaling activated by biophysical cues within the ISC niche and leads to variability in Dll1 expression in primitive Paneth cells[33,38]. It was also observed that ISCs cluster which failed to produce Paneth cells turned into ISC-absent enterocysts with a limited life-span, which contain only enterocytes[33]. In addition to Dll1-induced inhibition against secretory differentiation, Paneth cells generate endogenous growth factors including Wnt-3, EGF and TGF-α, facilitating niche environment reconstitution and budding organoid maturation[30,39]. Accordingly, Paneth cells are regarded as essential niche cells for the ISC niche.

Table 1 Organoid culture conditions for multiform reconstitution of the intestinal stem cell niche

	Media for canonical organoid culture	Media for organoids on 2D monolayer or 3D scaffold
Human small intestinal organoids	Wnt, R-spondin, EGF, IGF-1, FGF, Noggin, TGF-βi, p38i, gastrin[43]	Noggin, EGF, R-spondin, GSK-3i, ROCKi[44]
Human colon intestinal organoids	Wnt, R-spondin, EGF, Noggin, TGF-βi, p38i, gastrin[43]	Wnt, R-spondin, EGF, Noggin, EGF, TGF-βi, p38i, gastrin[45]
Mouse small intestinal organoid	R-spondin, EGF, Noggin[24]	Noggin, EGF, R-spondin, ROCKi[46]
Mouse colon intestinal organoid	Wnt, R-spondin, EGF, Noggin[24]	Wnt, R-spondin, EGF, Noggin, TGF-βi[47]

TGF-βi: Transforming growth factor-β inhibitor; p38i: p38 inhibitor; GSK-3: Downstream of Lrp5 receptor, transduce nuclear translocation when suppressed; EGF: Epidermal growth factor; IGF-1: insulin-like growth factor 1; FGF: Fibroblast growth factor.

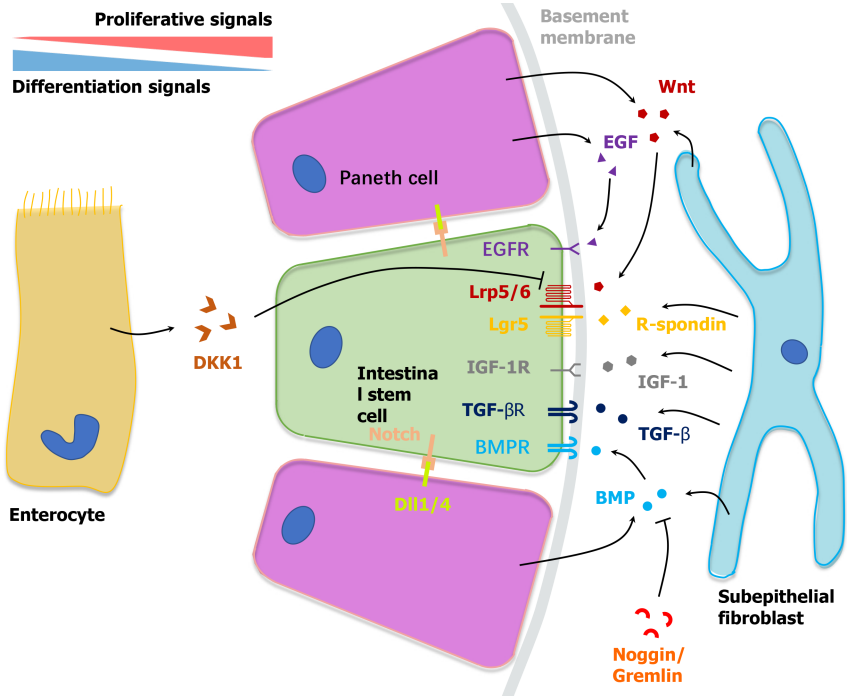

Figure 1 Niche microenvironment of intestinal stem cells at the crypt bottom. Bidirectional gradients of biochemical signals established by neighboring cells including Paneth cells, subepithelial cells and enterocytes regulate the self-renewal and differentiation of intestinal stem cells synergistically. TGF-β: Transforming growth factor-β; TGF-α: transforming growth factor-α; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; IGF: insulin-like growth factor; BMPR: Bone morphogenetic protein receptor; BMP: Bone morphogenetic protein; DKK1: Dickkkopf-1; Dll1/4: Delta-like 1/4.

Stromal production of Wnts in the submucosa is necessary for maintaining murine and human intestinal epithelium homeostasis[17,40]. Fibroblasts and myofibroblasts are known to be a source of Wnt ligands, R-spondin and TGF-β around the ISC niche *in vivo*. In experiments *in vitro*, organoid formation was rescued by the co-culture with embryo fibroblasts in the absence of supplemental Wnts and R-spondin, which indicates the fundamental role of stroma cells in intestinal homeostasis and crypts proliferation[22]. In addition, a recent publication also revealed endothelial cells and macrophages as Wnt ligand sources[22]. Although stroma cells especially fibroblasts and myofibroblasts can synergistically generate niche factor gradients and support the ISC microenvironment, single cell or crypt-based organoid culture lack submucosal components. In induced pluripotent stem cells (iPSCs) oriented induced human intestinal organoid, iPSCs can partially differentiate into mesodermal cells under activin A stimulation[41,42]. These mesodermal cells can further generate a fibroblast-enriched mesenchymal layer surrounding the epithelium[24,43-47] (Table 1).

Mechanical cues

Substantial research has proved that the mechanical properties of ECM or the stiffness and elasticity of biomaterials are key parameters impacting cell behaviors[48].

Organoid formation and ISCs growth, proliferation, differentiation and migration are impacted by such mechanical cues in organoid-ECM or cell-ECM interactions[49]. The incorporation of physiological relevant mechanical cues can contribute to ISCs proliferation and maturation. Stiffness refers to the degree of flexibility of the tissue microenvironment. Plastic dishes used in traditional cell culture provide a hard surface which is much stiffer than native tissues. However, Matrigel (approximately 100 Pa) provides organoids and ISCs with a softer microenvironment than intestinal tissue (approximately 800 Pa). High stiffness (approximately 1 kPa) of the matrix was demonstrated to facilitate ISC expansion *via* the YAP1 molecule[33,50]. In contrast, a soft matrix promoted cell differentiation and organoid maturation. By inserting compressed nitinol springs in transplanted human organoids to introduce strain forces, Poling *et al*[51] observed enlarged tissue size, complexity and more similarities to native intestine. In addition, native ECM and synthesized matrix biomaterial also exhibit complex viscoelasticity, which describes a time-dependent response to loading or deformation[52]. Studies carried out using 2D and 3D culture systems indicated that matrix viscoelasticity might influence gene expression and differentiation pattern.

Epithelium of the intestinal tract especially the small intestine is constantly immersed in digestive juice *in vivo*. Dynamic fluid cues are often ignored in intestinal tissue models at the early stages. It was unrealistic to investigate such luminal mechanical force in structure-lacking cell line culture and canonical sealed-off organoids. Using the organoid culture system on monolayer or chips, luminal stream added through constant shaking was found to be an inductive cue for villus formation [53]. However, colon-derived organoids failed to exhibit similar sensitivity to dynamic fluid cues. This fluid cue stimulation is related to activation of an inherent villus-forming program within the small intestine, which markedly improves villus formation and increases villus density[54].

CULTURE MATRICES

The novel organoid culture systems rely on hydrogels as matrix to provide soft mechanical support to facilitate the proliferation and differentiation of ISCs. However, in consideration of the appearing limitations, tissue-mimicking hydrogels with tunable properties based on well-defined natural biomaterials or customized synthetic polymers are needed. Among different kinds of biomaterials, hyaluronic acid (HA), silk protein, collagen gels, and various polyethylene glycol (PEG)-based hydrogels are found to have potential in organoid cultures.

Matrigel

As a basement membrane extract from mouse EHS tumor and a natural ECM analogue, Matrigel (also named Cultrex or EHS matrix) has been applied in the majority of cellular experiments for nearly half a century, such as cell culture, tumor invasion, lineage differentiation and gene expression[9]. Matrigel also serves as a vital and seemingly exclusive matrix that has been used in organoid culture since the early 2010s[55].

Canonical Matrigel consists of 4 major ECM proteins, which include approximately 60% laminin, approximately 30% collagen IV, approximately 8% nidogen and approximately 2% heparin sulfate proteoglycan perlecan (Figure 2A)[56]. Moreover, Matrigel contains several tumor-derived growth factors, such as TGF-β and fibroblast growth factor as well as enzymes such as matrix metalloproteinases (MMPs), which synergistically lead to its first-class bioactivity. In the temperature range of 25-37 ℃, Matrigel undergoes rapid gelation driven by entactin-mediated strong crosslinking between laminin and collagen IV as well as relatively weak hydrogen bonds within collagen molecules. Gelated hydrogel in the form of a dome or coating provides concise and optimal 3D or 2D platforms, which are suitable for multi-type cell culture. Although they vary from batch to batch, the poor mechanical properties of Matrigel (approximately 100 Pa) facilitate stem cell differentiation and organoid maturation rather than stem cell proliferation[20,57].

When ISCs or other epithelial cells *in vitro* lose intimate integrin attachment to the basal membrane, cells easily cease propagation and undergo anoikis. Different laminin subtypes within Matrigel offer a great number of adhesion sites for integrins in the ISC niche, which is mostly made up by α2β1, α7β1, and α5β1 subtypes[50]. Upon binding to laminin binding peptides, anoikis of ISCs *in vitro* induced by Rho-ROCK kinase activation is markedly inhibited. The uniqueness and ubiquitousness of bioactive Matrigel make it the most preferred culture matrix for organoid, based on which

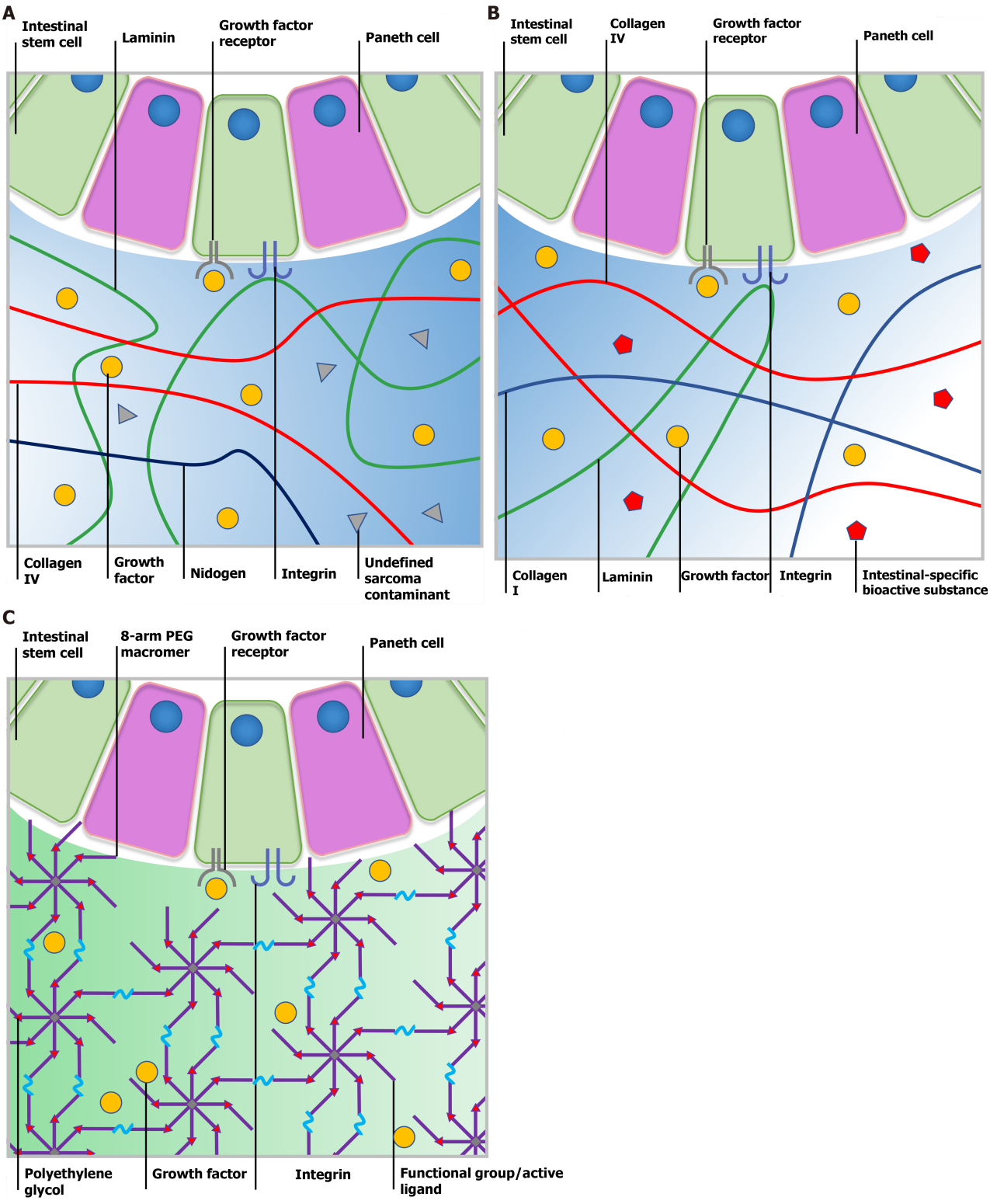

Figure 2 Comparison of Matrigel, decellularized extracellular matrix and a synthetic matrix. A: Ill-defined Matrigel is variable in composition. Although certain bioactive factors exist, Matrigel still contains undefined contamination from the tumor microenvironment, which leads to low reproducibility and biosecurity; B: Submucosa-derived decellularized extracellular matrix (dECM) provides intestinal stem cells (ISCs) with a natural microenvironment closest to native tissue and the crypt niche. Tissue-specific bioactive substances could help maintain the physical phenotype of cultured ISCs. However, the composition of dECM may vary from batch to batch and be affected by the age, gender and health status of source animals; C: Polyethylene glycol is one of the most frequently used synthetic material for cell culture as it is bioinert and tunable. Following modification with diverse functional groups or active ligands, researchers can manipulate the physical and chemical parameters to further support organoid formation. PEG: Polyethylene glycol.

canonical intestinal organoids culturing pattern is fully established. After being encapsulated in Matrigel, disassociated intestinal crypts or ISCs form cyst structures in the first 3 d. During days 4-5, the first batch of Paneth cells develop, thus forming heterogeneous Wnt activation among cysts and proliferating buds[41].

Although commonly used, Matrigel has limitations in the application of organoid techniques. Firstly, variations exist in component contents and biochemical properties from batch to batch or even within a single batch of Matrigel, which have caused a lack of homogeneity and reproducibility in cell culture results. Proteomic analyses of Matrigel are consistent between each other along with the growing number of identified different proteins (> 2000) and peptides (> 14000)[58]. Secondly, the mechanical properties of Matrigel also vary from batch to batch even between regions within a single dome. Heterogenicity among cross-linked materials such as multitype laminin and collagen peptides in part leads to uneven distribution of material density, causing variations in the stiffness of median and marginal areas. Thirdly, the tumor origin and natural resource of Matrigel restrict the promising potential of the organoid technique in tissue repair and regeneration. Although transplanted intestinal organoids carried by designed scaffolds show robust viability and origin-specific functions in animal experiments, concerns have arisen about Matrigel's uncertain tumorigenicity when it comes to clinical use[59]. Finally, Matrigel is not amenable to chemical modification or manipulation to adjust mechanical properties, which is attributed to the inconsistencies in the concentrations of its contents. As an ill-defined complex, the infinite variety of cues within Matrigel cannot be split to elaborate their specific function in different stem cell niches. These undesirable characteristics of Matrigel require further research on synthetic or natural alternatives with highly tunable biochemical and biophysical properties to reconstitute the ISC niche[60].

Decellularized ECM

The ideal matrix for cell culture should mimic the native microenvironment and provide original tissue-specific ECM contents, sites for adhesion, stiffness *etc*.[61]. This concept initiated research into decellularized ECM (dECM), which has emerged as a promising material for regenerative medicine and tissue engineering. Recent studies have also highlighted dECM as a promising natural hydrogel material for organoid culture[62].

dECM is a biological scaffold derived from native tissues in which cellular components, preserved structural components, functional enzymes and partial factors have been removed[63]. Three decellularizing strategies are commonly used to produce dECM based on different natural tissues such as cartilage, liver, kidney, breast, intestine, prostate and heart. (1) Physical strategies include freeze-thaw, agitation and rinsing to break up the cell membrane and strip off cells; (2) By using chemical agents such as acid (acetic acid), base (sodium hydroxide), chelating agent (EDTA), hypotonic detergents (Tris-HCL), ionic detergents (sodium dodecyl sulfate) or non-ionic detergents (Triton-X-100), cytoarchitecture is easily disrupted and undergoes disaggregation; and (3) Biological strategies utilize enzymes to break up ribonucleotide and deoxyribonucleotide chains specifically[64,65]. After multistep decellularization, dECM eliminates most xenogenic antigens and acquires minor immunogenicity and assured biosecurity. As an FDA approved biosafe material and medicinal product in use, different types of dECM vary greatly in major constituent concentrations such as collagens, fibronectin, elastin, laminin, proteoglycan and glycoprotein according to the tissue source and decellularization strategy. Despite these differences, the dECM material provides a truly biomimetic environment which retains native structural, signaling components and specific cell-ECM interactions (Figure 2B). Digested dECM powder can undergo collagen-based gelation in response to external conditions such as temperature, ionic concentration and pH with tunable contents and stiffness, which shows more intelligence and maneuverability over Matrigel[66].

A study by Giobbe *et al*[67] identified that dECM gel from porcine small intestine mucosa/submucosa enables the formation and growth of multiple types of endoderm-derived human organoids with equivalent efficiency. In dECM gel, gastric, hepatic, pancreatic and small intestinal organoids showed regular proliferation and differentiation capacities. However, transcriptomic analysis of small intestinal organoids in dECM gel revealed a higher expression of ISC and Paneth cell markers, such as OLFM4, SMOC2 and LYZ, and a reduction in the expression of differentiation markers, such as EZR, VIL1 and MUC12, compared to Matrigel. This discrepancy was caused by culture matrices possessing a differential biochemical signature and environmental niche, which lead to varied effects on cellular behavior and experimental results[68]. Interestingly, the dECM scaffold carrying small intestinal organoids survived two months after transplantation, which was superior to Matrigel[67]. In addition, application of dECM in organoid culture promotes physiological function. Saheli *et al*[69] seeded human hepatocarcinoma (Huh7) cells, human umbilical cord vein endothelial cells (HUVEC) and human bone marrow-derived mesenchymal stem cells (MSCs) in sheep live-derived dECM to produce liver organoids. Mixed cells not

only formed self-organized liver organoids, but also exhibited enhanced hepatic functions with significant upregulation of transcripts of albumin, CYP3A4 and CYP3A7 compared to Matrigel and collagen I. A recent study also used dECM as a tool to determine the exact interactions between environmental cues and stem cell behavior. By applying rat pancreatic ECM gel, the study identified collagen V as the key cue within the dECM that boosted the formation of cultured human pluripotent stem cells (iPSCs) towards islet-like organoids and functional α, β, δ type pancreatic endocrine cells[70]. Similarly, dECM may act as the bridge between poorly-defined Matrigel material and pinpoint biochemical contents that are adequate for the propagation of ISC. In addition, chemically defined dECM gel can be tailored to have tunable mechanical properties and viscoelasticity by chemical modification or appending a compound hydrogel system[71]. By slightly enhancing viscosity or utilizing multi-step crosslinking, organoid-laden dECM ink may be used directly to fabricate biomimetic crypt-villus structures or a sophisticated bioreactor *via* 3D printing[72].

PEG

The great potential of organoids in research and therapy remains restricted due to ill-defined matrices derived from animals. Therefore, efforts have been made to design and synthetize chemically defined hydrogel networks that enable ISC propagation and organoid formation by recapitulating key cues from the ECM[73]. To fully recognize the key cues that dominate ISC expansion, Gjorevski *et al*[74] created a well-defined 3D matrix based on PEG and peptides from fibronectin, laminin and collagen IV, which were enriched within the ISC microenvironment. As a biocompatible and enzymatically biodegradable polymeric substance, after reaction with diverse nucleophiles, PEG can bond to reactive groups such as vinyl sulfone (VS) or acrylate, to form multiarmed-PEG macromers. A subsequent Michael-type addition with thiol-reactive peptides allows the formation of PEG-based hydrogel networks (Figure 2C)[75].

Synthetic inert and soft PEG scaffolds were not sufficient to maintain ISC expansion and organoid formation, similar to sodium alginate or gelatin methacrylate (GelMA) hydrogel. Interestingly, by replacing VS reactive groups on 8-arm PEG monomers with fibronectin-derived RGD (Arg-Gly-Asp) peptides to target integrins on ISCs, intestinal crypts embedded in such modified RGD-functionalized PEG gels (PEG RGD) exhibited long-term propagation and colony formation abilities, suggesting that both physical support and biochemical signals from the matrix are involved in ISC survival[50,74]. Thus, PEG RGD is regarded as a synthetic hydrogel with tunable mechanical, biochemical properties that promotes intestinal organoids growth *in vitro*.

In intestinal organoid culture, stiffness of the matrix has been depicted to play a critical role in ISC fate and organoid formation as described previously[76]. For PEG hydrogel formation, by blending 8-arm PEG macromers of 20- or 40-kDa at various ratios and modulating final PEG content, the storage modulus of PEG gel ranged between 110-1034 Pa[77]. Adjusting the network's crosslink level also enabled tunable biophysical properties. By incorporating peptide sequences, which are sensitive to cell-secreted MMP, the PEG gel could acquire degradability and increase stiffness, which stimulates cell proliferation in the early phase of culture[78]. Following partially enzymatic gel degradation over time, softened PEG gel (approximately 300 Pa) promoted ISC and TA cell differentiation which is needed for organoid maturation. Compared to stable PEG gel, degradable PEG gel showed abundant expression of differential markers and higher organoid formation efficiency.

In addition, the 8-arm PEG monomer can be modified with customized peptides according to different integrin subunits[79]. The most frequently used fibronectin-derived peptide Arg-Gly-Asp binds to αvβ3 and αvβ5 integrins. It was also reported that the presence of a collagen-derived peptide (GFOGER) targeting α2β1 integrin exhibited outstanding culture efficiency of human duodenal and colon enteroids[50]. Also, collagen-like peptide grafted PEG gel promoted the spontaneous organization of other primary stem cells into clusters.

Although PEG-RGD gel has shortcomings such as inferior organoid culture efficiency (50%) compared with Matrigel, PEG materials fill the vacancies in the synthetic microenvironment by mimicking ECM composition which are devoid of unknown factors and enable standardized ISC culture[80]. Modification of 8-arm/4-arm PEG macromers with multiple chemical groups or peptides, which are sensitive to MMPs, pH, temperature or chemical irritation may endow the synthetic matrix with more interesting characteristics for organoid culture and achieve a fully-designed stem cell niche.

BIOENGINEERED MODELS OF THE INTESTINE

As described earlier, the first generation of organoid culture is characterized by encapsulated single ISCs, fresh crypts or disassociated organoids within a dome-like matrix. After days of proliferation and self-assembly, cell clusters grow into sealed-off difform spheroids hosting 7 intestinal epithelial cell types. Although encapsulated organoids exhibit a crypt-villus structure and mesenchyme-free ISC niche, they still have a number of dissimilarities compared to intestinal tissue. To fully enhance the maneuverability and adaptability to high-throughput screening and microfabrication, many technical approaches have been established to reproduce the stem cell niche and intestine model *in vitro*.

Two-dimensional monolayers

The monolayer system on a dish or porous transwell insert provides an intestinal tissue model that offers access to both luminal and basal sides, which enables the establishment of biochemical gradients of growth factors and observation of host-pathogen interactions (Figure 3A). By introducing extrinsic Wnt and BMP to the monolayer system, Thorne *et al*[19] was able to evaluate the contribution of epithelial-intrinsic and extrinsic Wnt to epithelial homeostasis. In a colonoid monolayer, the lack of Paneth cells in crypt-like zones explained why extrinsic Wnt is an essential factor for colonoid culture. For diarrheal pathogen study, enteroaggregative Escherichia coli revealed aggregative adherence to enteroids from the duodenum and ileum, which revealed unique patterns of intestinal segment-specific adherence of various pathogens. It is also worth noting that the self-organized monolayer showed integrated and effective barrier function with a physiological transepithelial electrical resistance (55 ohms.cm^2) and dextran permeability[46].

Although the monolayer system offers a culture platform that mimics *in vivo*-like cell distribution and is compatible with high-throughput drug absorption or host-pathogen interactions, these simple systems lack crypt-villus architecture and mesenchymal components. In addition, static culture cannot provide ISCs with the dynamic mechanical forces of peristalsis in the native microenvironment which is believed to affect cellular behavior and organoids self-assembly[44,81].

Three-dimensional scaffolds

The intestinal epithelium is a highly polarized tissue containing crypt-villus topography, while canonical organoids within Matrigel are much more like heterogeneous and difform spheroids. Thus, 3D scaffolds mimicking physiologic morphology have been fabricated to study stem cell behavior influenced by complex architecture, in which organoids showed robust proliferation and differentiation (Figure 3B).

In the first study, photolithography (5 μm resolution) was introduced into the microfabrication of 3D scaffold for enteroids, Costello *et al*[82] used laser ablation to create an array of 500 μm deep holes in polymethyl methacrylate (PMMA). Through multistep casting, a porous villous poly-lactic-glycolic acid (PLGA) scaffold (villi height = 500 μm) was fabricated, which supported the propagation of primary intestinal crypts and Caco-2 cells. Wang *et al*[83] generated a polydimethylsiloxane (PDMS) stamp with both crypt and villi architectures *via* two times of ultraviolet (UV) irradiation. Cross-linked collagen-I hydrogel was used as the culture matrix[84]. After applying the PDMS stamp, micromolded collagen in a transwell insert resembled physical intestinal lamina propria structure and a crypt-villus micropattern (crypt depth = 132 μm, villi height = 477 μm). In addition, biochemical gradients of factors were established along with crypt-villus axis which promoted the formation of a stem cell-abundant zone at the crypt bottom and differentiated an enterocyte-abundant zone around the villi. ISCs also revealed tunable migration and differentiation capacities in response to changes in extrinsic biochemical gradients.

As a cutting-edge technique wide-spread in biomedicine, 3D printing can provide ISCs and organoids with a designed niche microenvironment and delicate architecture that restores intestinal epithelium. By horizontally slicing the digital objective with a computer aided-design and solidifying materials layer by layer, 3D printing can create intestinal models or culture scaffolds with arranged parameters, which include a degradable ink-based soluble microenvironment, an insoluble microenvironment, shape, external force and additive components[85]. In 3D printed tubular scaffolds composed of collagen or silk fibroin, multiple cell types such as like fibroblasts, myofibroblasts, macrophages and neurocytes were appended in the organoid system to build mesenchymal, immune and nerve components, respectively. Notably, adapted biomaterials such as described collagen, PEG-DA, dECM and silk have made possible

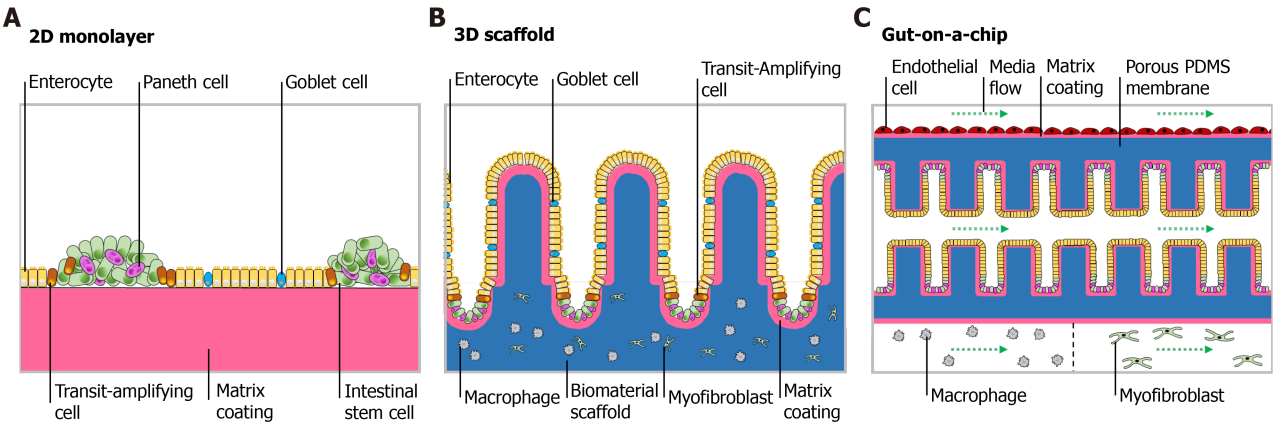

Figure 3 Multiform reconstitution of the intestinal stem cell niche. A: On matrix coating with relatively high substrate stiffness, intestinal stem cells (ISCs) generate an epithelial monolayer that recapitulates polarized cell distribution and barrier function; B: Micropatterned scaffold with suitable extracellular matrix coating enables ISC self-renewal, differentiation and epithelial cell migration and resembles distinct crypt-villus or multilayer architecture; C: Gut-on-a-chip allows incorporation of vasculature and lymph-vessels into the organoid technique and provides an effective platform for high-throughput drug screening.

the fabrication of 3D architecture directly from bioinks containing living ISCs, which is also called 3D bioprinting (200 μm resolution). In 2020, Brassard[86] and colleagues embedded human ISCs into a viscous Matrigel/collagen precursor solution and applied it to bioprinting. By controlling printing geometry and spatial deposition of cells, bioprinted ISCs within the Matrigel demonstrated spontaneous self-organization into centimeter-scale tubular tissue incorporating intestinal features such as continuous lumen, branched vasculature and crypt-villus domains. Bright-field images confirmed obvious growth and expansion over time. On day 6, printed tubes showed multiple differential markers like Lyz (Paneth cells) and L-FABP (enterocytes). In addition, Sox9+ ISCs were well enriched in the self-organized crypt-like region, which was not found in other areas[87].

Taken together, these studies indicate that 3D morphology obviously contributes to ISC differentiation and tissue function. Also, macro-scale organoid systems on scaffolds have huge therapeutic potential for short bowel syndrome and genetic intestinal diseases such as multiple familial polyposis coli or cystic fibrosis *via* transplantation. On the other hand, 3D organoid scaffolds have emerged as promising bioengineering tools to construct multicellular systems comprising epithelium, mesenchyme, vasculature, lymph-vessels, nerves and smooth muscles, which may reproducibly direct the fate of ISC into a coordinated and collective behavior *in vitro* [88]. For instance, small-diameter vascular grafts produced by non-degradable materials or decellularized vessels may assist in the construction and functionalization of large-scale organoid systems[89].

Gut-on-a-chip

The term organ-on-a-chip was first proposed in 2010, describing microfluidic devices containing designed micrometer sized chambers for cell culture[90]. Organ-on-a-chip uses channels tens to hundreds of micrometers wide, in which fluid flow generates gradients by passive diffusion. At the junctions of channels, chambers perfused continuously are seeded with cells (Figure 3C). This type of culture system has been used to create a continuous digestive epithelial tube composed of stem cells from different segments of the digestive tract and mimic dynamic fluid mechanical stimulation and peristaltic motions[91].

"Gut-on-a-chip" was first presented as a microdevice composed of two microfluidic channels which were separated by a 30 μm ECM-coated PDMS porous membrane and lined by the Caco-2 cancer cell line. When exposed to a low flow rate (30 μL/h) and low shear stress (0.02 dyne/cm2), Caco-2 cells not only commenced with villus morphogenesis and the expression of differentiation markers expression such as mucin and villin, but also formed a proliferative cell-enriched zone. By replacing seeded Caco-2 cells with primary ISCs, this dynamic condition induced self-organization and villus formation was amplified. In this field, Brandenberg *et al*[92] and Nikolaev *et al*[93] and colleague have made outstanding contributions in attention to achieve full control of ISCs behaviors on designed chips. Shin *et al*[53] elaborated the mechanism behind villi morphogenesis induced by dynamic fluid flow by means of assisted computational simulation. They identified Dickkkopf-1 (Dkk1) as a

regulator of ISC Wnt signaling activation. As an epithelial cell-secreted Wnt antagonist toward the basolateral direction, Dkk1 accumulates around basal cells and thus inhibits villi morphogenesis under static conditions. However, the cell chamber within chips allows constant removal of Dkk1 which established a transepithelial gradient of Dkk1 and corresponding spatially heterogeneous proliferation activation.

As the forefront of organoid research, gut-on-a-chip has emerged as a multicellular system to mimic organ-like features. Kim *et al*[94] isolated human peripheral blood mononuclear cells to seed the lower capillary channel and cocultured commensal microbes contacting epithelial cells. Analysis of epithelial inflammation indicated secretion of proinflammatory cytokines (IL-8, IL-6, IL-1β and TNF-α) induced by mixed immune cells and lipopolysaccharide. This human gut-on-a-chip microdevice resembled impaired villi and compromised intestinal barrier function, mimicking the pathophysiology observed in patients with inflammatory bowel disease and ileus. Thus, gut-on-a-chip can be used to investigate the interaction between intestinal epithelial cells, immune cells, microbe etc. in a tunable microdevice, which has major implications for intestinal disease research.

CONCLUSION

Identification of intestinal ISCs by exquisite and specific Lgr5 expression enables access to these stem cells through a minimal isolation process. As non-classical rapid-cycling stem cells, ISCs undergo heterogeneous differentiation towards Paneth cells upon asymmetrical YAP1 activation. It seems that ISCs are inclined to build their own niche environment rather than depend on an exogenous one to guarantee lifelong proliferation, which has resulted in a revolutionary advance in basic science and translational therapy. In addition, it is now known that the biochemical signals especially Wnt ligands secreted by mesenchymal cells are crucial to transepithelial gradient establishment[2]. The influence of mesenchyme cell such as fibroblasts, myofibroblasts, smooth muscle cells, innate macrophages and nerve cells upon ISC and other epithelial cells fate and behavior is not yet fully elaborated, which highlights the development of an intestinal model containing mesenchyme, immune components, microbiome as well as epithelium. The advent of tissue engineering and microfabrication based on HA or PLGA hydrogels has enabled the development of multilayer or tubular co-culture systems[95]. 3D engineered scaffolds or chips composed of patient-derived ISCs and immune cells offer powerful models to study fundamental biochemical mechanisms or disease pathophysiology. Newly developed bioprinting approaches such as bioprinting-assisted tissue emergence contribute to macroscale organoid tissue that could be applied in regenerative therapy to treat short bowel disease[86]. However, the lack of standardization in culture conditions and interventions restricts its clinical application. The production of reproducible and easily manageable platforms that recapitulate the key features of native tissue is of great significance[96]. In addition, the organ-on-a-chip technique offers a new approach that can reproduce dynamic fluid cues and peristaltic forces and allows multicellular culture at the same time. Simultaneous control over cell distribution, biochemical gradients and mechanical cues can be achieved in such microfluidic systems, which have set a trend to reproduce the complexity of the digestive tract.

ISC proliferation and organoid generation entail appropriate mechanical support and adhesion sites. The conventional widely-used culture matrix Matrigel, extracted from native ECM, has gradually revealed its drawbacks. Matrigel cannot fully resemble intestinal ECM components or provide microenvironmental cues within the ISC niche, which may alter cellular behavior and limit the reliability of organoids as platforms for disease modeling and transplantation therapy. Well-defined or engineered materials have been established to replenish or replace conventional matrices. For instance, several natural polymer materials such as dECM, collagen and laminin also offer ISCs with a porous, fibrillar environment and structural properties of ECM proteins. Synthetized multiarmed-PEG macromers produce a structure with tunable adhesion sites and degradability to mimic ECM characteristics *in vivo*. The user-defined tunability of engineered biomaterials allows intervention during organoid culture by changing their physicochemical properties to determine the interaction between organoid morphogenesis and adhesive ligand or physicochemical cues. Equipping the matrix with light sensitivity by incorporating light-sensitive moieties could enable external control over cell differentiation level or investigate matrix-stiffness relevant disease, such as fibrosis.

In conclusion, to dispel concerns regarding biosecurity and enable further drug-screening or transplantation therapy, the conventional Matrigel-based organoid system requires optimization. Reliability, reproducibility, culture effectiveness and biosecurity of the natural or synthetized hydrogel for ISC culture and organoid generation need to be tested and verified. The development of biomaterial-based bioink for ISCs is also significant, which requires specific viscosity and bioactivity. However, technique challenges need to be overcomed to reach the designed cell deposition and fabricate refined tissue models *via* 3D printing. Gut-on-a-chip incorporating blood and lymph vasculature and nerves may further advance organoid in future pathophysiological studies or functional intestine tissue reconstitution *in vitro* .

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ORIGINAL ARTICLE

Basic Study

Effect of glycyrrhizic acid and 18β-glycyrrhetinic acid on the differentiation of human umbilical cord-mesenchymal stem cells into hepatocytes

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Author contributions: Fatima A performed all experiments, analyzed the data and wrote the first draft; Malick TS and Ishaque A performed part of the experiments and analysis of the data; Khan I provided technical guidance for all experiments and data analysis; Salim A designed the research study, supervised the research and wrote the manuscript in its final form; all authors have read and approved the final manuscript.

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Abstract

BACKGROUND

End-stage liver disease is a global health complication with high prevalence and limited treatment options. Cell-based therapies using mesenchymal stem cells (MSCs) emerged as an alternative approach to support hepatic regeneration. *In vitro* preconditioning strategies have been employed to strengthen the regenerative and differentiation potential of MSCs towards hepatic lineage. Chemical compounds of the triterpene class; glycyrrhizic acid (GA) and 18β-glycyrrhetinic acid (GT) possess diverse therapeutic properties including hepato-protection and anti-fibrosis characteristics. They are capable of modulating several signaling pathways that are crucial in hepatic regeneration. Preconditioning with hepato-protective triterpenes may stimulate MSC fate transition towards hepatocytes.

AIM

To explore the effect of GA and GT on hepatic differentiation of human umbilical cord-MSCs (hUC-MSCs).

METHODS

hUC-MSCs were isolated and characterized phenotypically by flow cytometry and immunocytochemistry for the expression of MSC-associated surface molecules. Isolated cells were treated with GA, GT, and their combination for 24 h and then analyzed at three time points; day 7, 14, and 21. qRT-PCR was performed for the expression of hepatic genes. Expression of hepatic proteins was analyzed by immunocytochemistry at day 21. Periodic acid Schiff staining was performed to determine the functional ability of treated cells.

RESULTS

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The fusiform-shaped morphology of MSCs in the treatment groups in comparison with the untreated control, eventually progressed towards the polygonal morphology of hepatocytes with the passage of time. The temporal transcriptional profile of preconditioned MSCs displayed significant expression of hepatic genes with increasing time of differentiation. Preconditioned cells showed positive expression of hepatocyte-specific proteins. The results were further corroborated by positive periodic acid Schiff staining, indicating the presence of glycogen in their cytoplasm. Moreover, bi-nucleated cells, which is the typical feature of hepatocytes, were also seen in the preconditioned cells.

CONCLUSION

Preconditioning with glycyrrhizic acid, 18β-glycyrrhetinic acid and their combination, successfully differentiates hUC-MSCs into hepatic-like cells. These MSCs may serve as a better therapeutic option for degenerative liver diseases in future.

Key Words: Glycyrrhizic acid; 18β-glycyrrhetinic acid; Hepatocyte differentiation; Human umbilical cord-MSCs; Mesenchymal stem cells

Core Tip: This study focuses on exploring the effect of two triterpenes, glycyrrhizic acid and 18β-glycyrrhetinic acid to enhance the differentiation potential of mesenchymal stem cells (MSCs) into hepatocytes to ensure a potent and valuable cell source for cellular therapy for end-stage liver disease. Preconditioning of human umbilical cord-MSCs with these compounds enhances expression of both early and late hepatic markers, regulated with time of differentiation. The significant expression of hepatocyte markers, the ability to store glycogen, and presence of bi-nucleated cells, suggest successful hepatic differentiation. Preconditioned MSCs may help in the replacement of damaged hepatocytes, and improve liver function post-transplantation in impaired hepatic tissues.

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INTRODUCTION

Liver is a complex organ, designed to fulfil variety of tissue-dependent tasks such as metabolic, excretory, circulatory and secretory functions[1]. Parenchymal hepatocytes are polarized polygonal epithelial cells with remarkable proliferation ability, which efficiently repopulates liver cells in response to the injurious stimuli. Tissue mass is restored by their mitotic division and increase in the size of cells termed as compensatory hyperplasia and hypertrophy[2]. Even though liver is a pivotal organ for homeostasis with considerable inherent regenerative ability, massive injury to the liver may prevent regeneration due to the accumulation of extracellular matrix proteins and scar tissue formation (fibrosis) which progress to cirrhosis, a common pathological condition contributing to end-stage liver disease (ESLD)[3]. To prevent ESLD progression, stem cell therapy has also shown promising clinical outcomes and considered as a potential alternative to conventional therapies. Mesenchymal stem cells (MSCs) have gained considerable attention because of their multi-lineage potential, immune-privileged and paracrine properties, making them valuable candidates for translational approach. These cells can be obtained from different sources, such as bone marrow, adipose tissue, peripheral blood, cartilage, skeletal muscle, umbilical cord blood and tissue[4]. Although MSC-mediated therapeutic approach has been effective in ESLD, the major setback is the low cell viability and resistance in impaired tissues post-transplantation[5]. To overcome this consequence,

MSCs can be differentiated into functional hepatocytes *in vitro*, to enhance their therapeutic potential prior to transplantation. Various extrinsic factors including mechanical induction, growth factors, and chemical compounds promote differentiation of stem cells. They are capable of triggering several signaling pathways that control fate of stem cells, directing them towards a desired cell lineage[6]. In recent years, researchers explored the role of phytochemical compounds such as triterpenes in inducing stem cell differentiation. In a clinical finding, it is reported that pentacyclic triterpene, oleanolic acid has a potential to enhance osteogenic differentiation of MSCs by inhibiting Notch signaling pathway[7]. A study conducted on triterpene compound, ginsenosides has highlighted their role in enhancing hepatic gene expression during the induction of hepatic differentiation of MSCs[8].

Triterpenes, structurally similar to steroids, are diversified class of chemical compounds containing isoprene units. They can occur as glycosides or aglycones and categorized as pentacyclic and tetracyclic[9]. They are abundantly found in vegetables, fruit peels, medicinal plants, and stem bark[10,11]. Glycyrrhizic acid (GA) and 18β-glycyrrhetinic acid (GT) are potent triterpenes possessing variety of therapeutic properties for liver diseases. This includes anti-hepatotoxic, anti-fibrotic, anti-tumor, anti-inflammatory, anti-arthiritic, anti-allergic and anti-viral properties[12,13]. Their beneficial effects in cell culture and animal models of liver toxicity have been investigated. Interestingly, these triterpenes have been used in the treatment of hepatitis, liver cirrhosis, and cancer and have shown to improve liver function[14]. Considering the characteristics of these compounds in hepatic anomalies, we hypothesized that these triterpenes may have the ability to directly differentiate hUC-MSCs into hepatocytes or aid in the process of differentiation.

MATERIALS AND METHODS

Ethics committee approval and human umbilical cord collection

Present study was approved by the Independent Ethics Committee, International Center for Chemical and Biological Sciences (IEC, ICCBS) under protocol #: ICCBS/IEC-037-HT-2018/Protocol/1.0. The umbilical cord samples (n = 10) were collected from Zainab Panjwani Memorial Hospital (ZPMH) with an informed consent from healthy donors following full-term cesarean delivery. Using aseptic techniques, cord samples (approximately 8-10 cm in length) were collected in a phosphate buffered saline (PBS) transfer medium containing anticoagulant (5 g/L EDTA) in a sterile bottle and kept at 4 °C until transferred to the experimental research facility in ice box and processed within 2-4 h of collection.

Processing and culturing of human umbilical cord tissue using explant culture

Sample processing was carried out in a biosafety cabinet class II, type A2 (ESCO, United States). Cord tissue sample was placed in a culture dish and rinsed multiple times with sterile PBS to remove blood clots and debris. Cord tissue was chopped into small pieces of about 1-3 mm in size and plated in T-75 flasks having 10-13 mL of DMEM (GIBCO, United States) supplied with 100 mL/L FBS, 0.1 g/L streptomycin, 100000 Units/L penicillin, and 0.001 mol/L sodium pyruvate. Explants were placed at 37 °C in humidified CO_2 incubator (Hera Cell, Germany). Tissue culture was observed for cell growth and medium was replaced after every 72 h. hUC-MSCs migrated out from the small pieces of umbilical cord Wharton's jelly to the surface of the culture flask during 10-14 d of initial culture. After sufficient attachment of hUC-MSCs, explants were removed and fresh medium was added for further expansion. At this stage, cells were termed as P0 passage. Once the adherent cells reached 80% confluence, they were sub-cultured for next passages using 2.5 g/L trypsin. hUC-MSCs of P4 passage were used in all subsequent experiments.

Characterization of hUC-MSCs by immunocytochemical analysis and flow cytometry

Isolated hUC-MSCs were characterized by immunocytochemistry for the expression of MSC associated surface molecules, as reported previously[15]. Approximately, 8000 cells were seeded on cover slips placed in 24-well plate with 200 µL DMEM per well. The cells were then kept at 37 °C in incubator containing 50 mL/L CO_2 for 24-48 h until the monolayer was formed. After confirming cell expansion, media was removed, and cells were washed with 1 × PBS. Cells were fixed with 200-300 µL 40 g/L PFA for 10 min at room temperature followed by washing with PBS. 300 µL of 0.1% Triton X-100 was added in each well for cell permeabilization for 10 min at room temperature and washed 3 times with PBS. Cells were then kept at 37 °C for 1 h in the blocking solution

containing PBS supplemented with 20 g/L BSA and 1 mL/L Tween 20 to prevent non-specific binding. Blocking solution was removed and cells were incubated with monoclonal primary antibodies against CD73, CD105, vimentin, and CD45. Plate was kept overnight at 4 °C. Subsequently, primary antibodies were removed and wells were washed with 1 × PBS. Alexa Fluor 546 goat anti mouse secondary antibody was added in each well and incubated at 37°C for 1 h followed by washing with PBS. Cells were counterstained with 0.0005 g/L solution of DAPI-PBS for 15 min at room temperature and again washed with PBS. Lastly, 5 μL mounting medium was added on the glass slide and coverslip (cell-side) was placed on it. Slides were analyzed using fluorescence microscope (TE2000-S, Nikon, Japan).

Flow cytometric analysis was performed according to the previously reported method with some modifications[16]. Cells were cultured in T-75 flasks. Upon reaching 80% confluence, cells were washed twice with 1 × PBS and treated with 5 mL cell dissociation buffer. Cells were incubated in 50 mL/L CO_2 at 37 °C for 30-40 min. Cell suspension was collected in 15 mL conical tube and centrifuged for 10 min at 400 × g. Supernatant was removed and pellet was mixed with 600 μL cold FACS solution (10 g/L BSA-1 × PBS, 0.001 mol/L EDTA and 1 g/L sodium azide) and transferred equally into 6 conical tubes. Each cell suspension was centrifuged at 400 × g for 5 min. Supernatant was discarded and blocking solution was added in each tube. 200 μL monoclonal primary antibodies against vimentin, CD73, CD105, and CD45 were added in separate tubes, and kept for 1 h at room temperature. Cell suspension was centrifuged at 400 × g at 5 min. Supernatant containing unbound primary antibodies were removed and pellet was washed twice with 500 μL ice cold FACS solution by centrifugation at 400 × g for 8 min each. After discarding the supernatant, 200 μL Alexa Fluor 546 goat anti mouse secondary antibody was added in each tube and incubated for 2 h at room temperature in dark. Cells were again washed with 500 μL FACS solution. Pellet was suspended in 300 μL FACS solution and transferred in 5 mL FACS tubes. Cells labeled only with secondary antibody were used as control. Single cell suspension was analyzed by flow cytometer (FACScaliber, Becton Dickinson) and data was interpreted using BD Cell Quest Pro software.

MTT assay for cell viability

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was conducted as previously reported[17], to find out the non-toxic concentration of GA, GT and their combination. The assay is based on the ability of converting water-soluble tetrazolium salt into water-insoluble formazan particles by metabolically active cells. Approximately, 5000 cells were seeded with 200 μL DMEM per well in a flat-bottom 96-well plate. Plate was kept in CO_2 incubator overnight at 37 °C. Next day, medium was removed and cells were incubated for 24 h with different concentrations of GA and GT in FBS-free medium. Following day, medium was removed and cells were incubated with 200 μL of 0.5 g/L MTT solution for 4 h in the CO_2 incubator. MTT solution was removed and 200 μL DMSO was added to dissolve formazan granules formed by the metabolically active cells. Absorbance was monitored at 570 nm using spectrophotometer (SpectraMax, United States).

Treatment of hUC-MSCs

To induce hepatic differentiation, 10 μM concentration of both compounds and their combination was selected based on MTT cell viability assay. hUC-MSCs were divided into the following experimental groups; untreated control, GA-treated, GT-treated, and combination group of (GT + GA)-treated hUC-MSCs. Working solutions were prepared from their 0.01 mol/L stock solutions. Cells grown in T-25 flasks were treated in FBS-free medium for 24 h. Subsequently, medium was removed, fresh medium was added and cells were kept in CO_2 incubator for different time-points; day 7, 14 and 21 for morphological examination and RNA isolation. Exhausted medium was replaced after every 72 h.

Evaluation of hepatic markers by gene and protein expression analysis

For gene expression analysis, total cellular RNA was isolated from untreated and treated cells at day 7, 14 and 21 by Trizol method according to the manufacturer's guidelines. Micro-volume UV-Vis spectrophotometer (Nanodrop 2000, Thermo Fisher, United States) was used to quantify and assess the purity of extracted RNA. Absorbance was measured at 260 nm in μg/μL and an absorbance ratio of 260/280 was calculated for sample purity. cDNA was synthesized equivalent to 1 μg of extracted RNA using RevertAid First Strand cDNA synthesis kit (Thermo Fisher, United States). Gene expression levels of hepatic markers were determined by qRT-

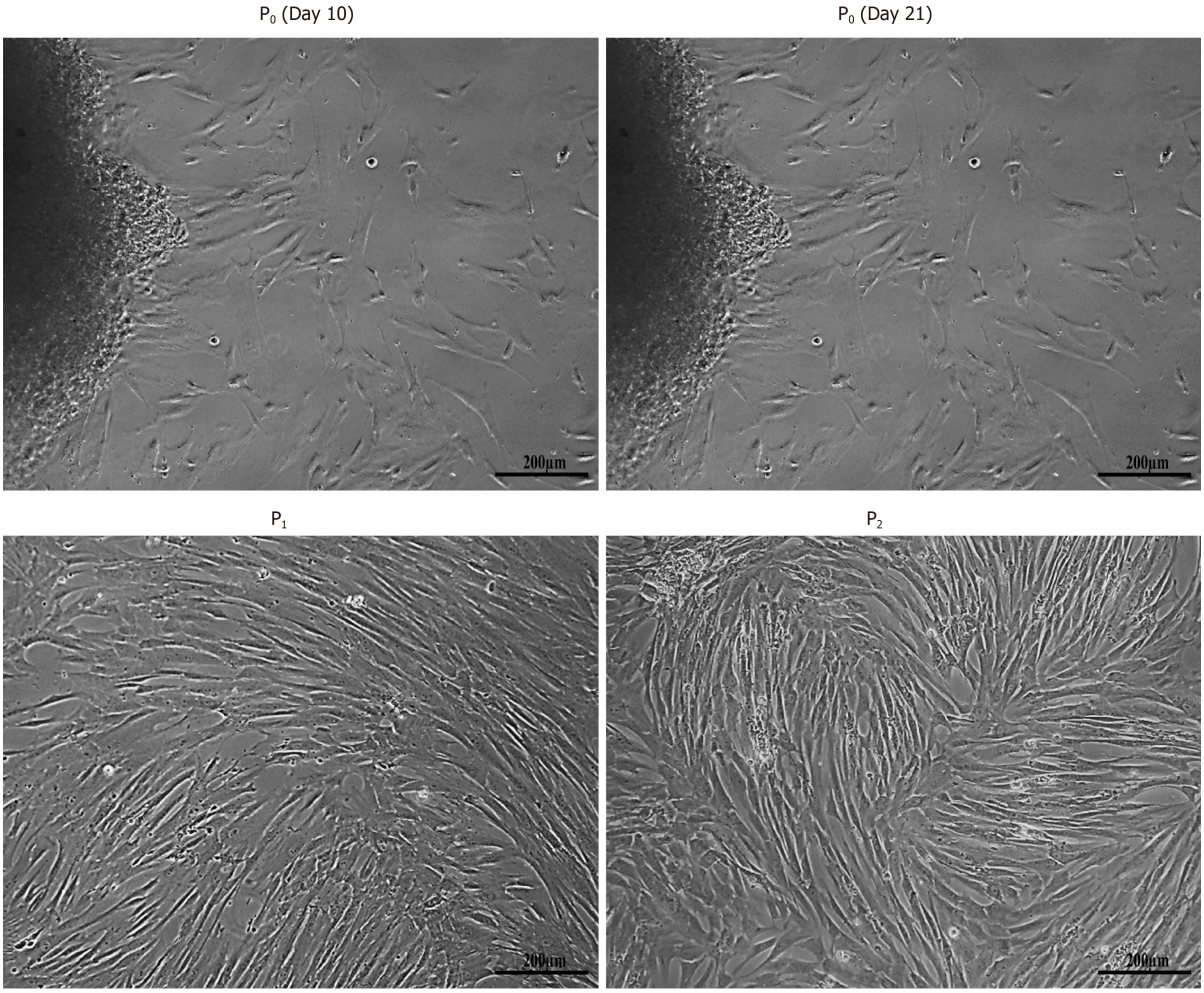

Figure 1 Isolation of human umbilical cord-mesenchymal stem cells. Morphological observations of human umbilical cord-mesenchymal stem cells showing passage 0 (P_0) cells at day 10 and day 21 with cells migrating towards the periphery of the adherent tissue, and passage 1 (P1) and passage 2 (P2) cells with homogeneous population showing fusiform- and fibroblast-like appearance after sub-culturing. All images were taken under phase contrast at 10× magnification.

PCR (Mastercycler ep realplex, Eppendorf, Germany) using primer sequences listed in Table 1. Total of 40 PCR cycles were run according to the program layout shown in Table 2. All reactions were carried out in triplicates to validate them statistically. Human specific *β-actin* gene was used as an internal control to normalize gene expression in all the experimental groups. Results obtained in the form of Ct values were used to find relative gene expression.

For protein expression, immunofluorescence staining of untreated and 21 day treated hUC-MSCs was performed using primary antibodies for hepatocyte specific proteins *i.e.*, alpha-fetoprotein (AFP), albumin (ALB), hepatocyte nuclear factor-3α (HNF-3α) and tyrosine-aminotransferase (TAT). Protein expression was examined under fluorescence microscope.

Periodic acid Schiff staining for glycogen detection

For functional characterization, presence of intracellular glycogen in 21 d treated hUC-MSCs was assessed by periodic acid Schiff staining in accordance with the protocol described previously[18]. Cells were fixed in 40 g/L PFA and permeabilized with 0.1% Triton X-100 for 10 min in 24-well plate. After multiple washings with PBS, cells were oxidized with 1 mL/L periodic acid for 5 min and then washed three times with PBS. Cells were then incubated with Schiff's reagent for 15 min and again washed with PBS. Subsequently, cells were counterstained with hematoxylin for 1 min and washed with PBS in the same manner. Finally, 10 mL/L mounting medium was added on the slides and cells were examined under light microscope (YS100, Nikon, Japan).

Statistical analysis

Statistical analysis of the data was performed by IBM SPSS software, version 22. Independent sample *t*-test, and One-way analysis of variance (ANOVA) with Post-Hoc

Table 1 Primer sequences of the targeted genes

Genes	Primer sequence (5'-3')	Annealing temperature (°C)
Beta- Actin	Forward: 5′-CACTGGCATCGTGATGGACT-3′	60
	Reverse: 5′-TGGCCATCTCTTGCTCGAAG-3′	
Alpha-Fetoprotein	Forward: 5′-CAGCATCGATCCCACTTTTCC-3′	62
	Reverse: 5′-ATTTTGTCATAGCGAGCAGCC-3′	
Albumin	Forward: 5′-CAAAGCATGGGCAGTGCTC-3′	60
	Reverse: 5′-GCCCTGTCATCAGCACATTC-3′	
Cytokeratin-18	Forward: 5′-CCAGCTTGGAGAACAGCCT-3′	60
	Reverse: 5′-AGCCTCCAGCTTGACCTTG-3′	
Tyrosine-aminotransferase	Forward: 5′-TCTGAGCTTCCTCAAGTCCAA-3′	63
	Reverse: 5′-CATGAGGTCATAGCCCCAGA-3′	
Cytochrome P450 2B6	Forward: 5′-ATTGTCACCCAACACACCAG -3′	60
	Reverse: 5′-CAGTCTTTTTCAGTGCCCCA -3′	
Hepatocyte nuclear; factor 4-α	Forward: 5′-GACTACATTGTCCCTCGGCA -3′	62
	Reverse: 5′-ATACTGGCGGTCGTTGATGT -3′	

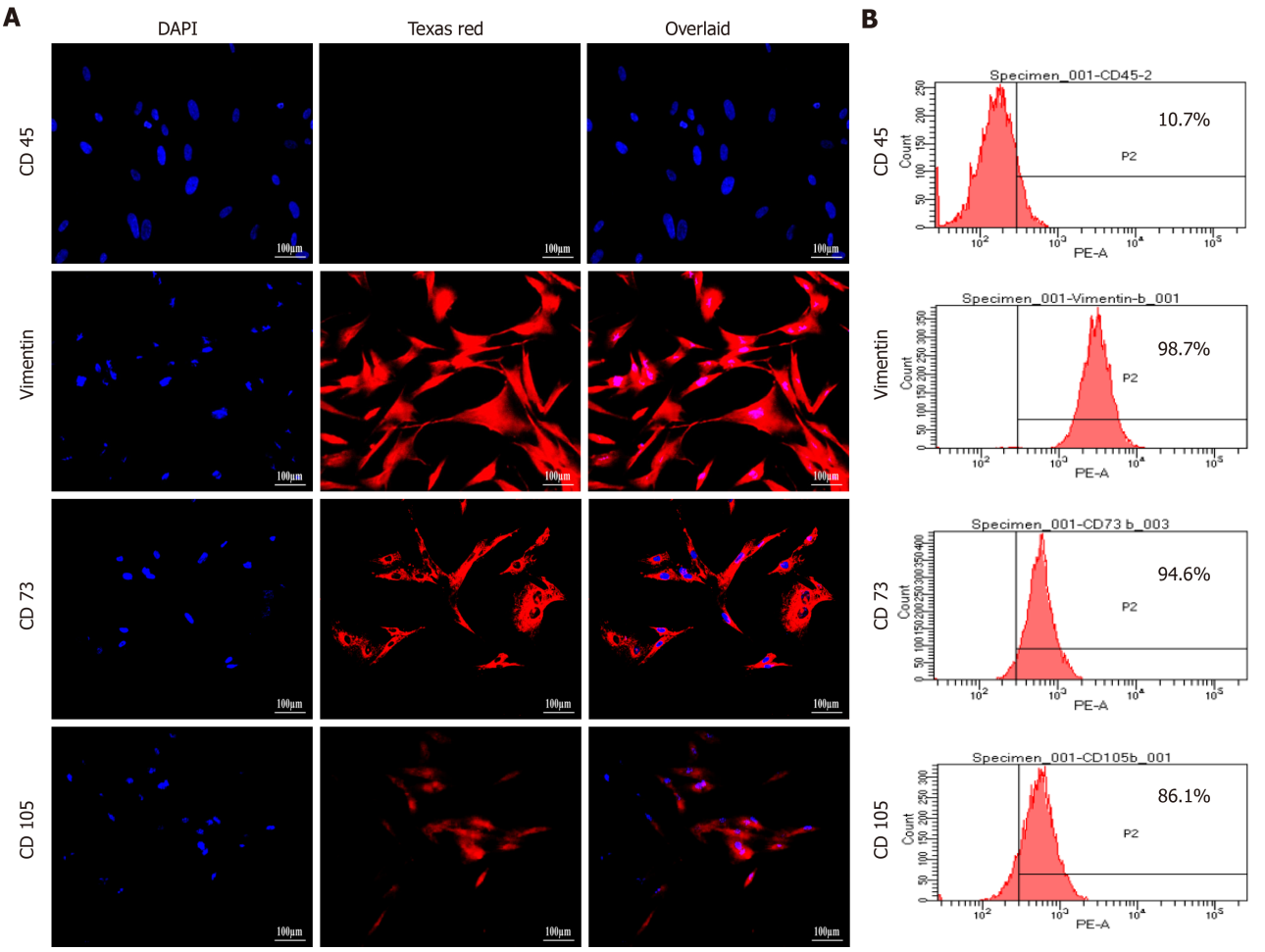

Figure 2 Characterization of human umbilical cord-mesenchymal stem cells by immunocytochemical analysis and flow cytometry. A: Immunocytochemical analysis showing positive expression of mesenchymal markers, CD73, CD105, and vimentin, and negative expression of hematopoietic marker CD45. Fluorescent micrographs were captured at 20 × magnification; B: Flow cytometric analysis showing mesenchymal markers CD73, CD105, and vimentin having more than 85% positive cells. Data were analyzed using Cell Quest Pro software.

Bonferroni corrections were done to assess statistical significance. All experiments we

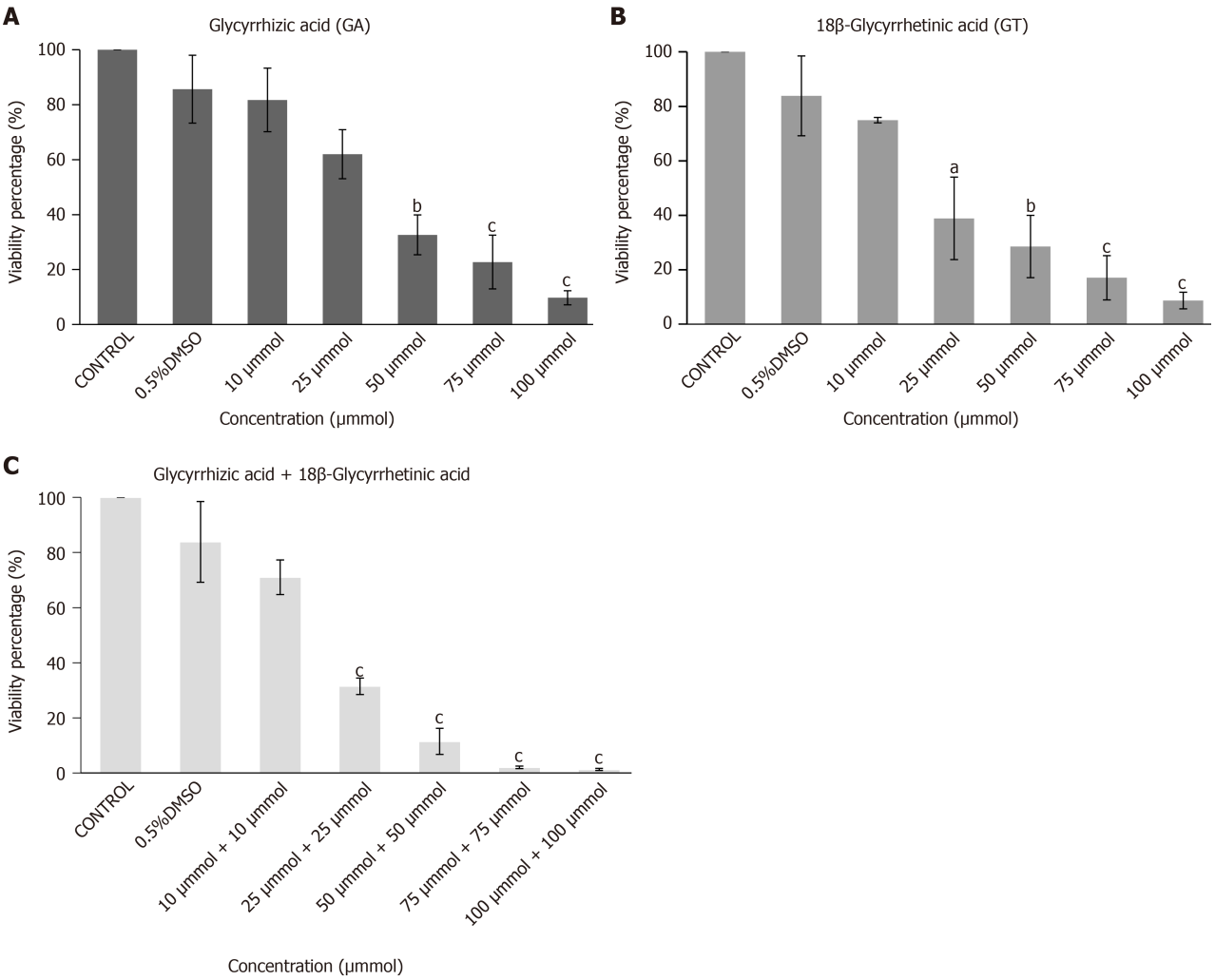

Figure 3 Cytotoxicity analysis of human umbilical cord-mesenchymal stem cells by MTT Assay. Bar graphs showing percent viability of human umbilical cord-mesenchymal stem cells treated with A: glycyrrhizic acid (GA), B: 18β-glycyrrhetinic acid (GT), and C: their combination (GA + GT) at different concentrations. A decrease in percent (%) cell viability is observed with increasing concentrations of GA, GT and their combination. Statistical analysis was conducted by One-way ANOVA and Post-Hoc Bonferroni tests. Results are expressed as mean ± SEM (n = 3); level of significance $^{a}P \leq 0.05$ (where $^{a}P \leq 0.05$, $^{b}P \leq 0.01$, and $^{c}P \leq 0.001$).

RESULTS

Morphological features of cultured hUC-MSCs

hUC-MSCs began to migrate from explants after approximately 8-10 d in culture and adhere to the surface of tissue culture flask. The adherent cells having fibroblast and fusiform appearance, exhibited distinct ability to expand following adherence. After sub-culturing, cells showed greater proliferative ability and maintained their morphology beyond several passages (Figure 1).

Phenotypic characterization of hUC-MSCs

Cultured hUC-MSCs were phenotypically characterized through immunocytochemistry and flow cytometry which confirmed increased number of cells expressing MSC-specific markers including CD73, CD105, and vimentin, while negative expression of hematopoietic marker, CD45 was observed (Figure 2).

Cell viability

Viability of hUC-MSCs was decreased in a concentration-dependent manner when cells were subjected to different concentrations of GA, GT and their combination. Concentration of 10 µM of both compounds was non-cytotoxic and therefore selected for cell treatment in all groups (Figure 3).

Morphological observation of preconditioned MSCs

hUC-MSCs were observed for the morphological changes at three different time points *i.e.*, day 7, 14 and 21. Cells lost their fusiform / spindle shaped morphology and progressed towards polygonal morphology of hepatocytes in a time-dependent manner. At day 7, GA- and GT-treated hUC-MSCs became slightly shorter, flattened, and broader in appearance with retracted ends, while in the combination group, cells also began to change their morphology and appeared as polygonal. Changes were more apparent at day 14, where cells decreased in size and transformed into an irregular or polygonal shape. By day 21, majority of the cells became more compact and polygonal in shape exhibiting tight interactions between the cells in all treatment groups (Figure 4).

Temporal gene expression analysis

Gene expression pattern of GA and GT-treated hUC-MSCs showed significant expression of early hepatocyte marker *AFP* at all time points. Highest expression was observed at day 7 but it decreased in a time-dependent manner. Significant expression of *ALB* and *CK-18* was detected at all time points. Late hepatic markers, *TAT*, *CYP450-2B6*, and *HNF-4α* were not detectable at day 7 but their expression levels were significantly up-regulated at day 14 and 21, as compared to the untreated control (Figure 5A and B). Gene expression pattern of the combination group showed similar results for *AFP*. Significant expression of *ALB, CK-18, TAT, CYP450-2B6,* and *HNF-4α* was observed at all time points and their expression increased with time of differentiation as compared to the untreated control (Figure 5C). These results suggest that MSCs were differentiated into hepatic lineage after treatment with these compounds separately or in combination.

Immunofluorescence analysis of hepatocyte specific proteins

Differentiation of treated hUC-MSCs towards hepatic lineage was further confirmed by immunocytochemical analysis of hepatocyte specific proteins. hUC-MSCs treated with GA, GT and their combination exhibited positive expression of hepatocyte specific proteins including, AFP, ALB, HNF-3α, and TAT as compared to the untreated control at day 21, demonstrating that hUC-MSCs successfully differentiated towards hepatic lineage (Figure 6A).

Quantification of fluorescence intensity

Fluorescence intensity of MSCs treated with GA, GT, and their combination was also measured and validated statistically. Significant up-regulation of hepatocyte specific proteins including, AFP, ALB, HNF-3α, and TAT was observed in all treatment groups as compared to the MSCs with no treatment (control) at day 21, demonstrating that MSCs differentiated towards hepatic lineage (Figure 6B).

Analysis of glycogen content

Treated hUC-MSCs were further evaluated for their functionality by periodic acid Schiff staining (PAS) to corroborate their hepatic differentiation. hUC-MSCs treated with GA, GT and their combination, showed strong PAS positive signals at day 21, indicating their capability to store glycogen as compared to the untreated control. Bi-nucleated cells were also observed, which is one of the prominent characteristics of functional hepatocytes (Figure 7).

DISCUSSION

In our study, we determined the effects of two triterpenes, GA and GT on differentiation of human umbilical cord-MSCs towards hepatic lineage. These compounds exhibit remarkable pharmacological characteristics. GA has a significant role in the treatment of liver fibrosis, hepatic steatosis, viral hepatitis, acute and chronic liver injury, and myocarditis[19-21]. In liver injury, GA exerts anti-inflammatory response by inhibiting TNF release, myeloperoxidase activity, and translocation of nuclear factor-κB in the nuclei. It also increases the expression of proliferating cell nuclear antigen, promoting the regeneration of damaged hepatic cells[22]. Pretreatment with GA significantly increases cytochrome P450 enzymes in liver to regulate oxidative metabolism of dietary toxins, drugs, mutagens and carcinogens, which lowers the risk of carcinomas[23]. GT exhibits numerous biological activities including anti-oxidative, anti-apoptotic, anti-inflammatory and anti-allergic effects[24]. GT treatment showed

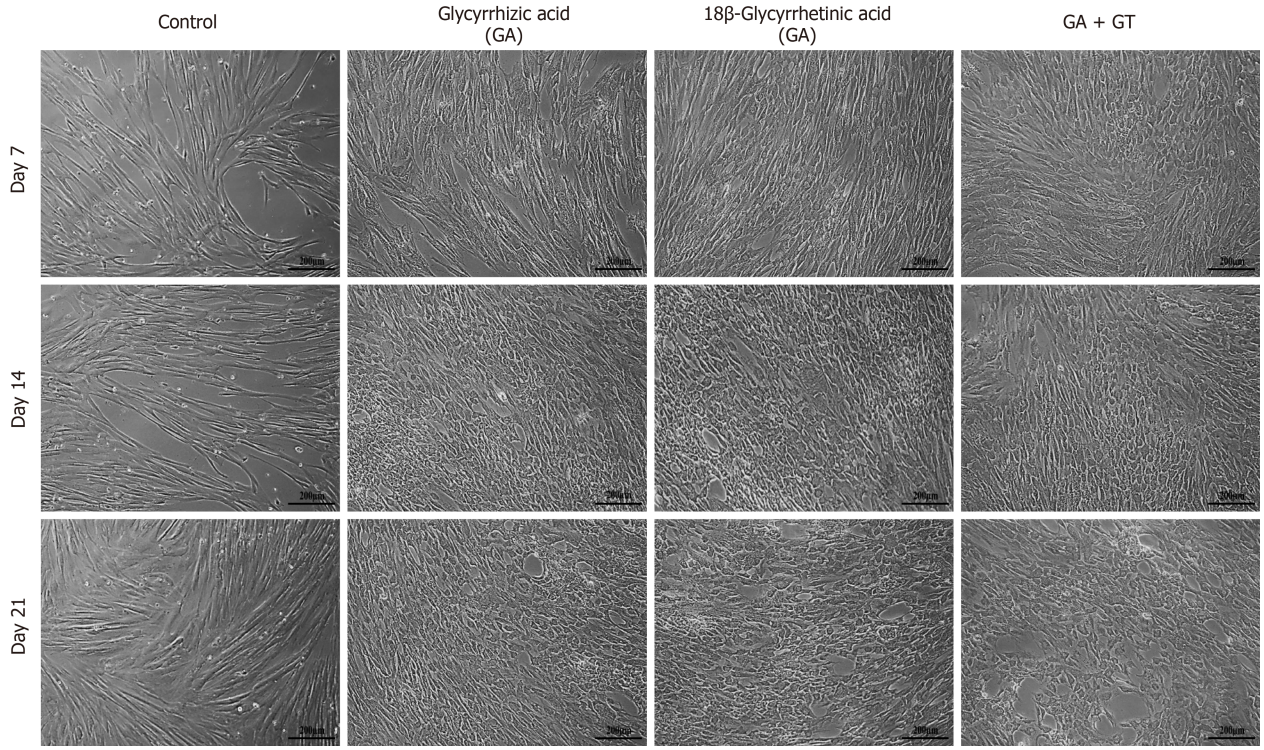

Figure 4 Morphological features of human umbilical cord-mesenchymal stem cells treated with glycyrrhizic acid (GA), 18β-glycyrrhetinic acid (GT), and their combination (GA + GT). At day 7, GA and GT treated MSCs became slightly shorter, flattened, and broader in appearance with retracted ends, while in the combination group, cells began to appear polygonal in shape. At day 14, changes were more apparent and cells progressively decreased in size and transformed into irregular or polygonal shape and by day 21, majority of the cells developed into more compact and polygonal morphology like that of hepatocytes exhibiting tight interactions between the cells in all treatment groups as compared to the untreated control. All images were taken under phase contrast at 10× magnification.

anti-diabetic effects by elevating plasma insulin levels, and reducing the levels of gluconeogenic enzymes in liver. Previously, a study showed hepato-protective effects of GT on drug-induced hepatotoxicity, by down-regulation of HMGB1-TLR4 (High-Mobility Group Protein B1- TollLike Receptor 4), Nrf2, PPARγ signaling pathway[25, 26].

On the basis of their therapeutic potential and beneficial role in modulating several physiological pathways in liver regeneration, we hypothesized that these chemical compounds may have the ability to either directly differentiate MSCs into hepatocytes or aid in the process of differentiation. For this purpose, MSCs were harvested from human umbilical cord tissue (hUC) using explant method[27]. The isolated cells expressed MSC markers *i.e.*, CD73, CD105, and vimentin, but were negative for hematopoietic marker, CD45. Therefore, the isolated cells possess the basic characteristics of MSCs in accordance with the specifications suggested by the Society of Cellular Therapy[28]. Next, we identified the non-toxic concentration of GA and GT by conducting MTT assay. For both compounds, 10 μM concentration was selected for subsequent experiments as this concentration was found to be non-toxic to cells. hUC-MSCs were treated with GA and GT separately and in combination for 24 h, and then examined at three different time points; day 7, 14, and 21. We observed fibroblast-like and fusiform-shaped morphology of MSCs in all treatment groups in comparison with the control group. This eventually progressed towards irregular or hepatocyte-like polygonal morphology with time of differentiation, in accordance with earlier findings [29,30].

To evaluate hepatic differentiation, we determined gene expression levels of hepatocyte specific markers such as *AFP, ALB, CK-18, TAT, CYP450-2B6*, and *HNF-4α* by qRT-PCR, as their expression is predominately regulated at the transcription level. The transcriptional profile of treated hUC-MSCs of GA, GT, and the combination group showed positive expression of early to mid-late hepatic markers including *AFP, ALB,* and *CK-18* at all time points. The expression of *AFP* was higher at day 7, indicating initiation of cell differentiation, however, it decreased at later time points; whereas the expression of *ALB* and *CK-18* enhanced with time of differentiation. The late hepatic markers, *HNF-4α, TAT,* and *CYP450-2B6* were detectable at day 14 and 21 in GA-, and GT-treated MSCs, while in the combination group, their significant up-

Table 2 qRT-PCR program layout

Process	Temperature (°C)	Time duration	Cycles
Initial denaturation	95	10 min	1
Cyclic denaturation	95	15 s	40
Annealing and extension	60	1 min	
Melting curve	95	15 s	-
	60	15 s	
	95	15 s	

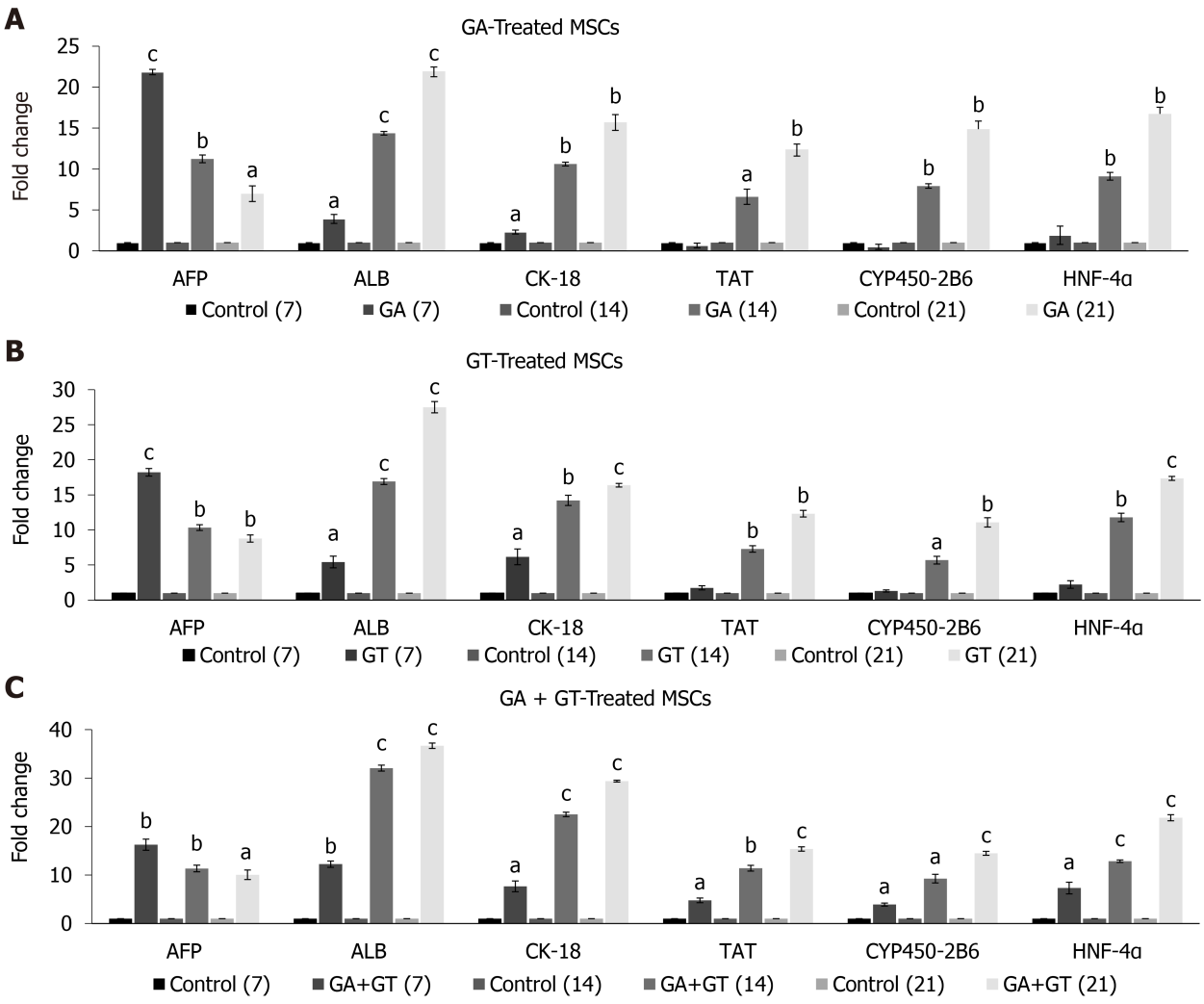

Figure 5 Expression of hepatic genes in human umbilical cord-mesenchymal stem cells treated with glycyrrhizic acid, 18β-glycyrrhetinic acid, and their combination. A and B: Glycyrrhizic acid (GA)- and 18β-glycyrrhetinic acid (GT)-treated mesenchymal stem cells showed significant expression of early hepatocyte marker *AFP* at all time points. Highest expression was observed at day 7 but it declined in a time-dependent manner. Significant expression levels of *ALB* and *CK-18* were detected at all time points. Late hepatic markers, *TAT*, *CYP450-2B6*, and *HNF-4α* were not detectable at day 7 but were significantly up-regulated at day 14 and 21, as compared to the untreated control; C: GA + GT-treated MSCs revealed significant up-regulation of *AFP* at all time points. Highest expression was observed at day 7 but it declined with treatment time. Significant expression levels of *ALB, CK-18, TAT, CYP450-2B6,* and *HNF-4α* were observed at all time points and their expression increased with time of differentiation, as compared to the untreated control. Statistical analysis was conducted by independent sample t-test. Results are expressed as mean ± SEM (n = 3); level of significance $^{a}P \leq 0.05$ (where $^{a}P \leq 0.05$, $^{b}P \leq 0.01$, and $^{c}P \leq 0.001$).

regulation was observed at all time points.

AFP is an endodermal marker as well as early developmental marker of hepatoblast formation; its level gradually reduces with time indicating that the immature hepatocytes progress towards adult phenotype[31]. ALB is the most abundant protein produced by adult hepatocytes; its synthesis begins in early fetal hepatocytes and

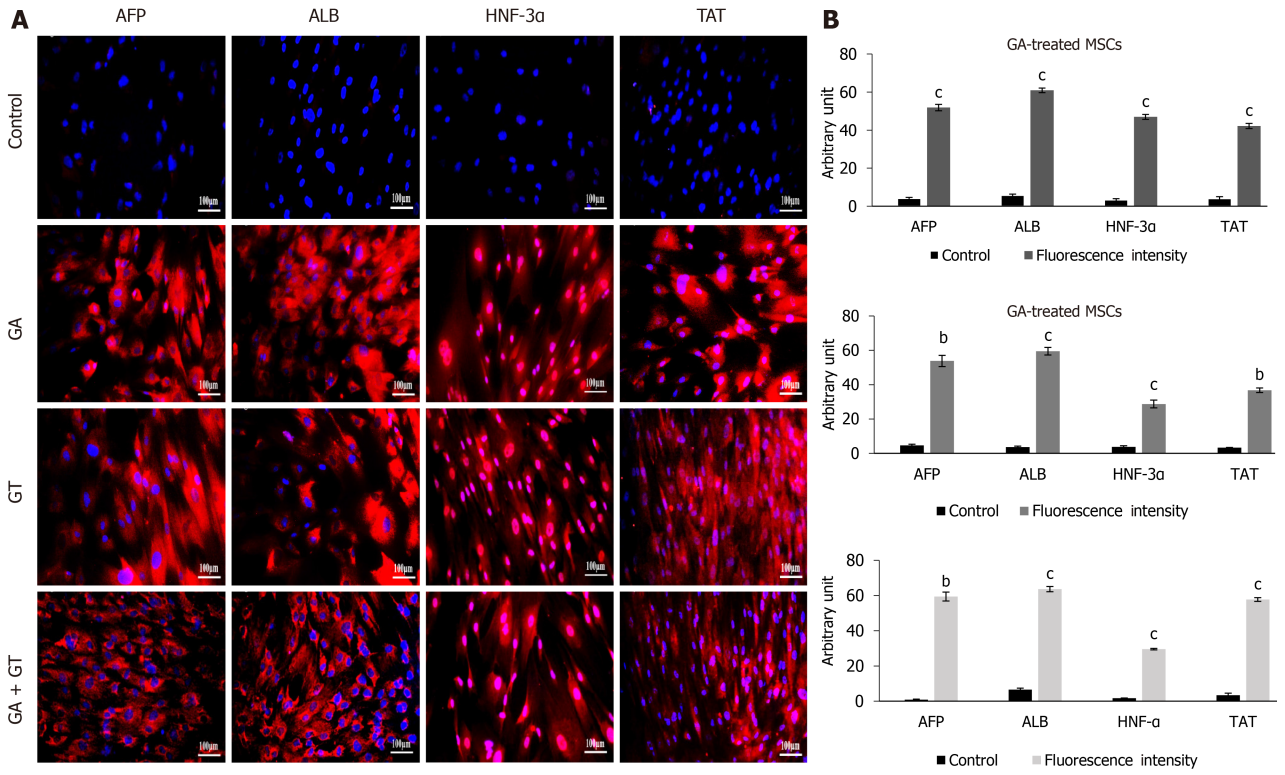

Figure 6 Immunocytochemical analysis and quantification of hepatic proteins in human umbilical cord-mesenchymal stem cells treated with glycyrrhizic acid, 18β-glycyrrhetinic acid and their combination. A: At day 21, treated mesenchymal stem cells exhibited positive expression for hepatocyte specific proteins including, alpha-fetoprotein, albumin, hepatocyte nuclear factor-3α, and tyrosine-aminotransferase in all treatment groups as compared to the untreated control; B: Fluorescence intensities of all treatment groups were measured using Image J software. Statistical analysis was conducted by independent sample *t*-test. Results are expressed as mean ± SEM (n = 3); level of significance $^{a}P \leq 0.05$ (where $^{b}P \leq 0.01$ and $^{c}P \leq 0.001$).

reaches the maximum level in the mature hepatocytes[32]. Similarly, cytokeratin-18 (CK-18) is a hepatic cytoskeletal protein and epithelial surface marker; it is weakly expressed during hepatoblast formation, while up-regulated at the maturation stage [33]. HNF-4α, a liver enriched transcription factor, maintains the hepatic differentiation status of MSCs, and is considered as a key regulator of hepatic functions and metabolic enzymes[34,35]. TAT and cytochrome P450-2B6 (CYP450-2B6) are enzymes, largely expressed in adult hepatocytes[36]. The gene expression pattern of all treatment groups suggests that GA, GT and their combination was able to direct hUC-MSCs towards hepatic lineage. Interestingly, in the combination group, positive expression of early and late hepatocyte markers at the initial days of treatment may indicate their synergistic effect in an early switch to hepatic differentiation.

Additionally, hepatic markers were evaluated at the protein level, where hUC-MSCs of GA, GT, and the combination groups showed positive immunostaining of AFP, ALB, TAT, and HNF-3α proteins at day 21 of treatment. HNF-3α and HNF-4α are transcription factors essential for hepatic specification, and primarily involved in hepatic metabolism[37,38]. To further validate the hepatic differentiation, we performed PAS to assess glycogen storage ability of the treated cells. In comparison to the control group, hUC-MSCs treated with GA, GT and their combination, stained purple-pink at day 21 of treatment, indicating the presence of glycogen in their cytoplasm, which is in line with earlier findings[39]. Furthermore, bi-nucleated cells were also seen in all treatment groups, which is the typical feature of functional hepatocytes[40].

Collectively, our experimental results imply that triterpenes, GA and GT, have the potential to enhance hepatic differentiation of hUC-MSCs. The preconditioned MSCs may serve as an effective cell source for hepatic diseases in clinical applications. Nevertheless, the underlying mechanism still needs to be investigated to understand the process of cellular differentiation to ensure a better hepatic induction strategy.

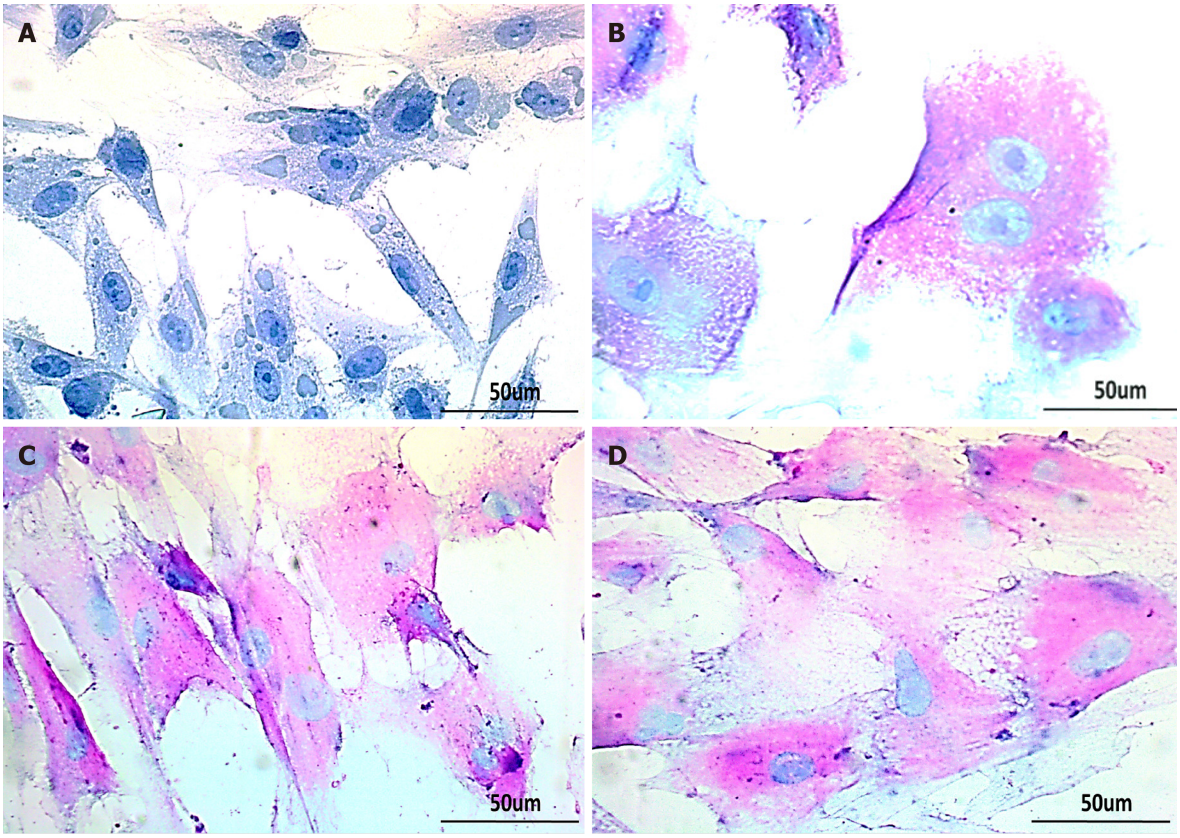

Figure 7 Glycogen detection in treated human umbilical cord-mesenchymal stem cells by periodic acid Schiff staining. Bright-field images of A: untreated control showing negative periodic acid Schiff staining (PAS); B: MSCs treated with glycyrrhizic acid (GA); C: 18β-glycyrrhetinic acid (GT); and D: GA+GT (combination group) at day 21 showing PAS positive cells stained as purple-pink, indicating the presence of glycogen in their cytoplasm. Bi-nucleated cells (a hepatocyte characteristic) were also observed. Images were taken at 40× magnification.

CONCLUSION

It is concluded from this study that triterpenes, GA and GT at 10 μM concentration were able to induce hepatic differentiation of hUC-MSCs. The early and late hepatic genes were significantly up-regulated in a time-dependent manner. Moreover, the combination of both compounds has strong synergistic effect as shown by the induction of hepatic differentiation at early time points after treatment. The significant expression of hepatocyte markers, the ability to store glycogen and presence of bi-nucleated cells, suggest hepatic differentiation in all treatment groups. However, further investigations are required to explore the molecular mechanism involved in the differentiation process and to assess the therapeutic effect of preconditioned hUC-MSCs in the *in vivo* model of end-stage liver disease. These findings will help in developing novel cell-mediated therapeutic strategies for regenerative medicine for end-stage liver disease.

ARTICLE HIGHLIGHTS

Research background

End-stage liver disease (ESLD) causes extensive healthcare burden with limited treatment options. Cell-based therapies using mesenchymal stem cells (MSCs) emerged as a potential approach to support hepatic regeneration in ESLD. However, effective translation of this therapy requires MSCs to have maximum regenerative potential. *In vitro* preconditioning strategies have been employed to strengthen the regenerative and differentiation potential of MSCs. Chemical compounds of the class triterpenes; glycyrrhizic acid and 18β-glycyrrhetinic acid present diverse therapeutic features including hepato-protection and anti-fibrotic characteristics. They are capable of modulating several physiological pathways that are crucial in hepatic regeneration. Preconditioning with hepato-protective triterpenes may stimulate MSC fate transition

towards hepatocytes.

Research motivation

Although mesenchymal stem cell-mediated therapy has proved as an effective approach for hepatic regeneration, the major challenge is low cell viability and resistance in the impaired tissue post-transplantation. To acquire a persistent therapeutic efficacy, *in vitro* preconditioning of MSCs with hepato-protective chemical compounds may facilitate their regenerative and differentiation potential towards hepatic lineage for better survival, homing, and migration ability at the site of injury.

Research objectives

Considering the characteristics of triterpenes, glycyrrhizic acid and 18β-glycyrrhetinic acid in hepatic anomalies, the objective of the study is to explore their role in hepatic differentiation of MSCs. Preconditioned cells may serve as a better source for tissue regeneration in liver injury.

Research methods

hUC-MSCs were harvested and characterized phenotypically by flow cytometry and immunocytochemistry for the expression of MSC associated surface molecules. Isolated cells were treated with glycyrrhizic acid, 18β-glycyrrhetinic acid, and their combination for 24 h, and then analyzed at three time points; day 7, 14, and 21. qRT-PCR was performed to evaluate the expression of hepatic genes. On day 21, hepatic proteins were analyzed by immunocytochemistry. Periodic acid Schiff staining was performed to determine the functional ability of treated cells.

Research results

The transcriptional profile of preconditioned MSCs displayed significant expression of hepatic genes with increasing time of differentiation. Preconditioned cells showed positive protein expression of hepatocyte specific proteins. The results were further corroborated by positive periodic acid Schiff staining, indicating the presence of glycogen in their cytoplasm. Moreover, bi-nucleated cells which is the typical feature of hepatocytes, were also seen in the preconditioned cells.

Research conclusions

Our data suggest that preconditioning of hUC-MSCs with triterpene compounds, glycyrrhizic acid, and 18β-glycyrrhetinic acid or both, successfully differentiates these cells into hepatic-like cells. The study would serve as an attempt to develop new targeted therapies using triterpenes in combination with stem cells for the treatment of end-stage liver diseases.

Research perspectives

The present study is an endeavor to augment cell based therapeutic approach by preconditioning hUC-MSCs with glycyrrhizic acid and 18β-glycyrrhetinic acid to promote their therapeutic and differentiation potential towards hepatic lineage. The preconditioned MSCs may serve as an effective source for cell therapy for injured hepatic tissue in clinical applications.

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ORIGINAL ARTICLE

Basic Study

Impact of senescence on the transdifferentiation process of human hepatic progenitor-like cells

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Abstract

BACKGROUND

Senescence is characterized by a decline in hepatocyte function, with impairment of metabolism and regenerative capacity. Several models that duplicate liver functions *in vitro* are essential tools for studying drug metabolism, liver diseases, and organ regeneration. The human HepaRG cell line represents an effective model for the study of liver metabolism and hepatic progenitors. However, the impact of senescence on HepaRG cells is not yet known.

AIM

To characterize the effects of senescence on the transdifferentiation capacity and mitochondrial metabolism of human HepaRG cells.

METHODS

We compared the transdifferentiation capacity of cells over 10 (passage 10 [P10]) *vs* P20. Aging was evaluated by senescence-associated (SA) beta-galactosidase activity and the comet assay. HepaRG transdifferentiation was analyzed by confocal microscopy and flow cytometry (expression of cluster of differentiation 49a [CD49a], CD49f, CD184, epithelial cell adhesion molecule [EpCAM], and cytokeratin 19 [CK19]), quantitative PCR analysis (expression of albumin, cytochrome P450 3A4 [CYP3A4], γ-glutamyl transpeptidase [γ-GT], and carcinoembryonic antigen [CEA]), and functional analyses (albumin secretion, CYP3A4, and γ-GT). Mitochondrial respiration and the ATP and nicotinamide adenine dinucleotide (NAD^+)/NAD with hydrogen (NADH) content were also measured.

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RESULTS

SA β-galactosidase staining was higher in P20 than P10 HepaRG cells; in parallel, the comet assay showed consistent DNA damage in P20 HepaRG cells. With respect to P10, P20 HepaRG cells exhibited a reduction of CD49a, CD49f, CD184, EpCAM, and CK19 after the induction of transdifferentiation. Furthermore, lower gene expression of albumin, CYP3A4, and γ-GT, as well as reduced albumin secretion capacity, CYP3A4, and γ-GT activity were reported in transdifferentiated P20 compared to P10 cells. By contrast, the gene expression level of CEA was not reduced by transdifferentiation in P20 cells. Of note, both cellular and mitochondrial oxygen consumption was lower in P20 than in P10 transdifferentiated cells. Finally, both ATP and NAD^+/NADH were depleted in P20 cells with respect to P10 cells.

CONCLUSION

SA mitochondrial dysfunction may limit the transdifferentiation potential of HepaRG cells, with consequent impairment of metabolic and regenerative properties, which may alter applications in basic studies.

Key Words: Senescence; HepaRG cells; Transdifferentiation; Mitochondria; Regeneration; Nicotinamide adenine dinucleotide

Core Tip: The human HepaRG cell line represents an effective model for the study of liver metabolism and hepatic progenitors. However, the impact of senescence on HepaRG cells is not known. We characterized the effects of senescence on the transdifferentiation capacity and mitochondrial metabolism of HepaRG cells. By using a replication protocol, we described higher senescence-associated markers and lower transdifferentiation markers in passage 20 (P20) than in P10 cells. Cellular and mitochondrial oxygen consumption, and ATP and nicotinamide adenine dinucleotide (NAD^+)/NAD with hydrogen (NADH) content were lower in P20 than in P10 transdifferentiated cells. To conclude, senescence-associated mitochondrial dysfunction may limit the transdifferentiation potential of HepaRG cells.

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INTRODUCTION

Primary human hepatocytes are the gold standard to study the biology, pharmacology, and toxicology of parenchymal liver cells[1]. Nevertheless, primary hepatocytes present with several limitations such as difficult isolation procedures, high variability between different donors, quick failure in biological function, and proliferation capacity[2]. To overcome these limitations, several human hepatoma cell lines were developed and characterized to provide a steady and unrestricted supply of hepatocyte-like cells. Of these, HepG2 and Huh-7 cells are the most widely used. HepG2 cells originated from an American patient[3], while Huh-7 were isolated from a Japanese patient[4], both affected by well-differentiated hepatocellular carcinoma. Even though these cell lines exhibit several hepatic functions, low expression of enzymes and cytochromes limit their use for metabolism and toxicity studies[5].

To study the metabolism, toxicology, and regeneration/differentiation processes, the HepaRG cell line was used as a replacement for primary hepatocytes, HepG2, and Huh-7 cells[6-8]. Isolated from an Edmonson grade I differentiated liver tumor, HepaRG cells exhibit a hepatocyte-like morphology and express hepatocyte-specific functions in defined culture conditions[9]. Nevertheless, HepaRG cells display features

of human oval ductular bipotent hepatic progenitors at confluence[10,11]. Supplementation of dimethyl sulfoxide (DMSO) to confluent HepaRG cells triggers differentiation toward hepatocytes[10,12]. Thus, the behavior of HepaRG cells is exclusive; these cells can be cultured for several passages to proliferate, or stimulated to differentiate towards fully functional hepatocyte-like cells[13].

Cellular senescence consists of a steady cell cycle block occurring because of different harmful events, which include DNA damage, oxidative stress, or even replication[14]. Stem/progenitor cells undergoing senescence cause impairment of tissue homeostasis and regeneration, caused by defective stemness and differentiation processes[15]. The induction of senescence in both HepG2 and Huh-7 cells is associated with altered gene signature, which includes changes in cell cycle regulation, signal transduction, and metabolism[16,17]. Nevertheless, to the best of our knowledge, the impact of senescence on HepaRG cells has not yet been investigated.

Thus, this study investigated whether a replication protocol would induce senescence in HepaRG cells. In addition, we characterized the effects of senescence on the transdifferentiation capacity and mitochondrial metabolism.

MATERIALS AND METHODS

Cell line and culture

The human cell line HepaRG was purchased by Merck Millipore (MMHPR116; Merck KGaA, Darmstadt, Germany). Undifferentiated HepaRG cells exhibit a fibroblast-like morphology, and the differentiation process induces both hepatocyte- and biliary-like epithelial phenotypes at confluence, indicating bipotent progenitor features[11,18]. HepaRG cells were seeded at 27000 cell/cm^2 confluence in a base medium composed by William's E Medium + GlutaMAX (3255-020; Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10% fetal bovine serum (F7524; Merck KGaA), 100 U/mL penicillin (13752; Merck KGaA), and 100 μg/mL streptomycin (P4333; Merck KGaA). Medium was changed twice a week and cells were passaged once every 7 d. Cells in passage 10 (P10, young cells) and P20 (senescent cells) were used for assays and compared. To obtain HepaRG differentiation, a two-step procedure was used as previously described. Cells were cultured in the medium for 2 wk and then in the presence of 2% DMSO for an additional 2 wk[11].

Senescence-associated β-galactosidase activity assay

The senescence-associated (SA) β-galactosidase (SA-β-gal) activity assay was performed according to the manufacturer's protocol (#9860; Cell Signaling Technology, Inc. Danvers, MA, United States). Briefly, P10 and P20 HepaRG cells grown on 6-well plates were fixed in 1X fixative solution containing 2% formaldehyde and 2% glutaraldehyde for 10 min, and then stained overnight at 37°C with the β-galactosidase staining solution at pH 6.0 for 15 h. Images were acquired using the Nikon Eclipse Ni-U microscope (Nikon, Tokyo, Japan).

Comet assay

The comet assay was performed as previously described[19]. DNA was stained with SYBR green (172-5271; Bio-Rad Laboratories, Hercules, CA, United States) just before blind slide scoring with the Nikon Eclipse Ni-U fluorescence microscope equipped with the CCD-200E video camera. At least 100 cells per sample were analyzed using the Comet Assay IV analysis software (Perceptive Instruments, Haverhill, Suffolk, United Kingdom). The extent of DNA damage in single cells was evaluated by the percentage of tail DNA.

Confocal microscopy and flow cytometry

Cells (1.5 × 10^5 cells/well) were seeded on a glass coverslip in a 24-multiwell plate. The next day, cells were washed three times with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min at room temperature (RT), and washed twice with PBS. Cells were first permeabilized with PBS + 0.1% X-100 Triton (93418; Merck KGaA) for 10 min, and then incubated in blocking buffer (3% bovine serum albumin, A7906; Merck KGaA) + 0.3 M glycine (G-7126-500-50; Merck KGaA) for 30 min at RT. Subsequently, cells were treated for 1.5 h in the dark at RT with the following labeled antibodies: anti-cluster of differentiation 49a (CD49a) (130-101-397), anti-CD49f (130-097-246), anti-CD184 (130-098-354), anti-epithelial cell adhesion molecule (EpCAM) (130-091-253), and anti-cytokeratin 19 (CK19; 130-080-101). All antibodies were

purchased by Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, North Rhine-Westphalia, Germany), and labeled with phycoerythrin, except anti-CK19, which was labeled with fluorescein isothiocyanate. Nuclei were counterstained with 4',6-diamidino-2-phenylindole included in mounting medium (ab104139; Abcam plc, Cambridge, United Kingdom). Cells were analyzed using the Nikon Eclipse Ti-E confocal microscope and by flow cytometry analysis using the FlowSight Cytometer (Amnis; Merck Millipore) and the IDEAS software.

Gene expression analysis

To study the levels of genes expressed in P10 and P20 HepaRG cells after differentiation, RNA was extracted from 1.0 × 10^6 cells/sample and converted into cDNA, which was used as a template in the following RT-PCR. RNA extraction was performed by the "Pure Link RNA Mini Kit" (12183025; Thermo Fisher Scientific), according to the manufacturer's protocol. RNA concentration was determined by spectrophotometer method at Nanodrop, measuring absorbance at λ = 260 nm. A260/A280 > 2 was evaluated to guarantee protein-free samples. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (4368814; Thermo Fisher Scientific), and SYBR Green (172-5271; Bio-Rad Laboratories, Hercules, CA, United States) was used as a fluorescent probe. The sequences of the forward and reverse primers for all of the genes studied are provided in Table 1.

Albumin secretion analyses

To assess the secretion of human albumin, supernatants of P10 and P20 HepaRG cells in basal conditions and after DMSO treatment were collected after 24 h of culture and assessed using a Human Albumin ELISA Kit, according to the manufacturer's instructions (ab108788; Abcam, Cambridge, United Kingdom).

CYP3A4 and γ-glutamyl transpeptidase activities

P10 and P20 HepaRG cells in basal conditions and after DMSO treatment were washed with 1× PBS, and 50 µL of 3 µM P450-Glo™ substrate (V8801; Promega, Waldorf, Germany) was added and incubated for 1 h at 37°C, 5% CO_2. Then, 25 µL substrate medium was transferred to a 96-white plate, and CYP3A4 activity was measured according to the manufacturer's protocol. HepaRG cells were homogenized in 200 µL ice-cold γ-glutamyl transpeptidase (γ-GT) assay buffer, and the γ-GT Activity Colorimetric Assay Kit (MAK089; Merck KGaA) was used according to the manufacturer's protocol.

Cell respirometry

HepaRG cells (5.0 × 10^6 cells) were washed with PBS and resuspended in 10 mmol/L KH_2PO_4, 27 mmol/L KCl, 1 mmol/L $MgCl_2$, 40 mmol/L HEPES, 0.5 mmol/L EGTA buffer (pH 7.1), and assayed for oxygen consumption by the Oxygraph Plus System (Hansatech Instruments, Norfolk, UK) at 37°C under continuous stirring. Oligomycin (8 µg/mL) was added followed the addition of valinomycin (2 µg/mL) after 5 min. The rates of oxygen consumption were corrected for 3 mmol/L potassium cyanide (KCN)-insensitive respiration and normalized to the cell number. Each experiment was repeated in triplicate.

Intracellular ATP content

Total intracellular ATP was quantified using the ENLITEN® ATP Assay System (FF2000; Promega Corporation, Madison, WI, United States), according to the manufacturer's instructions. This assay is based on luciferase, and used as the catalyzing enzyme of the ATP reaction with d-Luciferin. ATP extraction is carried out with trichloroacetic acid reagent, which releases ATP from cells, preventing its enzymatic degradation. After addition of the enzyme reagent to the intracellular extract, light emission was detected by a luminometer at 560 nm (DTX 880 Microplate Reader; Beckman Coulter, Brea, CA, United States).

Intracellular NAD^+/NADH content

Total intracellular NAD^+/NADH was quantified using the NAD/NADH-Glo Bioluminescent Assay Kit (G9072; Promega Corporation, United States), according to the manufacturer's instructions. HepaRG cells were first lysed with dodecyl trimethyl ammonium bromide and treated to neutralize their counterparts. To measure NAD^+, the extract was treated with 25 µL of 0.4 N HCl and heated at 60°C for 15 min, incubated at RT for 10 min, following the addition of 25 µL Trizma base. To quantify NADH, the extract was incubated at 60°C for 15 min followed by further incubation

Table 1 Sequences of forward and reverse primers of the genes studied

Actin	Human	FOR	5′-TGGACATCCGCAAAGACCTG-3′
		REV	5′-GCCGATCCACACGGAGTACTT-3′
Albumin	Human	FOR	5′-CCTGTTGCCAAAGCTCGATG-3′
		REV	5′-GAAATCTCTGGCTCAGGCGA-3′
CYP3A4	Human	FOR	5′-CTTCATCCAATGGACTGCATAAAT-3′
		REV	5′-TCCCAAGTATAACACTCTACACAG-3′
CEA	Human	FOR	5′-GGTCTTCAACCCAATCAGTAAGAAC-3′
		REV	5′-ATGGCCCCAGGTGAGAGG-3′
γ-GT	Human	FOR	5′-TTTGGTGTGCTGCTGGATGAC-3′
		REV	5′-ACCTGAGCTTCCCCACCTATG-3′

CEA: Carcinoembryonic antigen; CYP3A4: Cytochrome P350 3A4; FOR: Forward; γ-GT: Gamma-glutamyl transpeptidase; REV: Reverse.

for 10 min at RT. Then 50 mL HCl/Trizma solution was added to the extract. In the presence of each species, a reductase reduced a pro-luciferin reductase to luciferin. The intensity of light (proportional to the amount of each metabolite) was detected by a luminometer (DTX 880 Microplate Reader; Beckman Coulter).

Statistical analyses

Data are expressed as the mean ± standard deviation of three different experiments. Within-group variability was analyzed using Levene's test for homogeneity of variances. Differences between two groups (P10 *vs* P20) were determined by the Student's *t*-test, while two-way analysis of variance was used to test the main effects of senescence (S, P10 *vs* P20) or transdifferentiation (T, Basal *vs* DMSO) as between-subject factors; the interaction S × T was studied, and a Tukey's test was used as a post hoc test for multiple comparisons. Statistical significance was accepted when $P < 0.05$. GraphPad Prism 6.0 software was used to perform the analyses.

RESULTS

Replication induces the expression of senescence markers in HepaRG cells

Replication-induced senescence was first described in human fibroblasts because of serial culture passages[20]. Since increased SA-β-gal activity is a well-recognized marker of senescence[21], positive SA-β-gal-stained cells were counted to validate senescence in HepaRG passaged up to P10 compared to cells passaged up to P20. As shown in Figure 1A and B, replication-induced senescence in P20 HepaRG cells resulted in increased numbers of cells positive for SA-β-gal staining compared with P10 cells. A further marker of senescence is represented by the extent of DNA damage [22], determined by the comet assay, which measures the prevalence of single- and double-strand breaks. The level of DNA damage was calculated from the percentage of DNA in the tail of the comet formed after single-cell gel electrophoresis, as broken DNA moves faster in the current. The percentage of DNA in the tails *vs* the core was analyzed using Comet Assay IV software, and the results showed that P20 HepaRG cells presented with higher levels of damaged DNA than P10 cells (Figure 1C and D).

Transdifferentiation towards bipotent progenitors is altered in senescent HepaRG cells

HepaRG cells are able to actively proliferate and commit toward hepatocyte and biliary differentiation pathway, reaching maximum cell differentiation after a 2-wk DMSO exposure[11]. Then we exposed P10 and P20 HepaRG cells to DMSO, and studied the expression of markers of both progenitor and differentiated cells.

The expression of adhesion molecules such as CD49a (limited to hepatocyte-like cells) and CD49f (associated with biliary-like cells) after the differentiation process induced by DMSO was lower in P20 than in P10 HepaRG cells. Even CD184/C-X-C motif chemokine receptor 4, a known definitive endoderm marker, was less expressed

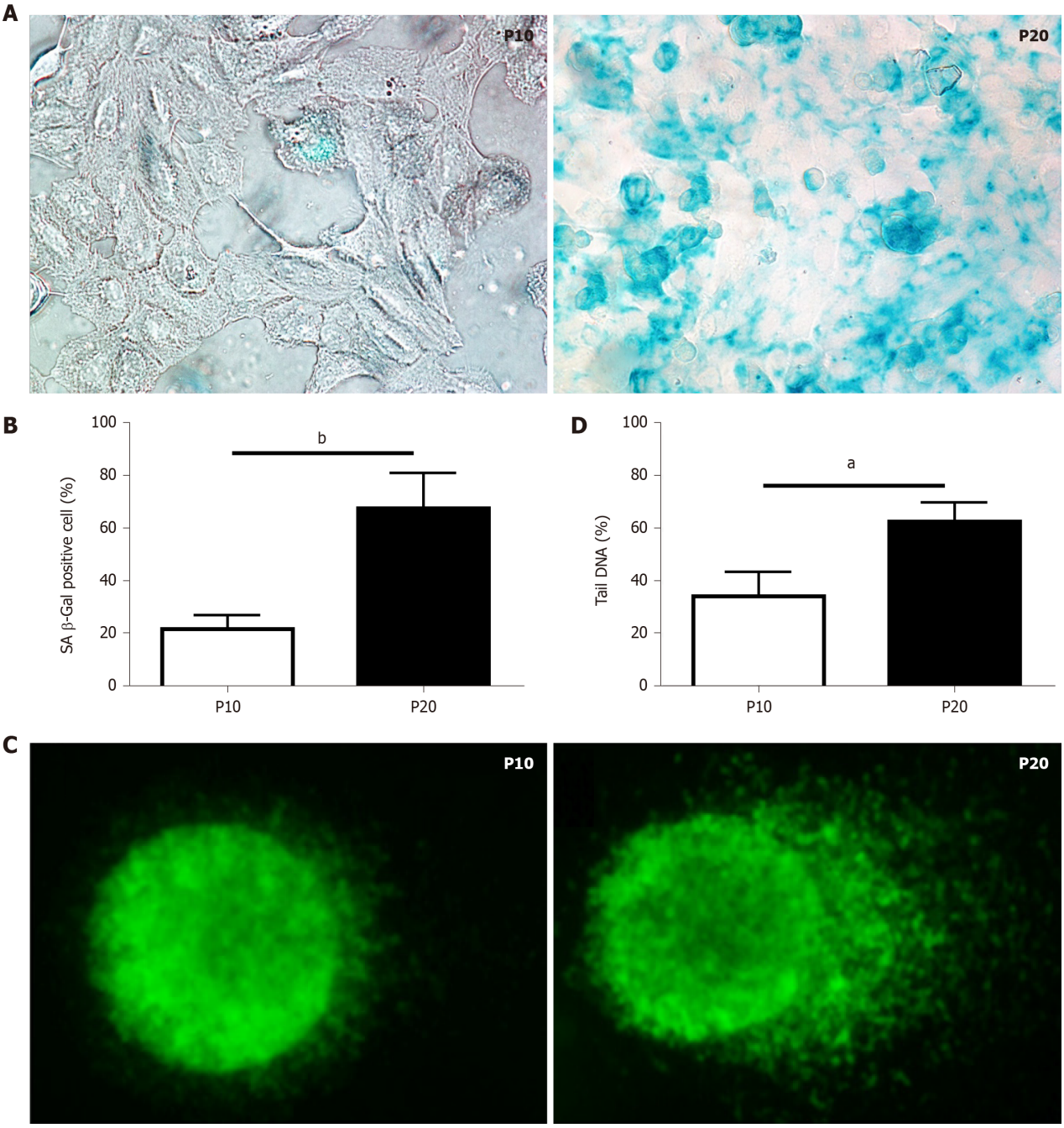

Figure 1 Characterization of markers of cellular senescence in HepaRG cells. A: Senescence-associated β-galactosidase staining of HepaRG cells at passage 10 (P10) or P20. After staining, the cells were imaged by phase contrast microscopy. Micrographs are shown at magnification 200 ×; B: Quantitative analysis of positive β-galactosidase-stained cells in P10 and P20 HepaRG cells; C: Representative images of DNA damage analysis by the comet assay in P10 and P20 HepaRG cells. After staining, the cells were imaged by fluorescence microscopy. Micrographs are shown at magnification 600 ×; D: Quantitative analysis of DNA tails from 100 cells. Data in the graphs are represented as the mean ± SD of three independent experiments. Statistical differences were assessed by the Student's *t*-test. [a]*P* < 0.05 *vs* P10; [b]*P* < 0.01 *vs* P10.

in P20 than in P10 HepaRG cells exposed to DMSO. Moreover, the differentiation protocol resulted in lower expression of EpCAM and CK19 (markers of hepatic progenitor cells[23]) in P20 than in P10 HepaRG cells (Figures 2 and 3).

The expression of genes typical of a differentiated status, such as albumin, CYP3A4 and γ-GT, and of CEA as marker of undifferentiation was next determined in HepaRG cells before and after DMSO exposure. The results obtained by statistical analyses aimed to test the main effects of S, of T, and the interaction S × T. The main effect of S was significant for the expression of all the genes studied (albumin: $F_{(1,8)}$ = 44.54, *P* = 0.0002; CYP3A4: $F_{(1,8)}$ = 24.22, *P* = 0.0012; γ-GT: $F_{(1,8)}$ = 46.82, *P* = 0.0001; CEA: $F_{(1,8)}$ = 12.24, *P* = 0.0081). The main effect of T was significant for the expression of albumin ($F_{(1,8)}$ = 243.3, *P* < 0.0001), CYP3A4 ($F_{(1,8)}$ = 37.50, *P* = 0.0003), and γ-GT ($F_{(1,8)}$ = 190.2, *P* < 0.0001), but not for CEA. The interaction between S and T was significant for the

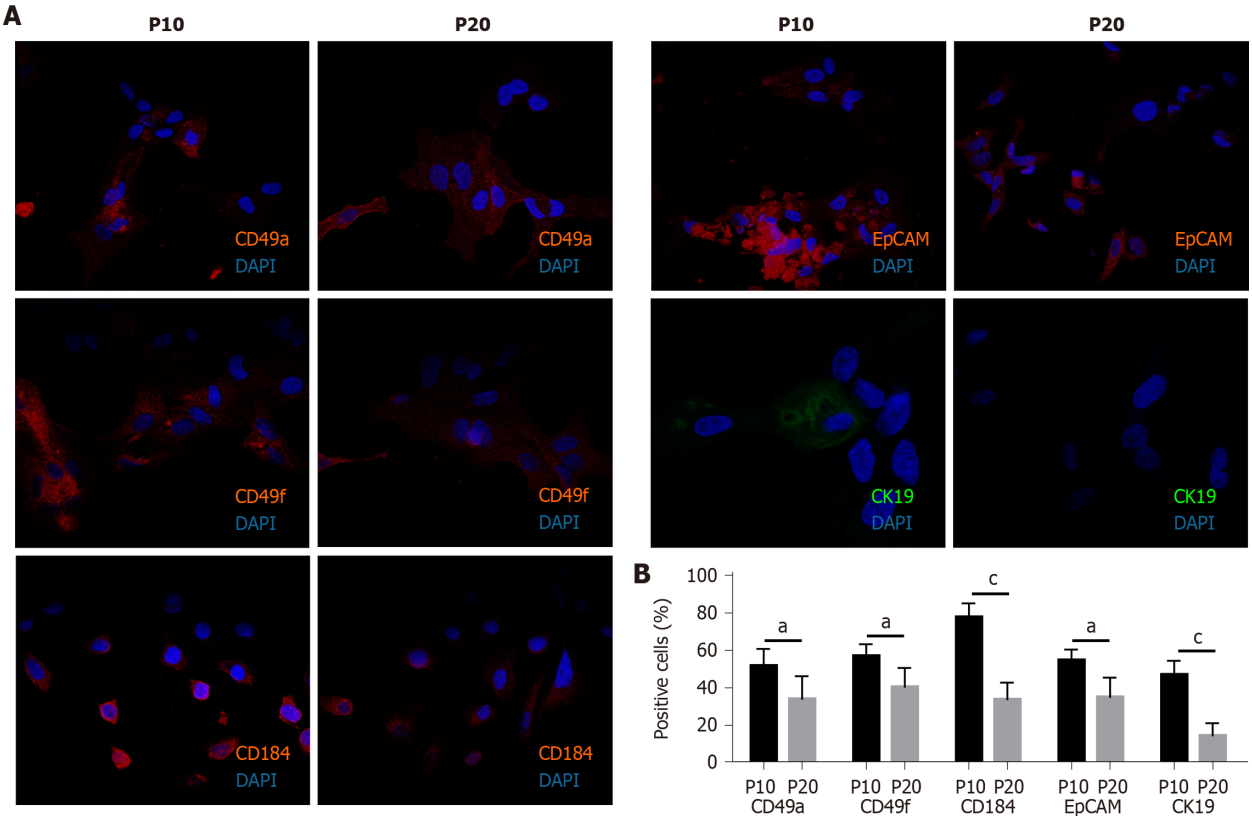

Figure 2 Expression of progenitor markers in HepaRG cells after the transdifferentiation process. A: Representative immunofluorescence images of HepaRG cells at passage 10 (P10) or P20. After staining, the cells were imaged by confocal microscopy. Micrographs are shown at magnification 200 ×; B: Quantitative analysis of positively stained cells in P10 and P20 HepaRG cells. Data in the graphs are represented as the mean ± standard deviation of three independent experiments. Statistical differences were assessed by the Student's *t*-test. $^{a}P < 0.05$ *vs* P10; $^{c}P < 0.001$ *vs* P10.

expression of all the genes studied (albumin: $F_{(1,8)} = 43.95$, $P = 0.0002$; CYP3A4: $F_{(1,8)} = 24.22$, $P = 0.0012$; γ-GT: $F_{(1,8)} = 60.71$, $P < 0.0001$; CEA: $F_{(1,8)} = 10.13$, $P = 0.0129$). Post hoc analysis showed that the expression of albumin, CYP3A4 and γ-GT was induced by DMSO exposure both in P10 and in P20 HepaRG cells, supporting the transdifferentiation process; however, mRNA levels were lower in DMSO-treated P20 rather than P10 HepaRG cells (Figure 4A-C). Finally, the expression of CEA was reduced by DMSO exposure in P10 but not in P20 HepaRG cells (Figure 4D).

The same analysis was performed on specific functional activities of HepaRG cells, studying albumin secretion, CYP3A4 and γ-GT activity, and the results are represented in Figure 5. In particular, we found a main effect of S (albumin secretion: $F_{(1,8)} = 20.95$, $P = 0.0018$; CYP3A4 activity: $F_{(1,8)} = 10.24$, $P = 0.0126$; γ-GT activity: $F_{(1,8)} = 18.13$, $P = 0.0028$), T (albumin secretion: $F_{(1,8)} = 120.2$, $P < 0.0001$, CYP3A4 activity: $F_{(1,8)} = 9.15$, $P = 0.0164$; γ-GT activity: $F_{(1,8)} = 68.25$, $P < 0.0001$), and interaction between S and T (albumin secretion: $F_{(1,8)} = 20.53$, $P = 0.0019$; CYP3A4 activity: $F_{(1,8)} = 7.181$, $P = 0.0233$; γ-GT activity: $F_{(1,8)} = 19.36$, $P = 0.0023$). Post hoc analysis showed that albumin secretion, CYP3A4 and γ-GT activities were reduced in DMSO-treated P20 rather than P10 HepaRG cells.

Taken together, these results suggest that the transdifferentiation process triggered by DMSO in HepaRG cells is hampered by the replication-induced senescence.

Senescence-induced impairment of HepaRG transdifferentiation is associated with mitochondrial dysfunction

Mitochondrial dysfunction is recognized as one of the hallmarks of senescence[24]. Thus, we performed respirometry analyses in HepaRG by high-resolution oximetry. Figure 6A details the protocol used and is representative of the oxygraphic profiles in young (P10) and senescent (P20) HepaRG cells in basal conditions and after the transdifferentiation protocol (DMSO). The resting respiration (RR), which depends on endogenous substrates, was impacted by both the S and the T factor (S: $F_{(1,8)} = 78.38$, $P < 0.0001$; T: $F_{(1,8)} = 57.12$, $P < 0.0001$), and by the interaction S×T ($F_{(1,8)} = 60.86$, $P < 0.0001$). Addition of oligomycin (a F_oF_1-ATP synthase inhibitor) reduced the oxygen uptake, suggesting that most of the mitochondrial respiration was coupled to the

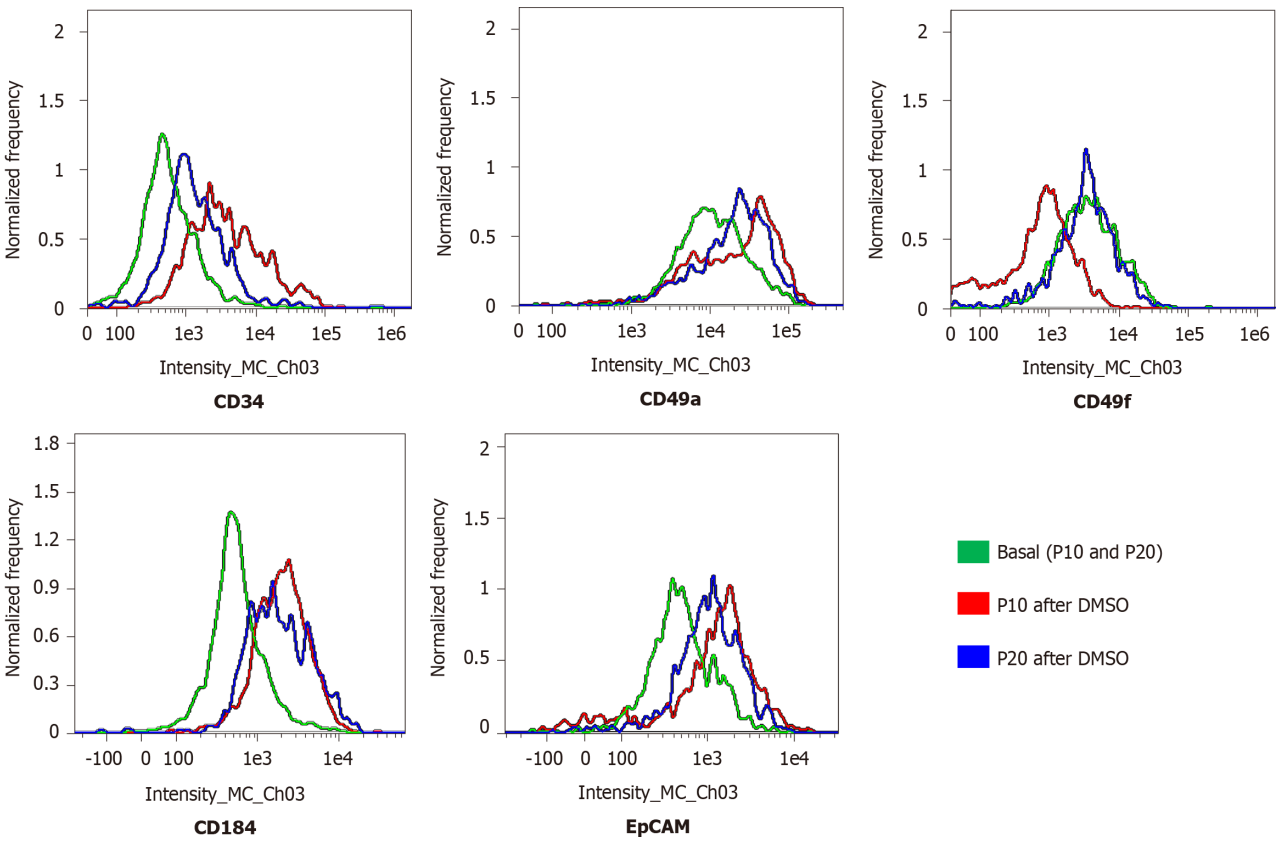

Figure 3 Expression of progenitor markers in HepaRG cells before and after the transdifferentiation process. Flow cytometry histograms of HepaRG cells at passage 10 (P10) or P20 in basal conditions or after the transdifferentiation protocol (dimethyl sulfoxide). After staining, the cells were analyzed with flow cytometry.

synthesis of ATP. Nevertheless, both the S and the T factor impacted this parameter (S: $F_{(1,8)}$ = 10.83, P = 0.0018; T: $F_{(1,8)}$ = 6.847, P = 0.0308), as well as the interaction S × T ($F_{(1,8)}$ = 8.104, P = 0.0216). Restoration of the oxygen uptake by the addition of valinomycin (a K^+ ionophore which uncouples oxygen consumption from ATP synthesis) was also impacted by both the S and the T factor (S: $F_{(1,8)}$ = 59.58, P < 0.0001; T: $F_{(1,8)}$ = 53.09, P < 0.0001), and the interaction S × T ($F_{(1,8)}$ = 55.97, P < 0.0001). The post hoc analysis showed that the oxygen uptake in all the examined conditions was higher in P10 HepaRG cells after the transdifferentiation protocol with respect to the other samples (Figure 6B). The effects of both S and T, and their interaction, was observed on the ATP-dependent oxygen uptake, calculated as the difference between RR and oligomycin-induced respiration (S: $F_{(1,8)}$ = 28.94, P = 0.0007; T: $F_{(1,8)}$ = 25.35, P = 0.001; S × T: $F_{(1,8)}$ = 27.36, P = 0.0008). The post hoc analysis showed that the P10 HepaRG cells after the transdifferentiation protocol exhibited a higher ATP-dependent oxygen uptake than the other samples (Figure 6C). The respiratory control ratio (RCR), calculated as the ratio between RR and oligomycin-induced respiration, was also influenced by both S and T (S: $F_{(1,8)}$ = 14.76, P = 0.0049; T: $F_{(1,8)}$ = 13.43, P = 0.0064), and their interaction (S × T: $F_{(1,8)}$ = 11.38, P = 0.0097). The post hoc analysis resulted in a higher RCR for the P10 HepaRG cells after the transdifferentiation protocol compared to the other samples (Figure 6D). Taken together, these data suggest that the transdifferentiation induced by DMSO increases mitochondrial respiration in HepaRG cells; however, this does not occur in senescent cells.

To confirm a defect in mitochondrial dysfunction of senescent HepaRG, the cellular ATP concentration was further measured. We observed a significant effect of both S and T (S:$F_{(1,8)}$ = 14.89, P = 0.0048; T:$F_{(1,8)}$ = 12.15, P = 0.0083), and the post hoc analysis showed that the ATP content of transdifferentiated P10 HepaRG cells was higher than the other samples (Figure 7A). SA mitochondrial dysfunction can be consequent to an exhaustion of the oxidized form of NAD^+[25]. The analysis of NAD^+/NADH content in HepaRG cells revealed no changes in NADH; nevertheless, the impact of S, T, and their interaction was observed on both NAD^+ (S:$F_{(1,8)}$ = 13.84, P = 0.0059; T:$F_{(1,8)}$ = 26.00, P = 0.0009; S × T: $F_{(1,8)}$ = 10.47, P = 0.0120) and NAD^+/NADH (S:$F_{(1,8)}$ = 22.23, P = 0.0015; T:$F_{(1,8)}$ = 19.75, P = 0.0022; S × T: $F_{(1,8)}$ = 10.02, P = 0.0133). The post hoc analysis resulted

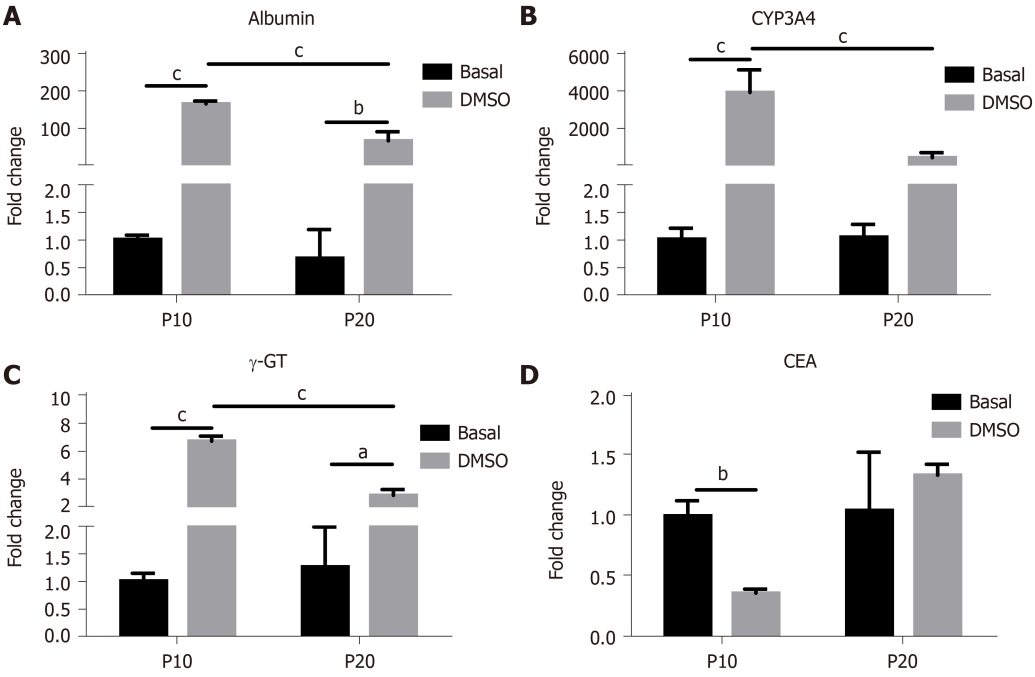

Figure 4 Gene expression analysis in HepaRG cells at passage 10 or passage 20 in basal conditions or after the transdifferentiation protocol (dimethyl sulfoxide). A: mRNA level of albumin; B: Cytochrome P350 3A4 (CYP3A4); C: Gamma-glutamyl transpeptidase (γ-GT); D: Carcinoembryonic antigen (CEA) measured by reverse transcription-polymerase chain reaction. Data in the graphs are represented as the mean ± standard deviation of three independent experiments. Statistical differences were assessed by two-way analysis of variance followed by the Tukey test as the post hoc test. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. P10: Passage 10; P20: Passage 20; DMSO: Dimethyl sulfoxide.

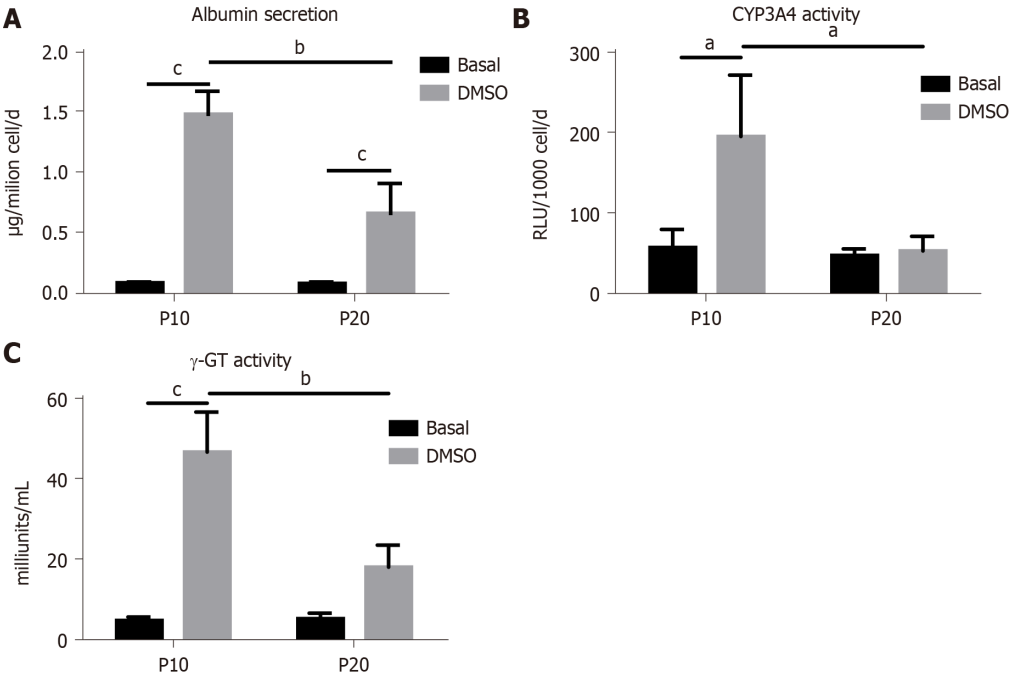

Figure 5 Functional tests in HepaRG cells at passage 10 or passage 20) in basal conditions or after the transdifferentiation protocol (dimethyl sulfoxide). A: Albumin secretion; B: Cytochrome P350 3A4 (CYP3A4); C: Gamma-glutamyl transpeptidase (γ-GT) activities. Data in the graphs are represented as the mean ± standard deviation of three independent experiments. Statistical differences were assessed by two-way analysis of variance followed by the Tukey test as the post hoc test. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. P10: Passage 10; P20: Passage 20; DMSO: Dimethyl sulfoxide.

in higher NAD^+ and NAD^+/NADH in transdifferentiated P10 HepaRG cells with respect to the other samples (Figure 7B).

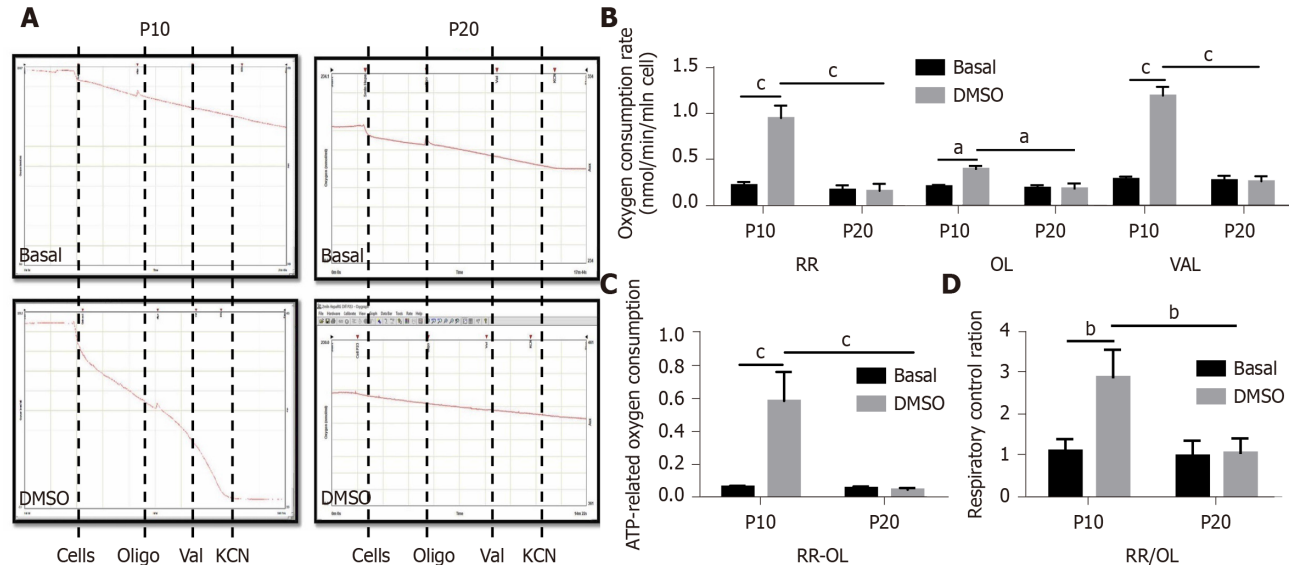

Figure 6 Measurement of mitochondrial respiration in HepaRG cells at passage 10 or passage 20 in basal conditions or after the transdifferentiation protocol (dimethyl sulfoxide). A: Representative oxymetric traces of mitochondrial respiration in HepaRG cells. Where indicated, the following were added: 4 × 10^6 HepaRG cells, 8 μg/mL oligomycin (OL), 2 μg/mL valinomycin (VAL), 3 mmol/L potassium cyanide (KCN). The continuous line represents the oxygen concentration measured every 0.1 s throughout the time-course of the assay; B: Representative graph of the normalized and KCN-insensitive-corrected oxygen consumption rates measured under resting respiration (RR) conditions, in the presence of OL and in the presence of VAL (see panel A); C: ATP-dependent oxygen consumption measured as absolute difference between that obtained in the absence and that in the presence of oligomycin (RR-OL); D: Respiratory control ratios obtained dividing the oxygen consumption rates measured under resting conditions by that in the presence of oligomycin (RR/OL). Data in the graphs are represented as the mean ± standard deviation of three independent experiments. Statistical differences were assessed by two-way analysis of variance followed by the Tukey's test as the post hoc test. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$. DMSO: Dimethyl sulfoxide; P10: Passage 10; P20: Passage 20.

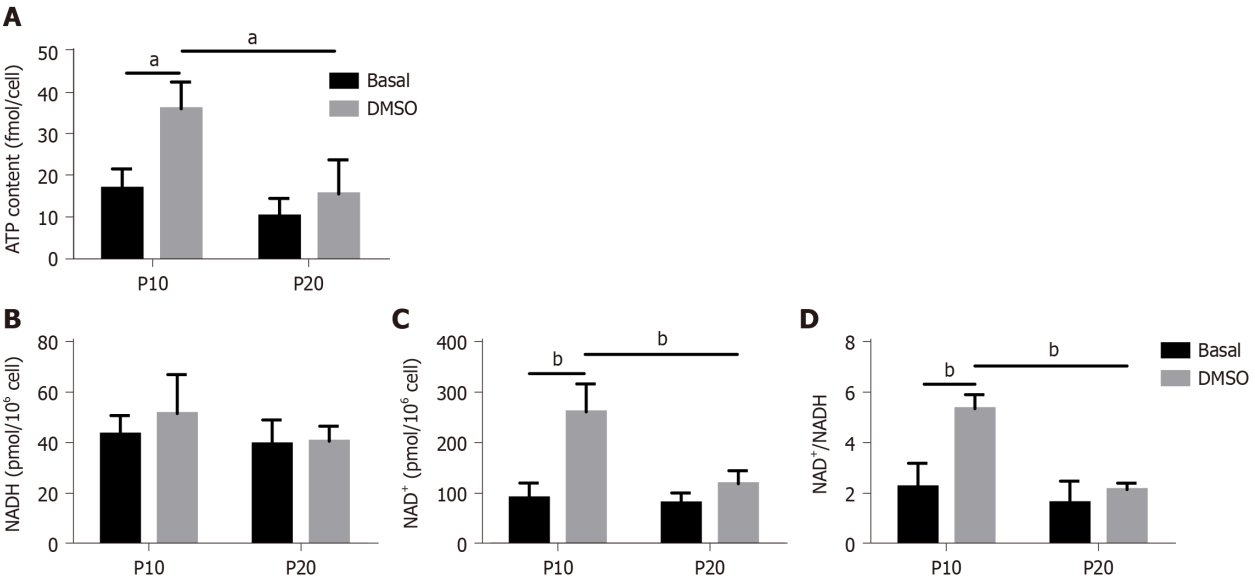

Figure 7 ATP and nicotinamide adenine dinucleotide (NAD)/NAD with hydrogen content in HepaRG cells at passage 10 or passage 20 in basal conditions or after the transdifferentiation protocol (dimethyl sulfoxide). A: Cellular ATP content was expressed as ATP concentration/cells; B: Intracellular nicotinamide adenine dinucleotide (NAD^+) and NAD with hydrogen (NADH) were extracted, and NAD^+ and NADH levels were measured using a microplate reader. NAD^+/NADH ratio was calculated based on the concentration of NAD^+ and NADH. Data in the graphs are represented as the mean ± standard deviation of three independent experiments. Statistical differences were assessed by two-way analysis of variance followed by the Tukey's test as the post hoc test. [a]$P < 0.05$; [b]$P < 0.01$. DMSO: Dimethyl sulfoxide; P10: Passage 10; P20: Passage 20.

DISCUSSION

This study demonstrates that cellular replication in HepaRG cells is associated with both the expression of senescence markers and the reduction in transdifferentiation potential. Indeed, data related to phenotype, gene expression, functional analysis, and

mitochondrial function suggest that the transdifferentiation process of HepaRG is preserved after a few passages, but it is altered by continuous replication.

HepaRG represents a unique cell line characterized by high expression of detoxifying and metabolizing enzymes, transport proteins, and nuclear receptors, as well as the ability to transdifferentiate toward hepatocyte-like and cholangiocyte-like cells[9-11]. Other than representing a viable tool for cell biology, drug metabolism, and virology studies, differentiated HepaRG cells are suitable to generate humanized liver in rodent models, allowing *in vivo* studies of liver development and physiology[26,27].

Cultured cells stop proliferating after a finite number of divisions, a phenomenon defined to as replicative senescence[28,29]. However, cells can escape replicative senescence by several mechanisms, including overexpression of viral genes such as simian virus 40 large T antigen (which inactivates p53), or telomerase reverse transcriptase protein (TERT, which elongates telomeres)[30,31]. Proliferating HepaRG cells show over-expression and hyper-activation of human TERT, as well as inhibition of p21 and p27, which in turn inhibit the catalytic activity of cyclin-dependent kinases and stop the cell cycle[32]. Nevertheless, our data demonstrate that a replication protocol induces the expression of senescence markers in HepaRG cells, such as positive SA-β-gal staining and DNA alterations. Thus, we hypothesize that the mechanisms that induce senescence in HepaRG cells are different from telomere shortening and p21/p27 activation.

Senescence impairs the transdifferentiation of several cell lines[33-36]. Senescent cells secrete a large variety of molecules that change the surrounding microenvironment, with consequent alterations of differentiation and tissue regeneration[37,38]. These compounds include a wide range of cytokines, growth factors, and signaling molecules that are included in the SA secretory phenotype (SASP)[39]. Our data clearly demonstrate that replicative senescence alters the transdifferentiation process of HepaRG cells. Nevertheless, we did not analyze the SASP in our study, since this marker is strictly linked with telomere shortening[40]. By contrast, we focused on mitochondria, since the homeostasis of these organelles is crucial for several aspects of senescence including SASP[41]. Indeed, the impairment of mitochondrial oxidative phosphorylation (OxPhos) is mainly involved in the early steps of cell senescence[42]. Moreover, senescent cells exhibit severe metabolic alterations associated with mitochondrial metabolites, such as oxidized to reduced NAD ratios (NAD+/NADH)[43]. The novel and straight findings of this study demonstrate the higher mitochondria-related respiration in transdifferentiated HepaRG cells, which was not observed after induction of replicative senescence. Indeed, this respiration was suppressed by oligomycin, a specific inhibitor of ATP synthase, suggesting that transdifferentiated HepaRG cells in resting conditions oxidize substrates to synthetize ATP through the OxPhos system. On the other hand, senescent HepaRG cells subjected to the transdifferentiation protocol exhibited lower oxygen consumption which was insensitive to oligomycin. These results are consistent with a transition from a glycolytic to an oxidative metabolism in transdifferentiated HepaRG cells, which did not occur in senescent cells. Of interest, the RR in transdifferentiated HepaRG cells was lower than that reached in the presence of an uncoupler (maximal respiratory rate), indicating the existence of a respiratory reserve. By contrast, the RR of senescent HepaRG cells undergoing transdifferentiation was similar to the maximal respiratory rate, suggesting the absence of a respiratory reserve. Mitochondrial dysfunction in senescent HepaRG cells after transdifferentiation was further confirmed by a reduction in cellular ATP concentration.

The concentration of the oxidized form of NAD^+ is determinant for mitochondrial homeostasis[44,45]. Moreover, NAD^+ is crucial in several metabolic pathways, including glycolysis, citric acid cycle, and OxPhos, with consequent implications for both stemness/differentiation and cell senescence[46]. Cells that maintain a physiological quiescent state to preserve long-term self-renewal capacity are characterized by low NAD^+ levels to obtain energy from glycolysis; on the contrary, cell differentiation leads to reduced glycolysis and increased OhPhos, which require high NAD^+ levels[47]. Age-related reductions of both NAD^+ levels and NAD^+/NADH ratio are evolutionarily preserved, and consistent evidence for low NAD^+ has been provided for several old mammalian tissues[48]. Our data show that HepaRG transdifferentiation is associated with increased NAD^+ and relatively stable NADH, with consequent high NAD^+/NADH. However, the raise of both NAD^+ and NAD^+/NADH is not observed in HepaRG undergoing replicative senescence. Even though the effect of a treatment goes beyond the scope of our study, it is conceivable that a replenishment of NAD^+ would protect mitochondria and improve the transdifferentiation process of senescent HepaRG cells, as already described for other cell types[49-51].

CONCLUSION

In conclusion, the present report demonstrates that HepaRG cells undergo replicative senescence, which is associated with impairment in transdifferentiation, mitochondrial dysfunction, and NAD^+ depletion. Further investigations are required to refine the molecular mechanism underlying such observation. The limitations in the transdifferentiation potential of senescent HepaRG cells, with consequent alteration of metabolic and regenerative properties, may have serious implications when this cell line is applied for basic studies.

ARTICLE HIGHLIGHTS

Research background

The HepaRG cell line is used to study metabolism, toxicology, and the regeneration/differentiation processes, as a replacement to primary hepatocytes, HepG2, and Huh-7 cells. These cells exhibit a hepatocyte-like morphology and express hepatocyte-specific functions in defined culture conditions; furthermore, HepaRG display features of human oval ductular bipotent hepatic progenitors.

Research motivation

Cellular senescence consists in a steady cell cycle block occurring because of different harmful events, leading to defective stemness and differentiation processes, as well as changes in cell cycle regulation, signal transduction, and metabolism. The impact of senescence on HepaRG cells has not yet been investigated.

Research objectives

This study investigated whether a replication protocol would induce senescence in HepaRG cells. In addition, we characterized the effects of senescence on transdifferentiation capacity and mitochondrial metabolism.

Research methods

The transdifferentiation capacity of HepaRG cells over passage 10 (P10) *vs* passage 20 (P20) was compared. To stimulate transdifferentiation, HepaRG cells were treated with dimethyl sulfoxide (DMSO). Aging was evaluated by senescence-associated (SA) β-galactosidase activity and the comet assay. HepaRG transdifferentiation was analyzed by confocal microscopy and flow cytometry (expression of cluster of differentiation 49a [CD49a], CD49f, CD184, epithelial cell adhesion molecule [EpCAM], and cytokeratin 19 [CK19]), by quantitative PCR analysis (expression of albumin, cytochrome P450 3A4 [CYP3A4], γ-glutamyl transpeptidase [γ-GT] and carcinoembryonic antigen [CEA]) and functional analysis (albumin secretion, CYP3A4 and γ-GT). Mitochondrial respiration, the ATP and the NAD^+/NADH content were also measured.

Research results

We first observed that replication induces the expression of senescence markers in HepaRG cells, since SA β-galactosidase staining was higher in P20 than in P10 HepaRG cells, and the comet assay showed a consistent DNA damage in P20 HepaRG cells. We further reported that transdifferentiation towards bipotent progenitors is altered in senescent HepaRG cells, as P20 HepaRG cells exhibited a reduction of CD49a, CD49f, CD184, EpCAM and CK19 – with respect to P10 – after DMSO treatment. Furthermore, the lower gene expression of albumin, CYP3A4, and γ-GT, as well as the reduced albumin secretion capacity, CYP3A4, and γ-GT activity were reported in transdifferentiated P20 compared to P10 cells. By contrast, the gene expression level of CEA was not reduced by transdifferentiation in P20 cells. Finally, we show that senescence-induced impairment of HepaRG transdifferentiation is associated with mitochondrial dysfunction, since both cellular and mitochondrial oxygen consumption were lower in P20 than in P10 transdifferentiated cells, and both ATP and NAD^+/NADH were depleted in P20 cells with respect to P10 cells.

Research conclusions

The present study demonstrates that HepaRG cells undergo replicative senescence, with consequent impairment in transdifferentiation, functional activity, mitochondrial dysfunction, and NAD^+ depletion.

Research perspectives

Further studies will define the molecular mechanisms underlying our observations. The limitations in the transdifferentiation potential of senescent HepaRG cells, with consequent alteration of metabolic and regenerative properties, may have serious implications when this cell line is applied for basic studies.

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