

# World Journal of *Stem Cells*

*World J Stem Cells* 2020 August 26; 12(8): 706-896



**THERAPEUTIC AND DIAGNOSTIC GUIDELINES**

- 706** Hunting down the dominating subclone of cancer stem cells as a potential new therapeutic target in multiple myeloma: An artificial intelligence perspective  
*Lee LX, Li SC*

**OPINION REVIEW**

- 721** Off-the-shelf mesenchymal stem cells from human umbilical cord tissue can significantly improve symptoms in COVID-19 patients: An analysis of evidential relations  
*Pham PV, Vu NB*

**REVIEW**

- 731** Mesenchymal stromal cells as potential immunomodulatory players in severe acute respiratory distress syndrome induced by SARS-CoV-2 infection  
*Mallis P, Michalopoulos E, Chatzistamatiou T, Stavropoulos-Giokas C*
- 752** Practical choice for robust and efficient differentiation of human pluripotent stem cells  
*Fang M, Liu LP, Zhou H, Li YM, Zheng YW*
- 761** Human embryonic stem cells as an *in vitro* model for studying developmental origins of type 2 diabetes  
*Chen ACH, Lee KF, Yeung WSB, Lee YL*
- 776** Autophagy in fate determination of mesenchymal stem cells and bone remodeling  
*Chen XD, Tan JL, Feng Y, Huang LJ, Zhang M, Cheng B*
- 787** Stem cell therapy for Alzheimer's disease  
*Liu XY, Yang LP, Zhao L*
- 803** Exosomes derived from stem cells as an emerging therapeutic strategy for intervertebral disc degeneration  
*Hu ZL, Li HY, Chang X, Li YY, Liu CH, Gao XX, Zhai Y, Chen YX, Li CQ*
- 814** Mesenchymal stem cell-derived exosomes: Toward cell-free therapeutic strategies in regenerative medicine  
*Ma ZJ, Yang JJ, Lu YB, Liu ZY, Wang XX*

**ORIGINAL ARTICLE****Basic Study**

- 841** Assessment of tobacco heating system 2.4 on osteogenic differentiation of mesenchymal stem cells and primary human osteoblasts compared to conventional cigarettes  
*Aspera-Werz RH, Ehnert S, Müller M, Zhu S, Chen T, Weng W, Jacoby J, Nussler AK*



- 857 Human embryonic stem cell-derived mesenchymal stem cells improved premature ovarian failure

*Bahrebar K, Rezazadeh Valojerdi M, Esfandiari F, Fathi R, Hassani SN, Baharvand H*

### SYSTEMATIC REVIEWS

- 879 Role of mesenchymal stem cell derived extracellular vesicles in autoimmunity: A systematic review

*Wang JH, Liu XL, Sun JM, Yang JH, Xu DH, Yan SS*

**ABOUT COVER**

Editorial Board member of *World Journal of Stem Cells*, Dr. Perez-Campo is currently an Associate Professor in the Department of Molecular Biology at the University of Cantabria (Spain). She obtained her degree in Biological Sciences from the University of Salamanca (Spain), where she then went on to complete her PhD in 1999. Dr. Perez-Campo undertook her postdoctoral research at the Paterson Institute for Cancer Research (United Kingdom; currently known as Cancer Research UK Manchester Institute) under the supervision of Prof. Lacaud, where she remained for more than 10 years working in the field of stem cell biology. Upon returning to Spain, she joined the University of Cantabria and focused her research efforts on the molecular mechanisms that control mesenchymal stem cell (MSC) differentiation towards the osteoblastic and adipogenic lineages, and how those mechanisms are altered in osteoporosis. (L-Editor: Filipodia)

**AIMS AND SCOPE**

The primary aim of *World Journal of Stem Cells* (WJSC, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. WJSC publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, etc.

**INDEXING/ABSTRACTING**

The WJSC is now indexed in Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports/Science Edition, Biological Abstracts, BIOSIS Previews, PubMed, and PubMed Central. The 2020 Edition of Journal Citation Reports® cites the 2019 impact factor (IF) for WJSC as 3.231; IF without journal self cites: 3.128; Ranking: 18 among 29 journals in cell and tissue engineering; Quartile category: Q3; Ranking: 113 among 195 journals in cell biology; and Quartile category: Q3.

**RESPONSIBLE EDITORS FOR THIS ISSUE**

Production Editor: Yan-Xia Xing; Production Department Director: Yun-Xiao Jian Wu; Editorial Office Director: Jin-Lai Wang.

**NAME OF JOURNAL**

*World Journal of Stem Cells*

**ISSN**

ISSN 1948-0210 (online)

**LAUNCH DATE**

December 31, 2009

**FREQUENCY**

Monthly

**EDITORS-IN-CHIEF**

Carlo Ventura

**EDITORIAL BOARD MEMBERS**

<https://www.wjgnet.com/1948-0210/editorialboard.htm>

**PUBLICATION DATE**

August 26, 2020

**COPYRIGHT**

© 2020 Baishideng Publishing Group Inc

**INSTRUCTIONS TO AUTHORS**

<https://www.wjgnet.com/bpg/gerinfo/204>

**GUIDELINES FOR ETHICS DOCUMENTS**

<https://www.wjgnet.com/bpg/GerInfo/287>

**GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH**

<https://www.wjgnet.com/bpg/gerinfo/240>

**PUBLICATION ETHICS**

<https://www.wjgnet.com/bpg/GerInfo/288>

**PUBLICATION MISCONDUCT**

<https://www.wjgnet.com/bpg/gerinfo/208>

**ARTICLE PROCESSING CHARGE**

<https://www.wjgnet.com/bpg/gerinfo/242>

**STEPS FOR SUBMITTING MANUSCRIPTS**

<https://www.wjgnet.com/bpg/GerInfo/239>

**ONLINE SUBMISSION**

<https://www.f6publishing.com>



## Hunting down the dominating subclone of cancer stem cells as a potential new therapeutic target in multiple myeloma: An artificial intelligence perspective

Lisa X Lee, Shengwen Calvin Li

**ORCID number:** Lisa X Lee 0000-0002-0664-2253; Shengwen Calvin Li 0000-0002-9699-9204.

**Author contributions:** Lee LX and Li SC conceived and wrote the manuscript; both revised the manuscript; and all authors approved the final version submitted.

**Supported by** the CHOC-UCI Joint Research Award (in part).

**Conflict-of-interest statement:** The authors declare no conflict of interest.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Invited manuscript

**Lisa X Lee**, Division of Hematology/Oncology, Department of Medicine, Chao Family Comprehensive Cancer Center, UCI Health, Orange, CA 92868, United States

**Shengwen Calvin Li**, Neuro-oncology and Stem Cell Research Laboratory, CHOC Children's Research Institute, Children's Hospital of Orange County, Orange, CA 92868, United States

**Shengwen Calvin Li**, Department of Neurology, University of California-Irvine School of Medicine, Orange, CA 92868, United States

**Corresponding author:** Shengwen Calvin Li, PhD, Professor, Neuro-oncology and Stem Cell Research Laboratory, CHOC Children's Research Institute, Children's Hospital of Orange County, 1201 W La Veta Ave., Orange, CA 92868, United States. [shengwel@uci.edu](mailto:shengwel@uci.edu)

### Abstract

The development of single-cell subclones, which can rapidly switch from dormant to dominant subclones, occur in the natural pathophysiology of multiple myeloma (MM) but is often "pressed" by the standard treatment of MM. These emerging subclones present a challenge, providing reservoirs for chemoresistant mutations. Technological advancement is required to track MM subclonal changes, as understanding MM's mechanism of evolution at the cellular level can prompt the development of new targeted ways of treating this disease. Current methods to study the evolution of subclones in MM rely on technologies capable of phenotypically and genotypically characterizing plasma cells, which include immunohistochemistry, flow cytometry, or cytogenetics. Still, all of these technologies may be limited by the sensitivity for picking up rare events. In contrast, more incisive methods such as RNA sequencing, comparative genomic hybridization, or whole-genome sequencing are not yet commonly used in clinical practice. Here we introduce the epidemiological diagnosis and prognosis of MM and review current methods for evaluating MM subclone evolution, such as minimal residual disease/multiparametric flow cytometry/next-generation sequencing, and their respective advantages and disadvantages. In addition, we propose our new single-cell method of evaluation to understand MM's mechanism of evolution at the molecular and cellular level and to prompt the development of new targeted ways of treating this disease, which has a broad prospect.

**Received:** May 18, 2020**Peer-review started:** May 18, 2020**First decision:** June 5, 2020**Revised:** July 8, 2020**Accepted:** August 14, 2020**Article in press:** August 14, 2020**Published online:** August 26, 2020**P-Reviewer:** Lin JM, Vatansever M**S-Editor:** Wang JL**L-Editor:** A**P-Editor:** Wu YXJ

**Key words:** Multiple myeloma; Single cells; Single-cell transcriptome; Subclonal evolution; Cancer stem cells; Systemic tracking of single-cell landscape; Artificial intelligence medicine

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Current methods for determining prognosis in multiple myeloma are limited. The prototype device called Multi-Phase Laser-cavitation Single Cell Analyzer can perform reverse transcriptase polymerase chain reaction (RT-PCR) on single cells in a one-step microfluidics chip platform. The ability of the microfluidics chip platform to enrich plasma cell content by depleting CD45+ white blood cells has been demonstrated. Further studies will need to combine single-cell selection with RT-PCR to further enhance the diagnostic capabilities of this technology. This platform has the potential to be used for clinical risk stratification in multiple myeloma as well as minimal residual disease monitoring and selection of therapies to modulate the development of resistance.

**Citation:** Lee LX, Li SC. Hunting down the dominating subclone of cancer stem cells as a potential new therapeutic target in multiple myeloma: An artificial intelligence perspective. *World J Stem Cells* 2020; 12(8): 706-720

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/706.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.706>

## INTRODUCTION

### Epidemiology

With approximately 31000 new cases of multiple myeloma (MM) diagnosed in the United States (US) per year, the impact of this incurable disease on individual patients and society as a whole is profound. The median age at diagnosis is 70 years old<sup>[1]</sup>. All diagnoses of MM are believed to be preceded by a state of clonal expansion of plasma cells (PCs), including monoclonal gammopathy of unknown significance (MGUS) and smoldering myeloma (SM). The duration of these precursor conditions of MM has been demonstrated to be present up to 15 years prior to the diagnosis of MM<sup>[2]</sup>.

### Diagnosis and disease prognostication

The current diagnosis of MM requires a bone marrow biopsy and aspirate, which is used to enumerate plasma cell content and to characterize PCs by immunohistochemical staining, cytogenetics, and flow cytometry. Detection of cytogenetic alterations, in particular, are paramount to provide prognostication and direct therapy and have been incorporated into the standardized staging system for MM<sup>[3]</sup>. For example, the presence of high-risk cytogenetics, including del17p, t(4,14), and t(14;16) prognosticates for survival 1/5<sup>th</sup> that of standard-risk cytogenetics<sup>[4]</sup>. However, the identification of such cytogenetic features may be used to guide therapy such as in patients with t(4;14), who have traditionally had significantly inferior outcome may be able to have an overall survival (OS) similar to patients with standard-risk MM when treated with bortezomib-containing regimens and autologous stem cell transplantation<sup>[5]</sup>.

## CURRENT SOLUTIONS TO OVERCOME THERAPEUTIC RESISTANCE

Initial treatment incorporating conventional drugs such as Dexamethasone (Dex) effectively induces MM cell death; however, prolonged drug exposures result in the development of chemoresistance. Thus, individual patients' survival within a risk category remains variable, and the patients relapse despite achieving a "complete response," reflecting persistent disease that cannot be detected using the currently recommended disease evaluation techniques. It is becoming apparent that static cytogenetic categories alone are not sufficient to define subclone formation and stage<sup>[6]</sup>. Several methods are being evaluated to enhance further our ability to individualize treatment.



First, response assessment using minimal residual disease (MRD) at varying time points in a patient's disease process can further fine-tune response-adapted treatment strategies. MRD negativity at any given time point is closely correlated with more prolonged progression-free survival (PFS). It has been incorporated into the International Myeloma Working Group recommendation for response assessment, and ongoing studies are studying adaptive treatment strategies based on achieving MRD negativity<sup>[7]</sup>. Current methods for minimal residual disease testing include flow cytometry or next-generation sequencing. Multiparametric flow cytometry (MFC) MRD testing in MM has quite low sensitivity, detecting one cell in  $10^4$  cells and requires at least  $2 \times 10^6$ , preferably greater than  $5 \times 10^6$  bone marrow cells to be measured<sup>[8]</sup>, as recommended by the International Clinical Cytometry Society and the European Society for Clinical Cell Analysis. In addition to low sensitivity, MFC may be unable to differentiate between the dominant clone and various subclones<sup>[9]</sup>. In addition, there is heterogeneity between laboratories (cross-platform flow cytometry), which depends on instrumentation used and initial gating parameters (CD38, CD138, CD45, forward, and sideward light scatter)<sup>[10]</sup>, within the same aliquot and is therefore entirely subjective. Next-generation sequencing (NGS) of immunoglobulin gene sequences is an alternative method for MRD assessment. While a more sensitive technique compared with MFC, detecting one tumor cell in  $10^6$ , NGS cannot detect mutations that are present within individual cells<sup>[11]</sup>. Therefore, based on our current technologies for MRD detection, we can only say whether a patient is positive or negative without genuinely understanding the temporal and spatial heterogeneity within a given plasma cell population.

Second, we recognize the temporal and spatial heterogeneity in MM, as clinical observations revealed that several subclones of PCs exist at diagnosis and that there is selective therapeutic pressure for the evolution of individual subclones. This phenomenon can be tracked utilizing the whole-genome sequencing of paired tumor/normal samples. In one study, from 203 MM patients revealed frequent mutations in KRAS, NRAS, BRAF, FAM46C, TP53, and DIS3. Mutations were often present in subclonal populations, and multiple mutations within the same pathway (e.g., KRAS, NRAS, and BRAF) were observed in the same patient<sup>[12]</sup>. However, a more recent study utilizing plasma samples found mutations in the KRAS-MAPK pathway in 70% of samples in addition to multiple mutations within subclones including a notable mutation in PIK3CA<sup>[13]</sup> signifying perhaps the relative insensitivity of a one site biopsy in addition to the development of more mutated clones with the escape of PCs from the bone marrow microenvironment. Liquid biopsy utilizing cfDNA can provide us with information on targetable mutations, but is a way to study spatial and temporal heterogeneity present. The drawbacks for above current testing mistaken population phenomena for real physiological events happening only within a single-cell (*i.e.*, subclone) – mutations exist in different cells may not cross-talk – thereby not being able to manifest as clinically treatable phenotypes – which would not give early insight into the evolution of a given patient's MM<sup>[14]</sup>.

Third, the identification of chemoresistant biomarkers offers a trace to the subclones, *e.g.*, the oligonucleotide array analysis demonstrates that heat shock protein-27 (Hsp27) is upregulated in Dex-resistant, but not in Dex-sensitive MM cells. Proteomics analysis of Hsp27-immunocomplexes revealed the presence of actin in Dex-resistant, but not in Dex-sensitive cells. The activator protein-1 transcription factor family (JUNB) driving the JunB-mediated phenotype in MM cells: knockdown of JUNB restored the response to dexamethasone in dexamethasone-resistant MM cells. When JunB-ER fusion protein in dexamethasone-sensitive MM cells is activated by 4-hydroxytamoxifen, Dex-sensitive cells become to be resistant to dexamethasone- and bortezomib-induced cytotoxicity<sup>[15]</sup>.

Thus, the ability to track mutations within a single cell subclone lends to the study of mechanisms of drug resistance, possibly leading to a better selection of targeted therapies. To that end, new technology must be developed and raised its sensitivity sufficient to evaluate the low burden of MM cells, which is currently being investigated as a way to detect pre-biochemical relapse<sup>[16]</sup>. We propose to develop a technique that combines the detection of low-frequency events combines with the in-depth characterization of the remaining subclones.

## DEVELOPMENT OF AN INNOVATIVE SINGLE-CELL MOLECULAR PROFILING PLATFORM

While single-cell proteomics is still uncertain, single-cell RNA-seq is a widespread

practice in research laboratories now. Many microfluidic devices, including ours, have been developed for single-cell transcriptome analysis<sup>[17]</sup> but clinical application of single-cell transcriptome is still not common, especially in cancer characterization and classification. US Food and Drug Administration approved CellSearch™ (Janssen, Raritan, NJ) of circulating tumor cells (CTC) for predicting PFS and OS in metastatic breast cancer for clinical use in 2005. However, CellSearch® data correlate negatively with survival in patients with metastatic breast, colorectal, or prostate cancer<sup>[18]</sup>. CellSearch™, along with several other CTC enrichment techniques, relies on only fluorescent imaging analysis.

At least 50 competitor circulating tumor cell platforms exist (Table 1)<sup>[19,20]</sup>, only one, “CellSearch™” (Janssen, Raritan, NJ) was cleared by the US Food and Drug Administration in October 2005. As we have a prototype Multi-Phase Laser-cavitation Single Cell Analyzer (MLSCA) device for single-cell transcriptome analysis (Figure 1), we develop a necessary commercialization 510k, and the CAP/CLIA component of this proposal must be side by side comparison of the applicant's technology to the CellSearch™ technology. This feature differentiates it from numerous competitor platforms used for single-cell counting. CellSearch™ can be used for predicting PFS and OS in metastatic breast cancer<sup>[19]</sup>, however; CellSearch™ cannot generate consistent results for routine clinical use<sup>[21]</sup>, due to the limit of the sensitivity of these devices<sup>[22]</sup>. CellSearch™ captures CTCs from blood using magnetic particles coated with anti-EpCAM (CD326; 17-1A antigen) antibodies<sup>[23]</sup>, which relies on an antibody that binds to the protein EpCAM (epithelial cell adhesion molecule), present on the surface of malignant epithelial cells but not of blood cells. As CellSearch®, along with several other CTC enrichment techniques, relies on the presence of epithelial cell markers, CTCs that do not express EpCAM, such as those that have undergone an epithelial to mesenchymal transition may be missed<sup>[24,25]</sup>.

Thus, CellSearch™ cannot generate consistent results for routine clinical use<sup>[21]</sup>, and there is an unmet need to combine image analysis with RNA-seq for cancer classification. To fill this unmet need, we developed a microfluidic prototype device to connect FACS and imaging analysis (FISH) with molecular analysis (e.g., single-cell transcriptomes) - whose prototype device is called MLSCA (Figure 1).

Our MLSCA can overcome the limitation of “CellSearch™” [multiple-step processing, *i.e.*, separated reverse transcriptase polymerase chain reaction (RT-PCR)], and the strength is the ability of our MLSCA to perform RT-PCR on a single cell isolated on the chip (one-step processing), eliminating process errors. Our microfluidic system is equipped with both single-cell isolation and cDNA synthesis capabilities. Thus, our MLSCA enables (1) microscale fluorescence-activated cell selection for separation of rare cell subpopulations; and (2) generation of the high-quality single-cell transcriptome with nano-droplets. We have conducted and published a small Phase I clinical trial with our devices in myeloma risk stratification of MM<sup>[26]</sup>. As a proof-of-concept for instrumentation of our prototype device, we published the clinical trial using MLSCA/MF-CD45-TACs on MM 48 patients<sup>[17]</sup>, which shed new light for scale-up applications in clinics.

Conventional single-cell isolation techniques with microliter carry-over volumes cannot be used for sensitive nano-liter RT directly. Unlike PCR, which is a repetitive event that itself may introduce bias, RT is a single biochemical reaction for which starting mRNA concentration is critical. Further, the resultant cDNA population is thought to be unbiased. The innovations of our device include: (1) The single cell analyzer (MLSCA) combines single-cell fluorescent-activated cell selection, reverse transcript synthesis of high-quality cDNA, transcriptome analysis - all in 0.1 nano-liter droplets (50 pico-liter), thereby reducing procedure errors to improve single-cell cDNA quality and to yield reliable single-cell transcriptomes down-stream; (2) The ability to create single artificial cells, a highly controllable test system, for evaluation of platform performance, using homogeneous droplets of known low abundant RNA; (3) Demonstration of such MLSCA analysis clinical specimens; (4) Development of statistical methods for defining and quantifying molecular heterogeneity in populations of cells; and (5) Linkage to an RNAseq platform (Helicos and Ion Torrent) that does not require PCR amplification of the input cDNA is crucial for ensuring the fidelity of the measured transcriptome: read counts can be directly related to RNA abundance, with no possible distortion due to differential PCR efficiencies. Thus, the strength is the ability of the MLSCA to perform RT-PCR on a single cell on the chip. This strength differentiates it from numerous competitor platforms only used for CTC counting. We tested the MLSCA by addressing MM heterogeneity to identify underlying tumor initiation, and relapse biomarkers in MM, which was inspired by that fact that single-cell transcriptomic analysis in medulloblastomas led to mapping oncogenic networks including HIPPO-YAP/TAZ and AURORA-A/MYC/N

**Table 1 Multi-Phase Laser-cavitation Single Cell Analyzer can perform both circulating tumor cells enumeration and single-cell molecular characterization**

Technology for CTC	CTC count	Molecular profile	Single CTC molecular profile	Ref.
MF				
Proposed MLSCA (uFACS and on-chip RT/PCR)	Yes	Yes	Yes	
CTC chip micropost	Yes	Yes	No	[47,48]
Isolate CTC by size with filter	Yes	Yes	No	[49]
Cytometer				
Flow cytometry ( <i>i.e.</i> FACS)	Yes	Yes	No	[50]
Multiphoton intravital flow cytometry	Yes	No	No	[51]
laser scanning cytometry	Yes	No	No	[52]
Photoacoustic flowmetry	Yes	No	No	[53]
Fiber-optic array scanning technology (FAST)	Yes	No	No	[54]
ICC (Ab)				
CellSearch™ (Immunomagnetic enrichment, FDA approved)	Yes	Yes	No	[55]
Immunomagnetic cell sorting for positive or negative selection	Yes	Yes	No	[56]
Epithelial immunospot (EPISPOT) of CTC secreted proteins	Yes	No	No	[57]
Others				
Density gradient centrifugation	Yes	Yes	No	[58]
Dielectrophoresis	Yes	Yes	No	[59]
Collagen adhesion matrix ingestion assay	Yes	No	No	[60]
PCR detection of tumor-derived nucleic acid in serum/plasma	No	Yes	No	[61]
RT/PCR detection of tumor-specific markers in nucleated blood cells	No	Yes	No	[62]
Membrane arrays for detecting multiple tumor-specific mRNA	No	Yes	No	[63]

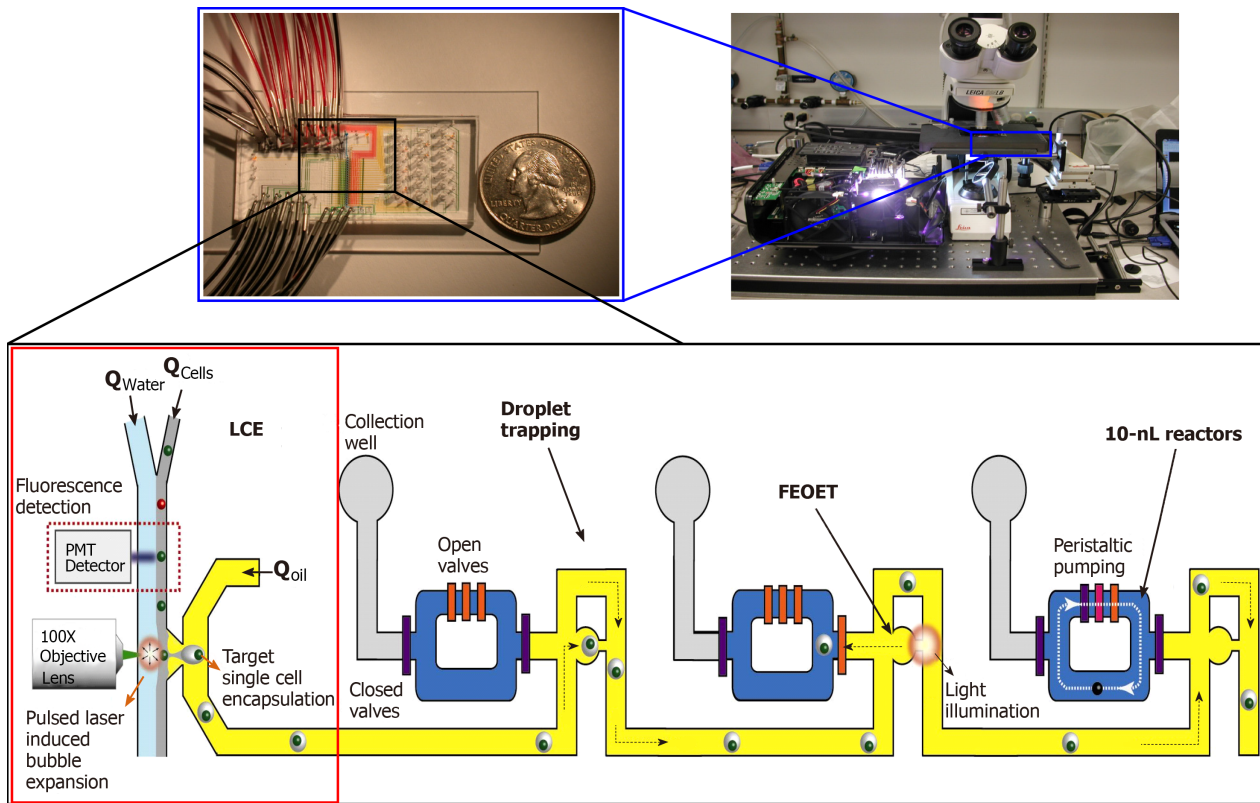
MLSCA: Multi-Phase Laser-cavitation Single Cell Analyzer; ICC: Immunocytochemical staining; MEMS: Microelectromechanical system; MC: Microfluidic channels; MF: Multiparametric flow; CTC: Circulating tumor cells; RT: Reverse transcriptase; PCR: Polymerase chain reaction.

pathways<sup>[27]</sup> and to identify pathways of drug resistance<sup>[28]</sup>.

We applied this MLSCA for a clinical trial of risk stratification of MM<sup>[17]</sup>. As our MF-CD45-TACs differs from CellSearch™, we expected single-cell features (FACS, biomarkers) of CellSearch™ differ from those of our MF-CD45-TACs (Figure 2). Specifically, our MF-CD45-TACs-based technology distinguished CD45<sup>+</sup> cells from MM PCs, which improved the detection of rare genetic alternation in PCs, which was a significant improvement over direct flow cytometry and FISH, and led to more precise diagnosis and prognosis of MM.

This attribute is of significance as MM is an incurable neoplasm of PCs that affects more than 20000 people annually in the United States. Risk stratification, primarily based on cytogenetic abnormalities, has emerged as essential for its management<sup>[29]</sup>. Thalidomide, lenalidomide, and pomalidomide, first to the third generation of immunomodulatory drugs (IMiDs), respectively, are used for maintenance therapy of MM. Cytogenetic alterations are the base of risk stratification for MM and the selection of which IMiDs for MM therapy<sup>[30]</sup>.

The rarity and sporadic distribution of PCs in bone marrow often lead to false-negative results of FACS and cytogenetic detection performed directly on a bone marrow biopsy sample. Target cell enrichment could overcome the rarity and sporadic distribution of PCs in the bone marrow. Density gradient centrifugation and



**Figure 1** A prototype LSCAT device for single-cell transcriptome analysis.

magnetically labeled antibodies [DG/magnetic-activated cell sorting (MACS)] with MACS have been widely used to enrich target cells in blood samples. MACS enrichment of CD138<sup>+</sup> cells for FISH in MM diagnosis has been reported<sup>[31]</sup>. However, MM cells with low levels of CD138 have also been associated with poor prognosis<sup>[32]</sup>. Therefore, a better enrichment method is needed. Here, we report a novel microfluidic approach, combining microfluidic size selection and CD45 depletion with tetrameric antibody complexes (TACs) for the enrichment of MM cells (MF-CD45-TACs) in bone marrow samples. Our study showed that this approach significantly improves the detection of rare genetic alternations in PCs. Parallel diagnosis performed for 48 patients (Figure 3) showed that the microfluidic enrichment approach represents a significant improvement over direct flow cytometry and FISH and leads to more precise diagnosis and prognosis<sup>[17]</sup>. Implementation of this modified diagnostic assay in clinics could improve the current clinical outcomes of MM (Figure 4 and Figure 5).

## APPLICATION OF SINGLE CELL SUBCLONE TRACKING TO THE FUTURE TREATMENT OF MM

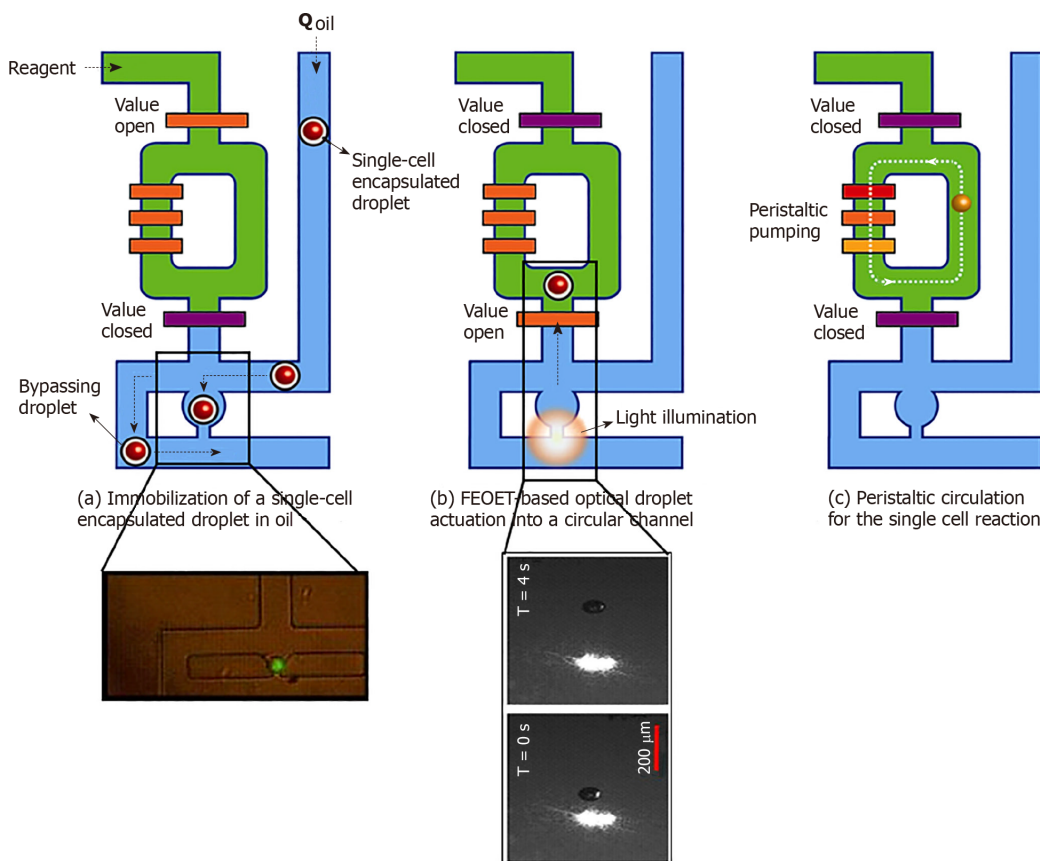
### At the MGUS/Smoldering stage

While all MM is preceded by an asymptomatic MGUS/smoldering myeloma stage<sup>[33]</sup>, only a fraction of these individuals will evolve to symptomatic MM. Currently, some high-risk features such as high bone-marrow plasma cell burden, light chain ratios, and predates the development of symptomatic MM. Still, we do not understand the oncogenesis of MM and, therefore, cannot accurately determine who will progress and who will remain asymptomatic. Using circulating tumor cell technology, we could track the occurrence of trigger genetic events in pre-symptomatic patients without the need to perform repeated invasive procedures and potentially intervene to eradicate these emerging malignant subclones using targeted therapies prior to the development of symptomatic disease or the acquisition of additional potent genetic mutations.

### MRD monitoring

MRD monitoring has become one of the most relevant prognostic factors for MM. It has been shown that persistent MRD is associated with improved progression-free



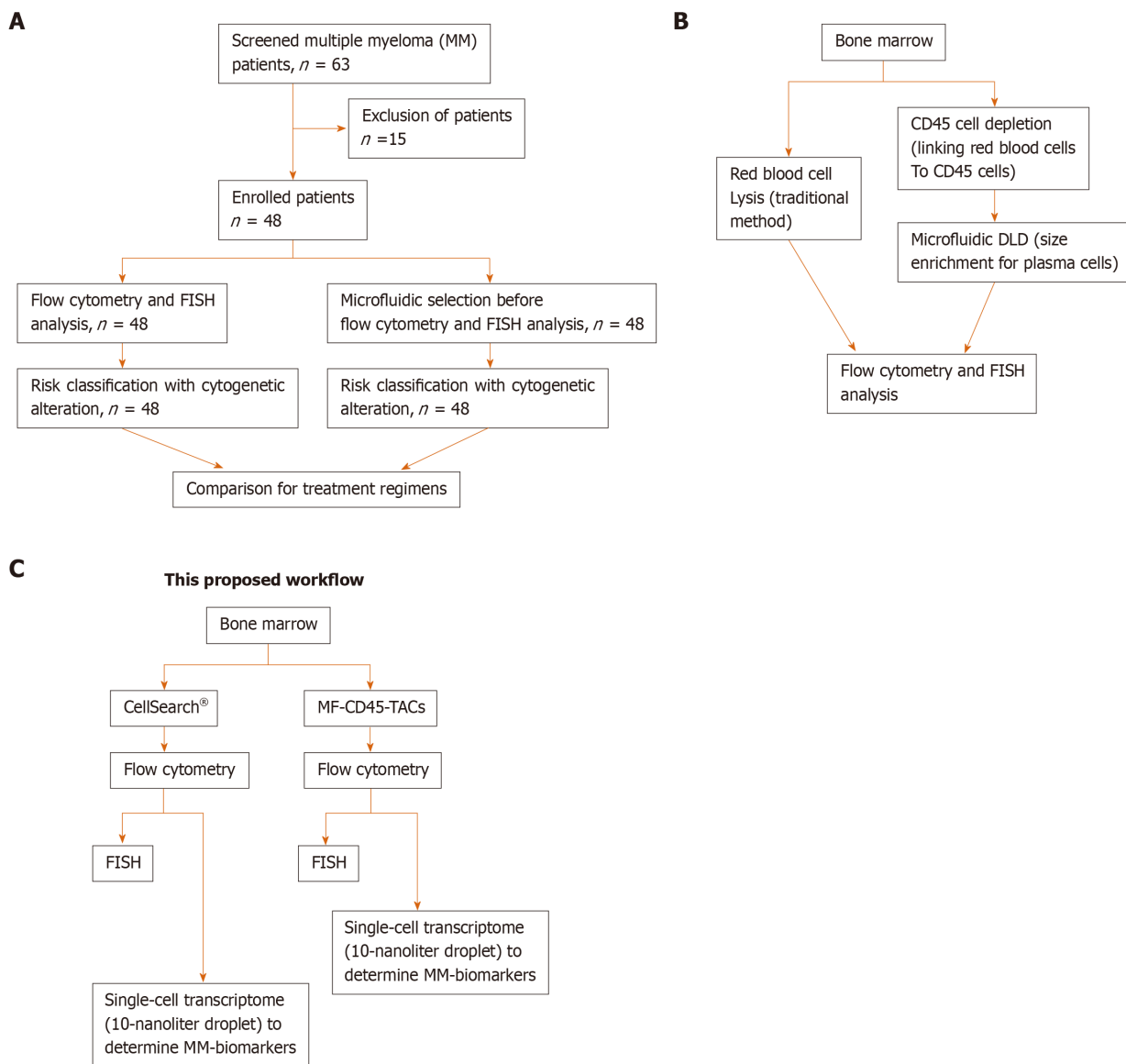


**Figure 2 Processing of single-cell droplets.** A: A single-cell in an oil droplet travels to a trapping module of the 10-nl reactor (green, in inset). Other cells are forced to bypass to the next unit; B: FEOET push the droplet (with a cell) past the open valve (orange bar), which closes, locking the droplet into the 10-nl ring with RT/RT-PCR master mix (green); and C: The ring's peristaltic pump breaks the droplet to mix the cell with master-mix. When the reaction is finished, oil (blue) pushes the product (cDNA) out of the ring in the form of a droplet (10-nl) for downstream molecular analysis (Refer to<sup>[17]</sup> for details).

survival and overall survival<sup>[33]</sup>. Not only is single cell tracking methodology as described in this article a sensitive method to detect MRD, but the characteristics of the residual cells will also be able to be elucidated. As such, one can detect the emergence of a “dangerous” clone.

### **Determining the sequencing of therapies including immunotherapy**

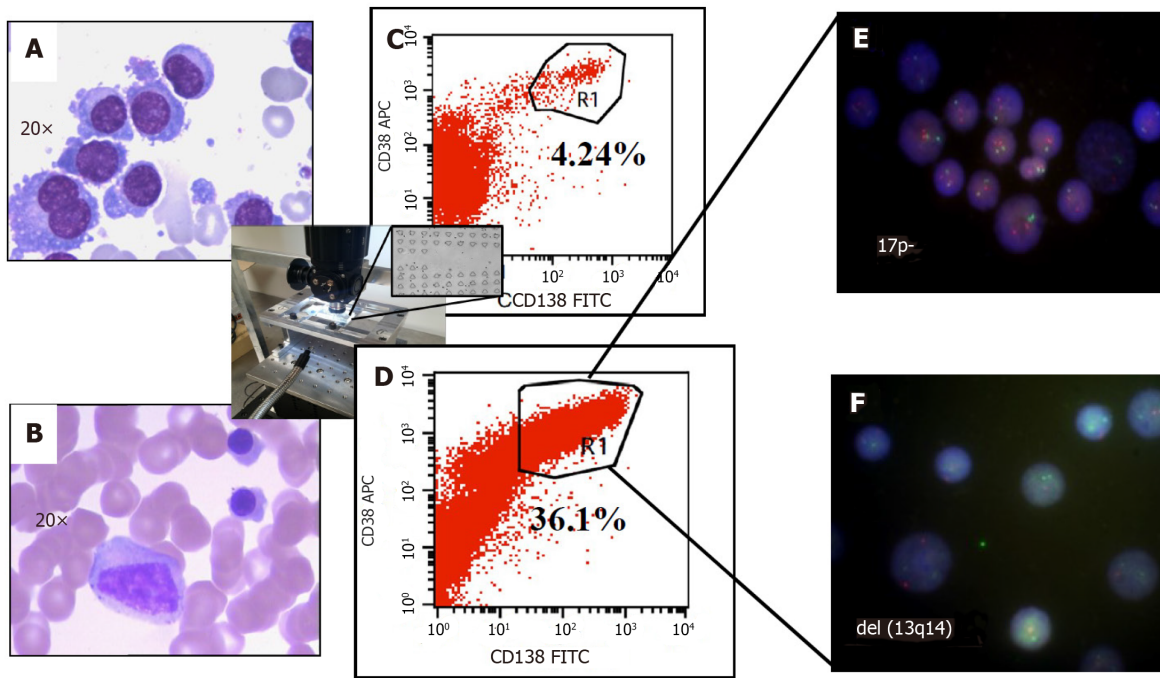
Currently, we have more than 14 unique treatments for the MM, which, when used in combination, yields dozens of combination options for patients. Clinical trials using antibody drug-conjugate and bispecific antigen-directed CD3 T-cell engager targeting, by checkpoint inhibitors and an anti-T-cell immunoglobulin and ITIM domains antibody are currently underway and has the potential to further prolong survival times<sup>[34]</sup>. Chimeric antigen receptor T cell therapy targeting B-cell maturation antigen, immunoglobulin kappa chain, SLAM family member 7, or G-protein coupled receptor family C group 5 member D, the activated integrin beta7 is a promising treatment modality which can often give long progression-free survival in heavily treated patients<sup>[35,36]</sup>. Despite this arsenal of treatments, the elusive cure for MM has yet to be found, and the current approaches to the treatment of refractory disease produce progressively short-lived efficacy. Perhaps this is because the sequencing of these treatments is often borne out of trial and error and do not take into consideration the temporal changes and spatial relationships of MM subclones. The development of chemoresistance, leading to shorter progression-free with each subsequent treatment and overall survival, lies in understanding how therapies drive the evolution of subclones. Using sensitive methods for detection and characterization of subclones, we can understand whether therapy induces molecular alterations within myeloma cells or selects for the survival of specific clones over others. Knowing how the treatments we use drive the process of evolution can allow clinicians to choose combinations of therapies that will modulate the development of chemoresistance.



**Figure 3 Schematic designs of the proposed workflow.** A: Consolidated Standards of Reporting Trials diagram. A total of 63 patients were screened for eligibility. Only 48 patients were newly diagnosed with multiple myeloma before receiving any treatment. These patients were enrolled, and their bone marrow obtained at diagnosis was divided into two aliquots: One aliquot underwent traditional flow cytometry and FISH analysis, and the other aliquot was subjected to microfluidic selection for enrichment of CD45-PCs, then subjected to flow cytometry and FISH analysis. Results from both methods were compared; B: Comparison of traditional method to microfluidic method (MF-CD45-TACs). MF-CD45-TACs significantly enrich plasma cells for flow cytometry and FISH assays and improve the accuracy of these assays; C: This proposed workflow (Note that we can use both bone marrow and circulating multiple myeloma cells<sup>[76]</sup>).

## CONCLUSION

We envision that single-cell technology will innovate cancer stem cell subclonal evolution on time-space landscaping of heterogeneity and imply the lineage-tracking pathway-based prediction of therapeutic efficacies of cancer treatment. Accurately, temporal development and spatial distribution of quantitative subclonal measurement of MM will reveal therapeutic sensitizing mutations, thereby moving closer to developing a therapeutic window<sup>[37]</sup> of cancer in the advent of new, more productive, and less toxic therapies. We hypothesize that subclonal evolution, in conjunction with current standard care, will improve outcomes in patients with heterogeneous pathologies (Figure 6)<sup>[38]</sup>. Circulating MM counts and Cav-1 molecules early during radiotherapy are independently predictive of recurrence in MM. Physicians assert that every time that there is a reference to, visual or spoken, the patient view of the landscape of an MM diagnostics that they claim to predict the outcomes legitimately, it has to be as comprehensive and individualized as possible given the data package generated from AI-Med algorithms. Recently, we applied our microfluidic devices to myeloma risk stratification. However, like many current microfluidic devices, the device only enhanced and improved traditional FACS and FISH. The gap of



**Figure 4 Improved clinical outcomes with microfluidic CD45 depletion (Patient 1).** A: Bone marrow smear at the time of initial diagnosis; B: Bone marrow smear after effective treatment (complete remission); C: Flow-cytometry without microfluidic enrichment. Plasma cells (CD38+/CD138+) is only 4.24% and no FISH cytogenetic abnormalities were found (low risk); D: After microfluidic enrichment (center inset) plasma cells (CD38+/CD138+) increased to 36.1%; E: FISH on enriched plasma cells show 17p- (Red: D13S319; Green: P53); F: FISH showed del(13q14) in enriched PC (Red: D13S319; Green: RB1). With enriched plasma cell for FISH, the patient was reclassified and treated as high-risk multiple myeloma which leads to complete remission (Refer to<sup>[17]</sup> for details).

integrating genomic profiles into cancer characterization is still not filled. Therefore, it is a logical and necessary step for us to integrate our single-cell RNA-seq technology into cancer characterization, specifically molecular classification of MM in cancer genome landscape such as the Pan-Cancer Analysis of Whole Genomes consortium<sup>[39]</sup>. "Timing analyses suggest that driver mutations often precede diagnosis by many years, if not decades. Together, these results determine the evolutionary trajectories of cancer and highlight opportunities for early cancer detection"<sup>[40]</sup>. All of these must rely on "A deep learning system accurately classifies primary and metastatic cancers using passenger mutation patterns"<sup>[41]</sup> for integration of dynamic space-time changes. One such integrated platform was to scaffold the diverse datasets together, allowing them to interface not only across single-cell transcriptomics (scRNA-seq), but also across distinct cellular modalities – e.g., a bone marrow atlas to characterize lymphocyte populations<sup>[42]</sup> – to better understand cellular identity and function beyond the taxonomic listing of clusters of cellular heterogeneity<sup>[43]</sup>.

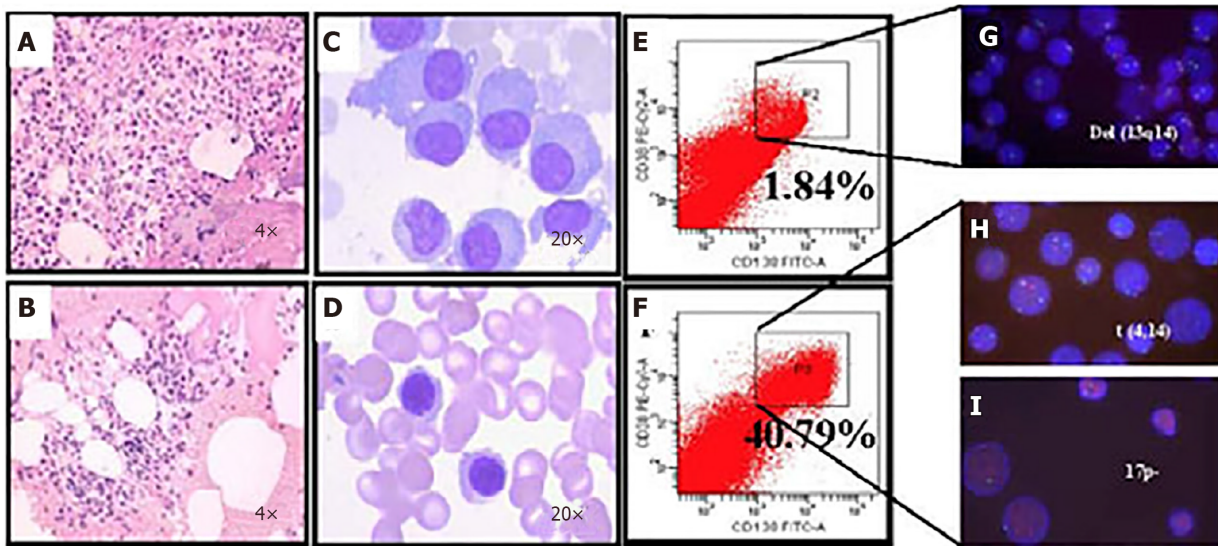
Implementation of this modified diagnostic device in clinics proven to improve clinical outcomes of MM (Figure 3C). Our microfluidic-assisted stratification of single cancer cells may help understand the mechanisms underlying the temporal and spatial heterogeneities in solid tumors like brain cancer as testing is underway<sup>[44-46]</sup>, thereby holding promise for using the single-cell analysis to guide treatment for targeted therapy (Table 2), as governed by artificial intelligence-based integration of genome, epigenome, and pathological measurements.

**Table 2 Therapeutics targets and corresponding agents in multiple myeloma and artificial intelligence medicine**

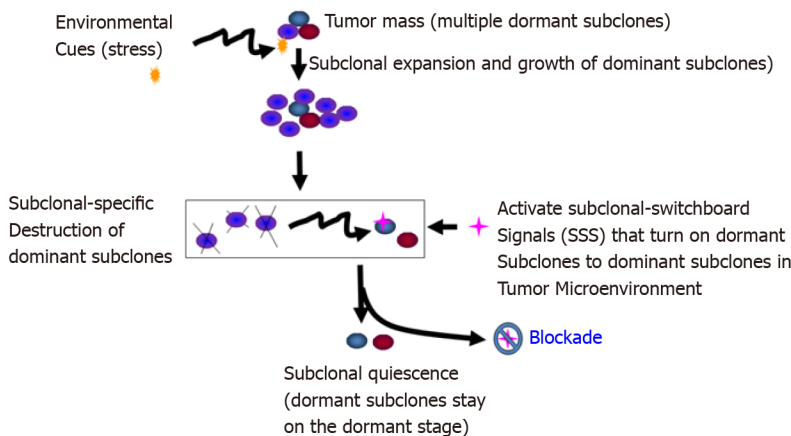
Mechanism of action (clinical phenotype)	Target	Agent	Ref.
Resistance to chemotherapy in MM	Bcl-2/Bcl-X(L)/Bcl-w (antiapoptotic proteins)	Inhibitor ABT-737 (with bortezomib-, dexamethasone-(Dex) and thalidomide)	[64]
Dexamethasone-resistance in MM	Heat shock protein-27	2-methoxyestadiol and bortezomib/proteasome-inhibitor	[65]
JunB-mediated phenotype in dexamethasone-resistant MM cells	JunB: AP-1 transcription factor family	Knockdown AP-1/JunB to down-regulate MM cell proliferation, survival and drug resistance	[15]
Cyclin D dysregulation and overexpression/growth arrest or caspase-dependent apoptosis in MM cells	cyclin D1	P276-00, a novel small-molecule cyclin-dependent kinase inhibitor	[66]
Sensitivity to bortezomib in MM cells	Cav-1	Sensitivity to bortezomib of RPIM8226 MM cells after co-cultured with down-regulated Cav-1 expression HUVECs	[67]
Heartbeat/pulse patterns – AI relevance		Flattening of the flow velocity (pulse) patterns correlates with the local severity of arteriosclerotic disease	[68]
	Preventive medicine using pulse oximetry screening		
		Pulse transit time (PTT) is the time it takes a pulse wave to travel between two arterial sites R-wave-gated photo-plethysmography as of measurement of PTT as a surrogate for intra-thoracic pressure changes in obstructive sleep apnea)	[69]
		Pulse oximetry screening for critical congenital heart defects	[70]
AI-Medicine algorithm	Algorithm to track changes in cardiorespiratory interactions (heartbeat intervals and respiratory recordings under dynamic breathing patterns)		[71]
		Respiratory sinus arrhythmia (RSA) with an algorithm for quantifying instantaneous RSA as applied to heartbeat interval and respiratory recordings to track changes in cardiorespiratory interactions elicited during meditation, otherwise not evidenced in control resting states)	[72]
	The tongue is a critical organ for respiration and speech		[73]
		18 voice features with posttraumatic stress disorder	[74]
	Breathing pattern parameters: Peak airway pressure ( $P_{aw_{peak}}$ ), mean airway pressure ( $P_{aw_{mean}}$ ), tidal volume ( $V_T$ , mL/kg), minute volume, respiratory muscle unloading (peak electricity of diaphragm ( $EAdi_{peak}$ ), $P_{0.1}$ , $V_T/EAdi$ ), clinical outcomes (ICU mortality, duration of ventilation days, ICU stay time, hospital stay time		[75]

Cited Literature. MM: Multiple myeloma; AP-1: Activator protein-1; ICU: Intensive care unit.





**Figure 5 Microfluidic risk-stratification improves clinical outcomes of multiple myeloma (Patient 2).** A: Bone marrow at diagnosis. Active granulocyte hyperplasia; B: Partial remission was achieved with revised risk-stratification; C: At diagnosis, bone marrow plasma cell (PC) abnormalities included clustered and scattered distribution of primitive and immature PCs, with large cell body, fine chromatin, visible nucleolus, and abundant cytoplasm; D: After treatment for high-risk multiple myeloma, PCs were rare and had normal morphology; no typical abnormal PCs were observed; E: At diagnosis without microfluidic enrichment, PCs (CD38+/CD138+) were only 1.84 %; F: After microfluidic enrichment, PCs (CD38+/CD138+) increased to 40.79%; G: Without microfluidic enrichment, FISH showed IgH rearrangement and del(13q14), leading to classification as intermediate risk (Red: D13S319; Green: P53); H and I: After microfluidic enrichment, in addition to del(13q14), FISH showed t(4,14) fusion (yellow dots) and 17p- (Red: D13S319; Green: P53), patient was reclassified and treated as high-risk, which led to efficacious treatment (Refer to<sup>[17]</sup> for details).



**Figure 6 Blockade of the dominating subclonal switchboard signals in cancer stem cells as a new therapeutic strategy to suppress the dominating subclone shift to control cancer progression and post-treatment cancer recurrence.** Showed is the proposed new treatment paradigm that should target the subclonal-switchboard signals (SSS). Blocking the dominating subclonal SSS leads to subclonal quiescence, so keeping tumors alive but small and manageable (dormant/quiescent subclone). Note that SSS as mechanisms for leading to shifting dominating subclones as triggered by environmental cues (stress) for cancer progression and post-treatment. A cancer subclone may gain a mutation that, in the appropriate environment cue, leads to dominating subclonal activation due to positive selection. Showed lettering and lines/ arrows in the black color is the current concept of a treatment strategy for cancer- dominant subclonal cells (cancer stem cells) that may acquire a mutation in a suitable environment, triggering to dominating subclonal expansion and growth. When this dominating subclone is explicitly destroyed, it sends out dominating subclonal-SSS to a dormant/quiescent subclonal cell, which gets activated for dominating subclonal expansion and growth (adopted from<sup>[38]</sup>).

## ACKNOWLEDGEMENTS

We thank Maria Minon, MD; Brent A Dethlefs; Mustafa H Kabeer, MD; William G Loudon, MD, PhD; Leonard S Sender, MD; Anthony Christopher Chang, MD, MBA, MPH; Edward Nelson, MD; Richard A van Etten, MD, PhD; Dan Cooper, MD; and Jiang F Zhong, PhD; for their support and enthusiasm.

## REFERENCES

- Kazandjian D.** Multiple myeloma epidemiology and survival: A unique malignancy. *Semin Oncol* 2016; **43**: 676-681 [PMID: 28061985 DOI: 10.1053/j.seminoncol.2016.11.004]
- Weiss BM, Abadie J, Verma P, Howard RS, Kuehl WM.** A monoclonal gammopathy precedes multiple myeloma in most patients. *Blood* 2009; **113**: 5418-5422 [PMID: 19234139 DOI: 10.1182/blood-2008-12-195008]
- Fonseca R, Bergsagel PL, Drach J, Shaughnessy J, Gutierrez N, Stewart AK, Morgan G, Van Ness B, Chesi M, Minvielle S, Neri A, Barlogie B, Kuehl WM, Liebisch P, Davies F, Chen-Kiang S, Durie BG, Carrasco R, Sezer O, Reiman T, Pilarski L, Avet-Loiseau H; International Myeloma Working Group.** International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. *Leukemia* 2009; **23**: 2210-2221 [PMID: 19798094 DOI: 10.1038/leu.2009.174]
- Hebraud B, Magrangeas F, Cleynen A, Lauwers-Cances V, Chretien ML, Hulin C, Leleu X, Yon E, Marit G, Karlin L, Roussel M, Stoppa AM, Belhadj K, Voillat L, Garderet L, Macro M, Caillot D, Mohty M, Facon T, Moreau P, Attal M, Munshi N, Corre J, Minvielle S, Avet-Loiseau H.** Role of additional chromosomal changes in the prognostic value of t(4;14) and del(17p) in multiple myeloma: the IFM experience. *Blood* 2015; **125**: 2095-2100 [PMID: 25636340 DOI: 10.1182/blood-2014-07-587964]
- Siegel DS, Dimopoulos M, Jagannath S, Goldschmidt H, Durrant S, Kaufman JL, Leleu X, Nagler A, Offner F, Graef T, Eid JE, Hou P, Gause C, Vuocolo S, Anderson KC.** VANTAGE 095: An International, Multicenter, Open-Label Study of Vorinostat (MK-0683) in Combination With Bortezomib in Patients With Relapsed and Refractory Multiple Myeloma. *Clin Lymphoma Myeloma Leuk* 2016; **16**: 329-334.e1 [PMID: 27025160 DOI: 10.1016/j.clml.2016.02.042]
- Bochtler T, Merz M, Hielscher T, Granzow M, Hoffmann K, Krämer A, Raab MS, Hillengass J, Seckinger A, Kimmich C, Dittrich T, Müller-Tidow C, Hose D, Goldschmidt H, Hegenbart U, Jauch A, Schönland SO.** Cytogenetic intraclonal heterogeneity of plasma cell dyscrasia in AL amyloidosis as compared with multiple myeloma. *Blood Adv* 2018; **2**: 2607-2618 [PMID: 30327369 DOI: 10.1182/bloodadvances.2018023200]
- Kumar S, Paiva B, Anderson KC, Durie B, Landgren O, Moreau P, Munshi N, Lonial S, Bladé J, Mateos MV, Dimopoulos M, Kastritis E, Boccadoro M, Orłowski R, Goldschmidt H, Spencer A, Hou J, Chng WJ, Usmani SZ, Zamagni E, Shimizu K, Jagannath S, Johnsen HE, Terpos E, Reiman A, Kyle RA, Sonneveld P, Richardson PG, McCarthy P, Ludwig H, Chen W, Cavo M, Harousseau JL, Lentzsch S, Hillengass J, Palumbo A, Orfao A, Rajkumar SV, Miguel JS, Avet-Loiseau H.** International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. *Lancet Oncol* 2016; **17**: e328-e346 [PMID: 27511158 DOI: 10.1016/S1470-2045(16)30206-6]
- Soh KT, Tario JD Jr, Wallace PK.** Diagnosis of Plasma Cell Dyscrasias and Monitoring of Minimal Residual Disease by Multiparametric Flow Cytometry. *Clin Lab Med* 2017; **37**: 821-853 [PMID: 29128071 DOI: 10.1016/j.cll.2017.08.001]
- Berger N, Kim-Schulze S, Parekh S.** Minimal Residual Disease in Multiple Myeloma: Impact on Response Assessment, Prognosis and Tumor Heterogeneity. *Adv Exp Med Biol* 2018; **1100**: 141-159 [PMID: 30411265 DOI: 10.1007/978-3-319-97746-1\_9]
- Arroz M, Came N, Lin P, Chen W, Yuan C, Lagoo A, Monreal M, de Tute R, Vergilio JA, Rawstron AC, Paiva B.** Consensus guidelines on plasma cell myeloma minimal residual disease analysis and reporting. *Cytometry B Clin Cytom* 2016; **90**: 31-39 [PMID: 25619868 DOI: 10.1002/cyto.b.21228]
- Bai Y, Orfao A, Chim CS.** Molecular detection of minimal residual disease in multiple myeloma. *Br J Haematol* 2018; **181**: 11-26 [PMID: 29265356 DOI: 10.1111/bjh.15075]
- Lohr JG, Stojanov P, Carter SL, Cruz-Gordillo P, Lawrence MS, Auclair D, Sougnez C, Knoechel B, Gould J, Saksena G, Cibulskis K, McKenna A, Chapman MA, Straussman R, Levy J, Perkins LM, Keats JJ, Schumacher SE, Rosenberg M; Multiple Myeloma Research Consortium, Getz G, Golub TR.** Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. *Cancer Cell* 2014; **25**: 91-101 [PMID: 24434212 DOI: 10.1016/j.ccr.2013.12.015]
- Hocking J, Mithraprabhu S, Kalf A, Spencer A.** Liquid biopsies for liquid tumors: emerging potential of circulating free nucleic acid evaluation for the management of hematologic malignancies. *Cancer Biol Med* 2016; **13**: 215-225 [PMID: 27458529 DOI: 10.20892/j.issn.2095-3941.2016.0025]
- Terpos E, Suzan F, Goldschmidt H.** Going the distance: Are we losing patients along the multiple myeloma treatment pathway? *Crit Rev Oncol Hematol* 2018; **126**: 19-23 [PMID: 29759561 DOI: 10.1016/j.critrevonc.2018.03.021]
- Fan F, Bashari MH, Morelli E, Tonon G, Malvestiti S, Vallet S, Jarahian M, Seckinger A, Hose D, Bakiri L, Sun C, Hu Y, Ball CR, Glimm H, Sattler M, Goldschmidt H, Wagner EF, Tassone P, Jaeger D, Podar K.** The AP-1 transcription factor JunB is essential for multiple myeloma cell proliferation and drug resistance in the bone marrow microenvironment. *Leukemia* 2017; **31**: 1570-1581 [PMID: 27890927 DOI: 10.1038/leu.2016.358]
- He R, Yang N, Zhang P, Liu J, Li J, Zhou F, Zhang W.** Identification and expression of MMSA-8, and its clinical significance in multiple myeloma. *Oncol Rep* 2017; **37**: 3235-3243 [PMID: 28498418 DOI: 10.3892/or.2017.5609]
- Zeng Y, Gao L, Luo X, Chen Y, Kabeer MH, Chen X, Stucky A, Loudon WG, Li SC, Zhang X, Zhong JF.** Microfluidic enrichment of plasma cells improves treatment of multiple myeloma. *Mol Oncol* 2018; **12**: 1004-1011 [PMID: 29638042 DOI: 10.1002/1878-0261.12201]
- Miller MC, Doyle GV, Terstappen LW.** Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. *J Oncol* 2010; **2010**: 617421 [PMID: 20016752 DOI: 10.1155/2010/617421]
- Lianidou ES, Markou A.** Circulating tumor cells in breast cancer: detection systems, molecular characterization, and future challenges. *Clin Chem* 2011; **57**: 1242-1255 [PMID: 21784769 DOI: 10.1373/clinchem.2011.165068]
- Lianidou ES.** Circulating tumor cell isolation: a marathon race worth running. *Clin Chem* 2014; **60**: 287-289 [PMID: 24323980 DOI: 10.1373/clinchem.2013.216010]

- 21 **Parkinson DR**, Dracopoli N, Petty BG, Compton C, Cristofanilli M, Deisseroth A, Hayes DF, Kapke G, Kumar P, Lee JSh, Liu MC, McCormack R, Mikulski S, Nagahara L, Pantel K, Pearson-White S, Punnoose EA, Roadcap LT, Schade AE, Scher HI, Sigman CC, Kelloff GJ. Considerations in the development of circulating tumor cell technology for clinical use. *J Transl Med* 2012; **10**: 138 [PMID: [22747748](#) DOI: [10.1186/1479-5876-10-138](#)]
- 22 **Liu MC**. By the numbers: does circulating tumor cell enumeration have a role in metastatic breast cancer? *J Clin Oncol* 2014; **32**: 3479-3482 [PMID: [25245442](#) DOI: [10.1200/JCO.2014.56.6851](#)]
- 23 **Münz M**, Murr A, Kvesic M, Rau D, Mangold S, Pflanz S, Lumsden J, Volkland J, Fagerberg J, Riethmüller G, Rüttinger D, Kufer P, Baeuerle PA, Raum T. Side-by-side analysis of five clinically tested anti-EpCAM monoclonal antibodies. *Cancer Cell Int* 2010; **10**: 44 [PMID: [21044305](#) DOI: [10.1186/1475-2867-10-44](#)]
- 24 **Gorges TM**, Tinhofer I, Drosch M, Röse L, Zollner TM, Krahn T, von Ahnen O. Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC Cancer* 2012; **12**: 178 [PMID: [22591372](#) DOI: [10.1186/1471-2407-12-178](#)]
- 25 **Königsberg R**, Obermayr E, Bises G, Pfeiler G, Gneist M, Wrba F, de Santis M, Zeillinger R, Hudc M, Dittrich C. Detection of EpCAM positive and negative circulating tumor cells in metastatic breast cancer patients. *Acta Oncol* 2011; **50**: 700-710 [PMID: [21261508](#) DOI: [10.3109/0284186X.2010.549151](#)]
- 26 **Chen X**, Wen Q, Stucky A, Zeng Y, Gao S, Loudon WG, Ho HW, Kabeer MH, Li SC, Zhang X, Zhong JF. Relapse pathway of glioblastoma revealed by single-cell molecular analysis. *Carcinogenesis* 2018; **39**: 931-936 [PMID: [29718126](#) DOI: [10.1093/carcin/bgy052](#)]
- 27 **Zhang L**, He X, Liu X, Zhang F, Huang LF, Potter AS, Xu L, Zhou W, Zheng T, Luo Z, Berry KP, Pribnow A, Smith SM, Fuller C, Jones BV, Fouladi M, Drissi R, Yang ZJ, Gustafson WC, Remke M, Pomeroy SL, Girard EJ, Olson JM, Morrissy AS, Vladoiu MC, Zhang J, Tian W, Xin M, Taylor MD, Potter SS, Roussel MF, Weiss WA, Lu QR. Single-Cell Transcriptomics in Medulloblastoma Reveals Tumor-Initiating Progenitors and Oncogenic Cascades during Tumorigenesis and Relapse. *Cancer Cell* 2019; **36**: 302-318.e7 [PMID: [31474569](#) DOI: [10.1016/j.ccell.2019.07.009](#)]
- 28 **Bertrand KC**, Faria CC, Skowron P, Luck A, Garzia L, Wu X, Agnihotri S, Smith CA, Taylor MD, Mack SC, Rutka JT. A functional genomics approach to identify pathways of drug resistance in medulloblastoma. *Acta Neuropathol Commun* 2018; **6**: 146 [PMID: [30591080](#) DOI: [10.1186/s40478-018-0652-8](#)]
- 29 **Mikhael JR**, Dingli D, Roy V, Reeder CB, Buadi FK, Hayman SR, Dispenzieri A, Fonseca R, Sher T, Kyle RA, Lin Y, Russell SJ, Kumar S, Bergsagel PL, Zeldenrust SR, Leung N, Drake MT, Kapoor P, Ansell SM, Witzig TE, Lust JA, Dalton RJ, Gertz MA, Stewart AK, Rajkumar SV, Chanan-Khan A, Lacy MQ; Mayo Clinic. Management of newly diagnosed symptomatic multiple myeloma: updated Mayo Stratification of Myeloma and Risk-Adapted Therapy (mSMART) consensus guidelines 2013. *Mayo Clin Proc* 2013; **88**: 360-376 [PMID: [23541011](#) DOI: [10.1016/j.mayocp.2013.01.019](#)]
- 30 **Nathwani N**, Larsen JT, Kapoor P. Consolidation and Maintenance Therapies for Newly Diagnosed Multiple Myeloma in the Era of Novel Agents. *Curr Hematol Malig Rep* 2016; **11**: 127-136 [PMID: [26893062](#) DOI: [10.1007/s11899-016-0310-9](#)]
- 31 **Chen L**, Li J, Xu W, Qiu H, Zhu Y, Zhang Y, Duan L, Qian S, Lu H. Molecular cytogenetic aberrations in patients with multiple myeloma studied by interphase fluorescence in situ hybridization. *Exp Oncol* 2007; **29**: 116-120 [PMID: [17704743](#)]
- 32 **Kawano Y**, Fujiwara S, Wada N, Izaki M, Yuki H, Okuno Y, Iyama K, Yamasaki H, Sakai A, Mitsuya H, Hata H. Multiple myeloma cells expressing low levels of CD138 have an immature phenotype and reduced sensitivity to lenalidomide. *Int J Oncol* 2012; **41**: 876-884 [PMID: [22766978](#) DOI: [10.3892/ijo.2012.1545](#)]
- 33 **Landgren O**, Kyle RA, Pfeiffer RM, Katzmman JA, Caporaso NE, Hayes RB, Dispenzieri A, Kumar S, Clark RJ, Baris D, Hoover R, Rajkumar SV. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. *Blood* 2009; **113**: 5412-5417 [PMID: [19179464](#) DOI: [10.1182/blood-2008-12-194241](#)]
- 34 **Tamura H**, Ishibashi M, Sunakawa M, Inokuchi K. Immunotherapy for Multiple Myeloma. *Cancers (Basel)* 2019; **11**: 2009 [PMID: [31842518](#) DOI: [10.3390/cancers11122009](#)]
- 35 **Hosen N**. Chimeric Antigen Receptor T-Cell Therapy for Multiple Myeloma. *Cancers (Basel)* 2019; **11**: 2024 [PMID: [31847470](#) DOI: [10.3390/cancers11122024](#)]
- 36 **Hosen N**. Chimeric antigen receptor T-cell therapy for multiple myeloma. *Int J Hematol* 2020; **111**: 530-534 [PMID: [31981097](#) DOI: [10.1007/s12185-020-02827-8](#)]
- 37 **Li SC**, Han YP, Dethlefs BA, Loudon WG. Therapeutic window, a critical developmental stage for stem cell therapies. *Curr Stem Cell Res Ther* 2010; **5**: 297-293 [PMID: [20528752](#) DOI: [10.2174/157488810793351730](#)]
- 38 **Li SC**, Lee KL, Luo J. Control dominating subclones for managing cancer progression and posttreatment recurrence by subclonal switchboard signal: implication for new therapies. *Stem Cells Dev* 2012; **21**: 503-506 [PMID: [21933025](#) DOI: [10.1089/scd.2011.0267](#)]
- 39 **ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium**. Pan-cancer analysis of whole genomes. *Nature* 2020; **578**: 82-93 [PMID: [32025007](#) DOI: [10.1038/s41586-020-1969-6](#)]
- 40 **Gerstung M**, Jolly C, Leshchiner I, Dentre SC, Gonzalez S, Rosebrock D, Mitchell TJ, Rubanova Y, Anur P, Yu K, Tarabichi M, Deshwar A, Wintersinger J, Kleinheinz K, Vázquez-García I, Haase K, Jerman L, Sengupta S, Macintyre G, Malikic S, Donmez N, Livitz DG, Cmero M, Demeulemeester J, Schumacher S, Fan Y, Yao X, Lee J, Schlesner M, Boutros PC, Bowtell DD, Zhu H, Getz G, Imielinski M, Beroukhi R, Sahinalp SC, Ji Y, Peifer M, Markowitz F, Mustonen V, Yuan K, Wang W, Morris QD; PCAWG Evolution & Heterogeneity Working Group, Spellman PT, Wedge DC, Van Loo P; PCAWG Consortium. The evolutionary history of 2,658 cancers. *Nature* 2020; **578**: 122-128 [PMID: [32025013](#) DOI: [10.1038/s41586-019-1907-7](#)]
- 41 **Jiao W**, Atwal G, Polak P, Karlic R, Cuppen E; PCAWG Tumor Subtypes and Clinical Translation Working Group, Danyi A, de Ridder J, van Herpen C, Lolkema MP, Steeghs N, Getz G, Morris Q, Stein LD; PCAWG Consortium. A deep learning system accurately classifies primary and metastatic cancers using passenger mutation patterns. *Nat Commun* 2020; **11**: 728 [PMID: [32024849](#) DOI: [10.1038/s41467-019-13825-8](#)]
- 42 **Stuart T**, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM 3rd, Hao Y, Stoeckius M, Smibert

- P, Satija R. Comprehensive Integration of Single-Cell Data. *Cell* 2019; **177**: 1888-1902.e21 [PMID: 31178118 DOI: 10.1016/j.cell.2019.05.031]
- 43 **Cao J**, Packer JS, Ramani V, Cusanovich DA, Huynh C, Daza R, Qiu X, Lee C, Furlan SN, Steemers FJ, Adey A, Waterston RH, Trapnell C, Shendure J. Comprehensive single-cell transcriptional profiling of a multicellular organism. *Science* 2017; **357**: 661-667 [PMID: 28818938 DOI: 10.1126/science.aam8940]
  - 44 **Li Z**, Hao P, Wu Q, Li F, Zhao J, Wu K, Qu C, Chen Y, Li M, Chen X, Stucky A, Zhong J, Li L, Zhong JF. Genetic mutations associated with metastatic clear cell renal cell carcinoma. *Oncotarget* 2016; **7**: 16172-16179 [PMID: 26908440 DOI: 10.18632/oncotarget.7473]
  - 45 **Chen X**, Chakravarty T, Zhang Y, Li X, Zhong JF, Wang C. Single-cell transcriptome and epigenomic reprogramming of cardiomyocyte-derived cardiac progenitor cells. *Sci Data* 2016; **3**: 160079 [PMID: 27622691 DOI: 10.1038/sdata.2016.79]
  - 46 **Zhang Y**, Zhong JF, Qiu H, MacLellan WR, Marbán E, Wang C. Epigenomic Reprogramming of Adult Cardiomyocyte-Derived Cardiac Progenitor Cells. *Sci Rep* 2015; **5**: 17686 [PMID: 26657817 DOI: 10.1038/srep17686]
  - 47 **Nagrath S**, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, Tompkins RG, Haber DA, Toner M. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* 2007; **450**: 1235-1239 [PMID: 18097410 DOI: 10.1038/nature06385]
  - 48 **Stott SL**, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, Rothenberg SM, Shah AM, Smas ME, Korir GK, Floyd FP Jr, Gilman AJ, Lord JB, Winokur D, Springer S, Irimia D, Nagrath S, Sequist LV, Lee RJ, Isselbacher KJ, Maheswaran S, Haber DA, Toner M. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc Natl Acad Sci USA* 2010; **107**: 18392-18397 [PMID: 20930119 DOI: 10.1073/pnas.1012539107]
  - 49 **Zheng S**, Lin H, Liu JQ, Balic M, Datar R, Cote RJ, Tai YC. Membrane microfilter device for selective capture, electrolysis and genomic analysis of human circulating tumor cells. *J Chromatogr A* 2007; **1162**: 154-161 [PMID: 17561026 DOI: 10.1016/j.chroma.2007.05.064]
  - 50 **Simpson SJ**, Vachula M, Kennedy MJ, Kaizer H, Coon JS, Ghalie R, Williams S, Van Epps D. Detection of tumor cells in the bone marrow, peripheral blood, and apheresis products of breast cancer patients using flow cytometry. *Exp Hematol* 1995; **23**: 1062-1068 [PMID: 7544737]
  - 51 **He W**, Wang H, Hartmann LC, Cheng JX, Low PS. In vivo quantitation of rare circulating tumor cells by multiphoton intravital flow cytometry. *Proc Natl Acad Sci USA* 2007; **104**: 11760-11765 [PMID: 17601776 DOI: 10.1073/pnas.0703875104]
  - 52 **Pachmann K**, Clement JH, Schneider CP, Willen B, Camara O, Pachmann U, Höfken K. Standardized quantification of circulating peripheral tumor cells from lung and breast cancer. *Clin Chem Lab Med* 2005; **43**: 617-627 [PMID: 16006258 DOI: 10.1515/CCLM.2005.107]
  - 53 **Weight RM**, Dale PS, Viator JA. Detection of circulating melanoma cells in human blood using photoacoustic flowmetry. *Conf Proc IEEE Eng Med Biol Soc* 2009; **2009**: 106-109 [PMID: 19965119 DOI: 10.1109/IEMBS.2009.5335145]
  - 54 **Marrinucci D**, Bethel K, Bruce RH, Curry DN, Hsieh B, Humphrey M, Krivacic RT, Kroener J, Kroener L, Ladanyi A, Lazarus NH, Nieva J, Kuhn P. Case study of the morphologic variation of circulating tumor cells. *Hum Pathol* 2007; **38**: 514-519 [PMID: 17188328 DOI: 10.1016/j.humpath.2006.08.027]
  - 55 **Attard G**, Swennenhuis JF, Olmos D, Reid AH, Vickers E, A'Hern R, Levink R, Coumans F, Moreira J, Riisnaes R, Oommen NB, Hawche G, Jameson C, Thompson E, Sipkema R, Carden CP, Parker C, Dearnaley D, Kaye SB, Cooper CS, Molina A, Cox ME, Terstappen LW, de Bono JS. Characterization of ERG, AR and PTEN gene status in circulating tumor cells from patients with castration-resistant prostate cancer. *Cancer Res* 2009; **69**: 2912-2918 [PMID: 19339269 DOI: 10.1158/0008-5472.CAN-08-3667]
  - 56 **Yang L**, Lang JC, Balasubramanian P, Jatana KR, Schuller D, Agrawal A, Zborowski M, Chalmers JJ. Optimization of an enrichment process for circulating tumor cells from the blood of head and neck cancer patients through depletion of normal cells. *Biotechnol Bioeng* 2009; **102**: 521-534 [PMID: 18726961 DOI: 10.1002/bit.22066]
  - 57 **Alix-Panabières C**, Vendrell JP, Pellé O, Rebillard X, Riethdorf S, Müller V, Fabbro M, Pantel K. Detection and characterization of putative metastatic precursor cells in cancer patients. *Clin Chem* 2007; **53**: 537-539 [PMID: 17327507 DOI: 10.1373/clinchem.2006.079509]
  - 58 **Müller V**, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, Jänicke F, Pantel K. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. *Clin Cancer Res* 2005; **11**: 3678-3685 [PMID: 15897564 DOI: 10.1158/1078-0432.CCR-04-2469]
  - 59 **Gascoyne PR**, Noshari J, Anderson TJ, Becker FF. Isolation of rare cells from cell mixtures by dielectrophoresis. *Electrophoresis* 2009; **30**: 1388-1398 [PMID: 19306266 DOI: 10.1002/elps.200800373]
  - 60 **Lu J**, Fan T, Zhao Q, Zeng W, Zaslavsky E, Chen JJ, Frohman MA, Golightly MG, Madajewicz S, Chen WT. Isolation of circulating epithelial and tumor progenitor cells with an invasive phenotype from breast cancer patients. *Int J Cancer* 2010; **126**: 669-683 [PMID: 19662651 DOI: 10.1002/ijc.24814]
  - 61 **Leary RJ**, Kinde I, Diehl F, Schmidt K, Clouser C, Duncan C, Antipova A, Lee C, McKernan K, De La Vega FM, Kinzler KW, Vogelstein B, Diaz LA Jr, Velculescu VE. Development of personalized tumor biomarkers using massively parallel sequencing. *Sci Transl Med* 2010; **2**: 20ra14 [PMID: 20371490 DOI: 10.1126/scitranslmed.3000702]
  - 62 **Xi L**, Nicastri DG, El-Hefnawy T, Hughes SJ, Luketich JD, Godfrey TE. Optimal markers for real-time quantitative reverse transcription PCR detection of circulating tumor cells from melanoma, breast, colon, esophageal, head and neck, and lung cancers. *Clin Chem* 2007; **53**: 1206-1215 [PMID: 17525108 DOI: 10.1373/clinchem.2006.081828]
  - 63 **Wu CH**, Lin SR, Yu FJ, Wu DC, Pan YS, Hsieh JS, Huang SY, Wang JY. Development of a high-throughput membrane-array method for molecular diagnosis of circulating tumor cells in patients with gastric cancers. *Int J Cancer* 2006; **119**: 373-379 [PMID: 16477642 DOI: 10.1002/ijc.21856]
  - 64 **Chauhan D**, Velankar M, Brahmandam M, Hideshima T, Podar K, Richardson P, Schlossman R, Ghobrial I, Raje N, Munshi N, Anderson KC. A novel Bcl-2/Bcl-X(L)/Bcl-w inhibitor ABT-737 as therapy in multiple



- myeloma. *Oncogene* 2007; **26**: 2374-2380 [PMID: [17016430](#) DOI: [10.1038/sj.onc.1210028](#)]
- 65 **Chauhan D**, Li G, Auclair D, Hideshima T, Podar K, Mitsiades N, Mitsiades C, Chen LB, Munshi N, Saxena S, Anderson KC. 2-Methoxyestadiol and bortezomib/proteasome-inhibitor overcome dexamethasone-resistance in multiple myeloma cells by modulating Heat Shock Protein-27. *Apoptosis* 2004; **9**: 149-155 [PMID: [15004512](#) DOI: [10.1023/B:APPT.0000018797.66067.6c](#)]
- 66 **Raje N**, Hideshima T, Mukherjee S, Raab M, Vallet S, Chhetri S, Cirstea D, Pozzi S, Mitsiades C, Rooney M, Kiziltepe T, Podar K, Okawa Y, Ikeda H, Carrasco R, Richardson PG, Chauhan D, Munshi NC, Sharma S, Parikh H, Chabner B, Scadden D, Anderson KC. Preclinical activity of P276-00, a novel small-molecule cyclin-dependent kinase inhibitor in the therapy of multiple myeloma. *Leukemia* 2009; **23**: 961-970 [PMID: [19151776](#) DOI: [10.1038/leu.2008.378](#)]
- 67 **Chen L**, Ju SG, Wang ZY, Li J, Yuan YQ, Fu JX. [Sensitivity to bortezomib of RPM8226 cells after co-cultured with down-regulated Cav-1 expression HUVECs]. *Zhonghua Xue Ye Xue Za Zhi* 2013; **34**: 946-951 [PMID: [24294850](#) DOI: [10.3760/cma.j.issn.0253-2727.2013.11.008](#)]
- 68 **Krug B**, Kugel H, Harnischmacher U, Heindel W, Schmidt R, Krings F. MR pulsatility measurements in peripheral arteries: preliminary results. *Magn Reson Med* 1995; **34**: 698-705 [PMID: [8544690](#) DOI: [10.1002/mrm.1910340508](#)]
- 69 **Naschitz JE**, Bezobchuk S, Mussafia-Priselac R, Sundick S, Dreyfuss D, Khorshidi I, Karidis A, Manor H, Nagar M, Peck ER, Peck S, Storch S, Rosner I, Gaitini L. Pulse transit time by R-wave-gated infrared photoplethysmography: review of the literature and personal experience. *J Clin Monit Comput* 2004; **18**: 333-342 [PMID: [15957624](#) DOI: [10.1007/s10877-005-4300-z](#)]
- 70 **Oddie S**, McGuire W. Response to the Letter "RE: Commentary on 'Pulse Oximetry Screening for Critical Congenital Heart Defects'". *Neonatology* 2019; **116**: 392 [PMID: [31473740](#) DOI: [10.1159/000502014](#)]
- 71 **Kodituwakku S**, Lazar SW, Indic P, Chen Z, Brown EN, Barbieri R. Point process time-frequency analysis of dynamic respiratory patterns during meditation practice. *Med Biol Eng Comput* 2012; **50**: 261-275 [PMID: [22350435](#) DOI: [10.1007/s11517-012-0866-z](#)]
- 72 **Kodituwakku S**, Lazar SW, Indic P, Brown EN, Barbieri R. Point process time-frequency analysis of respiratory sinus arrhythmia under altered respiration dynamics. *Conf Proc IEEE Eng Med Biol Soc* 2010; **2010**: 1622-1625 [PMID: [21096135](#) DOI: [10.1109/IEMBS.2010.5626648](#)]
- 73 **Ye C**, Murano E, Stone M, Prince JL. A Bayesian approach to distinguishing interdigitated tongue muscles from limited diffusion magnetic resonance imaging. *Comput Med Imaging Graph* 2015; **45**: 63-74 [PMID: [26296155](#) DOI: [10.1016/j.compmedimag.2015.07.005](#)]
- 74 **Marmar CR**, Brown AD, Qian M, Laska E, Siegel C, Li M, Abu-Amara D, Tsiartas A, Richey C, Smith J, Knoth B, Vergyi D. Speech-based markers for posttraumatic stress disorder in US veterans. *Depress Anxiety* 2019; **36**: 607-616 [PMID: [31006959](#) DOI: [10.1002/da.22890](#)]
- 75 **Chen C**, Wen T, Liao W. Neurally adjusted ventilatory assist versus pressure support ventilation in patient-ventilator interaction and clinical outcomes: a meta-analysis of clinical trials. *Ann Transl Med* 2019; **7**: 382 [PMID: [31555696](#) DOI: [10.21037/atm.2019.07.60](#)]
- 76 **Swennenhuis JF**, van Dalum G, Zeune LL, Terstappen LW. Improving the CellSearch® system. *Expert Rev Mol Diagn* 2016; **16**: 1291-1305 [PMID: [27797592](#) DOI: [10.1080/14737159.2016.1255144](#)]



## Off-the-shelf mesenchymal stem cells from human umbilical cord tissue can significantly improve symptoms in COVID-19 patients: An analysis of evidential relations

Phuc Van Pham, Ngoc Bich Vu

**ORCID number:** Phuc Van Pham 0000-0001-7254-0717; Ngoc Bich Vu 0000-0003-4447-9212.

**Author contributions:** Pham PV and Vu NB equally contributed to this work.

**Conflict-of-interest statement:** The author(s) declare that they have no competing interests.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Unsolicited manuscript

**Received:** May 6, 2020

**Peer-review started:** May 6, 2020

**First decision:** May 15, 2020

**Revised:** May 21, 2020

**Phuc Van Pham, Ngoc Bich Vu,** Stem Cell Institute, University of Science, Ho Chi Minh 08000, Viet Nam

**Phuc Van Pham, Ngoc Bich Vu,** Vietnam National University, Ho Chi Minh 08000, Viet Nam

**Corresponding author:** Phuc Van Pham, PhD, Associate Professor, Director, Lecturer, Senior Scientist, Stem Cell Institute, University of Science, No. 227, Nguyen Van Cu, No. 5, District, Ho Chi Minh 08000, Viet Nam. [pvphuc@hcmuns.edu.vn](mailto:pvphuc@hcmuns.edu.vn)

### Abstract

Coronavirus disease-2019 (COVID-19) has affected more than 200 countries worldwide. This disease has hugely affected healthcare systems as well as the economy to an extent never seen before. To date, COVID-19 infection has led to about 165000 deaths in 150 countries. At present, there is no specific drug or efficient treatment for this disease. In this analysis based on evidential relationships of the biological characteristics of MSCs, especially umbilical cord (UC)-derived MSCs as well as the first clinical trial using MSCs for COVID-19 treatment, we discuss the use of UC-MSCs to improve the symptoms of COVID-19 in patients.

**Key words:** Coronavirus; COVID-19; Mesenchymal stem cells; Umbilical cord-derived mesenchymal stem cells

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Based on the biological characteristics of mesenchymal stem cells (MSCs), especially umbilical cord (UC)-derived MSCs, and the first clinical trial using MSCs for coronavirus disease-2019 (COVID-19) treatment, we discuss the use of UC-MSCs to improve COVID-19 symptoms. UC-MSCs are suitable therapeutic candidates for COVID-19. Indeed, they are the strongest immunomodulatory MSCs and exhibit low immunogenicity compared with bone marrow-derived MSCs, adipose tissue-derived MSCs, and other MSCs. UC-MSCs are easily collected and expanded *in vitro* with minimal ethical concerns. In summary, UC-MSCs can be used to improve symptoms in patients with severe COVID-19 by suppressing inflammation and stimulating wound

**Accepted:** August 1, 2020**Article in press:** August 1, 2020**Published online:** August 26, 2020**P-Reviewer:** Chen CM, Elhamid SA, Fatkhudinov TK, Tanabe S**S-Editor:** Zhang H**L-Editor:** Filipodia**P-Editor:** Xing YX

healing.

**Citation:** Pham PV, Vu NB. Off-the-shelf mesenchymal stem cells from human umbilical cord tissue can significantly improve symptoms in COVID-19 patients: An analysis of evidential relations. *World J Stem Cells* 2020; 12(8): 721-730

**URL:** <https://www.wjnet.com/1948-0210/full/v12/i8/721.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.721>

## INTRODUCTION

Coronaviruses express spike glycoprotein on their surfaces, which facilitates binding and entry into host cells *via* angiotensin I-converting enzyme 2 (ACE2) receptor expressed on host cells<sup>[1,2]</sup>. Although this receptor exists in almost all cells of the human body, it is highly expressed in alveolar type II (AT2) cells, the capillary endothelium, endothelial cells, and smooth muscle cells<sup>[3]</sup>. Therefore, all of these cells can be infected by a novel coronavirus (nCoV).

Similar to other viral infectious diseases, virus-infected cells are recognized and killed by the immune system of patients. With multiple affected organs, the host immune system is activated to kill the virus and virus-infected cells through activation of immune cells, such as natural killer (NK) cells, which produce a large number of inflammatory factors. This so-called severe cytokine storm entails the release of cytokines such as interleukin 2 (IL-2), IL-6, IL-7, granulocyte-colony stimulating factor (G-CSF), interferon-inducible protein of 10 kD, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-alpha (MIP-1A), and tumor necrosis factor alpha (TNF- $\alpha$ ). In addition to affecting the virus and virus-infected cells, these immune cells and their cytokines have an extreme effect on normal cells, causing edema, dysfunction of air exchange, acute respiratory distress syndrome (ARDS), and acute cardiac injury. As a result, there are multiple injuries in multiple organs and tissues. Moreover, these injured tissues and organs can be infected by other microorganisms. After entry of coronavirus into ACE2 receptor-expressing cells, multiple organs are injured in patients, which subsequently leads to death. Examinations of peripheral blood from coronavirus disease-2019 (COVID-19) patients have revealed increases of inflammatory factors such as IL-6, IL-10, and TNF- $\alpha$ <sup>[4]</sup>. In intensive care unit patients, the plasma levels of IL-2, IL-7, IL-10, G-CSF, IP-10, MCP-1, MIP-1A, and TNF- $\alpha$  also increase<sup>[5]</sup>.

It appears that the death of nCoV-infected patients is related to the injury and failure of multiple organs, which is triggered by the host immune system. The positive feedback regulation of the immune system not only allows attack of the virus and virus-infected cells but can also destroys healthy cells, leading to severe injury in some vital organs such as the lungs, heart, liver, and kidneys. In two reports about the clinical features of COVID-19 patients in Wuhan, China, COVID-19 was found to cause complications such as ARDS, arrhythmia, shock<sup>[6]</sup>, severe kidney injury, acute cardiac injury, liver dysfunction, and secondary infection<sup>[7]</sup>.

Current treatments for COVID-19 are mainly focused on symptomatic and respiratory support. Almost all patients receive oxygen therapy and rescue treatment with convalescent plasma, and in some critical cases, immunoglobulin G is applied. In addition to some anti-viral drugs, chloroquine has emerged as a repurposed drug with a great potential to treat COVID-19<sup>[8,9]</sup>. Indeed, chloroquine inhibits the replication of several viruses<sup>[10-12]</sup> and has immunomodulatory effects such as suppressing the production and release of TNF- $\alpha$  and IL-6<sup>[13]</sup>. Although the use of chloroquine/hydroxychloroquine may be promising, their use should be restricted to clinical trials. Indeed, to date, there have been 17 clinical trials regarding the use of chloroquine/hydroxychloroquine for COVID-19 worldwide, but none have been completed<sup>[14]</sup>. Thus, there is insufficient evidence to recommend them for routine treatment of COVID-19.

## HOW MESENCHYMAL STEM CELLS CAN BE USED IN COVID-19 TREATMENT

Mesenchymal stem cells (MSCs) are the most common stem cells in the human body. They exist in almost all tissues and organs, especially bone marrow and adipose tissue<sup>[15,16]</sup>. These cells have been isolated from umbilical cord tissue<sup>[17-19]</sup> and umbilical cord blood<sup>[20,21]</sup>. They are characterized by their shape when adhered onto the surface of a tissue culture vessel and the expression profile of markers (positive for cluster of differentiation 44 [CD44], CD73, CD90, and CD105, while negative for CD14, CD34, CD45, and human leukocyte antigen DR [HLA-DR]). They are also unique in their potential to differentiate into mesodermal cells including adipocytes, osteoblasts, and chondrocytes<sup>[22,23]</sup>.

MSCs have been used clinically to treat more than 10 different diseases<sup>[24,25]</sup>. Importantly, MSC transplantations have been approved in some countries to treat various inflammatory diseases<sup>[26,27]</sup>. In Canada, since 2012, bone marrow-derived MSCs have been approved for graft-versus-host disease (GVHD) treatment (Prochymal)<sup>[28,29]</sup>. Also in that year, umbilical cord blood-derived MSCs (UC-MSCs) were used in South Korea to treat knee osteoarthritis (Cartistem)<sup>[30]</sup>. Recently, the Japanese government has approved HS Temcell, which contains allogeneic MSCs from bone marrow for GVHD treatment<sup>[31,32]</sup>. In Europe, adipose-derived MSCs have been approved for Crohn's disease<sup>[33]</sup>. Indeed, there have been many products related to clinical MSC transplantation, which have been used or are being investigated in clinical trials in several countries<sup>[34]</sup>.

MSC transplantation for pulmonary diseases has also been carried out with positive outcomes<sup>[35,36]</sup>. In our recent publication, we showed that transplantation of UC-MSCs significantly improved symptoms of chronic obstructive pulmonary disease (COPD) in late-stage patients<sup>[37]</sup>.

Lastly, a clinical trial by Leng *et al*<sup>[38]</sup> (2020) showed that transplantation of MSCs was a safe and effective treatment for patients with COVID-19, especially critically severe patients. Seven patients in the study were cured or showed significant improvement of pulmonary functions with symptoms of COVID-19, but without observed adverse effects. Interestingly, C-reactive protein (CRP) and TNF- $\alpha$  were decreased, and cytokine-secreting immune cells, such as CXCR3<sup>+</sup>CD4<sup>+</sup> T cells, CXCR3<sup>+</sup>CD8<sup>+</sup> T cells, and CXCR3<sup>+</sup> NK cells, disappeared after 3-6 d of treatment<sup>[38]</sup>.

## POSSIBLE MECHANISMS OF MSCS IN COVID-19 TREATMENT

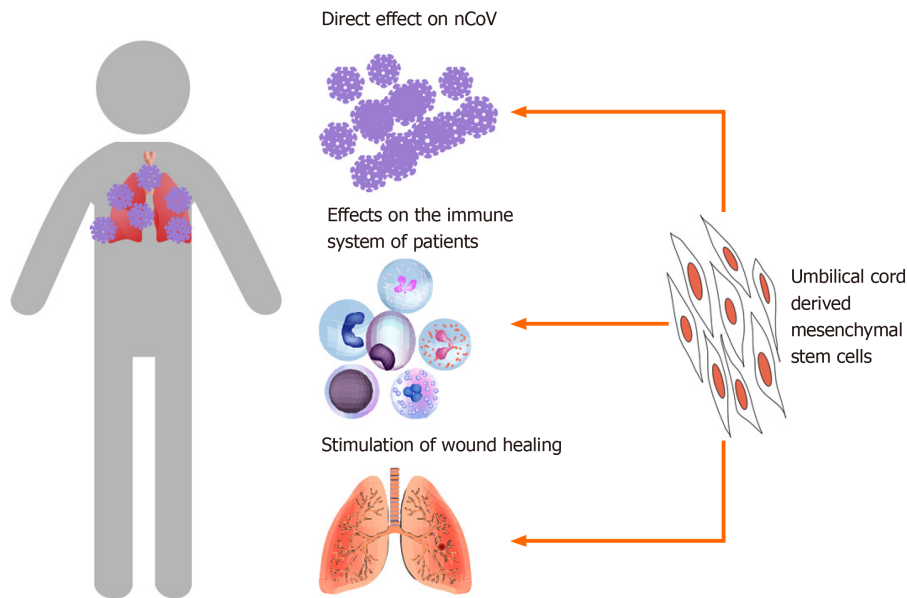
### **Direct effect on nCoV**

Although the role of MSCs in killing nCoV has not been reported, some published studies have shown that MSCs attack bacteria and viruses<sup>[39-44]</sup>. In 2009, Gonzalez *et al*<sup>[39]</sup> reported that MSC transplantation reduces the number of bacterial colony-forming units in the blood, liver, spleen, and peritoneal fluid of septic mice. The mechanism of the anti-bacterial activity of MSCs remains unclear. Some reports have suggested that MSCs enhance phagocytosis of monocytes<sup>[40,41]</sup>. An increase of C5a complement was found by Krasnodembskaya *et al*<sup>[41]</sup> (2012). The anti-bacterial activity is also enhanced by triggering the phagocytic index of macrophages by transferring MSC-derived mitochondria to macrophages<sup>[42,43]</sup>.

The patent WO2010053350A1 from Erasmus University Medical Center in Rotterdam, Netherlands, describes methods to produce concentrated MSC-conditioned medium (C-MSC-CM) that retains potent anti-viral activity. C-MSC-CM inhibits hepatitis C virus (HCV) and hepatitis B virus replication. Recent studies have suggested that MSCs also display anti-viral activities. For example, the study by Qian *et al*<sup>[44]</sup> (2016) was the first to demonstrate that MSCs inhibit HCV infection *via* their exosomes. The authors showed that exosomes from MSCs contained functional miRNAs, mainly let-7f, miR-145, miR-199a, and miR-221. These microRNAs possess binding sites for HCV RNA.

### **Effects on the immune system of patients**

The primary mechanism by which MSCs improve the symptoms of COVID-19 is related to their immunomodulation potential (Figure 1). MSCs efficiently regulate inflammation by suppression of effector immune cells, upregulation of regulatory T cells, and inhibition of T-cell activation<sup>[45-47]</sup>. Therefore, MSCs have great potential to treat immune diseases, especially acute and chronic inflammation<sup>[48-51]</sup>.



**Figure 1 Possible mechanisms of mesenchymal stem cells in coronavirus disease-2019 treatment.** Mesenchymal stem cells directly affect novel coronavirus, affect the immune system of patients, and stimulate wound healing.

In the report by Leng *et al*<sup>[38]</sup> (2020), the levels of CRP and TNF- $\alpha$  were both reduced in treated COVID-19 patients. These observations are similar to those previously published on MSC transplantation for immunological diseases<sup>[52,53]</sup>. MSCs also inhibit the maturation of T cells and prevent them from becoming too active<sup>[54,55]</sup>. This may be why some cytokine-secreting immune cells decrease significantly in treated COVID-19 patients.

Indeed, under *in vitro* conditions, MSCs have been observed to inhibit the production of TNF- $\alpha$  by immune cells. Zheng *et al*<sup>[56]</sup> (2008) reported that allogenic MSCs inhibit both CD4<sup>+</sup> and CD8<sup>+</sup> T cells from producing interferon gamma (IFN- $\gamma$ ) and TNF- $\alpha$ . Another study showed that MSCs inhibit macrophages from producing proinflammatory cytokines such as IFN- $\gamma$ , IL-6, and TNF- $\alpha$ <sup>[57]</sup>.

Similar to sepsis and septic shock, patients infected with COVID-19 experience a severe cytokine storm that is harmful and ultimately leads to death. Preclinical trials in rodents showed that MSC transplantation improves sepsis and septic shock<sup>[58-61]</sup>. In a meta-analysis, Lalu *et al*<sup>[62]</sup> (2016) analyzed 20 controlled comparison experiments (980 animals from 18 publications) of *in vivo* sepsis models and found that MSC transplantation significantly reduced mortality under a range of experimental conditions. Some current clinical trials have reported that MSC transplantation is safe in severe sepsis patients<sup>[63]</sup>.

### Stimulation of wound healing

Several tissues and organs affected by activated immune cells and severe cytokine storms can be injured. MSCs contribute to stimulating the wound healing process. In preclinical and clinical trials, MSC administration facilitated healing a range of injuries. Some publications have shown that MSC transplantation triggers lung injury healing. In models of radiation-induced lung injury in rats and mice, MSC transplantation significantly reduced pulmonary radiation fibrosis<sup>[64]</sup>, reduced serum levels of IL-1, IL-6, and TNF- $\alpha$ <sup>[65]</sup>, and inhibited fibrosis<sup>[66]</sup>.

The roles of MSCs in wound healing appear to be related to the growth factors that they produce and secrete into the medium. These factors include epidermal growth factor, fibroblast growth factor, and insulin-like growth factor. All of these growth factors strongly stimulate wound healing. Moreover, MSCs promote wound healing *via* their exosomes (extracellular vesicles) that are secreted and targeted to injured tissues. These growth factors and exosomes produced by MSCs enable MSCs to exert their anti-apoptosis effects, rescuing apoptotic cells from traumatic exposure to hypoxia, chemicals/acidity, and mechanical damage or radiation<sup>[67-69]</sup>.

Although MSCs provide benefits for COVID-19 patients *via* these possible mechanisms, the treatment efficacy of COVID-19 by MSC transplantation is variable among patients because of the complexity of COVID-19. The stage of COVID-19 may



be the main issue affecting treatment efficacy. MSCs appear to be effective to control inflammation and the cytokine storm by immunomodulation. This effect also reduces the immune response to virus-infected cells and bacteria. Therefore, the risk of bacterial infection can be increased in MSC-transplanted COVID-19 patients. The rejection response of recipients *versus* transplanted allogenic MSCs should also be considered and monitored. Theoretically, MSCs do not express HLA. Hence, they can be used in allogenic transplantation. However, expression of HLA can be upregulated after MSCs are transplanted into patients and differentiate into specialized cells. Indeed, chronic rejection of transplanted allogenic MSCs decreases the treatment efficacy and causes slight fever for a long time, even after successful treatment of COVID-19.

The treatment efficacy also depends on several factors included the MSC type, quality of MSCs, and dose of MSCs. MSCs can be obtained from various tissues for clinical treatment, but MSCs from different sources usually exhibit different biological characteristics, especially immunomodulation, as well as angiogenic characteristics. These characteristics play important roles in COVID-19 treatment. The therapeutic characteristics can also be affected by the biological status, especially the senescent status, tissue donation, and culture conditions for *in vitro* expansion. Indeed, some studies have shown that immunomodulation of MSCs is reduced significantly in the senescent phase and in MSCs derived from older individuals. *In vitro* expansion conditions significantly affect MSC immunomodulation. Chen *et al*<sup>[70]</sup> (2017) showed that MSCs expanded in a 3D-stirred tank bioreactor/microcarrier culture system display better immunomodulation than those expanded in flasks. Some recent studies have shown MSCs expanded in a platelet lysate have a superior capacity to suppress the proliferation of allogeneic lymphocytes than those expanded in fetal bovine serum<sup>[71,72]</sup>. Kabat *et al*<sup>[73]</sup> (2020) analyzed 914 MSC trials reported through 2018, concerning the MSC dose for transplantation. They found that the minimal effective dose (MED) of MSCs ranged from 70 to 190 million MSCs/patient/dose. Lower or higher intravenous doses of MSC transplantation were less effective. Indeed, the dose of MSCs directly affected the immunomodulation response in recipients. At a lower dose of MSCs than the MED, the immunomodulation response was insufficient to induce therapeutic effects in patients. However, at higher doses, MSCs caused too much immunomodulation that increased side effects. Therefore, determining the optimal dose of MSCs for transplantation is important for effective treatment with the lowest side effects.

## OFF-THE-SHELF MSCs FROM UMBILICAL CORD TISSUE ARE A SUITABLE SOURCE FOR COVID-19 TREATMENT

Although MSCs can be obtained from several tissue sources, it appears that UC-MSCs are optimal to treat COVID-19. The first reason is that transplantation of UC-MSCs is safe and effective in humans for sepsis and chronic inflammation. He *et al*<sup>[63]</sup> (2018) reported no serious adverse events associated with infusion of UC-MSCs in 15 patients. At a high dose of UC-MSCs ( $3 \times 10^6$  cells/kg), the allogenic UC-MSCs were also found to be safe and well tolerated in the 15 patients with severe sepsis. Additionally, in chronic inflammation such as COPD, we confirmed that infusion of allogenic UC-MSCs is safe<sup>[37]</sup>.

UC-MSCs have great potential and suitability for COVID-19 treatment because of their useful properties. A publication in 2019 demonstrated that MSCs from Wharton's Jelly (a part of the UC) improve bacterial clearance and survival in sepsis mouse models, whereas bone marrow-derived MSCs did not have these effects<sup>[74]</sup>. Several studies have suggested that UC-MSCs perform their immunomodulation better than bone marrow-derived MSCs and adipose-derived MSCs<sup>[75,76]</sup>. This therapeutic effect is obtained by the activatable status of UC-MSCs. Selich *et al*<sup>[77]</sup> (2019) demonstrated that UC-MSCs are activatable for immunomodulation, whereas bone marrow-derived MSCs<sup>[78,79]</sup> and adipose-tissue derived MSCs<sup>[80]</sup> are activated by TNF- $\alpha$ <sup>[80]</sup>, IFN- $\gamma$ <sup>[79]</sup>, and lymphocyte extracts<sup>[78]</sup> to trigger immunomodulation. UC-MSCs also express higher levels of immunomodulatory surface proteins, such as CD200, CD273, and CD274, and cytokines such as IL-1 $\beta$ , IL-8, leukemia inhibitory factor, and TGF- $\beta$ 2 compared with bone marrow-derived MSCs<sup>[81]</sup>, which makes them the strongest immunomodulatory MSCs.

UC-MSCs are suitable sources of allogenic MSCs with low immunogenicity and high yield manufacturing. In 2018, Kim *et al*<sup>[76]</sup> compared the immunological characteristics of MSCs derived from the periodontal ligament, umbilical cord, and

adipose tissue. They found that UC-MSCs expressed minimal levels of HLA-DR and HLA-ABC after activation by IFN- $\gamma$ <sup>[76]</sup> compared with adipose tissue- and periodontal ligament-derived MSCs. Li *et al*<sup>[82]</sup> (2018) also obtained similar findings in which UC-MSCs displayed the strongest immunomodulatory ability compared with MSCs from exfoliated deciduous teeth, bone marrow, and gingival tissues. After treatment with IFN- $\gamma$ , UC-MSCs expressed HLA-DR at a low level compared with those from other sources, whereas bone marrow-derived MSCs expressed HLA-DR at the highest level compared with those from other sources<sup>[82]</sup>. High expression of HLA-DR or HLA-ABC is a major obstacle for allogeneic transplantation.

Moreover, UC-MSCs are easily collected and expanded *in vitro* with minimal ethical concerns. UC-MSCs also grow faster than adipose tissue- and periodontal ligament-derived MSCs<sup>[76]</sup>. UC-MSCs can be obtained easily from the umbilical cord, according to GMP-compliant conditions for clinical usage<sup>[19,37]</sup> with established high yield manufacturing<sup>[83]</sup> (Figure 2). These characteristics suggest that UC-MSCs are suitable MSC therapeutic candidates for COVID-19.

## OFF-THE-SHELF UC-MSCS FOR COVID-19 TREATMENT

Clinically, patients with COVID-19 have two phases of immunoresponses after nCoV infection. The first phase is the incubation and non-severe stages in which the immunoresponse of patients is triggered to eliminate the virus. The second phase occurs when the immune system fails to eliminate the severe acute respiratory syndrome coronavirus 2, which will cause the severe stage because of severely damaged lungs. These differences in the immunoresponse suggest that different approaches are needed to treat COVID-19 at incubation and severe stages.

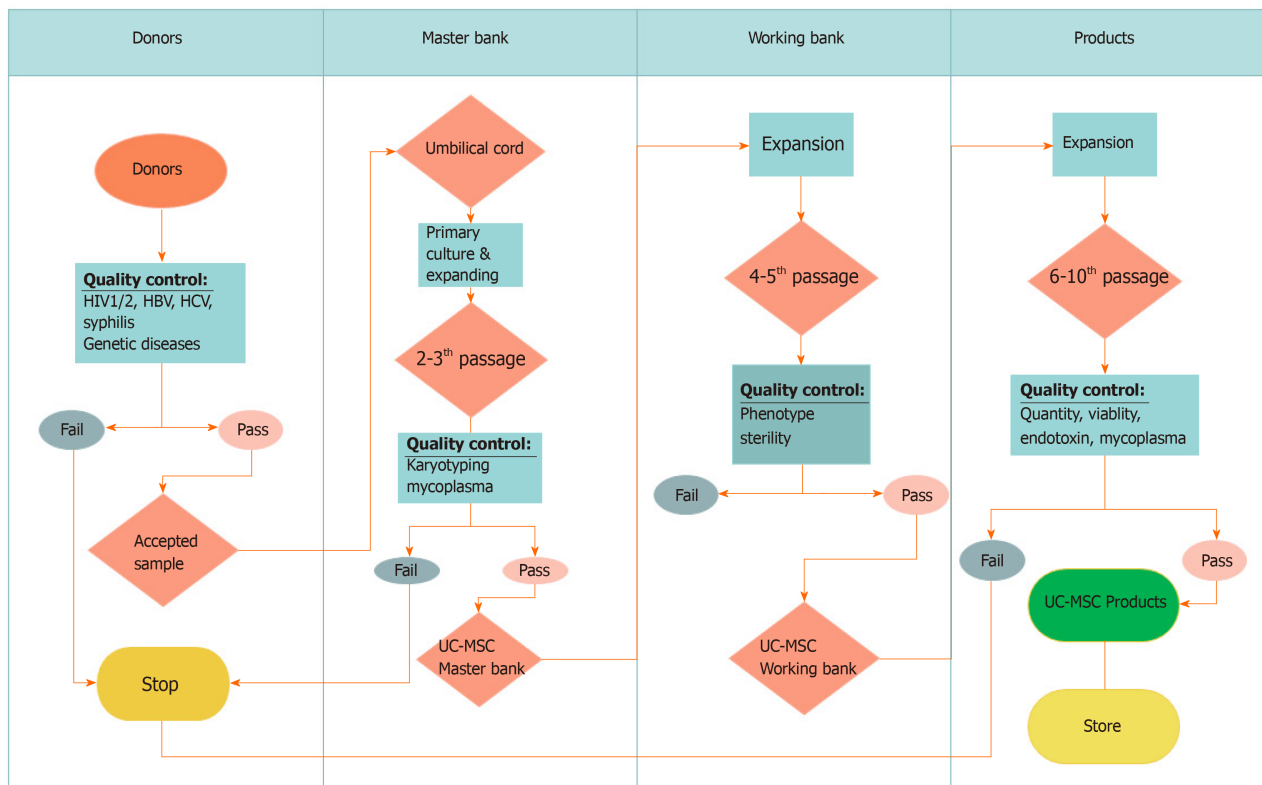
In the first stage, some strategies related to boosting the patient's immune system may provide good outcomes, whereas lung inflammation control is important for severe-stage patients. Xu *et al*<sup>[84]</sup> (2020) reported that lung inflammation is the main cause of life-threatening respiratory disorders. UC-MSC transplantation that suppresses inflammation and manages symptoms appears to be effective to treat severe-stage COVID-19 patients.

COVID-19 patients should be confirmed by a real-time reverse transcription polymerase chain reaction assay and classified as the severe stage of COVID-19 that released by National Health Commission of China included respiratory distress, RR  $\geq$  30/min; oxygen saturation  $\leq$  93% at rest; arterial partial pressure of oxygen/fraction of inspiration  $O_2 \leq$  300 mmHg, 1 mmHg = 0.133 kPa<sup>[88]</sup>.

Based on the above analyses, severe-stage COVID-19 patients would be transfused intravenously with 1 million thawed off-the-shelf UC-MSCs per kilogram of body weight. Inflammatory markers, including cytokines (CRP and TNF- $\alpha$ ) and cytokine-secreting immune cells (CXCR3<sup>+</sup>CD4<sup>+</sup> T cells, CXCR3<sup>+</sup>CD8<sup>+</sup> T cells, and CXCR3<sup>+</sup> NK cells) should be monitored everyday post-stem cell transplantation. The UC-MSC transplantation should be repeated with the same dose of off-the-shelf UC-MSCs after 2 wk. Decreases of inflammatory cytokine concentrations in peripheral blood are a good indicator of reduced inflammation in combination with other improved symptoms. Other therapies should be maintained during UC-MSC transplantation until COVID-19 symptoms clearly improve.

## CONCLUSION

COVID-19 has spread rapidly across the globe. The World Health Organization officially declared COVID-19 as a public health emergency of international concern. In addition to anti-viral drugs and oxygen therapy, MSC transplantation is a therapeutic option for the treatment of COVID-19. Based on the related evidence of COVID-19, the biology of UC-MSCs, and the initial results of a clinical trial using MSCs for COVID-19 treatment, we believe that off-the-shelf UC-MSC transplantation may be an additional therapy to improve treatment efficacy, especially in critically ill COVID-19 patients.



**Figure 2 Workflow of umbilical cord-derived mesenchymal stem cell manufacturing.** Donors are screened for some viruses and genetic disorders. Then, accepted umbilical cords are used to isolate umbilical cord-derived mesenchymal stem cells (UC-MSCs) for harvest at passage 2–3 to produce the master cell bank. A working bank of UC-MSCs is produced from the master bank before expanding UC-MSCs to obtain enough cells for transplantation. HIV: Human immunodeficiency virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

## REFERENCES

- Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol* 2019; **17**: 181-192 [PMID: 30531947 DOI: 10.1038/s41579-018-0118-9]
- Tortorici MA, Veesler D. Structural insights into coronavirus entry. *Adv Virus Res* 2019; **105**: 93-116 [PMID: 31522710 DOI: 10.1016/bs.aivir.2019.08.002]
- Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol* 2004; **203**: 631-637 [PMID: 15141377 DOI: 10.1002/path.1570]
- Gao G, Chen L, Huang C. Anti-cancer drug discovery: update and comparisons in yeast, Drosophila, and zebrafish. *Curr Mol Pharmacol* 2014; **7**: 44-51 [PMID: 24993385 DOI: 10.2174/1874467207666140702113629]
- Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]
- Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 2020 [PMID: 32031570 DOI: 10.1001/jama.2020.1585]
- Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* 2020; **395**: 1054-1062 [PMID: 32171076 DOI: 10.1016/S0140-6736(20)30566-3]
- Gao J, Tian Z, Yang X. Breakthrough: Chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. *Biosci Trends* 2020; **14**: 72-73 [PMID: 32074550 DOI: 10.5582/bst.2020.01047]
- Colson P, Rolain JM, Lagier JC, Brouqui P, Raoult D. Chloroquine and hydroxychloroquine as available weapons to fight COVID-19. *Int J Antimicrob Agents* 2020; **55**: 105932 [PMID: 32145363 DOI: 10.1016/j.ijantimicag.2020.105932]
- Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: an old drug against today's diseases? *Lancet Infect Dis* 2003; **3**: 722-7 [PMID: 14592603 DOI: 10.1016/S1473-3099(03)00806-5]
- Cortegiani A, Ingoglia G, Ippolito M, Giarratano A, Einav S. A systematic review on the efficacy and safety of chloroquine for the treatment of COVID-19. *J Crit Care* 2020; **57**: 279-283 [PMID: 32173110 DOI: 10.1016/j.jcrc.2020.03.005]
- Devaux CA, Rolain JM, Colson P, Raoult D. New insights on the antiviral effects of chloroquine against

- coronavirus: what to expect for COVID-19? *Int J Antimicrob Agents* 2020; **55**: 105938 [PMID: [32171740](#) DOI: [10.1016/j.ijantimicag.2020.105938](#)]
- 13 **Jang CH**, Choi JH, Byun MS, Jue DM. Chloroquine inhibits production of TNF-alpha, IL-1beta and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. *Rheumatology (Oxford)* 2006; **45**: 703-710 [PMID: [16418198](#) DOI: [10.1093/rheumatology/kei282](#)]
- 14 **Palmeira VA**, Costa LB, Perez LG, Ribeiro VT, Lanza K, Silva ACSE. Do we have enough evidence to use chloroquine/hydroxychloroquine as a public health panacea for COVID-19? *Clinics (Sao Paulo)* 2020; **75**: e1928 [PMID: [32401962](#) DOI: [10.6061/clinics/2020/e1928](#)]
- 15 **Keating A**. Mesenchymal stromal cells: new directions. *Cell Stem Cell* 2012; **10**: 709-716 [PMID: [22704511](#) DOI: [10.1016/j.stem.2012.05.015](#)]
- 16 **Stewart MC**, Stewart AA. Mesenchymal stem cells: characteristics, sources, and mechanisms of action. *Vet Clin North Am Equine Pract* 2011; **27**: 243-261 [PMID: [21872757](#) DOI: [10.1016/j.cveq.2011.06.004](#)]
- 17 **Han YF**, Tao R, Sun TJ, Chai JK, Xu G, Liu J. Optimization of human umbilical cord mesenchymal stem cell isolation and culture methods. *Cytotechnology* 2013; **65**: 819-827 [PMID: [23306781](#) DOI: [10.1007/s10616-012-9528-0](#)]
- 18 **Li T**, Xia M, Gao Y, Chen Y, Xu Y. Human umbilical cord mesenchymal stem cells: an overview of their potential in cell-based therapy. *Expert Opin Biol Ther* 2015; **15**: 1293-1306 [PMID: [26067213](#) DOI: [10.1517/14712598.2015.1051528](#)]
- 19 **Pham PV**, Truong NC, Le PT, Tran TD, Vu NB, Bui KH, Phan NK. Isolation and proliferation of umbilical cord tissue derived mesenchymal stem cells for clinical applications. *Cell Tissue Bank* 2016; **17**: 289-302 [PMID: [26679929](#) DOI: [10.1007/s10561-015-9541-6](#)]
- 20 **Pham PV**, Vu NB, Pham VM, Truong NH, Pham TL, Dang LT, Nguyen TT, Bui AN, Phan NK. Good manufacturing practice-compliant isolation and culture of human umbilical cord blood-derived mesenchymal stem cells. *J Transl Med* 2014; **12**: 56 [PMID: [24565047](#) DOI: [10.1186/1479-5876-12-56](#)]
- 21 **Fujii S**, Miura Y, Iwasa M, Yoshioka S, Fujishiro A, Sugino N, Kaneko H, Nakagawa Y, Hirai H, Takaori-Kondo A, Ichinohe T, Maekawa T. Isolation of mesenchymal stromal/stem cells from cryopreserved umbilical cord blood cells. *J Clin Exp Hematop* 2017; **57**: 1-8 [PMID: [28420812](#) DOI: [10.3960/jslrt.16019](#)]
- 22 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: [16923606](#) DOI: [10.1080/14653240600855905](#)]
- 23 **Horwitz EM**, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A; International Society for Cellular Therapy. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 2005; **7**: 393-395 [PMID: [16236628](#) DOI: [10.1080/14653240500319234](#)]
- 24 **Squillaro T**, Peluso G, Galderisi U. Clinical Trials With Mesenchymal Stem Cells: An Update. *Cell Transplant* 2016; **25**: 829-848 [PMID: [26423725](#) DOI: [10.3727/096368915X689622](#)]
- 25 **Galipeau J**, Sensébé L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell* 2018; **22**: 824-833 [PMID: [29859173](#) DOI: [10.1016/j.stem.2018.05.004](#)]
- 26 **Pham PV**. Stem cell drugs: The next generation of pharmaceutical products. *Biomed Res Ther* 2016; **3**: 857-871 [DOI: [10.15419/bmrat.v3i10.128](#)]
- 27 **Vu NB**, Le PT-B, Truong NC, Pham PV. Off-the-shelf mesenchymal stem cell technology. Stem cell drugs-a new generation of biopharmaceuticals. *Springer* 2018; 119-141
- 28 **Reicin C**, McMahon E, Chung C. Stem cell therapy regulation in canada: Implications of the prochymal approval. *Westlaw J* 2012; **28**: 1-4
- 29 **Rattue P**. Prochymal-first stem cell drug approved. *Medical News Today* 2012; 22
- 30 **Song JS**, Hong KT, Kim NM, Jung JY, Park HS, Lee SH, Cho YJ, Kim SJ. Implantation of allogenic umbilical cord blood-derived mesenchymal stem cells improves knee osteoarthritis outcomes: Two-year follow-up. *Regen Ther* 2020; **14**: 32-39 [PMID: [31988992](#) DOI: [10.1016/j.reth.2019.10.003](#)]
- 31 **Konishi A**, Sakushima K, Isobe S, Sato D. First Approval of Regenerative Medical Products under the PMD Act in Japan. *Cell Stem Cell* 2016; **18**: 434-435 [PMID: [27058934](#) DOI: [10.1016/j.stem.2016.03.011](#)]
- 32 **Najima Y**, Ohashi K. Mesenchymal stem cells: The first approved stem cell drug in japan. *Journal of Hematopoietic Cell Transplantation* 2017; **6**: 125-132 [DOI: [10.7889/hct-16-031](#)]
- 33 **Sheridan C**. First off-the-shelf mesenchymal stem cell therapy nears European approval. *Nat Biotechnol* 2018; **36**: 212-214 [PMID: [29509727](#) DOI: [10.1038/nbt0318-212a](#)]
- 34 **Pham PV**, Nguyen HT, Vu NB. Evolution of stem cell products in medicine: Future of off-the-shelf products. Stem cell drugs-a new generation of biopharmaceuticals. *Stem Cell Drugs-A New Generation of Biopharmaceuticals* 2018; 93-118 [DOI: [10.1007/978-3-319-99328-7\\_6](#)]
- 35 **Harrell CR**, Sadikot R, Pascual J, Fellabaum C, Jankovic MG, Jovicic N, Djonov V, Arsenijevic N, Volarevic V. Mesenchymal Stem Cell-Based Therapy of Inflammatory Lung Diseases: Current Understanding and Future Perspectives. *Stem Cells Int* 2019; **2019**: 4236973 [PMID: [31191672](#) DOI: [10.1155/2019/4236973](#)]
- 36 **Cruz FF**, Rocco PRM. The potential of mesenchymal stem cell therapy for chronic lung disease. *Expert Rev Respir Med* 2020; **14**: 31-39 [PMID: [31608724](#) DOI: [10.1080/17476348.2020.1679628](#)]
- 37 **Le Thi Bich P**, Nguyen Thi H, Dang Ngo Chau H, Phan Van T, Do Q, Dong Khac H, Le Van D, Nguyen Huy L, Mai Cong K, Ta Ba T, Do Minh T, Vu Bich N, Truong Chau N, Van Pham P. Allogeneic umbilical cord-derived mesenchymal stem cell transplantation for treating chronic obstructive pulmonary disease: a pilot clinical study. *Stem Cell Res Ther* 2020; **11**: 60 [PMID: [32054512](#) DOI: [10.1186/s13287-020-1583-4](#)]
- 38 **Leng Z**, Zhu R, Hou W, Feng Y, Yang Y, Han Q, Shan G, Meng F, Du D, Wang S, Fan J, Wang W, Deng L, Shi H, Li H, Hu Z, Zhang F, Gao J, Liu H, Li X, Zhao Y, Yin K, He X, Gao Z, Yang B, Jin R, Stambler I, Lim LW, Su H, Moskalev A, Cano A, Chakrabarti S, Min KJ, Ellison-Hughes G, Caruso C, Jin K, Zhao RC. Transplantation of ACE2<sup>+</sup> Mesenchymal Stem Cells Improves the Outcome of Patients with COVID-19 Pneumonia. *Aging Dis* 2020; **11**: 216-228 [PMID: [32257537](#) DOI: [10.14336/AD.2020.0228](#)]
- 39 **Gonzalez-Rey E**, Anderson P, González MA, Rico L, Büscher D, Delgado M. Human adult stem cells



- derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009; **58**: 929-939 [PMID: 19136511 DOI: 10.1136/gut.2008.168534]
- 40 **Mei SH**, Haitisma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, Stewart DJ. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010; **182**: 1047-1057 [PMID: 20558630 DOI: 10.1164/rccm.201001-0010OC]
  - 41 **Krasnodembskaya A**, Samarani G, Song Y, Zhuo H, Su X, Lee JW, Gupta N, Petrini M, Matthay MA. Human mesenchymal stem cells reduce mortality and bacteremia in gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes. *Am J Physiol Lung Cell Mol Physiol* 2012; **302**: L1003-L1013 [PMID: 22427530 DOI: 10.1152/ajplung.00180.2011]
  - 42 **Jackson MV**, Morrison TJ, Doherty DF, McAuley DF, Matthay MA, Kissenpfennig A, O'Kane CM, Krasnodembskaya AD. Mitochondrial Transfer via Tunneling Nanotubes is an Important Mechanism by Which Mesenchymal Stem Cells Enhance Macrophage Phagocytosis in the In Vitro and In Vivo Models of ARDS. *Stem Cells* 2016; **34**: 2210-2223 [PMID: 27059413 DOI: 10.1002/stem.2372]
  - 43 **Morrison TJ**, Jackson MV, Cunningham EK, Kissenpfennig A, McAuley DF, O'Kane CM, Krasnodembskaya AD. Mesenchymal Stromal Cells Modulate Macrophages in Clinically Relevant Lung Injury Models by Extracellular Vesicle Mitochondrial Transfer. *Am J Respir Crit Care Med* 2017; **196**: 1275-1286 [PMID: 28598224 DOI: 10.1164/rccm.201701-0170OC]
  - 44 **Qian X**, Xu C, Fang S, Zhao P, Wang Y, Liu H, Yuan W, Qi Z. Exosomal MicroRNAs Derived From Umbilical Mesenchymal Stem Cells Inhibit Hepatitis C Virus Infection. *Stem Cells Transl Med* 2016; **5**: 1190-1203 [PMID: 27496568 DOI: 10.5966/sctm.2015-0348]
  - 45 **Jiang W**, Xu J. Immune modulation by mesenchymal stem cells. *Cell Prolif* 2020; **53**: e12712 [PMID: 31730279 DOI: 10.1111/cpr.12712]
  - 46 **Yarygin KN**, Lupatov AY, Sukhikh GT. Modulation of Immune Responses by Mesenchymal Stromal Cells. *Bull Exp Biol Med* 2016; **161**: 561-565 [PMID: 27590761 DOI: 10.1007/s10517-016-3461-8]
  - 47 **Abumaree MH**, Abomaray FM, Alshabibi MA, AlAskar AS, Kalionis B. Immunomodulatory properties of human placental mesenchymal stem/stromal cells. *Placenta* 2017; **59**: 87-95 [PMID: 28411943 DOI: 10.1016/j.placenta.2017.04.003]
  - 48 **Ansboro S**, Roelofs AJ, De Bari C. Mesenchymal stem cells for the management of rheumatoid arthritis: immune modulation, repair or both? *Curr Opin Rheumatol* 2017; **29**: 201-207 [PMID: 27941390 DOI: 10.1097/BOR.0000000000000370]
  - 49 **Shi Y**, Wang Y, Li Q, Liu K, Hou J, Shao C, Wang Y. Immunoregulatory mechanisms of mesenchymal stem and stromal cells in inflammatory diseases. *Nat Rev Nephrol* 2018; **14**: 493-507 [PMID: 29895977 DOI: 10.1038/s41581-018-0023-5]
  - 50 **Liang J**, Zhang H, Kong W, Deng W, Wang D, Feng X, Zhao C, Hua B, Wang H, Sun L. Safety analysis in patients with autoimmune disease receiving allogeneic mesenchymal stem cells infusion: a long-term retrospective study. *Stem Cell Res Ther* 2018; **9**: 312 [PMID: 30428931 DOI: 10.1186/s13287-018-1053-4]
  - 51 **Lu X**, Wang X, Nian H, Yang D, Wei R. Mesenchymal stem cells for treating autoimmune dacryoadenitis. *Stem Cell Res Ther* 2017; **8**: 126 [PMID: 28583168 DOI: 10.1186/s13287-017-0593-3]
  - 52 **Gu X**, Yu X, Zhao C, Duan P, Zhao T, Liu Y, Li S, Yang Z, Li Y, Qian C, Yin Z, Wang Y. Efficacy and Safety of Autologous Bone Marrow Mesenchymal Stem Cell Transplantation in Patients with Diabetic Retinopathy. *Cell Physiol Biochem* 2018; **49**: 40-52 [PMID: 30134223 DOI: 10.1159/000492838]
  - 53 **Karamini A**, Bakopoulou A, Andreadis D, Gkiouras K, Kritis A. Therapeutic Potential of Mesenchymal Stromal Stem Cells in Rheumatoid Arthritis: a Systematic Review of In Vivo Studies. *Stem Cell Rev Rep* 2020; **16**: 276-287 [PMID: 31950339 DOI: 10.1007/s12015-020-09954-z]
  - 54 **Niu J**, Yue W, Le-Le Z, Bin L, Hu X. Mesenchymal stem cells inhibit T cell activation by releasing TGF- $\beta$ 1 from TGF- $\beta$ 1/GARP complex. *Oncotarget* 2017; **8**: 99784-99800 [PMID: 29245940 DOI: 10.18632/oncotarget.21549]
  - 55 **Laing AG**, Fanelli G, Ramirez-Valdez A, Lechler RI, Lombardi G, Sharpe PT. Mesenchymal stem cells inhibit T-cell function through conserved induction of cellular stress. *PLoS One* 2019; **14**: e0213170 [PMID: 30870462 DOI: 10.1371/journal.pone.0213170]
  - 56 **Zheng ZH**, Li XY, Ding J, Jia JF, Zhu P. Allogeneic mesenchymal stem cell and mesenchymal stem cell-differentiated chondrocyte suppress the responses of type II collagen-reactive T cells in rheumatoid arthritis. *Rheumatology (Oxford)* 2008; **47**: 22-30 [PMID: 18077486 DOI: 10.1093/rheumatology/kem284]
  - 57 **Zhang QZ**, Su WR, Shi SH, Wilder-Smith P, Xiang AP, Wong A, Nguyen AL, Kwon CW, Le AD. Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing. *Stem Cells* 2010; **28**: 1856-1868 [PMID: 20734355 DOI: 10.1002/stem.503]
  - 58 **Pedraza L**, Lunardelli A, Luft C, Cruz CU, de Mesquita FC, Bitencourt S, Nunes FB, de Oliveira JR. Mesenchymal stem cells decrease splenocytes apoptosis in a sepsis experimental model. *Inflamm Res* 2014; **63**: 719-728 [PMID: 24888322 DOI: 10.1007/s00011-014-0745-1]
  - 59 **Rocheteau P**, Chatre L, Briand D, Mebarki M, Jouvion G, Bardon J, Crochemore C, Serrani P, Lecci PP, Latil M, Matot B, Carlier PG, Latronico N, Huchet C, Lafoux A, Sharshar T, Ricchetti M, Chrétien F. Sepsis induces long-term metabolic and mitochondrial muscle stem cell dysfunction amenable by mesenchymal stem cell therapy. *Nat Commun* 2015; **6**: 10145 [PMID: 26666572 DOI: 10.1038/ncomms10145]
  - 60 **Rojas M**, Cárdenas N, Kocyildirim E, Tedrow JR, Cáceres E, Deans R, Ting A, Bermúdez C. Human adult bone marrow-derived stem cells decrease severity of lipopolysaccharide-induced acute respiratory distress syndrome in sheep. *Stem Cell Res Ther* 2014; **5**: 42 [PMID: 24670268 DOI: 10.1186/sct430]
  - 61 **Zhao X**, Liu D, Gong W, Zhao G, Liu L, Yang L, Hou Y. The toll-like receptor 3 ligand, poly(I:C), improves immunosuppressive function and therapeutic effect of mesenchymal stem cells on sepsis via inhibiting MiR-143. *Stem Cells* 2014; **32**: 521-533 [PMID: 24105952 DOI: 10.1002/stem.1543]
  - 62 **Lalu MM**, Sullivan KJ, Mei SH, Moher D, Straus A, Fergusson DA, Stewart DJ, Jazi M, MacLeod M, Winston B, Marshall J, Hutton B, Walley KR, McIntyre L. Evaluating mesenchymal stem cell therapy for sepsis with preclinical meta-analyses prior to initiating a first-in-human trial. *Elife* 2016; **5** [PMID: 27870924 DOI: 10.7554/eLife.17850]
  - 63 **He X**, Ai S, Guo W, Yang Y, Wang Z, Jiang D, Xu X. Umbilical cord-derived mesenchymal stem (stromal)



- cells for treatment of severe sepsis: aphase 1 clinical trial. *Transl Res* 2018; **199**: 52-61 [PMID: 30044959 DOI: 10.1016/j.trsl.2018.04.006]
- 64 **Wei L**, Zhang J, Yang ZL, You H. Extracellular superoxide dismutase increased the therapeutic potential of human mesenchymal stromal cells in radiation pulmonary fibrosis. *Cytotherapy* 2017; **19**: 586-602 [PMID: 28314668 DOI: 10.1016/j.jcyt.2017.02.359]
- 65 **Jiang X**, Jiang X, Qu C, Chang P, Zhang C, Qu Y, Liu Y. Intravenous delivery of adipose-derived mesenchymal stromal cells attenuates acute radiation-induced lung injury in rats. *Cytotherapy* 2015; **17**: 560-570 [PMID: 25791071 DOI: 10.1016/j.jcyt.2015.02.011]
- 66 **Wang H**, Yang YF, Zhao L, Xiao FJ, Zhang QW, Wen ML, Wu CT, Peng RY, Wang LS. Hepatocyte growth factor gene-modified mesenchymal stem cells reduce radiation-induced lung injury. *Hum Gene Ther* 2013; **24**: 343-353 [PMID: 23458413 DOI: 10.1089/hum.2012.177]
- 67 **Cselenyák A**, Pankotai E, Horváth EM, Kiss L, Lacza Z. Mesenchymal stem cells rescue cardiomyoblasts from cell death in an in vitro ischemia model via direct cell-to-cell connections. *BMC Cell Biol* 2010; **11**: 29 [PMID: 20406471 DOI: 10.1186/1471-2121-11-29]
- 68 **Li N**, Sarojini H, An J, Wang E. Prosaposin in the secretome of marrow stroma-derived neural progenitor cells protects neural cells from apoptotic death. *J Neurochem* 2010; **112**: 1527-1538 [PMID: 20050969 DOI: 10.1111/j.1471-4159.2009.06565.x]
- 69 **Kim SY**, Lee JH, Kim HJ, Park MK, Huh JW, Ro JY, Oh YM, Lee SD, Lee YS. Mesenchymal stem cell-conditioned media recovers lung fibroblasts from cigarette smoke-induced damage. *Am J Physiol Lung Cell Mol Physiol* 2012; **302**: L891-L908 [PMID: 22307909 DOI: 10.1152/ajplung.00288.2011]
- 70 **Chen C**, Tseng P, Lo W, Wang F, Lee C. Comparing the impact of 3d bioreactor and 2d culture system on immunomodulation potency of warton's jelly derived-msc. *Cytotherapy* 2017; **19**: S186 [DOI: 10.1016/j.jcyt.2017.02.261]
- 71 **Kandoi S**, L PK, Patra B, Vidyasekar P, Sivanesan D, S V, K R, Verma RS. Evaluation of platelet lysate as a substitute for FBS in explant and enzymatic isolation methods of human umbilical cord MSCs. *Sci Rep* 2018; **8**: 12439 [PMID: 30127445 DOI: 10.1038/s41598-018-30772-4]
- 72 **Naskou MC**, Sumner SM, Chocallo A, Kemelmakher H, Thoresen M, Copland I, Galipeau J, Peroni JF. Platelet lysate as a novel serum-free media supplement for the culture of equine bone marrow-derived mesenchymal stem cells. *Stem Cell Res Ther* 2018; **9**: 75 [PMID: 29566772 DOI: 10.1186/s13287-018-0823-3]
- 73 **Kabat M**, Bobkov I, Kumar S, Grumet M. Trends in mesenchymal stem cell clinical trials 2004-2018: Is efficacy optimal in a narrow dose range? *Stem Cells Transl Med* 2020; **9**: 17-27 [PMID: 31804767 DOI: 10.1002/sctm.19-0202]
- 74 **Laroye C**, Boufenzar A, Jolly L, Cunat L, Alauzet C, Merlin JL, Yguel C, Bensoussan D, Reppel L, Gibot S. Bone marrow vs Wharton's jelly mesenchymal stem cells in experimental sepsis: a comparative study. *Stem Cell Res Ther* 2019; **10**: 192 [PMID: 31248453 DOI: 10.1186/s13287-019-1295-9]
- 75 **Van Pham P**, Vu NB, Phan NK. Umbilical cord-derived stem cells (modulatistm) show strong immunomodulation capacity compared to adipose tissue-derived or bone marrow-derived mesenchymal stem cells. *Biomed Res Ther* 2016; **3**: 687-696 [DOI: 10.7603/s40730-016-0029-1]
- 76 **Kim JH**, Jo CH, Kim HR, Hwang YI. Comparison of Immunological Characteristics of Mesenchymal Stem Cells from the Periodontal Ligament, Umbilical Cord, and Adipose Tissue. *Stem Cells Int* 2018; **2018**: 8429042 [PMID: 29760736 DOI: 10.1155/2018/8429042]
- 77 **Selich A**, Zimmermann K, Tenspolde M, Dittrich-Breiholz O, von Kaisenberg C, Schambach A, Rothe M. Umbilical cord as a long-term source of activatable mesenchymal stromal cells for immunomodulation. *Stem Cell Res Ther* 2019; **10**: 285 [PMID: 31547865 DOI: 10.1186/s13287-019-1376-9]
- 78 **Saldaña L**, Bensiamar F, Vallés G, Mancebo FJ, García-Rey E, Vilaboa N. Immunoregulatory potential of mesenchymal stem cells following activation by macrophage-derived soluble factors. *Stem Cell Res Ther* 2019; **10**: 58 [PMID: 30760316 DOI: 10.1186/s13287-019-1156-6]
- 79 **Cuerquis J**, Romieu-Mourez R, François M, Routy JP, Young YK, Zhao J, Eliopoulos N. Human mesenchymal stromal cells transiently increase cytokine production by activated T cells before suppressing T-cell proliferation: effect of interferon- $\gamma$  and tumor necrosis factor- $\alpha$  stimulation. *Cytotherapy* 2014; **16**: 191-202 [PMID: 24438900 DOI: 10.1016/j.jcyt.2013.11.008]
- 80 **Kwon YW**, Heo SC, Jeong GO, Yoon JW, Mo WM, Lee MJ, Jang IH, Kwon SM, Lee JS, Kim JH. Tumor necrosis factor- $\alpha$ -activated mesenchymal stem cells promote endothelial progenitor cell homing and angiogenesis. *Biochim Biophys Acta* 2013; **1832**: 2136-2144 [PMID: 23959047 DOI: 10.1016/j.bbdis.2013.08.002]
- 81 **Bárcia RN**, Santos JM, Filipe M, Teixeira M, Martins JP, Almeida J, Água-Doce A, Almeida SC, Varela A, Pohl S, Dittmar KE, Calado S, Simões SI, Gaspar MM, Cruz ME, Lindenmaier W, Graça L, Cruz H, Cruz PE. What Makes Umbilical Cord Tissue-Derived Mesenchymal Stromal Cells Superior Immunomodulators When Compared to Bone Marrow Derived Mesenchymal Stromal Cells? *Stem Cells Int* 2015; **2015**: 583984 [PMID: 26064137 DOI: 10.1155/2015/583984]
- 82 **Li J**, Xu SQ, Zhao YM, Yu S, Ge LH, Xu BH. Comparison of the biological characteristics of human mesenchymal stem cells derived from exfoliated deciduous teeth, bone marrow, gingival tissue, and umbilical cord. *Mol Med Rep* 2018; **18**: 4969-4977 [PMID: 30272340 DOI: 10.3892/mmr.2018.9501]
- 83 **Mennan C**, García J, Roberts S, Hulme C, Wright K. A comprehensive characterisation of large-scale expanded human bone marrow and umbilical cord mesenchymal stem cells. *Stem Cell Res Ther* 2019; **10**: 99 [PMID: 30885254 DOI: 10.1186/s13287-019-1202-4]
- 84 **Xu Z**, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med* 2020; **8**: 420-422 [PMID: 32085846 DOI: 10.1016/S2213-2600(20)30076-X]

# Mesenchymal stromal cells as potential immunomodulatory players in severe acute respiratory distress syndrome induced by SARS-CoV-2 infection

Panagiotis Mallis, Efstathios Michalopoulos, Theofanis Chatzistamatiou, Catherine Stavropoulos-Giokas

**ORCID number:** Panagiotis Mallis 0000-0001-9429-190X; Efstathios Michalopoulos 0000-0002-1901-6294; Theofanis Chatzistamatiou 0000-0003-4895-0155; Catherine Stavropoulos-Giokas 0000-0003-0698-6061.

**Author contributions:** Mallis P designed and retrieved the data and prepared the whole manuscript; Michalopoulos E and Chatzistamatiou T made critical revisions related to the content of the manuscript; Stavropoulos-Giokas C performed the final approval of the manuscript.

**Conflict-of-interest statement:** The authors declare no conflict of interest.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Panagiotis Mallis, Efstathios Michalopoulos, Theofanis Chatzistamatiou, Catherine Stavropoulos-Giokas**, Hellenic Cord Blood Bank, Biomedical Research Foundation Academy of Athens, Athens 11527, Greece

**Corresponding author:** Panagiotis Mallis, MSc, PhD, Associate Research Scientist, Teaching Assistant, Hellenic Cord Blood Bank, Biomedical Research Foundation Academy of Athens, 4 Soranou Ephessiou Street, Athens 11527, Greece. [pmallis@bioacademy.gr](mailto:pmallis@bioacademy.gr)

## Abstract

Severe acute respiratory syndrome coronavirus-2 and the related coronavirus disease-19 (COVID-19) is a worldwide emerging situation, which was initially reported in December 2019 in Wuhan, China. Currently, more than 7258842 new cases, and more than 411879 deaths have been reported globally. This new highly transmitted coronavirus is responsible for the development of severe acute respiratory distress syndrome. Due to this disorder, a great number of patients are hospitalized in the intensive care unit followed by connection to extracorporeal membrane oxygenation for breath supporting and survival. Severe acute respiratory distress syndrome is mostly accompanied by the secretion of proinflammatory cytokines, including interleukin (IL)-2, IL-6, IL-7, granulocyte colony-stimulating factor (G-CSF), interferon-inducible protein 10 (IP10), monocyte chemoattractant protein-1 (MCP1), macrophage inflammatory protein 1A (MIP1A), and tumor necrosis factor alpha (TNF- $\alpha$ ), an event which is known as "cytokine storm". Further disease pathology involves a generalized modulation of immune responses, leading to fatal multiorgan failure. Currently, no specific treatment or vaccination against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been developed. Mesenchymal stromal cells (MSCs), which are known for their immunosuppressive actions, could be applied as an alternative co-therapy in critically-ill COVID-19 patients. Specifically, MSCs can regulate the immune responses through the conversion of Th1 to Th2, activation of M2 macrophages, and modulation of dendritic cells maturation. These key immunoregulatory properties of MSCs may be exerted either by produced soluble factors or by cell-cell contact interactions. To date, several clinical trials have been registered to assess the safety, efficacy, and therapeutic potential of MSCs in COVID-19. Moreover, MSC treatment may be effective for the reversion of ground-glass opacity of damaged lungs and reduce the tissue fibrosis. Taking into account the multifunctional properties of MSCs, the proposed stem-cell-based

[/by-nc/4.0/](#)**Manuscript source:** Invited manuscript**Received:** May 15, 2020**Peer-review started:** May 15, 2020**First decision:** June 3, 2020**Revised:** June 10, 2020**Accepted:** July 19, 2020**Article in press:** July 19, 2020**Published online:** August 26, 2020**P-Reviewer:** Gentile P, Ullah M**S-Editor:** Yan JP**L-Editor:** Filipodia**P-Editor:** Wu YXJ

therapy may be proven significantly effective in critically-ill COVID-19 patients. The current therapeutic strategy may improve the patient's overall condition and in parallel may decrease the mortality rate of the current disease.

**Key words:** SARS-CoV-2; COVID-19; Respiratory syndrome; Cytokine storm; Mesenchymal stromal cells; Immunoregulation; Lungs; Th2 response; Dendritic cells; Natural killer cells

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** In this review, the therapeutic potential of mesenchymal stromal cells (MSCs) towards coronavirus disease-19 (COVID-19), will be highlighted. Importantly, MSCs exert key immunoregulatory/immunosuppressive properties that can alter significantly the immune responses. Also, MSCs share regenerative abilities, reducing in this way the lung tissue damage. These unique MSCs features, which may significantly improve the overall condition of COVID-19 patients, will be presented in the following article.

**Citation:** Mallis P, Michalopoulos E, Chatzistamatiou T, Stavropoulos-Giokas C. Mesenchymal stromal cells as potential immunomodulatory players in severe acute respiratory distress syndrome induced by SARS-CoV-2 infection. *World J Stem Cells* 2020; 12(8): 731-751

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/731.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.731>

## INTRODUCTION

In December 2019, a new highly transmitted coronavirus, which was provisionally named 2019-novel coronavirus, was spread in Wuhan, China<sup>[1]</sup>. In March 2020, the International Committee on Taxonomy of Viruses (ICTV) has renamed it as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)<sup>[2]</sup>. The zoonotic transmission in a seafood market of Wuhan is speculated until now as the initial route of transmission to humans<sup>[2,3]</sup>. By the time that this publication is prepared, more than 7258842 cases have been reported, and more than 411879 deaths have been occurred worldwide. Due to the global transmission of SARS-CoV-2, the World Health Organization recognized it as a serious public health issue and in January 2020 declared coronavirus disease-19 (COVID-19) as pandemic<sup>[4]</sup>. Despite the fact that China was initially affected by COVID-19, accounting more than 80000 cases and 77000 deaths, the United States is now the leading country in confirmed SARS-CoV-2 cases<sup>[4]</sup>. It is estimated that more than 1979893 people have been infected in the United States<sup>[4]</sup>. The European Union, Italy, and Spain have been stricken hard by COVID-19, accounting for more than 32500 deaths<sup>[4]</sup>.

Epidemiological studies have shown that SARS-CoV-2 infection and disease severity vary between the two genders. Currently, it has been reported that males are more susceptible to COVID-19 infection than females<sup>[5,6]</sup>. In addition, SARS-CoV-2 can affect all group ages, while 15% of patients may suffer from the severe form of this disorder<sup>[3]</sup>. Moreover, the probability for severe disorder occurrence increases dramatically in those aged over 65 years<sup>[3,5,7]</sup>. The average mortality of COVID-19 is under 3%, however, in highly affected countries such as Italy, Spain and France, the mortality rate may exceed 6%<sup>[4]</sup>. Additionally, mortality rate can be varied and is dependent on underlying disease and age<sup>[3]</sup>. Recently, a mortality rate of 10%-27% was reported in patients aged over 85 years, 3%-11% in ages of 65-84 years, 1%-3% in ages of 55-64 years, and below 1% in ages of 20-54 years<sup>[3,5,7]</sup>.

SARS-CoV-2 is responsible for inducing severe acute respiratory distress syndrome (ARDS) in infected patients, which promotes further lung damage and tissue fibrosis<sup>[8,9]</sup>. Its pathophysiology is related with elevated levels of inflammatory cytokines such as IL-2, IL-6, IL-7, GSCF, IP10, MCP1, MIP1A, and TNF- $\alpha$ , an event which is known as "cytokine storm"<sup>[9-11]</sup>. Except for lung damage, SARS-CoV-2 may infiltrate other organs such as heart, kidney, and brain, causing cardiomyopathy, arrhythmias, kidney failure, and encephalitis, respectively<sup>[3,12,13]</sup>. Also, the placental transmission of SARS-CoV-2 from mothers to their fetuses is currently being investigated<sup>[13,14]</sup>. In the study of Schwartz<sup>[13]</sup>, no SARS-CoV-2 intrauterine transmission

from infected mothers to their fetuses was reported. Additionally, no maternal deaths from SARS-CoV-2 has been reported to date, which is in contrast to previous related viruses such as SARS and Middle East respiratory syndrome (MERS)<sup>[13-15]</sup>.

Until now, several treatments have been tested, including prophylactic hydroxychloroquine and colchicine administration, antiviral agents, monoclonal antibodies against SARS-CoV-2, and transfusion of convalescent plasma<sup>[16-19]</sup>. The majority of the above treatments have Food and Drug Administration (FDA) approval for their application to other diseases, while their use in COVID-19 patients is still under investigation. Remdesivir, an experimental drug to treat Ebola, has been tested in clinical trials for its efficacy in SARS-CoV-2 infection, while globally a great effort has been performed for the production of satisfactory vaccines against the current virus<sup>[19-24]</sup>. Recently, remdesivir was approved by the FDA for application in COVID-19 patients.

Knowing that COVID-19 can cause significant modifications to the patient's immune system, alternative strategies should also be tested. In this way, the mesenchymal stromal cells (MSCs), a mesodermal cellular population originated mostly from bone marrow (BM), Wharton's Jelly tissue (WJ-tissue), and adipose tissue (AT), could be potentially applied in COVID-19 patients<sup>[25-28]</sup>. MSCs are known for their immunoregulatory/immunosuppressive properties, which are exerted in several ways<sup>[25]</sup>. MSCs are currently applied in severe autoimmune disorders such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Crohn's disease, diabetes mellitus, *etc.*, thus reducing disease manifestations<sup>[29]</sup>. In this way, MSCs can be applied mostly as cotherapy in combination with the above pharmaceutical agents to reverse the severe manifestations induced by SARS-CoV-2 infections. In this review, we will highlight the specific immunomodulation aspects of MSCs and their potential application in patients infected by SARS-CoV-2. Furthermore, the immune response against COVID-19, MSCs origin, immunoregulatory properties, and their possible application in COVID-19 will be presented. For the purposes of the current review article, we searched initially over 300 published articles focused on COVID-19 pathogenesis and MSC biology. During the eligibility process, 166 studies were excluded and the remaining 134 articles were finally included in this review. Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram describes the methodological framework that was followed in the current article ( **Supplementary Figure 1**). Based on the above data, the scientific community might consider the broad use of MSCs in critically ill patients, improving their overall condition.

## INVASION MECHANISM OF SARS-COV-2

SARS-CoV-2, according to the ICTV, is placed within the *Coronaviridae* and specifically is a member of the subgenus *Sarbecovirus*<sup>[2,30]</sup>. SARS-CoV-2 consists of four structural proteins, spike (S), envelope (E), membrane (M), and nucleocapsid (N), and its diameter is approximately 50-150 nm<sup>[31,32]</sup>. It is a positive-sense single-stranded RNA virus (++ssRNA), while its genome contains 29903 bases<sup>[33]</sup>. The use of molecular methods has revealed that SARS-CoV-2 (Genbank accession No. NC\_045512) is similar to bat-SL-CoVZC45 (88% similarity, Genbank accession No. MG772933.1) and bat-SL-CoVZXC21 (89% similarity, Genbank accession No. MG772934.1)<sup>[33-35]</sup>. Also, it is related more with the original SARS-CoV (80% similarity, Genbank accession No. FJ588686) than the MERS coronavirus (MERS-CoV, 50% similarity, Genbank accession No. NC\_019843)<sup>[36]</sup>.

The infection of SARS-CoV-2 begins with its proper adaption to the angiotensin I converting enzyme 2 receptor (ACE2), a process where the S protein is involved<sup>[17]</sup>. Zhou *et al.*<sup>[37]</sup> showed with the use of reverse genetics that ACE2 is the main receptor that SARS-CoV-2 uses for its fusion with the cellular membrane. Moreover, Wrapp *et al.*<sup>[38,39]</sup> showed that SARS-CoV-2 can bind ACE2 with higher affinity than the original SARS-CoV. Furthermore, the entrance of the virus to host cells is performed by the S protein primed by transmembrane protease serine 2 (TMPRSS2)<sup>[40]</sup>. TMPRSS2 is a cysteine-rich protease domain, and its expression is regulated by androgenic hormones<sup>[41]</sup>. Overexpression of TMPRSS2 is mostly observed in prostate cancer<sup>[41]</sup>. Its relation with the pathophysiological mechanism of SARS-Cov-2 led researchers to associate possibly its function with lung damage severity and tumorigenesis.

ACE2 and TMPRSS2 are mostly found in alveolar type II cells<sup>[42]</sup>. Additionally, capillary endothelial cells (ECs) of other tissues such as heart, liver, kidney, and intestine also express the above proteins<sup>[43]</sup>. For this reason, patients suffering from



COVID-19, besides pneumonia, which is the most common manifestation, may exhibit other disorders such as cardiomyopathy, impaired liver and kidney function, and encephalitis.

## IMMUNE SYSTEM AND COVID-19

After the infiltration of SARS-CoV-2 to the host cells, a specific series of events occur. COVID-19 is characterized by three-phase immunopathology, where the patient's immune system has a central role. Among patients, fever (92.8% of the patients), cough (69.8%), dyspnea (34.5%), myalgia (27%), headache (7%), and diarrhea (6%) were the most common symptoms<sup>[3]</sup>. Additionally, there is an increasing tendency that younger persons (ages < 20-years-old) can be asymptomatic carriers of SARS-CoV-2, who can transmit effectively the virus to others<sup>[5,7]</sup>.

The first phase is the incubation – non-severe stage, where the virus is recognized by the immune system<sup>[5,44]</sup>. In this way, the innate immune response is performed to limit the virus infection. If the virus escapes the host's defense, then the second phase is initiated<sup>[44,45]</sup>. For this reason, immunocompromised patients or patients with underlying disorders characterized by impaired immune function, may have increased probabilities for severe disease occurrence. Chen *et al*<sup>[5]</sup> showed that the most common underlying diseases, which were related with the severe outcome, were cardiovascular disease, hypertension, and diabetes mellitus.

In the second phase, the virus induces severe damage to the infected cells through its replication cycle<sup>[46]</sup>. This event leads to the activation of dendritic cells (DCs), epithelial cells, and macrophages, which are producing IL-1 $\beta$ , IL-2, IL-6, IL-8, interferon (IFN)- $\alpha/\beta$ , TNF- $\alpha$ , C-C motif chemokine 3 (CCL3), CCL5, CCL2, and IP-10. These inflammatory molecules can efficiently stimulate T helper cell (Th)1, Th2, and Th17 responses. Indeed, the overactivation of CXCR3<sup>+</sup>CD4<sup>+</sup> T cells, CXCR3<sup>+</sup>CD8<sup>+</sup> T cells, and CXCR3<sup>+</sup> natural killer (NK) cells has been observed in COVID-19<sup>[47]</sup>. These cells are responsible for the production of inflammatory cytokines. Previous studies in COVID-19 patients have shown increased levels of IL-1B, IL-1RA, IL-7, IL-8, IL-9, fibroblast growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- $\gamma$ , G-CSF, IP10, MCP1, MIP1A, platelet-derived growth factor (PDGF), TNF- $\alpha$ , vascular endothelial growth factor (VEGF)<sup>[48]</sup>. Among them, IL-2, IL-6, IL-7, G-CSF, IP10, MCP1, MIP1A, and TNF- $\alpha$  were significantly higher in severely conditioned patients<sup>[9-11,47,49]</sup>. In addition, thrombocytopenia, lymphopenia, respiratory failure, pneumonitis, shock, and organ failure have been observed in COVID-19 patients<sup>[11]</sup>. In addition, Wan *et al*<sup>[50]</sup> reported that COVID-19 patients were characterized by overactivated lymphocytes despite the occurred lymphocytopenia that was evident in the peripheral blood. This study showed that CD8<sup>+</sup> T cells were reduced at a percentage of 28.4% and 61.9% in mild and severe group patients, respectively. Also, NK cell reduction was evident in both groups (34.1% in mild, 47.6% in severe)<sup>[50]</sup>. These findings were accompanied by significantly high IL-6 levels in both mild and severe groups. Additionally, Xu *et al*<sup>[51]</sup> reported the substantial reduction of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in peripheral blood of COVID-19 patients, although it appeared that lymphocytes were overactivated due to high expression of human leukocyte antigen DR-isotype (HLA-DR) (34.7%) in CD4<sup>+</sup> and CD38 (39.4%) in CD8<sup>+</sup> T cells<sup>[51]</sup>.

The third phase involves the prolonged lung damage that can cause severe ARDS. These patients must enter the intensive care unit (ICU), followed by connection to extracorporeal membrane oxygenation in order to survive<sup>[46]</sup>. It has been reported in COVID-19 patients that hyaluronan (HA) is produced due to the "cytokine storm", thus promoting pneumonia accompanied by pulmonary ground-glass opacity<sup>[52]</sup>. Indeed, IL1 and TNF- $\alpha$  can strongly induce the HA production through overactivation of HA-synthase-2 of CD31<sup>+</sup> cells and lung alveolar epithelial cells. A series of studies showed that damaged lungs are infiltrated mostly by macrophages, monocytes, and giant cells, while few overactivated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were present<sup>[52,53]</sup>. Furthermore, inflammatory cytokines can activate fibroblasts, which in turn can overproduce collagen, promoting even more lung fibrosis<sup>[53,54]</sup>.

Currently, the satisfactory administration of COVID-19 is a great challenge. Th1 and Th2 responses are playing a central role in disease severity of SARS-CoV-2 in the same way as MERS-CoV and SARS-CoV. Moreover, from previous experience, it is known that most neutralizing antibodies are against the S and E proteins<sup>[11,55]</sup>. However, the manufacturing of COVID-19 specific drugs and vaccines is still an ongoing process. Alternative strategies should also be tested for the suppression of the hyperacute immune response, which can lead to patient survival. To date, a great number of



pharmaceutical agents, including antiviral agents, monoclonal antibodies against inflammatory cytokines, and convalescent plasma, are under investigation for COVID-19 (Table 1). The majority of these agents have current FDA approval for other diseases, including treatment of hepatitis C and rheumatic disease, while they are under evaluation for the safety, tolerability, and efficacy in COVID-19 patients. Most of these pharmaceutical agents have specific targets (*e.g.*, S protein, IL-6R, and IL-6) but also are accompanied by adverse reactions. Moderate adverse effects may be observed in patients, including fever, headache, nausea, skin rash, diarrhea, and impairment of liver function. In addition to the use of pharmaceutical agents, it has been suggested that MSCs can effectively suppress the patient's overactivated immune system by utilizing their immunoregulatory properties. MSCs have been used extensively in a wide number of registered clinical trials, supporting their tolerability, safety, and efficacy<sup>[56]</sup>. No severe adverse reactions have been observed after MSCs infusion to patients. In this way, MSCs could be employed as an alternative therapeutic strategy or as cotherapy in severely conditioned COVID-19 patients.

## ORIGIN AND PROPERTIES OF MSCS

MSCs, a mesodermal multipotent cellular population, can mostly be derived from BM, WJ-tissue, umbilical cord blood, AT, amniotic fluid (AF), and dental pulp<sup>[57]</sup>. MSCs also have been isolated from solid organs such as the liver, lungs and kidney. Friedenstein, 40 years ago, was the first to isolate a fibroblastic-like cell population from BM with plastic adherent and differentiation properties<sup>[58]</sup>. In 2006, the International Society for Cellular Therapy (ISCT) provided the minimum criteria for the proper identification of MSCs<sup>[59]</sup>. Based on ISCT, the following criteria must be fulfilled to define MSCs: (1) Spindle-shaped plastic adherent cells; (2) Expression of specific antigens. Specifically, positive expression ( $\geq 95\%$ ) of CD73, CD90, CD105, and low expression ( $\leq 2\%$ ) of CD34, CD45, CD11a, CD19 and HLA-DR; and (3) Mesodermal multilineage differentiation abilities towards "osteocytes", "adipocytes", and "chondrocytes".

Additionally, ISCT proposed the term "multipotent mesenchymal stromal cells" be used when this cellular population is applied in experimental approaches<sup>[59]</sup>. In 2019, the ISCT's MSC committee reported that "mesenchymal stromal cells" have specific secretory, immunomodulatory, and homing properties and are distinguished from "mesenchymal stem cells", which have restricted properties focused on self-renewal and differentiation<sup>[60]</sup>.

Until date, BM is the most common source for MSCs isolation, estimating that MSCs represent 0.01%-0.001% of total nucleated cells<sup>[25]</sup>. BM-MSCs are located to the abluminal surface of sinusoidal blood vessels in BM. These cells represent the stroma, where can efficiently regulate the differentiation of the resident hematopoietic stem cells (HSCs)<sup>[25]</sup>. Specifically, MSCs support HSC differentiation through the expression of c-Kit, CXCR4, and Tie2. It has been shown in the literature that CXCR4/CXCL12, and CXCR7/CXCL12 interplay has a significant impact on the regulation of HSC homing and differentiation<sup>[61]</sup>. Additionally, the Notch-Delta, Wnt, Shh signaling pathways have been reported to be involved in this differentiation process<sup>[61]</sup>.

Unlike BM harvesting, MSCs can be non-invasively isolated from other sources, including the umbilical cord's WJ-tissue, a discarded material after gestation<sup>[62]</sup>. In recent years, there is a tendency to use MSCs derived from the stromal vascular fraction (SVF) of liposuction aspirates or subcutaneous AT in regenerative medicine applications<sup>[63]</sup>. Typically, MSCs represents 1%-10% of AT resident cells, making AT a greater source than BM. MSCs originated from AT are known as adipose derived stem cells or adipose derived MSCs (AD-MSCs) and exhibit similar immunophenotypic, differentiation potential and immunoregulatory properties as MSCs from other sources<sup>[63]</sup>.

MSCs have been used in a great number of approaches, including tissue engineering and regenerative medicine applications, graft *vs* host disease (GvHD), co-transplantation with HSCs, and administration of autoimmune disorders such as MS, ALS, and Crohn's disease<sup>[64-66]</sup>. MSCs can exert their functions through the production of cytokines, chemokines, exosomes, and miRNAs, which can act in a paracrine manner on targeted cellular populations<sup>[25]</sup>. Moreover, MSCs have proven their regenerative properties when applied to injured lungs, liver, kidney, and heart<sup>[67]</sup>. Recently, it is under evaluation the beneficial regenerative effect of MSCs in erectile dysfunction<sup>[68,69]</sup>. MSCs, due to their plasticity, have been differentiated successfully to ECs, vascular smooth muscle cells, hepatocytes, insulin-producing cells, *etc.* Much effort has been put into the transdifferentiation of MSCs to ectodermal cell lineages,

**Table 1 Most common therapeutic compounds used for the treatment of coronavirus disease-19**

	Agents	Mechanism of action	Approval
Antimalarial agent	Chloroquine/hydroxychloroquine	Inhibition of RNA-dependent RNA polymerase, Increase of endosomal and lysosomal pH	FDA approved (under investigation for COVID-19)
Antiviral agent	Ribavirin	Inducing mutations in RNA-dependent replication in RNA viruses, stop viral RNA synthesis	FDA approved (under investigation for COVID-19)
	Nelfinavir	Inhibitor of viral proteases	FDA approved (under investigation for COVID-19)
	Remdesivir	Inhibition of viral RNA-dependent RNA polymerase and proofreading	FDA approved (for COVID-19)
Anti-rheumatic agent	Baricitinib/ruxolitinib	JAK inhibitor/suppression of immune cells	Clinical Trial phase III
Monoclonal antibody	Tocilizumab	Humanized monoclonal antibody against the interleukin-6 receptor (IL-6R) and IL-6	FDA approved (under investigation for COVID-19)
	Anakinra	Humanized monoclonal antibody against IL-1R	FDA approved (under investigation for COVID-19)
Vaccination	Ad5-nCoV	Recombinant adenovirus type 5	Clinical Trials Phase II
	ChAdOx1 nCoV-19	Adenovirus vector	Clinical Trials Phase I-II
	INO-4800	DNA plasmid delivered by electroporation	Clinical Trials Phase I-II
	NVX-CoV2373	Recombinant S protein of SARS-CoV-2	Clinical Trial Phase I
	LV-SMENP-DC	Minigene vaccination in combination with modified DCs	Clinical Trial Phase I
Human antibodies	Convalescent plasma	Plasma enriched with antibodies specific to SARS-CoV-2, derived from convalescent patients	Clinical Trials Phase I
Stem cells	MSCs and derivatives (exosomes)	Suppression of the overactivated immune response through cell-cell contact and secretion of soluble factors	Clinical Trials Phase I

COVID-19: Coronavirus disease-19; MSCs: Mesenchymal stromal cells; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; DCs: Dendritic cells; FDA: Food and Drug Administration.

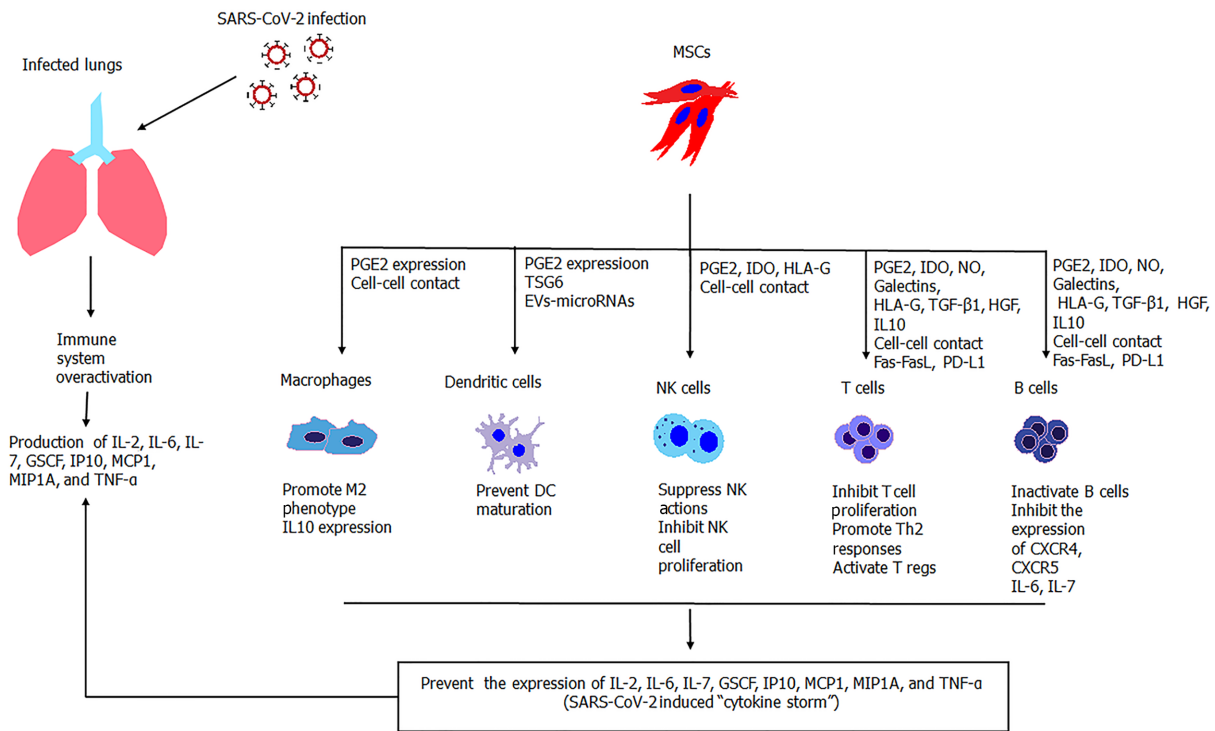
such as neurons, by utilizing the induced pluripotent stem cell technology<sup>[70]</sup>.

MSCs are immune privileged cells, and thus can be used either allogeneically or autologously in large scale clinical trials. Most often, a great number of clinical grade MSCs is required, which has been proven a quite demanding task. In this way, the autologous isolation and expansion of MSCs in significant numbers may be hampered by an individual's characteristics, such as age and disease severity. On the other hand, allogeneic pooled MSCs may be a better option for regenerative medicine approaches. Le Blanc *et al*<sup>[71]</sup> have reported that MSCs expressed low levels of HLA class II intracellularly, which can be presented to their membrane surface after IFN- $\gamma$  induction. However, MSCs do not express the costimulatory molecules B7-1, B7-2, CD40, and CD40L. Studies in non-human primates have revealed that MSCs escape T cell recognition and are well tolerated<sup>[71,72]</sup>. Besides the above properties, MSCs have unique immunoregulatory properties. Specifically, there is evidence that MSCs have a role in the antigen presentation process and are actively involving in organism homeostasis<sup>[29]</sup>.

## IMMUNOMODULATORY PROPERTIES OF MSCS

Despite the therapeutic potential of MSCs in regenerative medicine applications, these cells have shown promising results in the regulation of immune responses<sup>[25,29]</sup>. MSCs can act both in immune activation and immune suppression, depending on the microenvironment stimuli (Figure 1). The significant immunoregulatory/immunosuppressive abilities of MSCs have been exploited for therapeutic applications in autoimmune disorders and GvHD<sup>[65,66]</sup>. Taking into account that SARS-CoV-2 can induce significant alterations to innate and adaptive immune responses, leading to the "cytokine storm", the transfusion of MSCs to COVID-19 patients may be beneficial.

It has been described in the literature that MSCs can effectively modulate DC maturation, T cells (naïve and effector T cells – Th1/ Th2/ Th17 cells), and NK cell



**Figure 1 Immunomodulatory properties of activated MSCs against overactivated immune cells during SARS-CoV-2 infection.** MSCs efficiently suppress the immune responses through the secretion of soluble molecules or cell-cell contact interactions. MSCs have a broad effect on the immune responses exerted by macrophages, dendritic cells, natural killer cells, and T and B cells. SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; MSCs: Mesenchymal stromal cells; NK cells: Natural killer cells; NO: Nitric oxide; IDO: Indoleamine 2,3-dioxygenase; EVs: Extracellular vesicles.

responses<sup>[25,29]</sup>. In the presence of low IFN-γ levels, MSCs can translocate the intracellular HLA class II to their membrane, thus playing a role in innate immunity. On the other hand, high IFN-γ levels can induce MSCs to exert their immunoregulatory properties, mostly through paracrine signaling pathways. In addition, there is evidence that MSCs under specific circumstances may act as antigen-presenting cells<sup>[25]</sup>. The immunomodulation abilities of MSCs can be exerted by delivered soluble factors (cytokines, chemokines, growth factors, modulators of enzyme function), direct cell-cell contact, or a combination of those (Table 2)<sup>[29]</sup>.

### Interplay between MSCs and macrophages

MSCs can effectively modulate the responses of neutrophils and macrophages. Both cells are playing central roles to the antigen presentation process to DCs, leading to specific immune responses by the cells of innate immunity<sup>[73]</sup>. Macrophages are distinguished to M1 (classically activated) and M2 (alternatively activated) macrophages<sup>[29]</sup>. M1 macrophages are responsible for pathogen phagocytosis and presentation of antigen epitopes to DCs. In this process, a set of inflammatory cytokines are produced by M1 macrophages, such as TNF-α, IL-1α/β, IL-6, and IL-12, thus activating and promoting Th1 responses. On the other hand, M2 macrophages promote Th2 responses and are considered immunosuppressive cells<sup>[74]</sup>. These cells exhibit low expression of inflammatory cytokines and high production of anti-inflammatory IL-10, which is associated with tissue remodeling, wound repair, and clearance of apoptotic cells<sup>[74]</sup>. It has been reported that MSCs can modulate the M1/M2 macrophage phenotype through cell-cell or paracrine interactions<sup>[29,75]</sup>.

Activation of MSCs with IFN-γ leads to the production of TNF-α, MCP1, and IL-1β, which can further promote the M1 macrophage phenotype. Alternatively, MSCs, through the expression of prostaglandin E2 (PGE2), can induce M2 macrophage phenotype switch<sup>[29]</sup>. Further evidence for the role of MSCs in promoting the M2 macrophage phenotype arises from the study of Wahnou *et al*<sup>[76]</sup>. Specifically, they reported that signal transducer activators of transcription-3 (STAT3) is activated through cell-cell interactions between MSCs and macrophages<sup>[76]</sup>. Moreover, the same study showed that the STAT3 transcription factor is responsible for IL-10 production by macrophages and DCs, promoting further their immunosuppressive functions<sup>[76]</sup>.

**Table 2 Immunomodulation mechanisms of mesenchymal stromal cells**

	Implicated biomolecules	Mechanism of action	Implicated immune cells	Result
Cell-cell contact	Fas/fas ligand	Fas/fas ligand death signaling pathway/FADD/caspases activation or TRAIL signaling pathway	Macrophages, DCs, T and B cells	Apoptosis
	PD-L1/ PD-1	PD-L1 induced death through binding with the inhibitory checkpoint protein PD-1	T and B cells	Reducing cell proliferation, reducing apoptosis of T reg
	HLA-G/LIRB2 (ILT4/CD85d) and KIR2DL4 (CD158d)	HLA-G/LIRB2 interaction /phosphorylation of TIMS/SHP phosphatases activation/ MAPK downregulation	DCs, NK cells and T cells	Inhibition of cellular proliferation
Soluble factors	PGE2	PGE2/cAMP production/ downregulation of IL-2, IL-2R expression. PGE2/ downregulation of PtdIn/ suppression of T cell receptor signaling	Macrophages, DCs, T and B cells	M2 macrophages switching; Prevention of DCs maturation, T and B cell inactivation
	IDO	IDO blocks the conversion of tryptophan to kynurenin/in combination with TGF- $\beta$ 1 and HGF	DCs NK cells, T and B cells	G0/G1 cell cycle arrest
	NO	NO/suppression of STAT5 phosphorylation	Macrophages, T and B cells	Inhibition of cellular proliferation
	Galectins	Crosslinking with TCR/clustering prevention	T and B cells	Inhibition of T and B cell proliferation
	Soluble HLA-G isoforms	Similar interaction mechanism as membrane bound HLA-G isoforms	DCs, NK cells and T cells	Inhibition of cellular proliferation
EVs	miR-21-5p, miR-142-3p, miR-223-3p, and miR-126-3p	Interaction with JAG1, PDCD4, IL-12p35, downregulation of IL-6 expression	DCs	Inhibition of DC maturation
	miR-145, miR-146 and miR-155	Suppression of TRAF6 and IL-1 IRAK1 expression/ down-regulation of NF- $\kappa$ B p65 phosphorylation/decrease in TNF- $\alpha$ , IL-1 $\beta$ and IL-6 production	Macrophages, T cells	Inactivation of M1 macrophages; Switching from Th1 to Th2 responses

FADD: Fas-associated death domain; PtdIn: Phosphatidylinositol; DCs: Dendritic cells; NK cells: Natural killer cells; PGE2: Prostaglandin E2; IL: Interleukin; TNF: Tumor necrosis factor; NO: Nitric oxide; STAT5: signal transducer and activator of transcription 5; TCR: T cell receptor; TRAIL: TNF-related apoptosis-inducing ligand; IDO: Indoleamine-2,3-dioxygenase; EVs: Extracellular vesicles; SHP: Src-homology 2 domain-containing protein tyrosine phosphatases; TRAF6: TNF receptor-associated factor 6; IRAK1: IL-1 receptor-associated kinase 1.

### Regulation of DC maturation

Immature DCs (imDCs), the immune sentinels of the periphery, can be activated after inflammatory cytokine production by macrophages. Cleaved antigenic epitopes are loaded to HLA molecules of imDCs, followed by the presentation to T cells of the draining lymph nodes<sup>[77]</sup>. Then, DCs undergo maturation, which can induce specific Th1 and Th2 responses against specific antigenic epitopes. It has been found that TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, produced either by M1 macrophages or IFN- $\gamma$  activated MSCs, can drive DC maturation<sup>[78]</sup>. The prevention of DC maturation is of great importance, especially when this leads to prolonged T cell responses, causing host cell and tissue damage. Several studies have shown that MSCs can interfere with DCs maturation through the production of soluble factors<sup>[29,78,79]</sup>. Mostly, it has been suggested that secreted PGE2 by activated MSCs plays an important role in the inhibition of DCs maturation<sup>[29]</sup>. This has been confirmed by results from co-culture studies using MSCs and DCs<sup>[80]</sup>. These studies, were characterized by low expression of CD38, CD80, CD86, IL-6, and IL-12, which are significant molecules for T cell activation<sup>[81]</sup>. Moreover, DCs exhibited low migratory ability, exerted by CCR7-CCL21 interaction. Liu *et al*<sup>[82]</sup> suggested that the production of TNF- $\alpha$ -stimulating gene 6 by MSCs can suppress DCs maturation though the inactivation of signaling cascades mediated by mitogen activated protein kinase (MAPK) and nuclear factor-kappa B (NF- $\kappa$ B).

The inhibitory effect of MSCs in DC maturation can be increased by the production of specific miRNAs, including miR-21-5p, miR-142-3p, miR-223-3p, and miR-126-3p<sup>[83]</sup>. In this process, extracellular vesicles (EVs) of MSCs are enriched with the above miRNAs, suggesting an additional way of regulating DC maturation<sup>[84]</sup>.

### MSCs and T cell responses

Immunomodulation of T cell responses can be exerted by MSCs in several ways. MSCs secrete a set of molecules that can act either positively or negatively to the T cell responses. Also, inhibition of T cell proliferation can be performed *via* cell-cell contact with MSCs<sup>[85]</sup>.

Dependent on the microenvironment stimuli, MSCs can effectively inhibit T cell proliferation through the production of PGE2, indoleamine-2,3-dioxygenase (IDO), TGF- $\beta$ , and hepatocyte growth factor (HGF)<sup>[25,29]</sup>. The effect of PGE2 inhibition of T cell proliferation was reported for the first time in 1971<sup>[86]</sup>. Several years later, the specific mechanism of action by which PGE2 can exert its immunosuppressive effects on T cells was revealed. PGE2 is a prostanoid, which is synthesized by arachidonic acid through the action of cyclooxygenase-1<sup>[87]</sup>. PGE2 is responsible for the production of cAMP in activated T cells. cAMP plays a key role in the downregulation of IL-2 and IL-2R expression and abrogation of Ca<sup>2+</sup> after T cell receptor (TCR) activation. Also, PGE2 negatively regulates the hydrolysis of phosphatidylinositol and the production of diacylglycerol and inositol phosphate (IP), resulting in T cell inactivation<sup>[87]</sup>. Recently, it was reported that PGE2 may be involved in T cell polarization, promoting further Th2 responses. In addition, PGE2 produced by MSCs can orchestrate the CD4<sup>+</sup> CD25<sup>+</sup>FOXP3 T reg responses, influencing even more the immunosuppression of hyperactivated T cells<sup>[29]</sup>.

IDO also is a strong immunosuppressive agent of T cell responses<sup>[85]</sup>. Specifically, IDO blocks the metabolism of tryptophan to kynurenine in T cells. Kynurenine is an essential amino acid for the cell cycle of T cells, and its absence leads to G0/G1 cell cycle arrest. In addition, Ryan *et al*<sup>[88]</sup> reported that IFN- $\gamma$  activated MSCs can produce TGF- $\beta$ 1 and HGF and, in combination with IDO, can significantly suppress alloreactive T cell proliferation. MSCs *via* the secretion of nitric oxide (NO) can inhibit T cell proliferation<sup>[89]</sup>. NO is another potent immunosuppressive agent that can effectively downregulate immune responses. Specifically, NO is responsible for the suppression of signal transducer and activator of transcription 5 phosphorylation, which further results in the inhibition of TCR-mediated T cell proliferation and inflammatory cytokine production. Additionally, it was found that galectins 1 and 3 secreted by MSCs can effectively suppress T cell proliferation by preventing TCR clustering through a crosslink interaction mechanism<sup>[89]</sup>.

Additionally, MSCs can exert their immunosuppressive properties through T cell apoptosis mediated by cell-cell interactions<sup>[90]</sup>. In this way, the Fas/Fas ligand death signaling pathway can induce apoptosis to T cells, through downstream activation of Fas-associated death domain and caspases. It has been found that MSCs, upon inflammatory stimuli, can express the Fas ligand, binding in this way to Fas receptor of hyperactivated T cells<sup>[90,91]</sup>. Another potential activator of Fas-associated death domain is the TNF-related apoptosis-inducing ligand (TRAIL)/death receptor (DR) signaling pathway. IFN- $\gamma$  activated MSCs produce high amounts of TRAIL, which binds to DRs expressed in T cells, thus leading to their apoptosis. Also, MSCs can reduce T cell proliferation *via* programmed death ligand-1 (PD-L1)/programmed death-1 (PD-1) interaction. Specifically, PD-L1 expressed by MSCs binds to the inhibitory checkpoint molecule PD-1 of T cells, followed by Src-homology 2 domain-containing protein tyrosine phosphatases (SHP)-1 and SHP-2 phosphorylation. Finally, inhibition of MAPK is performed, which leads to inhibition of cellular proliferation. The PD-L1 can inhibit cellular proliferation and may have a broad effect on T cell subpopulations, including CD4<sup>+</sup>, CD8<sup>+</sup>, and Th17 cells<sup>[91]</sup>. Remarkably, Luz-Crawford *et al*<sup>[92]</sup> showed that the PD-L1/PD-1 interaction also could induce inhibition of Th17 cells *via* cell-cell contact. On the other hand, this mechanism has a positive association with CD4<sup>+</sup> CD25<sup>+</sup>FOXP3 T reg proliferation.

### MSCs and B cell responses

Similarly, to T cell modulation, B cell responses can be regulated by MSCs *via* the secretion of soluble factors and through cell-cell contact interactions. IDO and PGE2 alongside the production of TGF- $\beta$ 1 and HGF can lead to cell cycle arrest of B cells (G0/G1)<sup>[93]</sup>. Additionally, direct cell contact interactions, mostly utilizing the Fas/Fas ligand, TRAIL/DR death signaling, and PD-L1/PD-1 pathways, can promote apoptosis in B cells<sup>[94,95]</sup>. Recent evidence also showed that coculture of MSCs with B cells resulted in downregulation of CXCR4 and CXCR5, thus preventing the migratory ability and homing of B cells towards CXCL12 and CXCL13 chemoattractant agents. It has been shown that BM-MSCs *via* secretion of GM-CSF can inhibit the production of CXCR4, CXR5, IL-6, and IL-7 by activated B cells<sup>[95]</sup>. Indeed, the MSC interaction with plasma B cells results in impaired secretion of immunoglobulin (Ig)M, IgG, and IgA by



plasma B cells. However, MSCs cannot downregulate the expression of the costimulatory molecules CD80, CD86, and HLA-DR in activated B cells<sup>[96]</sup>. Additionally, MSCs did not cause any negative effect on IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-10 expression by B cells<sup>[96]</sup>.

### **Modulation of NK responses**

NK cells are the responsible cellular population for the elimination of virus-infected and tumorigenic cells. In addition, these cells of the innate immunity bear inhibitory and activating receptors to their surface<sup>[97]</sup>. Killer cell immunoglobulin-like receptors (KIRs) exert inhibitory effects, while natural cytotoxicity receptors, NKp46, NKp30, and NKp44, are responsible for NK activation<sup>[98]</sup>. Due to virus infection or malignant transformation, the affected cells initially decrease the expression of HLA class I, thus becoming susceptible by NK cells. In this way, NK cells are activated to exhibit their strong cytolytic activity against their targets<sup>[98]</sup>.

A remarkably complex interplay between NK cells and MSCs has been reported<sup>[99]</sup>. Indeed, NK cells can exert cytolytic actions against MSCs due to the low expression of HLA class I<sup>[100]</sup>. Alternatively, activation of MSCs with IFN- $\gamma$  leads to upregulation of HLA class I expression, which can strongly interact with KIRs, inhibiting in this way the MSCs cytotoxicity mediated by NK cells<sup>[100]</sup>. In this context, toll-like receptors (TLRs), presented in MSCs, seem to play a significant role. Activation of TLR3 in MSCs leads to increased immunosuppression against NK cells<sup>[101]</sup>.

Further immunosuppression of NK cells is mediated by the secretion of soluble factors from MSCs<sup>[29]</sup>. Indeed, IDO and PGE2 can inhibit IL-2 induced NK responses, while TGF- $\beta$ 1 and HGF act cooperatively with the above-described molecules<sup>[29,102]</sup>. Depending on the microenvironment stimuli, MSCs can either activate or inhibit the action of NK cells. It has been shown that WJ-MSCs can stimulate the proliferation of NK cells *via* the production of inflammatory cytokines, including TNF- $\alpha$ , IL1 $\alpha$  /  $\beta$ , IL-6, and others<sup>[103]</sup>. More research is required regarding the interplay of MSCs and NK cells in order to understand better their association.

### **HLA-G and immune responses**

The HLA system has an essential key role in immune responses<sup>[104]</sup>. The HLA system includes the HLA class I and class II molecules, which are located within chromosome 6p21<sup>[105]</sup>. In this complex system, the HLA class I involves the classical A, B, and C genes and the non-classical genes E, F, and G, while the HLA class II involves the genes DP, DQ, DM, DO, and DR. Both, HLA class I and II are expressed in all cells, particularly in immune cells, and thus play a significant role in antigen recognition and presentation. On the other hand, the exact role of non-classical HLA genes has not yet been completely elucidated. Among them, HLA-G seems to share key immunoregulatory properties<sup>[104,105]</sup>.

For the first time, the role of HLA-G was investigated in pregnancy. The placental and fetus region of the umbilical cord express significant amounts of HLA-G, thus preventing the rejection of the semi-allogeneic fetus by the mother<sup>[104,106]</sup>. HLA-G involves the non-secreted membrane bound isoforms HLA-G1-4 and the soluble isoforms HLA-G5-7. It is speculated that HLA-G5 occurs after specific cleavage of membrane-bound HLA-G1 mediated by metalloproteinase-2. It seems that HLA-G exerts its anti-inflammatory and immunoregulatory properties in a wide variety of immune cells, including monocytes, DCs, NK cells, and T cells<sup>[104]</sup>.

Importantly, the HLA-G isoforms can interact with leukocyte immunoglobulin like receptor B1 (LILRB1, ILT2/CD88j), LIRB2 (ILT4/CD85d), and KIR2DL4 (CD158d), which are expressed in monocytes, DCs, T cells, and NK cells<sup>[104,107]</sup>. Indeed, the interaction of HLA-G with LILRB1 and LIRB2 induces the phosphorylation of the immunoreceptor tyrosine-base inhibitory motifs, followed by activation of SHP phosphatases, which lead to downregulation of MAPK and inhibition of cell proliferation<sup>[107]</sup>. Alongside IDO and IL10, HLA-G can suppress the proliferation of hyperacute T cells and can prevent the differentiation of monocytes to DCs<sup>[80,85]</sup>. In this way, the secreted cytokines, including TNF- $\alpha$ , IL1- $\alpha$ ,  $\beta$ , IL-6, IL-7, IL-8, IL-9, GM-CSF, and IFN- $\gamma$ , can be blocked indirectly *via* the HLA-G mediated inhibition of DCs and T cells. In a high inflammatory environment, HLA-G interactions can mediate the recruitment and the expansion of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cells (T reg), which in turn can inhibit effectively the proliferation of alloreactive T cells. Additionally, soluble HLA-G5-7 isoforms, can induce the secretion of IL-10 and TGF- $\beta$ 1, which further promotes the inhibition of CD8<sup>+</sup> T cell proliferation<sup>[104,108]</sup>. Several reports have indicated that HLA-G can influence the Th2 response, leading to increased production of anti-inflammatory cytokine IL-10, TGF- $\beta$ 1, and HGF. HLA-G can also modulate the function of NK cells by interacting with KIR2DL4. It has been described in the

literature that endocytosis of soluble HLA-G with KIR2DL4 is required for the inhibition of NK cells and cytokine production<sup>[109]</sup>. On the other hand, it has been reported that HLA-G homodimers are required for the secretion of TNF- $\alpha$ , IL-4, and IL-6<sup>[110]</sup>. This specific set of cytokines is required for successful trophoblast implantation. In this way, more experiments must be performed in order to elucidate better the interplay between HLA-G and NK cells.

MSCs, as part of their immunomodulation, are capable of producing both the membrane bound and soluble HLA-G isoforms. MSCs can be derived efficiently from placental and WJ-tissue, which is located within umbilical cord. Also, it has been shown that placental and WJ-MSCs may exert advanced immunoregulatory/ immunosuppressive properties compared to BM or AD -MSCs due to their embryonic origin<sup>[106]</sup>. Evidence from our previous work confirmed the expression of HLA-G isoforms by WJ-MSCs<sup>[106]</sup>. Moreover, HLA-G expression was retained after long term storage of WJ-MSCs with the vitrification approach. Vitrified WJ-MSCs after 1 year of storage at -196 °C successfully inhibited monocyte proliferation in mixed lymphocyte reaction studies<sup>[106]</sup>. In this way, MSCs can be isolated, expanded and stored long term without any negative effect to their immunomodulatory properties. Thus, they can be ready for use on demand.

## MSCS AND COVID-19

Until date, MSCs have been applied in large scale cell based therapies, where safety and treatment efficacy have been well documented<sup>[56]</sup>. Their unique role in immune modulation led to the use of MSCs as potential treatment in immunological based diseases, including GvHD, MS, ALS, SLE, *etc*<sup>[25,29]</sup>. Currently, no specific drugs against COVID-19 exist, while the development of satisfactory vaccines is a challenging process. Therefore, a safe and effective treatment is still under investigation. Taking into consideration that SARS-CoV-2 is responsible for an extended immunomodulation, caused by “cytokine storm”, the administration of MSCs may have a considerable therapeutic benefit, especially in patients with increased COVID-19 severity<sup>[8,9]</sup>. In addition, MSCs are negative for ACE2 receptor and TMPRSS2, and thus cannot be a target for SARS-CoV-2, making their application even safer<sup>[47]</sup>.

Currently, over 30 clinical trials, where MSCs can be potentially applied in COVID-19 as therapeutic agents, have been officially registered ([www.clinicaltrials.gov](http://www.clinicaltrials.gov))<sup>[111]</sup>. The majority of the clinical trials involve intravenous administration of MSCs to COVID-19 patients, but most of them are currently under the recruitment phase (Table 3).

Among them, a study of Leng *et al*<sup>[47]</sup> showed promising results in severe diseased COVID-19 patients. This study was conducted in a great number of hospitals, mostly in China, and was approved by the National Health Commission of China. In this study, clinical grade MSCs were intravenously infused in seven patients diagnosed with SARS-CoV-2. Importantly, one patient was in critical condition and entered the ICU, four patients exhibited severe type of the disease, and the last two were in better condition. Also, a placebo group was used, consisting of three severe type patients. The MSCs were administrated in a number of  $1 \times 10^6$  cells/kg with an infusion rate of 40 drops/min. After 2-4 d of MSC administration, the severe symptoms, including fever, oxygen saturation, and cough, were improved, and the majority of the patients were discharged. Also, the critically severe patients exited the ICU and successfully recovered. On the other hand, one patient from the placebo group was died, one developed ARDS, and one was in stable condition<sup>[47]</sup>.

Leng *et al*<sup>[47]</sup> showed that MSCs exerted their immunoregulatory properties and were capable of tolerating the hyperacute immune responses of patients, thus improving significantly the patients' overall condition (Figure 1). Indeed, the levels of C-reactive protein in the study group were declined, accompanied by a decrease in the TNF- $\alpha$  concentration. Mass cytometry analysis revealed that the overactivated CXCR3<sup>+</sup> CD4<sup>+</sup> T cells, CXCR3<sup>+</sup> CD8<sup>+</sup> T cells, and CXCR3<sup>+</sup> NK cells disappeared and that the CD14<sup>+</sup> CD11c<sup>+</sup> CD11b<sup>mid</sup> DCs reversed to normal conditions. Leukopenia was also reversed in all patients within 1 or 2 wk after MSCs administration. Furthermore, the ground glass opacity of lungs seemed to have subsided in patients<sup>[47]</sup>.

Another important issue that should be mentioned is the time point of MSC administration to COVID-19 patients. The exact time point for MSC administration varied among the studies. In a number of clinical trials, the MSCs are infused at specific time points (0, 2, 6, 10, 14 wk) after disease onset. In other studies, MSCs are infused in severe conditioned COVID-19 patients as a co-therapy alongside with their

**Table 3 Clinical trials associated with the use of mesenchymal stromal cells in coronavirus disease-19**

NCT number	Title	Status	MSCs origin	Route of infusion
NCT04252118	Mesenchymal stem cell treatment for pneumonia patients infected with COVID-19	Recruiting	Not specified	Intravenously
NCT04313322	Treatment of COVID-19 patients using Wharton's Jelly-mesenchymal stem cells	Recruiting	Wharton's Jelly MSCs	Intravenously
NCT04336254	Safety and efficacy study of allogeneic human dental pulp mesenchymal stem cells to treat severe COVID-19 patients	Recruiting	Allogeneic human dental pulp stem cells	Intravenously
NCT04288102	Treatment with mesenchymal stem cells for severe COVID-19	Recruiting	Not specified	Intravenously
NCT04346368	Bone marrow-derived mesenchymal stem cell treatment for severe patients with COVID-19	Not yet recruiting	BM-MSCs	Intravenously
NCT04366323	Clinical trial to assess the safety and efficacy of intravenous administration of allogeneic adult mesenchymal stem cells of expanded adipose tissue in patients with severe pneumonia due to COVID-19	Not yet recruiting	Allogeneic and expanded adipose tissue-derived MSCs	Intravenously
NCT04276987	A pilot clinical study on inhalation of mesenchymal stem cells exosomes treating severe novel coronavirus pneumonia	Not yet recruiting	MSCs-derived exosomes	5 times aerosol inhalation of MSCs derived exosomes
NCT04269525	Umbilical cord (UC)-derived mesenchymal stem cells (MSCs) treatment for the 2019-novel coronavirus (nCoV) pneumonia	Recruiting	UC-MSCs	Intravenously
NCT04348461	Battle against COVID-19 using mesenchymal stromal cells	Not yet recruiting	Allogeneic and expanded adipose tissue-derived MSCs	Intravenously
NCT03042143	Repair of acute respiratory distress syndrome by stromal cell administration (REALIST) (COVID-19)	Recruiting	Human umbilical cord derived CD362 enriched MSCs	Intravenously
NCT04333368	Cell therapy using umbilical cord-derived mesenchymal stromal cells in SARS-CoV-2-related ARDS	Recruiting	Umbilical cord Wharton's jelly-derived human	Intravenously
NCT04352803	Adipose mesenchymal cells for abatement of SARS-CoV-2 respiratory compromise in COVID-19 disease	Not yet recruiting	Autologous adipose MSC's	Intravenously

COVID-19: Coronavirus disease-19; MSCs: Mesenchymal stromal cells; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; ARDS: Acute respiratory distress syndrome; UC-MSCs: Umbilical cord-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells.

primary therapeutic protocol. Severe COVID-19 condition is established through the determination of specific clinical manifestations. Severe clinical manifestations include fever, respiratory distress (respiratory rate  $\geq 30/\text{min}$ ), low oxygen saturation ( $\leq 93\%$  at rest state), low arterial partial pressure of oxygen/fraction of inspiration  $\text{O}_2 \leq 300$  mmHg, while respiratory failure (connection of patients to extracorporeal membrane oxygenation), shock, and organ failure compromise the critically severe manifestations. The cytokine levels of IL-2, IL-6, IL-7, G-CSF, IP10, MCP1, MIP1A, and TNF- $\alpha$  are also determined and were mostly found to be elevated when patients are suffering from the severe manifestations and critically severe manifestations.

MSCs can exert their immunoregulatory properties by activating inflammatory cytokines, including IL-1, IL-6, IFN- $\gamma$  and TNF- $\alpha$ . At disease onset, patients are not characterized by increased alveolar epithelium damage due to the low virus replication. On the other hand, when virus load is high, severe alveolar epithelium damage can occur due to the production of inflammatory cytokines and the initiation of "cytokine storm". MSCs can now be activated (by inflammatory cytokines), which can lead to immunoregulation of the overactivated immune system. Besides these facts, MSCs could also have a prophylactic effect, and this could be the primary reason for their use at disease onset. Taking into consideration the above data, the specific time point for the administration of MSCs should be established that leads to patients' condition improvement.

Similar results regarding the immunomodulatory benefits of MSCs have been reported by other research groups around the world. Indeed, in the study of Petrou *et al*<sup>[112]</sup> the ALS severity score was decreased in all patients after MSCs infusion, while the treatment was proven safe and well tolerated.

In this way, MSCs can modulate effectively a series of immune responses, including

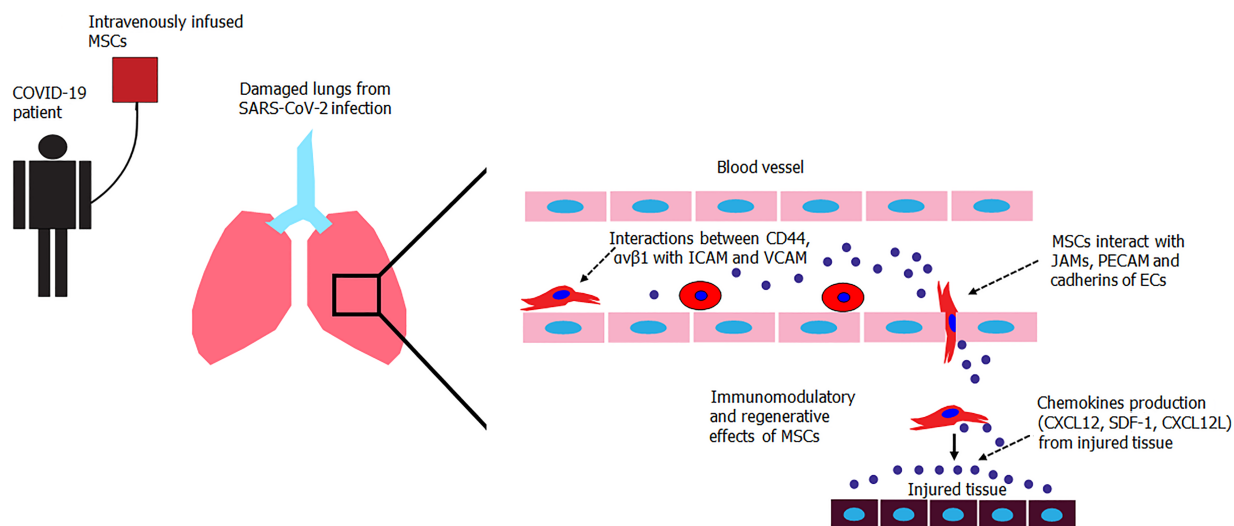
macrophages, DCs, NK, T, and B cells. Especially in COVID-19, the latest evidence supports that IL-6 is the main inflammatory cytokine for the immune system overactivation, and MSCs may be used as potential immunoregulatory players and inhibitors of IL-6 production<sup>[113]</sup>. MSCs can secrete anti-inflammatory cytokines, including IL-10. In addition, MSCs can induce apoptosis to overactivate T and B cells, promote Th2 responses, and activate M2 macrophages, regulating even more the inflammation.

Besides its well documented immunoregulatory properties, MSCs may exert key regenerative properties towards damaged alveolar epithelium and lung fibrosis<sup>[114]</sup>. In previous studies, it has been shown that MSCs accumulate in lung capillary vessels after intravenous injections<sup>[115]</sup>. MSCs engraft to the injury site in a chemokine-gradient dependent manner. Upon chemokine detection (CXCL12, SDF-1, CXCL12L), MSCs can upregulate CD44 and  $\alpha_v\beta_1$  integrins, and through the interaction with P/E selectins, vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) can perform the rolling adhesion to endothelium surface until reaching the injury site<sup>[25]</sup>. Then, transendothelial migration of MSCs occurs in a process mediated by junctional adhesion molecules, platelet-EC adhesion molecule-1, and cadherins (Figure 2). MSCs produce several growth factors, such as TGF- $\beta$ 1, VEGF, HGF, EGF, and IGF, that could induce the differentiation of alveolar epithelial cells from progenitor cells, attracting the M2 macrophages while reducing the scar tissue formation process<sup>[25]</sup>. This has been further confirmed by evidence regarding the reversion of ground glass opacity in lungs of COVID-19 patients after MSC infusion<sup>[47]</sup>.

The safe and tolerability of allogeneically infused MSCs have been reported in the past. There was evidence that intravenously infused MSCs could be accumulated in capillary vessels of lungs, thus raising the possibility of pulmonary embolism occurrence. In a phase I clinical trial performed by Iacobaeus *et al*<sup>[115]</sup>, no long-term engraftment of MSCs was performed in patients with MS. MSCs were further cleared from the circulation through the secondary lymphoid organs<sup>[115]</sup>. In another phase I clinical trial evaluating the possible treatment of ARDS, allogeneic BM-MSCs were well tolerated by the patients, and no infusion or treatment related adverse events were reported<sup>[116]</sup>. Moreover, Matthay *et al*<sup>[117]</sup> reported that intravenous injection of MSCs in a number of  $10 \times 10^6$  cells/kg was safe in patients with moderate ARDS. In both studies, after MSC infusion, the levels of IL-6 and IL-8 and the in-hospital mortality of patients were critically decreased, promoting even more the therapeutic potential of MSCs<sup>[47,117]</sup>. In recent years, AD-MSCs have gained significant interest by the scientific society for their regenerative and immunoregulatory properties. Recently, Gentile *et al*<sup>[26,27]</sup> reported that AD-MSCs exert key immunosuppressive properties *via* the secretion of TGF- $\beta$ 1, HGF, and IFN- $\gamma$  and remarkable regenerative properties through the secretion of VEGF and PDGF<sup>[26,27]</sup>. Indeed, besides their immunosuppressive properties, AD-MSCs can secrete pro-angiogenic factors such as VEGF and PDGF, which can stimulate the ECs, promoting in this way the vascularization and the regeneration of damaged lung tissue<sup>[26-28]</sup>. Moreover, a phase I clinical trial using autologous or allogenic AD-MSCs was conducted since April 2020 in COVID-19 patients, determining the safety and tolerability of these cells<sup>[26]</sup>.

Moreover, MSCs, upon environmental stimuli, are able to secrete EVs containing biomolecules, which effectively can promote key function processes, such as local immunomodulation and tissue regeneration<sup>[118]</sup>. EVs are composed of lipid bilayer membranes and can be classified into exosomes (microvesicles (MVs) with diameter of 30-100 nm), apoptotic bodies, and the recently described nanovesicles with diameter of 8-12 nm<sup>[119,120]</sup>. MVs mostly contain mRNAs, microRNAs, cytokines, and chemokines that can regulate a wide number of biological processes, including the function and homing of immune cells<sup>[121,122]</sup>. It has been shown that MVs derived from BM-MSCs suppress the expression of IL-1 $\beta$  and TNF- $\alpha$  and can promote the conversion of Th1 to Th2 responses<sup>[120,123]</sup>. Also, MVs contain the miRNAs miR-145, miR-146, and miR-155, which can effectively suppress the expression of TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1, followed by down-regulation of NF- $\kappa$ B p65 phosphorylation<sup>[120,122,124]</sup>. These events further lead to the impaired production of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 and is accompanied by inhibition of proliferation of M1 macrophages<sup>[120,122]</sup>. To date, a clinical trial that involves the application of inhaled exosomes derived from MSCs in patients with COVID-19 has been registered (NCT04276987). Moreover, MSC derived exosomes have been used as therapeutic agents in lung inflammatory diseases and tissue protection from fibrosis. In this context, WJ-MSCs derived exosomes significantly suppressed TGF- $\beta$ 1 expression and its downstream signaling pathway involving the inactivation of SMAD4 and SMAD5, leading to decreased collagen production and attenuation of scar tissue formation<sup>[120,125]</sup>. Exosomes can be produced under large scale good manufacturing





**Figure 2 Transendothelial migration of intravenously infused MSCs in COVID-19 patients.** Proposed mechanism of MSCs trafficking towards to chemokine stimuli produced from affected cells. MSCs perform endothelial rolling through interactions of CD44 and  $\alpha v \beta 1$  with intercellular cell adhesion molecule and vascular cell adhesion molecule. Transendothelial migration of MSCs is mediated through interactions of junctional adhesion molecular, platelet endothelial cell adhesion molecule PECAM, and cadherins. Upon arrival to their destination, MSCs exert their immunomodulatory and regenerative potential to the damaged tissue. COVID-19: Coronavirus disease-19; ICAM: Intercellular cell adhesion molecule; MSCs: Mesenchymal stromal cells; VCAM: Vascular cell adhesion molecule; PECAM: Platelet endothelial cell adhesion molecule; JAM: Junctional adhesion molecular; ECs: Endothelial cells.

practices (GMPs) conditions and could act beneficially in COVID-19 patients.

## CURRENT LEGISLATION DESCRIBING THE USE OF MSCS

The use of MSCs in COVID-19 patients is categorized as stem cell-based therapies, and therefore the guidelines of International Society for Stem Cell Research must be strictly followed<sup>[126]</sup>. Moreover, International Society for Stem Cell Research released the “The Guidelines for the Clinical Translation of Stem Cells”, which involves the current standards for the development of stem cell-based therapies, leading to proven therapeutic potential for the patient<sup>[126]</sup>.

To promote the therapeutic outcome for the patients, MSC based therapies must overcome a great number of challenges, including expansion time, required cell number-dosage, cell cultivation, and cell exposure to animal-derived products, which can affect significantly the safety and efficacy. Furthermore, prolonged *in vitro* cultured stem cells may lose their key function characteristics such as stemness and plasticity, or even more could undergo malignant tumorigenic transformation<sup>[126]</sup>.

According to the “Guidance of Human Somatic Cell therapy and Gene Therapy” established for human cells, tissues, and cellular, tissue-based products (HCT/Ps), stem cell-based products must be developed under GMPs conditions. Also, additional regulations exist depending on the cell manipulation or either if HCT/Ps are intended to be used in homologous or allogeneic setting<sup>[126]</sup>. FDA has defined the minimal manipulation as the process, which is not altering the biological characteristics of stem cells. Moreover, processes including density-gradient separation, cell selection, centrifugation, and cryopreservation are defined as minimal manipulation HCT/Ps<sup>[126]</sup>.

All other processes involving cell cultivation, expansion, exposure to animal derived substances, activation, and gene modifications are considered as more-than-minimal-manipulated HCT/Ps<sup>[126]</sup>. Furthermore, the use of MSCs in allogeneic setting must fulfill donor eligibility (donor screening and testing for communicable diseases), Quality Assurance program (GMPs conditions and Quality Control system), proper storage conditions (long-term stem cell stability under cryopreserved conditions), and distributing-release criteria. The aforementioned information must be specifically defined in Standard Operation Procedure documentation in order to reduce the risk occurrence for the patient. Trials regarding the homologous or allogeneic use of MSCs must be approved by the corresponding national health commission<sup>[126]</sup>.

All MSC based therapies performed in United States must be registered to the FDA. Accordingly, the European Medicines Agency and the Therapeutic Goods



Administration is responsible for the cell-based therapies performed in Europe and in Australia, respectively. According to Committee for Advanced Therapies, which has been established by the European Medicines Agency, all MSC-based products in the European Union will be defined as Advanced Therapeutic Medicinal Products, with the only exception being the aforementioned minimally manipulated stem cells<sup>[126]</sup>. The above requirements and the developed authorities make the MSC based therapies even safer for the patients, bringing stem cell based therapy one step closer to broad clinical utility.

## CONCLUDING REMARKS

The above evidence clearly indicate that MSCs could be used as potential therapeutic agents in patients suffering from COVID-19. MSCs can modulate effectively the overactivated immune responses caused by SARS-CoV-2 through inactivation of macrophages and NK, T, and B cells, while at the same time could promote the Th2 response and the activation of M2 macrophages<sup>[25,29]</sup>. In addition, MSCs could have a beneficial regenerative effect in damaged lungs and alveolar epithelial cells, reversing in this way the ground glass opacity in patient's lungs<sup>[67]</sup>.

Until now, several treatment protocols have been proposed, such as the administration of remdesivir, hydrochloroquine, colchicine, and vaccination of high risk patients<sup>[20,22]</sup>. However, most of these treatments are accompanied by significant adverse reactions, limiting their application to patients. The induced "cytokine storm" by SARS-CoV-2 infection has attracted the attention of the scientific society. This phenomenon, and specifically IL-6, may be responsible for the immune system overactivation and also is associated with poor prognosis in critically ill patients.

On the other hand, MSCs, which are negative for ACE2 receptor and TMPRSS2, could be effective in regulating the "cytokine storm"<sup>[47]</sup>. Moreover, WJ-MSCs may possess better immunoregulatory properties through the elevated expression of HLA-G, the existence of longer telomeres, and fewer epigenetic modifications compared to MSCs from the other sources<sup>[106]</sup>. AD-MSCs that are extensively used in a great series of personalized regenerative medicine applications, including breast reconstruction with fat graft, androgenic alopecia, *etc*, exhibited similar properties as the MSCs from other sources<sup>[127-133]</sup>.

Recently, the application of AD-MSCs as immunoregulatory agents in COVID-19 patients is gaining day by day more attention by the scientific society, worldwide<sup>[26,27]</sup>. AD-MSCs can be efficiently isolated in great quantities from SVF or subcutaneous AT, expanded under clinical grade GMP conditions, and applied through intravenously infusion to critically-ill COVID-19 patients<sup>[26,27]</sup>. AD-MSCs exert equal immunoregulatory properties as MSCs from BM and WJ tissue through secretion of soluble factors or cell-cell contact interactions, as has been described previously<sup>[26,27]</sup>. Furthermore, AD-MSCs can be efficiently differentiated towards "chondrogenic" and "osteogenic" lineages and can produce significant amounts of proangiogenic factors, indicating their significant regenerative potential<sup>[26,27,127]</sup>. These regenerative properties of AD-MSCs may prove to be of great importance in restoration of lung alveolar epithelium and reversion of ground-glass opacity in critically-ill COVID-19 patients. Taking into account the above data, AD-MSCs may also represent a safe and effective therapeutic strategy for COVID-19 patients, and may be the best cellular population option when a great number of MSCs are required for immediate use.

## CONCLUSION

MSCs can be potentially applied in COVID-19 as co-therapy without having severe adverse reactions. The exact time point of MSCs administration should be established in order to obtain the best outcome for patients<sup>[134]</sup>. It has been shown in literature that MSCs can suppress the overactivated immune system of severe and critically severe patients. Simultaneously, MSCs may also possess a prophylactic effect if used at disease onset. Existing evidence suggest that MSCs are important immunoregulatory players, but more research is needed, in order to obtain safer conclusions. The ultimate goal will be to decrease the COVID-19 mortality rate, and this is where MSCs can assist significantly.

## REFERENCES

- 1 **Lu H**, Stratton CW, Tang YW. Outbreak of pneumonia of unknown etiology in Wuhan, China: The mystery and the miracle. *J Med Virol* 2020; **92**: 401-402 [PMID: 31950516 DOI: 10.1002/jmv.25678]
- 2 **Coronaviridae Study Group of the International Committee on Taxonomy of Viruses**. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* 2020; **5**: 536-544 [PMID: 32123347 DOI: 10.1038/s41564-020-0695-z]
- 3 **Lai CC**, Shih TP, Ko WC, Tang HJ, Hsueh PR. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): The epidemic and the challenges. *Int J Antimicrob Agents* 2020; **55**: 105924 [PMID: 32081636 DOI: 10.1016/j.ijantimicag.2020.105924]
- 4 **Johns Hopkins University of Medicine**. COVID-19 Map. 2020 [cited 7 May 2020]. Available from: <https://coronavirus.jhu.edu/map.html>
- 5 **Chen N**, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020; **395**: 507-513 [PMID: 32007143 DOI: 10.1016/S0140-6736(20)30211-7]
- 6 **Yuan S**, Liao Z, Huang H, Jiang B, Zhang X, Wang Y, Zhao M. Comparison of the Indicators of Psychological Stress in the Population of Hubei Province and Non-Endemic Provinces in China During Two Weeks During the Coronavirus Disease 2019 (COVID-19) Outbreak in February 2020. *Med Sci Monit* 2020; **26**: e923767 [PMID: 32294078 DOI: 10.12659/MSM.923767]
- 7 **Chang**, Lin M, Wei L, Xie L, Zhu G, Dela Cruz CS, Sharma L. Epidemiologic and Clinical Characteristics of Novel Coronavirus Infections Involving 13 Patients Outside Wuhan, China. *JAMA* 2020; **323**: 1092-1093 [PMID: 32031568 DOI: 10.1001/jama.2020.1623]
- 8 **Ye Q**, Wang B, Mao J. The pathogenesis and treatment of the 'Cytokine Storm' in COVID-19. *J Infect* 2020; **80**: 607-613 [PMID: 32283152 DOI: 10.1016/j.jinf.2020.03.037]
- 9 **Mehta P**, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ; HLH Across Speciality Collaboration, UK. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet* 2020; **395**: 1033-1034 [PMID: 32192578 DOI: 10.1016/S0140-6736(20)30628-0]
- 10 **Law HK**, Cheung CY, Ng HY, Sia SF, Chan YO, Luk W, Nicholls JM, Peiris JS, Lau YL. Chemokine up-regulation in SARS-coronavirus-infected, monocyte-derived human dendritic cells. *Blood* 2005; **106**: 2366-2374 [PMID: 15860669 DOI: 10.1182/blood-2004-10-4166]
- 11 **Promptchara E**, Ketloy C, Palaga T. Immune responses in COVID-19 and potential vaccines: Lessons learned from SARS and MERS epidemic. *Asian Pac J Allergy Immunol* 2020; **38**: 1-9 [PMID: 32105090 DOI: 10.12932/AP-200220-0772]
- 12 **Li T**, Lu H, Zhang W. Clinical observation and management of COVID-19 patients. *Emerg Microbes Infect* 2020; **9**: 687-690 [PMID: 32208840 DOI: 10.1080/22221751.2020.1741327]
- 13 **Schwartz DA**. An Analysis of 38 Pregnant Women with COVID-19, Their Newborn Infants, and Maternal-Fetal Transmission of SARS-CoV-2: Maternal Coronavirus Infections and Pregnancy Outcomes. *Arch Pathol Lab Med* 2020 [PMID: 32180426 DOI: 10.5858/arpa.2020-0901-SA]
- 14 **Chen D**, Yang H, Cao Y, Cheng W, Duan T, Fan C, Fan S, Feng L, Gao Y, He F, He J, Hu Y, Jiang Y, Li Y, Li J, Li X, Li X, Lin K, Liu C, Liu J, Liu X, Pan X, Pang Q, Pu M, Qi H, Shi C, Sun Y, Sun J, Wang X, Wang Y, Wang Z, Wang Z, Wang C, Wu S, Xin H, Yan J, Zhao Y, Zheng J, Zhou Y, Zou L, Zeng Y, Zhang Y, Guan X. Expert consensus for managing pregnant women and neonates born to mothers with suspected or confirmed novel coronavirus (COVID-19) infection. *Int J Gynaecol Obstet* 2020; **149**: 130-136 [PMID: 32196655 DOI: 10.1002/ijgo.13146]
- 15 **Lu Q**, Shi Y. Coronavirus disease (COVID-19) and neonate: What neonatologist need to know. *J Med Virol* 2020; **92**: 564-567 [PMID: 32115733 DOI: 10.1002/jmv.25740]
- 16 **Gendelman O**, Amital H, Bragazzi NL, Watad A, Chodick G. Continuous hydroxychloroquine or colchicine therapy does not prevent infection with SARS-CoV-2: Insights from a large healthcare database analysis. *Autoimmun Rev* 2020; **19**: 102566 [PMID: 32380315 DOI: 10.1016/j.autrev.2020.102566]
- 17 **Zhou G**, Zhao Q. Perspectives on therapeutic neutralizing antibodies against the Novel Coronavirus SARS-CoV-2. *Int J Biol Sci* 2020; **16**: 1718-1723 [PMID: 32226289 DOI: 10.7150/ijbs.45123]
- 18 **Dzik S**. COVID-19 Convalescent Plasma: Now Is the Time for Better Science. *Transfus Med Rev* 2020 [PMID: 32359789 DOI: 10.1016/j.tmr.2020.04.002]
- 19 **Ullah M**. Novel coronavirus (covid-19) treatment options. 2020 [DOI: 10.31219/osf.io/dv6u3]
- 20 **Sheahan TP**, Sims AC, Leist SR, Schäfer A, Won J, Brown AJ, Montgomery SA, Hogg A, Babusis D, Clarke MO, Spahn JE, Bauer L, Sellers S, Porter D, Feng JY, Cihlar T, Jordan R, Denison MR, Baric RS. Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat Commun* 2020; **11**: 222 [PMID: 31924756 DOI: 10.1038/s41467-019-13940-6]
- 21 **Wang M**, Cao R, Zhang L, Yang X, Liu J, Xu M, Shi Z, Hu Z, Zhong W, Xiao G. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res* 2020; **30**: 269-271 [PMID: 32020029 DOI: 10.1038/s41422-020-0282-0]
- 22 **Agostini ML**, Andres EL, Sims AC, Graham RL, Sheahan TP, Lu X, Smith EC, Case JB, Feng JY, Jordan R, Ray AS, Cihlar T, Siegel D, Mackman RL, Clarke MO, Baric RS, Denison MR. Coronavirus Susceptibility to the Antiviral Remdesivir (GS-5734) Is Mediated by the Viral Polymerase and the Proofreading Exoribonuclease. *mBio* 2018; **9**: e00221-18 [PMID: 29511076 DOI: 10.1128/mBio.00221-18]
- 23 **Tseng CT**, Sbrana E, Iwata-Yoshikawa N, Newman PC, Garron T, Atmar NL, Peters CJ, Couch RB. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PLoS One* 2012; **7**: e35421 [PMID: 22536382 DOI: 10.1371/journal.pone.0035421]
- 24 **Ahn DG**, Shin HJ, Kim MH, Lee S, Kim HS, Myoung J, Kim BT, Kim SJ. Current Status of Epidemiology, Diagnosis, Therapeutics, and Vaccines for Novel Coronavirus Disease 2019 (COVID-19). *J Microbiol Biotechnol* 2020; **30**: 313-324 [PMID: 32238757 DOI: 10.4014/jmb.2003.03011]
- 25 **Salem HK**, Thiernemann C. Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* 2010; **28**: 585-596 [PMID: 19967788 DOI: 10.1002/stem.269]

- 26 **Gentile P**, Sterodimas A. Adipose-derived stromal stem cells (ASCs) as a new regenerative immediate therapy combating coronavirus (COVID-19)-induced pneumonia. *Expert Opin Biol Ther* 2020; **20**: 711-716 [PMID: [32329380](#) DOI: [10.1080/14712598.2020.1761322](#)]
- 27 **Gentile P**, Sterodimas A. Adipose Stem Cells (ASCs) and Stromal Vascular Fraction (SVF) as a Potential Therapy in Combating (COVID-19)-Disease. *Aging Dis* 2020; **11**: 465-469 [PMID: [32489692](#) DOI: [10.14336/AD.2020.0422](#)]
- 28 **Rogers CJ**, Harman RJ, Bunnell BA, Schreiber MA, Xiang C, Wang FS, Santidrian AF, Minev BR. Rationale for the clinical use of adipose-derived mesenchymal stem cells for COVID-19 patients. *J Transl Med* 2020; **18**: 203 [PMID: [32423449](#) DOI: [10.1186/s12967-020-02380-2](#)]
- 29 **Yagi H**, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N, Yarmush ML. Mesenchymal stem cells: Mechanisms of immunomodulation and homing. *Cell Transplant* 2010; **19**: 667-679 [PMID: [20525442](#) DOI: [10.3727/096368910X508762](#)]
- 30 **International Committee on Taxonomy of Viruses**. 2020 [cited 7 May 2020]. Available from: <https://talk.ictvonline.org/information/w/news/1300/page>
- 31 **Wang Q**, Zhang Y, Wu L, Niu S, Song C, Zhang Z, Lu G, Qiao C, Hu Y, Yuen KY, Wang Q, Zhou H, Yan J, Qi J. Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. *Cell* 2020; **181**: 894-904.e9 [PMID: [32275855](#) DOI: [10.1016/j.cell.2020.03.045](#)]
- 32 **Walls AC**, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell* 2020; **181**: 281-292.e6 [PMID: [32155444](#) DOI: [10.1016/j.cell.2020.02.058](#)]
- 33 **Chen L**, Liu W, Zhang Q, Xu K, Ye G, Wu W, Sun Z, Liu F, Wu K, Zhong B, Mei Y, Zhang W, Chen Y, Li Y, Shi M, Lan K, Liu Y. RNA based mNGS approach identifies a novel human coronavirus from two individual pneumonia cases in 2019 Wuhan outbreak. *Emerg Microbes Infect* 2020; **9**: 313-319 [PMID: [32020836](#) DOI: [10.1080/22221751.2020.1725399](#)]
- 34 **Jiang S**, Du L, Shi Z. An emerging coronavirus causing pneumonia outbreak in Wuhan, China: calling for developing therapeutic and prophylactic strategies. *Emerg Microbes Infect* 2020; **9**: 275-277 [PMID: [32005086](#) DOI: [10.1080/22221751.2020.1723441](#)]
- 35 **Lu R**, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 2020; **395**: 565-574 [PMID: [32007145](#) DOI: [10.1016/S0140-6736\(20\)30251-8](#)]
- 36 **Ren LL**, Wang YM, Wu ZQ, Xiang ZC, Guo L, Xu T, Jiang YZ, Xiong Y, Li YJ, Li XW, Li H, Fan GH, Gu XY, Xiao Y, Gao H, Xu JY, Yang F, Wang XM, Wu C, Chen L, Liu YW, Liu B, Yang J, Wang XR, Dong J, Li L, Huang CL, Zhao JP, Hu Y, Cheng ZS, Liu LL, Qian ZH, Qin C, Jin Q, Cao B, Wang JW. Identification of a novel coronavirus causing severe pneumonia in human: a descriptive study. *Chin Med J (Engl)* 2020; **133**: 1015-1024 [PMID: [32004165](#) DOI: [10.1097/CM9.0000000000000722](#)]
- 37 **Zhou P**, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, Guo H, Jiang RD, Liu MQ, Chen Y, Shen XR, Wang X, Zheng XS, Zhao K, Chen QJ, Deng F, Liu LL, Yan B, Zhan FX, Wang YY, Xiao GF, Shi ZL. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020; **579**: 270-273 [PMID: [32015507](#) DOI: [10.1038/s41586-020-2012-7](#)]
- 38 **Wrapp D**, De Vlieger D, Corbett KS, Torres GM, Wang N, Van Breedam W, Roose K, van Schie L; VIB-CMB COVID-19 Response Team, Hoffmann M, Pöhlmann S, Graham BS, Callewaert N, Schepens B, Saelens X, McLellan JS. Structural Basis for Potent Neutralization of Betacoronaviruses by Single-Domain Camelid Antibodies. *Cell* 2020; **181**: 1004-1015.e15 [PMID: [32375025](#) DOI: [10.1016/j.cell.2020.04.031](#)]
- 39 **Wrapp D**, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, Graham BS, McLellan JS. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 2020; **367**: 1260-1263 [PMID: [32075877](#) DOI: [10.1126/science.abb2507](#)]
- 40 **Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280.e8 [PMID: [32142651](#) DOI: [10.1016/j.cell.2020.02.052](#)]
- 41 **Chen YW**, Lee MS, Lucht A, Chou FP, Huang W, Havighurst TC, Kim K, Wang JK, Antalis TM, Johnson MD, Lin CY. TMPRSS2, a serine protease expressed in the prostate on the apical surface of luminal epithelial cells and released into semen in prostasomes, is misregulated in prostate cancer cells. *Am J Pathol* 2010; **176**: 2986-2996 [PMID: [20382709](#) DOI: [10.2353/ajpath.2010.090665](#)]
- 42 **Song H**, Seddighzadeh B, Cooperberg MR, Huang FW. Expression of ACE2, the SARS-CoV-2 receptor, and TMPRSS2 in prostate epithelial cells. 2020 Preprint. Available from: [bioRxiv](https://doi.org/10.1101/2020.04.24.056259) [DOI: [10.1101/2020.04.24.056259](#)]
- 43 **Vaara MH**, Porvari KS, Kellokumpu S, Kyllönen AP, Vihko PT. Expression of transmembrane serine protease TMPRSS2 in mouse and human tissues. *J Pathol* 2001; **193**: 134-140 [PMID: [11169526](#) DOI: [10.1002/1096-9896\(2000\)9999:9999::AID-PATH743>3.0.CO;2-T](#)]
- 44 **Lin L**, Lu L, Cao W, Li T. Hypothesis for potential pathogenesis of SARS-CoV-2 infection-a review of immune changes in patients with viral pneumonia. *Emerg Microbes Infect* 2020; **9**: 727-732 [PMID: [32196410](#) DOI: [10.1080/22221751.2020.1746199](#)]
- 45 **Chen Y**, Li L. SARS-CoV-2: virus dynamics and host response. *Lancet Infect Dis* 2020; **20**: 515-516 [PMID: [32213336](#) DOI: [10.1016/S1473-3099\(20\)30235-8](#)]
- 46 **Shereen MA**, Khan S, Kazmi A, Bashir N, Siddique R. COVID-19 infection: Origin, transmission, and characteristics of human coronaviruses. *J Adv Res* 2020; **24**: 91-98 [PMID: [32257431](#) DOI: [10.1016/j.jare.2020.03.005](#)]
- 47 **Leng Z**, Zhu R, Hou W, Feng Y, Yang Y, Han Q, Shan G, Meng F, Du D, Wang S, Fan J, Wang W, Deng L, Shi H, Li H, Hu Z, Zhang F, Gao J, Liu H, Li X, Zhao Y, Yin K, He X, Gao Z, Wang Y, Yang B, Jin R, Stambler I, Lim LW, Su H, Moskalev A, Cano A, Chakrabarti S, Min KJ, Ellison-Hughes G, Caruso C, Jin K, Zhao RC. Transplantation of ACE2<sup>+</sup> Mesenchymal Stem Cells Improves the Outcome of Patients with

- COVID-19 Pneumonia. *Aging Dis* 2020; **11**: 216-228 [PMID: [32257537](#) DOI: [10.14336/AD.2020.0228](#)]
- 48 **Conti P**, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, Kritas SK. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. *J Biol Regul Homeost Agents* 2020; **34**: 1 [PMID: [32171193](#) DOI: [10.23812/CONTI-E](#)]
- 49 **Rahmati M**, Moosavi MA. Cytokine-targeted therapy in severely ill covid-19 patients: Options and cautions. *E. ur J Med Oncol* 2020; **4**: 179-180 [DOI: [10.14744/ejmo.2020.72142](#)]
- 50 **Wan S**, Yi Q, Fan S, Lv J, Zhang X, Guo L, Lang C, Xiao Q, Xiao K, Yi Z, Qiang M, Xiang J, Zhang B, Chen Y. Characteristics of lymphocyte subsets and cytokines in peripheral blood of 123 hospitalized 3 patients with 2019 novel coronavirus pneumonia (NCP). 2020 Preprint. Available from: [medRxiv](#) [DOI: [10.1101/2020.02.10.20021832](#)]
- 51 **Xu Z**, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med* 2020; **8**: 420-422 [PMID: [32085846](#) DOI: [10.1016/S2213-2600\(20\)30076-X](#)]
- 52 **Shi Y**, Wang Y, Shao C, Huang J, Gan J, Huang X, Bucci E, Piacentini M, Ippolito G, Melino G. COVID-19 infection: the perspectives on immune responses. *Cell Death Differ* 2020; **27**: 1451-1454 [PMID: [32205856](#) DOI: [10.1038/s41418-020-0530-3](#)]
- 53 **Bell TJ**, Brand OJ, Morgan DJ, Salek-Ardakani S, Jagger C, Fujimori T, Cholewa L, Tilakaratna V, Östling J, Thomas M, Day AJ, Snelgrove RJ, Hussell T. Defective lung function following influenza virus is due to prolonged, reversible hyaluronan synthesis. *Matrix Biol* 2019; **80**: 14-28 [PMID: [29933044](#) DOI: [10.1016/j.matbio.2018.06.006](#)]
- 54 **Collum SD**, Chen NY, Hernandez AM, Hanmandlu A, Sweeney H, Mertens TCJ, Weng T, Luo F, Molina JG, Davies J, Horan IP, Morrell NW, Amione-Guerra J, Al-Jabbari O, Youker K, Sun W, Rajadas J, Bollyky PL, Akkanti BH, Jyothula S, Sinha N, Guha A, Karmouty-Quintana H. Inhibition of hyaluronan synthesis attenuates pulmonary hypertension associated with lung fibrosis. *Br J Pharmacol* 2017; **174**: 3284-3301 [PMID: [28688167](#) DOI: [10.1111/bph.13947](#)]
- 55 **Jiang S**, Hillyer C, Du L. Neutralizing Antibodies against SARS-CoV-2 and Other Human Coronaviruses. *Trends Immunol* 2020; **41**: 355-359 [PMID: [32249063](#) DOI: [10.1016/j.it.2020.03.007](#)]
- 56 **Kabat M**, Bobkov I, Kumar S, Grumet M. Trends in mesenchymal stem cell clinical trials 2004-2018: Is efficacy optimal in a narrow dose range? *Stem Cells Transl Med* 2020; **9**: 17-27 [PMID: [31804767](#) DOI: [10.1002/sctm.19-0202](#)]
- 57 **Mushahary D**, Spittler A, Kasper C, Weber V, Charwat V. Isolation, cultivation, and characterization of human mesenchymal stem cells. *Cytometry A* 2018; **93**: 19-31 [PMID: [29072818](#) DOI: [10.1002/cyto.a.23242](#)]
- 58 **Bianco P**, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008; **2**: 313-319 [PMID: [18397751](#) DOI: [10.1016/j.stem.2008.03.002](#)]
- 59 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz Z. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: [16923606](#) DOI: [10.1080/14653240600855905](#)]
- 60 **Viswanathan S**, Shi Y, Galipeau J, Krampera M, Leblanc K, Martin I, Nolta J, Phinney DG, Sensebe L. Mesenchymal stem versus stromal cells: International Society for Cell & Gene Therapy (ISCT®) Mesenchymal Stromal Cell committee position statement on nomenclature. *Cytotherapy* 2019; **21**: 1019-1024 [PMID: [31526643](#) DOI: [10.1016/j.jcyt.2019.08.002](#)]
- 61 **Saleh M**, Shamsasanjan K, Movassaghpourakbari A, Akbarzadehlaleh P, Molaeipour Z. The Impact of Mesenchymal Stem Cells on Differentiation of Hematopoietic Stem Cells. *Adv Pharm Bull* 2015; **5**: 299-304 [PMID: [26504750](#) DOI: [10.15171/apb.2015.042](#)]
- 62 **Troyer DL**, Weiss ML. Wharton's jelly-derived cells are a primitive stromal cell population. *Stem Cells* 2008; **26**: 591-599 [PMID: [18065397](#) DOI: [10.1634/stemcells.2007-0439](#)]
- 63 **Baer PC**, Geiger H. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. *Stem Cells Int* 2012; **2012**: 812693 [PMID: [22577397](#) DOI: [10.1155/2012/812693](#)]
- 64 **Marion NW**, Mao JJ. Mesenchymal stem cells and tissue engineering. *Methods Enzymol* 2006; **420**: 339-361 [PMID: [17161705](#) DOI: [10.1016/S0076-6879\(06\)20016-8](#)]
- 65 **Galipeau J**. Mesenchymal Stromal Cells for Graft-versus-Host Disease: A Trilogy. *Biol Blood Marrow Transplant* 2020; **26**: e89-e91 [PMID: [32156632](#) DOI: [10.1016/j.bbmt.2020.02.023](#)]
- 66 **Figuerola FE**, Carrión F, Villanueva S, Khoury M. Mesenchymal stem cell treatment for autoimmune diseases: a critical review. *Biol Res* 2012; **45**: 269-277 [PMID: [23283436](#) DOI: [10.4067/S0716-97602012000300008](#)]
- 67 **Han Y**, Li X, Zhang Y, Han Y, Chang F, Ding J. Mesenchymal Stem Cells for Regenerative Medicine. *Cells* 2019; **8**: 886 [PMID: [31412678](#) DOI: [10.3390/cells8080886](#)]
- 68 **Protogerou V**, Beshari SE, Michalopoulos E, Mallis P, Chrysikos D, Samolis AA, Stavropoulos-Giokas C, Troupis T. The Combined Use of Stem Cells and Platelet Lysate Plasma for the Treatment of Erectile Dysfunction: A Pilot Study-6 Months Results. *Medicines (Basel)* 2020; **7**: 14 [PMID: [32197323](#) DOI: [10.3390/medicines7030014](#)]
- 69 **Protogerou V**, Michalopoulos E, Mallis P, Gontika I, Dimou Z, Liakouras C, Stavropoulos-Giokas C, Kostakopoulos N, Chrisofos M, Deliveliotis C. Administration of Adipose Derived Mesenchymal Stem Cells and Platelet Lysate in Erectile Dysfunction: A Single Center Pilot Study. *Bioengineering (Basel)* 2019; **6**: 21 [PMID: [30841525](#) DOI: [10.3390/bioengineering6010021](#)]
- 70 **Steens J**, Klein D. Current Strategies to Generate Human Mesenchymal Stem Cells In Vitro. *Stem Cells Int* 2018; **2018**: 6726185 [PMID: [30224922](#) DOI: [10.1155/2018/6726185](#)]
- 71 **Le Blanc K**, Tammik C, Rosendahl K, Zetterberg E, Ringdén O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003; **31**: 890-896 [PMID: [14550804](#) DOI: [10.1016/s0301-472x\(03\)00110-3](#)]
- 72 **Devine SM**, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, Sturgeon C, Hewett T, Chung T,



- Stock W, Sher D, Weissman S, Ferrer K, Mosca J, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol* 2001; **29**: 244-255 [PMID: [11166464](#) DOI: [10.1016/s0301-472x\(00\)00635-4](#)]
- 73 **Németh K**, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009; **15**: 42-49 [PMID: [19098906](#) DOI: [10.1038/nm.1905](#)]
- 74 **Shapouri-Moghaddam A**, Mohammadian S, Vazini H, Taghadossi M, Esmaili SA, Mardani F, Seifi B, Mohammadi A, Afshari JT, Sahebkar A. Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol* 2018; **233**: 6425-6440 [PMID: [29319160](#) DOI: [10.1002/jcp.26429](#)]
- 75 **Bernardo ME**, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* 2013; **13**: 392-402 [PMID: [24094322](#) DOI: [10.1016/j.stem.2013.09.006](#)]
- 76 **Gur-Wahnon D**, Borovsky Z, Beyth S, Liebergall M, Rachmilewitz J. Contact-dependent induction of regulatory antigen-presenting cells by human mesenchymal stem cells is mediated via STAT3 signaling. *Exp Hematol* 2007; **35**: 426-433 [PMID: [17309823](#) DOI: [10.1016/j.exphem.2006.11.001](#)]
- 77 **Mellman I**, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 2001; **106**: 255-258 [PMID: [11509172](#) DOI: [10.1016/s0092-8674\(01\)00449-4](#)]
- 78 **Djouad F**, Charbonnier LM, Bouffi C, Louis-Plence P, Bony C, Apparailly F, Cantos C, Jorgensen C, Noël D. Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells* 2007; **25**: 2025-2032 [PMID: [17510220](#) DOI: [10.1634/stemcells.2006-0548](#)]
- 79 **English K**, Barry FP, Mahon BP. Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. *Immunol Lett* 2008; **115**: 50-58 [PMID: [18022251](#) DOI: [10.1016/j.imlet.2007.10.002](#)]
- 80 **Spaggiari GM**, Abdelrazik H, Becchetti F, Moretta L. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. *Blood* 2009; **113**: 6576-6583 [PMID: [19398717](#) DOI: [10.1182/blood-2009-02-203943](#)]
- 81 **van Megen KM**, van 't Wout ET, Lages Motta J, Dekker B, Nikolic T, Roep BO. Activated Mesenchymal Stromal Cells Process and Present Antigens Regulating Adaptive Immunity. *Front Immunol* 2019; **10**: 694 [PMID: [31001285](#) DOI: [10.3389/fimmu.2019.00694](#)]
- 82 **Liu Y**, Yin Z, Zhang R, Yan K, Chen L, Chen F, Huang W, Lv B, Sun C, Jiang X. MSCs inhibit bone marrow-derived DC maturation and function through the release of TSG-6. *Biochem Biophys Res Commun* 2014; **450**: 1409-1415 [PMID: [25014173](#) DOI: [10.1016/j.bbrc.2014.07.001](#)]
- 83 **Scalavino V**, Liso M, Serino G. Role of microRNAs in the Regulation of Dendritic Cell Generation and Function. *Int J Mol Sci* 2020; **21**: 1319 [PMID: [32075292](#) DOI: [10.3390/ijms21041319](#)]
- 84 **Chen W**, Huang Y, Han J, Yu L, Li Y, Lu Z, Li H, Liu Z, Shi C, Duan F, Xiao Y. Immunomodulatory effects of mesenchymal stromal cells-derived exosome. *Immunol Res* 2016; **64**: 831-840 [PMID: [27115513](#) DOI: [10.1007/s12026-016-8798-6](#)]
- 85 **Haddad R**, Saldanha-Araujo F. Mechanisms of T-cell immunosuppression by mesenchymal stromal cells: what do we know so far? *Biomed Res Int* 2014; **2014**: 216806 [PMID: [25025040](#) DOI: [10.1155/2014/216806](#)]
- 86 **Sreeramkumar V**, Fresno M, Cuesta N. Prostaglandin E2 and T cells: friends or foes? *Immunol Cell Biol* 2012; **90**: 579-586 [PMID: [21946663](#) DOI: [10.1038/icb.2011.75](#)]
- 87 **Ricciotti E**, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* 2011; **31**: 986-1000 [PMID: [21508345](#) DOI: [10.1161/ATVBAHA.110.207449](#)]
- 88 **Ryan JM**, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin Exp Immunol* 2007; **149**: 353-363 [PMID: [17521318](#) DOI: [10.1111/j.1365-2249.2007.03422.x](#)]
- 89 **Ren G**, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008; **2**: 141-150 [PMID: [18371435](#) DOI: [10.1016/j.stem.2007.11.014](#)]
- 90 **Wang L**, Zhao Y, Shi S. Interplay between mesenchymal stem cells and lymphocytes: implications for immunotherapy and tissue regeneration. *J Dent Res* 2012; **91**: 1003-1010 [PMID: [22988011](#) DOI: [10.1177/0022034512460404](#)]
- 91 **Davies LC**, Heldring N, Kadri N, Le Blanc K. Mesenchymal Stromal Cell Secretion of Programmed Death-1 Ligands Regulates T Cell Mediated Immunosuppression. *Stem Cells* 2017; **35**: 766-776 [PMID: [27671847](#) DOI: [10.1002/stem.2509](#)]
- 92 **Luz-Crawford P**, Noël D, Fernandez X, Khoury M, Figueroa F, Carrión F, Jorgensen C, Djouad F. Mesenchymal stem cells repress Th17 molecular program through the PD-1 pathway. *PLoS One* 2012; **7**: e45272 [PMID: [23028899](#) DOI: [10.1371/journal.pone.0045272](#)]
- 93 **Franquesa M**, Hoogduijn MJ, Bestard O, Grinyó JM. Immunomodulatory effect of mesenchymal stem cells on B cells. *Front Immunol* 2012; **3**: 212 [PMID: [22833744](#) DOI: [10.3389/fimmu.2012.00212](#)]
- 94 **Fan L**, Hu C, Chen J, Cen P, Wang J, Li L. Interaction between Mesenchymal Stem Cells and B-Cells. *Int J Mol Sci* 2016; **17**: 650 [PMID: [27164080](#) DOI: [10.3390/ijms17050650](#)]
- 95 **Asari S**, Itakura S, Ferreri K, Liu CP, Kuroda Y, Kandeel F, Mullen Y. Mesenchymal stem cells suppress B-cell terminal differentiation. *Exp Hematol* 2009; **37**: 604-615 [PMID: [19375651](#) DOI: [10.1016/j.exphem.2009.01.005](#)]
- 96 **Machado Cde V**, Telles PD, Nascimento IL. Immunological characteristics of mesenchymal stem cells. *Rev Bras Hematol Hemoter* 2013; **35**: 62-67 [PMID: [23580887](#) DOI: [10.5581/1516-8484.20130017](#)]
- 97 **Biassoni R**. Natural killer cell receptors. *Adv Exp Med Biol* 2008; **640**: 35-52 [PMID: [19065782](#) DOI: [10.1007/978-0-387-09789-3\\_4](#)]
- 98 **Finton KA**, Strong RK. Structural insights into activation of antiviral NK cell responses. *Immunol Rev* 2012; **250**: 239-257 [PMID: [23046134](#) DOI: [10.1111/j.1600-065X.2012.01168.x](#)]
- 99 **Casado JG**, Tarazona R, Sanchez-Margallo FM. NK and MSCs crosstalk: the sense of immunomodulation and their sensitivity. *Stem Cell Rev Rep* 2013; **9**: 184-189 [PMID: [23397451](#) DOI: [10.1007/s12015-013-9430-y](#)]



- 100 **Najar M**, Fayyad-Kazan M, Meuleman N, Bron D, Fayyad-Kazan H, Lagneaux L. Mesenchymal stromal cells of the bone marrow and natural killer cells: cell interactions and cross modulation. *J Cell Commun Signal* 2018; **12**: 673-688 [PMID: 29350342 DOI: 10.1007/s12079-018-0448-4]
- 101 **Sangiorgi B**, Panepucci RA. Modulation of Immunoregulatory Properties of Mesenchymal Stromal Cells by Toll-Like Receptors: Potential Applications on GVHD. *Stem Cells Int* 2016; **2016**: 9434250 [PMID: 27738438 DOI: 10.1155/2016/9434250]
- 102 **Holt D**, Ma X, Kundu N, Fulton A. Prostaglandin E(2) (PGE (2)) suppresses natural killer cell function primarily through the PGE(2) receptor EP4. *Cancer Immunol Immunother* 2011; **60**: 1577-1586 [PMID: 21681369 DOI: 10.1007/s00262-011-1064-9]
- 103 **Najar M**, Fayyad-Kazan M, Meuleman N, Bron D, Fayyad-Kazan H, Lagneaux L. Immunological impact of Wharton's Jelly mesenchymal stromal cells and natural killer cell co-culture. *Mol Cell Biochem* 2018; **447**: 111-124 [PMID: 29380244 DOI: 10.1007/s11010-018-3297-9]
- 104 **Alegre E**, Rizzo R, Bortolotti D, Fernandez-Landázuri S, Fainardi E, González A. Some basic aspects of HLA-G biology. *J Immunol Res* 2014; **2014**: 657625 [PMID: 24818168 DOI: 10.1155/2014/657625]
- 105 **Shiina T**, Hosomichi K, Inoko H, Kulski JK. The HLA genomic loci map: expression, interaction, diversity and disease. *J Hum Genet* 2009; **54**: 15-39 [PMID: 19158813 DOI: 10.1038/jhg.2008.5]
- 106 **Mallis P**, Boulari D, Michalopoulos E, Dinou A, Spyropoulou-Vlachou M, Stavropoulos-Giokas C. Evaluation of HLA-G Expression in Multipotent Mesenchymal Stromal Cells Derived from Vitriified Wharton's Jelly Tissue. *Bioengineering (Basel)* 2018; **5**: 95 [PMID: 30388848 DOI: 10.3390/bioengineering5040095]
- 107 **Rajagopalan S**, Long EO. KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front Immunol* 2012; **3**: 258 [PMID: 22934097 DOI: 10.3389/fimmu.2012.00258]
- 108 **Rizzo R**. Immunosuppressive properties of hla-g molecules produced by mesenchymal stromal cells. *J Transplant Technol Res* 2013 [DOI: 10.4172/2161-0991.1000e127]
- 109 **Rajagopalan S**, Bryceson YT, Kuppusamy SP, Geraghty DE, van der Meer A, Joosten I, Long EO. Activation of NK cells by an endocytosed receptor for soluble HLA-G. *PLoS Biol* 2006; **4**: e9 [PMID: 16366734 DOI: 10.1371/journal.pbio.0040009]
- 110 **Roussev RG**, Coulam CB. HLA-G and its role in implantation (review). *J Assist Reprod Genet* 2007; **24**: 288-295 [PMID: 17629722 DOI: 10.1007/s10815-007-9148-3]
- 111 **ClinicalTrials.gov**. Available from: <https://clinicaltrials.gov/>
- 112 **Petrou P**, Gothelf Y, Argov Z, Gotkine M, Levy YS, Kassir I, Vaknin-Dembinsky A, Ben-Hur T, Offen D, Abramsky O, Melamed E, Karussis D. Safety and Clinical Effects of Mesenchymal Stem Cells Secreting Neurotrophic Factor Transplantation in Patients With Amyotrophic Lateral Sclerosis: Results of Phase 1/2 and 2a Clinical Trials. *JAMA Neurol* 2016; **73**: 337-344 [PMID: 26751635 DOI: 10.1001/jamaneurol.2015.4321]
- 113 **Zhang C**, Wu Z, Li JW, Zhao H, Wang GQ. Cytokine release syndrome in severe COVID-19: interleukin-6 receptor antagonist tocilizumab may be the key to reduce mortality. *Int J Antimicrob Agents* 2020; **55**: 105954 [PMID: 32234467 DOI: 10.1016/j.ijantimicag.2020.105954]
- 114 **Lee JW**, Fang X, Krasnodemskaia A, Howard JP, Matthay MA. Concise review: Mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. *Stem Cells* 2011; **29**: 913-919 [PMID: 21506195 DOI: 10.1002/stem.643]
- 115 **Iacobaeus E**, Kadri N, Lefsihane K, Boberg E, Gavin C, Törnqvist Andrén A, Lilja A, Brundin L, Blanc KL. Short and Long Term Clinical and Immunologic Follow up after Bone Marrow Mesenchymal Stromal Cell Therapy in Progressive Multiple Sclerosis-A Phase I Study. *J Clin Med* 2019; **8**: 2102 [PMID: 31810187 DOI: 10.3390/jcm8122102]
- 116 **Wilson JG**, Liu KD, Zhuo H, Caballero L, McMillan M, Fang X, Cosgrove K, Vojnik R, Calfee CS, Lee JW, Rogers AJ, Levitt J, Wiener-Kronish J, Bajwa EK, Leavitt A, McKenna D, Thompson BT, Matthay MA. Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial. *Lancet Respir Med* 2015; **3**: 24-32 [PMID: 25529339 DOI: 10.1016/S2213-2600(14)70291-7]
- 117 **Matthay MA**, Calfee CS, Zhuo H, Thompson BT, Wilson JG, Levitt JE, Rogers AJ, Gotts JE, Wiener-Kronish JP, Bajwa EK, Donahoe MP, McVerry BJ, Ortiz LA, Exline M, Christman JW, Abbott J, Delucchi KL, Caballero L, McMillan M, McKenna DH, Liu KD. Treatment with allogeneic mesenchymal stromal cells for moderate to severe acute respiratory distress syndrome (START study): a randomised phase 2a safety trial. *Lancet Respir Med* 2019; **7**: 154-162 [PMID: 30455077 DOI: 10.1016/S2213-2600(18)30418-1]
- 118 **Gowen A**, Shahjin F, Chand S, Odegaard KE, Yelamanchili SV. Mesenchymal Stem Cell-Derived Extracellular Vesicles: Challenges in Clinical Applications. *Front Cell Dev Biol* 2020; **8**: 149 [PMID: 32226787 DOI: 10.3389/fcell.2020.00149]
- 119 **Raposo G**, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013; **200**: 373-383 [PMID: 23420871 DOI: 10.1083/jcb.201211138]
- 120 **Harrell CR**, Jovicic N, Djonov V, Arsenijevic N, Volarevic V. Mesenchymal Stem Cell-Derived Exosomes and Other Extracellular Vesicles as New Remedies in the Therapy of Inflammatory Diseases. *Cells* 2019; **8**: 1605 [PMID: 31835680 DOI: 10.3390/cells8121605]
- 121 **Hulsmans M**, Holvoet P. MicroRNA-containing microvesicles regulating inflammation in association with atherosclerotic disease. *Cardiovasc Res* 2013; **100**: 7-18 [PMID: 23774505 DOI: 10.1093/cvr/cvt161]
- 122 **Yin K**, Wang S, Zhao RC. Exosomes from mesenchymal stem/stromal cells: a new therapeutic paradigm. *Biomark Res* 2019; **7**: 8 [PMID: 30992990 DOI: 10.1186/s40364-019-0159-x]
- 123 **Xie M**, Xiong W, She Z, Wen Z, Abdirahman AS, Wan W, Wen C. Immunoregulatory Effects of Stem Cell-Derived Extracellular Vesicles on Immune Cells. *Front Immunol* 2020; **11**: 13 [PMID: 32117221 DOI: 10.3389/fimmu.2020.00013]
- 124 **Saba R**, Sorensen DL, Booth SA. MicroRNA-146a: A Dominant, Negative Regulator of the Innate Immune Response. *Front Immunol* 2014; **5**: 578 [PMID: 25484882 DOI: 10.3389/fimmu.2014.00578]
- 125 **Zhao T**, Sun F, Liu J, Ding T, She J, Mao F, Xu W, Qian H, Yan Y. Emerging Role of Mesenchymal Stem Cell-derived Exosomes in Regenerative Medicine. *Curr Stem Cell Res Ther* 2019; **14**: 482-494 [PMID: 30455077 DOI: 10.1016/S2213-2600(18)30418-1]

- 30819086 DOI: [10.2174/1574888X14666190228103230](https://doi.org/10.2174/1574888X14666190228103230)]
- 126 **Deasy BM**, Anderson JE, Zeli S. Regulatory issues in the therapeutic use of stem cells. *Regen Med Tissue Eng* 2013 [DOI: [10.5772/55945](https://doi.org/10.5772/55945)]
  - 127 **Scioli MG**, Bielli A, Gentile P, Cervelli V, Orlandi A. Combined treatment with platelet-rich plasma and insulin favours chondrogenic and osteogenic differentiation of human adipose-derived stem cells in three-dimensional collagen scaffolds. *J Tissue Eng Regen Med* 2017; **11**: 2398-2410 [PMID: [27074878](https://pubmed.ncbi.nlm.nih.gov/27074878/) DOI: [10.1002/term.2139](https://doi.org/10.1002/term.2139)]
  - 128 **Gentile P**, Scioli MG, Bielli A, Orlandi A, Cervelli V. Concise Review: The Use of Adipose-Derived Stromal Vascular Fraction Cells and Platelet Rich Plasma in Regenerative Plastic Surgery. *Stem Cells* 2017; **35**: 117-134 [PMID: [27641055](https://pubmed.ncbi.nlm.nih.gov/27641055/) DOI: [10.1002/stem.2498](https://doi.org/10.1002/stem.2498)]
  - 129 **Gentile P**. Autologous Cellular Method Using Micrografts of Human Adipose Tissue Derived Follicle Stem Cells in Androgenic Alopecia. *Int J Mol Sci* 2019; **20**: 3446 [PMID: [31337037](https://pubmed.ncbi.nlm.nih.gov/31337037/) DOI: [10.3390/ijms20143446](https://doi.org/10.3390/ijms20143446)]
  - 130 **Gentile P**, Kothari A, Casella D, Calabrese C. Fat Graft Enhanced With Adipose-Derived Stem Cells in Aesthetic Breast Augmentation: Clinical, Histological, and Instrumental Evaluation. *Aesthet Surg J* 2019 [PMID: [31637416](https://pubmed.ncbi.nlm.nih.gov/31637416/) DOI: [10.1093/asj/sjz292](https://doi.org/10.1093/asj/sjz292)]
  - 131 **Gentile P**, Casella D, Palma E, Calabrese C. Engineered Fat Graft Enhanced with Adipose-Derived Stromal Vascular Fraction Cells for Regenerative Medicine: Clinical, Histological and Instrumental Evaluation in Breast Reconstruction. *J Clin Med* 2019; **8**: 504 [PMID: [31013744](https://pubmed.ncbi.nlm.nih.gov/31013744/) DOI: [10.3390/jcm8040504](https://doi.org/10.3390/jcm8040504)]
  - 132 **Gentile P**, De Angelis B, Pasin M, Cervelli G, Curcio CB, Floris M, Di Pasquali C, Bocchini I, Balzani A, Nicoli F, Insalaco C, Tati E, Lucarini L, Palla L, Pascali M, De Logu P, Di Segni C, Bottini DJ, Cervelli V. Adipose-derived stromal vascular fraction cells and platelet-rich plasma: basic and clinical evaluation for cell-based therapies in patients with scars on the face. *J Craniofac Surg* 2014; **25**: 267-272 [PMID: [24406591](https://pubmed.ncbi.nlm.nih.gov/24406591/) DOI: [10.1097/01.scs.0000436746.21031.ba](https://doi.org/10.1097/01.scs.0000436746.21031.ba)]
  - 133 **Cervelli V**, Bocchini I, Di Pasquali C, De Angelis B, Cervelli G, Curcio CB, Orlandi A, Scioli MG, Tati E, Delogu P, Gentile P. P.R.L. platelet rich lipotransfert: our experience and current state of art in the combined use of fat and PRP. *Biomed Res Int* 2013; **2013**: 434191 [PMID: [24191244](https://pubmed.ncbi.nlm.nih.gov/24191244/) DOI: [10.1155/2013/434191](https://doi.org/10.1155/2013/434191)]
  - 134 **Ullah M**. The pandemic of novel coronavirus disease 2019 (covid-19): Need for an immediate action. *OAJBS* 2020; **2**: 301-302 [DOI: [10.38125/oajbs.000168](https://doi.org/10.38125/oajbs.000168)]



## Practical choice for robust and efficient differentiation of human pluripotent stem cells

Mei Fang, Li-Ping Liu, Hang Zhou, Yu-Mei Li, Yun-Wen Zheng

**ORCID number:** Mei Fang 0000-0002-9435-3119; Li-Ping Liu 0000-0003-4445-8403; Hang Zhou 0000-0001-8659-5030; Yu-Mei Li 0000-0002-9501-5314; Yun-Wen Zheng 0000-0001-9002-3190.

**Author contributions:** Fang M, Liu LP, and Zheng YW designed the study; Fang M drafted the manuscript; Zheng YW, Li YM, Liu LP, and Zhou H contributed to reviewing and revising the manuscript; all authors approved the final manuscript; Fang M and Liu LP contributed equally to this work; Li YM and Zheng YW are senior authors and co-correspondents of this work.

**Supported by** National Natural Science Foundation of China, No. 81770621; Ministry of Education, Culture, Sports, Science, and Technology of Japan, KAKENHI, No. 18H02866; Natural Science Foundation of Jiangsu Province, No. BK20180281.

**Conflict-of-interest statement:** The authors declare no potential financial interests.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution

**Mei Fang, Li-Ping Liu, Hang Zhou, Yu-Mei Li, Yun-Wen Zheng**, Institute of Regenerative Medicine, Affiliated Hospital of Jiangsu University, Jiangsu University, Zhenjiang 212001, Jiangsu Province, China

**Yun-Wen Zheng**, School of Biotechnology and Health Sciences, Wuyi University, Jiangmen 529020, Guangdong Province, China

**Yun-Wen Zheng**, Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, University of Tsukuba Faculty of Medicine, Tsukuba, Ibaraki 305-8575, Japan

**Yun-Wen Zheng**, Yokohama City University School of Medicine, Yokohama, Kanagawa 234-0006, Japan

**Yun-Wen Zheng**, Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, the University of Tokyo, Tokyo 108-8639, Japan

**Corresponding author:** Yun-Wen Zheng, PhD, Associate Professor, Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, University of Tsukuba Faculty of Medicine, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8575, Japan. [ywzheng@md.tsukuba.ac.jp](mailto:ywzheng@md.tsukuba.ac.jp)

### Abstract

Human pluripotent stem cells (hPSCs) have the distinct advantage of being able to differentiate into cells of all three germ layers. Target cells or tissues derived from hPSCs have many uses such as drug screening, disease modeling, and transplantation therapy. There are currently a wide variety of differentiation methods available. However, most of the existing differentiation methods are unreliable, with uneven differentiation efficiency and poor reproducibility. At the same time, it is difficult to choose the optimal method when faced with so many differentiation schemes, and it is time-consuming and costly to explore a new differentiation approach. Thus, it is critical to design a robust and efficient method of differentiation. In this review article, we summarize a comprehensive approach in which hPSCs are differentiated into target cells or organoids including brain, liver, blood, melanocytes, and mesenchymal cells. This was accomplished by employing an embryoid body-based three-dimensional (3D) suspension culture system with multiple cells co-cultured. The method has high stable differentiation efficiency compared to the conventional 2D culture and can meet the requirements of clinical application. Additionally, *ex vivo* co-culture models might be able to constitute organoids that are highly similar or mimic human organs for

NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Invited manuscript

**Received:** March 28, 2020

**Peer-review started:** March 28, 2020

**First decision:** April 22, 2020

**Revised:** April 30, 2020

**Accepted:** July 1, 2020

**Article in press:** July 1, 2020

**Published online:** August 26, 2020

**P-Reviewer:** Bai G

**S-Editor:** Ma YJ

**L-Editor:** Filipodia

**P-Editor:** Wu YXJ



potential organ transplantation in the future.

**Key words:** Human pluripotent stem cells; Three dimensional; Embryoid body; Differentiation; Efficient; Three germ layers

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Identifying a practical way to efficiently differentiate pluripotent stem cells is essential in regenerative medicine. After considering the advantages and limitations of current approaches, we summarize the ideal conditions and systems. We also provide potential choices for efficiently and robustly differentiating human pluripotent stem cells into target cells and tissues in different germ layers.

**Citation:** Fang M, Liu LP, Zhou H, Li YM, Zheng YW. Practical choice for robust and efficient differentiation of human pluripotent stem cells. *World J Stem Cells* 2020; 12(8): 752-760

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/752.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.752>

## INTRODUCTION

The first five lines of human embryonic stem cells (hESCs) were obtained in 1998 from the inner cell mass of a 3- to 5-day-old fertilized embryo<sup>[1]</sup>. Subsequently, induced pluripotent stem cells (iPSCs) were created by reprogramming fibroblasts<sup>[2]</sup>. Human pluripotent stem cells (hPSCs), including hESCs and human (h)iPSCs, have the ability to self-renew and differentiate into any cell type from all germ layers<sup>[3]</sup>, driving the development of regenerative medicine. The cells and organoids derived from hPSCs have various potential applications including complex diseases studies, cell-based drug screening, and limitless transplantation treatments<sup>[4,5]</sup>. With the rapid development of regenerative medicine technology, many differentiation approaches based on hPSCs have been explored. However, some challenges remain. To meet the needs of clinical application and basic research, high efficiency and stability are the key objectives during hPSC differentiation into high-quality target cells. Thus, it is important to identify an efficient and robust differentiation approach that can increase the differentiation ratio of target cells, produce stronger functions in cells, generate more complete structural organoids, or be reproduced in different cell lines or in other laboratories. Currently, there are great differences in these experimental schemes. Differentiation efficiency<sup>[6]</sup> and stability are impacted by whether the method is based on an embryoid body (EB) or a two-dimensional (2D) or 3D system, or if single or multiple cell co-cultures are used.

In this review article, we combine the experiences of our laboratory with a summary of existing mainstream approaches involving hPSC differentiation, with the goal of providing a reference and time-saving guide for future experimental design.

## DIFFERENTIATION INDUCTION FROM HPSCS

### EB-based differentiation system

EB has been a very common model of *in vitro* hPSC differentiation for more than 50 years<sup>[7]</sup>. The EB-based method is widely used to differentiate majority of cell lineages from the three germ layers (Table 1) and has an obvious advantage in improving the differentiation efficiency of some cells<sup>[8-10]</sup>, such as hematopoietic progenitors<sup>[11]</sup> and melanocytes<sup>[12]</sup>. Combined with suspension bioreactor technology, this advantage can be further amplified for large-scale production<sup>[13]</sup>. Additionally, EB formation provides an excellent way to assess and manipulate developmental potential<sup>[7]</sup>. Differentiation predictions can be made in the early stage of EB to predict which germ layer hPSC is likely to differentiate into, which can save on the cost for subsequent differentiation and indirectly improve differentiation efficiency. For example, Spalt like transcription factor 3 (*SALL3*) expression in EB indicates a high probability of differentiating into the ectoderm and a low chance of differentiating into the mesoderm/endoderm<sup>[14]</sup>. Our study also confirmed these findings, and we found that iPSC lines that expressed

**Table 1 Summary of current approaches for human pluripotent stem cells differential direction into targeted cells or tissues**

Targeted cells or tissues	Cultural approaches				Check points of differential status			Ref.
	EB formation	2D or 3D	2D Surface or 3D system	Multiple cells co-culture	Gene expression	Protein level	Under <i>in vitro</i> or <i>in vivo</i>	
Neural progenitor	-	2D	Matrigel	-	<i>PAX6, TBR2</i>	-	<i>In vitro</i>	[21]
Brain	EB	3D	Low attachment plate, Matrigel	-	<i>PAX6, SOX2, FOXG1, TBR2, ARHGAP11B</i>	Nestin	<i>In vitro</i>	[21]
Midbrain	EB	3D	Matrigel	-	<i>SOX1, VMAT2, TH, NURR1, DAT, GIRK2, PITX3, AADC, ANLN, FAH, MBP, GLA35ST1, ACSL1, CLDN11, CHAT, MAPT, GFAP, S100B, ALDH1L1</i>	-	<i>In vitro</i>	[30]
Brain	-	3D	Microwell, bioreactor	Neuronal, astrocyte	-	-	<i>In vitro</i>	[54]
Retinae	-	3D	Matrigel	-	<i>SIX3, PAX6, RAX, OTX2, VSX2, PRKCZ, MITF</i>	TJP1, LAMB1, RHO, OPN1LW/OPN1MW, OPN1SW	<i>In vitro</i>	[31]
Retinae	EB	3D	Low attachment plate	-	<i>BRN3B, PAX6, RAX, SIX3, LHX2, CHX10, OTX2</i>	RHO, PKC $\alpha$ , Arl13b, OPN1SW, OPN1MW	<i>In vitro</i>	[37]
Retinae	-	3D	Matrigel	RPE, retinal organoid	<i>CRX, RCVRN, NRL, GNAT1, RHO, CHX10, OPN1LW/MW, OTX2, RLBP1, PROX1</i>	-	<i>In vitro</i>	[32]
Melanocyte	EB	3D	Microwell, low attachment plate	-	<i>MITF, PAX3, SOX10, KIT, TYR, TYRP, DCT, PEARL</i>	Melanin	<i>In vivo</i>	[12]
Hepatic stellate cells	-	2D	Matrigel	-	<i>NCAM, KDR, PDGFR<math>\alpha</math>, P75NTR, ALCAM, ACTA2, COL1a1, LRAT, RELN, PCDH7, PDGFR<math>\beta</math>, SYP, GFAP, PPAR<math>\gamma</math>, NGF, <math>\alpha</math>-SMA</i>	Desmin, PDGFR $\beta$ , P75NTR, ALCAM, PDGFR $\alpha$ , CD73, KDR, NCAM	<i>In vitro</i>	[23]
Liver	EB	3D	Microwell	iPSC endoderm cell, HUVEC, BM-MSC	<i>ALB, G6PC, CYP2C9, CYP2C19, CYP3A4, CYP3A7</i>	CYP3A4, ALB, Urea, NTCP	<i>In vitro</i>	[52]
Liver	-	3D	Microwell	iPSC-tHE, iPSC-EC, iPSC-STM	<i>TBX3, ADRA1B</i>	AFP, ALB, Complement factor H, Coagulation factor VIII, Transferrin, AAT	<i>In vivo</i>	[50]
Intestinal	-	3D	Matrigel	-	<i>KLF5, ECAD, SOX9, Klf6</i>	Villin	<i>In vivo</i>	[33]
Entersphere	-	3D	Matrigel	Pan-epithelium cell, HLF, HUVEC	<i>SOX9, CK20, CDX2, NNKX2.1, LGR5, OLFM4, TACSTD2, VIL1, APOA1, FABP2</i>	E-cadherin, Cytokeratin18, $\alpha$ -SMA	<i>In vitro</i>	[34]
Cardiomyocyte	-	2D	Matrigel	-	-	TNNT2, ACTN2	<i>In vitro</i>	[67]
Cardiomyocyte	EB	3D	Low attachment plate	-	<i>TBX5, NKX2.5, GATA4</i>	TNNT2, TNNT3, MYH6, MYL7	<i>In vitro</i>	[47]
Cardiomyocyte	EB	3D	Suspension bioreactor	-	-	TNNT2, $\alpha$ -Actinin, MLC-2v, MLC-2a	<i>In vitro</i>	[13]
Heart	EB	3D	Matrigel	hESC-CPC, hESC-MSC, HUVEC	<i>KDR, MESP1, NKX2.5, TBX5, GATA4, ISL1, PDGFR-<math>\alpha</math>, MEF2C, CD90, CD73, CD105, CD44, CD31, cTNT, <math>\beta</math>-MHC, MLC2v, KCNA4, KCNJ2, KCNH2</i>	-	<i>In vivo</i>	[35]
Hematopoietic cell	EB	3D	-	-	<i>RUNX1, SCL/TAL1</i>	CD34, CD43, CD45	<i>In vitro</i>	[68]



T Cell	-	2D	-	-	<i>TRA, TRB, RAG1, RAG2</i>	CD8ab, LMP2, TCR, TCRab-CD3	<i>In vivo</i>	[69]
Macrophage	EB	3D	Low attachment plate	-	<i>MAF, CSFR1, FLT3, CCR2</i>	CD14, CD45, CD11b, CD16, TNF-α	<i>In vitro</i>	[11]
Liver sinusoidal endothelial cell	EB	3D	Low-cluster plate	-	<i>CD31, CDH5, CD34, F8, STAB2, LYVE1, FLK1, FLT4, FCGR2B</i>	-	<i>In vitro</i>	[55]
Platelet	-	3D	Ultra-low attachment plate	-	<i>CD41a, CD13, CD42b, CD31, CD34, CD43, CD41b</i>	Thrombospondin4, Platelet factor 4	<i>In vivo</i>	[49]
Mesenchymal cell	-	2D	Matrigel	-	<i>CD146, CD73, CD140a, CD90, CD105, CD44, PDGFRβ, CSPG4, NES, LEPR, ADRB2, KITLG, IGFBP2, TNC, CXCL12, ADRB3</i>	-	<i>In vitro</i>	[26]

-. None; EB: Embryoid body; 2D: Two-dimensional; 3D: Three-dimensional; RPE: Retinal pigment epithelium; iPSCs: Induced pluripotent stem cells; HUVECs: Human umbilical cord vein endothelial cells; BM-MSC: Bone marrow mesenchymal stem cell.

higher levels of *SALL3* on day 7 of EB formation showed greater potential for melanocyte differentiation<sup>[15]</sup>. Additionally, miR-371-3 plays both a predictive and functional role in neurogenic differentiation<sup>[16]</sup>, and the low expression of fibroblast growth factor 1 (commonly known as *FGF-1*), ras homolog family member U (commonly known as *RHOU*), and thymidine phosphorylase (commonly known as *TYMP*) genes are associated with low hepatic differentiation<sup>[17]</sup>, which can be used to predict the differentiation potential in early EB. Therefore, EB-based differentiation systems not only help to increase the percentage of target cells, but also contribute to the prediction of differentiation potentials in the early stage, which improves efficiency directly and indirectly, respectively.

### Matrigel-mediated system

Matrigel, a natural extracellular matrix, is widely used in hPSC maintenance and can also be used in 2D and 3D hPSC differentiation (Table 1). During 2D differentiation, the culture vessels are first coated with Matrigel, followed by single cell or cell cluster inoculation. The role of Matrigel in 2D is adherence of cells or cell clumps to a culture vessel. Furthermore, the major component of Matrigel is laminin, which promotes the formation of a rigid neuroepithelial structure<sup>[18]</sup>. Laminin-positive basement membranes are crucial for continuous epithelial integrity<sup>[19]</sup>. A massive volume increase of the human neocortex results from expansion of the cortical area and the related emergence of extensive cortical folding<sup>[20]</sup>, which is thought to be due to the increase of the proliferative potential of neural progenitors (NPs)<sup>[21]</sup>. As this study shows, two human ESC lines were differentiated into NPs in Matrigel-coated 2D adherent culture. Jaenisch and his colleagues constituted human cerebral organoids in an EB-based 3D system, which displayed markedly increased outgrowth of neuroepithelial tissue surrounding ventricle-like structures<sup>[21]</sup>. Other desired cells can also be differentiated in a Matrigel-coated 2D culture system such as hepatocytes<sup>[22]</sup>, hepatic stellate cells<sup>[23]</sup>, intestinal epithelium<sup>[24,25]</sup>, mesenchymal cells<sup>[26]</sup>, cardiomyocytes (CMs)<sup>[27]</sup>, monocytes, and macrophages<sup>[28]</sup>. Thus, the Matrigel-based 2D culture approach is a basic method for the directed induced differentiation of hPSCs.

Matrigel can also be used for 3D differentiation of hPSCs. In addition to coating the substrate, the Matrigel-based 3D construct is formed by adding mixed Matrigel and special differentiation medium<sup>[29]</sup> in the hPSC differentiation process, resulting in differentiation in the solution of a suspended system. A 3D differentiation system provides enough space for establishing an organoid, and promotes cellular communication and interaction among cells compared to a 2D approach. Currently, many target cell lineages or tissues can be differentiated in this way including the brain<sup>[30]</sup>, retinae<sup>[31,32]</sup>, intestinal organoids<sup>[33,34]</sup>, and heart<sup>[35]</sup>. Interestingly, after adding Matrigel, retinal induction cells increase by up to 30%-70% of the total cells in the low cell adhesion plate<sup>[18]</sup>. Because this gel promotes the epithelialization of hPSCs toward retinal differentiation, researchers have tried to use 3D Matrigel methods for differentiating hPSCs. Epithelialized cysts are obtained by floating culture clumps of Matrigel/hESCs and the subsequent floating culture results in self-formation of retinal organoids<sup>[31]</sup>. This includes patterned neuroretina, ciliary margin, and retinal pigment epithelium, which autonomously generates stratified retinal tissues, comprising photoreceptors with ultrastructure of outer segments in long-term culture. This system

has been validated in two lines of human hPSCs<sup>[31]</sup>. Clearly, the use of Matrigel is common in 2D or 3D differentiation of hPSCs into target cells. However, the Matrigel-embedded 3D differentiation system has distinct advantages in self-organizing and generating organoids with a more complete structure when compared to a 2D culture.

### 3D suspension system

During hPSC differentiation, there are many decisions in creating a 3D floating state such as a non- or ultra-low attachment plate, microwell plate, and suspended bioreactors. At present, a variety of cell lineages have been generated by using 3D suspension system such as eye<sup>[36-38]</sup>, skin<sup>[39]</sup>, brain<sup>[40-43]</sup>, liver<sup>[44,45]</sup>, heart<sup>[46,47]</sup> and blood<sup>[11]</sup>. For example, during the 3D differentiation process, the authors generated iPSC-derived fully functional hepatocyte-like organoids in gene expression, protein secretion, and biotransformation<sup>[48]</sup>. Likewise, iPSC-derived platelets can be harvested by using a 3D differentiation system, and it is very similar to human platelets in terms of both ultrastructural features and *in vivo* and *in vitro* functional characterizations<sup>[49]</sup>. Thus, the 3D differentiation system can produce cells with ideal functions. The yield of differentiated cells is also important. The omni-well-array culture platform can produce massive and miniaturized iPSC-derived liver buds on a clinically relevant large scale ( $> 10^8$ )<sup>[50]</sup>. Hama *et al.*<sup>[13]</sup> designed a protocol that generated  $> 90\%$  hiPSC-derived CMs that yielded on average 72 million cells per 100 mL in a 3D bioreactor. These results show that the yield from the 3D suspension system is remarkable in contrast to the 2D system. To test the reproducibility of the CM 3D differentiation protocol, a previous study compared biologically independent experiments with various passage numbers of iPSCs, and found minor inter-experimental variations<sup>[13]</sup>. Overall, the 3D differentiation culture appears to have advantages in differentiation efficiency and stability over the 2D system. This indicates that the 3D differentiation method is optimal when hPSCs differentiation experiments are conducted.

### Multiple cells co-culture system

Each organ has a variety of cell components with a certain structure and its own specific functions. Because of the communication and interaction among cells, co-culturing with different supportive and tissue-constructive cells has become attractive. The benefits of co-culturing multiple cells are that they can facilitate communication and interaction among different cells, enhance the hPSCs differentiation efficiency<sup>[51]</sup>, and better simulate the environment *in vivo*. It can bring surprises when used in a co-culture system to self-organize and generate an organoid. For example, to recapitulate hepatitis B virus-host interactions in liver organoids, Nie *et al.*<sup>[52]</sup> co-cultured iPSC endoderm cells, human umbilical cord vein endothelial cells (HUVECs), and human bone marrow mesenchymal stem cells to form liver organoids in a 3D microwell plate, which exhibits stronger hepatic functions than iPSC-derived hepatic like cells. Furthermore, the co-culture pattern also has a higher differentiation yield<sup>[48]</sup> and organoids with more complex functions<sup>[53]</sup>. There are co-culture combinations in other studies such as co-culturing hPSC-derived neurons and astrocytes<sup>[54]</sup>; co-culturing iPSC-derived hepatic parenchymal and non-parenchymal cells<sup>[55]</sup>; co-culturing hiPSC-derived retinal pigment epithelium and retinal organoids<sup>[32]</sup>; and co-culturing HUVECs, hESC-derived MSCs, and hESC-derived cardiac progenitor cells<sup>[35]</sup>. Human PSC-derived organoids with multiple cell components have a complete structure and sturdy function similar to a human organ, which may provide an alternative source for organ transplantation. Therefore, the 3D culture method is a better choice for organoid generation.

### Transcription factor-directed differentiation

Transcription factors (TFs) play an important role in pluripotent stem cell induction and transdifferentiation<sup>[56]</sup>. Recently, they have been used to differentiate hPSCs into desired cells or tissues such as neural<sup>[57]</sup>, liver<sup>[58,59]</sup>, and cardiac muscle<sup>[60,61]</sup>. A growing body of TF-directed differentiation method of hPSCs has demonstrated that efficient cell fate is reprogrammed *via* forced expression of single or multiple TFs<sup>[62]</sup>. Sun *et al.*<sup>[63]</sup> used the technique to design a single-step protocol for forebrain GABAergic neuron differentiation, which could generate cells similar to rodent cortical interneurons with  $> 80\%$  efficiency, and the target cells showed mature functional properties within 6-8 wk. By contrast, other process takes as long as 30 wk<sup>[64]</sup>. The TF-mediated method can differentiate hPSCs into terminal cells directly, and the experimental procedure is relatively brief.

## LIMITATION AND CHOICE

Current methods for hPSC differentiation described above have various limitations. 2D differentiation culturing is performed on the surface of the culture vessel and the limited contact area limits the yield of the target cells. Furthermore, all structural components of organoids cannot be generated<sup>[37,65]</sup>. Without 3D contact with Matrigel, Lowe *et al.*<sup>[31]</sup> reported that most cells died and the few surviving cells formed solid cell masses on 2D culturing. Most 3D culture methods involve various intermediate stages requiring varying combinations of recombinant factors and small molecules<sup>[63]</sup>, thus rendering the method cumbersome to repeat. Although TF-mediated methods improve the differentiation efficiency of hPSCs, numerous tools for TF transfection, including plasmids and viruses, have led to the integration of exogenous genes<sup>[66]</sup> into the target cells, thus presenting a remote prospects for their clinical application<sup>[56]</sup>. In this situation, EB-based 3D culture systems allow for large-scale directional differentiation of hPSCs, and the co-culture method seems to constitute highly functional organoids *in vitro* to compensate for organ transplantation insufficiency.

## CONCLUSION

Although only a few articles have compared the differences between 2D and 3D differentiation, it can be concluded that 3D system with EB has obvious advantages for hPSC differentiation compared to 2D culture. The details of the differentiation approaches are shown in the “cultural approaches” of Table 1. Regarding future studies, there are some key recommendations. First, the ability of EB not only can scale up culture systems and differentiation, but also predict the fate of hPSCs differentiation for reducing unnecessary waste. Second, the 3D differentiation system also has significant improvement in differentiation efficiency, and 3D space is necessary for organoid formation. Finally, it is a promising and challenging task that co-cultures multiple kinds of cells, supportive, structured, vascularized and further neurovascularized for organoid organization in 3D suspension system. Simply put, an EB-based 3D differentiation culture system is an efficient and powerful choice for hPSCs to meet the demand in clinical applications and basic research.

## ACKNOWLEDGEMENTS

We thank the kind support and help suggestions from Drs. Ning-Ning Guo and Qian Zhang, and other Lab members.

## REFERENCES

- 1 Croze RH, Clegg DO. Differentiation of pluripotent stem cells into retinal pigmented epithelium. *Dev Ophthalmol* 2014; **53**: 81-96 [PMID: 24732763 DOI: 10.1159/000357361]
- 2 Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408 DOI: 10.1016/j.cell.2007.11.019]
- 3 Studer L. Derivation of dopaminergic neurons from pluripotent stem cells. *Prog Brain Res* 2012; **200**: 243-263 [PMID: 23195422 DOI: 10.1016/B978-0-444-59575-1.00011-9]
- 4 Bock C, Kiskinis E, Verstappen G, Gu H, Boulting G, Smith ZD, Ziller M, Croft GF, Amoroso MW, Oakley DH, Gnirke A, Eggan K, Meissner A. Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. *Cell* 2011; **144**: 439-452 [PMID: 21295703 DOI: 10.1016/j.cell.2010.12.032]
- 5 Liu LP, Zheng YW. Predicting differentiation potential of human pluripotent stem cells: Possibilities and challenges. *World J Stem Cells* 2019; **11**: 375-382 [PMID: 31396366 DOI: 10.4252/wjsc.v11.i7.375]
- 6 Ohta S, Imaizumi Y, Okada Y, Akamatsu W, Kuwahara R, Ohyama M, Amagai M, Matsuzaki Y, Yamanaka S, Okano H, Kawakami Y. Generation of human melanocytes from induced pluripotent stem cells. *PLoS One* 2011; **6**: e16182 [PMID: 21249204 DOI: 10.1371/journal.pone.0016182]
- 7 Brickman JM, Serup P. Properties of embryoid bodies. *Wiley Interdiscip Rev Dev Biol* 2017; **6** [PMID: 27911036 DOI: 10.1002/wdev.259]
- 8 Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA. Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol* 2010; **148**: 3-15 [PMID: 20097238 DOI: 10.1016/j.jbiotec.2010.01.012]
- 9 Ungrin MD, Joshi C, Nica A, Bauwens C, Zandstra PW. Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. *PLoS One* 2008; **3**: e1565 [PMID: 18270562 DOI: 10.1371/journal.pone.0001565]

- 10 **Guo NN**, Liu LP, Zheng YW, Li YM. Inducing human induced pluripotent stem cell differentiation through embryoid bodies: A practical and stable approach. *World J Stem Cells* 2020; **12**: 25-34 [PMID: [32110273](#) DOI: [10.4252/wjsc.v12.i1.25](#)]
- 11 **Buchrieser J**, James W, Moore MD. Human Induced Pluripotent Stem Cell-Derived Macrophages Share Ontogeny with MYB-Independent Tissue-Resident Macrophages. *Stem Cell Reports* 2017; **8**: 334-345 [PMID: [28111278](#) DOI: [10.1016/j.stemcr.2016.12.020](#)]
- 12 **Liu LP**, Li YM, Guo NN, Li S, Ma X, Zhang YX, Gao Y, Huang JL, Zheng DX, Wang LY, Xu H, Hui L, Zheng YW. Therapeutic Potential of Patient iPSC-Derived iMelanocytes in Autologous Transplantation. *Cell Rep* 2019; **27**: 455-466.e5 [PMID: [30970249](#) DOI: [10.1016/j.celrep.2019.03.046](#)]
- 13 **Hamad S**, Derichsweiler D, Papadopoulos S, Nguemo F, Šarić T, Sachinidis A, Brockmeier K, Hescheler J, Boukens BJ, Pfannkuche K. Generation of human induced pluripotent stem cell-derived cardiomyocytes in 2D monolayer and scalable 3D suspension bioreactor cultures with reduced batch-to-batch variations. *Theranostics* 2019; **9**: 7222-7238 [PMID: [31695764](#) DOI: [10.7150/thno.32058](#)]
- 14 **Kuroda T**, Yasuda S, Tachi S, Matsuyama S, Kusakawa S, Tano K, Miura T, Matsuyama A, Sato Y. SALL3 expression balance underlies lineage biases in human induced pluripotent stem cell differentiation. *Nat Commun* 2019; **10**: 2175 [PMID: [31092818](#) DOI: [10.1038/s41467-019-09511-4](#)]
- 15 **Guo NN**, Liu LP, Zhang YX, Cai YT, Guo Y, Zheng YW, Li YM. Early prediction of the differentiation potential during the formation of human iPSC-derived embryoid bodies. *Biochem Biophys Res Commun* 2019; **516**: 673-679 [PMID: [31248595](#) DOI: [10.1016/j.bbrc.2019.06.081](#)]
- 16 **Kim H**, Lee G, Ganat Y, Papapetrou EP, Lipchina I, Socci ND, Sadelain M, Studer L. miR-371-3 expression predicts neural differentiation propensity in human pluripotent stem cells. *Cell Stem Cell* 2011; **8**: 695-706 [PMID: [21624813](#) DOI: [10.1016/j.stem.2011.04.002](#)]
- 17 **Yanagihara K**, Liu Y, Kanie K, Takayama K, Kokunugi M, Hirata M, Fukuda T, Suga M, Nikawa H, Mizuguchi H, Kato R, Furue MK. Prediction of Differentiation Tendency Toward Hepatocytes from Gene Expression in Undifferentiated Human Pluripotent Stem Cells. *Stem Cells Dev* 2016; **25**: 1884-1897 [PMID: [27733097](#) DOI: [10.1089/scd.2016.0099](#)]
- 18 **Eiraku M**, Sasai Y. Mouse embryonic stem cell culture for generation of three-dimensional retinal and cortical tissues. *Nat Protoc* 2011; **7**: 69-79 [PMID: [22179593](#) DOI: [10.1038/nprot.2011.429](#)]
- 19 **Fujiwara H**, Hayashi Y, Sanzen N, Kobayashi R, Weber CN, Emoto T, Futaki S, Niwa H, Murray P, Edgar D, Sekiguchi K. Regulation of mesodermal differentiation of mouse embryonic stem cells by basement membranes. *J Biol Chem* 2007; **282**: 29701-29711 [PMID: [17690109](#) DOI: [10.1074/jbc.M611452200](#)]
- 20 **Sun T**, Hevner RF. Growth and folding of the mammalian cerebral cortex: from molecules to malformations. *Nat Rev Neurosci* 2014; **15**: 217-232 [PMID: [24646670](#) DOI: [10.1038/nrn3707](#)]
- 21 **Li Y**, Muffat J, Omer A, Bosch I, Lancaster MA, Sur M, Gehrke L, Knoblich JA, Jaenisch R. Induction of Expansion and Folding in Human Cerebral Organoids. *Cell Stem Cell* 2017; **20**: 385-396.e3 [PMID: [28041895](#) DOI: [10.1016/j.stem.2016.11.017](#)]
- 22 **Calabrese D**, Roma G, Bergling S, Carbone W, Mele V, Nuciforo S, Fofana I, Campana B, Szkolnicka D, Hay DC, Tchorz J, Bouwmeester T, Wieland S, Heim MH. Liver biopsy derived induced pluripotent stem cells provide unlimited supply for the generation of hepatocyte-like cells. *PLoS One* 2019; **14**: e0221762 [PMID: [31465481](#) DOI: [10.1371/journal.pone.0221762](#)]
- 23 **Coll M**, Perea L, Boon R, Leite SB, Vallverdú J, Mannaerts I, Smout A, El Taghdouini A, Blaya D, Rodrigo-Torres D, Graupera I, Aguilar-Bravo B, Chesne C, Najimi M, Sokal E, Lozano JJ, van Grunsven LA, Verfaillie CM, Sancho-Bru P. Generation of Hepatic Stellate Cells from Human Pluripotent Stem Cells Enables In Vitro Modeling of Liver Fibrosis. *Cell Stem Cell* 2018; **23**: 101-113.e7 [PMID: [30049452](#) DOI: [10.1016/j.stem.2018.05.027](#)]
- 24 **Münner JO**, Sundaram N, Rankin SA, Hill D, Watson C, Mahe M, Vallance JE, Shroyer NF, Sinagoga KL, Zarzoso-Lacoste A, Hudson JR, Howell JC, Chatuvedi P, Spence JR, Shannon JM, Zorn AM, Helmrath MA, Wells JM. Differentiation of Human Pluripotent Stem Cells into Colonic Organoids via Transient Activation of BMP Signaling. *Cell Stem Cell* 2017; **21**: 51-64.e6 [PMID: [28648364](#) DOI: [10.1016/j.stem.2017.05.020](#)]
- 25 **Mithal A**, Capilla A, Heinze D, Berical A, Villacorta-Martin C, Vedaie M, Jacob A, Abo K, Szymaniak A, Peasley M, Stuffer A, Mahoney J, Kotton DN, Hawkins F, Mostoslavsky G. Generation of mesenchyme free intestinal organoids from human induced pluripotent stem cells. *Nat Commun* 2020; **11**: 215 [PMID: [31924806](#) DOI: [10.1038/s41467-019-13916-6](#)]
- 26 **Chin CJ**, Li S, Corselli M, Casero D, Zhu Y, He CB, Hardy R, Péault B, Crooks GM. Transcriptionally and Functionally Distinct Mesenchymal Subpopulations Are Generated from Human Pluripotent Stem Cells. *Stem Cell Reports* 2018; **10**: 436-446 [PMID: [29307583](#) DOI: [10.1016/j.stemcr.2017.12.005](#)]
- 27 **Mills RJ**, Parker BL, Quaipe-Ryan GA, Voges HK, Needham EJ, Bornot A, Ding M, Andersson H, Polla M, Elliott DA, Drowley L, Clausen M, Plowright AT, Barrett IP, Wang QD, James DE, Porrello ER, Hudson JE. Drug Screening in Human PSC-Cardiac Organoids Identifies Pro-proliferative Compounds Acting via the Mevalonate Pathway. *Cell Stem Cell* 2019; **24**: 895-907.e6 [PMID: [30930147](#) DOI: [10.1016/j.stem.2019.03.009](#)]
- 28 **Cao X**, Yakala GK, van den Hil FE, Cochrane A, Mummery CL, Orlova VV. Differentiation and Functional Comparison of Monocytes and Macrophages from hiPSCs with Peripheral Blood Derivatives. *Stem Cell Reports* 2019; **12**: 1282-1297 [PMID: [31189095](#) DOI: [10.1016/j.stemcr.2019.05.003](#)]
- 29 **Lee GY**, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods* 2007; **4**: 359-365 [PMID: [17396127](#) DOI: [10.1038/nmeth1015](#)]
- 30 **Kim H**, Park HJ, Choi H, Chang Y, Park H, Shin J, Kim J, Lengner CJ, Lee YK, Kim J. Modeling G2019S-LRRK2 Sporadic Parkinson's Disease in 3D Midbrain Organoids. *Stem Cell Reports* 2019; **12**: 518-531 [PMID: [30799274](#) DOI: [10.1016/j.stemcr.2019.01.020](#)]
- 31 **Lowe A**, Harris R, Bhansali P, Cvekl A, Liu W. Intercellular Adhesion-Dependent Cell Survival and ROCK-Regulated Actomyosin-Driven Forces Mediate Self-Formation of a Retinal Organoid. *Stem Cell Reports* 2016; **6**: 743-756 [PMID: [27132890](#) DOI: [10.1016/j.stemcr.2016.03.011](#)]
- 32 **Akhtar T**, Xie H, Khan MI, Zhao H, Bao J, Zhang M, Xue T. Accelerated photoreceptor differentiation of hiPSC-derived retinal organoids by contact co-culture with retinal pigment epithelium. *Stem Cell Res* 2019;



- 39: 101491 [PMID: [31326746](#) DOI: [10.1016/j.scr.2019.101491](#)]
- 33 **Miura S**, Suzuki A. Generation of Mouse and Human Organoid-Forming Intestinal Progenitor Cells by Direct Lineage Reprogramming. *Cell Stem Cell* 2017; **21**: 456-471.e5 [PMID: [28943029](#) DOI: [10.1016/j.stem.2017.08.020](#)]
- 34 **Nadkarni RR**, Abed S, Cox BJ, Bhatia S, Lau JT, Surette MG, Draper JS. Functional Enterospheres Derived In Vitro from Human Pluripotent Stem Cells. *Stem Cell Reports* 2017; **9**: 897-912 [PMID: [28867347](#) DOI: [10.1016/j.stemcr.2017.07.024](#)]
- 35 **Varzideh F**, Pahlavan S, Ansari H, Halvaei M, Kostin S, Feiz MS, Latifi H, Aghdami N, Braun T, Baharvand H. Human cardiomyocytes undergo enhanced maturation in embryonic stem cell-derived organoid transplants. *Biomaterials* 2019; **192**: 537-550 [PMID: [30529872](#) DOI: [10.1016/j.biomaterials.2018.11.033](#)]
- 36 **Parfitt DA**, Lane A, Ramsden CM, Carr AJ, Munro PM, Jovanovic K, Schwarz N, Kanuga N, Muthiah MN, Hull S, Gallo JM, da Cruz L, Moore AT, Hardcastle AJ, Coffey PJ, Cheetham ME. Identification and Correction of Mechanisms Underlying Inherited Blindness in Human iPSC-Derived Optic Cups. *Cell Stem Cell* 2016; **18**: 769-781 [PMID: [27151457](#) DOI: [10.1016/j.stem.2016.03.021](#)]
- 37 **Deng WL**, Gao ML, Lei XL, Lv JN, Zhao H, He KW, Xia XX, Li LY, Chen YC, Li YP, Pan D, Xue T, Jin ZB. Gene Correction Reverses Ciliopathy and Photoreceptor Loss in iPSC-Derived Retinal Organoids from Retinitis Pigmentosa Patients. *Stem Cell Reports* 2018; **10**: 1267-1281 [PMID: [29526738](#) DOI: [10.1016/j.stemcr.2018.02.003](#)]
- 38 **Völkner M**, Zschätzsch M, Rostovskaya M, Overall RW, Busskamp V, Anastassiadis K, Karl MO. Retinal Organoids from Pluripotent Stem Cells Efficiently Recapitulate Retinogenesis. *Stem Cell Reports* 2016; **6**: 525-538 [PMID: [27050948](#) DOI: [10.1016/j.stemcr.2016.03.001](#)]
- 39 **Lee J**, Böske R, Tang PC, Hartman BH, Heller S, Koehler KR. Hair Follicle Development in Mouse Pluripotent Stem Cell-Derived Skin Organoids. *Cell Rep* 2018; **22**: 242-254 [PMID: [29298425](#) DOI: [10.1016/j.celrep.2017.12.007](#)]
- 40 **Bershteyn M**, Nowakowski TJ, Pollen AA, Di Lullo E, Nene A, Wynshaw-Boris A, Kriegstein AR. Human iPSC-Derived Cerebral Organoids Model Cellular Features of Lissencephaly and Reveal Prolonged Mitosis of Outer Radial Glia. *Cell Stem Cell* 2017; **20**: 435-449.e4 [PMID: [28111201](#) DOI: [10.1016/j.stem.2016.12.007](#)]
- 41 **Iefremova V**, Manikakis G, Krefft O, Jabali A, Weynans K, Wilkens R, Marsoner F, Brändl B, Müller FJ, Koch P, Ladewig J. An Organoid-Based Model of Cortical Development Identifies Non-Cell-Autonomous Defects in Wnt Signaling Contributing to Miller-Dieker Syndrome. *Cell Rep* 2017; **19**: 50-59 [PMID: [28380362](#) DOI: [10.1016/j.celrep.2017.03.047](#)]
- 42 **Linkous A**, Balamatsias D, Snuderl M, Edwards L, Miyaguchi K, Milner T, Reich B, Cohen-Gould L, Storaska A, Nakayama Y, Schenkein E, Singhanian R, Cirigliano S, Magdeldin T, Lin Y, Nanjangud G, Chadavalada K, Pisapia D, Liston C, Fine HA. Modeling Patient-Derived Glioblastoma with Cerebral Organoids. *Cell Rep* 2019; **26**: 3203-3211.e5 [PMID: [30893594](#) DOI: [10.1016/j.celrep.2019.02.063](#)]
- 43 **Xiang Y**, Tanaka Y, Cakir B, Patterson B, Kim KY, Sun P, Kang YJ, Zhong M, Liu X, Patra P, Lee SH, Weissman SM, Park IH. hESC-Derived Thalamic Organoids Form Reciprocal Projections When Fused with Cortical Organoids. *Cell Stem Cell* 2019; **24**: 487-497.e7 [PMID: [30799279](#) DOI: [10.1016/j.stem.2018.12.015](#)]
- 44 **Akbari S**, Sevinç GG, Ersoy N, Basak O, Kaplan K, Sevinç K, Ozel E, Sengun B, Enustun E, Ozcimen B, Bagriyanik A, Arslan N, Önder TT, Erdal E. Robust, Long-Term Culture of Endoderm-Derived Hepatic Organoids for Disease Modeling. *Stem Cell Reports* 2019; **13**: 627-641 [PMID: [31522975](#) DOI: [10.1016/j.stemcr.2019.08.007](#)]
- 45 **Nie YZ**, Zheng YW, Ogawa M, Miyagi E, Taniguchi H. Human liver organoids generated with single donor-derived multiple cells rescue mice from acute liver failure. *Stem Cell Res Ther* 2018; **9**: 5 [PMID: [29321049](#) DOI: [10.1186/s13287-017-0749-1](#)]
- 46 **Chauveau S**, Anyukhovsky EP, Ben-Ari M, Naor S, Jiang YP, Danilo P Jr, Rahim T, Burke S, Qiu X, Potapova IA, Doronin SV, Brink PR, Binah O, Cohen IS, Rosen MR. Induced Pluripotent Stem Cell-Derived Cardiomyocytes Provide In Vivo Biological Pacemaker Function. *Circ Arrhythm Electrophysiol* 2017; **10**: e004508 [PMID: [28500172](#) DOI: [10.1161/CIRCEP.116.004508](#)]
- 47 **Hoque A**, Sivakumaran P, Bond ST, Ling NXY, Kong AM, Scott JW, Bandara N, Hernández D, Liu GS, Wong RCB, Ryan MT, Hausenloy DJ, Kemp BE, Oakhill JS, Drew BG, Pébay A, Lim SY. Mitochondrial fission protein Drp1 inhibition promotes cardiac mesodermal differentiation of human pluripotent stem cells. *Cell Death Discov* 2018; **4**: 39 [PMID: [29531836](#) DOI: [10.1038/s41420-018-0042-9](#)]
- 48 **Pettinato G**, Lehoux S, Ramanathan R, Salem MM, He LX, Muse O, Flaumenhaft R, Thompson MT, Rouse EA, Cummings RD, Wen X, Fisher RA. Generation of fully functional hepatocyte-like organoids from human induced pluripotent stem cells mixed with Endothelial Cells. *Sci Rep* 2019; **9**: 8920 [PMID: [31222080](#) DOI: [10.1038/s41598-019-45514-3](#)]
- 49 **Feng Q**, Shabrani N, Thon JN, Huo H, Thiel A, Machlus KR, Kim K, Brooks J, Li F, Luo C, Kimbrel EA, Wang J, Kim KS, Italiano J, Cho J, Lu SJ, Lanza R. Scalable generation of universal platelets from human induced pluripotent stem cells. *Stem Cell Reports* 2014; **3**: 817-831 [PMID: [25418726](#) DOI: [10.1016/j.stemcr.2014.09.010](#)]
- 50 **Takebe T**, Sekine K, Kimura M, Yoshizawa E, Ayano S, Koido M, Funayama S, Nakanishi N, Hisai T, Kobayashi T, Kasai T, Kitada R, Mori A, Ayabe H, Ejiri Y, Amimoto N, Yamazaki Y, Ogawa S, Ishikawa M, Kiyota Y, Sato Y, Nozawa K, Okamoto S, Ueno Y, Taniguchi H. Massive and Reproducible Production of Liver Buds Entirely from Human Pluripotent Stem Cells. *Cell Rep* 2017; **21**: 2661-2670 [PMID: [29212014](#) DOI: [10.1016/j.celrep.2017.11.005](#)]
- 51 **Wang LY**, Liu LP, Ge JY, Yuan YY, Sun LL, Xu H, Huang PY, Hui LJ, Isoda H, Ohkohchi N, Li YM, Zheng YW. A Multiple-Cell Microenvironment in a 3-Dimensional System Enhances Direct Cellular Reprogramming Into Hepatic Organoids. *Transplant Proc* 2018; **50**: 2864-2867 [PMID: [30401413](#) DOI: [10.1016/j.transproceed.2018.03.076](#)]
- 52 **Nie YZ**, Zheng YW, Miyakawa K, Murata S, Zhang RR, Sekine K, Ueno Y, Takebe T, Wakita T, Ryo A, Taniguchi H. Recapitulation of hepatitis B virus-host interactions in liver organoids from human induced



- pluripotent stem cells. *EBioMedicine* 2018; **35**: 114-123 [PMID: [30120080](#) DOI: [10.1016/j.ebiom.2018.08.014](#)]
- 53 **Furuya K**, Zheng YW, Sako D, Iwasaki K, Zheng DX, Ge JY, Liu LP, Furuta T, Akimoto K, Yagi H, Hamada H, Isoda H, Oda T, Ohkohchi N. Enhanced hepatic differentiation in the subpopulation of human amniotic stem cells under 3D multicellular microenvironment. *World J Stem Cells* 2019; **11**: 705-721 [PMID: [31616545](#) DOI: [10.4252/wjsc.v11.i9.705](#)]
- 54 **Krencik R**, Seo K, van Asperen JV, Basu N, Cvetkovic C, Barlas S, Chen R, Ludwig C, Wang C, Ward ME, Gan L, Horner PJ, Rowitch DH, Ullian EM. Systematic Three-Dimensional Coculture Rapidly Recapitulates Interactions between Human Neurons and Astrocytes. *Stem Cell Reports* 2017; **9**: 1745-1753 [PMID: [29198827](#) DOI: [10.1016/j.stemcr.2017.10.026](#)]
- 55 **Kouji Y**, Kido T, Ito T, Oyama H, Chen SW, Katou Y, Shirahige K, Miyajima A. An In Vitro Human Liver Model by iPSC-Derived Parenchymal and Non-parenchymal Cells. *Stem Cell Reports* 2017; **9**: 490-498 [PMID: [28757162](#) DOI: [10.1016/j.stemcr.2017.06.010](#)]
- 56 **Ulasov AV**, Rosenkranz AA, Sobolev AS. Transcription factors: Time to deliver. *J Control Release* 2018; **269**: 24-35 [PMID: [29113792](#) DOI: [10.1016/j.jconrel.2017.11.004](#)]
- 57 **Pang ZP**, Yang N, Vierbuchen T, Ostermeier A, Fuentes DR, Yang TQ, Citri A, Sebastiano V, Marro S, Südhof TC, Wernig M. Induction of human neuronal cells by defined transcription factors. *Nature* 2011; **476**: 220-223 [PMID: [21617644](#) DOI: [10.1038/nature10202](#)]
- 58 **Tomizawa M**, Shinozaki F, Motoyoshi Y, Sugiyama T, Yamamoto S, Ishige N. Transcription Factors and Medium Suitable for Initiating the Differentiation of Human-Induced Pluripotent Stem Cells to the Hepatocyte Lineage. *J Cell Biochem* 2016; **117**: 2001-2009 [PMID: [26773721](#) DOI: [10.1002/jcb.25494](#)]
- 59 **Takayama K**, Inamura M, Kawabata K, Sugawara M, Kikuchi K, Higuchi M, Nagamoto Y, Watanabe H, Tashiro K, Sakurai F, Hayakawa T, Furue MK, Mizuguchi H. Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1 $\alpha$  transduction. *J Hepatol* 2012; **57**: 628-636 [PMID: [22659344](#) DOI: [10.1016/j.jhep.2012.04.038](#)]
- 60 **Kwon C**, Qian L, Cheng P, Nigam V, Arnold J, Srivastava D. A regulatory pathway involving Notch1/beta-catenin/Is11 determines cardiac progenitor cell fate. *Nat Cell Biol* 2009; **11**: 951-957 [PMID: [19620969](#) DOI: [10.1038/ncb1906](#)]
- 61 **Bai F**, Ho Lim C, Jia J, Santostefano K, Simmons C, Kasahara H, Wu W, Terada N, Jin S. Directed Differentiation of Embryonic Stem Cells Into Cardiomyocytes by Bacterial Injection of Defined Transcription Factors. *Sci Rep* 2015; **5**: 15014 [PMID: [26449528](#) DOI: [10.1038/srep15014](#)]
- 62 **Oh Y**, Jang J. Directed Differentiation of Pluripotent Stem Cells by Transcription Factors. *Mol Cells* 2019; **42**: 200-209 [PMID: [30884942](#) DOI: [10.14348/molcells.2019.2439](#)]
- 63 **Sun AX**, Yuan Q, Tan S, Xiao Y, Wang D, Khoo AT, Sani L, Tran HD, Kim P, Chiew YS, Lee KJ, Yen YC, Ng HH, Lim B, Je HS. Direct Induction and Functional Maturation of Forebrain GABAergic Neurons from Human Pluripotent Stem Cells. *Cell Rep* 2016; **16**: 1942-1953 [PMID: [27498872](#) DOI: [10.1016/j.celrep.2016.07.035](#)]
- 64 **Nicholas CR**, Chen J, Tang Y, Southwell DG, Chalmers N, Vogt D, Arnold CM, Chen YJ, Stanley EG, Elefanti AG, Sasai Y, Alvarez-Buylla A, Rubenstein JL, Kriegstein AR. Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development. *Cell Stem Cell* 2013; **12**: 573-586 [PMID: [23642366](#) DOI: [10.1016/j.stem.2013.04.005](#)]
- 65 **Osakada F**, Ikeda H, Sasai Y, Takahashi M. Stepwise differentiation of pluripotent stem cells into retinal cells. *Nat Protoc* 2009; **4**: 811-824 [PMID: [19444239](#) DOI: [10.1038/nprot.2009.51](#)]
- 66 **Ge JY**, Zheng YW, Liu LP, Isoda H, Oda T. Impelling force and current challenges by chemicals in somatic cell reprogramming and expansion beyond hepatocytes. *World J Stem Cells* 2019; **11**: 650-665 [PMID: [31616541](#) DOI: [10.4252/wjsc.v11.i9.650](#)]
- 67 **Sharma A**, McKeithan WL, Serrano R, Kitani T, Burridge PW, Del Álamo JC, Mercola M, Wu JC. Use of human induced pluripotent stem cell-derived cardiomyocytes to assess drug cardiotoxicity. *Nat Protoc* 2018; **13**: 3018-3041 [PMID: [30413796](#) DOI: [10.1038/s41596-018-0076-8](#)]
- 68 **Nakajima-Takagi Y**, Osawa M, Oshima M, Takagi H, Miyagi S, Endoh M, Endo TA, Takayama N, Eto K, Toyoda T, Koseki H, Nakauchi H, Iwama A. Role of SOX17 in hematopoietic development from human embryonic stem cells. *Blood* 2013; **121**: 447-458 [PMID: [23169777](#) DOI: [10.1182/blood-2012-05-431403](#)]
- 69 **Minagawa A**, Yoshikawa T, Yasukawa M, Hotta A, Kunitomo M, Iriguchi S, Takiguchi M, Kassai Y, Imai E, Yasui Y, Kawai Y, Zhang R, Uemura Y, Miyoshi H, Nakanishi M, Watanabe A, Hayashi A, Kawana K, Fujii T, Nakatsura T, Kaneko S. Enhancing T Cell Receptor Stability in Rejuvenated iPSC-Derived T Cells Improves Their Use in Cancer Immunotherapy. *Cell Stem Cell* 2018; **23**: 850-858.e4 [PMID: [30449714](#) DOI: [10.1016/j.stem.2018.10.005](#)]



## Human embryonic stem cells as an *in vitro* model for studying developmental origins of type 2 diabetes

Andy Chun-Hang Chen, Kai Fai Lee, William Shu Biu Yeung, Yin Lau Lee

**ORCID number:** Andy Chun-Hang Chen 0000-0002-7065-3192; Kai Fai Lee 0000-0001-5957-439X; William Shu Biu Yeung 0000-0003-0670-0879; Yin Lau Lee 0000-0003-0559-4381.

**Author contributions:** Chen ACH and Lee YL conceived the study and drafted the manuscript; Lee KF, Yeung WSB, and Lee YL contributed to critical revision and editing of the manuscript.

**Supported by** a small project grant from the University of Hong Kong, No. 201409176196.

**Conflict-of-interest statement:** The authors declare no conflict of interest.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Andy Chun-Hang Chen, Kai Fai Lee, Yin Lau Lee,** Department of Obstetrics and Gynaecology, The University of Hong Kong, Hong Kong, China

**Andy Chun-Hang Chen, Kai Fai Lee, William Shu Biu Yeung, Yin Lau Lee,** Shenzhen Key Laboratory of Fertility Regulation, The University of Hong Kong Shenzhen Hospital, Shenzhen 518053, Guangdong Province, China

**Corresponding author:** Yin Lau Lee, PhD, Research Scientist, Department of Obstetrics and Gynaecology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, China. [cherielee@hku.hk](mailto:cherielee@hku.hk)

### Abstract

The developmental origins of health and diseases (DOHaD) is a concept stating that adverse intrauterine environments contribute to the health risks of offspring. Since the theory emerged more than 30 years ago, many epidemiological and animal studies have confirmed that *in utero* exposure to environmental insults, including hyperglycemia and chemicals, increased the risk of developing noncommunicable diseases (NCDs). These NCDs include metabolic syndrome, type 2 diabetes, and complications such as diabetic cardiomyopathy. Studying the effects of different environmental insults on early embryo development would aid in understanding the underlying mechanisms by which these insults promote NCD development. Embryonic stem cells (ESCs) have also been utilized by researchers to study the DOHaD. ESCs have pluripotent characteristics and can be differentiated into almost every cell lineage; therefore, they are excellent *in vitro* models for studying early developmental events. More importantly, human ESCs (hESCs) are the best alternative to human embryos for research because of ethical concerns. In this review, we will discuss different maternal conditions associated with DOHaD, focusing on the complications of maternal diabetes. Next, we will review the differentiation protocols developed to generate different cell lineages from hESCs. Additionally, we will review how hESCs are utilized as a model for research into the DOHaD. The effects of environmental insults on hESC differentiation and the possible involvement of epigenetic regulation will be discussed.

**Key words:** Development origins of health and diseases; Maternal diabetes; Environmental insults; Type 2 diabetes; Human embryonic stem cells; Epigenetics

**Manuscript source:** Invited manuscript

**Received:** March 12, 2020

**Peer-review started:** March 12, 2020

**First decision:** April 25, 2020

**Revised:** April 28, 2020

**Accepted:** June 14, 2020

**Article in press:** June 14, 2020

**Published online:** August 26, 2020

**P-Reviewer:** El-Sayyad HIH

**S-Editor:** Wang JL

**L-Editor:** Wang TQ

**P-Editor:** Xing YX



©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** The study of the mechanisms by which the intrauterine environment regulates offspring health is important. In this review, we will discuss the use of human embryonic stem cells as an *in vitro* model for understanding the developmental origins of diseases such as type 2 diabetes.

**Citation:** Chen ACH, Lee KF, Yeung WSB, Lee YL. Human embryonic stem cells as an *in vitro* model for studying developmental origins of type 2 diabetes. *World J Stem Cells* 2020; 12(8): 761-775

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/761.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.761>

## INTRODUCTION

The increasing prevalence of diabetes is a serious global public health concern. According to the latest report from the International Diabetes Federation (Diabetes Atlas 2019), more than 400 million adults are thought to have diabetes<sup>[1]</sup>. More astonishingly, approximately half of them have not been diagnosed. The proportion of people with type 2 diabetes (T2D) has been increasing in most countries, including China. Indeed, the prevalence rate of diabetes in China has increased sharply in recent decades, from 1% in 1980<sup>[2]</sup> to 9.7% in 2008<sup>[3]</sup> and further to 10.9% in 2013<sup>[4]</sup>. Another report suggested that only one-fourth of the diabetes patients in China were diagnosed and treated, and among those treated, less than half of them had adequate glycemic control<sup>[5]</sup>. Diabetes is one of the biggest health issues in many countries. There is an urgent need for both national and international entities to tackle this problem.

T2D can be attributed to both genetic and environmental factors. For genetic factors, over 100 loci have been found to be associated with T2D. The susceptibility loci of T2D vary among ethnic groups. For instance, single nucleotide polymorphisms (SNPs) in *KCNQ1* are associated with T2D in both East Asian and European people<sup>[6]</sup>. *ARHGEF11* variants increase T2D risks in Pima Indian people<sup>[7]</sup>. On the other hand, SNPs of some loci (*TSPAN8-LGR5*, *THADA*, and *ADAMTS9*) are correlated with T2D susceptibility in Caucasian individuals but not in Chinese individuals<sup>[8]</sup>. Association studies suggested that genes such as *TCF7L2* and *KCNQ1* were related to pancreatic  $\beta$ -cell function and insulin secretion<sup>[9,10]</sup>. However, the causal relationship between genetic variants and disease phenotypes remains largely unclear. For environmental factors, in addition to personal lifestyle, maternal hyperglycemia also contributes to T2D risks. Approximately one-sixth of live births are affected by hyperglycemia during pregnancy<sup>[1]</sup>. Developmental epidemiological<sup>[11-13]</sup> and animal studies<sup>[14,15]</sup> indicated that *in utero* exposure to maternal diabetes increased the risks of developing T2D and insulin resistance in offspring. However, mechanistic studies on the inductive action of maternal hyperglycemic conditions on the development of T2D have been confined to animal models or pancreatic cell lines<sup>[16,17]</sup>. With the introduction of human embryonic stem cells (hESCs) in 1999<sup>[18]</sup>, early human embryo development can be studied *in vitro*. We and others have used hESCs as models for studying the *in utero* effects of maternal diabetes on early embryo development, which was previously not possible in other pancreatic cell lines. In this review, we will discuss the long-term health consequences of fetal exposure to maternal diabetes and update the use of hESCs for studying the developmental origins of T2D.

## MATERNAL CONDITIONS ASSOCIATED WITH DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASES

The concept of developmental origins of health and diseases (DOHaD) was first proposed by Barker *et al*<sup>[19-21]</sup> more than 30 years ago; therefore, it is also known as "Barker's hypothesis". The epidemiological studies by Barker *et al*<sup>[21]</sup> revealed a high correlation between infant mortality rate and the incidence of ischemic heart disease later in life. Additionally, fetal malnutrition was associated with the risk of developing heart disease in adulthood<sup>[19]</sup>. Based on their observations, it was suggested that an

adverse intrauterine environment would affect fetal programming. These changes permanently shaped the offspring's organ function and metabolism, which would contribute to the adult onset of noncommunicable diseases (NCDs). Birthweight is the first and most common parameter predicting the health status of individuals at childhood and adulthood. Low birthweight is associated with many NCDs, including heart disease and T2D<sup>[19,22]</sup>.

### **Maternal malnutrition**

Early studies of DOHaD focused on maternal malnutrition. A famous example of this was the Dutch famine study. The offspring cohort who had prenatal exposure to Dutch famine (1944-1945) was traced. Studies have revealed a strong association between prenatal exposure to famine and glucose intolerance<sup>[23]</sup>, obesity<sup>[24]</sup>, heart disease<sup>[25]</sup>, and even breast cancer<sup>[26]</sup>. A follow-up study demonstrated a trans-generational effect leading to neonatal adiposity in the F2 generation from the famine offspring cohort<sup>[27]</sup>. On the other hand, high birthweight, which has become more prevalent recently due to maternal obesity and overnutrition, is correlated with obesity<sup>[28]</sup>, T1D<sup>[29]</sup>, breast cancer, and pancreatic cancer<sup>[30]</sup>.

### **Maternal exposure to endocrine disrupting chemicals**

*In utero* exposure to chemicals was found to be detrimental to long-term health in offspring. Animal studies have demonstrated that *in utero* exposure to endocrine disrupting chemicals (EDCs), such as bisphenol A (BPA), alters the development of the mammary gland, increasing the risk of breast cancer<sup>[31]</sup>. Prenatal exposure to BPA and diethylstilbestrol reduces the fertility of female mice, and the effect is transgenerational through the F3 generation<sup>[32]</sup>. In addition to affecting the reproductive system, *in utero* exposure to chemicals also contributes to an increase in T2D risk. Prenatal exposure to BPA induced leptin levels in female infants, and elevated leptin levels are correlated with insulin resistance<sup>[33]</sup>. A similar finding was observed in mice, where the administration of low-dose BPA (10 µg/kg) led to the development of chronic hyperinsulinemia and impaired glucose tolerance<sup>[34]</sup>. Another study traced the offspring born from individuals exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) due to explosion incidence in Italy in the 1970s. They found that *in utero* exposure to TCDD increased the risk for metabolic syndrome in male offspring<sup>[35]</sup>. To date, many maternal conditions have been identified to be associated with DOHaD, including maternal stress, hypertension, obesity, diabetes, smoking, infection, malnutrition, and even overnutrition<sup>[36]</sup>.

### **Maternal diabetes**

One-sixth of live births worldwide are affected by hyperglycemia during pregnancy, among which approximately 80% are related to gestational diabetes (GDM)<sup>[1]</sup>. It is therefore apparent that maternal obesity, T2D, and GDM have long-term impacts on offspring health. GDM is defined as women without previously diagnosed diabetes who exhibit high blood glucose levels during pregnancy, especially during the third trimester. The prevalence of GDM ranges from 7%-10% of all pregnancies<sup>[37,38]</sup>. There are several risk factors contributing to GDM, which include obesity and personal or family history of T2D or GDM. Severely obese women have an 8-fold higher risk of developing GDM than pregnant women with a healthy weight<sup>[39]</sup>. It should be noted that GDM not only increased the risks of insulin resistance and T2D in offspring but also in mothers<sup>[40]</sup>. With the increasing number of pregnancies complicated by diabetes, it is important to understand the long-term impacts on offspring health through epidemiological studies. We will discuss the possible mechanisms in the context of epigenetics.

**Epidemiological and animal studies:** Maternal diabetes is often characterized by increased glucose transport from the placenta to the developing fetus; therefore, fetal macrosomia is the most obvious outcome that is studied<sup>[40,41]</sup>. Macrosomia is defined as birthweight of infants above 90<sup>th</sup> percentile of relative gestational age. More than 40% of infants born from diabetic pregnancy develop macrosomia<sup>[42]</sup>, which is associated with increased neonatal morbidity rates. Macrosomic infants have an approximately 5-fold higher risk of glucose infusion and a 2-fold higher risk of neonatal jaundice than healthy infants<sup>[43]</sup>. A similar observation was found in an animal model in which rat offspring born from streptozotocin (STZ)-induced diabetic mothers developed macrosomia<sup>[44]</sup>. The mechanisms by which *in utero* hyperglycemia leads to macrosomia are not completely known. It has been suggested that GDM causes downregulation of adiponectin and upregulation of leptin. Macrosomic development has been linked to the modulation of cytokines<sup>[45]</sup>.

The pathologies of macrosomia and maternal diabetes are associated with metabolic defects in infants. Macrosomic infants, and those born from diabetic pregnancies, have altered lipid metabolism. Compared with healthy babies, macrosomic infants have elevated plasma cholesterol and triglyceride levels<sup>[46]</sup>. In an STZ-induced diabetic rat model, the resulting offspring have increased lipid contents in serum and the liver<sup>[47]</sup>. These findings suggest alteration of lipid metabolism in the fetus, which contributes to risks of obesity and T2D in adulthood. Another important metabolic defect in the fetus is insulin secretion. Fetal development in the diabetic environment is accompanied by increased insulin secretion. Hyperinsulinemia has been found in cord blood in mothers with T2D or GDM<sup>[48]</sup>. Increased insulin secretion leads to overstimulation and exhaustion of fetal pancreatic  $\beta$ -cells. There is evidence of degranulation of fetal insulin-producing  $\beta$ -cells in the hyperglycemic intrauterine environment<sup>[49]</sup>.

In addition to metabolic defects, abnormal organ development frequently occurs in offspring exposed to an intrauterine diabetic environment. At the beginning of gestation, impaired gene expression resulting from oxidative stress in the hyperglycemic environment can lead to embryopathy and an increased risk of cardiac, renal, and gastrointestinal malformations<sup>[50,51]</sup>. Early fetal exposure to a diabetic environment is correlated with higher risks of congenital abnormalities than what is observed when analyzing other exposure periods<sup>[52]</sup>. Reduced organ mass is another abnormality observed during development in hyperglycemic *in utero* environments. In rats born to diabetic mothers, there is a reduction in *Igf2* expression levels in pancreatic  $\beta$ -cells and a decreased  $\beta$ -cell mass in the fetus<sup>[53]</sup>. In addition, *in utero* exposure to hyperglycemia is associated with a reduction in the number of nephrons and alterations of *Igf* expression in the fetal kidney<sup>[54,55]</sup>.

**Epigenetic mechanisms:** It has long been suggested that epigenetic changes act as mediators between the early life exposure to environmental insults and the later onset of diseases. Epigenetic changes, such as DNA methylation and histone modifications, are actively involved in the course of embryo development. For example, there is global demethylation after fertilization, and DNA methylation is reestablished upon lineage specification<sup>[56]</sup>. Therefore, the early fetal development period is highly susceptible to epigenomic dysregulation with long-term implications for the health of the offspring<sup>[57]</sup>.

The relationship between dysregulation of the DNA methylome and the risk of T2D has been extensively studied. In rats, offspring born from intrauterine growth retardation have increased risks of T2D in adulthood. In these offspring, *Pdx1* transcription in pancreatic  $\beta$  cells is silenced due to DNA hypermethylation<sup>[58]</sup>. In humans, the *PDX1* promoter is hypermethylated in the islets of T2D patients and is associated with lowered *PDX1* expression in islet cells<sup>[59]</sup>. *Pdx1* is important for early pancreatic specification in mouse embryos<sup>[60]</sup>. Peroxisome proliferator activated receptor gamma coactivator-1 alpha (*PPARGC1A*), which regulates ATP production, is also hypermethylated in human islet cells from T2D patients, and knockdown of *PPARGC1A* decreased insulin secretion<sup>[61]</sup>.

Two independent studies utilized DNA methylation profiling on islet cells from T2D patients to determine the global dysregulation of the DNA methylome in diabetic pathology. Volkmar *et al.*<sup>[62]</sup> and Dayeh *et al.*<sup>[63]</sup> reported 254 and 853 differentially methylated genes, respectively, between T2D and normal samples, among which most were hypomethylated in T2D patients. Their studies also indicated that the differentially methylated genes were related to  $\beta$ -cell function, insulin secretion, and T2D pathogenesis. Another report also showed that GDM altered the placental DNA methylome of genes related to insulin signaling and endocrine disorders in both humans and rats<sup>[64]</sup>.

Dysregulation of chromatin modifications is also closely associated with diabetes. High glucose conditions induce p300 acetyltransferase in primary human endothelial cells. The elevated p300 level increases histone acetylation, which results in induced gene expression of vasoactive factors and extracellular matrix proteins such as endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), and fibronectin, leading to functional alterations in endothelial cells mimicking diabetic conditions<sup>[65]</sup>. Histone methylation of the H3K4 active mark and H3K9 repressive mark is responsible for gene expression regulation. In rats, offspring born under diabetic conditions exhibit dysregulated histone modification of the *Pdx1* promoter; there is a progressive loss of H3K4 methylation but a gain of H3K9 methylation on the *Pdx1* promoter, leading to silencing of this gene during development<sup>[58]</sup>.



## PLURIPOTENT STEM CELLS AS MODELS FOR STUDYING DOHaD

hESCs have pluripotent characteristics. They can spontaneously differentiate into three germ layers (mesoderm, endoderm, and ectoderm) during embryoid body (EB) formation<sup>[18]</sup>. Directed differentiation protocols of hESCs into specific cell types have been developed. These differentiated cells are excellent *in vitro* models for studying early human embryo development. With the introduction of induced pluripotent stem cells (iPSCs) by Yamanaka *et al.*<sup>[66]</sup> in 2007, advancements were made to the regenerative medicine field, as patient iPSCs could be used to produce specific functional cell types to be used in replacement therapy. Indeed, iPSC technology-based regenerative therapy for diabetes has been vigorously studied in the past 10 years (reviewed in<sup>[67]</sup>).

Environmental insults such as maternal diabetes have been shown to affect neuronal, cardiac, and pancreatic development in offspring<sup>[68,69]</sup>. There is also evidence indicating the transgenerational epigenetic effects of environmental insults through germ cells. The specific cell lineages differentiated from pluripotent stem cells not only are of benefit for therapeutic purposes but also provide excellent *in vitro* models for studying DOHaD and the underlying mechanisms. In this section, we will update the differentiation protocols of those related cell lineages from pluripotent stem cells. The use of the models, in particular the pancreatic cell lineage, for studying the mechanism of DOHaD will also be discussed.

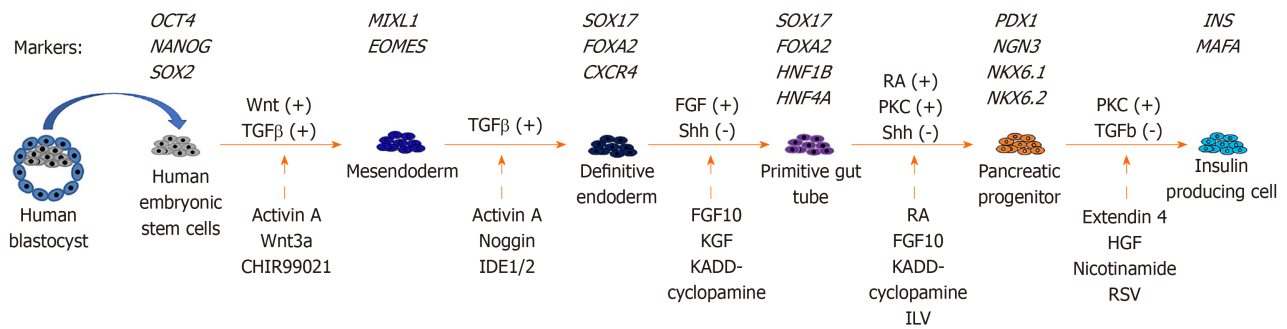
### Pancreatic cell lineage

**Pancreatic differentiation from hESCs:** Since hESCs were first established from human embryos in 1998<sup>[18]</sup>, there have been many studies on the production of glucose-responsive pancreatic  $\beta$  cells from hESCs for therapeutic purposes. The *in vitro* derivation of pancreatic  $\beta$  cells from hESCs involves stepwise inductions of cells representing mesendoderm (ME), definitive endoderm (DE), primitive gut tube (PGT), pancreatic progenitor (PP), and insulin-producing cell (IPC).

The stepwise differentiation of ESCs along the pancreatic lineage requires the activation of different signaling pathways (Figure 1). ME cells are bipotent in nature and are able to give rise to both the mesoderm and endoderm lineages during development<sup>[70]</sup>. In an early study of mouse embryonic development, ME cells were found to emerge from the anterior end of the primitive streak (APS)<sup>[71]</sup>. Brachyury (*T*)<sup>[72]</sup>, gooseoid (*GSC*)<sup>[73]</sup>, eomesodermin (*EOMES*)<sup>[74]</sup>, and *MIXL1*<sup>[75]</sup> are valuable mesendoderm markers. Activation of the Wnt and TGF $\beta$  pathways is important for the derivation of ME cells from hESCs *in vitro*<sup>[72,76]</sup>. Therefore, the differentiation of ME includes the use of recombinant activin A (AA), which mimics the action of Nodal as the ligand for the TGF $\beta$  signaling pathway<sup>[77]</sup>. In addition, treatment with recombinant Wnt3a or a glycogen synthase kinase 3 $\beta$  inhibitor (CHIR-99021) can be used to activate the Wnt pathway<sup>[76,78]</sup>.

DE can give rise to different endodermal cells, such as hepatocytes, epithelial cells of the respiratory tract, and the pancreas<sup>[79]</sup>. The efficient formation of DE cells is essential for subsequent differentiation into functional pancreatic cells<sup>[80]</sup>. The formation of the DE is marked by the expression of several transcription factors, including SRY (sex determining region Y)-box 17 (*SOX17*)<sup>[81]</sup>, forkhead box A2 (*FOXA2*), and chemokine (C-X-C Motif) receptor 4 (*CXCR4*)<sup>[82]</sup>. Similar to ME formation, activation of the TGF $\beta$  pathway is important for the induction of DE markers. Recombinant AA and noggin, which acts as a bone morphogenic protein (BMP) antagonist, are used for DE induction<sup>[83]</sup>. Small molecules, including induction of definitive endoderm 1/2 (*IDE1/IDE2*), can mimic the effects of AA. Treatment of hESCs and mESCs with *IDE1/2* induces DE formation, which is accompanied by an increase in *SOX17* expression<sup>[84]</sup>. Using a commercially available DE differentiation kit (STEMdiff DE kit), we have shown that ME cells can be induced after 2 d of differentiation with *T* and *MIXL1*, and we have shown the efficient generation of DE cells with *SOX17*, *FOXA2*, and *CXCR4* expression after 5 d of differentiation<sup>[85]</sup>.

The formation of a PGT follows after DE induction<sup>[86]</sup>. Growth factors, including FGF10 and keratinocyte growth factor, enhance the efficiency of PGT formation<sup>[76,87]</sup>. Inhibiting the sonic hedgehog (Shh) signaling pathway by cyclopamine-KAAD treatment efficiently induces PGT specification<sup>[76]</sup>. The action is concordant with inhibition of cells entering an intestinal differentiation pathway following knockout of Shh signaling during mouse pancreatic bud formation<sup>[88]</sup>. Further specification into PP cells requires the continuous activation of FGF and inhibition of Shh signaling. The addition of retinoic acid together with FGF10 and cyclopamine-KAAD enhances PP formation<sup>[76]</sup>. In addition, activation of the protein kinase C (PKC) signaling pathway aids the formation of PP cells from the DE stage. A small molecule, indolactam V,



**Figure 1 Stepwise *in vitro* differentiation of pancreatic cells from human embryonic stem cells.** Stage-specific markers, regulating pathways, recombinant proteins, and small molecules added at different stages are listed. +: Positive regulation; -: Negative regulation; IDE: Induction of definitive endoderm; Shh: Sonic hedgehog; KGF: Keratinocyte growth factor; RA: Retinoic acid; PKC: Protein kinase C; ILV: Indolactam V; HGF: Hepatocyte growth factor; RSV: Resveratrol.

which activates PKC signaling, was found to induce PP differentiation from hESCs<sup>[89]</sup>. The PP cells expressed several markers, including *PDX1*, *SOX9*, *NKX6.1*, and *NKX6.2*<sup>[76,90]</sup>.

For the final step of producing IPCs from hESCs, there are two major approaches. One approach is to transplant PP cells into the mouse kidney capsule and allow them to mature *in vivo*<sup>[91]</sup>. The other approach is the *in vitro* differentiation of IPCs from PP cells. Treatment with extendin 4, hepatocyte growth factor, BMP4, and nicotinamide increased insulin secretion by PP cells in response to high glucose levels. However, the *in vitro* differentiation protocols are not efficient, and only approximately 10% of cells are insulin positive<sup>[76,92]</sup>. Pagliuca *et al*<sup>[93]</sup> reported the use of Alk5 receptor inhibitor II, PKC signaling activator, and thyroid hormone in the formation of  $\beta$  cells. Their results demonstrated that the  $\beta$  cells that formed were functional, as transplantation into diabetic mice successfully restored blood glucose to normal levels<sup>[93]</sup>.

Three-dimensional organoid culture methods have recently been developed for the differentiation of hESCs. The organoids formed were reported to be structurally and functionally similar to their native tissue counterparts. For instance, pancreatic organoids were formed by aggregating hESC-derived PP cells in a novel hydrogel system named Amikagel. The resulting cells in the organoids closely mimicked pancreatic islet cells<sup>[94]</sup>.

**Pancreatic differentiation from hESCs as a model for studying DOHaD:** Diabetic pregnancy is known to increase the risks of insulin resistance and T2D in offspring in adulthood. Epigenetic dysregulation is associated with disease phenotypes. For instance, mice born from diabetic pregnancies exhibit hypermethylation of *pdx1* promoter DNA<sup>[58]</sup>. Diabetic pregnancies induce global changes in the DNA methylome related to insulin signaling in the human placenta<sup>[64]</sup>. However, studies on the effects of environmental insults on human fetal pancreas development are very limited. We used hESCs as an *in vitro* model to study the developmental origins of diabetes. Early pancreatic differentiation is mainly modulated by histone methylation<sup>[95,96]</sup>. We confirmed that the promoters of DE markers (*SOX17*, *FOXA2*, and *CXCR4*) were marked bivalently by both the activating mark H3K4me3 and the repressive mark H3K27me3 at the pluripotent stage. Upon differentiation into DE, the repressive mark H3K27me3 was removed, leading to active expression of DE markers. More importantly, our study was the first to discover that a hyperglycemic environment disrupted histone methylation patterns, resulting in retention of repressive H3K27me3 marks at DE promoters and a significant reduction in their expression compared to the control. The inhibition of DE specification is also observed in mice upon *in utero* exposure to hyperglycemia<sup>[85]</sup> (Table 1). Recently, studies have demonstrated active DNA methylation and hydroxymethylation during different stages of *in vitro* pancreatic differentiation from hESCs. DNA hydroxymethylation has been associated with chromatin accessibility, therefore allowing the binding of transcription factors for efficient pancreatic differentiation<sup>[97]</sup>. The above studies suggest the important roles of DNA methylation and hydroxymethylation in pancreatic development.

In addition to maternal diabetes, the effects of *in utero* exposure to chemicals such as EDCs have also been extensively studied in the later development of offspring. For instance, an animal study showed that *in utero* exposure to BPA increased glucagon secretion in fetal islets by affecting the  $\alpha$ -to- $\beta$  cell ratio<sup>[98]</sup>. Recently, we conducted

**Table 1** The use of pluripotent stem cells as *in vitro* models for studying the developmental origins of health and diseases

Environmental insult(s)	Type of pluripotent stem cells	Cell lineage	Effects on differentiation	Ref.
Hyperglycemia (25-50 mmol/L)	hESC	Pancreatic	(1) Inhibited differentiation into DE; and (2) retained repressive H3K27me3 mark on DE marker promoters	[85]
Hyperglycemia (25-50 mmol/L)	mESC	Pancreatic	Inhibited differentiation into DE	[85]
TCDD (10 pmol/L)	hESC	Pancreatic	Dysregulated DNA methylome of genes related to diabetes	[99]
TCDD (10-100 pmol/L)	hESC	Pancreatic	(1) Dysregulated DNA methylome of genes related to insulin signaling and diabetes; (2) inhibited differentiation into pancreatic progenitor; and (3) promoted DNA hypermethylation of <i>PRKAG1</i>	[100]
Hyperglycemia (10 mmol/L), endothelin-1 (ET-1) (10 nmol/L), and cortisol (1 $\mu$ mol/L)	hiPSC	Cardiac	(1) Inhibited cardiomyocyte differentiation; and (2) elevated oxidative stress in cardiomyocytes formed	[114]
Hyperglycemia (25 mmol/L)	mESC	Cardiac	Inhibited mesoderm and subsequent cardiomyocyte differentiation	[117]
Hyperglycemia (25 mmol/L)	mESC	Cardiac	Enhanced cardiomyocyte differentiation	[118]
TCDD (1 nmol/L)	mESC	Cardiac	Inhibited cardiomyocyte differentiation	[136]
TCDD (10-100 pmol/L)	hESC	Cardiac	Dysregulated DNA methylome of genes related to cardiomyopathy	[100]
BPA (1-8 $\mu$ g/mL); PFOS (5-40 $\mu$ g/mL); PFOA (10-80 $\mu$ g/mL)	mESC	Cardiac	Inhibited cardiomyocyte differentiation	[137]
Hyperglycemia (25 mmol/L)	mESC	Neural	Inhibited neural differentiation	[125]

hESC: Human embryonic stem cells; TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin; DE: Definitive endoderm; BPA: Bisphenol A.

transcriptomic and methylomic analyses on hESCs upon low-dose (10 pM) TCDD treatment. Our results revealed that the expression and DNA methylation status of a number of genes were dysregulated upon TCDD treatments. Among them, some of the genes, such as adenosine A1 receptor (*ADORA1*), *ADORA2A*, inhibin beta A subunit, and hemopexin, were associated with the pathogenesis of diabetes<sup>[99]</sup>. Low-dose TCDD (10-100 pM) treatment of hESCs also induced hypermethylation of a number of genes that are related to insulin signaling and T2D. Among them, *PRKAG1* remained hypermethylated even upon PP differentiation. *PRKAG1* knockdown in the pancreatic cell line INS-1E resulted in elevated levels of secreted insulin<sup>[100]</sup> (Table 1). In addition, our findings suggested that the dysregulated DNA methylation patterns induced by early chemical exposure might be maintained during early embryonic development. These changes might lead to pathology, such as insulin resistance and diabetes, in offspring.

### Cardiac cell lineage

**Cardiac differentiation from hESCs:** The human heart is often considered a nonregenerative organ due to the limited proliferative ability of adult cardiomyocytes (CMs). Following the first reports of hESCs<sup>[18]</sup> and iPSCs<sup>[66]</sup>, several approaches have been developed to differentiate these cells into functional CMs. This section will discuss the transcription factors and cell signaling pathways essential for CM development. We will also introduce various CM differentiation protocols that have been developed.

The heart is one of the organs that develops early in embryos. In the human embryo, the primordial heart begins to develop at 20 d after fertilization. Cardiac cell lineage emerges from the mesoderm. The induction of mesoderm formation is mainly controlled by three cell signaling pathways: the FGF, Wnt and TGF $\beta$  pathways. Mesoderm development can be marked with the expression of markers such as T-box transcription factor brachyury (*T*) and *EOMES*<sup>[101]</sup>. The mesodermal cell population expressing mesoderm posterior 1 (*MESP1*) further develop into cardiac progenitor cells *via* inhibition of the Wnt/ $\beta$ -catenin pathway<sup>[102,103]</sup>. The subsequent specification into CMs requires the action of signaling pathways such as retinoic acid (RA) and FGF pathways, where *MESP1* is the upstream regulator of cardiac-specific transcription factors, such as GATA binding protein 4 (*GATA4*) and NK2 homeobox 5 (*NKX2.5*)<sup>[104]</sup>. The *in vitro* differentiation of hESCs into CMs therefore involves stepwise manipulation of cell signaling pathways.

The successful derivation of CMs from hESCs was first reported through spontaneous differentiation of EBs. However, the efficiency was low, with 8.1% of the area exhibiting spontaneous beating and only 29.4% of cells expressing cardiac troponin I (*cTnI*) after 20 d of differentiation<sup>[105]</sup>. Several modified protocols have been subsequently reported. These reports also adopted the EB approach, but instead of spontaneous differentiation, and they mimicked *in vivo* signaling for directed differentiation. For instance, BMP4, Activin A, and bFGF were supplemented in culture for mesoderm induction. VEGF and DKK1 recombinant proteins were then added as Wnt/ $\beta$ -catenin inhibitors. The cells were further treated with VEGF, bFGF, and DKK1 to induce expansion and differentiation into CMs. With improved protocols, the efficiency of CM differentiation was increased (> 80% *cTnI*<sup>+</sup> cells), and it was achieved in a shorter period of time (8-10 d)<sup>[106,107]</sup>. Subsequently, different EB culturing tools were developed for scaling up CM production for therapeutic purposes. For instance, microwells allow the production of a large number of uniformly sized EBs<sup>[108]</sup>. On the other hand, researchers developed microcarriers that promoted the expansion of differentiating hESCs in spinner flasks and bioreactors for large-scale CM production<sup>[109,110]</sup>.

**Cardiac differentiation from hESCs as a model for studying DOHaD:** Diabetic cardiomyopathy (DCM) is a complication of T2D. Maternal diabetes also increases the risk of cardiomyopathy in infants<sup>[111,112]</sup>. An early animal study using streptozotocin-induced diabetic mice demonstrated a high rate of apoptosis in cardiomyocytes. An *in vitro* study using adult CMs also exhibited reduced myofibrillar formation under high glucose treatment<sup>[113]</sup>. However, the underlying mechanisms of the developmental origins of cardiomyopathy remain largely unknown. hESC-derived CMs can therefore serve as an excellent *in vitro* model for recapitulating major events during embryonic heart development.

Diabetic conditions, including high glucose (10 mmol/L), ET-1 (10 nmol/L), and cortisol (1  $\mu$ mol/L) treatments, induce hypertrophic stress with elevated expression of hypertrophic markers (*NPPA*, *NPPB*, *ACTA1*, and *MYH7*) during CM differentiation from hiPSCs. The treated CMs exhibit cardiomyopathy phenotypes such as disorganized sarcomere structures, accumulation of lipid contents, and oxidative stress<sup>[114]</sup> (Table 1). Defects in embryonic CM formation might lead to an increased risk of DCM in adulthood<sup>[114]</sup>. hESC-CMs are not extensively used as a DOHaD model for cardiomyopathy. This could be attributed to the fact that hESC-CMs do not represent fully mature CMs. The contractile function and cardiac marker expression of hESC-CMs are not comparable to those of fetal or adult CMs<sup>[115,116]</sup>. Notwithstanding, similar studies have been performed in a mESC model to understand the effects of *in utero* hyperglycemia on cardiac development. It was demonstrated that high glucose conditions (25 mmol/L) impaired cardiac differentiation from mESCs compared with what was observed in cells treated with physiological levels of glucose (5 mmol/L). There was a significant reduction in contracting CMs under high glucose levels. In addition, a significant reduction in the expression of mesoderm markers (*T* and *Mixl1*) and cardiac markers (*Gata4* and *Nkx2.5*)<sup>[117]</sup> was observed upon hyperglycemia treatment. However, opposite results from another study showed that CM formation from EBs was more efficient under high glucose treatment<sup>[118]</sup> (Table 1). The effects of hyperglycemia and other environmental insults on human CM differentiation require further investigation.

A recent epigenomic study on human CMs revealed that prenatal and postnatal heart development were regulated by DNA methylation and histone modifications. More importantly, active histone marks (H3K27ac, H3K4me3, H3K9ac, and H3K36me3) were found in the promoters of pathology-related genes such as connective tissue growth factor (*CTGF*) and natriuretic peptides A and B (*NPPA* and *NPPB*) in diseased CMs<sup>[119]</sup>. Another recent study demonstrated distinct DNA methylation patterns in atrial and ventricular subtypes of hiPSC-derived CMs<sup>[120]</sup>. These findings reveal that epigenetic regulation not only occurs during prenatal heart development but also is responsible for cardiomyopathy. The study of DOHaD in relation to cardiomyopathy in an epigenetic context warrants further investigation.

### Other lineage differentiation from hESCs as a model of DOHaD

**Neural lineage:** There is a strong clinical association between maternal diabetes and neural tube defects (NTDs). Maternal diabetes increases the risk of central nervous system malformation in fetuses by 10-15-fold over that of nondiabetic mothers<sup>[121,122]</sup>. Similarly, mouse offspring born from diabetic mothers have an approximately 10% chance of developing NTDs. A high level of oxidative stress leads to neural cell apoptosis in the affected offspring<sup>[123]</sup>. Maternal hyperglycemia also results in the



activation of apoptosis signal-regulating kinase 1 (*Ask1*) in the developing neural tubes of mouse embryos. The activation of *Ask1* is related to an increase in caspase 8 protein levels and apoptosis<sup>[124]</sup>.

Studies in animal models provide information on the effects of the *in utero* environment on early neural development. However, further studies remain challenging because of the limited number of cells in fetal neural tissues. Nevertheless, high glucose treatment (25 mmol/L) *in vitro* impedes neural differentiation, resulting in the downregulation of neuronal markers (*Sox1*, *Nestin*, and *Pax6*)<sup>[125]</sup> (Table 1). Folate deficiency was shown to induce inhibition of the DNA methylation cycle, leading to NTDs in animals<sup>[126]</sup>. Knockout of histone modifiers such as *Sirt1* and histone deacetylase 4 also causes NTDs in developing mouse embryos<sup>[127,128]</sup>. It should be noted that the effects of environmental insults on human neural development may be different from those observed in mice. Further mechanistic studies using hESCs as cell models can improve our understanding in the context of DOHaD. Indeed, treatment with noggin, which inhibits the BMP pathway, successfully enabled derivation of neuronal cells from hESCs. The neurospheres formed could further differentiate into mature neurons and glia<sup>[129]</sup>, providing a good cellular research model.

**Germ cell lineage:** Growing evidence suggests that the negative impacts of adverse intrauterine environments on offspring might be transgenerational, meaning that the disease phenotypes will be expressed in the F2 generation. Such transgenerational effects are evidenced in animal models. For example, vinclozolin (VCZ; 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione), one of the EDCs widely used as a fungicide, dysregulates the epigenome of primordial germ cells (PGCs) in mice from F1 to F3; the microRNA pattern in F1-F3 PGCs is disrupted following F0 animal exposure to VCZ<sup>[130]</sup>. The downregulation of *miR-23b* and *miR-21* in the treated mice disrupts the let7 pathway, leading to increased apoptosis of embryonic PGCs. Another EDC, TCDD, alters transcriptomes in the gonads of F1 and F2 zebrafish<sup>[131]</sup>. The genes with altered expression were related to lipid and glucose metabolism, oxidative stress, and sperm cell development.

In addition to EDC exposure, the transgenerational effects of maternal hyperglycemia have been extensively studied in animal models. Ding *et al.*<sup>[132]</sup> reported that the mating of F1 male mice from diabetic pregnancies with normal female mice resulted in F2 mice with increased birth weight and impaired glucose tolerance. They associated the above observations with DNA hypermethylation of imprinted genes *Igf2* and *H19* in the pancreatic islets of F1 and F2 mice<sup>[132]</sup>. A recent report revealed that maternal diabetes dysregulated the DNA methylome of embryonic F1 PGCs. The differentially methylated genes were related to obesity, insulin resistance, and T2D. More importantly, the same pattern was also observed in F2 somatic cells<sup>[133]</sup>. These studies demonstrated that environmental insults, such as chemicals or hyperglycemia, could be transmitted transgenerationally by changing the epigenomes of germ cells.

*In vitro* germ cell differentiation from ESCs has only recently been reported. Haploid germ cells can be generated by coculturing mESC-derived PGC-like cells with neonatal testicular somatic cells. *In vitro*-derived haploid spermatids are able to generate offspring when injected into oocytes<sup>[134]</sup>. In human culture systems, PGCs can be successfully derived from hESCs. The derivation protocol adopted a stepwise approach recapitulating *in vivo* developmental events, where Wnt and BMP pathways were activated to drive the formation of premesodermal cells. The specifications of PGCs were then induced by treatment with growth factors such as BMP2, stem cell factor, and epidermal growth factor<sup>[135]</sup>. Advances in germ cell differentiation using a human cell model also provide an opportunity for the study of the transgenerational effects of DOHaD.

## CONCLUSION

Much evidence supporting the idea of DOHaD has been obtained from animal models and observational studies of human. The mechanisms behind the long-term health consequences of fetal exposure to adverse maternal conditions are largely unknown in humans. Accumulating data from both hESCs and mESCs suggested that early cell lineage differentiation might be one of the vulnerable embryonic windows through which early exposure to adverse maternal conditions could exert its diabetogenic effects. While protocols for the differentiation of different cell types from ESCs still require further improvement to better mimic physiological development, it is expected that the information obtained from these cell models will provide valuable mechanistic insight into the mechanisms underlying the DOHaD.



## REFERENCES

- 1 **International Diabetes Federation.** IDF Diabetes Atlas, 2019. 9th ed. Brussels: International Diabetes Federation, 2019
- 2 [A mass survey of diabetes mellitus in a population of 300,000 in 14 provinces and municipalities in China (author's transl)]. *Zhonghua Nei Ke Za Zhi* 1981; **20**: 678-683 [PMID: [7341098](#)]
- 3 **Yang SH,** Dou KF, Song WJ. Prevalence of diabetes among men and women in China. *N Engl J Med* 2010; **362**: 2425-6; author reply 2426 [PMID: [20578276](#)]
- 4 **Wang L,** Gao P, Zhang M, Huang Z, Zhang D, Deng Q, Li Y, Zhao Z, Qin X, Jin D, Zhou M, Tang X, Hu Y, Wang L. Prevalence and Ethnic Pattern of Diabetes and Prediabetes in China in 2013. *JAMA* 2017; **317**: 2515-2523 [PMID: [28655017](#) DOI: [10.1001/jama.2017.7596](#)]
- 5 **Xu Y,** Wang L, He J, Bi Y, Li M, Wang T, Wang L, Jiang Y, Dai M, Lu J, Xu M, Li Y, Hu N, Li J, Mi S, Chen CS, Li G, Mu Y, Zhao J, Kong L, Chen J, Lai S, Wang W, Zhao W, Ning G; 2010 China Noncommunicable Disease Surveillance Group. Prevalence and control of diabetes in Chinese adults. *JAMA* 2013; **310**: 948-959 [PMID: [24002281](#) DOI: [10.1001/jama.2013.168118](#)]
- 6 **Unoki H,** Takahashi A, Kawaguchi T, Hara K, Horikoshi M, Andersen G, Ng DP, Holmkvist J, Borch-Johnsen K, Jørgensen T, Sandbaek A, Lauritzen T, Hansen T, Nurbaya S, Tsunoda T, Kubo M, Babazono T, Hirose H, Hayashi M, Iwamoto Y, Kashiwagi A, Kaku K, Kawamori R, Tai ES, Pedersen O, Kamatani N, Kadowaki T, Kikkawa R, Nakamura Y, Maeda S. SNPs in KCNQ1 are associated with susceptibility to type 2 diabetes in East Asian and European populations. *Nat Genet* 2008; **40**: 1098-1102 [PMID: [18711366](#) DOI: [10.1038/ng.208](#)]
- 7 **Ma L,** Hanson RL, Que LN, Cali AM, Fu M, Mack JL, Infante AM, Kobes S; International Type 2 Diabetes 1q Consortium, Bogardus C, Shuldiner AR, Baier LJ. Variants in ARHGEF11, a candidate gene for the linkage to type 2 diabetes on chromosome 1q, are nominally associated with insulin resistance and type 2 diabetes in Pima Indians. *Diabetes* 2007; **56**: 1454-1459 [PMID: [17287471](#) DOI: [10.2337/db06-0640](#)]
- 8 **Hu C,** Zhang R, Wang C, Wang J, Ma X, Lu J, Qin W, Hou X, Wang C, Bao Y, Xiang K, Jia W. PPARG, KCNJ11, CDKAL1, CDKN2A-CDKN2B, IDE-KIF11-HHEX, IGF2BP2 and SLC30A8 are associated with type 2 diabetes in a Chinese population. *PLoS One* 2009; **4**: e7643 [PMID: [19862325](#) DOI: [10.1371/journal.pone.0007643](#)]
- 9 **Müssig K,** Staiger H, Machicao F, Kirchhoff K, Guthoff M, Schäfer SA, Kantartzis K, Silbernagel G, Stefan N, Holst JJ, Gallwitz B, Häring HU, Fritsche A. Association of type 2 diabetes candidate polymorphisms in KCNQ1 with incretin and insulin secretion. *Diabetes* 2009; **58**: 1715-1720 [PMID: [19366866](#) DOI: [10.2337/db08-1589](#)]
- 10 **Gloyn AL,** Braun M, Rorsman P. Type 2 diabetes susceptibility gene TCF7L2 and its role in beta-cell function. *Diabetes* 2009; **58**: 800-802 [PMID: [19336690](#) DOI: [10.2337/db09-0099](#)]
- 11 **Meigs JB,** Cupples LA, Wilson PW. Parental transmission of type 2 diabetes: the Framingham Offspring Study. *Diabetes* 2000; **49**: 2201-2207 [PMID: [11118026](#) DOI: [10.2337/diabetes.49.12.2201](#)]
- 12 **Hunter WA,** Cundy T, Rabone D, Hofman PL, Harris M, Regan F, Robinson E, Cutfield WS. Insulin sensitivity in the offspring of women with type 1 and type 2 diabetes. *Diabetes Care* 2004; **27**: 1148-1152 [PMID: [15111536](#) DOI: [10.2337/diacare.27.5.1148](#)]
- 13 **Gautier JF,** Wilson C, Weyer C, Mott D, Knowler WC, Cavaghan M, Polonsky KS, Bogardus C, Pratley RE. Low acute insulin secretory responses in adult offspring of people with early onset type 2 diabetes. *Diabetes* 2001; **50**: 1828-1833 [PMID: [11473045](#) DOI: [10.2337/diabetes.50.8.1828](#)]
- 14 **Aerts L,** Holemans K, Van Assche FA. Maternal diabetes during pregnancy: consequences for the offspring. *Diabetes Metab Rev* 1990; **6**: 147-167 [PMID: [2091909](#) DOI: [10.1002/dmr.5610060303](#)]
- 15 **Boloker J,** Gertz SJ, Simmons RA. Gestational diabetes leads to the development of diabetes in adulthood in the rat. *Diabetes* 2002; **51**: 1499-1506 [PMID: [11978648](#) DOI: [10.2337/diabetes.51.5.1499](#)]
- 16 **Ishihara H,** Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki JI, Oka Y. Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* 1993; **36**: 1139-1145 [PMID: [8270128](#) DOI: [10.1007/bf00401058](#)]
- 17 **Merglen A,** Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P. Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 2004; **145**: 667-678 [PMID: [14592952](#) DOI: [10.1210/en.2003-1099](#)]
- 18 **Thomson JA,** Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: [9804556](#) DOI: [10.1126/science.282.5391.1145](#)]
- 19 **Barker DJ,** Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. *Lancet* 1993; **341**: 938-941 [PMID: [8096277](#) DOI: [10.1016/0140-6736\(93\)91224-a](#)]
- 20 **Barker DJ,** Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet* 1989; **2**: 577-580 [PMID: [2570282](#) DOI: [10.1016/s0140-6736\(89\)90710-1](#)]
- 21 **Barker DJ,** Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1986; **1**: 1077-1081 [PMID: [2871345](#) DOI: [10.1016/s0140-6736\(86\)91340-1](#)]
- 22 **Whincup PH,** Kaye SJ, Owen DG, Huxley R, Cook DG, Anazawa S, Barrett-Connor E, Bhargava SK, Birgisdottir BE, Carlsson S, de Rooij SR, Dyck RF, Eriksson JG, Falkner B, Fall C, Forsén T, Grill V, Gudnason V, Hulman S, Hyppönen E, Jeffreys M, Lawlor DA, Leon DA, Minami J, Mishra G, Osmond C, Power C, Rich-Edwards JW, Roseboom TJ, Sachdev HS, Syddall H, Thorsdottir I, Vanhala M, Wadsworth M, Yarbrough DE. Birth weight and risk of type 2 diabetes: a systematic review. *JAMA* 2008; **300**: 2886-2897 [PMID: [19109117](#) DOI: [10.1001/jama.2008.886](#)]
- 23 **Ravelli AC,** van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN, Bleker OP. Glucose tolerance in adults after prenatal exposure to famine. *Lancet* 1998; **351**: 173-177 [PMID: [9449872](#) DOI: [10.1016/s0140-6736\(97\)07244-9](#)]

- 24 **Ravelli AC**, van Der Meulen JH, Osmond C, Barker DJ, Bleker OP. Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* 1999; **70**: 811-816 [PMID: [10539740](#) DOI: [10.1093/ajcn/70.5.811](#)]
- 25 **Roseboom TJ**, van der Meulen JH, Osmond C, Barker DJ, Ravelli AC, Schroeder-Tanka JM, van Montfrans GA, Michels RP, Bleker OP. Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. *Heart* 2000; **84**: 595-598 [PMID: [11083734](#) DOI: [10.1136/heart.84.6.595](#)]
- 26 **Painter RC**, De Rooij SR, Bossuyt PM, Osmond C, Barker DJ, Bleker OP, Roseboom TJ. A possible link between prenatal exposure to famine and breast cancer: a preliminary study. *Am J Hum Biol* 2006; **18**: 853-856 [PMID: [17039469](#) DOI: [10.1002/ajhb.20564](#)]
- 27 **Painter RC**, Osmond C, Gluckman P, Hanson M, Phillips DI, Roseboom TJ. Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. *BJOG* 2008; **115**: 1243-1249 [PMID: [18715409](#) DOI: [10.1111/j.1471-0528.2008.01822.x](#)]
- 28 **Rugholm S**, Baker JL, Olsen LW, Schack-Nielsen L, Bua J, Sørensen TI. Stability of the association between birth weight and childhood overweight during the development of the obesity epidemic. *Obes Res* 2005; **13**: 2187-2194 [PMID: [16421354](#) DOI: [10.1038/oby.2005.271](#)]
- 29 **Harder T**, Roepke K, Diller N, Stechling Y, Dudenhausen JW, Plagemann A. Birth weight, early weight gain, and subsequent risk of type 1 diabetes: systematic review and meta-analysis. *Am J Epidemiol* 2009; **169**: 1428-1436 [PMID: [19363100](#) DOI: [10.1093/aje/kwp065](#)]
- 30 **Ahlgren M**, Wohlfahrt J, Olsen LW, Sørensen TI, Melbye M. Birth weight and risk of cancer. *Cancer* 2007; **110**: 412-419 [PMID: [17538980](#) DOI: [10.1002/cncr.22773](#)]
- 31 **Markey CM**, Luque EH, Munoz De Toro M, Sonnenschein C, Soto AM. In utero exposure to bisphenol A alters the development and tissue organization of the mouse mammary gland. *Biol Reprod* 2001; **65**: 1215-1223 [PMID: [11566746](#) DOI: [10.1093/biolreprod/65.4.1215](#)]
- 32 **Ziv-Gal A**, Wang W, Zhou C, Flaws JA. The effects of in utero bisphenol A exposure on reproductive capacity in several generations of mice. *Toxicol Appl Pharmacol* 2015; **284**: 354-362 [PMID: [25771130](#) DOI: [10.1016/j.taap.2015.03.003](#)]
- 33 **Ashley-Martin J**, Dodds L, Arbuckle TE, Ettinger AS, Shapiro GD, Fisher M, Morisset AS, Taback S, Bouchard MF, Monnier P, Dallaire R, Fraser WD. A birth cohort study to investigate the association between prenatal phthalate and bisphenol A exposures and fetal markers of metabolic dysfunction. *Environ Health* 2014; **13**: 84 [PMID: [25336252](#) DOI: [10.1186/1476-069X-13-84](#)]
- 34 **Alonso-Magdalena P**, Morimoto S, Ripoll C, Fuentes E, Nadal A. The estrogenic effect of bisphenol A disrupts pancreatic beta-cell function in vivo and induces insulin resistance. *Environ Health Perspect* 2006; **114**: 106-112 [PMID: [16393666](#) DOI: [10.1289/ehp.8451](#)]
- 35 **Warner M**, Rauch S, Ames J, Mocarelli P, Brambilla P, Signorini S, Eskenazi B. In utero dioxin exposure and cardiometabolic risk in the Seveso Second Generation Study. *Int J Obes (Lond)* 2019; **43**: 2233-2243 [PMID: [30659254](#) DOI: [10.1038/s41366-018-0306-8](#)]
- 36 **Mandy M**, Nyirenda M. Developmental Origins of Health and Disease: the relevance to developing nations. *Int Health* 2018; **10**: 66-70 [PMID: [29528398](#) DOI: [10.1093/inthealth/ihy006](#)]
- 37 **American Diabetes Association**. Gestational diabetes mellitus. *Diabetes Care* 2004; **27** Suppl 1: S88-S90 [PMID: [14693936](#)]
- 38 **Ross G**. Gestational diabetes. *Aust Fam Physician* 2006; **35**: 392-396 [PMID: [16751853](#)]
- 39 **Chu SY**, Callaghan WM, Kim SY, Schmid CH, Lau J, England LJ, Dietz PM. Maternal obesity and risk of gestational diabetes mellitus. *Diabetes Care* 2007; **30**: 2070-2076 [PMID: [17416786](#) DOI: [10.2337/dc06-2559a](#)]
- 40 **Ben-Haroush A**, Yogev Y, Hod M. Epidemiology of gestational diabetes mellitus and its association with Type 2 diabetes. *Diabet Med* 2004; **21**: 103-113 [PMID: [14984444](#) DOI: [10.1046/j.1464-5491.2003.00985.x](#)]
- 41 **Van Assche FA**, Horemans K, Aerts L. Long-term consequences for offspring of diabetes during pregnancy. *Br Med Bull* 2001; **60**: 173-182 [PMID: [11809625](#) DOI: [10.1093/bmb/60.1.173](#)]
- 42 **Kc K**, Shakya S, Zhang H. Gestational diabetes mellitus and macrosomia: a literature review. *Ann Nutr Metab* 2015; **66** Suppl 2: 14-20 [PMID: [26045324](#) DOI: [10.1159/000371628](#)]
- 43 **Hunter DJ**, Burrows RF, Mohide PT, Whyte RK. Influence of maternal insulin-dependent diabetes mellitus on neonatal morbidity. *CMAJ* 1993; **149**: 47-52 [PMID: [8319154](#)]
- 44 **Gauguier D**, Bihoreau MT, Picon L, Ktorza A. Insulin secretion in adult rats after intrauterine exposure to mild hyperglycemia during late gestation. *Diabetes* 1991; **40** Suppl 2: 109-114 [PMID: [1684164](#)]
- 45 **Atégbó JM**, Grissa O, Yessoufou A, Hichami A, Dramane KL, Moutairou K, Miled A, Grissa A, Jerbi M, Tabka Z, Khan NA. Modulation of adipokines and cytokines in gestational diabetes and macrosomia. *J Clin Endocrinol Metab* 2006; **91**: 4137-4143 [PMID: [16849405](#) DOI: [10.1210/jc.2006-0980](#)]
- 46 **Grissa O**, Atégbó JM, Yessoufou A, Tabka Z, Miled A, Jerbi M, Dramane KL, Moutairou K, Prost J, Hichami A, Khan NA. Antioxidant status and circulating lipids are altered in human gestational diabetes and macrosomia. *Transl Res* 2007; **150**: 164-171 [PMID: [17761369](#) DOI: [10.1016/j.trsl.2007.03.007](#)]
- 47 **Merzouk H**, Madani S, Hichami A, Prost J, Belleville J, Khan NA. Age-related changes in fatty acids in obese offspring of streptozotocin-induced diabetic rats. *Obes Res* 2002; **10**: 703-714 [PMID: [12105294](#) DOI: [10.1038/oby.2002.95](#)]
- 48 **Westgate JA**, Lindsay RS, Beattie J, Pattison NS, Gamble G, Mildenhall LF, Breier BH, Johnstone FD. Hyperinsulinemia in cord blood in mothers with type 2 diabetes and gestational diabetes mellitus in New Zealand. *Diabetes Care* 2006; **29**: 1345-1350 [PMID: [16732019](#) DOI: [10.2337/dc05-1677](#)]
- 49 **Van Assche FA**, Aerts L, de Prins F. Degranulation of the insulin-producing beta cells in an infant of a diabetic mother. Case report. *Br J Obstet Gynaecol* 1983; **90**: 182-185 [PMID: [6337622](#) DOI: [10.1111/j.1471-0528.1983.tb08906.x](#)]
- 50 **Zhao Z**, Reece EA. Experimental mechanisms of diabetic embryopathy and strategies for developing therapeutic interventions. *J Soc Gynecol Investig* 2005; **12**: 549-557 [PMID: [16325743](#) DOI: [10.1016/j.jsig.2005.07.005](#)]
- 51 **Loeken MR**. Advances in understanding the molecular causes of diabetes-induced birth defects. *J Soc Gynecol Investig* 2006; **13**: 2-10 [PMID: [16303321](#) DOI: [10.1016/j.jsig.2005.09.007](#)]

- 52 **Farrell T**, Neale L, Cundy T. Congenital anomalies in the offspring of women with type 1, type 2 and gestational diabetes. *Diabet Med* 2002; **19**: 322-326 [PMID: [11943005](#)]
- 53 **Serradas P**, Goya L, Lacorne M, Gangnerau MN, Ramos S, Alvarez C, Pascual-Leone AM, Portha B. Fetal insulin-like growth factor-2 production is impaired in the GK rat model of type 2 diabetes. *Diabetes* 2002; **51**: 392-397 [PMID: [11812746](#)]
- 54 **Amri K**, Freund N, Vilar J, Merlet-Bénichou C, Lelièvre-Pégorier M. Adverse effects of hyperglycemia on kidney development in rats: in vivo and in vitro studies. *Diabetes* 1999; **48**: 2240-2245 [PMID: [10535460](#)]
- 55 **Amri K**, Freund N, Duong Van Huyen JP, Merlet-Bénichou C, Lelièvre-Pégorier M. Altered nephrogenesis due to maternal diabetes is associated with increased expression of IGF-II/mannose-6-phosphate receptor in the fetal kidney. *Diabetes* 2001; **50**: 1069-1075 [PMID: [11334410](#)]
- 56 **Messerschmidt DM**, Knowles BB, Solter D. DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes Dev* 2014; **28**: 812-828 [PMID: [24736841](#) DOI: [10.1101/gad.234294.113](#)]
- 57 **Perera F**, Herbstman J. Prenatal environmental exposures, epigenetics, and disease. *Reprod Toxicol* 2011; **31**: 363-373 [PMID: [21256208](#) DOI: [10.1016/j.reprotox.2010.12.055](#)]
- 58 **Park JH**, Stoffers DA, Nicholls RD, Simmons RA. Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. *J Clin Invest* 2008; **118**: 2316-2324 [PMID: [18464933](#) DOI: [10.1172/JCI33655](#)]
- 59 **Yang BT**, Dayeh TA, Volkov PA, Kirkpatrick CL, Malmgren S, Jing X, Renström E, Wollheim CB, Nitert MD, Ling C. Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes. *Mol Endocrinol* 2012; **26**: 1203-1212 [PMID: [22570331](#) DOI: [10.1210/me.2012-1004](#)]
- 60 **Oliver-Krasinski JM**, Kasner MT, Yang J, Crutchlow MF, Rustgi AK, Kaestner KH, Stoffers DA. The diabetes gene Pdx1 regulates the transcriptional network of pancreatic endocrine progenitor cells in mice. *J Clin Invest* 2009; **119**: 1888-1898 [PMID: [19487809](#) DOI: [10.1172/JCI37028](#)]
- 61 **Ling C**, Del Guerra S, Lupi R, Rönn T, Granhall C, Luthman H, Masiello P, Marchetti P, Groop L, Del Prato S. Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. *Diabetologia* 2008; **51**: 615-622 [PMID: [18270681](#) DOI: [10.1007/s00125-007-0916-5](#)]
- 62 **Volkmar M**, Dedeurwaerder S, Cunha DA, Ndlovu MN, Defrance M, Deplus R, Calonne E, Volkmar U, Igoillo-Esteve M, Naamane N, Del Guerra S, Masini M, Bugliani M, Marchetti P, Cnop M, Eizirik DL, Fuks F. DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients. *EMBO J* 2012; **31**: 1405-1426 [PMID: [22293752](#) DOI: [10.1038/emboj.2011.503](#)]
- 63 **Dayeh T**, Volkov P, Salö S, Hall E, Nilsson E, Olsson AH, Kirkpatrick CL, Wollheim CB, Eliasson L, Rönn T, Bacos K, Ling C. Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion. *PLoS Genet* 2014; **10**: e1004160 [PMID: [24603685](#) DOI: [10.1371/journal.pgen.1004160](#)]
- 64 **Petropoulos S**, Guillemin C, Ergaz Z, Dimov S, Suderman M, Weinstein-Fudim L, Ornoy A, Szyf M. Gestational Diabetes Alters Offspring DNA Methylation Profiles in Human and Rat: Identification of Key Pathways Involved in Endocrine System Disorders, Insulin Signaling, Diabetes Signaling, and ILK Signaling. *Endocrinology* 2015; **156**: 2222-2238 [PMID: [25514087](#) DOI: [10.1210/en.2014-1643](#)]
- 65 **Chen S**, Feng B, George B, Chakrabarti R, Chen M, Chakrabarti S. Transcriptional coactivator p300 regulates glucose-induced gene expression in endothelial cells. *Am J Physiol Endocrinol Metab* 2010; **298**: E127-E137 [PMID: [19903865](#) DOI: [10.1152/ajpendo.00432.2009](#)]
- 66 **Takahashi K**, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: [18035408](#) DOI: [10.1016/j.cell.2007.11.019](#)]
- 67 **Kondo Y**, Toyoda T, Inagaki N, Osafune K. iPSC technology-based regenerative therapy for diabetes. *J Diabetes Investig* 2018; **9**: 234-243 [PMID: [28609558](#) DOI: [10.1111/jdi.12702](#)]
- 68 **Mills JL**. Malformations in infants of diabetic mothers. *Teratology* 25:385-94. 1982. *Birth Defects Res A Clin Mol Teratol* 2010; **88**: 769-778 [PMID: [20973049](#) DOI: [10.1002/bdra.20757](#)]
- 69 **Gabbay-Benziv R**, Reece EA, Wang F, Yang P. Birth defects in pregestational diabetes: Defect range, glycemic threshold and pathogenesis. *World J Diabetes* 2015; **6**: 481-488 [PMID: [25897357](#) DOI: [10.4239/wjdv6.i3.481](#)]
- 70 **Rodaway A**, Patient R. Mesendoderm. an ancient germ layer? *Cell* 2001; **105**: 169-172 [PMID: [11336666](#)]
- 71 **Wells JM**, Melton DA. Vertebrate endoderm development. *Annu Rev Cell Dev Biol* 1999; **15**: 393-410 [PMID: [10611967](#) DOI: [10.1146/annurev.cellbio.15.1.393](#)]
- 72 **Kubo A**, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. Development of definitive endoderm from embryonic stem cells in culture. *Development* 2004; **131**: 1651-1662 [PMID: [14998924](#) DOI: [10.1242/dev.01044](#)]
- 73 **Blum M**, Gaunt SJ, Cho KW, Steinbeisser H, Blumberg B, Bittner D, De Robertis EM. Gastrulation in the mouse: the role of the homeobox gene goosecoid. *Cell* 1992; **69**: 1097-1106 [PMID: [1352187](#)]
- 74 **Teo AK**, Arnold SJ, Trotter MW, Brown S, Ang LT, Chng Z, Robertson EJ, Dunn NR, Vallier L. Pluripotency factors regulate definitive endoderm specification through eomesodermin. *Genes Dev* 2011; **25**: 238-250 [PMID: [21245162](#) DOI: [10.1101/gad.607311](#)]
- 75 **Hart AH**, Hartley L, Sourris K, Stadler ES, Li R, Stanley EG, Tam PP, Elefanti AG, Robb L. Mixl1 is required for axial mesendoderm morphogenesis and patterning in the murine embryo. *Development* 2002; **129**: 3597-3608 [PMID: [12117810](#)]
- 76 **D'Amour KA**, Bang AG, Eliazar S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* 2006; **24**: 1392-1401 [PMID: [17053790](#) DOI: [10.1038/nbt1259](#)]
- 77 **Champeris Tsaniras S**, Jones PM. Generating pancreatic beta-cells from embryonic stem cells by manipulating signaling pathways. *J Endocrinol* 2010; **206**: 13-26 [PMID: [20385725](#) DOI: [10.1677/JOE-10-0073](#)]
- 78 **Mfopou JK**, Geeraerts M, Dejene R, Van Langenhoven S, Aberkane A, Van Grunsven LA, Bouwens L.

- Efficient definitive endoderm induction from mouse embryonic stem cell adherent cultures: a rapid screening model for differentiation studies. *Stem Cell Res* 2014; **12**: 166-177 [PMID: [24239964](#) DOI: [10.1016/j.scr.2013.10.004](#)]
- 79 **Lu CC**, Brennan J, Robertson EJ. From fertilization to gastrulation: axis formation in the mouse embryo. *Curr Opin Genet Dev* 2001; **11**: 384-392 [PMID: [11448624](#)]
  - 80 **Jaramillo M**, Mathew S, Task K, Barner S, Banerjee I. Potential for pancreatic maturation of differentiating human embryonic stem cells is sensitive to the specific pathway of definitive endoderm commitment. *PLoS One* 2014; **9**: e94307 [PMID: [24743345](#) DOI: [10.1371/journal.pone.0094307](#)]
  - 81 **Wang P**, Rodriguez RT, Wang J, Ghodasara A, Kim SK. Targeting SOX17 in human embryonic stem cells creates unique strategies for isolating and analyzing developing endoderm. *Cell Stem Cell* 2011; **8**: 335-346 [PMID: [21362573](#) DOI: [10.1016/j.stem.2011.01.017](#)]
  - 82 **D'Amour KA**, Agulnick AD, Eliazar S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* 2005; **23**: 1534-1541 [PMID: [16258519](#) DOI: [10.1038/nbt1163](#)]
  - 83 **Sumi T**, Tsuneyoshi N, Nakatsuji N, Suemori H. Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. *Development* 2008; **135**: 2969-2979 [PMID: [18667462](#) DOI: [10.1242/dev.021121](#)]
  - 84 **Borowiak M**, Maehr R, Chen S, Chen AE, Tang W, Fox JL, Schreiber SL, Melton DA. Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. *Cell Stem Cell* 2009; **4**: 348-358 [PMID: [19341624](#) DOI: [10.1016/j.stem.2009.01.014](#)]
  - 85 **Chen ACH**, Lee YL, Fong SW, Wong CCY, Ng EHY, Yeung WSB. Hyperglycemia impedes definitive endoderm differentiation of human embryonic stem cells by modulating histone methylation patterns. *Cell Tissue Res* 2017; **368**: 563-578 [PMID: [28283910](#) DOI: [10.1007/s00441-017-2583-2](#)]
  - 86 **Guney MA**, Gannon M. Pancreas cell fate. *Birth Defects Res C Embryo Today* 2009; **87**: 232-248 [PMID: [19750517](#) DOI: [10.1002/bdrc.20156](#)]
  - 87 **Kroon E**, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazar S, Young H, Richardson M, Smart NG, Cunningham J, Agulnick AD, D'Amour KA, Carpenter MK, Baetge EE. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol* 2008; **26**: 443-452 [PMID: [18288110](#) DOI: [10.1038/nbt1393](#)]
  - 88 **Apelqvist A**, Ahlgren U, Edlund H. Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Curr Biol* 1997; **7**: 801-804 [PMID: [9368764](#)]
  - 89 **Chen S**, Borowiak M, Fox JL, Maehr R, Osafune K, Davidow L, Lam K, Peng LF, Schreiber SL, Rubin LL, Melton D. A small molecule that directs differentiation of human ESCs into the pancreatic lineage. *Nat Chem Biol* 2009; **5**: 258-265 [PMID: [19287398](#) DOI: [10.1038/nchembio.154](#)]
  - 90 **Rezania A**, Bruin JE, Xu J, Narayan K, Fox JK, O'Neil JJ, Kieffer TJ. Enrichment of human embryonic stem cell-derived NKX6.1-expressing pancreatic progenitor cells accelerates the maturation of insulin-secreting cells in vivo. *Stem Cells* 2013; **31**: 2432-2442 [PMID: [23897760](#) DOI: [10.1002/stem.1489](#)]
  - 91 **Haller C**, Piccand J, De Franceschi F, Ohi Y, Bhoumik A, Boss C, De Marchi U, Jacot G, Metairon S, Descombes P, Wiederkehr A, Palini A, Bouche N, Steiner P, Kelly OG, R-C Kraus M. Macroencapsulated Human iPSC-Derived Pancreatic Progenitors Protect against STZ-Induced Hyperglycemia in Mice. *Stem Cell Reports* 2019; **12**: 787-800 [PMID: [30853374](#) DOI: [10.1016/j.stemcr.2019.02.002](#)]
  - 92 **Zhang D**, Jiang W, Liu M, Sui X, Yin X, Chen S, Shi Y, Deng H. Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. *Cell Res* 2009; **19**: 429-438 [PMID: [19255591](#) DOI: [10.1038/cr.2009.28](#)]
  - 93 **Pagliuca FW**, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, Peterson QP, Greiner D, Melton DA. Generation of functional human pancreatic  $\beta$  cells in vitro. *Cell* 2014; **159**: 428-439 [PMID: [25303535](#) DOI: [10.1016/j.cell.2014.09.040](#)]
  - 94 **Takahashi Y**, Sekine K, Kin T, Takebe T, Taniguchi H. Self-Condensation Culture Enables Vascularization of Tissue Fragments for Efficient Therapeutic Transplantation. *Cell Rep* 2018; **23**: 1620-1629 [PMID: [29742420](#) DOI: [10.1016/j.celrep.2018.03.123](#)]
  - 95 **Xie R**, Everett LJ, Lim HW, Patel NA, Schug J, Kroon E, Kelly OG, Wang A, D'Amour KA, Robins AJ, Won KJ, Kaestner KH, Sander M. Dynamic chromatin remodeling mediated by polycomb proteins orchestrates pancreatic differentiation of human embryonic stem cells. *Cell Stem Cell* 2013; **12**: 224-237 [PMID: [23318056](#) DOI: [10.1016/j.stem.2012.11.023](#)]
  - 96 **Astro V**, Adamo A. Epigenetic Control of Endocrine Pancreas Differentiation *in vitro*: Current Knowledge and Future Perspectives. *Front Cell Dev Biol* 2018; **6**: 141 [PMID: [30410880](#) DOI: [10.3389/fcell.2018.00141](#)]
  - 97 **Li J**, Wu X, Zhou Y, Lee M, Guo L, Han W, Mo W, Cao WM, Sun D, Xie R, Huang Y. Decoding the dynamic DNA methylation and hydroxymethylation landscapes in endodermal lineage intermediates during pancreatic differentiation of hESC. *Nucleic Acids Res* 2018; **46**: 2883-2900 [PMID: [29394393](#) DOI: [10.1093/nar/gky063](#)]
  - 98 **Whitehead R**, Guan H, Arany E, Cernea M, Yang K. Prenatal exposure to bisphenol A alters mouse fetal pancreatic morphology and islet composition. *Horm Mol Biol Clin Investig* 2016; **25**: 171-179 [PMID: [26812801](#) DOI: [10.1515/hmbci-2015-0052](#)]
  - 99 **Lai KP**, Li JW, Chan TF, Chen A, Lee CYL, Yeung WSB, Wong CKC. Transcriptomic and methylomic analysis reveal the toxicological effect of 2,3,7,8-Tetrachlorodibenzodioxin on human embryonic stem cell. *Chemosphere* 2018; **206**: 663-673 [PMID: [29778942](#) DOI: [10.1016/j.chemosphere.2018.05.058](#)]
  - 100 **Kubi JA**, Chen ACH, Fong SW, Lai KP, Wong CKC, Yeung WSB, Lee KF, Lee YL. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the differentiation of embryonic stem cells towards pancreatic lineage and pancreatic beta cell function. *Environ Int* 2019; **130**: 104885 [PMID: [31195220](#) DOI: [10.1016/j.envint.2019.05.079](#)]
  - 101 **Später D**, Hansson EM, Zangi L, Chien KR. How to make a cardiomyocyte. *Development* 2014; **141**: 4418-4431 [PMID: [25406392](#) DOI: [10.1242/dev.091538](#)]
  - 102 **Evans SM**, Yelon D, Conlon FL, Kirby ML. Myocardial lineage development. *Circ Res* 2010; **107**: 1428-1444 [PMID: [21148449](#) DOI: [10.1161/CIRCRESAHA.110.227405](#)]



- 103 **Foley AC**, Mercola M. Heart induction by Wnt antagonists depends on the homeodomain transcription factor Hex. *Genes Dev* 2005; **19**: 387-396 [PMID: [15687261](#) DOI: [10.1101/gad.1279405](#)]
- 104 **Bondue A**, Lapouge G, Paulissen C, Semeraro C, Iacovino M, Kyba M, Blanpain C. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* 2008; **3**: 69-84 [PMID: [18593560](#) DOI: [10.1016/j.stem.2008.06.009](#)]
- 105 **Kehat I**, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001; **108**: 407-414 [PMID: [11489934](#) DOI: [10.1172/JCI12131](#)]
- 106 **Yoon BS**, Yoo SJ, Lee JE, You S, Lee HT, Yoon HS. Enhanced differentiation of human embryonic stem cells into cardiomyocytes by combining hanging drop culture and 5-azacytidine treatment. *Differentiation* 2006; **74**: 149-159 [PMID: [16683985](#) DOI: [10.1111/j.1432-0436.2006.00063.x](#)]
- 107 **Kattman SJ**, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 2011; **8**: 228-240 [PMID: [21295278](#) DOI: [10.1016/j.stem.2010.12.008](#)]
- 108 **Mohr JC**, Zhang J, Azarin SM, Soerens AG, de Pablo JJ, Thomson JA, Lyons GE, Palecek SP, Kamp TJ. The microwell control of embryoid body size in order to regulate cardiac differentiation of human embryonic stem cells. *Biomaterials* 2010; **31**: 1885-1893 [PMID: [19945747](#) DOI: [10.1016/j.biomaterials.2009.11.033](#)]
- 109 **Ting S**, Chen A, Reuveny S, Oh S. An intermittent rocking platform for integrated expansion and differentiation of human pluripotent stem cells to cardiomyocytes in suspended microcarrier cultures. *Stem Cell Res* 2014; **13**: 202-213 [PMID: [25043964](#) DOI: [10.1016/j.scr.2014.06.002](#)]
- 110 **Dahlmann J**, Kensah G, Kempf H, Skvorc D, Gawol A, Elliott DA, Dräger G, Zweigerdt R, Martin U, Gruh I. The use of agarose microwells for scalable embryoid body formation and cardiac differentiation of human and murine pluripotent stem cells. *Biomaterials* 2013; **34**: 2463-2471 [PMID: [23332176](#) DOI: [10.1016/j.biomaterials.2012.12.024](#)]
- 111 **Hornberger LK**. Maternal diabetes and the fetal heart. *Heart* 2006; **92**: 1019-1021 [PMID: [16698822](#) DOI: [10.1136/hrt.2005.083840](#)]
- 112 **Narchi H**, Kulaylat N. Heart disease in infants of diabetic mothers. *Images Paediatr Cardiol* 2000; **2**: 17-23 [PMID: [22368579](#)]
- 113 **Dyntar D**, Sergeev P, Klisic J, Ambühl P, Schaub MC, Donath MY. High glucose alters cardiomyocyte contacts and inhibits myofibrillar formation. *J Clin Endocrinol Metab* 2006; **91**: 1961-1967 [PMID: [16522700](#) DOI: [10.1210/jc.2005-1904](#)]
- 114 **Drawnel FM**, Boccardo S, Prummer M, Delobel F, Graff A, Weber M, Gérard R, Badi L, Kam-Thong T, Bu L, Jiang X, Hoflack JC, Kiialainen A, Jeworutzki E, Aoyama N, Carlson C, Burcin M, Gromo G, Boehringer M, Stahlberg H, Hall BJ, Magnone MC, Kolaja K, Chien KR, Bailly J, Iacone R. Disease modeling and phenotypic drug screening for diabetic cardiomyopathy using human induced pluripotent stem cells. *Cell Rep* 2014; **9**: 810-821 [PMID: [25437537](#) DOI: [10.1016/j.celrep.2014.09.055](#)]
- 115 **Karakikes I**, Ameen M, Termglinchan V, Wu JC. Human induced pluripotent stem cell-derived cardiomyocytes: insights into molecular, cellular, and functional phenotypes. *Circ Res* 2015; **117**: 80-88 [PMID: [26089365](#) DOI: [10.1161/CIRCRESAHA.117.305365](#)]
- 116 **Lundy SD**, Zhu WZ, Regnier M, Laflamme MA. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells Dev* 2013; **22**: 1991-2002 [PMID: [23461462](#) DOI: [10.1089/scd.2012.0490](#)]
- 117 **Yang P**, Chen X, Kaushal S, Reece EA, Yang P. High glucose suppresses embryonic stem cell differentiation into cardiomyocytes : High glucose inhibits ES cell cardiogenesis. *Stem Cell Res Ther* 2016; **7**: 187 [PMID: [27938398](#) DOI: [10.1186/s13287-016-0446-5](#)]
- 118 **Mochizuki H**, Ohnuki Y, Kurosawa H. Effect of glucose concentration during embryoid body (EB) formation from mouse embryonic stem cells on EB growth and cell differentiation. *J Biosci Bioeng* 2011; **111**: 92-97 [PMID: [20869914](#) DOI: [10.1016/j.jbiosc.2010.09.001](#)]
- 119 **Gilsbach R**, Schwaderer M, Preissl S, Grüning BA, Kranzhöfer D, Schneider P, Nührenberg TG, Mulero-Navarro S, Weichenhan D, Braun C, Dreßen M, Jacobs AR, Lahm H, Doenst T, Backofen R, Krane M, Gelb BD, Hein L. Distinct epigenetic programs regulate cardiac myocyte development and disease in the human heart in vivo. *Nat Commun* 2018; **9**: 391 [PMID: [29374152](#) DOI: [10.1038/s41467-017-02762-z](#)]
- 120 **Hoff K**, Lemme M, Kahlert AK, Runde K, Audain E, Schuster D, Scheewe J, Attmann T, Pickardt T, Caliebe A, Siebert R, Kramer HH, Milting H, Hansen A, Ammerpohl O, Hitz MP. DNA methylation profiling allows for characterization of atrial and ventricular cardiac tissues and hiPSC-CMs. *Clin Epigenetics* 2019; **11**: 89 [PMID: [31186048](#) DOI: [10.1186/s13148-019-0679-0](#)]
- 121 **Becerra JE**, Khoury MJ, Cordero JF, Erickson JD. Diabetes mellitus during pregnancy and the risks for specific birth defects: a population-based case-control study. *Pediatrics* 1990; **85**: 1-9 [PMID: [2404255](#)]
- 122 **Correa A**, Gilboa SM, Besser LM, Botto LD, Moore CA, Hobbs CA, Cleves MA, Riehle-Colarusso TJ, Waller DK, Reece EA. Diabetes mellitus and birth defects. *Am J Obstet Gynecol* 2008; **199**: 237.e1-237.e9 [PMID: [18674752](#) DOI: [10.1016/j.ajog.2008.06.028](#)]
- 123 **Wu Y**, Wang F, Fu M, Wang C, Quon MJ, Yang P. Cellular Stress, Excessive Apoptosis, and the Effect of Metformin in a Mouse Model of Type 2 Diabetic Embryopathy. *Diabetes* 2015; **64**: 2526-2536 [PMID: [25720389](#) DOI: [10.2337/db14-1683](#)]
- 124 **Yang P**, Li X, Xu C, Eckert RL, Reece EA, Zielke HR, Wang F. Maternal hyperglycemia activates an ASK1-FoxO3a-caspase 8 pathway that leads to embryonic neural tube defects. *Sci Signal* 2013; **6**: ra74 [PMID: [23982205](#) DOI: [10.1126/scisignal.2004020](#)]
- 125 **Yang P**, Shen WB, Reece EA, Chen X, Yang P. High glucose suppresses embryonic stem cell differentiation into neural lineage cells. *Biochem Biophys Res Commun* 2016; **472**: 306-312 [PMID: [26940741](#) DOI: [10.1016/j.bbrc.2016.02.117](#)]
- 126 **Burren KA**, Savery D, Massa V, Kok RM, Scott JM, Blom HJ, Copp AJ, Greene ND. Gene-environment interactions in the causation of neural tube defects: folate deficiency increases susceptibility conferred by loss of Pax3 function. *Hum Mol Genet* 2008; **17**: 3675-3685 [PMID: [18753144](#) DOI: [10.1093/hmg/ddn262](#)]



- 127 **Vega RB**, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, McAnally J, Pomajzl C, Shelton JM, Richardson JA, Karsenty G, Olson EN. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* 2004; **119**: 555-566 [PMID: [15537544](#) DOI: [10.1016/j.cell.2004.10.024](#)]
- 128 **Cheng HL**, Mostoslavsky R, Saito S, Manis JP, Gu Y, Patel P, Bronson R, Appella E, Alt FW, Chua KF. Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. *Proc Natl Acad Sci U S A* 2003; **100**: 10794-10799 [PMID: [12960381](#) DOI: [10.1073/pnas.1934713100](#)]
- 129 **Dottori M**, Pera MF. Neural differentiation of human embryonic stem cells. *Methods Mol Biol* 2008; **438**: 19-30 [PMID: [18369746](#) DOI: [10.1007/978-1-59745-133-8\\_3](#)]
- 130 **Briño-Enríquez MA**, García-López J, Cárdenas DB, Guibert S, Cleroux E, Déd L, Hourcade Jde D, Pěkníková J, Weber M, Del Mazo J. Exposure to endocrine disruptor induces transgenerational epigenetic deregulation of microRNAs in primordial germ cells. *PLoS One* 2015; **10**: e0124296 [PMID: [25897752](#) DOI: [10.1371/journal.pone.0124296](#)]
- 131 **Meyer DN**, Baker BB, Baker TR. Ancestral TCDD Exposure Induces Multigenerational Histologic and Transcriptomic Alterations in Gonads of Male Zebrafish. *Toxicol Sci* 2018; **164**: 603-612 [PMID: [29788325](#) DOI: [10.1093/toxsci/kfy115](#)]
- 132 **Ding GL**, Wang FF, Shu J, Tian S, Jiang Y, Zhang D, Wang N, Luo Q, Zhang Y, Jin F, Leung PC, Sheng JZ, Huang HF. Transgenerational glucose intolerance with Igf2/H19 epigenetic alterations in mouse islet induced by intrauterine hyperglycemia. *Diabetes* 2012; **61**: 1133-1142 [PMID: [22447856](#) DOI: [10.2337/db11-1314](#)]
- 133 **Ren J**, Cheng Y, Ming ZH, Dong XY, Zhou YZ, Ding GL, Pang HY, Rahman TU, Akbar R, Huang HF, Sheng JZ. Intrauterine hyperglycemia exposure results in intergenerational inheritance via DNA methylation reprogramming on F1 PGCs. *Epigenetics Chromatin* 2018; **11**: 20 [PMID: [29801514](#) DOI: [10.1186/s13072-018-0192-2](#)]
- 134 **Zhou Q**, Wang M, Yuan Y, Wang X, Fu R, Wan H, Xie M, Liu M, Guo X, Zheng Y, Feng G, Shi Q, Zhao XY, Sha J, Zhou Q. Complete Meiosis from Embryonic Stem Cell-Derived Germ Cells In Vitro. *Cell Stem Cell* 2016; **18**: 330-340 [PMID: [26923202](#) DOI: [10.1016/j.stem.2016.01.017](#)]
- 135 **Kobayashi T**, Zhang H, Tang WWC, Irie N, Withey S, Klisch D, Sybirna A, Dietmann S, Contreras DA, Webb R, Allegrucci C, Alberio R, Surani MA. Principles of early human development and germ cell program from conserved model systems. *Nature* 2017; **546**: 416-420 [PMID: [28607482](#) DOI: [10.1038/nature22812](#)]
- 136 **Wang Q**, Kurita H, Carreira V, Ko CI, Fan Y, Zhang X, Biesiada J, Medvedovic M, Puga A. Ah Receptor Activation by Dioxin Disrupts Activin, BMP, and WNT Signals During the Early Differentiation of Mouse Embryonic Stem Cells and Inhibits Cardiomyocyte Functions. *Toxicol Sci* 2016; **149**: 346-357 [PMID: [26572662](#) DOI: [10.1093/toxsci/kfv246](#)]
- 137 **Zhou R**, Cheng W, Feng Y, Wei H, Liang F, Wang Y. Interactions between three typical endocrine-disrupting chemicals (EDCs) in binary mixtures exposure on myocardial differentiation of mouse embryonic stem cell. *Chemosphere* 2017; **178**: 378-383 [PMID: [28340460](#) DOI: [10.1016/j.chemosphere.2017.03.040](#)]

## Autophagy in fate determination of mesenchymal stem cells and bone remodeling

Xiao-Dan Chen, Jia-Li Tan, Yi Feng, Li-Jia Huang, Mei Zhang, Bin Cheng

**ORCID number:** Xiao-Dan Chen 0000-0003-2510-348X; Jia-Li Tan 0000-0001-8176-8318; Yi Feng 0000-0002-8691-3446; Li-Jia Huang 0000-0003-2818-940X; Mei Zhang 0000-0001-8514-0034; Bin Cheng 0000-0001-7288-806X.

**Author contributions:** Chen XD was involved in the conceptualization, funding acquisition, and writing of the original draft; Tan JL took part in the conceptualization, funding acquisition, and review and editing of the manuscript; Feng Y, Huang LJ, and Zhang M participated in the provision of resources, and review and editing of the manuscript; Cheng B took part in the conceptualization and funding acquisition, and participated in the supervision, and writing, review, and editing of the manuscript; all authors have read and approved the final manuscript.

**Supported by** National Natural Science Foundation of China, No. 81873710 and No. 81900976; Guangzhou Foundation for Science and Technology Planning Project, China, No. 201704030083 and No. 201704020063; and Guangdong Financial Fund for High-Caliber Hospital Construction, No. 174-2018-XMZZ-0001-03-0125/C-05.

**Conflict-of-interest statement:** The authors declare no conflict of

**Xiao-Dan Chen, Jia-Li Tan, Yi Feng, Li-Jia Huang, Mei Zhang, Bin Cheng,** Hospital of Stomatology, Sun Yat-sen University; Guangdong Provincial Key Laboratory of Stomatology; Guanghua School of Stomatology, Sun Yat-sen University, Guangzhou 510055, Guangdong Province, China

**Corresponding author:** Bin Cheng, DDS, PhD, Professor, Hospital of Stomatology, Sun Yat-sen University; Guangdong Provincial Key Laboratory of Stomatology; Guanghua School of Stomatology, Sun Yat-sen University, Guangzhou 510055, Guangdong Province, China. [chengbin@mail.sysu.edu.cn](mailto:chengbin@mail.sysu.edu.cn)

### Abstract

Mesenchymal stem cells (MSCs) have been widely exploited as promising candidates in clinical settings for bone repair and regeneration in view of their self-renewal capacity and multipotentiality. However, little is known about the mechanisms underlying their fate determination, which would illustrate their effectiveness in regenerative medicine. Recent evidence has shed light on a fundamental biological role of autophagy in the maintenance of the regenerative capability of MSCs and bone homeostasis. Autophagy has been implicated in provoking an immediately available cytoprotective mechanism in MSCs against stress, while dysfunction of autophagy impairs the function of MSCs, leading to imbalances of bone remodeling and a wide range of aging and degenerative bone diseases. This review aims to summarize the up-to-date knowledge about the effects of autophagy on MSC fate determination and its role as a stress adaptation response. Meanwhile, we highlight autophagy as a dynamic process and a double-edged sword to account for some discrepancies in the current research. We also discuss the contribution of autophagy to the regulation of bone cells and bone remodeling and emphasize its potential involvement in bone disease.

**Key words:** Mesenchymal stem cells; Autophagy; Cell self-renewal; Cell differentiation; Cytoprotection; Bone remodeling

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Autophagy is a dynamic recycling mechanism that fuels cellular renovation and homeostasis. Recent studies have shed light on an essential role of autophagy in orchestrating self-renewal and the multilineage differentiation potential of mesenchymal

interests for this article.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Invited manuscript

**Received:** February 28, 2020

**Peer-review started:** February 28, 2020

**First decision:** April 25, 2020

**Revised:** May 17, 2020

**Accepted:** June 20, 2020

**Article in press:** June 20, 2020

**Published online:** August 26, 2020

**P-Reviewer:** Arufe MC

**S-Editor:** Gong ZM

**L-Editor:** Wang TQ

**P-Editor:** Li JH



stem cells (MSCs), thus coordinating bone homeostasis. This review outlines the effects of autophagy on MSCs fate determination and cytoprotection under different kinds of stresses. Moreover, we emphasize that the involvement of autophagy ensures balanced bone remodeling, which will be of significance in facilitating its application as a therapeutic target in bone repair and regeneration.

**Citation:** Chen XD, Tan JL, Feng Y, Huang LJ, Zhang M, Cheng B. Autophagy in fate determination of mesenchymal stem cells and bone remodeling. *World J Stem Cells* 2020; 12(8): 776-786

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/776.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.776>

## INTRODUCTION

Mesenchymal stem cells (MSCs) are a heterogeneous cellular population that can be detected in and isolated from bone marrow, adipose, vascular, umbilical cord, placenta, skin, and kidney<sup>[1-3]</sup>. Characterized by their potential of self-renewal and differentiation into osteogenic, chondrogenic, and adipogenic lineages, they are considered promising therapeutic agents that confer a positive benefit to bone maintenance, repair, and regeneration<sup>[4]</sup>. Therefore, a thorough understanding of the underlying mechanisms regulating MSCs function would offer great promise in the field of bone regenerative medicine.

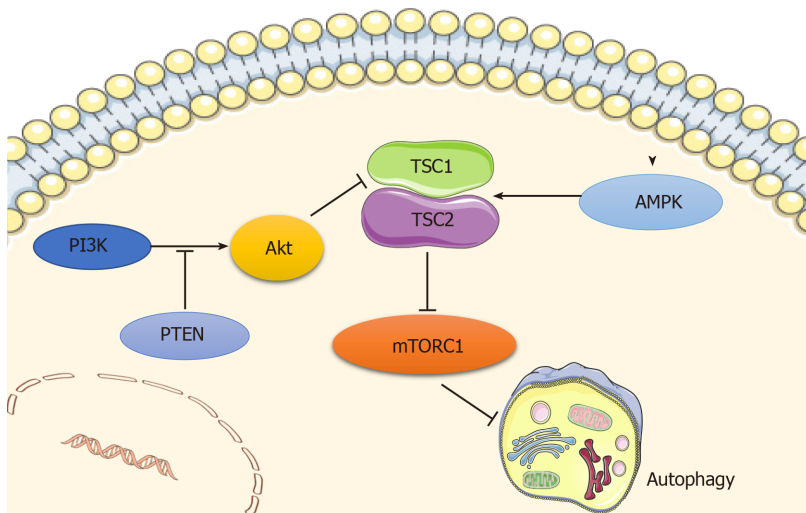
Autophagy is a conserved degradation process during which proteins and damaged organelles are engulfed by autophagosomes and then fused with lysosomes to be degraded for intracellular recycling to fuel cellular renovation<sup>[5,6]</sup>. There are three types of autophagy in mammals, including macroautophagy<sup>[7]</sup>, microautophagy<sup>[8]</sup>, and chaperone-mediated autophagy<sup>[9]</sup>, among which macroautophagy is in the spotlight for its crucial effects on cell biology and will be henceforth referred to as “autophagy” in this review.

Recent evidence has shed light on a fundamental role of autophagy in the fate determination of MSCs and the maintenance of bone homeostasis. In addition, autophagy has also been implicated as an immediately available cytoprotective mechanism in MSCs against stress<sup>[10,11]</sup>. Dysfunction of autophagy would impair the function of MSCs, leading to imbalances of bone remodeling and thus inducing a wide range of aging and degenerative bone diseases. Further delineation of the relationships among autophagy, MSCs function, and bone homeostasis would uncover new avenues for novel therapeutic strategies for bone repair and regeneration.

## REGULATORY MECHANISMS OF AUTOPHAGY

Autophagy is regulated by a number of signaling pathways, among which the most well-known are the adenosine monophosphate-activated protein kinase (AMPK) and the phosphoinositide3 kinase (PI3K)/AKT pathways, which converge on mammalian target of rapamycin (mTOR), a well-recognized negative regulator of autophagy that integrates nutrient signals<sup>[12]</sup> (Figure 1). mTOR recruits other regulatory proteins to form two distinct complexes, mTORC1 and mTORC2, and mTORC1 is involved in autophagy regulation<sup>[13]</sup>.

AMPK is a principal intracellular energy sensor, which conserves energy by inhibiting mTOR<sup>[14]</sup> by phosphorylating and potentiating tuberous sclerosis complex (TSC) or by directly binding to RAPTOR, a key subunit of mTORC1<sup>[15]</sup>, and consequently inducing autophagy<sup>[16]</sup>. Furthermore, the PI3K/AKT pathway is also an important mTOR modulator that inhibits the mTOR repressor TSC<sup>[17]</sup>, activates mTOR, and then blocks autophagy activity<sup>[12]</sup>. In addition, wnt/ $\beta$ -catenin has been shown to be a negative regulator of autophagy, while PTEN induces autophagy by inhibiting the PI3K/AKT/mTOR pathway, and activated EGFR/Ras/MEK/ERK, JUK/c-Jun, and p38 MAPK signaling pathways have also been revealed as stimulators of autophagy<sup>[18]</sup>.



**Figure 1 Main signaling pathways regulating autophagy.** Autophagy is regulated by the adenosine monophosphate-activated protein kinase (AMPK) and PI3K/AKT pathways, which converge at mammalian target of rapamycin (mTOR) that functions as a negative regulator of autophagy. AMPK inhibits mTOR by phosphorylating tuberous sclerosis complex (TSC), and consequently inducing autophagy. PI3K/AKT pathway inactivates TSC, phosphorylates mTOR, and then blocks autophagy activity, while PTEN acts as a brake upstream of Akt. Original elements used in this diagram are from Servier Medical Art (<http://smart.servier.com/>). AMPK: Adenosine monophosphate-activated protein kinase; mTOR: Mammalian target of rapamycin; TSC: Tuberous sclerosis complex.

## AUTOPHAGY AND MSCS FUNCTION

### *Autophagy and the lineage determination of MSCs*

Considerable evidence has shown a pivotal regulatory role of autophagy in self-renewal capacity and lineage determination of MSCs. Induction of autophagy in bone mesenchymal stem cells (BMSCs) may account for a decrease in their S-phase population and trigger their differentiation into neurons<sup>[19]</sup>. Despite some controversy, Isomoto *et al*<sup>[20]</sup> clarified that rapamycin does not have a spontaneous osteogenic effect on MSCs, while most studies have confirmed that autophagy contributes to the switch between osteogenesis and adipogenesis of BMSCs. More specifically, MSCs tend to accumulate undergraded autophagic vacuoles and undergo little autophagic turnover, while osteogenic differentiation of MSCs results in more autophagic turnover<sup>[21]</sup>. Induction of osteogenic differentiation of human gingiva-derived MSCs (HGMSCs) potentiates autophagy signaling, while inhibition of autophagy precludes osteoblast differentiation of HGMSCs<sup>[22]</sup>. The autophagy inducer rapamycin promotes osteoblast differentiation in human embryonic stem cells (ESC) by interfering with mTOR while augmenting the BMP/Smad signaling pathway<sup>[23]</sup>.

*Osterix*-expressing cells with a specific deletion of *TSC1*, a positive regulator of autophagy, have shown that *TSC1* deficiency is responsible for the reduction of bone mass, as characterized by inhibition of osteogenesis, enhancement of osteoclastogenesis, and elevation of bone marrow adiposity<sup>[24]</sup>. Consistently, *TSC1* deficiency in BMSCs results in decreased proliferation and a tendency to differentiate into adipocytes instead of osteoblasts<sup>[24]</sup>.

Other studies have provided evidence that early mTOR suppression accompanied by late Akt/mTOR activation contributes to osteoblast differentiation of MSCs<sup>[25]</sup>. Accordingly, activation of autophagy by mTOR inhibition facilitates osteoblast differentiation<sup>[25]</sup>, though whether late mTOR induction and subsequent autophagy inhibition would stimulate or interfere with osteogenesis remains to be elucidated. Another study in MC3T3 cells also revealed that early activation and subsequent inhibition of AMPK are indispensable for osteoblast differentiation<sup>[26]</sup>. Given that mTOR functions as an inhibitor and AMPK as a stimulator of autophagy, these two studies coincide in that autophagy is fueled at first and then abrogated during osteoblast differentiation. We theorized that such time-dependent catabolic dynamics seem fundamental to ensure the ever-changing energy demands during all stages of osteogenesis.

### *Autophagy in aging/senescence of MSCs*

Although several studies have revealed that autophagy is activated during aging in cells such as fibroblasts<sup>[27]</sup> and BMSCs<sup>[28]</sup>, the mainstream view currently is that with aging, autophagy decreases in different kinds of tissues, ranging from the kidney to

the brain<sup>[29,30]</sup>. Indeed, it has been reported that autophagy activity is significantly reduced in aged BMSCs compared with their young counterparts<sup>[31]</sup>. Basal autophagy has a crucial role in the maintenance of the young state of satellite cells, and dysfunction of autophagy leads to cell senescence as indicated by the decrease in satellite cell number and function<sup>[32]</sup>. In addition, blockage of autophagy converts young BMSCs to a relatively aged state by impairing their osteoblast differentiation and proliferation potential while promoting their adipocyte differentiation ability. Correspondingly, activation of autophagy turns aged BMSCs into a young state by strengthening osteoblast differentiation and proliferation potential while impairing adipocyte differentiation capacity<sup>[31]</sup>. Likewise, pretreatment with rapamycin remarkably alleviates MSC aging induced by D-gal and decreases of p-JNK, p-38, and ROS generation, supporting the concept that autophagy exerts a protective role in MSCs senescence<sup>[33]</sup>. This protective effect of rapamycin on MSCs senescence can be abolished by increasing the ROS level, and inhibition of p38 can rescue the H<sub>2</sub>O<sub>2</sub>-induced MSCs senescence, which suggests that ROS/JNK/p38 signaling contributes to mediating autophagy-delayed MSCs senescence<sup>[33]</sup>.

Collectively, autophagy is a surveillance pathway that tightly controls fate decisions of MSCs, and therefore it should be considered when searching for methods to maintain the pluripotency of MSCs.

## CYTOPROTECTION OF AUTOPHAGY IN MSCS UNDER STRESS

### *Hypoxic conditions*

Autophagy is known to exert cytoprotection for MSCs under stress conditions<sup>[34]</sup>. It has been demonstrated that hypoxia-pretreated MSCs exhibit AMPK/mTOR signaling activation, autophagy enhancement, and pro-angiogenic effect improvements<sup>[35]</sup>. Similarly, Zhang *et al.*<sup>[36]</sup> showed that the autophagy inhibitor 3-methyladenine (3-MA) promotes hypoxia-induced apoptosis, while a positive inducer of autophagy, rapamycin, decreases hypoxia-induced apoptosis, suggesting that autophagy seems to be a protective element in MSCs under hypoxic stress and that atorvastatin could improve BMSCs survival during hypoxia by enhancing autophagy *via* the AMPK/mTOR pathway. However, there are also studies showing that hypoxia activates the autophagic flux of BMSCs through the AMPK/mTOR pathway and that activation of the latter process plays an important role in hypoxia-induced apoptosis<sup>[37-39]</sup>. This complicated scenario might be due to the heterogeneity and site-specific properties of the MSCs. For instance, BMSCs derived from the mandible have higher expression of the stemness markers *Nanog*, *Oct-4*, and *Sox2*, as well as stronger autophagy and anti-aging capacities under normoxia or hypoxia, when compared to those derived from the tibia<sup>[40]</sup>.

### *Oxidative stress*

A recent study showed that oxidative stress-induced MSCs death could be prevented by carbon monoxide, and this protective effect is due to an increase of autophagy<sup>[41]</sup>. Autophagy facilitates the turnover of damaged cellular components, which may result in improved cellular survival in the setting of oxidative injury. Therefore, depletion of autophagy in MSCs exacerbates oxidative stress-induced MSCs death<sup>[41]</sup>. Augmenting autophagy by JNK activation also protects MSCs against oxidative damage, thereby improving MSCs survival<sup>[42]</sup>. Preconditioning or coconditioning with rapamycin alleviates, while 3-MA aggravates, H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis<sup>[34]</sup>. Likewise, H<sub>2</sub>O<sub>2</sub>-treated human MSCs (hMSCs) activates *FOXO3* and then induces autophagy in response to the elevated ROS level, thus preventing oxidative injury. In line with this, suppression of autophagy impairs ROS elimination and the osteogenic capacity of hMSCs<sup>[43]</sup>. However, it is worth noting that these cytoprotective effects of autophagy on MSCs in the context of oxidative damage seem to act in a stress severity- and duration-dependent manner. Autophagy flux is considered to be a self-defensive process during the early stage of MSCs injury induced by H<sub>2</sub>O<sub>2</sub>, and this protective effect would be abolished after sustained oxidative exposure (*i.e.*, 6 h), as demonstrated by increased levels of caspase-3 and caspase-6<sup>[34]</sup>, which indicates that adaptive autophagy contributes to an improved survival rate of MSCs under stress, while destructive autophagy is induced when it fails to manage excessive stress<sup>[44]</sup>.

### *Irradiation stress*

As the main mechanism by which cells initiate self-protection in a radiation microenvironment<sup>[45,46]</sup>, autophagy triggers a DNA damage response by regulating



DNA repair and checkpoint protein levels<sup>[47]</sup>. Some studies have reported that autophagy decreases after irradiation, suggesting an impairment in eliminating damaged cellular components<sup>[48]</sup>. Activation of autophagy in MSCs reduces radiation-generated ROS and DNA damage, leading to the maintenance of stemness and differentiation potential<sup>[34,49]</sup>, while suppression of autophagy results in more ROS generation, DNA damage, and worsening of self-renewal ability<sup>[49]</sup>. This radio-protective role of autophagy on MSCs is further supported by the observation that hypoxia increases both the autophagy level and MSCs radioresistance *via* ERK1/2 and mTOR signaling<sup>[50-52]</sup>, suggesting a positive relationship between autophagy and the radioresistance of MSCs.

### **Inflammatory stress**

Increasing evidence has shown that autophagy provides a crucial line of induction and modulation of the inflammatory status of MSCs. In a TNF- $\alpha$ /cycloheximide-induced inflammatory environment, enhancement of autophagy reverses the decreased survival rate of MSCs, while inhibition of autophagy aggravates apoptotic progression<sup>[53]</sup>. Nevertheless, there have been reports of the adverse regulatory effects of autophagy in MSCs. The inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  synergistically enhance autophagy in MSCs, as evidenced by increased expression of *BECN-1/Beclin-1*. Knockdown of *Beclin1* improves the therapeutic effects of MSCs and increases their survival by promoting *Bcl-2* expression *via* the ROS/MAPK1/3 pathway<sup>[54,55]</sup>. Wang *et al*<sup>[56]</sup> showed that autophagy is triggered in MSCs in response to a liver fibrosis (LF) microenvironment. Of note, autophagy suppression can improve the antifibrotic potential of MSCs and this contributes to their inhibitory effects on T lymphocyte infiltration as well as the production of inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$ <sup>[56]</sup>. Additionally, inhibition of autophagy increases ROS accumulation and MAPK 1/3 activation in MSCs, which are essential for prostaglandin E2 expression to exert an immunoregulatory function, thus resulting in enhanced suppression upon activation and expansion of CD4<sup>+</sup> T cells and leading to upregulation of the immunosuppressive function of MSCs<sup>[54]</sup>. This implies that autophagy may not always be beneficial in protecting the reparative effect of MSCs. Hence, modulating the multifaceted effects of autophagy in MSCs would provide a novel strategy to improve MSCs-based therapy.

---

## **AUTOPHAGY AND BONE REMODELING**

---

Bone remodeling is dynamic process that helps to maintain bone integrity and mineral homeostasis. There are three kinds of cell types involved in bone remodeling: Osteoclasts, osteoblasts, and osteocytes<sup>[57]</sup>. Among them, both osteoblasts and osteocytes are derived from BMSCs, while osteoclasts have a hematopoietic origin<sup>[58]</sup>. Osteoclasts are multinucleated cells that initiate bone remodeling by digesting old bone, whereas osteoblasts are responsible for synthesizing and secreting bone matrix to form new bone<sup>[58]</sup>. Osteocytes, as the most abundant cell type in bone tissue, are pivotal in bone remodeling by coupling osteoblasts and osteoclasts activities<sup>[59]</sup> *via* the receptor activator of NF-kappa B (RANK)/receptor activator of NF-kappa B ligand (RANKL)/osteoprotegerin (OPG) system<sup>[57]</sup>. Though still in its infancy, growing evidence has clarified that autophagy is closely related to bone remodeling mediated by osteoclasts, osteoblasts, and osteocytes, by which it exerts a critical role in coupling bone formation and bone resorption, thus maintaining normal postnatal bone homeostasis<sup>[60]</sup>.

### **Autophagy in osteoclasts**

Previous research has demonstrated that activation of autophagy by AMPK signaling inhibits osteoclast differentiation<sup>[61]</sup>. Moreover, autophagy induced by OPG attenuates osteoclast bone resorption *via* the AKT/mTOR/ULK1 axis<sup>[62]</sup>. Similarly, autophagy favors OPG-mediated inhibition of osteoclast differentiation and bone resorption through the AMPK/mTOR/p70S6K signaling pathway<sup>[63]</sup>. These data highlight a negative regulation of autophagy in osteoclastogenesis. However, Cao *et al*<sup>[64]</sup> showed that inhibiting autophagy suppresses TRPV4-induced osteoclast differentiation and osteoporosis *via* the Ca<sup>2+</sup>-calcinertin-NFATc1 pathway. In addition, JNK1-induced autophagy decreases apoptosis of osteoclast progenitors and stimulates RANKL-mediated osteoclastogenesis<sup>[65]</sup>, which shows a positive effect of autophagy on osteoclast activity, suggesting a potential role of autophagy in initiating bone remodeling.

### Autophagy in osteoblasts

Moreover, autophagy promoted by estradiol protects osteoblasts from apoptosis *via* the ER-ERK-mTOR axis<sup>[66]</sup>. Interestingly, both early proliferation and differentiation are not interfered by inactivation of autophagy by *FIP200* ablation, a fundamental element of mammalian autophagy, while osteoblast terminal differentiation is adversely affected, as shown by defective nodule formation<sup>[67]</sup>, which suggests a positive role of autophagy in nodule formation. Consistently, osteoblastic mineralization is found to be accompanied by activation of autophagy, in which vacuoles could act as vehicles for crystals secretion. Thus, osteoblast specific autophagy deficient mice exhibit a significant reduction of mineralization and bone mass<sup>[68]</sup>. Bone mass in osteoblast-specific *Atg7* conditional knockout (cKO) mice is significantly decreased compared with the control, the phenotype of which is caused by a decrease of osteoblast number and mineralization, as well as an increase of osteoclast number and osteoclast activity<sup>[69]</sup>. These results mean that autophagy exerts a critical role in osteoblast differentiation.

### Autophagy in osteocytes

Yang *et al.*<sup>[69]</sup> suggested a negative correlation between osteocyte autophagy and an ovariectomy (OVX) induced oxidative stress condition and bone loss. Reduction of autophagy by estrogen deficiency promotes the apoptosis of osteocytes, whereas restoration of autophagy strengthens the anti-apoptotic effects to improve osteocyte viability<sup>[70]</sup>. Osteocytes-specific cKO of *Atg7*, a key gene involved in autophagy, results in reduced bone mass, decreased cancellous and cortical bone thickness, and increased cortical bone porosity at 6 mo for both male and female mice, which contributes to decreases in osteoblast number, bone formation rate, and osteoclast number<sup>[71]</sup>. In addition, EphrinB2 in osteocytes limits autophagy to ensure bone quality by controlling mineral accumulation, while dysfunction of the osteocytic EphrinB2-autophagy signal results in bone fragility<sup>[72]</sup>. These findings emphasize a central role of autophagy in regulating osteocyte biology as well as bone remodeling.

---

## AUTOPHAGY AND BONE DISEASE

---

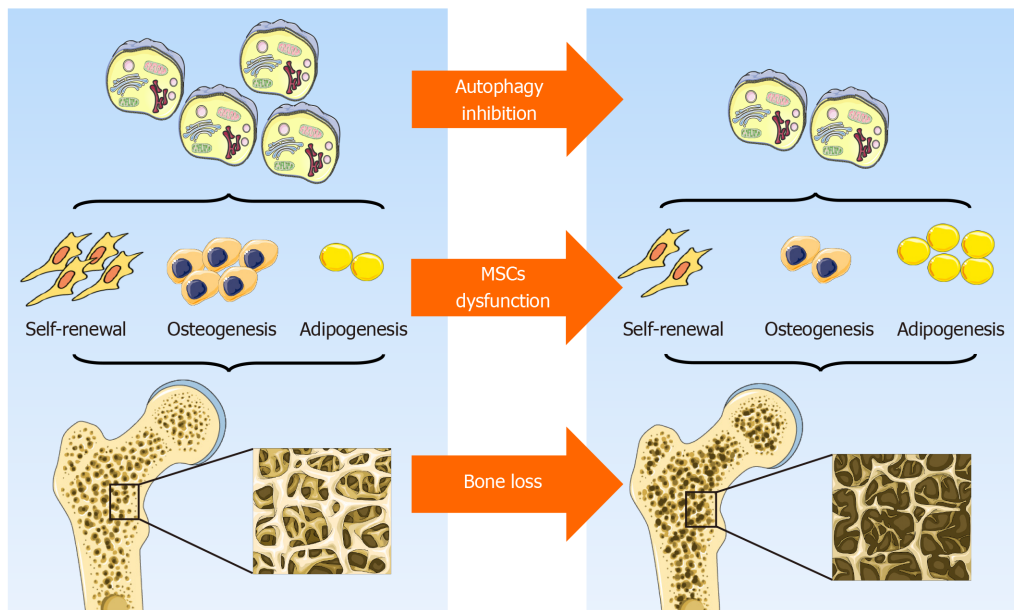
Increasing numbers of studies have revealed a crucial role of autophagy in the development and progression of many kinds of bone disease, such as osteopetrosis, Paget's disease, and osteoporosis<sup>[73-75]</sup>. Recently, a genome-wide association study of wrist bone mineral density caught our attention since it revealed a close relationship between osteoporosis and autophagy<sup>[76]</sup>. Further research demonstrated that MSCs from an osteoporosis mouse model induced by estrogen deficiency exhibit reduced autophagy, which is associated with abnormal regenerative function<sup>[73]</sup>. Interestingly, restoration of autophagy by administering rapamycin rescues the regenerative function of MSCs and protects OVX mice from osteoporotic development<sup>[73]</sup>. A similar decreased level of autophagy is also observed in OVX rats, while restoration of autophagy in osteoblasts by overexpressing autophagy gene damage-regulated autophagy modulator (*DRAM*) inhibits osteoblast proliferation and promotes their apoptosis<sup>[77]</sup>. In addition, activation of autophagy restored bone loss in aged mice<sup>[31]</sup>, whereas blockade of autophagy alleviated glucocorticoid-induced and OVX-induced bone loss by interfering with osteoclastogenesis<sup>[75]</sup>. What's more, defective autophagy in osteoblasts results in mouse osteopenia<sup>[67]</sup>. In experimental models of arthritis, rapamycin treatment can reduce the number of osteoclasts and osteoclast formation, thus inhibiting bone absorption in young rats<sup>[78]</sup>. Furthermore, rapamycin reduces bone resorption in renal transplant patients<sup>[79]</sup>, enhances osteogenic differentiation in a mouse model of osteopenia<sup>[80,81]</sup>, and ameliorates age-induced bone defects in aged rats<sup>[82]</sup>. Further study is warranted to explore the potential application of autophagy modulators as preventive or therapeutic strategies in bone disease.

---

## CONCLUSION AND FUTURE PERSPECTIVES

---

In general, although the prevailing views currently support the hypothesis that autophagy contributes to the maintenance of MSCs integrity by preserving their self-renewal and osteoblast differentiation potential while inhibiting adipocyte differentiation, thus orchestrating bone homeostasis (Figure 2), some data are still somewhat controversial. To some extent, autophagy is a dynamic process that



**Figure 2 Role of autophagy in mesenchymal stem cells integrity and bone homeostasis.** Autophagy contributes to the maintenance of mesenchymal stem cells integrity by preserving their self-renewal and osteoblast differentiation potential while inhibiting adipocyte differentiation, thus orchestrating bone homeostasis. Original elements used in this diagram are from Servier Medical Art (<http://smart.servier.com/>). MSCs: Mesenchymal stem cells.

depends on immediate cellular energy demands. Thus, it is necessary to investigate its biological role along a timeline instead of at a single isolated time point. In addition, autophagy may act as a double-edged sword, the effects of which are modified in response to the features, severity, and duration of a specific stress. Furthermore, the latest study emphasizes a critical role of mitochondrial autophagy, or mitophagy, in stem cell fate plasticity and determination<sup>[83,84]</sup>. Effects of an underlying crosstalk between autophagy and endoplasmic reticulum stress in MSCs and bone biology regulation is also beginning to be uncovered<sup>[85]</sup>. Further study is needed to lift the veil on the pleiotropy of autophagy, its reciprocal and functional interactions with other organelles, and their role in MSCs functional orchestration and bone biology modulation.

## REFERENCES

- 1 Murray IR, West CC, Hardy WR, James AW, Park TS, Nguyen A, Tawonsawatruk T, Lazzari L, Soo C, Péault B. Natural history of mesenchymal stem cells, from vessel walls to culture vessels. *Cell Mol Life Sci* 2014; **71**: 1353-1374 [PMID: 24158496 DOI: 10.1007/s00018-013-1462-6]
- 2 Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom SC. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* 2008; **26**: 300-311 [PMID: 17975221 DOI: 10.1634/stemcells.2007-0594]
- 3 Levi B, Longaker MT. Concise review: adipose-derived stromal cells for skeletal regenerative medicine. *Stem Cells* 2011; **29**: 576-582 [PMID: 21305671 DOI: 10.1002/stem.612]
- 4 Chanda D, Kumar S, Ponnazhagan S. Therapeutic potential of adult bone marrow-derived mesenchymal stem cells in diseases of the skeleton. *J Cell Biochem* 2010; **111**: 249-257 [PMID: 20506559 DOI: 10.1002/jcb.22701]
- 5 Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. *Nat Cell Biol* 2010; **12**: 814-822 [PMID: 20811353 DOI: 10.1038/ncb0910-814]
- 6 Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell* 2011; **147**: 728-741 [PMID: 22078875 DOI: 10.1016/j.cell.2011.10.026]
- 7 Gomes LC, Scorrano L. Mitochondrial morphology in mitophagy and macroautophagy. *Biochim Biophys Acta* 2013; **1833**: 205-212 [PMID: 22406072 DOI: 10.1016/j.bbamer.2012.02.012]
- 8 Dubouloz F, Deloche O, Wanke V, Cameroni E, De Virgilio C. The TOR and EGO protein complexes orchestrate microautophagy in yeast. *Mol Cell* 2005; **19**: 15-26 [PMID: 15989961 DOI: 10.1016/j]
- 9 Majeski AE, Dice JF. Mechanisms of chaperone-mediated autophagy. *Int J Biochem Cell Biol* 2004; **36**: 2435-2444 [PMID: 15325583 DOI: 10.1016/j.biocel.2004.02.013]
- 10 Salemi S, Yousefi S, Constantinescu MA, Fey MF, Simon HU. Autophagy is required for self-renewal and differentiation of adult human stem cells. *Cell Res* 2012; **22**: 432-435 [PMID: 22184008 DOI: 10.1038/cr.2011.200]

- 11 **Oliver L**, Hue E, Priault M, Vallette FM. Basal autophagy decreased during the differentiation of human adult mesenchymal stem cells. *Stem Cells Dev* 2012; **21**: 2779-2788 [PMID: [22519885](#) DOI: [10.1089/scd.2012.0124](#)]
- 12 **Jung CH**, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *FEBS Lett* 2010; **584**: 1287-1295 [PMID: [20083114](#) DOI: [10.1016/j.febslet.2010.01.017](#)]
- 13 **Wang H**, Liu Y, Wang D, Xu Y, Dong R, Yang Y, Lv Q, Chen X, Zhang Z. The Upstream Pathway of mTOR-Mediated Autophagy in Liver Diseases. *Cells* 2019; **8** [PMID: [31835352](#) DOI: [10.3390/cells8121597](#)]
- 14 **Shaw RJ**. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. *Acta Physiol (Oxf)* 2009; **196**: 65-80 [PMID: [19245654](#) DOI: [10.1111/j.1748-1716.2009.01972.x](#)]
- 15 **Wang Y**, Zhang H. Regulation of Autophagy by mTOR Signaling Pathway. *Adv Exp Med Biol* 2019; **1206**: 67-83 [PMID: [31776980](#) DOI: [10.1007/978-981-15-0602-4\\_3](#)]
- 16 **Yang Z**, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* 2010; **22**: 124-131 [PMID: [20034776](#) DOI: [10.1016/j.ceb.2009.11.014](#)]
- 17 **Hay N**. The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* 2005; **8**: 179-183 [PMID: [16169463](#) DOI: [10.1016/j.ccr.2005.08.008](#)]
- 18 **Xu Z**, Han X, Ou D, Liu T, Li Z, Jiang G, Liu J, Zhang J. Targeting PI3K/AKT/mTOR-mediated autophagy for tumor therapy. *Appl Microbiol Biotechnol* 2020; **104**: 575-587 [PMID: [31832711](#) DOI: [10.1007/s00253-019-10257-8](#)]
- 19 **Li B**, Duan P, Li C, Jing Y, Han X, Yan W, Xing Y. Role of autophagy on bone marrow mesenchymal stem-cell proliferation and differentiation into neurons. *Mol Med Rep* 2016; **13**: 1413-1419 [PMID: [26676567](#) DOI: [10.3892/mmr.2015.4673](#)]
- 20 **Isomoto S**, Hattori K, Ohgushi H, Nakajima H, Tanaka Y, Takakura Y. Rapamycin as an inhibitor of osteogenic differentiation in bone marrow-derived mesenchymal stem cells. *J Orthop Sci* 2007; **12**: 83-88 [PMID: [17260122](#) DOI: [10.1007/s00776-006-1079-9](#)]
- 21 **Nuschke A**, Rodrigues M, Stolz DB, Chu CT, Griffith L, Wells A. Human mesenchymal stem cells/multipotent stromal cells consume accumulated autophagosomes early in differentiation. *Stem Cell Res Ther* 2014; **5**: 140 [PMID: [25523618](#) DOI: [10.1186/s12930-014-0050-3](#)]
- 22 **Vidoni C**, Ferraresi A, Secomandi E, Vallino L, Gardin C, Zavan B, Mortellaro C, Isidoro C. Autophagy drives osteogenic differentiation of human gingival mesenchymal stem cells. *Cell Commun Signal* 2019; **17**: 98 [PMID: [31426798](#) DOI: [10.1186/s12964-019-0414-7](#)]
- 23 **Lee KW**, Yook JY, Son MY, Kim MJ, Koo DB, Han YM, Cho YS. Rapamycin promotes the osteoblastic differentiation of human embryonic stem cells by blocking the mTOR pathway and stimulating the BMP/Smad pathway. *Stem Cells Dev* 2010; **19**: 557-568 [PMID: [19642865](#) DOI: [10.1089/scd.2009.0147](#)]
- 24 **Choi HK**, Yuan H, Fang F, Wei X, Liu L, Li Q, Guan JL, Liu F. Tsc1 Regulates the Balance Between Osteoblast and Adipocyte Differentiation Through Autophagy/Notch1/ $\beta$ -Catenin Cascade. *J Bone Miner Res* 2018; **33**: 2021-2034 [PMID: [29924882](#) DOI: [10.1002/jbmr.3530](#)]
- 25 **Pantovic A**, Krstic A, Janjetovic K, Kocic J, Harhaji-Trajkovic L, Bugarski D, Trajkovic V. Coordinated time-dependent modulation of AMPK/Akt/mTOR signaling and autophagy controls osteogenic differentiation of human mesenchymal stem cells. *Bone* 2013; **52**: 524-531 [PMID: [23111315](#) DOI: [10.1016/j.bone.2012.10.024](#)]
- 26 **Xi G**, Rosen CJ, Clemmons DR. IGF-I and IGFBP-2 Stimulate AMPK Activation and Autophagy, Which Are Required for Osteoblast Differentiation. *Endocrinology* 2016; **157**: 268-281 [PMID: [26556533](#) DOI: [10.1210/en.2015-1690](#)]
- 27 **Demirovic D**, Nizard C, Rattan SI. Basal level of autophagy is increased in aging human skin fibroblasts in vitro, but not in old skin. *PLoS One* 2015; **10**: e0126546 [PMID: [25950597](#) DOI: [10.1371/journal.pone.0126546](#)]
- 28 **Zheng Y**, Hu CJ, Zhuo RH, Lei YS, Han NN, He L. Inhibition of autophagy alleviates the senescent state of rat mesenchymal stem cells during long-term culture. *Mol Med Rep* 2014; **10**: 3003-3008 [PMID: [25310478](#) DOI: [10.3892/mmr.2014.2624](#)]
- 29 **Kume S**, Uzu T, Horiike K, Chin-Kanasaki M, Isshiki K, Araki S, Sugimoto T, Haneda M, Kashiwagi A, Koya D. Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney. *J Clin Invest* 2010; **120**: 1043-1055 [PMID: [20335657](#) DOI: [10.1172/JCI41376](#)]
- 30 **Lipinski MM**, Zheng B, Lu T, Yan Z, Py BF, Ng A, Xavier RJ, Li C, Yankner BA, Scherzer CR, Yuan J. Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. *Proc Natl Acad Sci USA* 2010; **107**: 14164-14169 [PMID: [20660724](#) DOI: [10.1073/pnas.1009485107](#)]
- 31 **Ma Y**, Qi M, An Y, Zhang L, Yang R, Doro DH, Liu W, Jin Y. Autophagy controls mesenchymal stem cell properties and senescence during bone aging. *Aging Cell* 2018; **17** [PMID: [29210174](#) DOI: [10.1111/acer.12709](#)]
- 32 **García-Prat L**, Martínez-Vicente M, Perdiguero E, Ortet L, Rodríguez-Ubreva J, Rebollo E, Ruiz-Bonilla V, Gutarra S, Ballestar E, Serrano AL, Sandri M, Muñoz-Cánoves P. Autophagy maintains stemness by preventing senescence. *Nature* 2016; **529**: 37-42 [PMID: [26738589](#) DOI: [10.1038/nature16187](#)]
- 33 **Zhang D**, Chen Y, Xu X, Xiang H, Shi Y, Gao Y, Wang X, Jiang X, Li N, Pan J. Autophagy inhibits the mesenchymal stem cell aging induced by D-galactose through ROS/JNK/p38 signalling. *Clin Exp Pharmacol Physiol* 2020; **47**: 466-477 [PMID: [31675454](#) DOI: [10.1111/1440-1681.13207](#)]
- 34 **Song C**, Song C, Tong F. Autophagy induction is a survival response against oxidative stress in bone marrow-derived mesenchymal stromal cells. *Cytotherapy* 2014; **16**: 1361-1370 [PMID: [24980657](#) DOI: [10.1016/j.jcyt.2014.04.006](#)]
- 35 **Liu J**, Hao H, Huang H, Tong C, Ti D, Dong L, Chen D, Zhao Y, Liu H, Han W, Fu X. Hypoxia regulates the therapeutic potential of mesenchymal stem cells through enhanced autophagy. *Int J Low Extrem Wounds* 2015; **14**: 63-72 [PMID: [25759412](#) DOI: [10.1177/1534734615573660](#)]
- 36 **Zhang Q**, Yang YJ, Wang H, Dong QT, Wang TJ, Qian HY, Xu H. Autophagy activation: a novel mechanism of atorvastatin to protect mesenchymal stem cells from hypoxia and serum deprivation via AMP-



- activated protein kinase/mammalian target of rapamycin pathway. *Stem Cells Dev* 2012; **21**: 1321-1332 [PMID: 22356678 DOI: 10.1089/scd.2011.0684]
- 37 **Molaei S**, Roudkenar MH, Amiri F, Harati MD, Bahadori M, Jaleh F, Jalili MA, Mohammadi Roushandeh A. Down-regulation of the autophagy gene, ATG7, protects bone marrow-derived mesenchymal stem cells from stressful conditions. *Blood Res* 2015; **50**: 80-86 [PMID: 26157777 DOI: 10.5045/br.2015.50.2.80]
  - 38 **Zhang Z**, Yang M, Wang Y, Wang L, Jin Z, Ding L, Zhang L, Zhang L, Jiang W, Gao G, Yang J, Lu B, Cao F, Hu T. Autophagy regulates the apoptosis of bone marrow-derived mesenchymal stem cells under hypoxic condition via AMP-activated protein kinase/mammalian target of rapamycin pathway. *Cell Biol Int* 2016; **40**: 671-685 [PMID: 27005844 DOI: 10.1002/cbin.10604]
  - 39 **Maiuri MC**, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* 2007; **8**: 741-752 [PMID: 17717517 DOI: 10.1038/nrm2239]
  - 40 **Dong W**, Zhang P, Fu Y, Ge J, Cheng J, Yuan H, Jiang H. Roles of SATB2 in site-specific stemness, autophagy and senescence of bone marrow mesenchymal stem cells. *J Cell Physiol* 2015; **230**: 680-690 [PMID: 25200657 DOI: 10.1002/jcp.24792]
  - 41 **Ghanta S**, Tsoyi K, Liu X, Nakahira K, Ith B, Coronata AA, Fredenburgh LE, Englert JA, Piantadosi CA, Choi AM, Perrella MA. Mesenchymal Stromal Cells Deficient in Autophagy Proteins Are Susceptible to Oxidative Injury and Mitochondrial Dysfunction. *Am J Respir Cell Mol Biol* 2017; **56**: 300-309 [PMID: 27636016 DOI: 10.1165/rncmb.2016-0061OC]
  - 42 **Liu GY**, Jiang XX, Zhu X, He WY, Kuang YL, Ren K, Lin Y, Gou X. ROS activates JNK-mediated autophagy to counteract apoptosis in mouse mesenchymal stem cells in vitro. *Acta Pharmacol Sin* 2015; **36**: 1473-1479 [PMID: 26592514 DOI: 10.1038/aps.2015.101]
  - 43 **Gómez-Puerto MC**, Verhagen LP, Braat AK, Lam EW, Coffey PJ, Lorenowicz MJ. Activation of autophagy by FOXO3 regulates redox homeostasis during osteogenic differentiation. *Autophagy* 2016; **12**: 1804-1816 [PMID: 27532863 DOI: 10.1080/15548627.2016.1203484]
  - 44 **Hu C**, Zhao L, Wu D, Li L. Modulating autophagy in mesenchymal stem cells effectively protects against hypoxia- or ischemia-induced injury. *Stem Cell Res Ther* 2019; **10**: 120 [PMID: 30995935 DOI: 10.1186/s13287-019-1225-x]
  - 45 **Braunstein S**, Badura ML, Xi Q, Formenti SC, Schneider RJ. Regulation of protein synthesis by ionizing radiation. *Mol Cell Biol* 2009; **29**: 5645-5656 [PMID: 19704005 DOI: 10.1128/MCB.00711-09]
  - 46 **Feng Z**, Zhang H, Levine AJ, Jin S. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci USA* 2005; **102**: 8204-8209 [PMID: 15928081 DOI: 10.1073/pnas.0502857102]
  - 47 **Alexander A**, Cai SL, Kim J, Nanez A, Sahin M, MacLean KH, Inoki K, Guan KL, Shen J, Person MD, Kusewitt D, Mills GB, Kastan MB, Walker CL. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *Proc Natl Acad Sci USA* 2010; **107**: 4153-4158 [PMID: 20160076 DOI: 10.1073/pnas.0913860107]
  - 48 **Alessio N**, Del Gaudio S, Capasso S, Di Bernardo G, Cappabianca S, Cipollaro M, Peluso G, Galderisi U. Low dose radiation induced senescence of human mesenchymal stromal cells and impaired the autophagy process. *Oncotarget* 2015; **6**: 8155-8166 [PMID: 25544750 DOI: 10.18632/oncotarget.2692]
  - 49 **Hou J**, Han ZP, Jing YY, Yang X, Zhang SS, Sun K, Hao C, Meng Y, Yu FH, Liu XQ, Shi YF, Wu MC, Zhang L, Wei LX. Autophagy prevents irradiation injury and maintains stemness through decreasing ROS generation in mesenchymal stem cells. *Cell Death Dis* 2013; **4**: e844 [PMID: 24113178 DOI: 10.1038/cddis.2013.338]
  - 50 **Sugrue T**, Lowndes NF, Ceredig R. Hypoxia enhances the radioresistance of mouse mesenchymal stromal cells. *Stem Cells* 2014; **32**: 2188-2200 [PMID: 24578291 DOI: 10.1002/stem.1683]
  - 51 **Wu J**, Niu J, Li X, Li Y, Wang X, Lin J, Zhang F. Hypoxia induces autophagy of bone marrow-derived mesenchymal stem cells via activation of ERK1/2. *Cell Physiol Biochem* 2014; **33**: 1467-1474 [PMID: 24854431 DOI: 10.1159/000358711]
  - 52 **Lee Y**, Jung J, Cho KJ, Lee SK, Park JW, Oh IH, Kim GJ. Increased SCF/c-kit by hypoxia promotes autophagy of human placental chorionic plate-derived mesenchymal stem cells via regulating the phosphorylation of mTOR. *J Cell Biochem* 2013; **114**: 79-88 [PMID: 22833529 DOI: 10.1002]
  - 53 **Yang R**, Ouyang Y, Li W, Wang P, Deng H, Song B, Hou J, Chen Z, Xie Z, Liu Z, Li J, Cen S, Wu Y, Shen H. Autophagy Plays a Protective Role in Tumor Necrosis Factor- $\alpha$ -Induced Apoptosis of Bone Marrow-Derived Mesenchymal Stem Cells. *Stem Cells Dev* 2016; **25**: 788-797 [PMID: 26985709 DOI: 10.1089/scd.2015.0387]
  - 54 **Dang S**, Xu H, Xu C, Cai W, Li Q, Cheng Y, Jin M, Wang RX, Peng Y, Zhang Y, Wu C, He X, Wan B, Zhang Y. Autophagy regulates the therapeutic potential of mesenchymal stem cells in experimental autoimmune encephalomyelitis. *Autophagy* 2014; **10**: 1301-1315 [PMID: 24905997 DOI: 10.4161/auto.28771]
  - 55 **Dang S**, Yu ZM, Zhang CY, Zheng J, Li KL, Wu Y, Qian LL, Yang ZY, Li XR, Zhang Y, Wang RX. Autophagy promotes apoptosis of mesenchymal stem cells under inflammatory microenvironment. *Stem Cell Res Ther* 2015; **6**: 247 [PMID: 26670667 DOI: 10.1186/s13287-015-0245-4]
  - 56 **Wang HY**, Li C, Liu WH, Deng FM, Ma Y, Guo LN, Kong H, Hu KA, Liu Q, Wu J, Sun J, Liu YL. Autophagy inhibition via Beclin1 downregulation improves the mesenchymal stem cells antifibrotic potential in experimental liver fibrosis. *J Cell Physiol* 2020; **235**: 2722-2737 [PMID: 31508820 DOI: 10.1002/jcp.29176]
  - 57 **Hadjidakis DJ**, Androurakis II. Bone remodeling. *Ann N Y Acad Sci* 2006; **1092**: 385-396 [PMID: 17308163 DOI: 10.1196/annals.1365.035]
  - 58 **Jaber FA**, Khan NM, Ansari MY, Al-Adlaan AA, Hussein NJ, Safadi FF. Autophagy plays an essential role in bone homeostasis. *J Cell Physiol* 2019; **234**: 12105-12115 [PMID: 30820954 DOI: 10.1002/jcp.27071]
  - 59 **Yan Y**, Wang L, Ge L, Pathak JL. Osteocyte-Mediated Translation of Mechanical Stimuli to Cellular Signaling and Its Role in Bone and Non-bone-Related Clinical Complications. *Curr Osteoporos Rep* 2020; **18**: 67-80 [PMID: 31953640 DOI: 10.1007/s11914-020-00564-9]
  - 60 **Li H**, Li D, Ma Z, Qian Z, Kang X, Jin X, Li F, Wang X, Chen Q, Sun H, Wu S. Defective autophagy in osteoblasts induces endoplasmic reticulum stress and causes remarkable bone loss. *Autophagy* 2018; **14**: 1726-1741 [PMID: 29962255 DOI: 10.1080/15548627.2018.1483807]



- 61 **Tong X**, Zhang C, Wang D, Song R, Ma Y, Cao Y, Zhao H, Bian J, Gu J, Liu Z. Suppression of AMP-activated protein kinase reverses osteoprotegerin-induced inhibition of osteoclast differentiation by reducing autophagy. *Cell Prolif* 2020; **53**: e12714 [PMID: [31696568](#) DOI: [10.1111/cpr.12714](#)]
- 62 **Zhao H**, Sun Z, Ma Y, Song R, Yuan Y, Bian J, Gu J, Liu Z. Antiosteoclastic bone resorption activity of osteoprotegerin via enhanced AKT/mTOR/ULK1-mediated autophagic pathway. *J Cell Physiol* 2020; **235**: 3002-3012 [PMID: [31535378](#) DOI: [10.1002/jcp.29205](#)]
- 63 **Tong X**, Gu J, Song R, Wang D, Sun Z, Sui C, Zhang C, Liu X, Bian J, Liu Z. Osteoprotegerin inhibit osteoclast differentiation and bone resorption by enhancing autophagy via AMPK/mTOR/p70S6K signaling pathway in vitro. *J Cell Biochem* 2018; Online ahead of print [PMID: [30256440](#) DOI: [10.1002/jcb.27468](#)]
- 64 **Cao B**, Dai X, Wang W. Knockdown of TRPV4 suppresses osteoclast differentiation and osteoporosis by inhibiting autophagy through  $Ca^{2+}$ -calcineurin-NFATc1 pathway. *J Cell Physiol* 2019; **234**: 6831-6841 [PMID: [30387123](#) DOI: [10.1002/jcp.27432](#)]
- 65 **Ke D**, Ji L, Wang Y, Fu X, Chen J, Wang F, Zhao D, Xue Y, Lan X, Hou J. JNK1 regulates RANKL-induced osteoclastogenesis via activation of a novel Bcl-2-Bcln1-autophagy pathway. *FASEB J* 2019; **33**: 11082-11095 [PMID: [31295022](#) DOI: [10.1096/fj.201802597RR](#)]
- 66 **Yang YH**, Chen K, Li B, Chen JW, Zheng XF, Wang YR, Jiang SD, Jiang LS. Estradiol inhibits osteoblast apoptosis via promotion of autophagy through the ER-ERK-mTOR pathway. *Apoptosis* 2013; **18**: 1363-1375 [PMID: [23743762](#) DOI: [10.1007/s10495-013-0867-x](#)]
- 67 **Liu F**, Fang F, Yuan H, Yang D, Chen Y, Williams L, Goldstein SA, Krebsbach PH, Guan JL. Suppression of autophagy by FIP200 deletion leads to osteopenia in mice through the inhibition of osteoblast terminal differentiation. *J Bone Miner Res* 2013; **28**: 2414-2430 [PMID: [23633228](#) DOI: [10.1002/jbmr.1971](#)]
- 68 **Nollet M**, Santucci-Darmanin S, Breuil V, Al-Sahlanee R, Cros C, Topi M, Momier D, Samson M, Pagnotta S, Caillaudeau L, Battaglia S, Farlay D, Dacquin R, Barois N, Jurdic P, Boivin G, Heymann D, Lafont F, Lu SS, Dempster DW, Carle GF, Pierrefite-Carle V. Autophagy in osteoblasts is involved in mineralization and bone homeostasis. *Autophagy* 2014; **10**: 1965-1977 [PMID: [25484092](#) DOI: [10.4161/auto.36182](#)]
- 69 **Yang Y**, Zheng X, Li B, Jiang S, Jiang L. Increased activity of osteocyte autophagy in ovariectomized rats and its correlation with oxidative stress status and bone loss. *Biochem Biophys Res Commun* 2014; **451**: 86-92 [PMID: [25063028](#) DOI: [10.1016/j.bbrc.2014.07.069](#)]
- 70 **Florencio-Silva R**, Sasso GRS, Sasso-Cerri E, Simões MJ, Cerri PS. Effects of estrogen status in osteocyte autophagy and its relation to osteocyte viability in alveolar process of ovariectomized rats. *Biomed Pharmacother* 2018; **98**: 406-415 [PMID: [29276969](#) DOI: [10.1016/j.biopha.2017.12.089](#)]
- 71 **Onal M**, Piemontese M, Xiong J, Wang Y, Han L, Ye S, Komatsu M, Selig M, Weinstein RS, Zhao H, Jilka RL, Almeida M, Manolagas SC, O'Brien CA. Suppression of autophagy in osteocytes mimics skeletal aging. *J Biol Chem* 2013; **288**: 17432-17440 [PMID: [23645674](#) DOI: [10.1074/jbc.M112.444190](#)]
- 72 **Vrahnas C**, Blank M, Dite TA, Tatarczuch L, Ansari N, Crimeen-Irwin B, Nguyen H, Forwood MR, Hu Y, Ikegame M, Bamberg KR, Petibois C, Mackie EJ, Tobin MJ, Smyth GK, Oakhill JS, Martin TJ, Sims NA. Increased autophagy in EphrinB2-deficient osteocytes is associated with elevated secondary mineralization and brittle bone. *Nat Commun* 2019; **10**: 3436 [PMID: [31366886](#) DOI: [10.1038/s41467-019-11373-9](#)]
- 73 **Bo T**, Yan F, Guo J, Lin X, Zhang H, Guan Q, Wang H, Fang L, Gao L, Zhao J, Xu C. Characterization of a Relatively Malignant Form of Osteopetrosis Caused by a Novel Mutation in the PLEKHM1 Gene. *J Bone Miner Res* 2016; **31**: 1979-1987 [PMID: [27291868](#) DOI: [10.1002/jbmr.2885](#)]
- 74 **Rea SL**, Walsh JP, Layfield R, Ratajczak T, Xu J. New insights into the role of sequestosome 1/p62 mutant proteins in the pathogenesis of Paget's disease of bone. *Endocr Rev* 2013; **34**: 501-524 [PMID: [23612225](#) DOI: [10.1210/er.2012-1034](#)]
- 75 **Lin NY**, Chen CW, Kagwiria R, Liang R, Beyer C, Distler A, Luther J, Engelke K, Schett G, Distler JH. Inactivation of autophagy ameliorates glucocorticoid-induced and ovariectomy-induced bone loss. *Ann Rheum Dis* 2016; **75**: 1203-1210 [PMID: [26113650](#) DOI: [10.1136/annrheumdis-2015-207240](#)]
- 76 **Zhang L**, Guo YF, Liu YZ, Liu YJ, Xiong DH, Liu XG, Wang L, Yang TL, Lei SF, Guo Y, Yan H, Pei YF, Zhang F, Papasian CJ, Recker RR, Deng HW. Pathway-based genome-wide association analysis identified the importance of regulation-of-autophagy pathway for ultradistal radius BMD. *J Bone Miner Res* 2010; **25**: 1572-1580 [PMID: [20200951](#) DOI: [10.1002/jbmr.36](#)]
- 77 **Tang N**, Zhao H, Zhang H, Dong Y. Effect of autophagy gene DRAM on proliferation, cell cycle, apoptosis, and autophagy of osteoblast in osteoporosis rats. *J Cell Physiol* 2019; **234**: 5023-5032 [PMID: [30203495](#) DOI: [10.1002/jcp.27304](#)]
- 78 **Cejka D**, Hayer S, Niederreiter B, Sieghart W, Fuereder T, Zwerina J, Schett G. Mammalian target of rapamycin signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from patients with rheumatoid arthritis. *Arthritis Rheum* 2010; **62**: 2294-2302 [PMID: [20506288](#) DOI: [10.1002/art.27504](#)]
- 79 **Westenfeld R**, Schlieper G, Wöltje M, Gawlik A, Brandenburg V, Rutkowski P, Floege J, Jahnchen-Dechent W, Ketteler M. Impact of sirolimus, tacrolimus and mycophenolate mofetil on osteoclastogenesis--implications for post-transplantation bone disease. *Nephrol Dial Transplant* 2011; **26**: 4115-4123 [PMID: [21622987](#) DOI: [10.1093/ndt/gfr214](#)]
- 80 **Darcy A**, Meltzer M, Miller J, Lee S, Chappell S, Ver Donck K, Montano M. A novel library screen identifies immunosuppressors that promote osteoblast differentiation. *Bone* 2012; **50**: 1294-1303 [PMID: [22421346](#) DOI: [10.1016/j.bone.2012.03.001](#)]
- 81 **Shimada M**, Greer PA, McMahon AP, Bouxsein ML, Schipani E. In vivo targeted deletion of calpain small subunit, Capn4, in cells of the osteoblast lineage impairs cell proliferation, differentiation, and bone formation. *J Biol Chem* 2008; **283**: 21002-21010 [PMID: [18515801](#) DOI: [10.1074/jbc.M710354200](#)]
- 82 **Luo D**, Ren H, Li T, Lian K, Lin D. Rapamycin reduces severity of senile osteoporosis by activating osteocyte autophagy. *Osteoporos Int* 2016; **27**: 1093-1101 [PMID: [26395886](#) DOI: [10.1007/s00198-015-3325-5](#)]
- 83 **Chandel NS**. Evolution of Mitochondria as Signaling Organelles. *Cell Metab* 2015; **22**: 204-206 [PMID: [26073494](#) DOI: [10.1016/j.cmet.2015.05.013](#)]
- 84 **Martínez-Reyes I**, Diebold LP, Kong H, Schieber M, Huang H, Hensley CT, Mehta MM, Wang T, Santos

- JH, Woychik R, Dufour E, Spelbrink JN, Weinberg SE, Zhao Y, DeBerardinis RJ, Chandel NS. TCA Cycle and Mitochondrial Membrane Potential Are Necessary for Diverse Biological Functions. *Mol Cell* 2016; **61**: 199-209 [PMID: [26725009](#) DOI: [10.1016/j.molcel](#)]
- 85 **Cinque L**, Forrester A, Bartolomeo R, Svelto M, Venditti R, Montefusco S, Polishchuk E, Nusco E, Rossi A, Medina DL, Polishchuk R, De Matteis MA, Settembre C. FGF signalling regulates bone growth through autophagy. *Nature* 2015; **528**: 272-275 [PMID: [26595272](#) DOI: [10.1038/nature16063](#)]



## Stem cell therapy for Alzheimer's disease

Xin-Yu Liu, Lin-Po Yang, Lan Zhao

**ORCID number:** Xin-Yu Liu 0000-0002-1520-4159; Lin-Po Yang 0000-0002-2814-8520; Lan Zhao 0000-0002-7449-2947.

**Author contributions:** Liu XY wrote the paper; Yang LP was involved in the data collection; Zhao L was responsible for the review design, revision, and research funding. All authors approved the final version of the manuscript.

**Supported by** the National Natural Science Foundation of China, No. 81202740; and Tianjin Natural Science Fund, No. 17JCYBJC26200.

**Conflict-of-interest statement:** The authors declare no conflict of interests for this article.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Invited manuscript

**Xin-Yu Liu, Lin-Po Yang, Lan Zhao,** First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin 300381, China

**Lan Zhao,** Tianjin Key Laboratory of Acupuncture and Moxibustion, Tianjin 300381, China

**Lan Zhao,** National Clinical Research Center for Chinese Medicine Acupuncture and Moxibustion, Tianjin 300381, China

**Corresponding author:** Lan Zhao, PhD, Research Fellow, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine; Tianjin Key Laboratory of Acupuncture and Moxibustion; National Clinical Research Center for Chinese Medicine Acupuncture and Moxibustion, No. 88 Changling Road, Xiqing District, Tianjin 300381, China. [lanzhao69@163.com](mailto:lanzhao69@163.com)

### Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by memory loss and cognitive impairment. It is caused by synaptic failure and excessive accumulation of misfolded proteins. To date, almost all advanced clinical trials on specific AD-related pathways have failed mostly due to a large number of neurons lost in the brain of patients with AD. Also, currently available drug candidates intervene too late. Stem cells have improved characteristics of self-renewal, proliferation, differentiation, and recombination with the advent of stem cell technology and the transformation of these cells into different types of central nervous system neurons and glial cells. Stem cell treatment has been successful in AD animal models. Recent preclinical studies on stem cell therapy for AD have proved to be promising. Cell replacement therapies, such as human embryonic stem cells or induced pluripotent stem cell-derived neural cells, have the potential to treat patients with AD, and human clinical trials are ongoing in this regard. However, many steps still need to be taken before stem cell therapy becomes a clinically feasible treatment for human AD and related diseases. This paper reviews the pathophysiology of AD and the application prospects of related stem cells based on cell type.

**Key words:** Alzheimer's disease; Stem cell; Therapy; Pathogenesis; Animal experiment; Clinical trial

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Received:** February 28, 2020**Peer-review started:** February 28, 2020**First decision:** April 2, 2020**Revised:** April 10, 2020**Accepted:** July 26, 2020**Article in press:** July 26, 2020**Published online:** August 26, 2020**P-Reviewer:** Bugaj AM, Chakrabarti S**S-Editor:** Zhang L**L-Editor:** Wang TQ**P-Editor:** Wang LL

**Core tip:** Alzheimer's disease (AD), a progressive neurodegenerative disorder featuring memory loss and cognitive impairment, is caused by synaptic failure and the excessive accumulation of misfolded proteins. Stem cell-based therapies cast a new hope for AD treatment as a replacement or regeneration strategy. The results from recent preclinical studies regarding stem cell-based therapies are promising. Human clinical trials are now underway. However, a number of questions remain to be answered prior to safe and effective clinical translation. This review explores the pathophysiology of AD and summarizes the relevant stem cell research according to cell type. We also briefly summarize related clinical trials. Finally, future perspectives are discussed with regard to their clinical applications.

**Citation:** Liu XY, Yang LP, Zhao L. Stem cell therapy for Alzheimer's disease. *World J Stem Cells* 2020; 12(8): 787-802

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/787.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.787>

## INTRODUCTION

Dementia is a neurodegenerative, debilitating, and fatal disease characterized by progressive cognitive impairment, behavioral disorders, and loss of function in daily life. Alzheimer's disease (AD) is the most common cause of dementia, accounting for 50%-70% of dementia cases worldwide<sup>[1]</sup>. The 2018 World Alzheimer's Disease Report shows that 50 million people worldwide have dementia. With a new case occurring every 3 s worldwide, AD has rapidly become an epidemic, with the number of cases predicted to be 152 million by 2050<sup>[2]</sup>.

AD has several neuropathological hallmarks, including the deposition of  $\beta$ -amyloid ( $A\beta$ ) peptides in the extracellular matrix between neurons (known as amyloid plaques), the intracellular formation of neurofibrillary tangles arising from the accumulation of hyperphosphorylated tau protein in neurons, neuronal loss, neuroinflammation, and oxidative stress. Despite advances in understanding the etiology of AD, treating the disease by retaining acetylcholine and reducing glutamate is limited to symptom management<sup>[3]</sup>. Although cerebrospinal fluid (CSF) and positron emission tomography (PET) biomarkers combined with some relatively new clinical standards can help diagnose alive patients, the certainty of diagnosis was achieved only by post-mortem autopsy<sup>[3]</sup>. These criteria highlight that the gold standard for the etiological diagnosis remains the neuropathological assessment. Accordingly, the results of CSF biomarkers for AD may provide explanatory evidence for neurocognitive symptoms and predict the type of evolution, especially when there are no other obvious causes of cognitive impairment. Reducing  $A\beta$  levels has been the dominant treatment strategy in development to halt, retard, or even reverse the progression of AD pathology. In fact, currently available treatments include three types of cholinesterase inhibitors, one N-methyl-daspartate receptor antagonist, and one combined drug therapy (memantine plus donepezil) are currently approved for clinical use<sup>[4]</sup>. However, it is unclear how valuable such a palliative drug-based approach can be.

Therefore, new and effective treatments, such as removing toxic deposits and replacing lost neurons, need to be developed to improve the pathological state of the disease, stimulate neural precursors, prevent nerve death, enhance structural neural plasticity, and so forth. At the same time, it is also necessary to provide a better environment for the remaining cells. Current breakthroughs in preclinical research and clinical trials of stem cells have ignited hope for the treatment of refractory neurodegenerative diseases such as AD. They are considered to be the most suitable choice to provide uniform and unique cells required for cell replacement therapy<sup>[5]</sup>. This review focuses on the mechanisms of AD pathogenesis and discusses clinical and preclinical findings on the role of stem cells in the treatment of AD.



## RESEARCH PROGRESS IN THE PATHOGENESIS OF AD

### **Genetics of AD pathogenesis**

Studies have shown that two typical misfolded proteins accumulate in the brain of patients with AD. The first is A $\beta$ , which is a pathological cleavage product of amyloid precursor protein (APP). The accumulation of A $\beta$  into plaques and smaller oligomers is one of the pathological features of AD<sup>[6]</sup>. APP mutations have been confirmed to be associated with hereditary familial AD. Familial AD is an early-onset autosomal dominant genetic disease. The age of onset is less than 65 years, but it only accounts for 2% of all AD cases<sup>[7]</sup>. Many failed clinical trials targeted this pathway directly or indirectly through small-molecule or antibody therapies to reduce A $\beta$  production or promote A $\beta$  clearance<sup>[6,8]</sup>. The second misfolded protein in AD is tau, a microtubule-associated protein that aggregates in cells in the form of neurofibrillary tangles. The most closely related pathological feature is AD cognitive decline<sup>[9,10]</sup>. However, the vast majority (> 98%) of cases of AD, which do not involve mutations in APP processing pathways, are sporadic, and the age of onset is more than 65 years<sup>[6]</sup>. For this population, the main predictive factor for AD is the genetic risk factor apolipoprotein (APO) E4, in addition to age<sup>[6]</sup>. APOE4 carriers account for 60%–75% of AD cases. Compared with noncarriers, patients with AD and APOE4 are younger<sup>[11]</sup>.

### **Tau protein and AD pathogenesis**

In AD neurons, the protein kinase/protein phosphatase phosphorylation system is imbalanced, resulting in abnormal and overphosphorylated tau protein. The human tau protein is encoded by a single gene containing 16 exons on chromosome 17<sup>[12]</sup>, which is expressed in the brain as six isomers that contain amino acid sequences at the carboxyl and amino ends, where the carboxyl end is repeated. The sequence is a microtubule-binding region, and the tau protein can enhance the stability of microtubules in axons<sup>[13,14]</sup>. Mitogen-activated protein kinases include the extracellular signal-related kinases, which are activated by multiple stimuli including growth factors, c-Jun N-terminal kinases, and p38 mitogen-activated protein kinases. These kinases cause neuronal tau protein phosphorylation and are closely related to AD disease progression<sup>[15]</sup>. The tau protein in AD is overphosphorylated and accumulates in cells in the form of double-helix filaments, straight filaments, and tangled skeletons. This hallmark damage is directly related to the degree of dementia<sup>[16]</sup>. Abnormal tau protein is found in hereditary Parkinson-like frontotemporal dementia related to chromosome 17; it leads to neurodegenerative diseases and dementia<sup>[17]</sup>. The composition of abnormally hyperphosphorylated tau protein can be used to measure p-tau protein levels in the cerebrospinal fluid<sup>[18]</sup>. Decreased phosphatase activity, especially reduced protein phosphatase-2A activity, plays a key regulatory role in abnormal hyperphosphorylation of tau protein<sup>[19]</sup>. PET brain imaging technology shows that the accumulation of tau protein more directly predicts future neurodegenerative changes in patients with AD. The progress of tau pathology and brain atrophy in different regions may reflect a phase shift. Local elevation in tau levels precedes atrophy<sup>[20]</sup>. Tau may be one of the targets for the early clinical treatment of AD<sup>[21]</sup>.

### **$\beta$ -amyloid protein and AD pathogenesis**

The A $\beta$  is an important hypothesis for the pathogenesis of AD. The relationship between APP and A $\beta$  explains the pathogenesis of the lesion. APP is first cleaved at beta-secretase (BACE) 1 site by  $\beta$ -secretase to produce soluble amyloid precursor protein and released outside the cell. Then, C99 remaining in the cell is cleaved by  $\gamma$ -secretase to produce A $\beta$  polypeptide and APP intracellular domain. A $\beta$  peptides, mainly A $\beta$ 1–40 and A $\beta$ 1–42, are released outside the cell, while APP intracellular domain remains inside the cell<sup>[22]</sup>. Neuronal damage or death is caused by the accumulation of toxic A $\beta$  in the brain, which causes senile plaques in cells. A $\beta$ 1–40 in the brain has the highest content of A $\beta$ , but A $\beta$ 1–42 is more likely to form fibers and oligomers. The highly toxic A $\beta$ 1–42 oligomers are an important cause of AD<sup>[23]</sup>. The accumulation of A $\beta$  in the brain and subsequent plaque formation are pathological features of AD<sup>[24]</sup>. The impaired ability of the central nervous system to export A $\beta$  to the periphery through the barrier is considered to be the cause of A $\beta$  accumulation in AD and eventual plaque formation<sup>[25]</sup>. Studies have shown that the expression levels of blood–brain barrier endothelial cell receptors change with age and the development of AD. The expression level of efflux receptors decreases, and the expression level of influx receptors increases<sup>[26]</sup>. Changes and dysfunctions increase the accumulation of A $\beta$ , and neuronal synaptic rupture and apoptosis occur<sup>[27]</sup>.

However, the central conclusion that either accumulation of tau protein or of A $\beta$  protein is the cause of AD, at very least, is premature. The recent failure of clinical trials based on the immunotherapeutic approach against A $\beta$  protein questioned the validity of the “amyloid cascade hypothesis” as the molecular machinery causing the disease. However, important suggestions come from the critical analysis of such flop. Although synapse dysfunction is a key early event and accurate correlate of AD progression, A $\beta$  plaque deposition can occur without synapse loss<sup>[28]</sup>. Conversely, synapse and dendritic tree loss can occur in areas where there is no A $\beta$  deposition, although synapse loss does usually appear exacerbated near A $\beta$  plaques<sup>[29]</sup>. Furthermore, synaptic gene dysregulation in early AD can occur independently of alterations in the expression of APP and regulators of APP metabolism<sup>[30]</sup>. Thus, the timing of an A $\beta$ - or tau-targeted intervention has proven critical for clinical response since once A $\beta$ -induced synaptic dysfunction and extensive neurodegeneration occur, they can no longer be reversed by simply reducing brain amyloid burden<sup>[31]</sup>. This paradigm has shifted clinical trials from late clinical AD dementia to the early, asymptomatic stages of the disease<sup>[32]</sup>.

In fact, A $\beta$  or tau may be a player in a more complex view of disease and, further, its role may even be variable. We conclude that it is essential to expand our view of pathogenesis beyond A $\beta$  and tau pathology. Current drug design strategies are based on “one drug-one target” paradigm<sup>[33]</sup>, which until now failed to provide effective treatments against AD, due to the multifactorial nature of the disease<sup>[34,35]</sup>. Reducing A $\beta$  or tau levels has been the dominant treatment strategy in development to halt, retard, or even reverse the progression of AD pathology. However, they are experiencing difficulties in clinical trials<sup>[36]</sup> as the effects appear independent from symptomatic improvement<sup>[37]</sup>.

The revolutionary discovery of stem cells has cast a new hope for the development of disease-modifying treatments for AD, in terms of their potency in the replenishment of lost cells *via* differentiating towards specific lineages, stimulating *in situ* neurogenesis, and delivering the therapeutic agents to the brain. Indeed, researchers have effectively treated AD in transgenic mouse models in more than 50 different ways<sup>[38]</sup>. A recently completed open-label phase I clinical trial evaluated the safety and tolerability of intracranially injected allogeneic human umbilical cord blood-derived mesenchymal stem cells (MSCs) (Trial identifier: NCT01297218, NCT01696591)<sup>[39]</sup>. Alternatively, due to the complex nature of AD pathophysiology, a multimodal approach may be required, incorporating pharmacological targeting of pathology, stimulation of endogenous neurogenesis and synaptogenesis, as well as exogenous neuroreplacement.

---

## STEM CELL CLASSIFICATION

---

In recent years, embryonic stem cells (ESCs), MSCs, brain-derived neural stem cells (NSCs), and induced pluripotent stem cells (iPSCs) are most commonly used in AD research.

---

## CLASSIFICATION BASED ON CELL ORIGIN

---

### ***Embryonic stem cells***

ESCs are derived from the inner cell mass of pluripotent blastocysts<sup>[40]</sup> and classified as pluripotent because of their ability to generate cell types from the ectoderm, mesoderm, and endoderm. Studies have shown that ESCs can improve spatial learning and memory in rats with AD by differentiating into basal forebrain cholinergic neurons and  $\gamma$ -aminobutyric acid neurons<sup>[41]</sup>. However, the clinical application of ESCs is limited due to the high risk of teratoma formation, abnormal immune response, and rejection. In addition, ethical disputes must be clarified before they can be used in Food and Drug Administration-approved clinical trials<sup>[42]</sup>. Several reports have explored the role of ESCs in rodent models of AD. Pluripotency is one of the greatest advantages of ESCs. It represents one of the major disadvantages of ESCs because their differentiation can occur in any direction and cause tumors or teratomas<sup>[43,44]</sup>. Therefore, current research strategies focus on establishing a differentiating agreement. Mouse ESCs (mESCs) were successfully used to produce basal forebrain cholinergic neurons (BFCNs), which were severely affected in patients with AD. These neurons, when transplanted into AD rat models, drive the derivation

of ESCs and induce neural precursor cell (NPC) differentiation<sup>[45]</sup>.

In addition, these rats showed significant behavioral improvements in memory deficits. Human ESCs (hESCs) can also produce cholinergic neurons in the vitreous and hippocampal tissues, which are connected to existing neural network<sup>[46]</sup>. Similarly, mESCs and hESCs were introduced into mature BFCNs, and improvements in learning and memory performance were observed after transplantation into mice with AD<sup>[47]</sup>. Another method is to differentiate hESCs into medial ganglion protrusion MGE-like progenitor cells because MGE is the origin of basal forebrain neurons (including BFCNs and  $\gamma$ -aminobutyric acid intermediate neurons) during development. The transplantation of these MGE-like progenitor cells into the hippocampus of mice produced results similar to the findings of the present study<sup>[41]</sup>.

### **Mesenchymal stem cells**

MSCs are involved in the development of mesenchymal tissue types, which can be obtained from umbilical cord blood (ucb-MSCs) or the Wharton jelly. They are also found in some adult stem cell pupae, including bone marrow and adipose tissue. MSCs are classified as pluripotent cells and are capable of producing multiple cell types. These cells have a common embryonic origin: The mesoderm germ layer. Nevertheless, the phenotypic expression and differentiation potential of bone marrow MSCs may vary depending on the source tissue<sup>[23]</sup>. Umbilical cord blood is the residual blood of the placenta and umbilical cord after childbirth. The blood is rich in hematopoietic stem cells and other stem cells such as MSCs<sup>[48]</sup>. Previous studies on ucb-MSCs (mainly MSCs) using murine models of AD have shown that ucb-MSCs can improve spatial learning and prevent memory decline. Many mechanisms have also been proposed, including reduction of A $\beta$  plaques, BACE and tau hyperphosphorylation, and reversal of microglial inflammation and promotion of anti-inflammatory cytokines<sup>[49]</sup>. Immunomodulatory and anti-inflammatory effects have also been observed by upregulating neuroprotection and downregulating pro-inflammatory cytokines. Another important way for MSCs to participate in tissue repair is the secretion of extracellular vesicles and microvesicles, which has been widely explored. Bone marrow MSCs can release extracellular vesicles that target A $\beta$  deposition through genetic modification and are supplemented with therapeutic drugs, including siRNAs and enzymes<sup>[50,51]</sup>. Alternatively, MSCs can be regulated to overexpress cytokines and vascular endothelial growth factor, and show regeneration effects in the AD model<sup>[52]</sup>. Despite ethical issues, especially commercial cord blood banks, MSCs are the most common source of stem cells used in AD research because they are relatively easy to pick and handle if harvested after normal delivery<sup>[53,54]</sup>.

### **Induced pluripotent stem cells**

iPSCs were first obtained from mouse fibroblasts in 2006. They are derived *in vitro* from mature somatic cells, usually adult dermal fibroblasts, by small-molecule therapy or viral vector-mediated upregulation of transcription factors. Genetic modification makes them pluripotent and ESC-like in terms of phenotypic and differentiation capacity<sup>[55]</sup>.

iPSCs are thought to differentiate into a variety of cells, including neurons<sup>[56]</sup> and neurospheres<sup>[57]</sup>. Both *in vitro* and posttransplantation into the rodent cortex studies have shown that iPSCs can be used to generate and automate neuronal subtypes<sup>[58-61]</sup>. For example, iPSC-derived glial cells can be used to study the inflammatory response of AD<sup>[62]</sup>. Another study with a mouse model of AD used iPSCs to obtain macrophages capable of expressing neprilysin, an A $\beta$ -degrading protease<sup>[63]</sup>. An iPSC model is a powerful tool for studying the APP treatment of tissue-specific cells in mutant individuals caused by FAD<sup>[64,65]</sup>. Yagi *et al*<sup>[66]</sup> found increased levels of A $\beta$ 42 secretion in neurons with presenilin1 (PSEN1) (A246E) and PSEN2 (N141I) mutations<sup>[66]</sup>. Further research on neurons with the pathogenic PSEN1 mutation showed an increase in the ratio of A $\beta$ 42:40<sup>[67-69]</sup>. Similarly, iPSC-derived neurons with the APP V717I genotype showed an increased A $\beta$ 42:40 ratio<sup>[70-72]</sup> and an increased A $\beta$ 42:38 ratio<sup>[73]</sup>. Arber *et al*<sup>[74]</sup> used multiple patient-derived iPSC neurons to simulate APP processing and A $\beta$  production in the context of fAD-APP and PSEN1 mutations, indicating that iPSCs provided a valuable model for studying potential cell dysfunction caused by genetic fAD mutations<sup>[74]</sup>.

However, the following unresolved questions about the use of iPSCs pose huge obstacles to their clinical application: Teratoma formation, long-term safety and effectiveness, tumorigenicity, immunogenicity, patient genetic defects, optimal reprogramming and so forth<sup>[75-78]</sup>.

## CLASSIFICATION BASED ON CELL TYPE

### Neural stem cells

NSCs are responsible for the production of all nerve cell types during development. They also exist in the adult brain and are confined to discrete neurogenic niches in the subventricular zone and the granular layer of the dentate gyrus of the hippocampus. Adult NSCs are located in the subgranular zone of the dentate gyrus and the subventricular zone of the lateral wall of the ventricle. They are self-renewing pluripotent cells that produce neurons, oligodendrocytes, or astrocytes<sup>[79]</sup>.

The paracrine effect of NSCs has significant therapeutic potential. In rodent AD models<sup>[80]</sup> and senile primate brains<sup>[81]</sup>, transplantation of growth factor-secreting NSCs can improve neurogenesis and cognitive function, while transplantation of human NSCs with high expression of choline acetyltransferase can reverse spatial memory and learning deficits in rodent models of alkaline neurotoxicity<sup>[82]</sup>. NSC transplantation may reduce neuroinflammation in AD rodent models through the paracrine release of neuroprotective or immunomodulatory factors and also mediate neuronal differentiation<sup>[83]</sup>. These cells reduce tau and A $\beta$  expression levels<sup>[84]</sup>, promote neurogenesis and synapse formation<sup>[85,86]</sup>, and reverse cognitive deficits<sup>[83,85,86]</sup>. However, non-glial cells widely produced from transplanted NSCs are the main limiting factor for neural replacement strategies<sup>[87]</sup>. Studies on rodent AD models have shown that human NSCs (hNSCs) from the embryonic telomere, when transplanted into the lateral ventricle of the brain of mice with AD, can migrate and differentiate into neurons and glial cells in the lateral ventricle. This phenomenon reduces tau phosphorylation and A $\beta$ -42 levels, decreases glial and astrocyte hyperplasia<sup>[84]</sup>, enhances endogenous synapse formation<sup>[86]</sup>, and increases neuronal, synaptic, and nerve fiber density<sup>[88]</sup>, ultimately improving spatial memory in mice with AD. These effects are achieved through a variety of mechanisms, including regulation of signaling pathways, metabolic activity, secretion of anti-inflammatory factors, and cell-to-cell contact. Brain-derived neurotrophic factor (BDNF) is an important neuroprotective factor derived from NSCs. By increasing the synaptic density of the hippocampus<sup>[80]</sup> and the number of cholinergic neurons<sup>[87,89]</sup>, BDNF can be used in AD rodent transplanted NSCs (obtained from the brain or hippocampus). Animal cognition plays an important role. The hNSC line that overexpresses choline acetyltransferase is transplanted into elderly Institute of Cancer Research mice. By directly producing acetylcholine and restoring the integrity of cholinergic neurons, hNSCs can increase the levels of BDNF and nerve growth factor (NGF) neurotrophins and improve the cognitive function and physical activity of elderly mice<sup>[90]</sup>. In addition, hNSCs can be genetically modified to express NGF and transplanted into mice with induced cognitive dysfunction to improve their learning and memory abilities<sup>[91]</sup>.

## STEM CELLS AND AD

### Animal experiments

**Neural stem cells:** Researchers have used methods such as brain injury, neurotoxin-induced brain cell loss, and intraventricular injection of A $\beta$  peptide to establish AD-like pathology and induce memory impairment models in rats and mice<sup>[92,93]</sup>. Martinez-serrano *et al*<sup>[94]</sup> transplanted forebrain cholinergic neurons into the host striatum and Meynert nuclei, and found that cells survived well in the host brain for a long time and induced hypertrophic responses of cholinergic neurons. Sinden *et al*<sup>[95]</sup> found that the transplantation of choline-rich NSCs could reduce AD symptoms in rats. Qu *et al*<sup>[96]</sup> injected human undifferentiated NSCs into the brain of 6-mo-old and 24-mo-old rats, revealing a significant improvement in cognitive function. Wu *et al*<sup>[97]</sup> found that human fetal brain-derived NSCs transplanted into adult rat brains could produce cholinergic neurons in specific regions. Wang *et al*<sup>[98]</sup> transplanted ESC-derived neurospheres into the frontal cortex of a mouse model of Meynert nuclear injury. The transplanted neurospheres survived, migrated, and differentiated into choline acetyltransferase-positive serotonin-positive neurons. The rate of working memory error in neuron- and neurosphere-transplanted mice was significantly reduced. On the contrary, ESCs in the control group developed teratomas, which did not express neurons, and the working memory significantly deteriorated.

Animal models related to A $\beta$ -induced memory loss are widely used in exploring the pathophysiology of AD and the efficacy of therapeutic targets. Prakash *et al*<sup>[99]</sup> used a lateral ventricular injection of A $\beta$  to observe the effect of peroxisome proliferator-



activated receptor  $\gamma$  agonist pioglitazone on BDNF and found that A $\beta$ -injured animals showed obvious memory impairment; BDNF levels were reduced, and this situation was reversed by pioglitazone<sup>[99]</sup>. Tang and others showed that the A $\beta$ -40 fiber was neurotoxic in the hippocampus of rats, characterized by Congo erythema and degeneration neurons at the injection site; the Morris water maze test showed impaired cognitive function in rats<sup>[100]</sup>. Transplanted cells improved A $\beta$ -induced cognitive dysfunction in rats; they further survived, integrated, and differentiated into neuronal cells 16 wk after transplantation<sup>[101]</sup>. Blurton Jones and others transplanted NSCs into aged transgenic mice expressing mutant presenilin, tau, and APP, and found that transplanted NSCs could improve spatial learning and memory function in mice with dementia without altering the pathology of A $\beta$ . In addition, NSCs underwent BDNF-mediated regeneration and promoted a decrease in synaptic density. When recombinant BDNF was additionally supplemented, memory loss was restored<sup>[80]</sup>. When NSCs were genetically engineered to stably release the A $\beta$ -degrading enzyme neprilysin, synaptic plasticity could be enhanced and the potential A $\beta$  pathological characteristics of transgenic mice could be improved<sup>[102]</sup>.

The cells either replace degenerated neurons or secrete trophic factors to provide a protective environment for endogenous cells. They secrete a variety of neurotrophic factors to regulate synaptic function in the brain. In particular, BDNF is synthesized by neurons and is highly expressed in the cerebral cortex and hippocampus; these regions are essential for brain learning and memory<sup>[103]</sup>. Therefore, it is reasonable to conclude that these preliminary studies point to a potentially viable treatment for AD and that the effect of stem cell transplantation into the brain is supported by a combination of methods and mediated, or at least significantly affected, by paracrine effects to a large extent.

**Mesenchymal stem cells:** MSCs have been widely studied due to their accessibility and relative ease of operation. They have three main roles in AD treatment: (1) Immune regulation; (2) Reduction of A $\beta$  plaque burden through internalization and A $\beta$  degradation of endosomal-lysosomal pathway oligomers; and (3) Neurotrophic/regenerative potential<sup>[104]</sup>. Systematic injection of green fluorescent protein-labeled bone marrow MSCs has been shown to reduce the size of A $\beta$  plaques in the hippocampus of animal models of AD<sup>[105]</sup> and function in an immunomodulatory manner. Transplantation of placental-derived MSCs in the lateral ventricle in A $\beta$ 1-42 perfused mice has also been shown to have beneficial effects, including improving memory deficit function and reducing A $\beta$ 1-42 levels, APP and BACE1 expression levels, alpha- and beta-secretase activity, and gliomas<sup>[106]</sup>. After injecting MSCs in AD animal models, NPCs were induced to differentiate into hippocampal mature neurons by activating the Wnt pathway, providing evidence for MSCs supporting the growth and differentiation of local stem and progenitor cells<sup>[107]</sup>. In another study, human MSCs transplanted into aged rats have been shown to reach the brain and differentiate into nerve cells, restoring motor and cognitive activity<sup>[90]</sup>. It is worth noting that the encouraging clinical results obtained under different pathological conditions and the preclinical results of MSCs in animal models of AD<sup>[108]</sup> facilitated the start of clinical trials of MSCs in patients with AD (<https://clinicaltrials.gov>, using MSCs and AD as keywords). One of these trials has completed the first phase of the study, confirming the feasibility and safety of MSC injection in human brains in nine patients<sup>[38]</sup>.

**Embryonic stem cells:** ESCs are totipotent and self-renewing. They can differentiate into NPCs *in vitro*, hence serving as therapeutics when transplanted into animal models of AD<sup>[38]</sup>. Generally, the direct transplantation of ESCs into animal models of AD results in the formation of teratomas rather than neurons. However, the safety level of ESC-derived NPC and neuron transplantation has been demonstrated<sup>[38]</sup>. They can differentiate into astrocytes and neuron-like cells and improve neurodegenerative diseases *in vivo*<sup>[101]</sup>. In the AD rat model, mESCs-derived NPCs were transferred to unilateral meynert basal nucleus with and without pretreatment, improving learning and memory abilities. The mainstream cells of transplanted NPCs maintain a neuronal phenotype, but nearly 40% of these cells show a cholinergic phenotype<sup>[45]</sup>. Despite no report on the potential of hESCs for treating AD, hESCs can be considered as a new factor in treating different types of neurodegenerative diseases and brain damage<sup>[110]</sup>. However, these cells are derived from pre-implantation human embryos, and therefore ethical issues must be addressed before using hESCs in AD clinical trials. In addition, the possibility of the use of immune rejection in ESC-based AD cell therapy remains a controversial issue<sup>[110]</sup>.

**Induced pluripotent stem cells:** In some AD models, attempts to regulate endogenous neurogenesis, replace lost neurons, or reverse pathological changes through iPSCs

have demonstrated early effectiveness. In a Parkinson (PD) APP transgenic mouse model, an ipsilateral injection of cholinergic neuron precursors in humans stimulated endogenous neurogenesis and reversed spatial memory disorders<sup>[111]</sup>. Human iPSC-derived macrophage-like cells were genetically modified to express neprilysin-2, an A $\beta$ -degrading protease, differentiate into functional neurons, and therapeutically reduce A $\beta$  levels in a five familial AD (5  $\times$  FAD) transgenic mouse model<sup>[63]</sup>. In addition, the inoculation of human iPSC-derived NSCs into the hippocampus of a mouse model of stroke could significantly improve neural function, which might be explained by the transition from the pro-inflammatory cytokine response to the anti-inflammatory cytokine response through neurotrophin-related reprogramming effects<sup>[112]</sup>. In a recent study, the use of protein-induced iPSCs and ferritin released by mESCs greatly promoted the differentiation and maturation of oligodendrocytes, thereby reducing plaque deposition and improving bilateral brain transplantation in 5  $\times$  FAD transgenic mice with AD. Cognitive dysfunction highlights the significance of stem cells that promote the differentiation of transplanted cells into different cell lines<sup>[113]</sup>. Despite these successful studies, autologous iPSCs may show genetic instability and phenotypic neuropathology, such as significant A $\beta$  load rates, shortened axon lengths, and increased tau phosphorylation, hindering their clinical application in AD<sup>[73,78,114]</sup>. However, healthy neurons can be transplanted into patients with AD by implementing genome-editing techniques, such as recombinant homologs, transcription activator-like effect nucleases, and regularly spaced short palindromic repeats (CRISPR-cas9)<sup>[115]</sup>. To date, the efficiency and repeatability of automated iPSC reprogramming procedures have resulted in stable, high-quality cell lines for major disease modeling or cell therapy. Studies have shown efficient production of neuronal subtypes, such as cortical pyramids and BFCNs<sup>[59]</sup>.

### Clinical trials

In 2015, human umbilical cord blood MSCs were used for the first phase of a clinical trial in nine patients with mild-to-moderate AD<sup>[38]</sup>. In an attempt to treat AD, patients were stereotactically injected with human umbilical cord blood MSCs into the hippocampus and anterior hippocampus, confirming that the method of stem cell administration was safe and feasible without any adverse reactions. However, the clinical effect of the method on the pathogenesis of AD needs to be further verified. Several clinical trials are ongoing on patients with AD; however, the results have not been published (Clinicaltrials.gov, NCT01547689, NCT02672306, NCT02054208, and NCT02600130). Since 2011, preclinical trials of bone marrow MSCs in animal models of AD have achieved good results and are sufficient to authorize patients with AD to begin clinical trials (Table 1). Intravenous infusion is the most ideal method for stem cell implantation, and cord blood stem cells are the most commonly used source of cells. Kim *et al.*<sup>[38]</sup> stereotactically transplanted human umbilical cord blood-derived MSCs into the hippocampus and anterior thalamus.

Despite no serious adverse events, no significant clinical effects on cognitive decline were observed (Clinicaltrials.gov, NCT01297218, NCT01696591)<sup>[116]</sup>. In addition, no pathological changes or neuroprotective effects have been observed<sup>[106,116,117]</sup>. These results might be partly attributed to neuroimaging, which is an insensitive method for detecting these changes compared with postmortem biochemical analysis. Stem cell therapy using both MSCs and iPSCs reveals great potential in the treatment of several neurodegenerative disorders (AD and PD). Their use has shown promising results with regard to modulation of inflammation. Moreover, they can promote other beneficial effects, such as neuronal growth. In a recent AD clinical trial, intracranially injected MSCs were evaluated for safety and tolerability. Nine patients participated in this study. The criteria for inclusion in the study involved a Mini-Mental Status Examination score range from 10 to 24, indicating mild to moderate dementia. The patients were also confirmed to have A $\beta$  pathology using PET scans. The patients were divided into two groups, one of them received a low dose while the other received a higher dose of the same injection. MSCs were directly injected into the hippocampus of the patients surgically. Follow-up examinations were taken at the 3- and 24-mo time points. No slowing of cognitive decline was found at the 24 mo, and no decrease of AB pathology was observed. None of the patients showed adverse side effects from the surgery and transplantation<sup>[118]</sup>. Although preclinical trials in animal models demonstrated neuroprotective effects, they did not translate clinically.

In addition, specific issues such as the specific cell stage to be transplanted, dose, route of administration, and duration of therapeutic effect must be solved<sup>[119]</sup>. Thus, there are still numerous open questions which have to be answered before clinical trials can be initiated. Preclinical evidence of the efficacy and safety of stem cells from

**Table 1 Completed clinical trial trials of stem cells in patients with Alzheimer's disease**

Study name (study date)	Current state	Length (phase)	Site	Subjects (age)	Design	Stem cell	Route (n)	Dosage (participants)	n <sup>1</sup>	Outcome measures	
										Primary	Secondary
NCT03117738 (4/2017- 9/2019)	Active, NR	32 wk (I/II)	United States	AD (> 50)	PBO- control, Double- blind	AD- MSC	IV (9)	NA	21	ADAS- cog	MMSE, CDR- SB, NPI, GDS, ADL, biomarkers (MRI, Aβ, <i>etc.</i> )
NCT04040348 (4/2019- 9/2021)	Recruiting	65 wk (I)	United States	AD (50-85)	Open label	H- MSC	IV (NA)	1 × 10 <sup>8</sup> (5), 2 × 10 <sup>8</sup> (5)	10	AE number	ADAS-cog, MMSE, GDS, ADL, NPI, diverse biomarkers
NCT02600130 (4/2019- 9/2021)	Active, NR	65 wk (II)	United States	AD (50-80)	PBO- control, Double- blind	L-MSC	IV (1)	2 × 10 <sup>7</sup> (10), 1 × 10 <sup>8</sup> (10), PBO (5)	25	AE number	ADAS-cog, ADL, biomarkers (CSF, Aβ)
NCT02672306 (10/2017- 10/2019)	Active, NR	36 wk (I/II)	China	AD (50-85)	PBO- control, Double- blind	HUC- MSC	IV (8)	0.5 × 10 <sup>6</sup> /kg (NA), PBO (NA)	16	ADAS- cog	ADAS-cog, CIBIC, CIBIC plus, MMSE, ADL, NPI biomarkers (plasma Aβ, <i>etc.</i> )
NCT03724136 (10/2018- 10/2022)	Recruiting	12 mo	United States	AD+ other neurological disease (> 18)	Open label, Three groups	B-MSC	IV (NA)	NA	100	MMSE, ASQ-SE	Activities of daily living
NCT01547689 (2012.3- 2016.12)	Unknown status	10 wk (I/II)	China	AD (50-85)	Open label, Single- center, Self- control	HUC- MSC	IV (8)	0.5 × 10 <sup>6</sup> /kg	30	AE number	ADAS-cog, MMSE, CIBIC, ADL, NPI biomarkers (Aβ, tau, <i>etc.</i> )

<sup>1</sup>Number of total participants. Aβ: Amyloid-beta; AD: Dementia due to Alzheimer's disease; ADAS-Cog: Alzheimer's Disease Assessment Scale-Cognitive Subscale; AE: Adverse events; NPI: Neuropsychiatric Inventory; CSF: Cerebrospinal fluid; AD-MSC: Autologous adipose tissue-derived mesenchymal stem cells; H-MSC: Human mesenchymal stem cells; L-MSC: Leukemia mesenchymal stem cells; B-MSC: Bone marrow mesenchymal stem cells; HUC-MSC: Human umbilical cord blood-derived mesenchymal stem cells; ADL: Activities of daily living; NA: Not available; CDR-SB: Clinical Dementia Rating-Sum of the Boxes scale; GDS: Geriatric Depression Scale; MMSE: Minimum Mental State Examination; CIDIC: Composite International Diagnostic Interview Core; PBO: Placebo; IV: Intravenous; ASQ-SE: Ages and stages questionnaires-social-emotional.

different sources is necessary for the development of clinically useful therapies. Extensive cell characterization, more efficient modelling of human diseases, and better comprehension of the interaction with resident and immune/scavenging cells are some of the key points that still need to be properly addressed by researchers.

## DISCUSSION

The field of cell therapy awaits the results of many ongoing clinical trials on AD. Scientists are still working to solve some of the small technical issues in this area to pave the way for effective treatment of AD and accelerate the pace of development. In addition to insufficient survey funding, the question of participant registration is undoubtedly the most critical obstacle to the development of clinical investigations. First, the appropriate timing of stem cell transplantation for AD has not been determined. Some intervention trials for AD have failed because they have not been conducted at the appropriate time. AD is a progressive chronic disease that usually begins several years before diagnosis. Therefore, an individual's brain is severely damaged by the time symptoms or signs appear and a large number of central neurons in the brain are dead, resulting in an irreversible loss. CSF biomarkers are being used more and more widely, to increase the diagnostic certainty, provide comprehensive patient information, and optimize management, from the beginning of clinical symptoms. Most ongoing therapeutic trials target subjects with MCI due to biomarker-confirmed AD, since many recent longitudinal studies have demonstrated

the ability of biomarkers to predict the progression of cognitive impairment and the development of overt dementia<sup>[120,121]</sup>. In MCI, identifying AD lesions helps to predict the progression towards AD dementia. The evidence that CSF biomarkers could identify or exclude AD is strong in patients with mild dementia, but weaker in ambiguous cases. However, there are still uncertainties regarding the individual course of cognitive decline, even though the biomarkers show a typical AD profile. There is no precise framework for the use of biomarkers with regards to the age and general health status of the patient. The multiple causes of cognitive impairment in elderly and very elderly subjects make their interpretation difficult and ethical and clinical reflection must be systematically conducted. Conversely, in some cases, brain imaging showed that a few study participants did not have a trial treatment plan for amyloidosis, suggesting an urgent need for early detection technology (ClinicalTrials.gov, Reg. No. NCT01163825). Given that clinical trials lasted for several years, patients with dementia received several injections and went through some difficult follow-up procedures; some participants withdrew before the end of the trial, making it difficult to evaluate the results (NCT02600130, NCT03117738, *etc.*). Unfortunately, another subtle point not considered in clinical trials was sex-related differences. It is estimated that women account for about two thirds of the patients with AD. Therefore, to obtain meaningful data and hence develop effective treatments, randomized controlled trials targeting specific populations need to adapt and evolve to cope with sex-related differences.

## CONCLUSION

Animal research is difficult to translate into human trials. The transgenic model used in preclinical research is based on the familial AD hypothesis, and the clinical distribution of AD has genetic heterogeneity. In addition, results from rodent models or from models using higher-order animals may not be sufficient to support the clinical use of stem cells in AD because of significant differences in neuronal function and anatomy in rodents and primates. None have successfully replicated the complex microenvironment of the human brain or the precise pathophysiological conditions of AD. Consequently, it is challenging to precisely characterize the beneficial effects of stem cells in AD.

Another important area that requires further research is the role of stem cells in the lymphatic system. This system, which is composed mainly of astrocytes, is a recently discovered macroscopic waste removal system<sup>[122]</sup>. It plays an important role in eliminating potentially neurotoxic waste, including A $\beta$ . In addition, previous studies have shown that A $\beta$  clearance disorder due to a dysfunctional lymphatic system is a cause of AD pathology<sup>[123]</sup>. Therefore, vigorous research is needed to elucidate the interactions between stem cells, astrocytes, A $\beta$  clearance, and the lymphatic system.

AD is a progressive neurodegenerative disease with no effective treatment currently. Because of their regenerative potential, stem cells may be an effective treatment option (compared with traditional therapies). Although the mechanism of action of stem cell therapy has not been fully elucidated, many preclinical studies have provided promising results. However, human clinical trials are still in their infancy. Further relevant animal research and clinical trials (with standardized protocols) are needed for the successful clinical transformation of this technology.

Stem cells used in AD and animal models have achieved certain results, but there are still many problems to be solved before they can be extended to clinical applications. One of the disadvantages of stem cell therapy is the requirement for a neurosurgical procedure and immunosuppression. Human and rodent studies have reported tumor formation resulting from autologous haematopoietic stem cell<sup>[124]</sup>, allogeneic fetal NSC<sup>[125]</sup>, and genetically engineered MSC<sup>[126]</sup> transplantation. At this point, the major concerns are related to controlling the proliferation and differentiation of stem cells, controlling the targeting of molecular markers, and developing cell delivery systems, as well as understanding and exploiting the heterogeneity of AD patients. Related to the heterogeneity of AD, transgenic animal models to date have been developed for the familial type of AD. However, most human AD cases are sporadic. Regarding these issues, researchers will continue to attempt to optimize cells by genetic engineering approaches to improve safety, efficacy, and patient-specific individualization of cell therapy. Furthermore, the recent technological developments of stem cells, involving the use of hydrogels, nano-technology, and light therapies have made drug delivery and regeneration treatments more efficient neural replacement, and regeneration therapy can soon be translated into the clinical setting



with further research combining these recent advancements. Stem cell therapy for AD carries enormous promise but remains under development. Many problems such as uncertainty about the amyloid hypothesis, differing objectives such as preventing progression from MCI to AD *vs* symptomatic treatment of established AD, and methodological designs of the trials themselves have been mentioned. Additionally, temporary recovery of behavior is relatively easily obtained, but often fail to be linked to a complete cure. Curative treatment is likely dependent upon sufficiently early diagnosis (MCI) to prevent further cell death and brain deterioration. A combination of NSC transplantation alongside administering existing approved medication and preventing further A $\beta$  aggregation may be the most effective way. It is important to note that whilst behavioral or cognitive improvement is interpreted as positive outcomes, it can be frequently misinterpreted as permanent arrest or even reversal of AD progression. Alternatively, due to the complex nature of AD pathophysiology, a multimodal approach may be required, incorporating pharmacological targeting of pathology, stimulation of endogenous neurogenesis and synaptogenesis, as well as exogenous neuroreplacement.

## ACKNOWLEDGEMENTS

The authors would like to thank all members of the Tianjin Institution of Acupuncture and Moxibustion who provided us with critical comments and assistance.

## REFERENCES

- 1 **Ferri CP**, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y, Jorm A, Mathers C, Menezes PR, Rimmer E, Scazufca M; Alzheimer's Disease International. Global prevalence of dementia: a Delphi consensus study. *Lancet* 2005; **366**: 2112-2117 [PMID: 16360788 DOI: 10.1016/S0140-6736(05)67889-0]
- 2 **Alzheimer's Disease International**. World Alzheimer Report 2018 (cited 30 September 2018). Available at: <https://www.alz.co.uk/research/world-report2018>
- 3 **Kang JM**, Yeon BK, Cho SJ, Suh YH. Stem Cell Therapy for Alzheimer's Disease: A Review of Recent Clinical Trials. *J Alzheimers Dis* 2016; **54**: 879-889 [PMID: 27567851 DOI: 10.3233/jad-160406]
- 4 **Briggs R**, Kennelly SP, O'Neill D. Drug treatments in Alzheimer's disease. *Clin Med (Lond)* 2016; **16**: 247-253 [PMID: 27251914 DOI: 10.7861/clinmedicine.16-3-247]
- 5 **Kumar V**, Jahan S, Singh S, Khanna VK, Pant AB. Progress toward the development of in vitro model system for chemical-induced developmental neurotoxicity: potential applicability of stem cells. *Arch Toxicol* 2015; **89**: 265-267 [PMID: 25537189 DOI: 10.1007/s00204-014-1442-0]
- 6 **Huang Y**, Mucke L. Alzheimer mechanisms and therapeutic strategies. *Cell* 2012; **148**: 1204-1222 [PMID: 22424230 DOI: 10.1016/j.cell.2012.02.040]
- 7 **Rygiel K**. Novel strategies for Alzheimer's disease treatment: An overview of anti-amyloid beta monoclonal antibodies. *Indian J Pharmacol* 2016; **48**: 629-636 [PMID: 28066098 DOI: 10.4103/0253-7613.194867]
- 8 **Golde TE**, Schneider LS, Koo EH. Anti-a $\beta$  therapeutics in Alzheimer's disease: the need for a paradigm shift. *Neuron* 2011; **69**: 203-213 [PMID: 21262461 DOI: 10.1016/j.neuron.2011.01.002]
- 9 **Iqbal K**, Alonso AC, Gong CX, Khatoon S, Pei JJ, Wang JZ, Grundke-Iqbal I. Mechanisms of neurofibrillary degeneration and the formation of neurofibrillary tangles. *J Neural Transm Suppl* 1998; **53**: 169-180 [PMID: 9700655 DOI: 10.1007/978-3-7091-6467-9\_15]
- 10 **Zhan Y**, Zheng H, Wang C, Rong Z, Xiao N, Ma Q, Zhang YW. A novel presenilin 1 mutation (F388L) identified in a Chinese family with early-onset Alzheimer's disease. *Neurobiol Aging* 2017; **50**: 168.e1-168.e4 [PMID: 27836335 DOI: 10.1016/j.neurobiolaging.2016.10.010]
- 11 **Farrer LA**, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *JAMA* 1997; **278**: 1349-1356 [PMID: 9343467]
- 12 **Neve RL**, Harris P, Kosik KS, Kurnit DM, Donlon TA. Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Brain Res* 1986; **387**: 271-280 [PMID: 3103857 DOI: 10.1016/0169-328x(86)90033-1]
- 13 **Scott CW**, Blowers DP, Barth PT, Lo MM, Salama AI, Caputo CB. Differences in the abilities of human tau isoforms to promote microtubule assembly. *J Neurosci Res* 1991; **30**: 154-162 [PMID: 1795399 DOI: 10.1002/jnr.490300116]
- 14 **Trinczek B**, Biernat J, Baumann K, Mandelkow EM, Mandelkow E. Domains of tau protein, differential phosphorylation, and dynamic instability of microtubules. *Mol Biol Cell* 1995; **6**: 1887-1902 [PMID: 8590813 DOI: 10.1091/mbc.6.12.1887]
- 15 **Kins S**, Kurosinski P, Nitsch RM, Götz J. Activation of the ERK and JNK signaling pathways caused by neuron-specific inhibition of PP2A in transgenic mice. *Am J Pathol* 2003; **163**: 833-843 [PMID: 12937125 DOI: 10.1016/s0002-9440(10)63444-x]
- 16 **An WL**, Cowburn RF, Li L, Braak H, Alafuzoff I, Iqbal K, Iqbal IG, Winblad B, Pei JJ. Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's

- disease. *Am J Pathol* 2003; **163**: 591-607 [PMID: [12875979](#) DOI: [10.1016/s0002-9440\(10\)63687-5](#)]
- 17 **Spillantini MG**, Murrell JR, Goedert M, Farlow MR, Klug A, Ghetti B. Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc Natl Acad Sci USA* 1998; **95**: 7737-7741 [PMID: [9636220](#) DOI: [10.1073/pnas.95.13.7737](#)]
- 18 **Jadhav S**, Cubinkova V, Zimova I, Brezovakova V, Madari A, Cigankova V, Zilka N. Tau-mediated synaptic damage in Alzheimer's disease. *Transl Neurosci* 2015; **6**: 214-226 [PMID: [28123806](#) DOI: [10.1515/tnsci-2015-0023](#)]
- 19 **Sontag E**, Nunbhakdi-Craig V, Lee G, Brandt R, Kamibayashi C, Kuret J, White CL 3rd, Mumby MC, Bloom GS. Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. *J Biol Chem* 1999; **274**: 25490-25498 [PMID: [10464280](#) DOI: [10.1074/jbc.274.36.25490](#)]
- 20 **La Joie R**, Visani AV, Baker SL, Brown JA, Bourakova V, Cha J, Chaudhary K, Edwards L, Iaccarino L, Janabi M, Lesman-Segev OH, Miller ZA, Perry DC, O'Neil JP, Pham J, Rojas JC, Rosen HJ, Seeley WW, Tsai RM, Miller BL, Jagust WJ, Rabinovici GD. Prospective longitudinal atrophy in Alzheimer's disease correlates with the intensity and topography of baseline tau-PET. *Sci Transl Med* 2020; **12** [PMID: [31894103](#) DOI: [10.1126/scitranslmed.aau5732](#)]
- 21 **Bakota L**, Brandt R. Tau Biology and Tau-Directed Therapies for Alzheimer's Disease. *Drugs* 2016; **76**: 301-313 [PMID: [26729186](#) DOI: [10.1007/s40265-015-0529-0](#)]
- 22 **Nikolaev A**, McLaughlin T, O'Leary DD, Tessier-Lavigne M. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature* 2009; **457**: 981-989 [PMID: [19225519](#) DOI: [10.1038/nature07767](#)]
- 23 **Hass R**, Kasper C, Böhm S, Jacobs R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal* 2011; **9**: 12 [PMID: [21569606](#) DOI: [10.1186/1478-811X-9-12](#)]
- 24 **Benilova I**, Karran E, De Strooper B. The toxic A $\beta$  oligomer and Alzheimer's disease: an emperor in need of clothes. *Nat Neurosci* 2012; **15**: 349-357 [PMID: [22286176](#) DOI: [10.1038/nn.3028](#)]
- 25 **Zlokovic BV**, Yamada S, Holtzman D, Ghiso J, Frangione B. Clearance of amyloid beta-peptide from brain: transport or metabolism? *Nat Med* 2000; **6**: 718-719 [PMID: [10888892](#) DOI: [10.1038/77397](#)]
- 26 **Silverberg GD**, Messier AA, Miller MC, Machan JT, Majmudar SS, Stopa EG, Donahue JE, Johanson CE. Amyloid efflux transporter expression at the blood-brain barrier declines in normal aging. *J Neuropathol Exp Neurol* 2010; **69**: 1034-1043 [PMID: [20838242](#) DOI: [10.1097/NEN.0b013e3181f46e25](#)]
- 27 **Deo AK**, Borson S, Link JM, Domino K, Eary JF, Ke B, Richards TL, Mankoff DA, Minoshima S, O'Sullivan F, Eyal S, Hsiao P, Maravilla K, Unadkat JD. Activity of P-Glycoprotein, a  $\beta$ -Amyloid Transporter at the Blood-Brain Barrier, Is Compromised in Patients with Mild Alzheimer Disease. *J Nucl Med* 2014; **55**: 1106-1111 [PMID: [24842892](#) DOI: [10.2967/jnumed.113.130161](#)]
- 28 **Boncrisiano S**, Calhoun ME, Howard V, Bondolfi L, Kaeser SA, Wiederhold KH, Staufenbiel M, Jucker M. Neocortical synaptic bouton number is maintained despite robust amyloid deposition in APP23 transgenic mice. *Neurobiol Aging* 2005; **26**: 607-613 [PMID: [15708435](#) DOI: [10.1016/j.neurobiolaging.2004.06.010](#)]
- 29 **Spires TL**, Meyer-Luehmann M, Stern EA, McLean PJ, Skoch J, Nguyen PT, Bacskai BJ, Hyman BT. Dendritic spine abnormalities in amyloid precursor protein transgenic mice demonstrated by gene transfer and intravital multiphoton microscopy. *J Neurosci* 2005; **25**: 7278-7287 [PMID: [16079410](#) DOI: [10.1523/JNEUROSCI.1879-05.2005](#)]
- 30 **Counts SE**, Alldred MJ, Che S, Ginsberg SD, Mufson EJ. Synaptic gene dysregulation within hippocampal CA1 pyramidal neurons in mild cognitive impairment. *Neuropharmacology* 2014; **79**: 172-179 [PMID: [24445080](#) DOI: [10.1016/j.neuropharm.2013.10.018](#)]
- 31 **Cao J**, Hou J, Ping J, Cai D. Advances in developing novel therapeutic strategies for Alzheimer's disease. *Mol Neurodegener* 2018; **13**: 64 [PMID: [30541602](#) DOI: [10.1186/s13024-018-0299-8](#)]
- 32 **Pinheiro L**, Faustino C. Therapeutic Strategies Targeting Amyloid- $\beta$  in Alzheimer's Disease. *Curr Alzheimer Res* 2019; **16**: 418-452 [PMID: [30907320](#) DOI: [10.2174/1567205016666190321163438](#)]
- 33 **Schneider LS**, Mangialasche F, Andreasen N, Feldman H, Giacobini E, Jones R, Mantua V, Mecocci P, Pani L, Winblad B, Kivipelto M. Clinical trials and late-stage drug development for Alzheimer's disease: an appraisal from 1984 to 2014. *J Intern Med* 2014; **275**: 251-283 [PMID: [24605808](#) DOI: [10.1111/joim.12191](#)]
- 34 **Kumar A**, Tiwari A, Sharma A. Changing Paradigm from one Target one Ligand Towards Multi-target Directed Ligand Design for Key Drug Targets of Alzheimer Disease: An Important Role of In Silico Methods in Multi-target Directed Ligands Design. *Curr Neuropharmacol* 2018; **16**: 726-739 [PMID: [29542413](#) DOI: [10.2174/1570159X16666180315141643](#)]
- 35 **Ibrahim MM**, Gabr MT. Multitarget therapeutic strategies for Alzheimer's disease. *Neural Regen Res* 2019; **14**: 437-440 [PMID: [30539809](#) DOI: [10.4103/1673-5374.245463](#)]
- 36 **Traynor K**. Effective drug therapy for Alzheimer's disease remains elusive. *Am J Health Syst Pharm* 2015; **72**: 516, 518 [PMID: [25788503](#) DOI: [10.2146/news150026](#)]
- 37 **Morris GP**, Clark IA, Vissel B. Inconsistencies and controversies surrounding the amyloid hypothesis of Alzheimer's disease. *Acta Neuropathol Commun* 2014; **2**: 135 [PMID: [25231068](#) DOI: [10.1186/s40478-014-0135-5](#)]
- 38 **Kim HJ**, Seo SW, Chang JW, Lee JI, Kim CH, Chin J, Choi SJ, Kwon H, Yun HJ, Lee JM, Kim ST, Choe YS, Lee KH, Na DL. Stereotactic brain injection of human umbilical cord blood mesenchymal stem cells in patients with Alzheimer's disease dementia: A phase 1 clinical trial. *Alzheimers Dement (N Y)* 2015; **1**: 95-102 [PMID: [29854930](#) DOI: [10.1016/j.trci.2015.06.007](#)]
- 39 **Cummings JL**, Morstorf T, Zhong K. Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimers Res Ther* 2014; **6**: 37 [PMID: [25024750](#) DOI: [10.1186/alzrt269](#)]
- 40 **Martello G**, Smith A. The nature of embryonic stem cells. *Annu Rev Cell Dev Biol* 2014; **30**: 647-675 [PMID: [25288119](#) DOI: [10.1146/annurev-cellbio-100913-013116](#)]
- 41 **Liu Y**, Weick JP, Liu H, Krencik R, Zhang X, Ma L, Zhou GM, Ayala M, Zhang SC. Medial ganglionic eminence-like cells derived from human embryonic stem cells correct learning and memory deficits. *Nat*

- Biotechnol* 2013; **31**: 440-447 [PMID: [23604284](#) DOI: [10.1038/nbt.2565](#)]
- 42 **Jin X**, Lin T, Xu Y. Stem Cell Therapy and Immunological Rejection in Animal Models. *Curr Mol Pharmacol* 2016; **9**: 284-288 [PMID: [26415913](#) DOI: [10.2174/1874467208666150928153511](#)]
  - 43 **Fujikawa T**, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol* 2005; **166**: 1781-1791 [PMID: [15920163](#) DOI: [10.1016/s0002-9440\(10\)62488-1](#)]
  - 44 **Richards M**, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol* 2002; **20**: 933-936 [PMID: [12161760](#) DOI: [10.1038/nbt726](#)]
  - 45 **Moghadam FH**, Alaie H, Karbalaie K, Tanhaei S, Nasr Esfahani MH, Baharvand H. Transplantation of primed or unprimed mouse embryonic stem cell-derived neural precursor cells improves cognitive function in Alzheimerian rats. *Differentiation* 2009; **78**: 59-68 [PMID: [19616885](#) DOI: [10.1016/j.diff.2009.06.005](#)]
  - 46 **Bissonnette CJ**, Lyass L, Bhattacharyya BJ, Belmadani A, Miller RJ, Kessler JA. The controlled generation of functional basal forebrain cholinergic neurons from human embryonic stem cells. *Stem Cells* 2011; **29**: 802-811 [PMID: [21381151](#) DOI: [10.1002/stem.626](#)]
  - 47 **Yue W**, Li Y, Zhang T, Jiang M, Qian Y, Zhang M, Sheng N, Feng S, Tang K, Yu X, Shu Y, Yue C, Jing N. ESC-Derived Basal Forebrain Cholinergic Neurons Ameliorate the Cognitive Symptoms Associated with Alzheimer's Disease in Mouse Models. *Stem Cell Reports* 2015; **5**: 776-790 [PMID: [26489896](#) DOI: [10.1016/j.stemcr.2015.09.010](#)]
  - 48 **Ding DC**, Chang YH, Shyu WC, Lin SZ. Human umbilical cord mesenchymal stem cells: a new era for stem cell therapy. *Cell Transplant* 2015; **24**: 339-347 [PMID: [25622293](#) DOI: [10.3727/096368915X686841](#)]
  - 49 **Lee HJ**, Lee JK, Lee H, Carter JE, Chang JW, Oh W, Yang YS, Suh JG, Lee BH, Jin HK, Bae JS. Human umbilical cord blood-derived mesenchymal stem cells improve neuropathology and cognitive impairment in an Alzheimer's disease mouse model through modulation of neuroinflammation. *Neurobiol Aging* 2012; **33**: 588-602 [PMID: [20471717](#) DOI: [10.1016/j.neurobiolaging.2010.03.024](#)]
  - 50 **Alvarez-Erviti L**, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011; **29**: 341-345 [PMID: [21423189](#) DOI: [10.1038/nbt.1807](#)]
  - 51 **Katsuda T**, Tsuchiya R, Kosaka N, Yoshioka Y, Takagaki K, Oki K, Takeshita F, Sakai Y, Kuroda M, Ochiya T. Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci Rep* 2013; **3**: 1197 [PMID: [23378928](#) DOI: [10.1038/srep01197](#)]
  - 52 **Garcia KO**, Ornellas FL, Martin PK, Patti CL, Mello LE, Frussa-Filho R, Han SW, Longo BM. Therapeutic effects of the transplantation of VEGF overexpressing bone marrow mesenchymal stem cells in the hippocampus of murine model of Alzheimer's disease. *Front Aging Neurosci* 2014; **6**: 30 [PMID: [24639647](#) DOI: [10.3389/fnagi.2014.00030](#)]
  - 53 **Yang H**, Yang H, Xie Z, Wei L, Bi J. Systemic transplantation of human umbilical cord derived mesenchymal stem cells-educated T regulatory cells improved the impaired cognition in A $\beta$ PPswe/PS1dE9 transgenic mice. *PLoS One* 2013; **8**: e69129 [PMID: [23935936](#) DOI: [10.1371/journal.pone.0069129](#)]
  - 54 **Lee HJ**, Lee JK, Lee H, Shin JW, Carter JE, Sakamoto T, Jin HK, Bae JS. The therapeutic potential of human umbilical cord blood-derived mesenchymal stem cells in Alzheimer's disease. *Neurosci Lett* 2010; **481**: 30-35 [PMID: [20600610](#) DOI: [10.1016/j.neulet.2010.06.045](#)]
  - 55 **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: [16904174](#) DOI: [10.1016/j.cell.2006.07.024](#)]
  - 56 **Cooper O**, Hargus G, Deleidi M, Blak A, Osborn T, Marlow E, Lee K, Levy A, Perez-Torres E, Yow A, Isacson O. Differentiation of human ES and Parkinson's disease iPSCs into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. *Mol Cell Neurosci* 2010; **45**: 258-266 [PMID: [20603216](#) DOI: [10.1016/j.mcn.2010.06.017](#)]
  - 57 **Nori S**, Okada Y, Yasuda A, Tsuji O, Takahashi Y, Kobayashi Y, Fujiyoshi K, Koike M, Uchiyama Y, Ikeda E, Toyama Y, Yamanaka S, Nakamura M, Okano H. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. *Proc Natl Acad Sci USA* 2011; **108**: 16825-16830 [PMID: [21949375](#) DOI: [10.1073/pnas.1108077108](#)]
  - 58 **Kim TG**, Yao R, Monnell T, Cho JH, Vasudevan A, Koh A, Peeyush KT, Moon M, Datta D, Bolshakov VY, Kim KS, Chung S. Efficient specification of interneurons from human pluripotent stem cells by dorsoventral and rostrocaudal modulation. *Stem Cells* 2014; **32**: 1789-1804 [PMID: [24648391](#) DOI: [10.1002/stem.1704](#)]
  - 59 **Paul D**, Sevilla A, Zhou H, Hahn AK, Kim H, Napolitano C, Tsankov A, Shang L, Krumholz K, Jagadeesan P, Woodard CM, Sun B, Vilboux T, Zimmer M, Forero E, Moroziewicz DN, Martinez H, Malicdan MC, Weiss KA, Vensand LB, Dusenberry CR, Polus H, Sy KT, Kahler DJ, Gahl WA, Solomon SL, Chang S, Meissner A, Eggan K, Noggle SA. Automated, high-throughput derivation, characterization and differentiation of induced pluripotent stem cells. *Nat Methods* 2015; **12**: 885-892 [PMID: [26237226](#) DOI: [10.1038/nmeth.3507](#)]
  - 60 **Maroof AM**, Keros S, Tyson JA, Ying SW, Ganat YM, Merkle FT, Liu B, Goulburn A, Stanley EG, Elefanty AG, Widmer HR, Eggan K, Goldstein PA, Anderson SA, Studer L. Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. *Cell Stem Cell* 2013; **12**: 559-572 [PMID: [23642365](#) DOI: [10.1016/j.stem.2013.04.008](#)]
  - 61 **Nicholas CR**, Chen J, Tang Y, Southwell DG, Chalmers N, Vogt D, Arnold CM, Chen YJ, Stanley EG, Elefanty AG, Sasai Y, Alvarez-Buylla A, Rubenstein JL, Kriegstein AR. Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development. *Cell Stem Cell* 2013; **12**: 573-586 [PMID: [23642366](#) DOI: [10.1016/j.stem.2013.04.005](#)]
  - 62 **Holtman IR**, Raj DD, Miller JA, Schaafsma W, Yin Z, Brouwer N, Wes PD, Möller T, Orre M, Kamphuis W, Hol EM, Boddeke EW, Eggen BJ. Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: a co-expression meta-analysis. *Acta Neuropathol Commun* 2015; **3**: 31 [PMID: [26001565](#) DOI: [10.1186/s40478-015-0203-5](#)]

- 63 **Takamatsu K**, Ikeda T, Haruta M, Matsumura K, Ogi Y, Nakagata N, Uchino M, Ando Y, Nishimura Y, Senju S. Degradation of amyloid beta by human induced pluripotent stem cell-derived macrophages expressing Neprilysin-2. *Stem Cell Res* 2014; **13**: 442-453 [PMID: [25460605](#) DOI: [10.1016/j.scr.2014.10.001](#)]
- 64 **Israel MA**, Yuan SH, Bardy C, Reyna SM, Mu Y, Herrera C, Hefferan MP, Van Gorp S, Nazor KL, Boscolo FS, Carson CT, Laurent LC, Marsala M, Gage FH, Remes AM, Koo EH, Goldstein LS. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. *Nature* 2012; **482**: 216-220 [PMID: [22278060](#) DOI: [10.1038/nature10821](#)]
- 65 **Raja WK**, Mungenast AE, Lin YT, Ko T, Abdurrob F, Seo J, Tsai LH. Self-Organizing 3D Human Neural Tissue Derived from Induced Pluripotent Stem Cells Recapitulate Alzheimer's Disease Phenotypes. *PLoS One* 2016; **11**: e0161969 [PMID: [27622770](#) DOI: [10.1371/journal.pone.0161969](#)]
- 66 **Yagi T**, Ito D, Okada Y, Akamatsu W, Nihei Y, Yoshizaki T, Yamanaka S, Okano H, Suzuki N. Modeling familial Alzheimer's disease with induced pluripotent stem cells. *Hum Mol Genet* 2011; **20**: 4530-4539 [PMID: [21900357](#) DOI: [10.1093/hmg/ddr394](#)]
- 67 **Mahairaki V**, Ryu J, Peters A, Chang Q, Li T, Park TS, Burrridge PW, Talbot CC Jr, Asnaghi L, Martin LJ, Zambidis ET, Koliatsos VE. Induced pluripotent stem cells from familial Alzheimer's disease patients differentiate into mature neurons with amyloidogenic properties. *Stem Cells Dev* 2014; **23**: 2996-3010 [PMID: [25027006](#) DOI: [10.1089/scd.2013.0511](#)]
- 68 **Sproul AA**, Jacob S, Pre D, Kim SH, Nestor MW, Navarro-Sobrinho M, Santa-Maria I, Zimmer M, Aubry S, Steele JW, Kahler DJ, Dranovsky A, Arancio O, Cray JF, Gandy S, Nogge SA. Characterization and molecular profiling of PSEN1 familial Alzheimer's disease iPSC-derived neural progenitors. *PLoS One* 2014; **9**: e84547 [PMID: [24416243](#) DOI: [10.1371/journal.pone.0084547](#)]
- 69 **Woodruff G**, Young JE, Martinez FJ, Buen F, Gore A, Kinaga J, Li Z, Yuan SH, Zhang K, Goldstein LS. The presenilin-1  $\Delta$ E9 mutation results in reduced  $\gamma$ -secretase activity, but not total loss of PS1 function, in isogenic human stem cells. *Cell Rep* 2013; **5**: 974-985 [PMID: [24239350](#) DOI: [10.1016/j.celrep.2013.10.018](#)]
- 70 **Moore S**, Evans LD, Andersson T, Portelius E, Smith J, Dias TB, Saurat N, McGlade A, Kirwan P, Blennow K, Hardy J, Zetterberg H, Livesey FJ. APP metabolism regulates tau proteostasis in human cerebral cortex neurons. *Cell Rep* 2015; **11**: 689-696 [PMID: [25921538](#) DOI: [10.1016/j.celrep.2015.03.068](#)]
- 71 **Ochalek A**, Mihalik B, Avci HX, Chandrasekaran A, Téglási A, Bock I, Giudice ML, Tancos Z, Molnár K, László L, Nielsen JE, Holst B, Freude K, Hyttel P, Kobolák J, Dinnyés A. Neurons derived from sporadic Alzheimer's disease iPSCs reveal elevated TAU hyperphosphorylation, increased amyloid levels, and GSK3B activation. *Alzheimers Res Ther* 2017; **9**: 90 [PMID: [29191219](#) DOI: [10.1186/s13195-017-0317-z](#)]
- 72 **Sun L**, Zhou R, Yang G, Shi Y. Analysis of 138 pathogenic mutations in presenilin-1 on the in vitro production of A $\beta$ 42 and A $\beta$ 40 peptides by  $\gamma$ -secretase. *Proc Natl Acad Sci USA* 2017; **114**: E476-E485 [PMID: [27930341](#) DOI: [10.1073/pnas.1618657114](#)]
- 73 **Muratore CR**, Rice HC, Srikanth P, Callahan DG, Shin T, Benjamin LN, Walsh DM, Selkoe DJ, Young-Pearse TL. The familial Alzheimer's disease APPV717I mutation alters APP processing and Tau expression in iPSC-derived neurons. *Hum Mol Genet* 2014; **23**: 3523-3536 [PMID: [24524897](#) DOI: [10.1093/hmg/ddu064](#)]
- 74 **Arber C**, Toombs J, Lovejoy C, Ryan NS, Paterson RW, Willumsen N, Gkanatsiou E, Portelius E, Blennow K, Heslegrave A, Schott JM, Hardy J, Lashley T, Fox NC, Zetterberg H, Wray S. Familial Alzheimer's disease patient-derived neurons reveal distinct mutation-specific effects on amyloid beta. *Mol Psychiatry* 2019 [PMID: [30980041](#) DOI: [10.1038/s41380-019-0410-8](#)]
- 75 **Araki R**, Uda M, Hoki Y, Sunayama M, Nakamura M, Ando S, Sugiura M, Ideno H, Shimada A, Nifuji A, Abe M. Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. *Nature* 2013; **494**: 100-104 [PMID: [23302801](#) DOI: [10.1038/nature11807](#)]
- 76 **Hibaoui Y**, Feki A. Human pluripotent stem cells: applications and challenges in neurological diseases. *Front Physiol* 2012; **3**: 267 [PMID: [22934023](#) DOI: [10.3389/fphys.2012.00267](#)]
- 77 **Lister R**, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, Antosiewicz-Bourget J, O'Malley R, Castanon R, Klugman S, Downes M, Yu R, Stewart R, Ren B, Thomson JA, Evans RM, Ecker JR. Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* 2011; **471**: 68-73 [PMID: [21289626](#) DOI: [10.1038/nature09798](#)]
- 78 **Tolosa L**, Pareja E, Gómez-Lechón MJ. Clinical Application of Pluripotent Stem Cells: An Alternative Cell-Based Therapy for Treating Liver Diseases? *Transplantation* 2016; **100**: 2548-2557 [PMID: [27495745](#) DOI: [10.1097/tp.0000000000001426](#)]
- 79 **Shimada IS**, LeCompte MD, Granger JC, Quinlan NJ, Spees JL. Self-renewal and differentiation of reactive astrocyte-derived neural stem/progenitor cells isolated from the cortical peri-infarct area after stroke. *J Neurosci* 2012; **32**: 7926-7940 [PMID: [22674268](#) DOI: [10.1523/NEUROSCI.4303-11.2012](#)]
- 80 **Blurton-Jones M**, Kitazawa M, Martinez-Coria H, Castello NA, Müller FJ, Loring JF, Yamasaki TR, Poon WW, Green KN, LaFerla FM. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc Natl Acad Sci USA* 2009; **106**: 13594-13599 [PMID: [19633196](#) DOI: [10.1073/pnas.0901402106](#)]
- 81 **Kordower JH**, Winn SR, Liu YT, Mufson EJ, Sladek JR Jr, Hammang JP, Baetge EE, Emerich DF. The aged monkey basal forebrain: rescue and sprouting of axotomized basal forebrain neurons after grafts of encapsulated cells secreting human nerve growth factor. *Proc Natl Acad Sci USA* 1994; **91**: 10898-10902 [PMID: [7971980](#) DOI: [10.1073/pnas.91.23.10898](#)]
- 82 **Park D**, Yang YH, Bae DK, Lee SH, Yang G, Kyung J, Kim D, Choi EK, Lee SW, Kim GH, Hong JT, Choi KC, Lee HJ, Kim SU, Kim YB. Improvement of cognitive function and physical activity of aging mice by human neural stem cells over-expressing choline acetyltransferase. *Neurobiol Aging* 2013; **34**: 2639-2646 [PMID: [23731954](#) DOI: [10.1016/j.neurobiolaging.2013.04.026](#)]
- 83 **Zhang Q**, Wu HH, Wang Y, Gu GJ, Zhang W, Xia R. Neural stem cell transplantation decreases neuroinflammation in a transgenic mouse model of Alzheimer's disease. *J Neurochem* 2016; **136**: 815-825 [PMID: [26525612](#) DOI: [10.1111/jnc.13413](#)]



- 84 **Lee IS**, Jung K, Kim IS, Lee H, Kim M, Yun S, Hwang K, Shin JE, Park KI. Human neural stem cells alleviate Alzheimer-like pathology in a mouse model. *Mol Neurodegener* 2015; **10**: 38 [PMID: [26293123](#) DOI: [10.1186/s13024-015-0035-6](#)]
- 85 **Lilja AM**, Malmsten L, Röjdner J, Voytenko L, Verkhatsky A, Ögren SO, Nordberg A, Marutle A. Neural Stem Cell Transplant-Induced Effect on Neurogenesis and Cognition in Alzheimer Tg2576 Mice Is Inhibited by Concomitant Treatment with Amyloid-Lowering or Cholinergic  $\alpha 7$  Nicotinic Receptor Drugs. *Neural Plast* 2015; **2015**: 370432 [PMID: [26257960](#) DOI: [10.1155/2015/370432](#)]
- 86 **Ager RR**, Davis JL, Agazaryan A, Benavente F, Poon WW, LaFerla FM, Blurton-Jones M. Human neural stem cells improve cognition and promote synaptic growth in two complementary transgenic models of Alzheimer's disease and neuronal loss. *Hippocampus* 2015; **25**: 813-826 [PMID: [25530343](#) DOI: [10.1002/hipo.22405](#)]
- 87 **Xuan AG**, Luo M, Ji WD, Long DH. Effects of engrafted neural stem cells in Alzheimer's disease rats. *Neurosci Lett* 2009; **450**: 167-171 [PMID: [19070649](#) DOI: [10.1016/j.neulet.2008.12.001](#)]
- 88 **Li X**, Zhu H, Sun X, Zuo F, Lei J, Wang Z, Bao X, Wang R. Human Neural Stem Cell Transplantation Rescues Cognitive Defects in APP/PS1 Model of Alzheimer's Disease by Enhancing Neuronal Connectivity and Metabolic Activity. *Front Aging Neurosci* 2016; **8**: 282 [PMID: [27932977](#) DOI: [10.3389/fnagi.2016.00282](#)]
- 89 **Xuan AG**, Long DH, Gu HG, Yang DD, Hong LP, Leng SL. BDNF improves the effects of neural stem cells on the rat model of Alzheimer's disease with unilateral lesion of fimbria-fornix. *Neurosci Lett* 2008; **440**: 331-335 [PMID: [18579298](#) DOI: [10.1016/j.neulet.2008.05.107](#)]
- 90 **Park D**, Yang G, Bae DK, Lee SH, Yang YH, Kyung J, Kim D, Choi EK, Choi KC, Kim SU, Kang SK, Ra JC, Kim YB. Human adipose tissue-derived mesenchymal stem cells improve cognitive function and physical activity in ageing mice. *J Neurosci Res* 2013; **91**: 660-670 [PMID: [23404260](#) DOI: [10.1002/jnr.23182](#)]
- 91 **Lee HJ**, Lim IJ, Park SW, Kim YB, Ko Y, Kim SU. Human neural stem cells genetically modified to express human nerve growth factor (NGF) gene restore cognition in the mouse with ibotenic acid-induced cognitive dysfunction. *Cell Transplant* 2012; **21**: 2487-2496 [PMID: [22526467](#) DOI: [10.3727/096368912X638964](#)]
- 92 **Anand A**, Banik A, Thakur K, Masters CL. The animal models of dementia and Alzheimer's disease for pre-clinical testing and clinical translation. *Curr Alzheimer Res* 2012; **9**: 1010-1029 [PMID: [22698073](#) DOI: [10.2174/156720512803569055](#)]
- 93 **Banik A**, Anand A. Preclinical non-human models to combat dementia. *Ann Neurosci* 2013; **20**: 24-29 [PMID: [25206006](#) DOI: [10.5214/ans.0972.7531.200109](#)]
- 94 **Martinez-Serrano A**, Hantzopoulos PA, Björklund A. Ex vivo gene transfer of brain-derived neurotrophic factor to the intact rat forebrain: neurotrophic effects on cholinergic neurons. *Eur J Neurosci* 1996; **8**: 727-735 [PMID: [9081624](#) DOI: [10.1111/j.1460-9568.1996.tb01258.x](#)]
- 95 **Sinden JD**, Stroemer P, Grigoryan G, Patel S, French SJ, Hodges H. Functional repair with neural stem cells. *Novartis Found Symp* 2000; **231**: 270-83; discussion 283-8, 302-306 [PMID: [11131543](#) DOI: [10.1002/0470870834.ch16](#)]
- 96 **Qu T**, Brannen CL, Kim HM, Sugaya K. Human neural stem cells improve cognitive function of aged brain. *Neuroreport* 2001; **12**: 1127-1132 [PMID: [11338178](#) DOI: [10.1097/00001756-200105080-00016](#)]
- 97 **Wu P**, Tarasenko YI, Gu Y, Huang LY, Coggeshall RE, Yu Y. Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. *Nat Neurosci* 2002; **5**: 1271-1278 [PMID: [12426573](#) DOI: [10.1038/nn974](#)]
- 98 **Wang Q**, Matsumoto Y, Shindo T, Miyake K, Shindo A, Kawanishi M, Kawai N, Tamiya T, Nagao S. Neural stem cells transplantation in cortex in a mouse model of Alzheimer's disease. *J Med Invest* 2006; **53**: 61-69 [PMID: [16537997](#) DOI: [10.2152/jmi.53.61](#)]
- 99 **Prakash A**, Kumar A. Role of nuclear receptor on regulation of BDNF and neuroinflammation in hippocampus of  $\beta$ -amyloid animal model of Alzheimer's disease. *Neurotox Res* 2014; **25**: 335-347 [PMID: [24277156](#) DOI: [10.1007/s12640-013-9437-9](#)]
- 100 **Seaberg RM**, van der Kooy D. Stem and progenitor cells: the premature desertion of rigorous definitions. *Trends Neurosci* 2003; **26**: 125-131 [PMID: [12591214](#) DOI: [10.1016/s0166-2236\(03\)00031-6](#)]
- 101 **Tang J**, Xu H, Fan X, Li D, Rancourt D, Zhou G, Li Z, Yang L. Embryonic stem cell-derived neural precursor cells improve memory dysfunction in A $\beta$ (1-40) injured rats. *Neurosci Res* 2008; **62**: 86-96 [PMID: [18634835](#) DOI: [10.1016/j.neures.2008.06.005](#)]
- 102 **Blurton-Jones M**, Spencer B, Michael S, Castello NA, Agazaryan AA, Davis JL, Müller FJ, Loring JF, Masliah E, LaFerla FM. Neural stem cells genetically-modified to express neprilysin reduce pathology in Alzheimer transgenic models. *Stem Cell Res Ther* 2014; **5**: 46 [PMID: [25022790](#) DOI: [10.1186/scrt440](#)]
- 103 **Ernfors P**, Wetmore C, Olson L, Persson H. Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* 1990; **5**: 511-526 [PMID: [2206535](#) DOI: [10.1016/0896-6273\(90\)90090-3](#)]
- 104 **Elia CA**, Losurdo M, Malosio ML, Coco S. Extracellular Vesicles from Mesenchymal Stem Cells Exert Pleiotropic Effects on Amyloid- $\beta$ , Inflammation, and Regeneration: A Spark of Hope for Alzheimer's Disease from Tiny Structures? *Bioessays* 2019; **41**: e1800199 [PMID: [30919493](#) DOI: [10.1002/bies.201800199](#)]
- 105 **Naaldijk Y**, Jäger C, Fabian C, Leovsky C, Blüher A, Rudolph L, Hinze A, Stolzing A. Effect of systemic transplantation of bone marrow-derived mesenchymal stem cells on neuropathology markers in APP/PS1 Alzheimer mice. *Neuropathol Appl Neurobiol* 2017; **43**: 299-314 [PMID: [26918424](#) DOI: [10.1111/nan.12319](#)]
- 106 **Yun HM**, Kim HS, Park KR, Shin JM, Kang AR, il Lee K, Song S, Kim YB, Han SB, Chung HM, Hong JT. Placenta-derived mesenchymal stem cells improve memory dysfunction in an A $\beta$ 1-42-infused mouse model of Alzheimer's disease. *Cell Death Dis* 2013; **4**: e958 [PMID: [24336078](#) DOI: [10.1038/cddis.2013.490](#)]
- 107 **Oh SH**, Kim HN, Park HJ, Shin JY, Lee PH. Mesenchymal Stem Cells Increase Hippocampal Neurogenesis and Neuronal Differentiation by Enhancing the Wnt Signaling Pathway in an Alzheimer's

- Disease Model. *Cell Transplant* 2015; **24**: 1097-1109 [PMID: [24612635](#) DOI: [10.3727/096368914X679237](#)]
- 108 **Galipeau J**, Sensébé L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell* 2018; **22**: 824-833 [PMID: [29859173](#) DOI: [10.1016/j.stem.2018.05.004](#)]
- 109 **Rikhtegar R**, Yousefi M, Dolati S, Kasmaei HD, Charsouei S, Nouri M, Shakouri SK. Stem cell-based cell therapy for neuroprotection in stroke: A review. *J Cell Biochem* 2019; **120**: 8849-8862 [PMID: [30506720](#) DOI: [10.1002/jcb.28207](#)]
- 110 **Schwartz SD**, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R. Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet* 2012; **379**: 713-720 [PMID: [22281388](#) DOI: [10.1016/S0140-6736\(12\)60028-2](#)]
- 111 **Fujioka K**, Hanada S, Inoue Y, Sato K, Hirakuri K, Shiraishi K, Kanaya F, Ikeda K, Usui R, Yamamoto K, Kim SU, Manome Y. Effects of silica and titanium oxide particles on a human neural stem cell line: morphology, mitochondrial activity, and gene expression of differentiation markers. *Int J Mol Sci* 2014; **15**: 11742-11759 [PMID: [24992594](#) DOI: [10.3390/ijms150711742](#)]
- 112 **Eckert A**, Huang L, Gonzalez R, Kim HS, Hamblin MH, Lee JP. Bystander Effect Fuels Human Induced Pluripotent Stem Cell-Derived Neural Stem Cells to Quickly Attenuate Early Stage Neurological Deficits After Stroke. *Stem Cells Transl Med* 2015; **4**: 841-851 [PMID: [26025980](#) DOI: [10.5966/sctm.2014-0184](#)]
- 113 **Cha MY**, Kwon YW, Ahn HS, Jeong H, Lee YY, Moon M, Baik SH, Kim DK, Song H, Yi EC, Hwang D, Kim HS, Mook-Jung I. Protein-Induced Pluripotent Stem Cells Ameliorate Cognitive Dysfunction and Reduce A $\beta$  Deposition in a Mouse Model of Alzheimer's Disease. *Stem Cells Transl Med* 2017; **6**: 293-305 [PMID: [28170178](#) DOI: [10.5966/sctm.2016-0081](#)]
- 114 **Balez R**, Steiner N, Engel M, Muñoz SS, Lum JS, Wu Y, Wang D, Vallotton P, Sachdev P, O'Connor M, Sidhu K, Münch G, Ooi L. Neuroprotective effects of apigenin against inflammation, neuronal excitability and apoptosis in an induced pluripotent stem cell model of Alzheimer's disease. *Sci Rep* 2016; **6**: 31450 [PMID: [27514990](#) DOI: [10.1038/srep31450](#)]
- 115 **Brookhouser N**, Raman S, Potts C, Brafman DA. May I Cut in? Gene Editing Approaches in Human Induced Pluripotent Stem Cells. *Cells* 2017; **6** [PMID: [28178187](#) DOI: [10.3390/cells6010005](#)]
- 116 **Kim KS**, Kim HS, Park JM, Kim HW, Park MK, Lee HS, Lim DS, Lee TH, Chopp M, Moon J. Long-term immunomodulatory effect of amniotic stem cells in an Alzheimer's disease model. *Neurobiol Aging* 2013; **34**: 2408-2420 [PMID: [23623603](#) DOI: [10.1016/j.neurobiolaging.2013.03.029](#)]
- 117 **Yang H**, Xie Z, Wei L, Yang H, Yang S, Zhu Z, Wang P, Zhao C, Bi J. Human umbilical cord mesenchymal stem cell-derived neuron-like cells rescue memory deficits and reduce amyloid-beta deposition in an A $\beta$ PP/PS1 transgenic mouse model. *Stem Cell Res Ther* 2013; **4**: 76 [PMID: [23826983](#) DOI: [10.1186/scrt227](#)]
- 118 **Brazzini A**, Cantella R, De la Cruz A, Yupanqui J, León C, Jorquiera T, Brazzini M, Ortega M, Saenz LN. Intraarterial autologous implantation of adult stem cells for patients with Parkinson disease. *J Vasc Interv Radiol* 2010; **21**: 443-451 [PMID: [20346882](#) DOI: [10.1016/j.jvir.2010.01.008](#)]
- 119 **Xiao J**, Yang R, Biswas S, Qin X, Zhang M, Deng W. Mesenchymal stem cells and induced pluripotent stem cells as therapies for multiple sclerosis. *Int J Mol Sci* 2015; **16**: 9283-9302 [PMID: [25918935](#) DOI: [10.3390/ijms16059283](#)]
- 120 **Vos SJ**, Verhey F, Frölich L, Kornhuber J, Wiltfang J, Maier W, Peters O, Rütger E, Nobili F, Morbelli S, Frisoni GB, Drzezga A, Didic M, van Berckel BN, Simmons A, Soininen H, Kloszewska I, Mecocci P, Tsolaki M, Vellas B, Lovestone S, Muscio C, Herukka SK, Salmon E, Bastin C, Wallin A, Nordlund A, de Mendonça A, Silva D, Santana I, Lemos R, Engelborghs S, Van der Mussele S; Alzheimer's Disease Neuroimaging Initiative, Freund-Levi Y, Wallin ÅK, Hampel H, van der Flier W, Scheltens P, Visser PJ. Prevalence and prognosis of Alzheimer's disease at the mild cognitive impairment stage. *Brain* 2015; **138**: 1327-1338 [PMID: [25693589](#) DOI: [10.1093/brain/awv029](#)]
- 121 **Villemagne VL**, Burnham S, Bourgeat P, Brown B, Ellis KA, Salvado O, Szeoke C, Macaulay SL, Martins R, Maruff P, Ames D, Rowe CC, Masters CL; Australian Imaging Biomarkers and Lifestyle (AIBL) Research Group. Amyloid  $\beta$  deposition, neurodegeneration, and cognitive decline in sporadic Alzheimer's disease: a prospective cohort study. *Lancet Neurol* 2013; **12**: 357-367 [PMID: [23477989](#) DOI: [10.1016/S1474-4422\(13\)70044-9](#)]
- 122 **Jessen NA**, Munk AS, Lundgaard I, Nedergaard M. The Glymphatic System: A Beginner's Guide. *Neurochem Res* 2015; **40**: 2583-2599 [PMID: [25947369](#) DOI: [10.1007/s11064-015-1581-6](#)]
- 123 **Peng W**, Achariyar TM, Li B, Liao Y, Mestre H, Hitomi E, Regan S, Kasper T, Peng S, Ding F, Benveniste H, Nedergaard M, Deane R. Suppression of glymphatic fluid transport in a mouse model of Alzheimer's disease. *Neurobiol Dis* 2016; **93**: 215-225 [PMID: [27234656](#) DOI: [10.1016/j.nbd.2016.05.015](#)]
- 124 **Thirabanasak D**, Tantiwongse K, Thorner PS. Angiomyeloproliferative lesions following autologous stem cell therapy. *J Am Soc Nephrol* 2010; **21**: 1218-1222 [PMID: [20558536](#) DOI: [10.1681/ASN.2009111156](#)]
- 125 **Amariglio N**, Hirshberg A, Scheithauer BW, Cohen Y, Loewenthal R, Trakhtenbrot L, Paz N, Koren-Michowitz M, Waldman D, Leider-Trejo L, Toren A, Constantini S, Rechavi G. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. *PLoS Med* 2009; **6**: e1000029 [PMID: [19226183](#) DOI: [10.1371/journal.pmed.1000029](#)]
- 126 **Fazel SS**, Angoulvant D, Butany J, Weisel RD, Li RK. Mesenchymal stem cells engineered to overexpress stem cell factor improve cardiac function but have malignant potential. *J Thorac Cardiovasc Surg* 2008; **136**: 1388-1389 [PMID: [19026843](#) DOI: [10.1016/j.jtcvs.2007.11.068](#)]

## Exosomes derived from stem cells as an emerging therapeutic strategy for intervertebral disc degeneration

Zhi-Lei Hu, Hai-Yin Li, Xian Chang, Yue-Yang Li, Chen-Hao Liu, Xiao-Xin Gao, Yu Zhai, Yu-Xuan Chen, Chang-Qing Li

**ORCID number:** Zhi-Lei Hu 0000-0002-8621-6050; Hai-Yin Li 0000-0002-4254-7974; Xian Chang 0000-0002-0983-8550; Yue-Yang Li 0000-0002-9964-226X; Chen-Hao Liu 0000-0003-2769-9571; Xiao-Xin Gao 0000-0003-1671-7025; Yu Zhai 0000-0003-1601-3458; Yu-Xuan Chen 0000-0002-7920-8191; Chang-Qing Li 0000-0002-9423-9105.

**Author contributions:** Hu ZL wrote the paper; Li HY, Chang X, Li YY, Liu CH, Gao XX, Zhai Y, Chen YX, and Li CQ collected the data.

**Supported by** the National Natural Science Foundation of China, No. 81572208.

**Conflict-of-interest statement:** Authors declare no conflict of interests for this article.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Zhi-Lei Hu, Hai-Yin Li, Xian Chang, Yue-Yang Li, Chen-Hao Liu, Xiao-Xin Gao, Yu Zhai, Chang-Qing Li, Department of Orthopedics, Xinqiao Hospital, Army Military Medical University, Chongqing 400037, China

Yu-Xuan Chen, Center of Traumatic Orthopedics, People's Liberation Army 990 Hospital, Xinyang 46400, Henan Province, China

**Corresponding author:** Chang-Qing Li, MD, PhD, Chief Doctor, Director, Professor, Department of Orthopedics, Xinqiao Hospital, Army Military Medical University, Xinqiao Main Street, Shapingba District, Chongqing 400037, China. [changqli@163.com](mailto:changqli@163.com)

### Abstract

Intervertebral disc (IVD) degenerative diseases are a common problem in the world, and they cause substantial social and economic burdens for people. The current methods for treating IVD degenerative diseases mainly include surgery and conservative treatment, which cannot fundamentally restore the normal structure of the disc. With continuous research on the mechanism of degeneration and the development of regenerative medicine, rapid progress has been made in the field of regenerative medicine regarding the use of stem cell-derived exosomes, which are active biological substances used in intercellular communication, because they show a strong effect in promoting tissue regeneration. The study of exosomes in the field of IVD degeneration has just begun, and many surprising achievements have been made. This paper mainly reviews the biological characteristics of exosomes and highlights the current status of exosomes in the field of IVD degeneration, as well as future developments regarding exosomes.

**Key words:** Exosomes; Intervertebral disc degeneration; Stem cells; MicroRNA; Regenerative medicine; Biological characteristic

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** This article mainly reviews the brief pathological process of disc degeneration and the biological characteristics and functions of exosomes. We highlight the current status and advancement of exosome research in the field of intervertebral disc

[p://creativecommons.org/licenses/by-nc/4.0/](https://creativecommons.org/licenses/by-nc/4.0/)

**Manuscript source:** Invited manuscript

**Received:** February 27, 2020

**Peer-review started:** February 27, 2020

**First decision:** May 26, 2020

**Revised:** June 9, 2020

**Accepted:** July 5, 2020

**Article in press:** July 5, 2020

**Published online:** August 26, 2020

**P-Reviewer:** Oltra E, Tanabe S

**S-Editor:** Yan JP

**L-Editor:** Wang TQ

**P-Editor:** Li JH



degeneration, analyze the possible mechanisms, and discuss the future development of exosomes in this field.

**Citation:** Hu ZL, Li HY, Chang X, Li YY, Liu CH, Gao XX, Zhai Y, Chen YX, Li CQ.

Exosomes derived from stem cells as an emerging therapeutic strategy for intervertebral disc degeneration. *World J Stem Cells* 2020; 12(8): 803-813

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/803.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.803>

## INTRODUCTION

Low back pain (LBP) is a common spinal health problem worldwide<sup>[1]</sup>. In a global systematic review, the mean prevalence of LBP at a given time in the general population was approximately 18%, and the 1-year prevalence was approximately 38%<sup>[2]</sup>. Therefore, a small reduction in health care or disability rates related to LBP could bring significant social and economic benefits<sup>[3,4]</sup>.

The causes of LBP are complex<sup>[5,6]</sup>, and although there is no direct evidence, IVD degeneration is considered a major cause<sup>[5]</sup>. The cause of intervertebral disc (IVD) degeneration is still not fully understood, but some factors, such as aging, abnormal mechanical stress, trauma, nutritional deficiencies, and heredity, are considered to be involved in this process<sup>[7]</sup>. The pathological process of disc degeneration includes the reduction of nucleus pulposus cells (NPCs)<sup>[8,9]</sup> and extracellular matrix (due to decreased synthesis and increased degradation), aging of the annulus fibrosus, and calcification of cartilage endplates<sup>[10]</sup>.

Current treatments for LBP caused by IVD degeneration include invasive surgery and conservative treatment<sup>[11]</sup>, which are mainly aimed at relieving symptoms rather than changing pathogenic mechanisms. Therefore, there is an urgent need for new therapies that treat disc degeneration by directly addressing causes and mechanisms to retain and/or restore disc structure and mechanical function.

Recently, an increasing number of studies have focused on degenerated disc regeneration, including studies related to bioactive molecular injection<sup>[12,13]</sup>, cell-based therapies<sup>[14-16]</sup>, tissue engineering<sup>[17,18]</sup>, and gene therapy<sup>[19,20]</sup>. Bioactive molecular injection is a biological therapy utilizing chemical molecules with the effect of recruitment of endogenous stem cells into the IVD or stimulation of their proliferation. Although the short-term effect is acceptable, the long-term maintenance of biological activity has become an unavoidable obstacle for this therapy. Cell-based therapies, as the most attractive method among these studies, involve the injection of extracted cells, such as NPCs or various stem cells, into the disc *in vivo* to restore IVD homeostasis following the proliferation, differentiation, and immune regulation of the transplanted cells<sup>[21]</sup>. Although some progress has been made, the complex environment of the degenerated IVD causes a low survival rate of stem cells and makes it difficult to accurately control cell viability and differentiation. Additionally, the sources and safety issues of stem cells need to be considered. Gene therapy refers to modification of genomes to increase the expression of effector genes and promote the continuous production of one or more biologically active factors in the IVD to promote cell proliferation, extracellular matrix production, and inhibition of apoptosis.

Studies on the mechanism of stem cell therapy have provided increasing evidence that the factors that play an important role in these treatments are the exosomes that are secreted by stem cells<sup>[22-24]</sup>. Exosomes were considered waste products from cells when they were first reported in 1983<sup>[25]</sup>. Currently, this nanoscale cell vesicle is known to be an important substance in intercellular communication that can transfer biomolecules such as proteins and nucleic acids from parent cells to recipient cells. Their applications in regenerative medicine are also increasing, including in the regeneration of NPCs and the maintenance of disc homeostasis<sup>[26-28]</sup>. This paper reviews the biological characteristics of exosomes and their research status in the field of disc degeneration, and gives outlook on their future applications in this field.



## BIOLOGICAL CHARACTERISTICS AND FUNCTIONS OF EXOSOMES

Exosomes are a type of extracellular vesicle; the other two main types of extracellular vesicles are microvesicles and apoptotic bodies<sup>[29,30]</sup>. The characteristics of the three main extracellular vesicles are shown in Table 1.

Exosomes have spheroid membranes of a uniform lipid bilayer with diameters of approximately 30-150 nm<sup>[31]</sup>. They typically can be detected in various body fluids, such as blood<sup>[32,33]</sup>, amniotic fluid<sup>[34-36]</sup>, breast milk<sup>[37,38]</sup>, urine<sup>[39,40]</sup>, synovial fluid<sup>[41,42]</sup>, and saliva<sup>[43,44]</sup>. They can be transported to corresponding target cells through the body fluids to perform a specific function. *In vitro*, exosomes also have been isolated from cell culture supernatant<sup>[45]</sup>. We now know that the mechanism of exosome formation involves the inward invagination of the endosomal membrane pathway. At the first stage, the inward budding of the plasma membrane with receptors leads to the formation of an endosome. Then, small vesicles can be formed by further inward budding of the limiting membrane to form a multivesicular body (MVB) with intraluminal vesicles. The vesicular contents in MVBs are finally degraded when MVBs fuse with the lysosome or are released into the extracellular space<sup>[46]</sup>. After being released into the extracellular space, exosomes play a biological role when they contact another membrane and are endocytosed into a recipient cell<sup>[46,47]</sup>.

The function of exosomes mainly depends on their contents. Among the components of exosomes, lipids, proteins, and nucleic acids are the three main substances that determine the biological function of exosomes<sup>[48,49]</sup>. Lipids in exosomes are mainly located in the membrane, including cholesterol, phosphatidylserine, sphingomyelin, *etc.* In addition to maintaining the biological stability of exosomes, lipids are also involved in biological processes such as the formation and release of exosomes<sup>[50]</sup>. Exosomes are also rich in a variety of proteins, including cytoskeleton components, tetraspanins, heat-shock proteins, and other types of proteins<sup>[51,52]</sup>. Among them, ALIX and tetraspanin proteins, such as CD81, CD9, and CD63, are markers of exosomes<sup>[51]</sup>. However, it is difficult to distinguish exosomes from other extracellular vesicles with overlapping size and density based solely on these markers.

Exosomes usually carry nucleic acids, including mRNAs<sup>[53]</sup>, microRNAs (miRNAs)<sup>[54,55]</sup>, and long noncoding RNAs (lncRNAs)<sup>[56]</sup>. MiRNAs are a class of endogenous noncoding RNAs found in eukaryotes that have a length of approximately 20-25 nucleotides. Mature miRNAs are produced from longer primary transcripts that undergo a series of nuclease-mediated cleavages; then, the miRNAs are assembled into RNA-induced silencing complexes by complementary base pairing to a target mRNA, which guides degradation of targets or suppresses translation of targets, based on the degree of complementarity<sup>[57]</sup>. According to the principle of base complementary pairing, a miRNA usually can target hundreds of corresponding genes, which implies that miRNAs carried in exosomes may play an important role in regulating gene transcription in target cells. LncRNAs are a class of RNA molecules longer than 200 bp that do not encode proteins. They are widely involved in the transcription, translation, and posttranslational regulation of genes. LncRNAs can participate in chromatin modification, transcription activation, and transcription interference in cells, or they can act as "bait molecules" that interact with proteins, DNA, and RNA<sup>[58,59]</sup>. As messengers of intercellular communication, exosomes are secreted by parent cells and taken up by target cells in the following ways: (1) Transmembrane proteins are fused to target cell membranes by binding to receptor proteins; (2) The exosomal membrane fuses directly with the cell membrane, releasing the contents; and (3) Target cells take up exosomes through endocytosis<sup>[60,61]</sup>.

## THERAPEUTIC APPLICATION OF STEM CELL-DERIVED EXOSOMES IN VARIOUS TISSUES

Recently, an increasing number of studies have shown that exosomes derived from stem cells play an important role in restoring tissue homeostasis and promoting tissue regeneration.

Exosomes from bone marrow mesenchymal stem cells (MSCs) can significantly enhance bone regeneration, promote vascular regeneration, and accelerate fracture healing in a rat femur nonunion model<sup>[62]</sup>. Exosomes from adipose stem cells promote the vascularization of endothelial cells<sup>[63]</sup>. Human umbilical cord MSC exosomes can promote angiogenesis and repair of second-degree burn wounds of the skin<sup>[64]</sup>. Exosomes from human stem cells can promote the repair of jaw joints and the synthesis of extracellular matrix in that tissue<sup>[24]</sup>. Increasing experimental results show

**Table 1 Characteristics of three main types of extracellular vesicles**

Types of vesicles	Diameter	Markers	Cargos	Density (g/mL)	Origin	Ref.
Exosomes	30-150 nm	CD63, CD81, CD9, HSP70, Flotillin, TSG101, <i>etc.</i>	mRNA, microRNA, lncRNA, circRNA, DNA lipid, protein, <i>etc.</i>	1.13-1.18	Endosomes pathway	[51,74-77]
Microvesicles	50-1000 nm	Integrins, selectins, CD40 ligand	mRNA, microRNA, other non-coding RNA, protein, <i>etc.</i>	1.16-1.19	Plasma membrane; outward budding	[51,75]
Apoptotic bodies	500-2000 nm	Phosphatidylserine, genomic DNA	Nuclear fractions, cell organelles, <i>etc.</i>	1.16-1.28	Plasma membrane	[47,51,78]

the potential regenerative ability of stem cell-derived exosomes through their promotion of cell proliferation, enhancement of angiogenesis, promotion of extracellular matrix homeostasis recovery, inhibition of inflammation, and other unknown effects. Some of these beneficial mechanisms can also be achieved in the repair of disc degeneration.

## THERAPEUTIC EFFECTS OF STEM CELL-DERIVED EXOSOMES ON IVD DEGENERATION

Stem cell transplantation for treatment of IVD degeneration has made great progress. *In vitro* and *in vivo* studies have revealed the great advantages of stem cells as seed cells for cell-based therapies. However, because of the complex and harsh *in vivo* environment of the IVD, there are obstacles to be overcome by IVD degeneration stem cell therapy approaches<sup>[65]</sup>. With continued research into stem cell therapies, it has been found that the exosomes secreted by stem cells play an important role in their therapeutic effect<sup>[26,66]</sup>. Therefore, exosomes have attracted more and more attention in some preclinical studies of promoting IVD regeneration (Table 2).

When exosomes derived from bone marrow MSCs were cocultured with NPCs from degenerated IVDs, cell proliferation was significantly accelerated by extending the incubation time with exosomes. Additionally, the expression of the extracellular matrix synthesis and protection genes *ACAN*, *COL2A1*, *SOX-9*, and *TIMP-1* increased with incubation time, while the degradation-related genes *MMP-1* and *MMP-3* were decreased. Therefore, it seems to indicate that MSC-derived exosomes promote the proliferation and extracellular matrix homeostasis of NPCs<sup>[26]</sup>.

Stem cell-derived exosomes not only promote the proliferation of NPCs but also inhibit their apoptosis. In a study by Cheng *et al.*<sup>[28]</sup>, human bone marrow MSCs and fibroblast-derived exosomes were used to treat TNF- $\alpha$ -induced apoptotic NPCs. The cells treated with the exosomes derived from the bone marrow MSC group had a significantly lower apoptotic rate than those of the other groups. *In vivo* experiments showed that the MSC-derived exosome treatment group had significantly lower Pfirrmann grade, histological grade, and apoptotic rate than the noninjection groups. Another *in vitro* study also confirmed the anti-apoptotic effect of stem cell exosomes. Liao *et al.*<sup>[27]</sup> co-incubated exosomes from MSCs with advanced glycation end products-induced NPCs and confirmed that the levels of apoptosis-related markers caspase-3 and caspase-12 decreased significantly. With the increase in exosomal concentration, the declining trend was greater. The above studies confirmed that exosomes have significant anti-apoptotic effects both *in vivo* and *in vitro*.

The accumulation of a large number of inflammatory factors and extracellular matrix-degrading enzymes in the IVD is an important cause of NPC apoptosis and loss of the extracellular matrix<sup>[67,68]</sup>. Xia *et al.*<sup>[69]</sup> collected the normal nucleus pulposus from trauma patients and the degenerated nucleus pulposus and then screened 150 proteins by gene ontology and KEGG analysis, of which 69 proteins were downregulated and 81 were upregulated. Most of the proteins were associated with inflammatory responses, showing enhanced inflammatory responses in degenerative discs. By adding MSC-derived exosomes to apoptotic NPCs, the expression of IL-1 $\beta$ , iNOS, COX-2, IL-6, MMP3, MMP13, and other inflammation- and extracellular matrix degradation-related enzymes was significantly reduced. *In vivo* experiments also demonstrated that the exosome injection group had significantly lower MMP13 expression at 2, 4, and 8 wk than the control group<sup>[69]</sup>.

The decrease of viable cells is a key factor in the process of disc degeneration;

Table 2 Studies on exosomes for intervertebral disc degeneration

Ref.	Experimental objective	Cargo analysis	Animal model	<i>In vitro</i> appraisalment	<i>In vivo</i> appraisalment	Inhibition test	Research type
HBMSCs; Lu <i>et al</i> <sup>[26]</sup>	To detect the role of exosomes derived from BM-MSCs in NPCs	Not mentioned	None	(1) Promoted proliferation; and (2) Increased synthesis of extracellular matrix and decrease in degradation	None	None	Cell experimentation
HBMSC; Cheng <i>et al</i> <sup>[28]</sup>	To explore the protective effect of MSC-exosomes on NPCs in a cell and rat model	Highly enrichment in miR-21	SD rat model of IVD degeneration by needle puncture	(1) Decreased apoptosis rate; and (2) Decreased cleaved caspase-3	(1) IVD degeneration score lower; (2) Decreased apoptosis rate; and (3) Lower histologic score	MiR-21 antagonist enhanced cell apoptosis	Cell and animal experimentation
Rat nucleus pulposus; Moen <i>et al</i> <sup>[79]</sup>	To study the role of extracellular miRNA in lumbar radicular pain	Increased miR-223	Lewis rat IVD herniation	None	MiR-223 increased after disc herniation	None	Animal experimentation
Porcine notochordal cells; Bach <i>et al</i> <sup>[80]</sup>	To explore the biologic effect of the NCCM-derived EVs on canine and human CLCs from degenerated IVDs <i>in vitro</i>	None	None	Increased glycosaminoglycan (GAG) deposition	None	None	Cell experimentation
HBMSCs; Liao <i>et al</i> <sup>[27]</sup>	To prove that the delivery of MSC-exos could modulate ER stress and inhibit excessive NP cell apoptosis during IDD	None	SD rat model of IVD degeneration by needle puncture	(1) Western blot and TUNEL assays indicated decreased apoptosis rate; and (2) Western blot and qPCR data indicated decreased reticulum stress	(1) Higher DHI; (2) Lower Pfirrmann grade; (3) Lower histological grades; and (4) Decreased apoptosis rate	Akt inhibitor LY294002; ERK inhibitor PD98059	Cell and animal experimentation
C57BL/6 mice BMSCs; Xia <i>et al</i> <sup>[69]</sup>	To investigate the therapeutic effect of exosomes for use as IVDD therapeutics	None	Rabbit model of IVD degeneration by needle puncture	(1) Decreased apoptosis rate; (2) Western blot and qPCR data indicated recovery of matrix homeostasis; (3) Decreased inflammatory marker expression; (4) Suppressed inflammasome; and (5) Recovery of mitochondrial-related proteins and attenuated mitochondrial dysfunction	(1) Higher DHI; (2) Lower Pfirrmann MRI grade; (3) Lower histological grades; and (4) Decreased apoptosis rate	None	Cell and animal experimentation

BMSC: Bone marrow stromal cells; IVDD: Intervertebral disc degeneration; MRI: Magnetic resonance imaging; IVD: Intervertebral disc; IDD: Intervertebral disc degeneration; MSC: Mesenchymal stem cell; HBMSC: Human bone marrow stromal cells; CLC: Cardiomyoblast-like cells; NPC: Nucleus pulposus cells.

conversely, in the process of disc regeneration, the recovery of cell numbers is the most important issue. These studies have confirmed that stem cell-derived exosomes could enhance cell proliferation and inhibit apoptosis, especially for stem cells remaining in the disc. Moreover, exosomes could also enhance the expression of the extracellular matrix in NPCs and inhibit the expression of matrix protein degrading enzymes, which is beneficial for maintaining the homeostasis of the extracellular matrix. During IVD degeneration, a large number of cytokines participate in and accelerate the degeneration of the IVD, leading to apoptosis and senescence of NPCs<sup>[70]</sup>. Exosomes have a significant inhibitory effect on inflammation, which induces the restoration of the microenvironment for the surviving cells and reduces the disturbance of the intracellular environment. Therefore, stem cell-derived exosomes have the potential to treat disc degeneration.

## POTENTIAL MECHANISM OF STEM CELL-DERIVED EXOSOMES FOR IVD DEGENERATION

With an increasing understanding of the mechanisms behind disc degeneration and with in-depth studies of exosomes, the application of exosomes in disc degeneration has achieved some new progress. This progress clearly shows the tremendous potential of exosomes in disc repair. However, the exact mechanism of how exosomes affect disc repair is still unclear.

In a study of Cheng *et al.*<sup>[26]</sup>, miRNA array hybridization and data analysis was performed to compare normal NPCs and TNF-induced apoptotic NPCs; five miRNAs (miR-18a, miR-21, miR-106b, miR-217, and miR-26a) were found at significantly lower levels in the TNF-induced NPC group than in the control group. Furthermore, only miR-21 was present in MSC-derived exosomes at higher levels than it was in fibroblast-derived exosomes. MiR-21 also decreased apoptosis and suppressed the expression of PTEN. Based on the above results, the researchers believe that the PTEN-PI3K-Akt pathway is a potential target of exosomal miR-21-mediated apoptosis protection in NPCs. However, they believe that there are still other extracellular vesicles or other components in exosomes that are involved in this procedure<sup>[28]</sup>.

Liao *et al.*<sup>[27]</sup> proposed another possible mechanism. The endoplasmic reticulum stress-related markers GRP78 and CHOP were significantly increased in degenerated discs, and their expression positively correlated with Pfirrmann classification. Then, exosomes were added to induce NPCs, and the expression of endoplasmic reticulum-related pathways and apoptosis markers was inhibited. This means that exosomes inhibit endoplasmic reticulum stress-mediated apoptosis through AKT and ERK signaling pathways by reducing the levels of CHOP, the key molecule of endoplasmic reticulum stress<sup>[27]</sup>.

In addition to the above studies, Xia *et al.*<sup>[69]</sup> found that 150 proteins differentially expressed in degenerative discs are closely related to enhanced inflammatory responses. Exosomes can significantly inhibit the inflammatory response of apoptotic NPCs and the formation of inflammatory bodies. The proteins found in bone marrow MSC exosomes mainly recovered the damage to mitochondria in NPCs, restored the normal structure of mitochondria, and reduced oxidative stress in mitochondria. The results indicate that exosomes can play a role in inhibiting disc degeneration by restoring mitochondrial homeostasis and the antioxidative response and inhibiting formation of inflammatory bodies<sup>[69]</sup>.

These potential mechanisms have mainly been studied in terms of promoting extracellular matrix production, inhibiting matrix degradation, promoting an anti-inflammatory response, and inhibiting apoptosis and other aspects of exosome-based promotion of IVD repair. Unfortunately, there is no study on how exosomes promote the proliferation mechanism of NPCs. One of the most important reasons for the degeneration of the disc is the decrease in the number of cells, and how to restore the number of cells in the disc is a key question in treatment. Alternatively, in the process of IVD degeneration, the senescence of NPCs is also an important factor. Some molecules have also been found in exosomes that can inhibit the senescence of cells. Previous research has mainly focused on specific miRNAs in exosome-mediated apoptosis, but are there additional miRNAs in exosomes that promote the proliferation and inhibit the aging of NPCs? Furthermore, how do exosomes inhibit inflammation and promote mitochondrial homeostasis, and how are other molecules in exosomes, including lipids, proteins, mRNAs, and lncRNAs, involved?

## DISCUSSION

The unique double-layered membrane structure of exosomes makes their contents difficult to degrade by various enzymes in body fluids. The unique shape, size, and density range of exosomes, as well as the special molecular markers on their surface, enable their identification and isolation. Animal experiments have confirmed that exosomes are more efficient at delivering effective content into cells and cause a lower immune response in recipients than other methods. By overexpressing miRNAs targeting specific mRNAs in donor cells, exosomes promote cell proliferation, inhibit apoptosis, and promote the production of the extracellular matrix<sup>[28]</sup>. Engineered exosomes can also be loaded with miRNA synthesized *in vitro* by electroporation and can then be injected into tissues to achieve therapeutic goals<sup>[71]</sup>. Exosomes may also be combined with scaffold material to promote IVD regeneration. In a study by Liu *et al.*<sup>[72]</sup>, photoinduced imine crosslinking hydrogel glue combined with stem cell-



derived exosomes promoted defective cartilage repair and regeneration<sup>[72]</sup>. Therefore, an increasing number of studies have shown that exosomes are a promising method for the treatment of disc degeneration. However, there are still many challenges and disadvantages.

First, the physiological environment of the IVD is complex. As the largest avascular tissue in the body, long-term internal high pressure, high permeability, low pH, low nutrition, and low oxygen make it not suitable for cell proliferation<sup>[73]</sup>. In the degenerated IVD, the complex inflammatory environment, the decrease in the number of cells, and fibrosis may affect *in vivo* as *in vitro* results being the same. Additionally, IVD degeneration is a pathological process involving multiple factors, and the exact mechanism has not yet been determined. Therefore, choosing the appropriate exosomes for specific causes is very important.

Second, the exact mechanism of exosomal biogenesis needs to be further investigated. Exosomes are derived from endosomes after cell endocytosis. After processing, they may join multivesicular bodies (MVBs) and may contain proteins, nucleic acids, lipids, cholesterol, and other biologically active molecules; further, they may be secreted by exocytosis or may be encountered by a lysosome and become degraded<sup>[29]</sup>. This is a complex set of biological process, and more research needs to be done to determine the specific mechanisms. An increasing number of studies have demonstrated that exosome-mediated effects are mainly due to the contents of the exosomes, such as miRNAs, lncRNAs, and other molecules. Understanding how cells assemble these molecules in exosomes will enable additional exosomes to be harvested. The isolation of exosomes also has limitations regardless of the current methods being used, such as ultracentrifugation, ultrafiltration, and chromatography. Therefore, new methods need to be developed to improve the isolation and purity of exosomes.

Moreover, exosomes, as a collection of various biologically active molecules, are also affected by various factors, such as the source of cells, the status of cell growth, the conditions of culture, and even the consistency and reproducibility of their effects. All of those factors need to be considered. As a special carrier in the treatment of diseases, the application of exosomes still faces a series of problems, such as dosage, mode of administration, and evaluation of the efficacy.

---

## CONCLUSION

---

Exosomes are attracting increasing attention because of their unique structures and diverse properties. Exosomes have shown favorable possibilities during the repair of IVD, since they can promote the proliferation of NPCs, promote the homeostasis of the extracellular matrix, and inhibit cell apoptosis (Figure 1). However, the detailed mechanisms behind these activities are still unclear, so further research is needed to explore the complex regulation mechanisms, optimize the culture and transplant conditions, and perform more preclinical trials to verify the safety of exosomes.

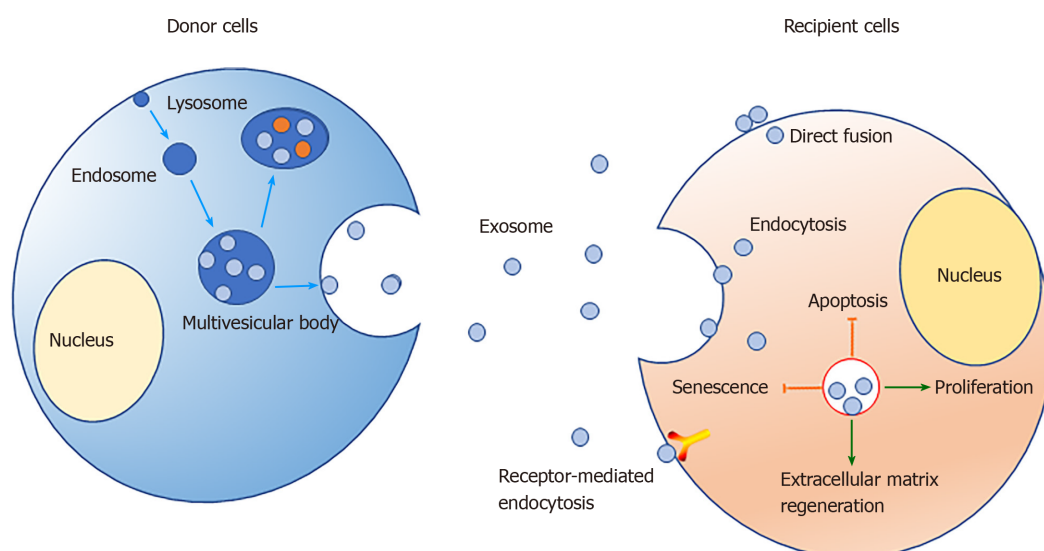


Figure 1 Exosome-mediated mechanism of stem cells regulating the activities of nucleus pulposus cells.

## REFERENCES

- 1 **Manchikanti L**, Singh V, Falco FJ, Benyamin RM, Hirsch JA. Epidemiology of low back pain in adults. *Neuromodulation* 2014; **17** Suppl 2: 3-10 [PMID: 25395111 DOI: 10.1111/ner.12018]
- 2 **Hoy D**, Bain C, Williams G, March L, Brooks P, Blyth F, Woolf A, Vos T, Buchbinder R. A systematic review of the global prevalence of low back pain. *Arthritis Rheum* 2012; **64**: 2028-2037 [PMID: 22231424 DOI: 10.1002/art.34347]
- 3 **Davis MA**, O'neal T, Weeks WB, Lurie JD. Where the United States spends its spine dollars: expenditures on different ambulatory services for the management of back and neck conditions. *Spine (Phila Pa 1976)* 2012; **37**: 1693-1701 [PMID: 22433497 DOI: 10.1097/BRS.0b013e3182541f45]
- 4 **Dunn A**, Grosse SD, Zuvekas SH. Adjusting Health Expenditures for Inflation: A Review of Measures for Health Services Research in the United States. *Health Serv Res* 2018; **53**: 175-196 [PMID: 27873305 DOI: 10.1111/1475-6773.12612]
- 5 **Urits I**, Burshtein A, Sharma M, Testa L, Gold PA, Orhurhu V, Viswanath O, Jones MR, Sidransky MA, Spektor B, Kaye AD. Low Back Pain, a Comprehensive Review: Pathophysiology, Diagnosis, and Treatment. *Curr Pain Headache Rep* 2019; **23**: 23 [PMID: 30854609 DOI: 10.1007/s11916-019-0757-1]
- 6 **Bikbov MM**, Kazakbaeva GM, Zainullin RM, Salavatova VF, Gilmanshin TR, Arslangareeva II, Nikitin NA, Mukhamadieva SR, Yakupova DF, Panda-Jonas S, Khikmatullin RI, Aminev SK, Nuriev IF, Zaynetdinov AF, Uziyanbaeva YV, Jonas JB. Prevalence of and factors associated with low Back pain, thoracic spine pain and neck pain in Bashkortostan, Russia: the Ural Eye and Medical Study. *BMC Musculoskelet Disord* 2020; **21**: 64 [PMID: 32007098 DOI: 10.1186/s12891-020-3080-4]
- 7 **Grunhagen T**, Shirazi-Adl A, Fairbank JC, Urban JP. Intervertebral disk nutrition: a review of factors influencing concentrations of nutrients and metabolites. *Orthop Clin North Am* 2011; **42**: 465-477, vii [PMID: 21944584 DOI: 10.1016/j.ocl.2011.07.010]
- 8 **Yang S**, Zhang F, Ma J, Ding W. Intervertebral disc ageing and degeneration: The antiapoptotic effect of oestrogen. *Ageing Res Rev* 2020; **57**: 100978 [PMID: 31669486 DOI: 10.1016/j.arr.2019.100978]
- 9 **Sakai D**, Nakamura Y, Nakai T, Mishima T, Kato S, Grad S, Alini M, Risbud MV, Chan D, Cheah KS, Yamamura K, Masuda K, Okano H, Ando K, Mochida J. Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc. *Nat Commun* 2012; **3**: 1264 [PMID: 23232394 DOI: 10.1038/ncomms2226]
- 10 **Urban JP**, Roberts S. Degeneration of the intervertebral disc. *Arthritis Res Ther* 2003; **5**: 120-130 [PMID: 12723977 DOI: 10.1186/ar629]
- 11 **Chen BL**, Guo JB, Zhang HW, Zhang YJ, Zhu Y, Zhang J, Hu HY, Zheng YL, Wang XQ. Surgical versus non-operative treatment for lumbar disc herniation: a systematic review and meta-analysis. *Clin Rehabil* 2018; **32**: 146-160 [PMID: 28715939 DOI: 10.1177/0269215517719952]
- 12 **Masuda K**, Imai Y, Okuma M, Muehleman C, Nakagawa K, Akeda K, Thonar E, Andersson G, An HS. Osteogenic protein-1 injection into a degenerated disc induces the restoration of disc height and structural changes in the rabbit annular puncture model. *Spine (Phila Pa 1976)* 2006; **31**: 742-754 [PMID: 16582847 DOI: 10.1097/01.brs.0000206358.66412.7b]
- 13 **Chujo T**, An HS, Akeda K, Miyamoto K, Muehleman C, Attawia M, Andersson G, Masuda K. Effects of growth differentiation factor-5 on the intervertebral disc--in vitro bovine study and in vivo rabbit disc degeneration model study. *Spine (Phila Pa 1976)* 2006; **31**: 2909-2917 [PMID: 17139221 DOI: 10.1097/01.brs.0000248428.22823.86]
- 14 **Ural IH**, Alptekin K, Ketenci A, Solakoglu S, Alpak H, Özyalçın S. Fibroblast Transplantation Results to the Degenerated Rabbit Lumbar Intervertebral Discs. *Open Orthop J* 2017; **11**: 404-416 [PMID: 28603572 DOI: 10.2174/1874325001711010404]
- 15 **Sheyn D**, Ben-David S, Tawackoli W, Zhou Z, Salehi K, Bez M, De Mel S, Chan V, Roth J, Avalos P, Giaconci JC, Yameen H, Hazanov L, Seliktar D, Li D, Gazit D, Gazit Z. Human iPSCs can be differentiated

- into notochordal cells that reduce intervertebral disc degeneration in a porcine model. *Theranostics* 2019; **9**: 7506-7524 [PMID: [31695783](#) DOI: [10.7150/thno.34898](#)]
- 16 **Jeong JH**, Jin ES, Min JK, Jeon SR, Park CS, Kim HS, Choi KH. Human mesenchymal stem cells implantation into the degenerated coccygeal disc of the rat. *Cytotechnology* 2009; **59**: 55-64 [PMID: [19363673](#) DOI: [10.1007/s10616-009-9192-1](#)]
  - 17 **Gloria A**, Russo T, D'Amora U, Santin M, De Santis R, Ambrosio L. Customised multiphasic nucleus/annulus scaffold for intervertebral disc repair/regeneration. *Connect Tissue Res* 2020; **61**: 152-162 [PMID: [31398999](#) DOI: [10.1080/03008207.2019.1650037](#)]
  - 18 **Doench I**, Torres-Ramos MEW, Montembault A, Nunes de Oliveira P, Halimi C, Viguier E, Heux L, Siadous R, Thiré RMSM, Osorio-Madrado A. Injectable and Gellable Chitosan Formulations Filled with Cellulose Nanofibers for Intervertebral Disc Tissue Engineering. *Polymers (Basel)* 2018; **10**: 1202 [PMID: [30961127](#) DOI: [10.3390/polym10111202](#)]
  - 19 **Sun W**, Zhang K, Liu G, Ding W, Zhao C, Xie Y, Yuan J, Sun X, Li H, Liu C, Tang T, Zhao J. Sox9 gene transfer enhanced regenerative effect of bone marrow mesenchymal stem cells on the degenerated intervertebral disc in a rabbit model. *PLoS One* 2014; **9**: e93570 [PMID: [24691466](#) DOI: [10.1371/journal.pone.0093570](#)]
  - 20 **Ren S**, Liu Y, Ma J, Liu Y, Diao Z, Yang D, Zhang X, Xi Y, Hu Y. Treatment of rabbit intervertebral disc degeneration with co-transfection by adeno-associated virus-mediated SOX9 and osteogenic protein-1 double genes in vivo. *Int J Mol Med* 2013; **32**: 1063-1068 [PMID: [24045878](#) DOI: [10.3892/ijmm.2013.1497](#)]
  - 21 **Barakat AH**, Elwell VA, Lam KS. Stem cell therapy in discogenic back pain. *J Spine Surg* 2019; **5**: 561-583 [PMID: [32043007](#) DOI: [10.21037/jss.2019.09.22](#)]
  - 22 **Takahashi A**, Okada R, Nagao K, Kawamata Y, Hanyu A, Yoshimoto S, Takasugi M, Watanabe S, Kanemaki MT, Obuse C, Hara E. Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. *Nat Commun* 2017; **8**: 15287 [PMID: [28508895](#) DOI: [10.1038/ncomms15287](#)]
  - 23 **Ma T**, Chen Y, Chen Y, Meng Q, Sun J, Shao L, Yu Y, Huang H, Hu Y, Yang Z, Yang J, Shen Z. MicroRNA-132, Delivered by Mesenchymal Stem Cell-Derived Exosomes, Promote Angiogenesis in Myocardial Infarction. *Stem Cells Int* 2018; **2018**: 3290372 [PMID: [30271437](#) DOI: [10.1155/2018/3290372](#)]
  - 24 **Zhang S**, Teo KYW, Chuah SJ, Lai RC, Lim SK, Toh WS. MSC exosomes alleviate temporomandibular joint osteoarthritis by attenuating inflammation and restoring matrix homeostasis. *Biomaterials* 2019; **200**: 35-47 [PMID: [30771585](#) DOI: [10.1016/j.biomaterials.2019.02.006](#)]
  - 25 **Pan BT**, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* 1983; **33**: 967-978 [PMID: [6307529](#) DOI: [10.1016/0092-8674\(83\)90040-5](#)]
  - 26 **Lu K**, Li HY, Yang K, Wu JL, Cai XW, Zhou Y, Li CQ. Exosomes as potential alternatives to stem cell therapy for intervertebral disc degeneration: in-vitro study on exosomes in interaction of nucleus pulposus cells and bone marrow mesenchymal stem cells. *Stem Cell Res Ther* 2017; **8**: 108 [PMID: [28486958](#) DOI: [10.1186/s13287-017-0563-9](#)]
  - 27 **Liao Z**, Luo R, Li G, Song Y, Zhan S, Zhao K, Hua W, Zhang Y, Wu X, Yang C. Exosomes from mesenchymal stem cells modulate endoplasmic reticulum stress to protect against nucleus pulposus cell death and ameliorate intervertebral disc degeneration in vivo. *Theranostics* 2019; **9**: 4084-4100 [PMID: [31281533](#) DOI: [10.7150/thno.33638](#)]
  - 28 **Cheng X**, Zhang G, Zhang L, Hu Y, Zhang K, Sun X, Zhao C, Li H, Li YM, Zhao J. Mesenchymal stem cells deliver exogenous miR-21 via exosomes to inhibit nucleus pulposus cell apoptosis and reduce intervertebral disc degeneration. *J Cell Mol Med* 2018; **22**: 261-276 [PMID: [28805297](#) DOI: [10.1111/jcmm.13316](#)]
  - 29 **Colombo M**, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 2014; **30**: 255-289 [PMID: [25288114](#) DOI: [10.1146/annurev-cellbio-101512-122326](#)]
  - 30 **Battistelli M**, Falcieri E. Apoptotic Bodies: Particular Extracellular Vesicles Involved in Intercellular Communication. *Biology (Basel)* 2020; **9**: 21 [PMID: [31968627](#) DOI: [10.3390/biology9010021](#)]
  - 31 **Phan J**, Kumar P, Hao D, Gao K, Farmer D, Wang A. Engineering mesenchymal stem cells to improve their exosome efficacy and yield for cell-free therapy. *J Extracell Vesicles* 2018; **7**: 1522236 [PMID: [30275938](#) DOI: [10.1080/20013078.2018.1522236](#)]
  - 32 **Zhang W**, Jiang H, Kong Y. Exosomes derived from platelet-rich plasma activate YAP and promote the fibrogenic activity of Müller cells via the PI3K/Akt pathway. *Exp Eye Res* 2020; **193**: 107973 [PMID: [32059976](#) DOI: [10.1016/j.exer.2020.107973](#)]
  - 33 **Ermakov KV**, Bukhvostov AA, Vedenkin AS, Stovbun SV, Dvornikov AS, Kuznetsov DA. Ultrashort ssDNA in Retinoblastoma Patients Blood Plasma Detected by a Novel High Resolution HPLC Technique: a Preliminary Report. *Acta Medica (Hradec Kralove)* 2019; **62**: 170-173 [PMID: [32036851](#) DOI: [10.14712/18059694.2020.8](#)]
  - 34 **Tracy SA**, Ahmed A, Tigges JC, Ericsson M, Pal AK, Zurakowski D, Fauza DO. A comparison of clinically relevant sources of mesenchymal stem cell-derived exosomes: Bone marrow and amniotic fluid. *J Pediatr Surg* 2019; **54**: 86-90 [PMID: [30361074](#) DOI: [10.1016/j.jpedsurg.2018.10.020](#)]
  - 35 **Dixon CL**, Sheller-Miller S, Saade GR, Fortunato SJ, Lai A, Palma C, Guanzon D, Salomon C, Menon R. Amniotic Fluid Exosome Proteomic Profile Exhibits Unique Pathways of Term and Preterm Labor. *Endocrinology* 2018; **159**: 2229-2240 [PMID: [29635386](#) DOI: [10.1210/en.2018-00073](#)]
  - 36 **Xiao GY**, Cheng CC, Chiang YS, Cheng WT, Liu IH, Wu SC. Exosomal miR-10a derived from amniotic fluid stem cells preserves ovarian follicles after chemotherapy. *Sci Rep* 2016; **6**: 23120 [PMID: [26979400](#) DOI: [10.1038/srep23120](#)]
  - 37 **Miyake H**, Lee C, Chusilp S, Bhalla M, Li B, Pitino M, Seo S, O'Connor DL, Pierro A. Human breast milk exosomes attenuate intestinal damage. *Pediatr Surg Int* 2020; **36**: 155-163 [PMID: [31713717](#) DOI: [10.1007/s00383-019-04599-7](#)]
  - 38 **Hock A**, Miyake H, Li B, Lee C, Ermini L, Koike Y, Chen Y, Määttänen P, Zani A, Pierro A. Breast milk-derived exosomes promote intestinal epithelial cell growth. *J Pediatr Surg* 2017; **52**: 755-759 [PMID: [28188035](#) DOI: [10.1016/j.jpedsurg.2017.01.032](#)]

- 39 **Danarto R**, Astuti I, Umbas R, Haryana SM. Urine miR-21-5p and miR-200c-3p as potential non-invasive biomarkers in patients with prostate cancer. *Turk J Urol* 2020; **46**: 26-30 [PMID: [31905122](#) DOI: [10.5152/tud.2019.19163](#)]
- 40 **Duan YR**, Chen BP, Chen F, Yang SX, Zhu CY, Ma YL, Li Y, Shi J. Exosomal microRNA-16-5p from human urine-derived stem cells ameliorates diabetic nephropathy through protection of podocyte. *J Cell Mol Med* 2019 [PMID: [31568645](#) DOI: [10.1111/jcmm.14558](#)]
- 41 **Domenis R**, Zanutel R, Caponnetto F, Toffoletto B, Cifù A, Pistis C, Di Benedetto P, Casero A, Pozzi M, Bassini F, Fabris M, Niazi KR, Soon-Shiong P, Curcio F. Characterization of the Proinflammatory Profile of Synovial Fluid-Derived Exosomes of Patients with Osteoarthritis. *Mediators Inflamm* 2017; **2017**: 4814987 [PMID: [28634420](#) DOI: [10.1155/2017/4814987](#)]
- 42 **Kolhe R**, Hunter M, Liu S, Jadeja RN, Pundkar C, Mondal AK, Mendhe B, Drewry M, Rojiani MV, Liu Y, Isaacs CM, Guldberg RE, Hamrick MW, Fulzele S. Gender-specific differential expression of exosomal miRNA in synovial fluid of patients with osteoarthritis. *Sci Rep* 2017; **7**: 2029 [PMID: [28515465](#) DOI: [10.1038/s41598-017-01905-y](#)]
- 43 **Ogawa Y**, Miura Y, Harazono A, Kanai-Azuma M, Akimoto Y, Kawakami H, Yamaguchi T, Toda T, Endo T, Tsubuki M, Yanoshita R. Proteomic analysis of two types of exosomes in human whole saliva. *Biol Pharm Bull* 2011; **34**: 13-23 [PMID: [21212511](#) DOI: [10.1248/bpb.34.13](#)]
- 44 **Michael A**, Bajracharya SD, Yuen PS, Zhou H, Star RA, Illei GG, Alevizos I. Exosomes from human saliva as a source of microRNA biomarkers. *Oral Dis* 2010; **16**: 34-38 [PMID: [19627513](#) DOI: [10.1111/j.1601-0825.2009.01604.x](#)]
- 45 **Théry C**, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006; **Chapter 3**: Unit 3.22 [PMID: [18228490](#) DOI: [10.1002/0471143030.cb0322s30](#)]
- 46 **Zhang Y**, Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci* 2019; **9**: 19 [PMID: [30815248](#) DOI: [10.1186/s13578-019-0282-2](#)]
- 47 **EL Andaloussi S**, Mäger I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov* 2013; **12**: 347-357 [PMID: [23584393](#) DOI: [10.1038/nrd3978](#)]
- 48 **Mashouri L**, Yousefi H, Aref AR, Ahadi AM, Molaei F, Alahari SK. Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. *Mol Cancer* 2019; **18**: 75 [PMID: [30940145](#) DOI: [10.1186/s12943-019-0991-5](#)]
- 49 **Conigliaro A**, Fontana S, Raimondo S, Alessandro R. Exosomes: Nanocarriers of Biological Messages. *Adv Exp Med Biol* 2017; **998**: 23-43 [PMID: [28936730](#) DOI: [10.1007/978-981-10-4397-0\\_2](#)]
- 50 **Skotland T**, Hessvik NP, Sandvig K, Llorente A. Exosomal lipid composition and the role of ether lipids and phosphoinositides in exosome biology. *J Lipid Res* 2019; **60**: 9-18 [PMID: [30076207](#) DOI: [10.1194/jlr.R084343](#)]
- 51 **Shao H**, Im H, Castro CM, Breakefield X, Weissleder R, Lee H. New Technologies for Analysis of Extracellular Vesicles. *Chem Rev* 2018; **118**: 1917-1950 [PMID: [29384376](#) DOI: [10.1021/acs.chemrev.7b00534](#)]
- 52 **Winczura K**, Domanski M, LaCava J. Affinity Proteomic Analysis of the Human Exosome and Its Cofactor Complexes. *Methods Mol Biol* 2020; **2062**: 291-325 [PMID: [31768983](#) DOI: [10.1007/978-1-4939-9822-7\\_15](#)]
- 53 **Wei Z**, Batagov AO, Schinelli S, Wang J, Wang Y, El Fatimy R, Rabinovsky R, Balaj L, Chen CC, Hochberg F, Carter B, Breakefield XO, Krichevsky AM. Coding and noncoding landscape of extracellular RNA released by human glioma stem cells. *Nat Commun* 2017; **8**: 1145 [PMID: [29074968](#) DOI: [10.1038/s41467-017-01196-x](#)]
- 54 **Fu Y**, Zhang L, Zhang F, Tang T, Zhou Q, Feng C, Jin Y, Wu Z. Exosome-mediated miR-146a transfer suppresses type I interferon response and facilitates EV71 infection. *PLoS Pathog* 2017; **13**: e1006611 [PMID: [28910400](#) DOI: [10.1371/journal.ppat.1006611](#)]
- 55 **Liu W**, Rong Y, Wang J, Zhou Z, Ge X, Ji C, Jiang D, Gong F, Li L, Chen J, Zhao S, Kong F, Gu C, Fan J, Cai W. Exosome-shuttled miR-216a-5p from hypoxic preconditioned mesenchymal stem cells repair traumatic spinal cord injury by shifting microglial M1/M2 polarization. *J Neuroinflammation* 2020; **17**: 47 [PMID: [32019561](#) DOI: [10.1186/s12974-020-1726-7](#)]
- 56 **Shyu KG**, Wang BW, Pan CM, Fang WJ, Lin CM. Hyperbaric oxygen boosts long noncoding RNA MALAT1 exosome secretion to suppress microRNA-92a expression in therapeutic angiogenesis. *Int J Cardiol* 2019; **274**: 271-278 [PMID: [30301563](#) DOI: [10.1016/j.ijcard.2018.09.118](#)]
- 57 **Tafrihi M**, Hasheminasab E. MiRNAs: Biology, Biogenesis, their Web-based Tools, and Databases. *Microrna* 2019; **8**: 4-27 [PMID: [30147022](#) DOI: [10.2174/2211536607666180827111633](#)]
- 58 **Khorkova O**, Hsiao J, Wahlestedt C. Basic biology and therapeutic implications of lncRNA. *Adv Drug Deliv Rev* 2015; **87**: 15-24 [PMID: [26024979](#) DOI: [10.1016/j.addr.2015.05.012](#)]
- 59 **Li X**, Wu Z, Fu X, Han W. lncRNAs: insights into their function and mechanics in underlying disorders. *Mutat Res Rev Mutat Res* 2014; **762**: 1-21 [PMID: [25485593](#) DOI: [10.1016/j.mrrev.2014.04.002](#)]
- 60 **Han C**, Sun X, Liu L, Jiang H, Shen Y, Xu X, Li J, Zhang G, Huang J, Lin Z, Xiong N, Wang T. Exosomes and Their Therapeutic Potentials of Stem Cells. *Stem Cells Int* 2016; **2016**: 7653489 [PMID: [26770213](#) DOI: [10.1155/2016/7653489](#)]
- 61 **Cocucci E**, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 2009; **19**: 43-51 [PMID: [19144520](#) DOI: [10.1016/j.tcb.2008.11.003](#)]
- 62 **Zhang L**, Jiao G, Ren S, Zhang X, Li C, Wu W, Wang H, Liu H, Zhou H, Chen Y. Exosomes from bone marrow mesenchymal stem cells enhance fracture healing through the promotion of osteogenesis and angiogenesis in a rat model of nonunion. *Stem Cell Res Ther* 2020; **11**: 38 [PMID: [31992369](#) DOI: [10.1186/s13287-020-1562-9](#)]
- 63 **An Y**, Zhao J, Nie F, Qin Z, Xue H, Wang G, Li D. Exosomes from Adipose-Derived Stem Cells (ADSCs) Overexpressing miR-21 Promote Vascularization of Endothelial Cells. *Sci Rep* 2019; **9**: 12861 [PMID: [31492946](#) DOI: [10.1038/s41598-019-49339-y](#)]
- 64 **Zhang B**, Wu X, Zhang X, Sun Y, Yan Y, Shi H, Zhu Y, Wu L, Pan Z, Zhu W, Qian H, Xu W. Human



- umbilical cord mesenchymal stem cell exosomes enhance angiogenesis through the Wnt4/ $\beta$ -catenin pathway. *Stem Cells Transl Med* 2015; **4**: 513-522 [PMID: 25824139 DOI: 10.5966/sctm.2014-0267]
- 65 **Wuertz K**, Godburn K, Neidlinger-Wilke C, Urban J, Iatridis JC. Behavior of mesenchymal stem cells in the chemical microenvironment of the intervertebral disc. *Spine (Phila Pa 1976)* 2008; **33**: 1843-1849 [PMID: 18670337 DOI: 10.1097/BRS.0b013e31817b8f53]
  - 66 **Zhang S**, Chu WC, Lai RC, Lim SK, Hui JH, Toh WS. Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. *Osteoarthritis Cartilage* 2016; **24**: 2135-2140 [PMID: 27390028 DOI: 10.1016/j.joca.2016.06.022]
  - 67 **Kadow T**, Sowa G, Vo N, Kang JD. Molecular basis of intervertebral disc degeneration and herniations: what are the important translational questions? *Clin Orthop Relat Res* 2015; **473**: 1903-1912 [PMID: 25024024 DOI: 10.1007/s11999-014-3774-8]
  - 68 **Risbud MV**, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. *Nat Rev Rheumatol* 2014; **10**: 44-56 [PMID: 24166242 DOI: 10.1038/nrrheum.2013.160]
  - 69 **Xia C**, Zeng Z, Fang B, Tao M, Gu C, Zheng L, Wang Y, Shi Y, Fang C, Mei S, Chen Q, Zhao J, Lin X, Fan S, Jin Y, Chen P. Mesenchymal stem cell-derived exosomes ameliorate intervertebral disc degeneration via anti-oxidant and anti-inflammatory effects. *Free Radic Biol Med* 2019; **143**: 1-15 [PMID: 31351174 DOI: 10.1016/j.freeradbiomed.2019.07.026]
  - 70 **Patil P**, Niedernhofer LJ, Robbins PD, Lee J, Sowa G, Vo N. Cellular senescence in intervertebral disc aging and degeneration. *Curr Mol Biol Rep* 2018; **4**: 180-190 [PMID: 30473991 DOI: 10.1007/s40610-018-0108-8]
  - 71 **Liang G**, Zhu Y, Ali DJ, Tian T, Xu H, Si K, Sun B, Chen B, Xiao Z. Engineered exosomes for targeted co-delivery of miR-21 inhibitor and chemotherapeutics to reverse drug resistance in colon cancer. *J Nanobiotechnology* 2020; **18**: 10 [PMID: 31918721 DOI: 10.1186/s12951-019-0563-2]
  - 72 **Liu X**, Yang Y, Li Y, Niu X, Zhao B, Wang Y, Bao C, Xie Z, Lin Q, Zhu L. Integration of stem cell-derived exosomes with in situ hydrogel glue as a promising tissue patch for articular cartilage regeneration. *Nanoscale* 2017; **9**: 4430-4438 [PMID: 28300264 DOI: 10.1039/c7nr00352h]
  - 73 **Gruber HE**, Ingram JA, Norton HJ, Hanley EN Jr. Senescence in cells of the aging and degenerating intervertebral disc: immunolocalization of senescence-associated beta-galactosidase in human and sand rat discs. *Spine (Phila Pa 1976)* 2007; **32**: 321-327 [PMID: 17268263 DOI: 10.1097/01.brs.0000253960.57051.de]
  - 74 **Zhang H**, Wang L, Li C, Yu Y, Yi Y, Wang J, Chen D. Exosome-Induced Regulation in Inflammatory Bowel Disease. *Front Immunol* 2019; **10**: 1464 [PMID: 31316512 DOI: 10.3389/fimmu.2019.01464]
  - 75 **Deng H**, Sun C, Sun Y, Li H, Yang L, Wu D, Gao Q, Jiang X. Lipid, Protein, and MicroRNA Composition Within Mesenchymal Stem Cell-Derived Exosomes. *Cell Reprogram* 2018; **20**: 178-186 [PMID: 29782191 DOI: 10.1089/cell.2017.0047]
  - 76 **Jan AT**, Rahman S, Khan S, Tasduq SA, Choi I. Biology, Pathophysiological Role, and Clinical Implications of Exosomes: A Critical Appraisal. *Cells* 2019; **8**: 99 [PMID: 30699987 DOI: 10.3390/cells8020099]
  - 77 **Huo C**, Li Y, Qiao Z, Shang Z, Cao C, Hong Y, Xiao H. [Proteomics analysis of serum exosomes and its application in osteoporosis]. *Se Pu* 2019; **37**: 863-871 [PMID: 31642257 DOI: 10.3724/SP.J.1123.2019.04022]
  - 78 **Akers JC**, Gonda D, Kim R, Carter BS, Chen CC. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol* 2013; **113**: 1-11 [PMID: 23456661 DOI: 10.1007/s11060-013-1084-8]
  - 79 **Moen A**, Jacobsen D, Phuyal S, Legfeldt A, Haugen F, Røe C, Gjerstad J. MicroRNA-223 demonstrated experimentally in exosome-like vesicles is associated with decreased risk of persistent pain after lumbar disc herniation. *J Transl Med* 2017; **15**: 89 [PMID: 28460630 DOI: 10.1186/s12967-017-1194-8]
  - 80 **Bach F**, Libregts S, Creemers L, Meij B, Ito K, Wauben M, Tryfonidou M. Notochordal-cell derived extracellular vesicles exert regenerative effects on canine and human nucleus pulposus cells. *Oncotarget* 2017; **8**: 88845-88856 [PMID: 29179481 DOI: 10.18632/oncotarget.21483]

## Mesenchymal stem cell-derived exosomes: Toward cell-free therapeutic strategies in regenerative medicine

Zhan-Jun Ma, Jing-Jing Yang, Yu-Bao Lu, Zhao-Yang Liu, Xue-Xi Wang

**ORCID number:** Zhan-Jun Ma 0000-0002-0825-0755; Jing-Jing Yang 0000-0002-6088-8529; Yu-Bao Lu 0000-0003-4375-8316; Zhao-Yang Liu 0000-0001-9150-8165; Xue-Xi Wang 0000-0003-3168-8816.

**Author contributions:** Ma ZJ wrote the manuscript; Yang JJ, Lu YB, and Liu ZY collected the literature; Wang XX revised the manuscript for important intellectual content.

**Supported by** the Chinese Medicine Administration Research Project of Gansu Province, No. GZK-2019-46; and the Cuiying Technology Innovation Project of Lanzhou University Second Hospital, No. CY2019-MS10.

**Conflict-of-interest statement:** The authors of this manuscript have no conflicts of interest to disclose.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Zhan-Jun Ma, Jing-Jing Yang, Yu-Bao Lu,** The Second Clinical Medical College, Lanzhou University, Lanzhou 730000, Gansu Province, China

**Zhao-Yang Liu,** Department of Medical Imaging, Shanxi Medical University, Jinzhong 030600, Shaanxi Province, China

**Xue-Xi Wang,** School of Basic Medical Sciences, Lanzhou University, Lanzhou 730000, Gansu Province, China

**Corresponding author:** Xue-Xi Wang, MD, Professor, School of Basic Medical Sciences, Lanzhou University, 205 South Tianshui Road, Lanzhou 730000, Gansu Province, China. [wangxuexi@lzu.edu.cn](mailto:wangxuexi@lzu.edu.cn)

### Abstract

Mesenchymal stem cells (MSCs) are multipotent stem cells with marked potential for regenerative medicine because of their strong immunosuppressive and regenerative abilities. The therapeutic effects of MSCs are based in part on their secretion of biologically active factors in extracellular vesicles known as exosomes. Exosomes have a diameter of 30-100 nm and mediate intercellular communication and material exchange. MSC-derived exosomes (MSC-Exos) have potential for cell-free therapy for diseases of, for instance, the kidney, liver, heart, nervous system, and musculoskeletal system. Hence, MSC-Exos are an alternative to MSC-based therapy for regenerative medicine. We review MSC-Exos and their therapeutic potential for a variety of diseases and injuries.

**Key words:** Exosomes; Mesenchymal stem cells; Cell-free therapy; Regenerative medicine; Mesenchymal stem cell-derived exosomes; Extracellular vesicles

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Mesenchymal stem cell-derived exosomes (MSC-Exos) contain a variety of functional proteins, mRNAs, microRNAs, and signaling lipids. MSC-Exos are more stable than their parent cells and do not have the safety issues of living cells, such as tumorigenesis and occlusion of the microvasculature. MSC-Exos represent an alternative to MSC-based therapies for regenerative medicine. In this review, we summarize the characteristics of MSC-Exos and highlight their functions and therapeutic potential for

[p://creativecommons.org/licenses/by-nc/4.0/](https://creativecommons.org/licenses/by-nc/4.0/)

**Manuscript source:** Invited manuscript

**Received:** March 15, 2020

**Peer-review started:** March 15, 2020

**First decision:** April 7, 2020

**Revised:** April 23, 2020

**Accepted:** June 27, 2020

**Article in press:** June 27, 2020

**Published online:** August 26, 2020

**P-Reviewer:** Goebel WS, Ramasamy T, Tanabe S

**S-Editor:** Dou Y

**L-Editor:** Wang TQ

**P-Editor:** Xing YX



tissue/organ regeneration and for kidney, liver, cardiovascular, neurological, and musculoskeletal diseases, as well as cutaneous wound healing.

**Citation:** Ma ZJ, Yang JJ, Lu YB, Liu ZY, Wang XX. Mesenchymal stem cell-derived exosomes: Toward cell-free therapeutic strategies in regenerative medicine. *World J Stem Cells* 2020; 12(8): 814-840

**URL:** <https://www.wjnet.com/1948-0210/full/v12/i8/814.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.814>

## INTRODUCTION

Regenerative medicine, aimed at promoting the repair and regeneration of tissues and organs, is multidisciplinary. It can be understood as the use of biology and tissue engineering to find effective and feasible treatments that promote self-repair and regeneration or the generation of new tissues or organs to maintain, improve, and repair damaged bodies. Stem cell transplantation is the main method for tissue regeneration. Stem cells are immature tissue precursor cells that are capable of self-renewal to form a cloned cell population and, thus, differentiate into multiple cell lineages<sup>[1,2]</sup>. Stem cells can be classified as (1) Embryonic stem cells derived from early embryos; (2) Induced pluripotent stem cells; and (3) Adult stem cells, including hematopoietic stem cells, neural stem cells, and mesenchymal stem cells (MSCs). The therapeutic potential of stem cells can be attributed to three key mechanisms<sup>[3]</sup>. The first is homing, the migration of stem cells to the site of injury; the mechanism is thought to be similar to that of leukocyte migration and to involve cell-surface receptors, such as chemotactic receptors. Integrins, vascular cell adhesion molecule 1, and G protein receptor signals are also likely to play important roles in this process. The second is differentiation into diverse cell types, enabling supplementation or replacement of damaged cells<sup>[4]</sup>. The third is secretion of biologically active factors that affect surrounding tissues. Adult stem cells promote the maintenance and repair of adult tissues and organs<sup>[5]</sup>. MSCs are one of the most important types of adult stem cell and have been used for cell-based therapy of diverse diseases<sup>[6]</sup>.

MSCs were discovered in 1968 by Friedenstein *et al*<sup>[7]</sup>, who described them as fibroblasts capable of secreting hematopoietic growth factors and cytokines. Later studies showed that MSCs are ubiquitous and can be isolated from a variety of tissues, including bone marrow, adipose tissue, dental pulp, umbilical cord, umbilical cord blood, placenta, amniotic fluid, Wharton's jelly, the brain, spleen, liver, kidney, lung, thymus, and pancreas. Moreover, MSCs have self-renewal ability and can differentiate into multiple cell types<sup>[8,9]</sup>. MSCs can be isolated and expanded from the stroma of many tissues, *e.g.*, bone marrow and subcutaneous adipose tissue<sup>[10]</sup>. MSCs show promise for cell therapy because of their ease of isolation, self-renewal and *in vitro* expansion ability, low immunogenicity, multidirectional differentiation, and release of trophic materials that promote tissue renovation or direct cell replacement<sup>[11]</sup>. However, the disadvantages of MSCs include the difficulty in producing cells with a stable phenotype, the deleterious effect of the presence of large cells in the pulmonary microvasculature, host cell rejection, ectopic tissue formation, and tumor formation. These disadvantages have restricted their clinical use<sup>[12-15]</sup>. Thus, alternative MSC-based and complication-free therapeutic strategies are needed. The therapeutic potential of MSCs is determined by their paracrine secretion of a range of growth factors, chemokines, and cytokines<sup>[16-18]</sup>. Therefore, finding a cell-free therapeutic strategy with the same output and efficacy seems to be necessary.

Research has focused on extracellular vesicles (EVs) secreted by MSCs as a possible non-cellular therapy<sup>[19]</sup>. MSCs release numerous EVs, including microvesicles (MVs), exosomes, and apoptotic bodies, which may act as paracrine mediators between MSCs and target cells<sup>[20]</sup>. MVs and exosomes exert a pro-regenerative effect, which is mediated by their protein, mRNA, and regulatory non-coding RNA (*e.g.*, microRNA [miRNA]) contents. Exosomes are the most prominent type of EV and have potential for cell-free therapy because of their biological activities and ability to mediate intercellular communication<sup>[21,22]</sup>. MSC-derived exosomes (MSC-Exos) replicate the biological activity of MSCs and are thus an alternative to whole-cell therapy<sup>[23,24]</sup>. In addition, the surface of exosomes can be modified to enable targeting of specific cell types, suggesting their promise for cell-free therapy.

MSC-Exos have potential for tissue engineering and regenerative therapy. In this review, we summarize the characteristics of MSC-Exos and highlight their functions and potential as a novel cell-free strategy for regenerative medicine.

## CHARACTERISTICS AND BIOLOGICAL FUNCTIONS OF MSCS

### Characteristics

MSCs are an undifferentiated adult stem cell population with self-renewal ability, low immunogenicity, and multilineage differentiation potential. MSCs have plastic adhesion properties and can be easily isolated from a variety of tissues, such as adipose tissue, umbilical cord blood, liver, amniotic fluid, placenta, and dental pulp<sup>[8,9]</sup>. The International Therapeutic Association of MSCs established the recognition characteristics of human MSCs in 2006. These include maintenance of adherence under standard culture conditions; expression of CD105, CD73, CD90, STRO-1, CD29, and CD44; no expression of CD45, CD34, CD14, CD11b, CD79a, CD19, or HLA-DR; and the ability to differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro*<sup>[25]</sup>. The ease of isolation and biological functions of MSCs make them suitable in preclinical and clinical trials of cell therapy (Figure 1).

### Biological functions

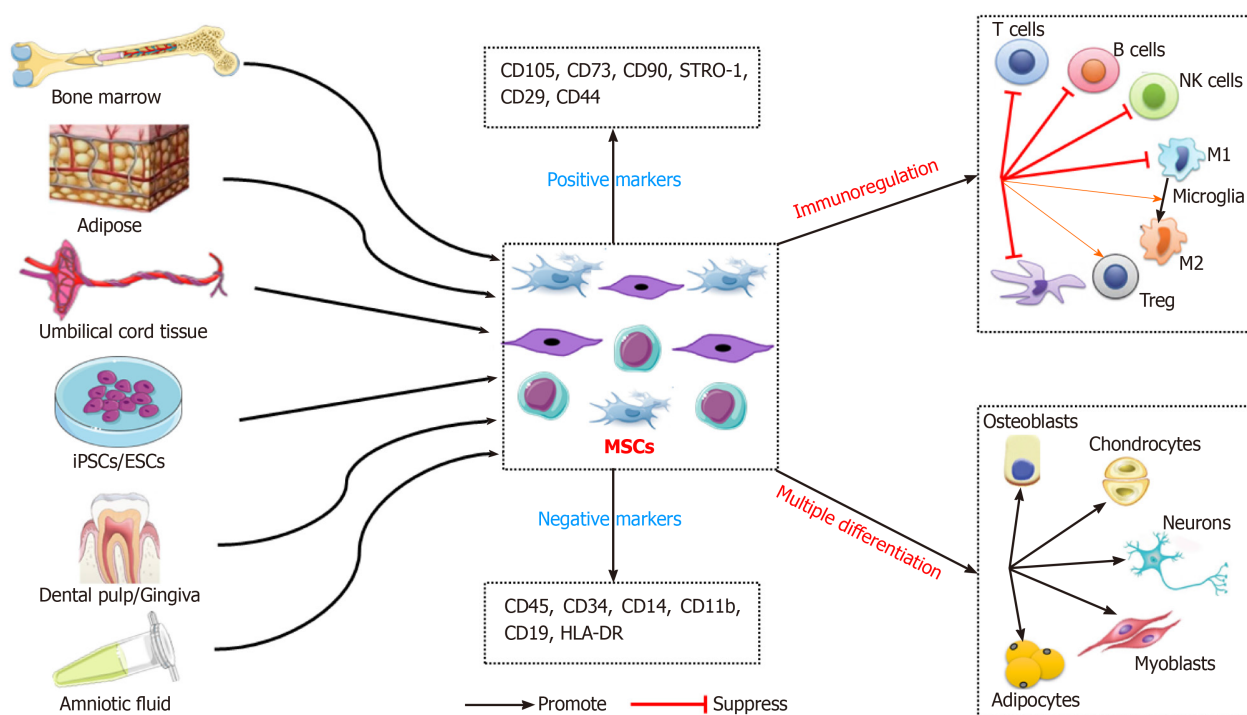
**Multilineage differentiation potential:** MSCs can differentiate into multiple mesenchymal (such as osteoblasts, chondrocytes, adipocytes, endothelial cells, and cardiomyocytes) and non-mesenchymal (such as neurons, glial cells, and hepatocytes) lineages. These characteristics make MSCs good seed cells for tissue engineering and regenerative medicine (*e.g.*, bone and cartilage reconstruction, nerve regeneration, and vascular tissue repair)<sup>[26]</sup>.

**Promotion of tissue repair:** After systemic adoptive transfer, MSCs may occur with lodging in non-specific tissues, homing to natural walls or migration into damaged and/or diseased tissues<sup>[27]</sup>. MSCs can migrate to injured tissue and release cytokines, inflammatory mediators, extracellular matrix (ECM) components, and antibacterial proteins, thereby generating a suitable microenvironment for tissue repair. MSCs are suitable for repair of tissue injury and treatment of, for instance, diabetes, graft-*vs*-host, cardiovascular, inflammatory, liver, lung, kidney, nerve, autoimmune, and bone and cartilage diseases<sup>[28,29]</sup>. In a rat model of lipopolysaccharide (LPS)-induced acute lung injury, allogeneic MSCs transplantation ameliorated the redox environment by upregulating heme oxygenase 1 and protected against lung injury<sup>[30]</sup>. In a clinical trial, autologous bone-marrow-derived MSCs (BMSCs) ameliorated the motor disability and cognitive impairment in stroke patients<sup>[31]</sup>.

**Immunosuppression:** The therapeutic effect of MSCs is mainly attributed to their immunoregulatory activity. MSCs exert immunomodulatory and anti-inflammatory effects by regulating lymphocytes associated with the innate and adaptive immune system<sup>[32]</sup>. MSCs modulate the immune response by inhibiting a wide range of immune cells, including T, B, and natural killer (NK) lymphocytes, and affecting the function of myeloid cells such as monocytes, dendritic cells (DCs), and macrophages<sup>[11,33]</sup>. Specifically, MSCs inhibit T-cell proliferation, activation, and secretion of inflammatory factors [such as interleukin (IL)-2, tumor necrosis factor (TNF)- $\alpha$ , and interferon- $\gamma$ ], reduce the Th1/Th2 ratio, and decrease the number of Th17 cells<sup>[33]</sup>. Also, MSCs induce the generation of regulatory T cells (Tregs), including classic CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> Tregs and non-classical Tregs (such as CD8<sup>+</sup> CD28<sup>+</sup> regulatory T cells), and IL-10<sup>+</sup> Tr1 cells<sup>[34,35]</sup>. In addition, MSCs suppress the differentiation of B lymphocytes into plasma cells and their secretion of immunoglobulins<sup>[36]</sup>. Furthermore, MSCs inhibit the cytotoxicity potential of NK lymphocytes, and promote the transformation of M1 macrophages (pro-inflammatory) to M2 macrophages (anti-inflammatory)<sup>[37,38]</sup>. MSCs modulate antigen presentation by antigen-presenting cells by downregulating MHC and co-stimulatory molecules (CD40, CD86, and CD80) and suppressing the maturation of DCs<sup>[38]</sup>. The immunomodulatory properties of MSCs suggest their therapeutic potential for a variety of diseases.

**Neuroprotective effect:** MSCs transdifferentiate into neural cells and secrete neurotrophic and anti-inflammatory factors following transplantation, thus exerting strong trophic and neuroprotective effects. The therapeutic role of MSCs has been evaluated in preclinical models of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Huntington disease, multiple sclerosis (MS), Parkinson disease,





**Figure 1 Schematic diagram of mesenchymal stem cells-based regenerative medicine.** Mesenchymal stem cells can be easily isolated from a variety of tissues, and the multiple differentiation and immunomodulatory properties of mesenchymal stem cells make them ideal candidates for cell therapy. ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; CD: Cluster of differentiation; MSCs: Mesenchymal stem cells; DC: Dendritic cells; NK cells: Natural killer cells; M1: Microglia M1 phenotype; M2: Microglia M2 phenotype; Treg: Regulatory cell.

and spinal cord injury (SCI)<sup>[39]</sup>. The neuroprotective effect of MSCs is mediated by production of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor, glial cell line-derived neurotrophic factor, nerve growth factor, and neurotrophin-3 (NT-3)<sup>[39,40]</sup>. The BDNF and NT-3 released by MSCs act on neural progenitor cells in the lesion, improving neurogenesis<sup>[40,41]</sup>.

### **Mechanisms underlying MSC based therapy**

MSCs have diverse functions but the underlying mechanisms are unclear. The therapeutic potential of MSCs is based mainly on their immunoregulatory activity and replacement of damaged tissue by differentiating into various cell lineages. It has long been thought that the effect of MSCs on damaged or diseased tissue is based on their immunoregulatory effect<sup>[11]</sup>. However, the therapeutic benefit of MSCs is attributable not only to their differentiation capacity but also their secretion of soluble factors that exert immunoregulatory, angiogenic, and ECM remodeling, and anti-apoptotic, anti-fibrotic, and antioxidant effects<sup>[8,16]</sup>. In this way, MSCs directly or in a paracrine manner rescue damaged cells, reduce tissue damage and, ultimately, accelerate organ repair<sup>[42]</sup>.

Haynesworth *et al*<sup>[42]</sup> in 1996 first reported the paracrine effect of MSCs. MSC-derived paracrine factors have been shown to promote angiogenesis, protect against acute renal, liver, and tissue injury, promote neovascularization, and enhance arteriogenesis<sup>[5,18,43]</sup>. MSCs secrete mediators that directly activate target cells or stimulate neighboring cells to secrete active factors<sup>[43]</sup>. Interestingly, MSC-derived EVs, including exosomes, exert other paracrine effects on tissue regeneration by transferring information to damaged cells or tissue and have biological activity similar to that of MSCs<sup>[19]</sup>. Moreover, compared with MSCs, MSC-Exos can cross biological barriers, can be modified to load molecular drugs, have fewer side effects and less immunogenicity, and remain active during storage<sup>[44]</sup>. Therefore, the regenerative potential of MSC-Exos as cell-free therapy has been evaluated.

## EXOSOMES

### **Definition and morphological characteristics of exosomes**

Exosomes are one of the main subclasses of EVs, which were discovered in sheep reticulocytes in 1983<sup>[45]</sup>; the term “exosome” was coined in 1987<sup>[46]</sup>. Exosomes are small lipid membrane vesicles, which are formed by endocytosis, integration, and efflux. Exosomes are secreted by a wide range of mammalian cell types, including MSCs, B cells, cytotoxic T cells, neurons, cancer cells, oligodendrocytes, platelets, epithelial cells, DCs, and mast cells<sup>[47]</sup>. Exosomes are present in body fluids such as saliva, blood, bile, urine, semen, cerebrospinal fluid, ascites fluid, amniotic fluid, and colostrum<sup>[48]</sup>. Morphologically, exosomes are described as cup-shaped or saucer-like when observed by transmission electron microscopy<sup>[48,49]</sup>. Similar to other lipid vesicles, exosomes float in a sucrose gradient and have a density of 1.13 g/mL (B-cell-derived exosomes) to 1.19 g/mL (intestinal cell-derived exosomes)<sup>[49,50]</sup>. B-cell exosomes are the most homogeneous in terms of size (60-80 nm)<sup>[50]</sup>.

EVs are classified as exosomes, MVs, or apoptotic bodies, depending on their origin. Exosomes are 30-100 nm in diameter, MVs are 100-1000 nm in diameter, and apoptotic bodies are 1-5  $\mu$ m in diameter<sup>[49,51,52]</sup>. There are overlaps in the sizes of EVs, and the lack of standardization is an issue. The major EV subtypes currently recognized, together with their basic characteristics, are summarized in Table 1.

### **Biogenesis of exosomes**

Exosomes originate from the endocytosis-exogenous pathway, while other EVs are derived directly from the plasma membrane. Exosome biogenesis occurs *via* the endocytosis-ectopic pathway when cells absorb a small amount of intracellular fluid in specific membrane regions and form early endosomes. Those early endosomes begin to mature and expand into late endosomes, which undergo inward germination to form intraluminal vesicles (ILVs) with a diameter of 30 nm to 100 nm. Late endosomes, often referred to as multivesicular bodies (MVBs) due to their inclusion of ILVs, fuse with lysosomes, resulting in degradation of their contents, or fuse with the cell membrane and are released into the extracellular environment – these are defined as exosomes<sup>[48,53]</sup>. The exosomes are subsequently taken up by recipient cells. Exosomes can be endocytosed or interact with recipient cells through ligand-receptor or direct binding<sup>[53]</sup> (Figure 2). Although the endosomal-dependent pathway is the main route of exosome biogenesis, direct budding of the plasma membrane can also produce exosomes. Two major MVB and ILV biogenesis pathways have been identified: The endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent pathways (Figure 2). The ESCRT comprises four complexes and their associated proteins, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, which are involved in identifying ubiquitinated proteins in the endosomal membrane, and budding and separating of the endosomal membrane then modulate the integration process that ultimately produces ILVs<sup>[54]</sup>. In contrast, the ESCRT-independent pathway integrates cellular content into exosomes *via* budding of ceramide-induced ILVs<sup>[55]</sup>. The classification of other proteins is mediated by variations in the normative ESCRT-dependent pathway<sup>[56]</sup>. In addition, there are other mechanisms in exosome biogenesis, and this finding suggests that ILV formation requires sphingolipid ceramide. Moreover, neutral sphingomyelinase enhances ILV formation by promoting MVB budding<sup>[48]</sup>.

### **Isolation of exosomes**

Various exosome separation techniques, including ultracentrifugation-based separation technology, size-based technology, precipitation technology, and immunoaffinity capture, as well as novel combinations of these, are available or under development (Table 2).

**Ultracentrifugation:** The method most commonly used to isolate exosomes is ultracentrifugation, frequently in combination with a sucrose density gradient or a sucrose cushion<sup>[57]</sup>. Cells and larger particles are removed by increasing the centrifugal force, and exosomes are pelleted by centrifugation at  $\geq 100000 \times g$  for > 2 h. This method is simple and cost-effective but requires specialized equipment and lacks specificity, so exosomes may be contaminated with other EVs of similar diameter<sup>[58]</sup>.

**Membrane filtration:** Exosomes can be isolated by membrane filtration<sup>[58]</sup>. After removing cell debris and macromolecules, the sample is ultrafiltered to remove contaminants. Membrane filtration is rapid and easy to perform. However, it can be difficult to separate exosomes from contaminants, such as apoptotic bodies or vesicles

**Table 1 Characteristics of different types of extracellular vesicles**

Feature	Exosomes	Microvesicles	Apoptotic bodies
Diameter and shape	30-100 nm, cup shape	100-1000 nm, irregular shape	1-5 $\mu$ m, heterogeneous shape
Sucrose gradient	1.13-1.19 g/mL	1.04-1.07 g/mL	1.18-1.28 g/mL
Sedimentation	100000 g	10000 g	16000 g
Protein markers	CD63, CD81, CD9, Alix, Tsg101, annexins, heat-shock proteins	Integrins, selectins, CD40, flotillins, CD40, ARF6, VCAMP3	TSP, C3b, histones
Origin	Fusion of multivesicular bodies with cell membrane	Outward budding of cell membrane	Outward budding of apoptotic cell membrane
Lipid content	Ceramide	Phosphatidylserine	Phosphatidylserine
Nucleic acids	DNA, mRNA, miRNA, non-coding RNA	DNA, mRNA, miRNA, non-coding RNA	Fragmented DNA, mRNA, miRNA, non-coding RNA

**Table 2 Summary of exosome isolation methods**

Methods	Mechanism	Advantages	Disadvantages
Ultracentrifugation	Physical method	A golden standard; low cost; a wide range of volumes	Low yield; low purity; time-consuming
Membrane filtration	Physical method using filters	Simple; fast; high yield; keeps exosomes intact	Low purity; deformation of exosomes
Precipitation	Physical/chemical method	High yield; easy; high recoveries	Low purity; contaminants
Size exclusion chromatography	Use columns packed with pore beads	High yield; reduces exosome aggregation; keeps exosomes intact	A small number of bands; time-consuming
Immunoaffinity capture technology	Magnetic beads bound to specific antibodies	High yield; high purity; specialty	Time-consuming; high cost

of similar diameter, depending on the pore size of the filter<sup>[59]</sup>.

**Precipitation:** Polyethylene glycols (PEGs) can be used for precipitation<sup>[60]</sup>. ExtraPEG was adapted from a PEG-based virus isolation method and can be applied to various vesicle types and biological fluids<sup>[61]</sup>. PEG-mediated exosome isolation involves low-speed centrifugation followed by a single small-volume filtration purification step. This method is rapid and inexpensive<sup>[58]</sup>, but the exosomes produced are of low purity and the technique is costly<sup>[57]</sup>.

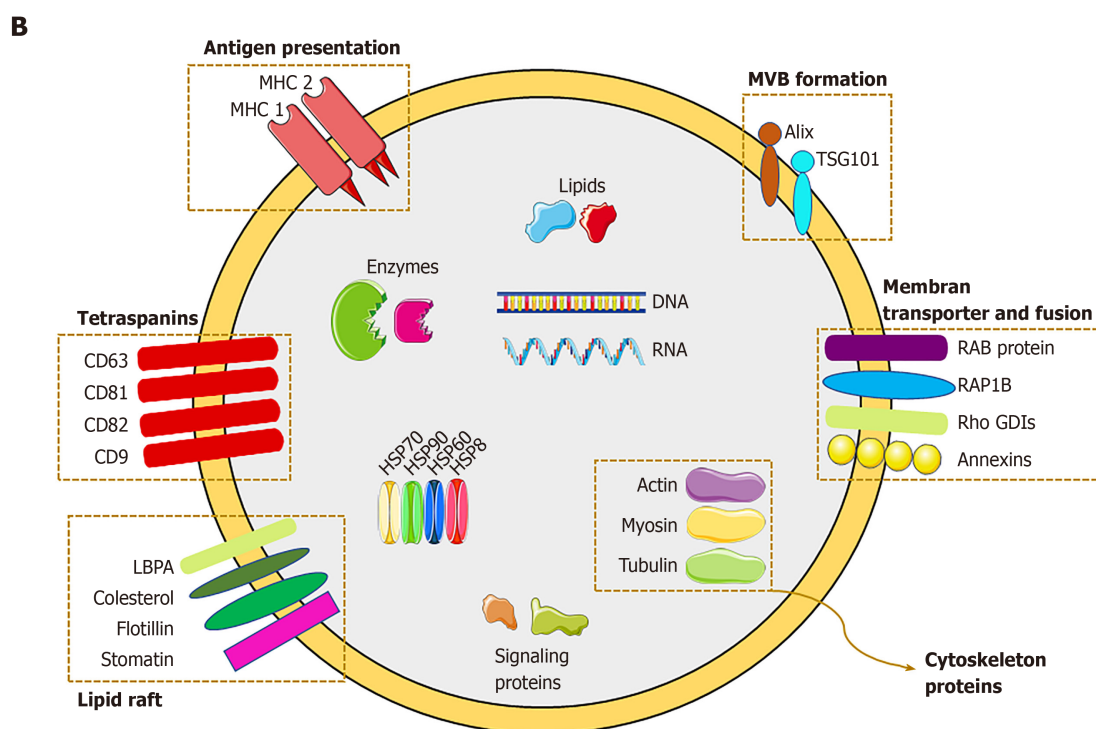
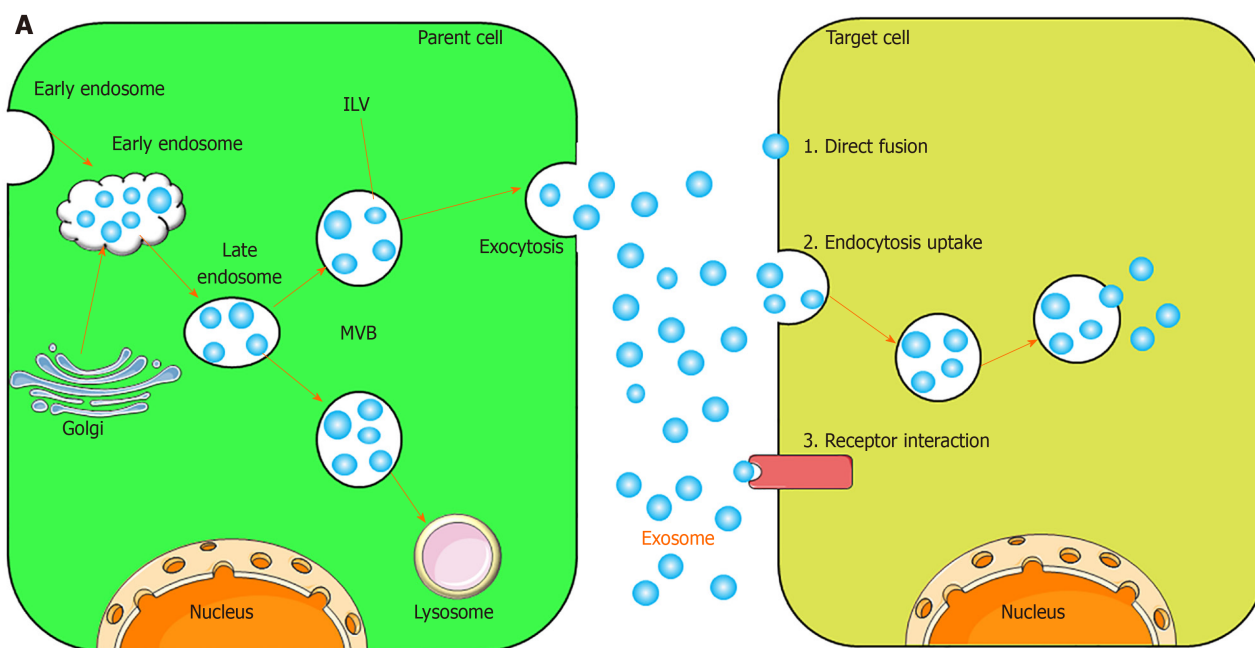
**Size exclusion chromatography:** Exosome isolation by size exclusion chromatography (SEC) involves a column packed with porous polymeric beads. SEC involves removal of cells and larger particles by low-speed centrifugation, followed by two filtration steps using 0.2  $\mu$ m pore filters with a 100 kDa molecular weight cut-off and purification by SEC<sup>[62]</sup>. High yield and no need for specialized equipment are the main advantages of this approach but the exosomes produced have low purity and clogging, vesicle capture, and exosome loss due to membrane attachment can occur<sup>[57,58]</sup>.

**Immunoaffinity capture:** Immunoaffinity capture of exosomes involves antibodies against exosome markers (including CD81, CD63, or CD9) and specialized lectins targeting mannose<sup>[62,63]</sup>. This method enables production of exosomes with high purity but is costly, has a low yield, and requires cell-free samples<sup>[57]</sup>.

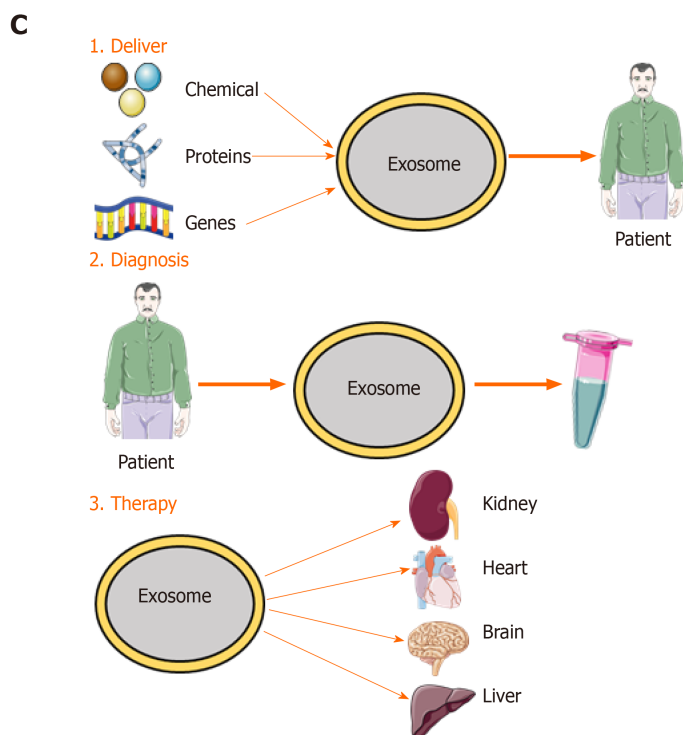
In addition, exosome isolation kits and precipitation solutions can be used to isolate exosomes. However, there is no one-size-fits-all technique, and it is impractical to separate exosomes completely from other components. Therefore, the most appropriate technique for isolating exosomes should be selected. After isolation, exosomes can be stored at -80 °C.

### Characterization and identification of exosomes

Exosomes are identified based on their morphology, size, and marker proteins. Methods for identifying exosomes include transmission electron microscopy (TEM),







**Figure 2 Exosome biogenesis and its application.** A: Exosome biogenesis and intercellular communication; B: Exosome components; C: Exosome application. The applications include: (1) Drug deliver. Therapeutic agents such as chemicals, peptides, and RNAs can be delivered into patients; (2) diagnosis: Exosomes derived from patients can be used for disease diagnosis; and (3) therapy: Exosomes derived from mesenchymal stem cells can be used for various diseases. MVB: Multivesicular body; ILV: Intraluminal vesicle; MCH 1, 2: Major histocompatibility complex 1, 2; TSG101: Tumor susceptibility gene 101; ALIX: ALG-2-Interacting Protein X; RAP1B: Member of RAS oncogene family.

scanning electron microscopy (SEM), atomic force microscopy (AFM), nanoparticle tracking analysis (NTA), flow cytometry analysis, Western blot, and enzyme-linked immunosorbent assay (ELISA)<sup>[47,62]</sup>. TEM, SEM, and AFM are used to determine the size and morphology of exosomes; of these, TEM is the most frequently used<sup>[64]</sup>. NTA is frequently applied to evaluate the size distribution and concentration of exosomes. Flow cytometry and Western blot can be used to identify exosome surface marker proteins<sup>[63]</sup>, for example, CD9, CD63, CD81, and CD82. The proteins in exosomes can be quantified by Bradford or bicinchoninic acid assay or ELISA<sup>[65]</sup>. Two or three of these methods are often used in combination to analyze exosomes.

### Exosome contents and function

Because exosomes are formed by budding from early endosomes, they have a lipid bilayer membrane, which protects the resident genetic material (DNA, mRNA, miRNA, pre-miRNA, and other non-coding RNAs), lipids, and proteins during transportation to target cells<sup>[66]</sup>. The most common exosomal surface proteins are members of the tetraspanin family, a group of scaffold membrane proteins including CD63, CD81, and CD9, which serve as markers. Other common proteins include membrane transporters and fusion proteins (such as GTPases and annexins), heat shock proteins (such as HSP60, 70, and 90), MVB biogenic proteins (such as ESCRT complex, Alix, and TSG101), lipid-related proteins, and phospholipases<sup>[21,67]</sup>. The exosome membrane also contains cholesterol, sphingomyelin, and ceramide in a large number of lipid rafts<sup>[68]</sup>. Exosomes also contain mRNA and miRNA, which, upon endocytosis by the recipient cell, modulate protein synthesis and cell function<sup>[69]</sup>. The protein, lipid, and nucleic acid contents of exosomes vary according to the identity and physiological condition of the source cell and the extracellular environment. Therefore, the content of exosomes serves as an indicator of their source cell. Unique exosomes containing different proteins and RNAs determine their various subpopulations and therefore exert different effects on recipient cells.

Exosomes have various functions. Depending on their characteristics, exosomes can be used for disease diagnosis, drug delivery, and as therapeutic agents (Figure 2). Exosomes engage in specific interactions with the recipient cells, promoting information and material exchange between widely separated anatomic sites<sup>[69]</sup>. Because they can cross the blood-brain barrier, exosomes have potential for drug

delivery to the brain<sup>[70]</sup>. The nanometer-scale size and stability of exosomes suggest their diagnostic potential. Finally, exosomes are a cell-free alternative to cellular therapy. Following injection, exosomes are safer and easier to control than live cells, which can undergo uncontrolled growth and tumor formation<sup>[24]</sup>. The roles of exosomes in immunology and cancer biology have been established<sup>[71]</sup>.

Unlike cells, exosomes do not undergo malignant transformation, do not replicate, and do not induce metastasis. In this review, we focus on the potential of MSC-Exos in regenerative medicine.

## MSC-EXOS

MSCs have been isolated from a variety of sources. Because of their ease of isolation, no ethical considerations, and low immunogenicity, MSCs have therapeutic potential for various diseases. However, injection of MSCs may cause malignant transformation and spread of tumors. Also, differentiation of MSCs induces tissue ossification or calcification in animal models. MSC-Exos have the same functions as MSCs, without the above complications.

### **Properties and functions of MSC-Exos**

MSC-Exos were first isolated by Lai *et al*<sup>[72]</sup> in 2010, and the purified exosomes reduced the infarct area in a mouse model of myocardial ischemia/reperfusion (I/R) injury. Therefore, exosomes represent novel biological agents for promoting tissue repair. MSCs are the most prolific exosome producers<sup>[73]</sup>, and the exosomes produced by MSCs have similar morphological characteristics, isolation methods, and preservation conditions to those from other cell types. The composition of exosomes depends on their cellular origin. MSC-Exos harbor membrane-bound proteins such as CD44, CD73, and CD29; the surface protein profile of exosomes is dependent on the medium used to culture the source MSCs. mRNAs and miRNAs are encapsulated in MSC-Exos; the miRNAs participate in the exchange of information between cells and modulate the function and fate of the recipient cell<sup>[73]</sup>.

MSC-Exos carry proteins, lipids, DNA, and RNA from MSCs, which is the basis for their therapeutic effect. MSC-Exos have biological functions similar to MSCs, but have a smaller volume, can penetrate biofilm, have low immunogenicity, and can be stored. The lipid bilayer of exosomes protects the contents and protects nucleic acids from RNases<sup>[74]</sup>. Furthermore, exosomes transport a variety of biologically active components and reflect the physiological and pathological state of the source cell; they transmit information, remove intracellular components, and can transport drugs<sup>[73]</sup>. Also, MSC-Exos can suppress apoptosis; promote cell regeneration and migration; regulate the immune and inflammatory responses; and promote angiogenesis, nerve regeneration, and tissue repair and regeneration (Figure 3). MSC-Exos have potential for regenerative medicine, as shown in animal models of disease and injury<sup>[75]</sup>.

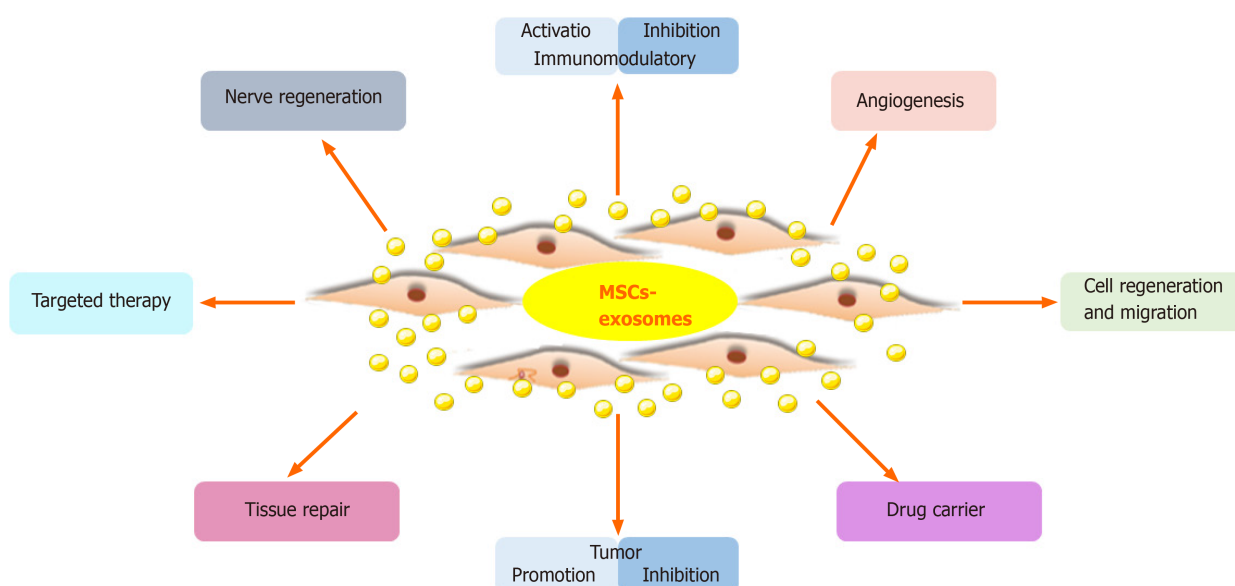
### **Regenerative advantages of exosomes over MSCs**

**Ease of collection:** Various types of MSCs secrete exosomes, and each produces 1000 to 10000 exosomes. Exosomes can be extracted from culture medium by, for example, ultracentrifugation. Exosomes can be produced on a large scale using specialized cell lines<sup>[76]</sup>. Compared with MSCs, the production of MSC-Exos is simpler and less costly and time-consuming.

**Stability for long-term storage:** The volume of exosomes is about one millionth that of MSCs, and they are less complex, have a stable structure, and are easy to produce and store. Exosomes are unaffected by storage at -20 °C for 1 wk, and their activity is maintained during long-term storage at -80 °C<sup>[73]</sup>.

**Safety:** MSC-based therapies have issues with cell survival, regenerative ability, immune rejection, and differentiation to tumors. These problems can be avoided by using exosomes as cell-free therapy. Due to the low content of exosome membrane-bound proteins, the possibility of immune rejection is very low even after allogeneic administration. In addition, exosomes do not proliferate, so there is no possibility of tumor formation<sup>[77]</sup>. Therefore, MSC-Exos have better safety than MSCs for clinical applications.

**Exosomes as ideal carriers:** Exosomes can transfer active substances into recipient cells for cell-to-cell information exchange. Therefore, exosomes can be used as carriers for drugs and biological macromolecules. Sun *et al*<sup>[78]</sup> in 2010 reported that curcumin



**Figure 3** Main functions of mesenchymal stem cell-derived exosomes. MSCs: Mesenchymal stem cells.

transported in exosomes had a stable structure, improved dissolution ability, a higher blood concentration, and greater anti-inflammatory activity. In animal models, curcumin in exosomes protected mice from LPS-induced septic shock. Furthermore, unlike non-host vehicles, exosomes have low immunogenicity and so do not induce immune rejection or other complications.

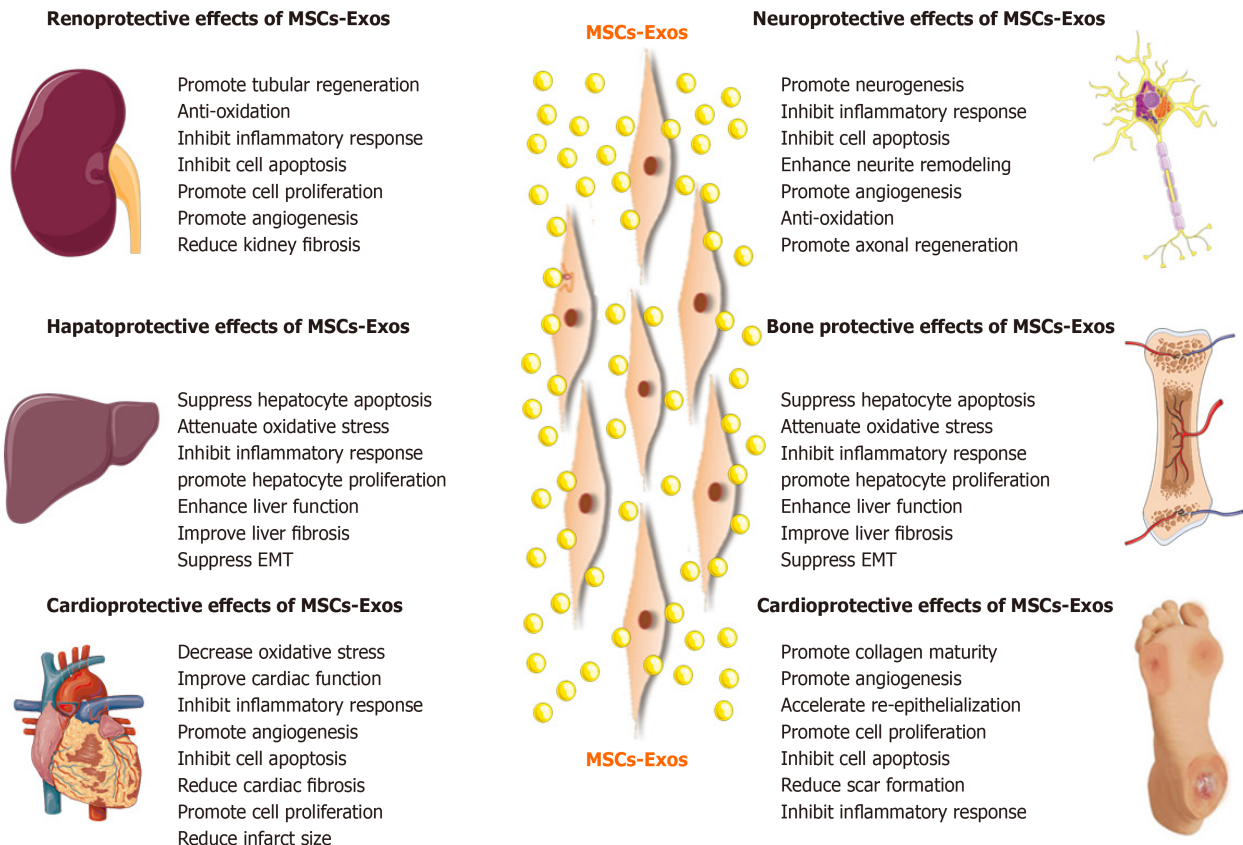
**Targeting:** The exosome membrane harbors proteins with binding affinity for the target cell membrane or a ligand in the ECM. These membrane-bound molecules facilitate the targeting of exosomes to specific tissues or microenvironments. The bilayer membrane of exosomes can be modified with specific factors to enable targeting of cells and tissues<sup>[48]</sup>.

## THERAPEUTIC POTENTIAL OF MSC-EXOS IN REGENERATIVE MEDICINE

Based on the advantages of MSC-Exos as cell-free therapy, their regenerative and therapeutic potential has been explored *in vitro* and *in vivo*. Below we will summarize recent studies on the effects of MSC-Exos on conditions of the kidney, liver, cardiovascular system, nervous system, skin, bone, and muscle (Figure 4).

### MSC-Exos in kidney diseases

**Acute kidney injury:** Renal I/R injury (I/RI) is one of the causes of acute kidney injury (AKI) and is caused by sudden obstruction of blood flow to the kidneys. It is associated with morbidity and mortality in patients with AKI<sup>[79]</sup>. In addition, AKI is a potential risk factor for progressive chronic kidney disease (CKD), and there is no effective treatment<sup>[79]</sup>. Wang *et al.*<sup>[80]</sup> assessed the effect of human bone marrow-derived MSC-Exos in rats with I/R-induced AKI. MSC-Exos improved renal I/RI and renal function by reducing the urea and creatinine levels and inhibiting inflammation and apoptosis. In a mouse model of I/RI, C-C motif chemokine receptor-2 (CCR2)-enriched mouse bone marrow-derived MSC-Exos strongly bound extracellular CCL2 and reduced its concentration, inhibiting the recruitment and activation of peripheral monocytes/macrophages. Importantly, CCR2 knockdown MSC-Exos failed to bind CCL2 and did not protect against renal I/RI<sup>[81]</sup>. Moreover, miRNAs in MSC-Exos also exert a reno-protective effect. Zhu *et al.*<sup>[82]</sup> studied the effect of human bone marrow-derived MSC-Exos containing miR 199a 3p on renal I/RI in a mouse model. Injection of MSC-Exos into mice with I/R injury induced recovery of renal function and histologic protection and reduced the cleaved caspase 3 and semaphorin 3A levels. An *in vitro* study by the same group showed that MSC-Exos increased the expression of the anti-apoptotic protein Bcl-2 and decreased that of the pro-apoptotic proteins Bax and caspase 8 by activating the AKT and ERK pathways in oxygen-glucose



**Figure 4** Therapeutic effects of mesenchymal stem cell-derived exosomes in kidney, liver, cardiovascular, neurological, and musculoskeletal diseases, as well as cutaneous wound healing. MSCs: Mesenchymal stem cells.

deprivation (OGD)-induced HK-2 cells. Co-culture with miR 199a 3p knockdown MSC-Exos reversed these effects<sup>[82]</sup>. Therefore, exosomal miR 199a 3p plays a crucial role in MSC-Exos mediated suppression of I/R induced apoptosis.

Drug-induced nephrotoxicity is a common cause of AKI, with an incidence as high as 60%. The anticancer drug cisplatin can induce kidney disease and elevate the levels of BUN and creatinine, inducing oxidative stress and apoptosis<sup>[79]</sup>. Zhou *et al*<sup>[83]</sup> showed that injection of human umbilical cord-derived MSC-Exos repaired cisplatin-induced AKI in rats and NRK-52E cell injury by ameliorating oxidative stress and apoptosis and promoting cell proliferation *in vivo* and *in vitro*. Human umbilical cord MSC-Exos promoted autophagy of renal tubule epithelial cells and in kidney tissue by inhibiting mTOR, thus alleviating apoptosis and inflammation<sup>[84]</sup>. Jia *et al*<sup>[85]</sup> reported that human umbilical cord-derived MSC-Exos-mediated delivery of 14-3-3 $\zeta$  enhanced autophagy by modulating ATG16L, thus preventing cisplatin-induced AKI.

**CKD:** CKD is a progressive disease with complex symptoms and multiple causes. Several factors influence the severity and rate of progression of CKD. AKI is associated with an increased risk of development of CKD, and no effective treatment is available. Zhu *et al*<sup>[86]</sup> investigated the effect of human adipose tissue-derived MSC-Exos on the AKI-CKD transition. MSCs upregulated the expression of Sox9 in the renal tubules, promoted the regeneration of renal tubules, ameliorated AKI, and reduced renal fibrosis. These effects were reversed by an inhibitor of MSC-Exos release. Further, the MSCs activated tubular Sox9 and prevented TGF- $\beta$ 1-induced transformation of tubular epithelial cells (TECs) into a pro-fibrotic phenotype *via* exosome shuttling *in vitro*. Therefore, MSC exosomes suppressed the AKI-CKD transition by TEC-dependent activation of Sox9<sup>[86]</sup>. Furthermore, MSC-Exos-mediated delivery of miR-let7c to injured kidneys improved kidney architecture and reduced collagen accumulation in unilateral ureteral obstruction-injured mice, ultimately ameliorating renal fibrosis<sup>[87]</sup>.

**Diabetic nephropathy:** Diabetic nephropathy (DN) is a serious complication of diabetes and a common cause of end-stage renal disease. Nagaishi *et al*<sup>[88]</sup> reported that BMSCs ameliorated DN *via* the paracrine effect of renal trophic factors, including



exosomes. Also, bone marrow-derived MSC-Exos markedly improved renal function, promoted histological restoration of renal tissue, significantly increased LC3 and Beclin-1 expression, and significantly decreased mTOR and fibrotic marker expression in renal tissue in a rat model of DN. These effects were in part abolished by the autophagy inhibitors chloroquine and 3-MA<sup>[89]</sup>. These findings suggest the therapeutic potential of MSC-Exos for DN.

### MSC-Exos in liver diseases

**Liver injury:** MSC-Exos can be used for treatment of liver injury. The liver injury caused by I/R affects liver function and increases mortality after liver transplantation and liver resection<sup>[90]</sup>. Nong *et al*<sup>[91]</sup> evaluated the effect of human-induced pluripotent stem cell (hiPSC)-derived MSC-Exos on a rat model of hepatic I/R injury. MSC-Exos markedly suppressed hepatocyte necrosis, sinusoidal congestion, and the levels of markers of hepatocyte injury [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)], inflammation (TNF- $\alpha$  and IL-6), apoptosis (caspase-3 and Bax), and oxidative stress (GSH, GSH-Px, and SOD). Therefore, the hiPSC-MSC-Exos alleviated hepatic I/R injury, possibly by inhibiting inflammation, oxidative stress, and apoptosis. Additionally, hiPSC-MSC-Exos alleviated hepatic I/R injury by activating the sphingosine kinase and sphingosine-1-phosphate pathway in hepatocytes and promoting cell proliferation *in vitro* and *in vivo*<sup>[92]</sup>. MiRNAs associated with MSC-Exos also exert a hepatoprotective effect. Zhang *et al*<sup>[90]</sup> reported that umbilical cord-derived MSC-Exos containing miR-20a alleviated liver I/R injury. Furthermore, MSC-Exos containing miR-20a stimulated the expression of miR-20a target genes, such as *Beclin 1* and *FAS*, in LO-2 cells. These target genes are involved in apoptosis and autophagy, which are implicated in the pathogenesis of liver I/R.

Drug-induced liver injury accounts for more than 50% of acute liver failure (ALF) cases in the United States and has become a major clinical problem<sup>[93]</sup>. Tan *et al*<sup>[93]</sup> found that human embryonic (HuES9.E1)-derived MSC-Exos exert a hepatoprotective effect in *in vitro* models of acetaminophen and H<sub>2</sub>O<sub>2</sub>-induced hepatocyte injury and in a mouse model of carbon tetrachloride (CCl<sub>4</sub>)-induced acute liver injury. The effect was mediated by increasing hepatocyte proliferation, as demonstrated by upregulation of two proliferation factors (PCNA and cyclin D1) and an anti-apoptotic factor (Bcl-xL). Also, the antioxidant activity of human umbilical cord-derived MSC-Exos reportedly suppresses CCl<sub>4</sub>-induced liver injury<sup>[96]</sup>. Importantly, MSC-Exos exerted a hepatoprotective effect *via* antioxidant defenses in the progression from initial liver injury to fibrosis and liver tumor<sup>[94]</sup>. Additionally, in a CCl<sub>4</sub>-induced liver injury mouse model, miR-455-3p-enriched exosomes from human umbilical cord MSCs attenuated macrophage infiltration and local liver damage and reduced the serum levels of inflammatory factors, thereby improving liver histology and ameliorating liver injury<sup>[95]</sup>.

Tamura *et al*<sup>[96]</sup> evaluated the effect of MSC-Exos on concanavalin-A-induced liver injury as a model of immune-induced liver injury. Bone marrow derived-MSC-Exos reduced the serum ALT level, decreased the hepatic necrotic area, apoptosis, and the production of proinflammatory cytokines, and increased the levels of anti-inflammatory cytokines and regulatory T cells, suggesting an anti-inflammatory effect.

**Liver fibrosis:** Liver fibrosis is a common outcome of severe chronic liver injury and is characterized by excessive accumulation of the ECM or scar tissue in the liver. If liver fibrosis is not well controlled, it can progress to cirrhosis but, in principle, it is reversible<sup>[97]</sup>. In a CCl<sub>4</sub>-induced liver injury model, transplantation of human umbilical cord derived-MSC-Exos reduced the surface fibrous capsules and softened their texture, alleviated hepatic inflammation and collagen production, and inhibited the epithelial-to-mesenchymal transition in the CCl<sub>4</sub>-induced fibrotic liver<sup>[98]</sup>. MSC-Exos significantly restored serum AST activity and inactivated the TGF- $\beta$ 1/Smad signaling pathway by decreasing collagen type I/III and TGF- $\beta$ 1 and the phosphorylation of Smad2<sup>[100]</sup>. Moreover, *in vivo* administration of human bone marrow derived-MSC-Exos alleviated liver fibrosis by reducing collagen accumulation, enhancing liver functionality, inhibiting inflammation, and increasing hepatocyte regeneration, by inhibiting hepatic stellate cell (HSC) activation through the Wnt/ $\beta$ -catenin pathway<sup>[99]</sup>. Also, exosomes containing miR-181-5p increased autophagy and ameliorated TGF- $\beta$ 1-induced liver fibrosis by inhibiting the STAT3/Bcl-2/Beclin 1 pathway in HSC cells and a CCl<sub>4</sub>-induced liver fibrosis mouse model<sup>[100]</sup>. Moreover, adipose tissue-derived MSC-Exos expressing miR-122 decreased the proliferation and activation of HSCs in a liver fibrosis model. Furthermore, MSC-Exos containing miR-122 stimulated the expression of miR-122 target genes such as insulin-like growth



factor receptor 1, cyclin G (1), and prolyl-4-hydroxylase  $\alpha 1$  in HSCs. These genes are involved in cell proliferation and collagen maturation<sup>[101]</sup>.

**Liver failure:** ALF is a clinical syndrome caused by inflammation-induced hepatocyte injury, apoptosis, and necrosis, and is a major challenge worldwide. The LPS/D-GalN-induced mouse is generally used as a model of ALF and reflects human liver failure precisely; also, LPS and D-GalN are used to create an animal model of ALF<sup>[102]</sup>. Jiang *et al.*<sup>[102]</sup> showed that human umbilical cord derived-MSC-Exos repaired damaged liver tissue and decreased the levels of ALT and AST and the expression of the NLRP3 inflammasome and downstream inflammatory factors in a LPS/D-GalN-induced mouse model of ALF. Moreover, pretreatment with exosomes from human umbilical cord derived-MSCs plus TNF- $\alpha$  alleviated ALF by inhibiting the activation of the NLRP3-related inflammatory pathway, at least in part, by increasing the expression of miRNA-299-3p<sup>[103]</sup>. In a model of hepatocyte injury and apoptosis induced by LPS/D-GalN, bone marrow derived-MSC-Exos increased the expression of the autophagy marker proteins LC3 and Beclin-1 and promoted the formation of autophagosomes. MSC-Exos significantly decreased the expression levels of the proapoptotic proteins Bax and cleaved caspase-3 and increased that of the anti-apoptotic protein Bcl-2. However, the autophagy inhibitor 3MA significantly reversed the inhibition of apoptosis by MSC-Exos. Therefore, MSC-Exos reduced hepatocyte apoptosis by promoting autophagy after ALF<sup>[104]</sup>.

### MSCs-Exos in cardiovascular diseases

**Myocardial ischemia-reperfusion injury:** Myocardial ischemia-reperfusion (MI/R) can induce apoptosis and necrosis of myocardial cells, and even cause cardiac arrest, thereby affecting the outcome of heart disease treatment. Practical and effective therapeutic modalities for MI/R injury are urgently needed. Lai *et al.*<sup>[72]</sup> reported that human embryonic stem cell-derived MSC-Exos reduced infarct size in a mouse model of MI/R injury. A subsequent study by the same group showed that MSC-Exos increased the levels of ATP and nicotinamide adenine dinucleotide (NADH), decreased oxidative stress, increased phosphorylated-Akt and phosphorylated-GSK-3 $\beta$  (anti-apoptotic factors), and reduced phosphorylated-c-JNK (proapoptotic factor) in I/R hearts, ultimately preventing left ventricular dilatation and improving cardiac performance. MSC-Exos also reduced neutrophil and macrophage infiltration<sup>[105]</sup>. Hence, MSC-Exos are a potential adjuvant to reperfusion therapy for myocardial infarction (MI). Liu *et al.*<sup>[106]</sup> found that rat bone marrow-derived MSC-Exos significantly reduced apoptosis and the myocardial infarct size, upregulated myocardial LC3B expression, and improved cardiac function in rats with I/R injury. Also, *in vitro*, MSC-Exos reduced H<sub>2</sub>O<sub>2</sub>-induced ROS production and apoptosis and enhanced autophagy *via* the AMPK/mTOR and Akt/mTOR pathways in rat H9C2 cardiomyocytes. Moreover, rat bone marrow-derived MSC-Exos reduced MI/R injury, possibly by inhibiting apoptosis and promoting autophagy<sup>[107]</sup>. Cui *et al.*<sup>[108]</sup> reported that adipose-derived MSC-Exos significantly attenuated I/R-induced MI, decreased the serum levels of creatine kinase-myocardial band, lactate dehydrogenase, and cardiac troponin I in a rat model of MI/R. MSC-Exos antagonized I/R-induced myocardial apoptosis, upregulated Bcl-2, and downregulated Bax and caspase-3 activity in rat myocardium. Furthermore, MSC-Exos activated Wnt/ $\beta$ -catenin signaling by attenuating the I/R-induced inhibition of Wnt3a, p-GSK-3 $\beta$  (Ser9), and  $\beta$ -catenin expression.

MiRNAs associated with MSC-Exos are also important in protecting against MI/R injury. In an MI/R injury model in which H9C2 cells are subjected to hypoxia/reoxygenation, Sun *et al.*<sup>[109]</sup> found that miR-486-5p carried by bone marrow-derived MSC-Exos suppressed PTEN expression, activated the PI3K/AKT signaling pathway, and inhibited the apoptosis of injured cardiomyocytes. MiR-125b reduced the myocardial infarct area and thus ameliorated MI/R. Chen *et al.*<sup>[110]</sup> loaded miR-125 into bone marrow-derived MSC-Exos, and the resulting MSC-Exos-miR-125b significantly increased cell viability; decreased apoptosis; downregulated Bax and caspase-3; upregulated Bcl-2; decreased the levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in cardiomyocytes; and restored the cardiac function of I/R rats by regulating SIRT7. Also, miRNA-181a delivery by human umbilical cord blood-derived MSC-Exos suppressed inflammation and increased the Treg ratio by inhibiting c-Fos, thus exerting a therapeutic effect on MI/R injury<sup>[111]</sup>. Therefore, MSC-Exos facilitate the targeted delivery of small RNAs to treat MI/R injury.

**Myocardial infarction:** MI typically results in irreversible loss of myocardial cells and heart failure due to limited blood supply and is a leading cause of death worldwide.

Zhao *et al*<sup>[112]</sup> used human umbilical cord derived-MSC-Exos in an acute MI (AMI) rat model. Administration of MSC-Exos significantly improved cardiac systolic function and reduced cardiac fibrosis. Moreover, MSC-Exos protected myocardial cells from apoptosis and promoted tube formation by, and migration of, human umbilical vein endothelial cells (EA.hy926 cells). Therefore, MSC-Exos improved cardiac systolic function by protecting myocardial cells from apoptosis and promoting angiogenesis. Subsequently, the same group reported that human umbilical cord derived-MSC-Exos promote Smad7 expression by suppressing miR-125b-5p to improve myocardial repair<sup>[113]</sup>. In a follow-up study, adipose-derived MSC-Exos alleviated MI-induced cardiac damage by inhibiting cardiac dysfunction, apoptosis, fibrosis, and inflammation *in vitro* and *in vivo* by activating the S1P/SK1/S1PR1 signaling pathway and promoting M2 macrophage polarization<sup>[114]</sup>. Xu *et al*<sup>[115]</sup> reported that exosomes from adipose tissue, bone marrow, and umbilical cord blood derived-MSCs inhibited cardiomyocyte apoptosis and promoted angiogenesis, thereby improving cardiac function and protecting the myocardium in rats with MI. Notably, adipose tissue derived MSC-Exos stimulated the production of cardioprotective factors<sup>[115]</sup>.

MSC-Exos have been genetically modified to enhance their protective effect against MI. Kang *et al*<sup>[116]</sup> produced CXCR4-enriched exosomes from rat bone marrow derived MSCs overexpressing CXCR4, which promoted cardiac functional recovery by increasing angiogenesis and cell survival, reducing infarct size, and improving cardiac remodeling by activating the PI3K/Akt signaling pathway following MI. Tissue matrix metalloproteinase inhibitor 2 (TIMP2) is a member of the tissue inhibitor family of metalloproteinases. Because TIMP2-mediated inhibition of matrix metalloproteinases is an important determinant of post-MI remodeling, Ni *et al*<sup>[117]</sup> analyzed the therapeutic effects of exosomes from TIMP2-overexpressing human umbilical cord derived-MSCs (MSC-Exos<sup>TIMP2</sup>) in a rat model of MI. MSC-Exos<sup>TIMP2</sup> improved cardiac function by alleviating MI-induced oxidative stress and cardiomyocyte apoptosis, and promoting angiogenesis and ECM remodeling, in part *via* the Akt/Sfrp2 pathway. Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, plays a key role in regulating cell homeostasis. Liu *et al*<sup>[118]</sup> found that bone marrow derived-MSC-Exos<sup>MIF</sup> are superior to MSC-Exos for ameliorating MI injury; the effect was mediated by enhancing cardiac function and reducing cardiac remodeling, cardiomyocyte mitochondrial fragmentation, reactive oxygen species generation, and apoptosis.

MiRNAs associated with MSC-Exos also protect against MI. Luther *et al*<sup>[119]</sup> demonstrated that bone marrow-derived MSC-Exos expressing miR-21a-5p downregulated the expression of the pro-apoptotic gene products PDCD4, PTEN, Peli1, and FasL, and reduced cardiomyocyte death in an AMI model. Moreover, miR-301 in exosomes secreted by bone marrow derived MSCs protected against MI by inhibiting myocardial autophagy. In a follow-up study, exosomes from human MSCs transfected with the lncRNA KLF3-AS1 were injected into rats with MI. The overexpression of KLF3-AS1 in exosomes reduced the MI area, apoptosis, and pyroptosis, and attenuated MI progression by acting as a competing endogenous RNA (ceRNA) to sponge miR-138-5p that can regulate Sirt1 so as to suppress cell pyroptosis and attenuate MI progression<sup>[120]</sup>. Moreover, human umbilical cord derived MSC-Exos protected cardiomyocytes from AMI injury by transferring miR-19a, targeting SOX6, activating AKT, and inhibiting JNK3/caspase-3 activation<sup>[121]</sup>. Also, bone marrow derived exosomal miR-185 suppressed ventricular remodeling, myocardial injury, and cardiomyocyte apoptosis, and improved the cardiac function of MI mice by inhibiting SOCS2<sup>[122]</sup>.

In summary, MSC-Exos improve cardiac function and myocardial remodeling by transporting specific factors with anti-apoptotic, anti-inflammatory, antioxidant, and pro-survival effects.

### MSC-Exos in neurological diseases

**Traumatic brain injury:** Traumatic brain injury (TBI) is characterized by functional and structural impairment. There is a need for modalities that improve the recovery rate. Zhang *et al*<sup>[123]</sup> found that rat bone marrow derived MSC-Exos improved functional recovery by promoting neurovascular remodeling (angiogenesis and neurogenesis) and by reducing inflammation in rats with TBI. Thus, MSC-Exos may be beneficial for TBI and possibly other neurological diseases. Subsequently, Ni *et al*<sup>[124]</sup> investigated the neuroprotective role of rat bone marrow derived MSC-Exos on early-stage controlled cortical impact (CCI)-induced TBI. Administration of MSC-Exos reduced the lesion size and improved neurobehavioral performance; they also inhibited the expression of a pro-apoptotic protein (Bax) and proinflammatory

cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) and increased the expression of an anti-apoptotic protein (Bcl-2). MSC-Exos also decreased the activation of microglia/M1 macrophages and increased that of M2 macrophages after TBI. Therefore, MSC-Exos exert a neuroprotective effect by inhibiting early neuroinflammation in mice with CCI-induced TBI *via* modulating microglia/macrophage M2 phenotype polarization<sup>[124]</sup>. Furthermore, human bone marrow derived MSC-Exos exerted a neuroprotective effect and improved the long-term neurologic outcomes in a porcine model of TBI<sup>[125]</sup>.

**Stroke:** Stroke is one of the leading causes of death and disability worldwide. Stroke can cause highly dynamic changes in neurovascular units and promote the development of brain injury. MSC-Exos play an important role in neurological and function recovery from stroke. Xin *et al*<sup>[126-128]</sup> investigated the effect of rat bone marrow derived MSC-Exos on stroke. The intravenous administration of MSC-Exos improved functional recovery and enhanced neurite remodeling, neurogenesis, and angiogenesis<sup>[126]</sup>. Also, miR-133b in the exosomes released from MSCs is transferred to neural cells, leading to regulation of gene expression, promotion of neurite remodeling, and improvement of functional recovery in a rat model of stroke<sup>[127]</sup>. Also, Xin *et al*<sup>[128]</sup> reported that MSC-Exos enriched with the miR-17-92 cluster increased neural plasticity and functional recovery from stroke in rats, possibly by inhibiting GSK-3 $\beta$  activity and targeting PTEN to activate the PI3K/AKT/mTOR signaling pathway. In a follow-up study, adipose-derived MSC-Exos promoted angiogenesis by brain microvascular endothelial cells after OGD *via* the miR-181b-5p/TRPM7 axis, suggesting their therapeutic potential for stroke<sup>[129]</sup>. Similarly, in the OGD induced rat oligodendrocyte (OL) injury model, miR 134 in rat bone marrow-derived MSC-Exos prevented OL apoptosis by negatively regulating the caspase 8 dependent apoptosis pathway and so have therapeutic potential for ischemic stroke<sup>[130]</sup>. Moreover, mouse BMSC-Exos promoted the proliferation, and inhibited the apoptosis of, astrocytes injured by OGD, accompanied by inhibition of the expression of inflammatory factors by downregulating lipocalin 2. More importantly, MSC-derived exosomal miR-138-5p reduced neuronal injury following stroke in mice<sup>[131]</sup>.

**Spinal cord injury:** SCI is a severe central nervous system (CNS) injury for which few efficacious drugs are available. Huang *et al*<sup>[132]</sup> reported that systemic administration of rat bone marrow-derived MSC-Exos significantly attenuated lesion size, apoptosis, and inflammation, and promoted angiogenesis, thus enhancing functional recovery from SCI in rats. Therefore, MSC-Exos show potential as a cell-free therapeutic strategy for SCI. Sun *et al*<sup>[133]</sup> reported that human umbilical cord-derived MSC-Exos significantly promoted locomotor functional recovery and reduced inflammation after SCI, possibly inducing macrophage polarization from the M1 (proinflammatory) to the M2 (anti-inflammatory) phenotype. In a rat model of traumatic SCI, Liu *et al*<sup>[134]</sup> showed that injection of rat bone marrow-derived MSC-Exos attenuated neuron apoptosis, suppressed glial scar formation and inflammation, and promoted axonal regeneration and angiogenesis, ultimately enhancing functional behavioral recovery after traumatic SCI. Administration of MSC-Exos suppressed the activation of A1 neurotoxic reactive astrocytes. Furthermore, rat bone marrow-derived MSC-Exos ameliorated SCI by inhibiting complement mRNA synthesis and release and inhibiting activation of NF- $\kappa$ B signaling by binding to microglial cells<sup>[135]</sup>. Additionally, rat bone marrow-derived MSC-Exos reduced tissue damage, promoted recovery of motor function, and inhibited neural cell apoptosis after SCI by activating the Wnt/ $\beta$ -catenin signaling pathway<sup>[136]</sup>. Accordingly, bone marrow-derived MSC-Exos show therapeutic potential for acute SCI.

MSC-Exos have been genetically modified (mainly by miRNAs) to enhance the protective effect against SCI. Li *et al*<sup>[137]</sup> found that systemic injection of exosomes from miR-133b-modified rat bone marrow-derived MSCs (MSC-Exos<sup>miR-133b</sup>) resulted in transfer of miR-133b into the injured spinal cord, promoting functional recovery after SCI. Also, tail vein injection of MSC-Exos<sup>miR-133b</sup> significantly improved the recovery of hindlimb function, reduced lesion volume, preserved neurons, and promoted axon regeneration after SCI, which was attributed in part to the activation of ERK1/2, STAT3, and CREB, and the inhibition of RhoA expression. Moreover, exosomes secreted from miRNA-29b-modified rat bone marrow-derived MSCs relieved SCI in rats, possibly by regulating proteins involved in neuronal regeneration, such as NF200, GAP-43, and GFAP<sup>[138]</sup>. Two consecutive studies assessed the effect of exosomes secreted from miRNA-126-modified rat bone marrow-derived MSCs on SCI in rat<sup>[139,140]</sup>. Huang *et al*<sup>[139]</sup> indicated that MSC-Exos<sup>miR-126</sup> induce angiogenesis and neurogenesis, inhibit apoptosis, and promote functional recovery after SCI. Yuan *et al*<sup>[140]</sup> indicated that MSC-Exos<sup>miR-126</sup> protect the neurons of rats with SCI, stimulate

axon regeneration, and improve the recovery of limb motor function after SCI, in part by activating ERK1/2, STAT3, and CREB and inhibiting RhoA expression. Therefore, exosomes from miRNA-modified MSCs is a novel therapeutic approach for SCI.

**Neurodegenerative diseases:** MSC-Exos play a pivotal role in neuroprotection and neuroregeneration in diverse neurodegenerative diseases. Alzheimer disease (AD) is one of the most common neurodegenerative diseases and causes cognitive and memory disorders. Amyloid- $\beta$  (A $\beta$ ) peptide induces neuroinflammatory processes in the CNS of AD patients, leading to excessive A $\beta$  accumulation. Lee *et al*<sup>[141]</sup> reported that human adipose-derived MSC-Exos reduced  $\beta$ -amyloid pathology, and reduced apoptosis of AD neurons. In an AD mouse model, human umbilical cord-derived MSC-Exos reversed cognitive impairment and cleared A $\beta$  deposits. Also, MSC-Exos modulated microglial activation, alleviating neuroinflammation<sup>[142]</sup>. Moreover, MSC-Exos stimulated neurogenesis in the subventricular zone and alleviated beta amyloid 1-42-induced cognitive impairment in a mouse model of AD<sup>[143]</sup>. Taken together, these findings demonstrate the therapeutic potential of MSC-Exos for AD. Huntington's disease (HD) is a hereditary neurodegenerative disease caused by the aggregation of mutant Huntingtin (mHtt). Lee *et al*<sup>[144]</sup> investigated the therapeutic role of exosomes from human adipose-derived MSC-Exos in an *in vitro* model of HD. MSC-Exos significantly decreased mHtt aggregates and reduced mitochondrial dysfunction and apoptosis in R6/2 mouse-derived neurons. ALS is a fatal neurodegenerative disease characterized by selective degeneration and death of upper and lower motor neurons. Treatment of neurons from G93A ALS mice with human adipose-derived MSC-Exos alleviated aggregation of superoxide dismutase 1, and normalized the cellular phenotype, restoring to normal the levels of mitochondrial proteins including p-CREB and PGC-1 $\alpha$ <sup>[145]</sup>. Subsequently, two studies by the same group showed that mouse adipose tissue-derived MSC-Exos exerted an anti-apoptotic effect and rescued the function of mitochondria in an *in vitro* model of ALS<sup>[146,147]</sup>. MS is a chronic demyelinating disease caused by CNS inflammation and immune dysfunction, which can result in severe physical disability. Li *et al*<sup>[148]</sup> reported that in a model of immune-induced demyelination, rat bone marrow-derived MSC-Exos improved motor function and reduced demyelination and neuroinflammation in rats by regulating M2 polarization of microglia. Thus, bone marrow-derived MSC-Exos have therapeutic potential for MS.

### MSC-Exos in musculoskeletal diseases

**Osteoarthritis:** Osteoarthritis (OA) is the most common chronic degenerative OA disease. Because of the limited self-healing ability of cartilage, there is no cure for OA. Exosomes secreted by MSCs show therapeutic potential for OA. Zhu *et al*<sup>[149]</sup> compared the effect of exosomes secreted by induced pluripotent stem cell-derived MSCs (iMSC-Exos) and those secreted by synovial membrane MSCs (SMMSC-Exos) on OA. Injection of iMSC-Exos and SMMSC-Exos attenuated OA in the collagenase-induced mouse model – iMSC-Exos had a superior therapeutic effect. Wang *et al*<sup>[150]</sup> examined the therapeutic potential for OA of exosomes from human embryonic stem cell-induced MSCs. *In vitro*, MSC-Exos maintained the phenotype of IL-1 $\beta$ -induced primary mouse chondrocytes by increasing collagen type II synthesis and reducing ADAMTS5 expression. In a mouse model of destabilization of the medial meniscus induced-knee joints, MSC-Exos prevented cartilage destruction<sup>[150]</sup>. Also, mouse bone marrow-derived MSC-Exos re-established chondrocyte homeostasis, prevented chondrocyte apoptosis, and stimulated macrophage polarization toward an anti-inflammatory phenotype *in vivo*. Moreover, MSC-Exos protected against cartilage and bone degradation *in vivo*<sup>[151]</sup>. Zhang *et al*<sup>[152]</sup> demonstrated that human embryonic stem cell-derived MSC-Exos alleviated subchondral bone deterioration, suppressed inflammation, and restored matrix homeostasis in a model of temporomandibular joint OA and, ultimately, promoted temporomandibular joint repair and regeneration. Lumbar facet joint OA (LFJ-OA) is a common cause of lower-back pain (LBP). Li *et al*<sup>[153]</sup> evaluated the effect of mouse bone marrow-derived MSC-Exos in an LFJ-OA mouse model. MSC-Exos relieved pain by abrogating aberrant CGRP positive nerves and abnormal H type vessel formation in the subchondral bone. Also, MSC-Exos attenuated cartilage degeneration and suppressed tartrate resistant acid phosphatase expression and RANKL RANK TRAF6 signaling activation to facilitate subchondral bone remodeling. Therefore, bone marrow-derived MSC-Exos can ameliorate LBP and LFJ-OA.

MiRNAs and long noncoding RNAs (lncRNAs) associated with MSC-Exos also protect against OA. Tao *et al*<sup>[154]</sup> overexpressed miR-140-5p in human synovial MSCs, and the resulting MSC-Exos<sup>miR-140-5p</sup> promoted chondrocyte proliferation and migration



and restored ECM secretion by rescuing SOX9, by inhibiting RalA. In an OA rat model, MSC-Exos<sup>miR-140-5p</sup> prevented OA and the severe damage to knee articular cartilage caused by instability of the knee joint. Also, human bone marrow-derived MSC-Exos increased the expression of the chondrogenic genes type II collagen alpha 1 and aggrecan and decreased that of the chondrocyte hypertrophy markers matrix metalloproteinase-13 and Runx2 (runt-related transcription factor 2) in chondrocytes from mice with OA. Furthermore, MSC-Exos attenuated the IL-1 $\beta$ -induced inhibition of chondrocyte proliferation and apoptosis *via* the lncRNA-KLF3-AS1/miR-206/GIT1 axis<sup>[155]</sup>. Liu *et al*<sup>[156]</sup> investigated the effect of human MSC-Exos on IL-1 $\beta$ -induced OA chondrocytes *in vitro* and in a collagenase-induced rat model of OA *in vivo*. The lncRNA KLF3-AS1 was markedly enriched in MSC-Exos, which ameliorated IL-1 $\beta$ -induced cartilage injury and suppressed IL-1 $\beta$ -induced apoptosis of chondrocytes *in vitro*. Also, the exosomal lncRNA KLF3-AS1 promoted cartilage repair and chondrocyte proliferation in a rat model of OA *in vivo*. Infrapatellar fat pad (IPFP)-derived MSC-Exos ameliorated OA *in vivo* and inhibited apoptosis, enhanced matrix synthesis, and reduced the expression of catabolic factors *in vitro*<sup>[157]</sup>. In addition, IPFP-MSC-Exos partially inhibited mTOR and significantly enhanced autophagy in chondrocytes. However, intra-articular injection of miR-100-5p antagonists significantly suppressed the IPFP-MSC-Exos-mediated protection of articular cartilage *in vivo*. In summary, IPFP-MSC-Exos improve OA by maintaining cartilage homeostasis, which is likely to be mediated by inhibiting miR-100-5p-regulated mTOR-dependent autophagy<sup>[157]</sup>. Moreover, human bone marrow-derived MSC-Exos carrying miR-26a-5p inhibited inflammation, proliferation, and migration and promoted apoptosis, thus attenuating OA progression<sup>[158]</sup>.

**Osteoporosis:** Osteoporosis is an age-related disease that results from an imbalance between bone formation and resorption and is characterized by systemic damage to bone mass and microstructure, ultimately increasing the risk of fragile fractures. Osteoporosis is particularly associated with postmenopausal estrogen deficiency. Qi *et al*<sup>[159]</sup> reported that *in vitro*, human induced pluripotent stem cell-derived MSC-Exos enhanced cell proliferation and alkaline phosphatase activity and upregulated the mRNA and protein levels of osteoblast-related factors in bone marrow MSCs from ovariectomized rats. *In vivo*, MSC-Exos stimulated bone regeneration and angiogenesis in critical-sized calvarial defects in ovariectomized rats. Zhao *et al*<sup>[160]</sup> investigated the effect of rat bone marrow-derived MSC-Exos on osteoblasts *in vitro*. Co-culture with MSC-Exos promoted the proliferation of hFOB 1.19 osteoblasts cells *via* the MAPK signaling pathway, alleviating the progression of osteoporosis. Rescue experiments indicated that MSC-Exos promoted the growth and cell cycle of hFOB 1.19 cells; these effects were reversed by p-JNK knockdown. Yang *et al*<sup>[161]</sup> showed that the human bone marrow-derived MSC-derived exosomal lncRNA MALAT1 enhanced osteogenic activity and alleviated symptoms of osteoporosis in a mouse model by acting as a miR-34c sponge to upregulate SATB2 expression. Radiotherapy for cancer causes damage to normal tissue, including bone. Radiation-induced bone marrow-derived MSC damage is the main cause of radiation-induced bone loss. Zuo *et al*<sup>[162]</sup> investigated the ability of bone marrow-derived MSC-Exos to restore the function of recipient bone marrow-derived MSCs and alleviate radiation-induced bone loss. MSC-Exos attenuated radiation-induced bone loss in a rat model by reducing oxidative stress, accelerating DNA damage repair, promoting proliferation, and increasing the levels of senescence-associated proteins.

### **MSC-Exos in cutaneous wound healing**

Skin wound healing is a complex pathophysiological process involving multiple cells and cytokines. MSC-Exos can accelerate skin healing and reduce excessive scar formation. Zhang *et al*<sup>[163]</sup> investigated the use of human induced pluripotent stem cell-derived MSC-Exos in cutaneous wound healing. Transplanting MSC-Exos to wound sites accelerated re-epithelialization, reduced scar width, and promoted collagen maturity. In addition, MSC-Exos not only promoted the formation of new blood vessels but also accelerated the maturation of the skin wound in a rat model. Also, MSC-Exos stimulated the proliferation and migration of human dermal fibroblasts and human umbilical vein endothelial cells and promoted the secretion of types I and III collagen and elastin<sup>[163]</sup>. Moreover, human umbilical cord-derived MSC-Exos significantly accelerated re-epithelialization, and increased expression of CK19, PCNA, and collagen I (compared to collagen III) *in vivo* in a rat model of skin burn. *In vivo* studies confirmed that MSC-Exos-mediated activation of Wnt/ $\beta$ -catenin promotes wound re-epithelialization and cell proliferation. Disruption of Wnt4 expression in MSC-Exos reduced the therapeutic effect *in vivo*<sup>[164]</sup>. Hu *et al*<sup>[165]</sup> investigated the roles of

human adipose-derived MSC-Exos in cutaneous wound healing. MSC-Exos were taken up and internalized by fibroblasts and stimulated their migration, proliferation, and collagen synthesis in a dose-dependent manner; also, the expression of N-cadherin, cyclin-1, PCNA, and collagens I and III was increased. Systemic administration of MSC-Exos increased collagens I and III production during the early stages of wound healing, while MSC-Exos inhibited collagen expression to reduce scar formation in the later stages. Therefore, MSC-Exos promote cutaneous wound healing by optimizing the characteristics of fibroblasts<sup>[167]</sup>. Ma *et al.*<sup>[166]</sup> exposed HaCaT keratinocytes to H<sub>2</sub>O<sub>2</sub> to establish a skin lesion model. Human adipose-derived MSC-Exos promoted the proliferation and migration of HaCaT cells and inhibited their apoptosis. In addition, activation of Wnt/ $\beta$ -catenin signaling was confirmed by an increased  $\beta$ -catenin protein level. Therefore, MSC-Exos promote cutaneous wound healing by modulating Wnt/ $\beta$  catenin signaling<sup>[168]</sup>. He *et al.*<sup>[167]</sup> found that human bone marrow-derived MSC-Exos accelerated cutaneous wound healing by inducing M2 polarization of macrophages in part by transferring donor exosome-derived miRNAs. Therefore, the miRNAs in MSC-Exos could be applied to enhance the healing of cutaneous wounds.

Diabetic foot ulcer (DFU) is a catastrophic medical problem caused by diabetes, which affects 15% of people with diabetes and increases the risk of amputation. MSC-Exos reportedly accelerate cutaneous wound healing in DFU. In the study of Dalirfardouei *et al.*<sup>[168]</sup>, a full-thickness excisional wound was established on the dorsal skin of streptozotocin induced diabetic mice. Menstrual blood derived MSC-Exos enhanced neoangiogenesis by upregulating vascular endothelial growth factor A, inhibited inflammation by inducing M1-M2 macrophage polarization, accelerated re-epithelialization, and reduced scar formation by decreasing the Col1:Col3 ratio. Therefore, menstrual blood derived MSC-Exos ameliorated cutaneous nonhealing DFUs. Moreover, Li *et al.*<sup>[169]</sup> showed that bone marrow-derived MSC-Exos carrying lncRNA H19 promoted wound healing in mice with DFU by promoting fibroblast proliferation and migration and suppressing apoptosis and inflammation by inhibiting miR-152-3p and promoting PTEN expression.

## CLINICAL STUDIES WITH MSC-EXOS

Although MSC-Exos had shown good clinical application prospects in preclinical studies, a limited number of human clinical studies are already available on the use of MSC-Exos products according to the ClinicalTrials.gov (Table 3). Among them, determining the optimal dose, the appropriate time window for MSC-Exos administration, and the route of administration to achieve maximum efficacy without side effects are the most important issues<sup>[170]</sup>. A preliminary study demonstrated that increasing dosage of MSC-Exos in a patient with severe treatment-refractory graft-*vs*-host grade IV disease, affecting the skin and intestinal tract, was well tolerated and showed a significant and sustainable improvement of symptoms, which remained stable for 5 mo<sup>[171]</sup>. Another clinical trial applied umbilical cord-blood-derived MSC-Exos for improving  $\beta$ -cell mass in type 1 diabetes mellitus patients (NCT02138331). Many more studies are expected to be initiated shortly (Table 3).

## LIMITATIONS OF MSC-EXOS

MSC-Exos are effective and safe for regenerative medicine. However, MSC-Exos have several limitations that should be mentioned<sup>[44,172]</sup>. First, ultracentrifugation can damage or destroy exosomes and the product is typically of low purity. There is no standardized technique for the isolation, quantification, and purification of MSC-Exos. The difficulty of extracting and purifying exosomes increases the cost of their application. Second, the potential of MSC-Exos in tissue repair and regeneration is unclear, as are the components/properties of MSC-Exos that promote tissue regeneration. Also, how to determine the amount of MSC-Exos needed for treatment is unknown, as is whether excess MSC-Exos cause irreversible tissue damage. Third, there is no guidance, supervision, or safety assessment of MSC-Exos. Fourth, it is worth exploring whether MSC exosomes are effective when administered systemically by the intravenous, subcutaneous, or intramuscular route<sup>[173]</sup>. As an emerging therapeutic agent, the safety, challenges, and risks of MSC-Exos need to be evaluated for their use in tissue repair and regenerative medicine.

Table 3 Clinical studies with mesenchymal stem cell-derived exosomes

Study title	Disease	Intervention	Phase	NCT
Allogenic mesenchymal stem cell derived exosome in patients with acute ischemic stroke	Cerebrovascular disorders	Biological: Exosome	Completed	NCT03384433
A pilot clinical study on inhalation of mesenchymal stem cells exosomes treating severe novel coronavirus pneumonia	Coronavirus	Biological: MSCs-derived exosomes	Phase 1	NCT04276987
Effect of microvesicles and exosomes therapy on $\beta$ -cell mass in type I diabetes mellitus (T1DM)	Diabetes mellitus type 1	Biological: MSC Exosomes	Phase 2, Phase 3	NCT02138331
iExosomes in treating participants with metastatic pancreas cancer with KrasG12D mutation	Metastatic pancreatic adenocarcinoma	Drug: Mesenchymal stromal cells-derived exosomes with KRAS G12D siRNA	Phase 1	NCT03608631
Effect of UMSCs Derived exosomes on dry eye in patients with cGVHD	Dry eye	Drug: Umbilical mesenchymal stem cells derived exosomes	Phase 1, Phase 2	NCT04213248
Evaluation of adipose derived stem cells exo.in treatment of periodontitis	Periodontitis	Biological: Adipose derived stem cells exosomes	Early phase 1	NCT04270006
A tolerance clinical study on aerosol inhalation of mesenchymal stem cells exosomes in healthy volunteers	Healthy	Biological: Low level of MSCs-Exo Biological: High level of MSCs-Exo	Phase 1	NCT04313647
MSC-Exos promote healing of MHs	Macular holes	Biological: Exosomes derived from mesenchymal stem cells (MSC-Exo)	Early Phase 1	NCT03437759

## CONCLUSION

MSC-based therapies are widely used worldwide, and the mechanisms underlying their effects may include induced differentiation, immune regulation, cell fusion, paracrine effects, carriage of mRNA or miRNA, and mitochondrial metastasis. MSC-Exos have therapeutic potential because they have most of the therapeutic effects of MSCs themselves. Exosomes can cross biological barriers, can be modified to load molecular drugs, have few side effects and are relatively non-immunogenic, and maintain their activity during storage. MSC-Exos have received attention because of their ability to, for example, promote tissue regeneration, suppress inflammation, and regulate the immune system. In addition, MSC-Exos do not have the safety implications of injecting live cells. The therapeutic efficacy of MSC-Exos against diseases of the kidney, liver, heart, brain, muscle, and skin has been demonstrated, and further research will enable their large-scale production.

The following issues related to MSC-Exos need to be overcome: (1) Lack of standardization of molecular characteristics, comparability, and reproducibility, and difficulty in obtaining high-purity exosomes of defined size; (2) Biodistribution, toxicity, and clearance of MSC-Exos after injection, and verification of their safety; and (3) Most studies of MSC-Exos are short-term, and so their long-term therapeutic effect is unknown, as is the safe dose for humans.

To promote the clinical application of exosomes, we suggest that: (1) Guidelines and standards for use of MSC-Exos are needed; (2) The pathways and recognition signals of MSC-Exos for target cells and organs need to be identified. Exosomal surface molecules can be modified to target MSC-Exos to particular cell types; (3) A standard method of collecting and enriching exosomes is needed, and the purity of MSC-Exos needs to be increased to enhance their therapeutic efficacy; and (4) Because they can pass biological barriers, loading of genes or drugs in MSC-Exos facilitates their targeted delivery.

In summary, MSC-Exos are theoretically superior to intact MSCs for regenerative medicine. However, a series of challenges and difficulties need to be addressed so that the therapeutic potential of exosomes can be realized.

## REFERENCES

- Graf T. Differentiation plasticity of hematopoietic cells. *Blood* 2002; **99**: 3089-3101 [PMID: 11964270 DOI: 10.1182/blood.v99.9.3089]
- Watt FM, Hogan BL. Out of Eden: stem cells and their niches. *Science* 2000; **287**: 1427-1430 [PMID: 10688781 DOI: 10.1126/science.287.5457.1427]
- Rüster B, Göttig S, Ludwig RJ, Bistran R, Müller S, Seifried E, Gille J, Henschler R. Mesenchymal stem

- cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 2006; **108**: 3938-3944 [PMID: 16896152 DOI: 10.1182/blood-2006-05-025098]
- 4 **Jiang W**, Ma A, Wang T, Han K, Liu Y, Zhang Y, Zhao X, Dong A, Du Y, Huang X, Wang J, Lei X, Zheng X. Intravenous transplantation of mesenchymal stem cells improves cardiac performance after acute myocardial ischemia in female rats. *Transpl Int* 2006; **19**: 570-580 [PMID: 16764636 DOI: 10.1111/j.1432-2277.2006.00307.x]
  - 5 **Gnecchi M**, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* 2008; **103**: 1204-1219 [PMID: 19028920 DOI: 10.1161/CIRCRESAHA.108.176826]
  - 6 **Abu-Rmeileh NM**, Hussein A, Capewell S, O'Flaherty M; MEDCHAMPS project. Preventing type 2 diabetes among Palestinians: comparing five future policy scenarios. *BMJ Open* 2013; **3**: e003558 [PMID: 24362011 DOI: 10.1136/bmjopen-2013-003558]
  - 7 **Friedenstein AJ**, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970; **3**: 393-403 [PMID: 5523063 DOI: 10.1111/j.1365-2184.1970.tb00347.x]
  - 8 **Teixeira FG**, Carvalho MM, Sousa N, Salgado AJ. Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration? *Cell Mol Life Sci* 2013; **70**: 3871-3882 [PMID: 23456256 DOI: 10.1007/s00018-013-1290-8]
  - 9 **Squillaro T**, Peluso G, Galderisi U. Clinical Trials With Mesenchymal Stem Cells: An Update. *Cell Transplant* 2016; **25**: 829-848 [PMID: 26423725 DOI: 10.3727/096368915X689622]
  - 10 **Crisan M**, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhning HJ, Giacobino JP, Lazzari L, Huard J, Péault B. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008; **3**: 301-313 [PMID: 18786417 DOI: 10.1016/j.stem.2008.07.003]
  - 11 **Karimineko S**, Movassaghpour A, Rahimzadeh A, Talebi M, Shamsasenjan K, Akbarzadeh A. Implications of mesenchymal stem cells in regenerative medicine. *Artif Cells Nanomed Biotechnol* 2016; **44**: 749-757 [PMID: 26757594 DOI: 10.3109/21691401.2015.1129620]
  - 12 **Wang S**, Guo L, Ge J, Yu L, Cai T, Tian R, Jiang Y, Zhao RCh, Wu Y. Excess Integrins Cause Lung Entrapment of Mesenchymal Stem Cells. *Stem Cells* 2015; **33**: 3315-3326 [PMID: 26148841 DOI: 10.1002/stem.2087]
  - 13 **Fennema EM**, Tchang LAH, Yuan H, van Blitterswijk CA, Martin I, Scherberich A, de Boer J. Ectopic bone formation by aggregated mesenchymal stem cells from bone marrow and adipose tissue: A comparative study. *J Tissue Eng Regen Med* 2018; **12**: e150-e158 [PMID: 28485099 DOI: 10.1002/term.2453]
  - 14 **Kusuma GD**, Menicanin D, Gronthos S, Manuelpillai U, Abumaree MH, Pertile MD, Brennecke SP, Kalonits B. Ectopic Bone Formation by Mesenchymal Stem Cells Derived from Human Term Placenta and the Decidua. *PLoS One* 2015; **10**: e0141246 [PMID: 26484666 DOI: 10.1371/journal.pone.0141246]
  - 15 **Jeong JO**, Han JW, Kim JM, Cho HJ, Park C, Lee N, Kim DW, Yoon YS. Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. *Circ Res* 2011; **108**: 1340-1347 [PMID: 21493893 DOI: 10.1161/CIRCRESAHA.110.239848]
  - 16 **Caplan AI**, Dennis JE. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 2006; **98**: 1076-1084 [PMID: 16619257 DOI: 10.1002/jcb.20886]
  - 17 **Spees JL**, Lee RH, Gregory CA. Mechanisms of mesenchymal stem/stromal cell function. *Stem Cell Res Ther* 2016; **7**: 125 [PMID: 27581859 DOI: 10.1186/s13287-016-0363-7]
  - 18 **Zhou Y**, Yamamoto Y, Xiao Z, Ochiya T. The Immunomodulatory Functions of Mesenchymal Stromal/Stem Cells Mediated via Paracrine Activity. *J Clin Med* 2019; **8** [PMID: 31336889 DOI: 10.3390/jcm8071025]
  - 19 **Rani S**, Ryan AE, Griffin MD, Ritter T. Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications. *Mol Ther* 2015; **23**: 812-823 [PMID: 25868399 DOI: 10.1038/mt.2015.44]
  - 20 **Heldring N**, Mäger I, Wood MJ, Le Blanc K, Andaloussi SE. Therapeutic Potential of Multipotent Mesenchymal Stromal Cells and Their Extracellular Vesicles. *Hum Gene Ther* 2015; **26**: 506-517 [PMID: 26153722 DOI: 10.1089/hum.2015.072]
  - 21 **Schorey JS**, Bhatnagar S. Exosome function: from tumor immunology to pathogen biology. *Traffic* 2008; **9**: 871-881 [PMID: 18331451 DOI: 10.1111/j.1600-0854.2008.00734.x]
  - 22 **Zhang W**, Wang Y, Kong J, Dong M, Duan H, Chen S. Therapeutic efficacy of neural stem cells originating from umbilical cord-derived mesenchymal stem cells in diabetic retinopathy. *Sci Rep* 2017; **7**: 408 [PMID: 28341839 DOI: 10.1038/s41598-017-00298-2]
  - 23 **Lou G**, Chen Z, Zheng M, Liu Y. Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. *Exp Mol Med* 2017; **49**: e346 [PMID: 28620221 DOI: 10.1038/emmm.2017.63]
  - 24 **Bagno L**, Hatzistergos KE, Balkan W, Hare JM. Mesenchymal Stem Cell-Based Therapy for Cardiovascular Disease: Progress and Challenges. *Mol Ther* 2018; **26**: 1610-1623 [PMID: 29807782 DOI: 10.1016/j.ymthe.2018.05.009]
  - 25 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]
  - 26 **Aurich H**, Sgoddard M, Kaltwasser P, Vetter M, Weise A, Liehr T, Brulport M, Hengstler JG, Dollinger MM, Fleig WE, Christ B. Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue in vitro promotes hepatic integration in vivo. *Gut* 2009; **58**: 570-581 [PMID: 19022918 DOI: 10.1136/gut.2008.154880]
  - 27 **Karp JM**, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* 2009; **4**: 206-216 [PMID: 19265660 DOI: 10.1016/j.stem.2009.02.001]
  - 28 **Castro-Manreza ME**, Montesinos JJ. Immunoregulation by mesenchymal stem cells: biological aspects and clinical applications. *J Immunol Res* 2015; **2015**: 394917 [PMID: 25961059 DOI: 10.1155/2015/394917]



- 10.1155/2015/394917]
- 29 **Wang J**, Liao L, Tan J. Mesenchymal-stem-cell-based experimental and clinical trials: current status and open questions. *Expert Opin Biol Ther* 2011; **11**: 893-909 [PMID: [21449634](#) DOI: [10.1517/14712598.2011.574119](#)]
  - 30 **Li J**, Li D, Liu X, Tang S, Wei F. Human umbilical cord mesenchymal stem cells reduce systemic inflammation and attenuate LPS-induced acute lung injury in rats. *J Inflamm (Lond)* 2012; **9**: 33 [PMID: [22974286](#) DOI: [10.1186/1476-9255-9-33](#)]
  - 31 **Tsang KS**, Ng CPS, Zhu XL, Wong GKC, Lu G, Ahuja AT, Wong KSL, Ng HK, Poon WS. Phase I/II randomized controlled trial of autologous bone marrow-derived mesenchymal stem cell therapy for chronic stroke. *World J Stem Cells* 2017; **9**: 133-143 [PMID: [28928910](#) DOI: [10.4252/wjsc.v9.i8.133](#)]
  - 32 **Li M**, Luo X, Lv X, Liu V, Zhao G, Zhang X, Cao W, Wang R, Wang W. In vivo human adipose-derived mesenchymal stem cell tracking after intra-articular delivery in a rat osteoarthritis model. *Stem Cell Res Ther* 2016; **7**: 160 [PMID: [27832815](#) DOI: [10.1186/s13287-016-0420-2](#)]
  - 33 **Shi M**, Liu ZW, Wang FS. Immunomodulatory properties and therapeutic application of mesenchymal stem cells. *Clin Exp Immunol* 2011; **164**: 1-8 [PMID: [21352202](#) DOI: [10.1111/j.1365-2249.2011.04327.x](#)]
  - 34 **González MA**, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009; **136**: 978-989 [PMID: [19135996](#) DOI: [10.1053/j.gastro.2008.11.041](#)]
  - 35 **Chen X**, Liu Q, Xiang AP. CD8+CD28- T cells: not only age-related cells but a subset of regulatory T cells. *Cell Mol Immunol* 2018; **15**: 734-736 [PMID: [29375130](#) DOI: [10.1038/cmi.2017.153](#)]
  - 36 **Corcione A**, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Risso M, Gualandi F, Mancardi GL, Pistoia V, Uccelli A. Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006; **107**: 367-372 [PMID: [16141348](#) DOI: [10.1182/blood-2005-07-2657](#)]
  - 37 **Spaggiari GM**, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 2008; **111**: 1327-1333 [PMID: [17951526](#) DOI: [10.1182/blood-2007-02-074997](#)]
  - 38 **Jiang XX**, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, Mao N. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005; **105**: 4120-4126 [PMID: [15692068](#) DOI: [10.1182/blood-2004-02-0586](#)]
  - 39 **Joyce N**, Annett G, Wirthlin L, Olson S, Bauer G, Nolta JA. Mesenchymal stem cells for the treatment of neurodegenerative disease. *Regen Med* 2010; **5**: 933-946 [PMID: [21082892](#) DOI: [10.2217/rme.10.72](#)]
  - 40 **Munoz JR**, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. *Proc Natl Acad Sci USA* 2005; **102**: 18171-18176 [PMID: [16330757](#) DOI: [10.1073/pnas.0508945102](#)]
  - 41 **Pollock K**, Dahlenburg H, Nelson H, Fink KD, Cary W, Hendrix K, Annett G, Torrest A, Deng P, Gutierrez J, Nacey C, Pepper K, Kalomoiris S, D Anderson J, McGee J, Gruenloh W, Fury B, Bauer G, Duffy A, Tempkin T, Wheelock V, Nolta JA. Human Mesenchymal Stem Cells Genetically Engineered to Overexpress Brain-derived Neurotrophic Factor Improve Outcomes in Huntington's Disease Mouse Models. *Mol Ther* 2016; **24**: 965-977 [PMID: [26765769](#) DOI: [10.1038/mt.2016.12](#)]
  - 42 **Haynesworth SE**, Baber MA, Caplan AI. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. *J Cell Physiol* 1996; **166**: 585-592 [PMID: [8600162](#) DOI: [10.1002/\(SICI\)1097-4652\(199603\)166:3<585::AID-JCP13>3.0.CO;2-6](#)]
  - 43 **Liang X**, Ding Y, Zhang Y, Tse HF, Lian Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant* 2014; **23**: 1045-1059 [PMID: [23676629](#) DOI: [10.3727/096368913X667709](#)]
  - 44 **Sun B**, Peng J, Wang S, Liu X, Zhang K, Zhang Z, Wang C, Jing X, Zhou C, Wang Y. Applications of stem cell-derived exosomes in tissue engineering and neurological diseases. *Rev Neurosci* 2018; **29**: 531-546 [PMID: [29267178](#) DOI: [10.1515/revneuro-2017-0059](#)]
  - 45 **Pan BT**, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* 1983; **33**: 967-978 [PMID: [6307529](#) DOI: [10.1016/0092-8674\(83\)90040-5](#)]
  - 46 **Johnstone RM**, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* 1987; **262**: 9412-9420 [PMID: [3597417](#)]
  - 47 **Zerlinger E**, Barta T, Li M, Vlassov AV. Strategies for isolation of exosomes. *Cold Spring Harb Protoc* 2015; **2015**: 319-323 [PMID: [25834266](#) DOI: [10.1101/pdb.top074476](#)]
  - 48 **Colombo M**, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 2014; **30**: 255-289 [PMID: [25288114](#) DOI: [10.1146/annurev-cellbio-101512-122326](#)]
  - 49 **Théry C**, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006; **Chapter 3**: Unit 3.22 [PMID: [18228490](#) DOI: [10.1002/0471143030.cb0322s30](#)]
  - 50 **Raposo G**, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 1996; **183**: 1161-1172 [PMID: [8642258](#) DOI: [10.1084/jem.183.3.1161](#)]
  - 51 **Heijnen HF**, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 1999; **94**: 3791-3799 [PMID: [10572093](#)]
  - 52 **Casado S**, Lobo MDVT, Paíno CL. Dynamics of plasma membrane surface related to the release of extracellular vesicles by mesenchymal stem cells in culture. *Sci Rep* 2017; **7**: 6767 [PMID: [28754913](#) DOI: [10.1038/s41598-017-07265-x](#)]
  - 53 **Kahroba H**, Hejazi MS, Samadi N. Exosomes: from carcinogenesis and metastasis to diagnosis and treatment of gastric cancer. *Cell Mol Life Sci* 2019; **76**: 1747-1758 [PMID: [30734835](#) DOI: [10.1007/s00018-019-03035-2](#)]

- 54 **Hanson PI**, Cashikar A. Multivesicular body morphogenesis. *Annu Rev Cell Dev Biol* 2012; **28**: 337-362 [PMID: 22831642 DOI: 10.1146/annurev-cellbio-092910-154152]
- 55 **Kosaka N**, Iguchi H, Hagiwara K, Yoshioka Y, Takeshita F, Ochiya T. Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. *J Biol Chem* 2013; **288**: 10849-10859 [PMID: 23439645 DOI: 10.1074/jbc.M112.446831]
- 56 **Hurley JH**, Odorizzi G. Get on the exosome bus with ALIX. *Nat Cell Biol* 2012; **14**: 654-655 [PMID: 22743708 DOI: 10.1038/ncb2530]
- 57 **Tauro BJ**, Greening DW, Mathias RA, Ji H, Mathivanan S, Scott AM, Simpson RJ. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods* 2012; **56**: 293-304 [PMID: 22285593 DOI: 10.1016/j.ymeth.2012.01.002]
- 58 **Li P**, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics* 2017; **7**: 789-804 [PMID: 28255367 DOI: 10.7150/thno.18133]
- 59 **Yu LL**, Zhu J, Liu JX, Jiang F, Ni WK, Qu LS, Ni RZ, Lu CH, Xiao MB. A Comparison of Traditional and Novel Methods for the Separation of Exosomes from Human Samples. *Biomed Res Int* 2018; **2018**: 3634563 [PMID: 30148165 DOI: 10.1155/2018/3634563]
- 60 **Yamamoto KR**, Alberts BM, Benzinger R, Lawhorne L, Treiber G. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* 1970; **40**: 734-744 [PMID: 4908735 DOI: 10.1016/0042-6822(70)90218-7]
- 61 **Rider MA**, Hurwitz SN, Meckes DG Jr. ExtraPEG: A Polyethylene Glycol-Based Method for Enrichment of Extracellular Vesicles. *Sci Rep* 2016; **6**: 23978 [PMID: 27068479 DOI: 10.1038/srep23978]
- 62 **Sokolova V**, Ludwig AK, Hornung S, Rotan O, Horn PA, Epple M, Giebel B. Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. *Colloids Surf B Biointerfaces* 2011; **87**: 146-150 [PMID: 21640565 DOI: 10.1016/j.colsurfb.2011.05.013]
- 63 **Xu Y**, Shen L, Li F, Yang J, Wan X, Ouyang M. microRNA-16-5p-containing exosomes derived from bone marrow-derived mesenchymal stem cells inhibit proliferation, migration, and invasion, while promoting apoptosis of colorectal cancer cells by downregulating ITGA2. *J Cell Physiol* 2019; **234**: 21380-21394 [PMID: 31102273 DOI: 10.1002/jcp.28747]
- 64 **Liu Y**, Song B, Wei Y, Chen F, Chi Y, Fan H, Liu N, Li Z, Han Z, Ma F. Exosomes from mesenchymal stromal cells enhance imatinib-induced apoptosis in human leukemia cells via activation of caspase signaling pathway. *Cytotherapy* 2018; **20**: 181-188 [PMID: 29269240 DOI: 10.1016/j.jcyt.2017.11.006]
- 65 **Yaghoubi Y**, Movassaghpour A, Zamani M, Talebi M, Mehdizadeh A, Yousefi M. Human umbilical cord mesenchymal stem cells derived-exosomes in diseases treatment. *Life Sci* 2019; **233**: 116733 [PMID: 31394127 DOI: 10.1016/j.lfs.2019.116733]
- 66 **Fu M**, Gu J, Jiang P, Qian H, Xu W, Zhang X. Exosomes in gastric cancer: roles, mechanisms, and applications. *Mol Cancer* 2019; **18**: 41 [PMID: 30876419 DOI: 10.1186/s12943-019-1001-7]
- 67 **Ohno S**, Ishikawa A, Kuroda M. Roles of exosomes and microvesicles in disease pathogenesis. *Adv Drug Deliv Rev* 2013; **65**: 398-401 [PMID: 22981801 DOI: 10.1016/j.addr.2012.07.019]
- 68 **Kowal J**, Tkach M, Théry C. Biogenesis and secretion of exosomes. *Curr Opin Cell Biol* 2014; **29**: 116-125 [PMID: 24959705 DOI: 10.1016/j.ccb.2014.05.004]
- 69 **Choi JY**, Kim S, Kwak HB, Park DH, Park JH, Ryu JS, Park CS, Kang JH. Extracellular Vesicles as a Source of Urological Biomarkers: Lessons Learned From Advances and Challenges in Clinical Applications to Major Diseases. *Int Neurol J* 2017; **21**: 83-96 [PMID: 28673066 DOI: 10.5213/inj.1734961.458]
- 70 **Stremersch S**, De Smedt SC, Raemdonck K. Therapeutic and diagnostic applications of extracellular vesicles. *J Control Release* 2016; **244**: 167-183 [PMID: 27491882 DOI: 10.1016/j.jconrel.2016.07.054]
- 71 **Azmi AS**, Bao B, Sarkar FH. Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer Metastasis Rev* 2013; **32**: 623-642 [PMID: 23709120 DOI: 10.1007/s10555-013-9441-9]
- 72 **Lai RC**, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010; **4**: 214-222 [PMID: 20138817 DOI: 10.1016/j.scr.2009.12.003]
- 73 **Yu B**, Zhang X, Li X. Exosomes derived from mesenchymal stem cells. *Int J Mol Sci* 2014; **15**: 4142-4157 [PMID: 24608926 DOI: 10.3390/ijms15034142]
- 74 **Joo HS**, Suh JH, Lee HJ, Bang ES, Lee JM. Current Knowledge and Future Perspectives on Mesenchymal Stem Cell-Derived Exosomes as a New Therapeutic Agent. *Int J Mol Sci* 2020; **21** [PMID: 31979113 DOI: 10.3390/ijms21030727]
- 75 **Hong P**, Yang H, Wu Y, Li K, Tang Z. The functions and clinical application potential of exosomes derived from adipose mesenchymal stem cells: a comprehensive review. *Stem Cell Res Ther* 2019; **10**: 242 [PMID: 31391108 DOI: 10.1186/s13287-019-1358-y]
- 76 **Mendt M**, Kamekar S, Sugimoto H, McAndrews KM, Wu CC, Gagea M, Yang S, Blanko EVR, Peng Q, Ma X, Marszalek JR, Maitra A, Yee C, Rezvani K, Shpall E, LeBleu VS, Kalluri R. Generation and testing of clinical-grade exosomes for pancreatic cancer. *JCI Insight* 2018; **3** [PMID: 29669940 DOI: 10.1172/jci.insight.99263]
- 77 **Marote A**, Teixeira FG, Mendes-Pinheiro B, Salgado AJ. MSCs-Derived Exosomes: Cell-Secreted Nanovesicles with Regenerative Potential. *Front Pharmacol* 2016; **7**: 231 [PMID: 27536241 DOI: 10.3389/fphar.2016.00231]
- 78 **Sun D**, Zhuang X, Xiang X, Liu Y, Zhang S, Liu C, Barnes S, Grizzle W, Miller D, Zhang HG. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol Ther* 2010; **18**: 1606-1614 [PMID: 20571541 DOI: 10.1038/mt.2010.105]
- 79 **Srisawat N**, Kellum JA. Acute kidney injury: definition, epidemiology, and outcome. *Curr Opin Crit Care* 2011; **17**: 548-555 [PMID: 22027404 DOI: 10.1097/MCC.0b013e32834cd349]
- 80 **Wang R**, Lin M, Li L, Li L, Qi G, Rong R, Xu M, Zhu T. [Bone marrow mesenchymal stem cell-derived exosome protects kidney against ischemia reperfusion injury in rats]. *Zhonghua Yi Xue Za Zhi* 2014; **94**: 3298-3303 [PMID: 25622627]

- 81 **Shen B**, Liu J, Zhang F, Wang Y, Qin Y, Zhou Z, Qiu J, Fan Y. CCR2 Positive Exosome Released by Mesenchymal Stem Cells Suppresses Macrophage Functions and Alleviates Ischemia/Reperfusion-Induced Renal Injury. *Stem Cells Int* 2016; **2016**: 1240301 [PMID: [27843457](#) DOI: [10.1155/2016/1240301](#)]
- 82 **Zhu G**, Pei L, Lin F, Yin H, Li X, He W, Liu N, Gou X. Exosomes from human-bone-marrow-derived mesenchymal stem cells protect against renal ischemia/reperfusion injury via transferring miR-199a-3p. *J Cell Physiol* 2019; **234**: 23736-23749 [PMID: [31180587](#) DOI: [10.1002/jcp.28941](#)]
- 83 **Zhou Y**, Xu H, Xu W, Wang B, Wu H, Tao Y, Zhang B, Wang M, Mao F, Yan Y, Gao S, Gu H, Zhu W, Qian H. Exosomes released by human umbilical cord mesenchymal stem cells protect against cisplatin-induced renal oxidative stress and apoptosis in vivo and in vitro. *Stem Cell Res Ther* 2013; **4**: 34 [PMID: [23618405](#) DOI: [10.1186/scrt194](#)]
- 84 **Wang B**, Jia H, Zhang B, Wang J, Ji C, Zhu X, Yan Y, Yin L, Yu J, Qian H, Xu W. Pre-incubation with hucMSC-exosomes prevents cisplatin-induced nephrotoxicity by activating autophagy. *Stem Cell Res Ther* 2017; **8**: 75 [PMID: [28388958](#) DOI: [10.1186/s13287-016-0463-4](#)]
- 85 **Jia H**, Liu W, Zhang B, Wang J, Wu P, Tandra N, Liang Z, Ji C, Yin L, Hu X, Yan Y, Mao F, Zhang X, Yu J, Xu W, Qian H. HucMSC exosomes-delivered 14-3-3 $\zeta$  enhanced autophagy via modulation of ATG16L in preventing cisplatin-induced acute kidney injury. *Am J Transl Res* 2018; **10**: 101-113 [PMID: [29422997](#)]
- 86 **Zhu F**, Chong Lee Shin OLS, Pei G, Hu Z, Yang J, Zhu H, Wang M, Mou J, Sun J, Wang Y, Yang Q, Zhao Z, Xu H, Gao H, Yao W, Luo X, Liao W, Xu G, Zeng R, Yao Y. Adipose-derived mesenchymal stem cells employed exosomes to attenuate AKI-CKD transition through tubular epithelial cell dependent Sox9 activation. *Oncotarget* 2017; **8**: 70707-70726 [PMID: [29050313](#) DOI: [10.18632/oncotarget.19979](#)]
- 87 **Wang B**, Yao K, Huuskes BM, Shen HH, Zhuang J, Godson C, Brennan EP, Wilkinson-Berka JL, Wise AF, Ricardo SD. Mesenchymal Stem Cells Deliver Exogenous MicroRNA-let7c via Exosomes to Attenuate Renal Fibrosis. *Mol Ther* 2016; **24**: 1290-1301 [PMID: [27203438](#) DOI: [10.1038/mt.2016.90](#)]
- 88 **Nagaishi K**, Mizue Y, Chikenji T, Otani M, Nakano M, Konari N, Fujimiya M. Mesenchymal stem cell therapy ameliorates diabetic nephropathy via the paracrine effect of renal trophic factors including exosomes. *Sci Rep* 2016; **6**: 34842 [PMID: [27721418](#) DOI: [10.1038/srep34842](#)]
- 89 **Ebrahim N**, Ahmed IA, Hussien NI, Dessouky AA, Farid AS, Elshazly AM, Mostafa O, Gazzar WBE, Sorour SM, Seleem Y, Hussein AM, Sabry D. Mesenchymal Stem Cell-Derived Exosomes Ameliorated Diabetic Nephropathy by Autophagy Induction through the mTOR Signaling Pathway. *Cells* 2018; **7** [PMID: [30467302](#) DOI: [10.3390/cells7120226](#)]
- 90 **Zhang L**, Song Y, Chen L, Li D, Feng H, Lu Z, Fan T, Chen Z, Livingston MJ, Geng Q. MiR-20a-containing exosomes from umbilical cord mesenchymal stem cells alleviates liver ischemia/reperfusion injury. *J Cell Physiol* 2020; **235**: 3698-3710 [PMID: [31566731](#) DOI: [10.1002/jcp.29264](#)]
- 91 **Nong K**, Wang W, Niu X, Hu B, Ma C, Bai Y, Wu B, Wang Y, Ai K. Hepatoprotective effect of exosomes from human-induced pluripotent stem cell-derived mesenchymal stromal cells against hepatic ischemia-reperfusion injury in rats. *Cytotherapy* 2016; **18**: 1548-1559 [PMID: [27592404](#) DOI: [10.1016/j.jcyt.2016.08.002](#)]
- 92 **Du Y**, Li D, Han C, Wu H, Xu L, Zhang M, Zhang J, Chen X. Exosomes from Human-Induced Pluripotent Stem Cell-Derived Mesenchymal Stromal Cells (hiPSC-MSCs) Protect Liver against Hepatic Ischemia/Reperfusion Injury via Activating Sphingosine Kinase and Sphingosine-1-Phosphate Signaling Pathway. *Cell Physiol Biochem* 2017; **43**: 611-625 [PMID: [28934733](#) DOI: [10.1159/000480533](#)]
- 93 **Tan CY**, Lai RC, Wong W, Dan YY, Lim SK, Ho HK. Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res Ther* 2014; **5**: 76 [PMID: [24915963](#) DOI: [10.1186/scrt465](#)]
- 94 **Jiang W**, Tan Y, Cai M, Zhao T, Mao F, Zhang X, Xu W, Yan Z, Qian H, Yan Y. Human Umbilical Cord MSC-Derived Exosomes Suppress the Development of CCl<sub>4</sub>-Induced Liver Injury through Antioxidant Effect. *Stem Cells Int* 2018; **2018**: 6079642 [PMID: [29686713](#) DOI: [10.1155/2018/6079642](#)]
- 95 **Shao M**, Xu Q, Wu Z, Chen Y, Shu Y, Cao X, Chen M, Zhang B, Zhou Y, Yao R, Shi Y, Bu H. Exosomes derived from human umbilical cord mesenchymal stem cells ameliorate IL-6-induced acute liver injury through miR-455-3p. *Stem Cell Res Ther* 2020; **11**: 37 [PMID: [31973730](#) DOI: [10.1186/s13287-020-1550-0](#)]
- 96 **Tamura R**, Uemoto S, Tabata Y. Immunosuppressive effect of mesenchymal stem cell-derived exosomes on a concanavalin A-induced liver injury model. *Inflamm Regen* 2016; **36**: 26 [PMID: [29259699](#) DOI: [10.1186/s41232-016-0030-5](#)]
- 97 **Guo Y**, Chen B, Chen LJ, Zhang CF, Xiang C. Current status and future prospects of mesenchymal stem cell therapy for liver fibrosis. *J Zhejiang Univ Sci B* 2016; **17**: 831-841 [PMID: [27819130](#) DOI: [10.1631/jzus.B1600101](#)]
- 98 **Li T**, Yan Y, Wang B, Qian H, Zhang X, Shen L, Wang M, Zhou Y, Zhu W, Li W, Xu W. Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev* 2013; **22**: 845-854 [PMID: [23002959](#) DOI: [10.1089/scd.2012.0395](#)]
- 99 **Rong X**, Liu J, Yao X, Jiang T, Wang Y, Xie F. Human bone marrow mesenchymal stem cells-derived exosomes alleviate liver fibrosis through the Wnt/ $\beta$ -catenin pathway. *Stem Cell Res Ther* 2019; **10**: 98 [PMID: [30885249](#) DOI: [10.1186/s13287-019-1204-2](#)]
- 100 **Qu Y**, Zhang Q, Cai X, Li F, Ma Z, Xu M, Lu L. Exosomes derived from miR-181-5p-modified adipose-derived mesenchymal stem cells prevent liver fibrosis via autophagy activation. *J Cell Mol Med* 2017; **21**: 2491-2502 [PMID: [28382720](#) DOI: [10.1111/jcmm.13170](#)]
- 101 **Lou G**, Yang Y, Liu F, Ye B, Chen Z, Zheng M, Liu Y. MiR-122 modification enhances the therapeutic efficacy of adipose tissue-derived mesenchymal stem cells against liver fibrosis. *J Cell Mol Med* 2017; **21**: 2963-2973 [PMID: [28544786](#) DOI: [10.1111/jcmm.13208](#)]
- 102 **Jiang L**, Zhang S, Hu H, Yang J, Wang X, Ma Y, Jiang J, Wang J, Zhong L, Chen M, Wang H, Hou Y, Zhu R, Zhang Q. Exosomes derived from human umbilical cord mesenchymal stem cells alleviate acute liver failure by reducing the activity of the NLRP3 inflammasome in macrophages. *Biochem Biophys Res Commun* 2019; **508**: 735-741 [PMID: [30528233](#) DOI: [10.1016/j.bbrc.2018.11.189](#)]
- 103 **Zhang S**, Jiang L, Hu H, Wang H, Wang X, Jiang J, Ma Y, Yang J, Hou Y, Xie D, Zhang Q. Pretreatment

- of exosomes derived from hUCMSCs with TNF- $\alpha$  ameliorates acute liver failure by inhibiting the activation of NLRP3 in macrophage. *Life Sci* 2020; **246**: 117401 [PMID: 32035931 DOI: 10.1016/j.lfs.2020.117401]
- 104 **Zhao S**, Liu Y, Pu Z. Bone marrow mesenchymal stem cell-derived exosomes attenuate D-GaIN/LPS-induced hepatocyte apoptosis by activating autophagy in vitro. *Drug Des Devel Ther* 2019; **13**: 2887-2897 [PMID: 31695322 DOI: 10.2147/DDDT.S220190]
- 105 **Arslan F**, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguor EN, Timmers L, van Rijen HV, Doevendans PA, Pasterkamp G, Lim SK, de Kleijn DP. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res* 2013; **10**: 301-312 [PMID: 23399448 DOI: 10.1016/j.scr.2013.01.002]
- 106 **Liu L**, Jin X, Hu CF, Li R, Zhou Z, Shen CX. Exosomes Derived from Mesenchymal Stem Cells Rescue Myocardial Ischaemia/Reperfusion Injury by Inducing Cardiomyocyte Autophagy Via AMPK and Akt Pathways. *Cell Physiol Biochem* 2017; **43**: 52-68 [PMID: 28848091 DOI: 10.1159/000480317]
- 107 **Zou L**, Ma X, Lin S, Wu B, Chen Y, Peng C. Bone marrow mesenchymal stem cell-derived exosomes protect against myocardial infarction by promoting autophagy. *Exp Ther Med* 2019; **18**: 2574-2582 [PMID: 31555366 DOI: 10.3892/etm.2019.7874]
- 108 **Cui X**, He Z, Liang Z, Chen Z, Wang H, Zhang J. Exosomes From Adipose-derived Mesenchymal Stem Cells Protect the Myocardium Against Ischemia/Reperfusion Injury Through Wnt/ $\beta$ -Catenin Signaling Pathway. *J Cardiovasc Pharmacol* 2017; **70**: 225-231 [PMID: 28582278 DOI: 10.1097/FJC.0000000000000507]
- 109 **Sun XH**, Wang X, Zhang Y, Hui J. Exosomes of bone-marrow stromal cells inhibit cardiomyocyte apoptosis under ischemic and hypoxic conditions via miR-486-5p targeting the PTEN/PI3K/AKT signaling pathway. *Thromb Res* 2019; **177**: 23-32 [PMID: 30844685 DOI: 10.1016/j.thromres.2019.02.002]
- 110 **Chen Q**, Liu Y, Ding X, Li Q, Qiu F, Wang M, Shen Z, Zheng H, Fu G. Bone marrow mesenchymal stem cell-secreted exosomes carrying microRNA-125b protect against myocardial ischemia reperfusion injury via targeting SIRT7. *Mol Cell Biochem* 2020; **465**: 103-114 [PMID: 31858380 DOI: 10.1007/s11010-019-03671-z]
- 111 **Wei Z**, Qiao S, Zhao J, Liu Y, Li Q, Wei Z, Dai Q, Kang L, Xu B. miRNA-181a over-expression in mesenchymal stem cell-derived exosomes influenced inflammatory response after myocardial ischemia-reperfusion injury. *Life Sci* 2019; **232**: 116632 [PMID: 31278944 DOI: 10.1016/j.lfs.2019.116632]
- 112 **Zhao Y**, Sun X, Cao W, Ma J, Sun L, Qian H, Zhu W, Xu W. Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells Relieve Acute Myocardial Ischemic Injury. *Stem Cells Int* 2015; **2015**: 761643 [PMID: 26106430 DOI: 10.1155/2015/761643]
- 113 **Wang XL**, Zhao YY, Sun L, Shi Y, Li ZQ, Zhao XD, Xu CG, Ji HG, Wang M, Xu WR, Zhu W. Exosomes derived from human umbilical cord mesenchymal stem cells improve myocardial repair via upregulation of Smad7. *Int J Mol Med* 2018; **41**: 3063-3072 [PMID: 29484378 DOI: 10.3892/ijmm.2018.3496]
- 114 **Deng S**, Zhou X, Ge Z, Song Y, Wang H, Liu X, Zhang D. Exosomes from adipose-derived mesenchymal stem cells ameliorate cardiac damage after myocardial infarction by activating S1P/SK1/S1PR1 signaling and promoting macrophage M2 polarization. *Int J Biochem Cell Biol* 2019; **114**: 105564 [PMID: 31276786 DOI: 10.1016/j.biocel.2019.105564]
- 115 **Xu H**, Wang Z, Liu L, Zhang B, Li B. Exosomes derived from adipose tissue, bone marrow, and umbilical cord blood for cardioprotection after myocardial infarction. *J Cell Biochem* 2020; **121**: 2089-2102 [PMID: 31736169 DOI: 10.1002/jcb.27399]
- 116 **Kang K**, Ma R, Cai W, Huang W, Paul C, Liang J, Wang Y, Zhao T, Kim HW, Xu M, Millard RW, Wen Z, Wang Y. Exosomes Secreted from CXCR4 Overexpressing Mesenchymal Stem Cells Promote Cardioprotection via Akt Signaling Pathway following Myocardial Infarction. *Stem Cells Int* 2015; **2015**: 659890 [PMID: 26074976 DOI: 10.1155/2015/659890]
- 117 **Ni J**, Liu X, Yin Y, Zhang P, Xu YW, Liu Z. Exosomes Derived from TIMP2-Modified Human Umbilical Cord Mesenchymal Stem Cells Enhance the Repair Effect in Rat Model with Myocardial Infarction Possibly by the Akt/Sfrp2 Pathway. *Oxid Med Cell Longev* 2019; **2019**: 1958941 [PMID: 31182988 DOI: 10.1155/2019/1958941]
- 118 **Liu X**, Li X, Zhu W, Zhang Y, Hong Y, Liang X, Fan B, Zhao H, He H, Zhang F. Exosomes from mesenchymal stem cells overexpressing MIF enhance myocardial repair. *J Cell Physiol* 2020 [PMID: 31960418 DOI: 10.1002/jcp.29456]
- 119 **Luther KM**, Haar L, McGuinness M, Wang Y, Lynch IV TL, Phan A, Song Y, Shen Z, Gardner G, Kuffel G, Ren X, Zilliox MJ, Jones WK. Exosomal miR-21a-5p mediates cardioprotection by mesenchymal stem cells. *J Mol Cell Cardiol* 2018; **119**: 125-137 [PMID: 29698635 DOI: 10.1016/j.yjmcc.2018.04.012]
- 120 **Mao Q**, Liang XL, Zhang CL, Pang YH, Lu YX. LncRNA KLF3-AS1 in human mesenchymal stem cell-derived exosomes ameliorates pyroptosis of cardiomyocytes and myocardial infarction through miR-138-5p/Sirt1 axis. *Stem Cell Res Ther* 2019; **10**: 393 [PMID: 31847890 DOI: 10.1186/s13287-019-1522-4]
- 121 **Huang L**, Yang L, Ding Y, Jiang X, Xia Z, You Z. Human umbilical cord mesenchymal stem cells-derived exosomes transfers microRNA-19a to protect cardiomyocytes from acute myocardial infarction by targeting SOX6. *Cell Cycle* 2020; **19**: 339-353 [PMID: 31924121 DOI: 10.1080/15384101.2019.1711305]
- 122 **Li Y**, Zhou J, Zhang O, Wu X, Guan X, Xue Y, Li S, Zhuang X, Zhou B, Miao G, Zhang L. Bone marrow mesenchymal stem cells-derived exosomal microRNA-185 represses ventricular remodeling of mice with myocardial infarction by inhibiting SOCS2. *Int Immunopharmacol* 2020; **80**: 106156 [PMID: 31945609 DOI: 10.1016/j.intimp.2019.106156]
- 123 **Zhang Y**, Chopp M, Meng Y, Katakowski M, Xin H, Mahmood A, Xiong Y. Effect of exosomes derived from multipotential mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. *J Neurosurg* 2015; **122**: 856-867 [PMID: 25594326 DOI: 10.3171/2014.11.JNS14770]
- 124 **Ni H**, Yang S, Siaw-Debrah F, Hu J, Wu K, He Z, Yang J, Pan S, Lin X, Ye H, Xu Z, Wang F, Jin K, Zhuge Q, Huang L. Exosomes Derived From Bone Mesenchymal Stem Cells Ameliorate Early Inflammatory Responses Following Traumatic Brain Injury. *Front Neurosci* 2019; **13**: 14 [PMID: 30733666 DOI: 10.3389/fn.2019.00014]



- DOI: [10.3389/fnins.2019.00014](https://doi.org/10.3389/fnins.2019.00014)]
- 125 **Williams AM**, Dennahy IS, Bhatti UF, Halaweish I, Xiong Y, Chang P, Nikolian VC, Chtraklin K, Brown J, Zhang Y, Zhang ZG, Chopp M, Buller B, Alam HB. Mesenchymal Stem Cell-Derived Exosomes Provide Neuroprotection and Improve Long-Term Neurologic Outcomes in a Swine Model of Traumatic Brain Injury and Hemorrhagic Shock. *J Neurotrauma* 2019; **36**: 54-60 [PMID: [29690826](https://pubmed.ncbi.nlm.nih.gov/29690826/) DOI: [10.1089/neu.2018.5711](https://doi.org/10.1089/neu.2018.5711)]
  - 126 **Xin H**, Li Y, Cui Y, Yang JJ, Zhang ZG, Chopp M. Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J Cereb Blood Flow Metab* 2013; **33**: 1711-1715 [PMID: [23963371](https://pubmed.ncbi.nlm.nih.gov/23963371/) DOI: [10.1038/jcbfm.2013.152](https://doi.org/10.1038/jcbfm.2013.152)]
  - 127 **Xin H**, Li Y, Liu Z, Wang X, Shang X, Cui Y, Zhang ZG, Chopp M. MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem Cells* 2013; **31**: 2737-2746 [PMID: [23630198](https://pubmed.ncbi.nlm.nih.gov/23630198/) DOI: [10.1002/stem.1409](https://doi.org/10.1002/stem.1409)]
  - 128 **Xin H**, Katakowski M, Wang F, Qian JY, Liu XS, Ali MM, Buller B, Zhang ZG, Chopp M. MicroRNA cluster miR-17-92 Cluster in Exosomes Enhance Neuroplasticity and Functional Recovery After Stroke in Rats. *Stroke* 2017; **48**: 747-753 [PMID: [28232590](https://pubmed.ncbi.nlm.nih.gov/28232590/) DOI: [10.1161/STROKEAHA.116.015204](https://doi.org/10.1161/STROKEAHA.116.015204)]
  - 129 **Yang Y**, Cai Y, Zhang Y, Liu J, Xu Z. Exosomes Secreted by Adipose-Derived Stem Cells Contribute to Angiogenesis of Brain Microvascular Endothelial Cells Following Oxygen-Glucose Deprivation In Vitro Through MicroRNA-181b/TRPM7 Axis. *J Mol Neurosci* 2018; **65**: 74-83 [PMID: [29705934](https://pubmed.ncbi.nlm.nih.gov/29705934/) DOI: [10.1007/s12031-018-1071-9](https://doi.org/10.1007/s12031-018-1071-9)]
  - 130 **Xiao Y**, Geng F, Wang G, Li X, Zhu J, Zhu W. Bone marrow-derived mesenchymal stem cells-derived exosomes prevent oligodendrocyte apoptosis through exosomal miR-134 by targeting caspase-8. *J Cell Biochem* 2018 [PMID: [30191592](https://pubmed.ncbi.nlm.nih.gov/30191592/) DOI: [10.1002/jcb.27519](https://doi.org/10.1002/jcb.27519)]
  - 131 **Deng Y**, Chen D, Gao F, Lv H, Zhang G, Sun X, Liu L, Mo D, Ma N, Song L, Huo X, Yan T, Zhang J, Miao Z. Exosomes derived from microRNA-138-5p-overexpressing bone marrow-derived mesenchymal stem cells confer neuroprotection to astrocytes following ischemic stroke via inhibition of LCN2. *J Biol Eng* 2019; **13**: 71 [PMID: [31485266](https://pubmed.ncbi.nlm.nih.gov/31485266/) DOI: [10.1186/s13036-019-0193-0](https://doi.org/10.1186/s13036-019-0193-0)]
  - 132 **Huang JH**, Yin XM, Xu Y, Xu CC, Lin X, Ye FB, Cao Y, Lin FY. Systemic Administration of Exosomes Released from Mesenchymal Stromal Cells Attenuates Apoptosis, Inflammation, and Promotes Angiogenesis after Spinal Cord Injury in Rats. *J Neurotrauma* 2017; **34**: 3388-3396 [PMID: [28665182](https://pubmed.ncbi.nlm.nih.gov/28665182/) DOI: [10.1089/neu.2017.5063](https://doi.org/10.1089/neu.2017.5063)]
  - 133 **Sun G**, Li G, Li D, Huang W, Zhang R, Zhang H, Duan Y, Wang B. hucMSC derived exosomes promote functional recovery in spinal cord injury mice via attenuating inflammation. *Mater Sci Eng C Mater Biol Appl* 2018; **89**: 194-204 [PMID: [29752089](https://pubmed.ncbi.nlm.nih.gov/29752089/) DOI: [10.1016/j.msec.2018.04.006](https://doi.org/10.1016/j.msec.2018.04.006)]
  - 134 **Liu W**, Wang Y, Gong F, Rong Y, Luo Y, Tang P, Zhou Z, Zhou Z, Xu T, Jiang T, Yang S, Yin G, Chen J, Fan J, Cai W. Exosomes Derived from Bone Mesenchymal Stem Cells Repair Traumatic Spinal Cord Injury by Suppressing the Activation of A1 Neurotoxic Reactive Astrocytes. *J Neurotrauma* 2019; **36**: 469-484 [PMID: [29848167](https://pubmed.ncbi.nlm.nih.gov/29848167/) DOI: [10.1089/neu.2018.5835](https://doi.org/10.1089/neu.2018.5835)]
  - 135 **Zhao C**, Zhou X, Qiu J, Xin D, Li T, Chu X, Yuan H, Wang H, Wang Z, Wang D. Exosomes Derived From Bone Marrow Mesenchymal Stem Cells Inhibit Complement Activation In Rats With Spinal Cord Injury. *Drug Des Devel Ther* 2019; **13**: 3693-3704 [PMID: [31695336](https://pubmed.ncbi.nlm.nih.gov/31695336/) DOI: [10.2147/DDDT.S209636](https://doi.org/10.2147/DDDT.S209636)]
  - 136 **Li C**, Jiao G, Wu W, Wang H, Ren S, Zhang L, Zhou H, Liu H, Chen Y. Exosomes from Bone Marrow Mesenchymal Stem Cells Inhibit Neuronal Apoptosis and Promote Motor Function Recovery via the Wnt/ $\beta$ -catenin Signaling Pathway. *Cell Transplant* 2019; **28**: 1373-1383 [PMID: [31423807](https://pubmed.ncbi.nlm.nih.gov/31423807/) DOI: [10.1177/0963689719870999](https://doi.org/10.1177/0963689719870999)]
  - 137 **Li D**, Zhang P, Yao X, Li H, Shen H, Li X, Wu J, Lu X. Exosomes Derived From miR-133b-Modified Mesenchymal Stem Cells Promote Recovery After Spinal Cord Injury. *Front Neurosci* 2018; **12**: 845 [PMID: [30524227](https://pubmed.ncbi.nlm.nih.gov/30524227/) DOI: [10.3389/fnins.2018.00845](https://doi.org/10.3389/fnins.2018.00845)]
  - 138 **Yu T**, Zhao C, Hou S, Zhou W, Wang B, Chen Y. Exosomes secreted from miRNA-29b-modified mesenchymal stem cells repaired spinal cord injury in rats. *Braz J Med Biol Res* 2019; **52**: e8735 [PMID: [31826179](https://pubmed.ncbi.nlm.nih.gov/31826179/) DOI: [10.1590/1414-431X20198735](https://doi.org/10.1590/1414-431X20198735)]
  - 139 **Huang JH**, Xu Y, Yin XM, Lin FY. Exosomes Derived from miR-126-modified MSCs Promote Angiogenesis and Neurogenesis and Attenuate Apoptosis after Spinal Cord Injury in Rats. *Neuroscience* 2020; **424**: 133-145 [PMID: [31704348](https://pubmed.ncbi.nlm.nih.gov/31704348/) DOI: [10.1016/j.neuroscience.2019.10.043](https://doi.org/10.1016/j.neuroscience.2019.10.043)]
  - 140 **Yuan B**, Pan S, Dong YQ, Zhang WW, He XD. Effect of exosomes derived from mir-126-modified mesenchymal stem cells on the repair process of spinal cord injury in rats. *Eur Rev Med Pharmacol Sci* 2020; **24**: 483-490 [PMID: [32016949](https://pubmed.ncbi.nlm.nih.gov/32016949/) DOI: [10.26355/eurev\\_202001\\_20025](https://doi.org/10.26355/eurev_202001_20025)]
  - 141 **Lee M**, Ban JJ, Yang S, Im W, Kim M. The exosome of adipose-derived stem cells reduces  $\beta$ -amyloid pathology and apoptosis of neuronal cells derived from the transgenic mouse model of Alzheimer's disease. *Brain Res* 2018; **1691**: 87-93 [PMID: [29625119](https://pubmed.ncbi.nlm.nih.gov/29625119/) DOI: [10.1016/j.brainres.2018.03.034](https://doi.org/10.1016/j.brainres.2018.03.034)]
  - 142 **Ding M**, Shen Y, Wang P, Xie Z, Xu S, Zhu Z, Wang Y, Lyu Y, Wang D, Xu L, Bi J, Yang H. Exosomes Isolated From Human Umbilical Cord Mesenchymal Stem Cells Alleviate Neuroinflammation and Reduce Amyloid-Beta Deposition by Modulating Microglial Activation in Alzheimer's Disease. *Neurochem Res* 2018; **43**: 2165-2177 [PMID: [30259257](https://pubmed.ncbi.nlm.nih.gov/30259257/) DOI: [10.1007/s11064-018-2641-5](https://doi.org/10.1007/s11064-018-2641-5)]
  - 143 **Reza-Zaldivar EE**, Hernández-Sapiéns MA, Gutiérrez-Mercado YK, Sandoval-Ávila S, Gomez-Pinedo U, Márquez-Aguirre AL, Vázquez-Méndez E, Padilla-Camberos E, Canales-Aguirre AA. Mesenchymal stem cell-derived exosomes promote neurogenesis and cognitive function recovery in a mouse model of Alzheimer's disease. *Neural Regen Res* 2019; **14**: 1626-1634 [PMID: [31089063](https://pubmed.ncbi.nlm.nih.gov/31089063/) DOI: [10.4103/1673-5374.255978](https://doi.org/10.4103/1673-5374.255978)]
  - 144 **Lee M**, Liu T, Im W, Kim M. Exosomes from adipose-derived stem cells ameliorate phenotype of Huntington's disease in vitro model. *Eur J Neurosci* 2016; **44**: 2114-2119 [PMID: [27177616](https://pubmed.ncbi.nlm.nih.gov/27177616/) DOI: [10.1111/ejn.13275](https://doi.org/10.1111/ejn.13275)]
  - 145 **Lee M**, Ban JJ, Kim KY, Jeon GS, Im W, Sung JJ, Kim M. Adipose-derived stem cell exosomes alleviate pathology of amyotrophic lateral sclerosis in vitro. *Biochem Biophys Res Commun* 2016; **479**: 434-439 [PMID: [27641665](https://pubmed.ncbi.nlm.nih.gov/27641665/) DOI: [10.1016/j.bbrc.2016.09.069](https://doi.org/10.1016/j.bbrc.2016.09.069)]

- 146 **Bonafede R**, Brandi J, Manfredi M, Scambi I, Schiaffino L, Merigo F, Turano E, Bonetti B, Marengo E, Ceccconi D, Mariotti R. The Anti-Apoptotic Effect of ASC-Exosomes in an In Vitro ALS Model and Their Proteomic Analysis. *Cells* 2019; 8 [PMID: 31540100 DOI: 10.3390/cells8091087]
- 147 **Calabria E**, Scambi I, Bonafede R, Schiaffino L, Peroni D, Potrich V, Capelli C, Schena F, Mariotti R. ASCs-Exosomes Recover Coupling Efficiency and Mitochondrial Membrane Potential in an *in vitro* Model of ALS. *Front Neurosci* 2019; 13: 1070 [PMID: 31680811 DOI: 10.3389/fnins.2019.01070]
- 148 **Li Z**, Liu F, He X, Yang X, Shan F, Feng J. Exosomes derived from mesenchymal stem cells attenuate inflammation and demyelination of the central nervous system in EAE rats by regulating the polarization of microglia. *Int Immunopharmacol* 2019; 67: 268-280 [PMID: 30572251 DOI: 10.1016/j.intimp.2018.12.001]
- 149 **Zhu Y**, Wang Y, Zhao B, Niu X, Hu B, Li Q, Zhang J, Ding J, Chen Y, Wang Y. Comparison of exosomes secreted by induced pluripotent stem cell-derived mesenchymal stem cells and synovial membrane-derived mesenchymal stem cells for the treatment of osteoarthritis. *Stem Cell Res Ther* 2017; 8: 64 [PMID: 28279188 DOI: 10.1186/s13287-017-0510-9]
- 150 **Wang Y**, Yu D, Liu Z, Zhou F, Dai J, Wu B, Zhou J, Heng BC, Zou XH, Ouyang H, Liu H. Exosomes from embryonic mesenchymal stem cells alleviate osteoarthritis through balancing synthesis and degradation of cartilage extracellular matrix. *Stem Cell Res Ther* 2017; 8: 189 [PMID: 28807034 DOI: 10.1186/s13287-017-0632-0]
- 151 **Cosenza S**, Ruiz M, Toupet K, Jorgensen C, Noël D. Mesenchymal stem cells derived exosomes and microparticles protect cartilage and bone from degradation in osteoarthritis. *Sci Rep* 2017; 7: 16214 [PMID: 29176667 DOI: 10.1038/s41598-017-15376-8]
- 152 **Zhang S**, Teo KYW, Chuah SJ, Lai RC, Lim SK, Toh WS. MSC exosomes alleviate temporomandibular joint osteoarthritis by attenuating inflammation and restoring matrix homeostasis. *Biomaterials* 2019; 200: 35-47 [PMID: 30771585 DOI: 10.1016/j.biomaterials.2019.02.006]
- 153 **Li J**, Ding Z, Li Y, Wang W, Wang J, Yu H, Liu A, Miao J, Chen S, Wu T, Cao Y. BMSCs-Derived Exosomes Ameliorate Pain Via Abrogation of Aberrant Nerve Invasion in Subchondral Bone in Lumbar Facet Joint Osteoarthritis. *J Orthop Res* 2020; 38: 670-679 [PMID: 31608495 DOI: 10.1002/jor.24497]
- 154 **Tao SC**, Yuan T, Zhang YL, Yin WJ, Guo SC, Zhang CQ. Exosomes derived from miR-140-5p-overexpressing human synovial mesenchymal stem cells enhance cartilage tissue regeneration and prevent osteoarthritis of the knee in a rat model. *Theranostics* 2017; 7: 180-195 [PMID: 28042326 DOI: 10.7150/thno.17133]
- 155 **Liu Y**, Lin L, Zou R, Wen C, Wang Z, Lin F. MSC-derived exosomes promote proliferation and inhibit apoptosis of chondrocytes via lncRNA-KLF3-AS1/miR-206/GIT1 axis in osteoarthritis. *Cell Cycle* 2018; 17: 2411-2422 [PMID: 30324848 DOI: 10.1080/15384101.2018.1526603]
- 156 **Liu Y**, Zou R, Wang Z, Wen C, Zhang F, Lin F. Exosomal KLF3-AS1 from hMSCs promoted cartilage repair and chondrocyte proliferation in osteoarthritis. *Biochem J* 2018; 475: 3629-3638 [PMID: 30341166 DOI: 10.1042/BCJ20180675]
- 157 **Wu J**, Kuang L, Chen C, Yang J, Zeng WN, Li T, Chen H, Huang S, Fu Z, Li J, Liu R, Ni Z, Chen L, Yang L. miR-100-5p-abundant exosomes derived from infrapatellar fat pad MSCs protect articular cartilage and ameliorate gait abnormalities via inhibition of mTOR in osteoarthritis. *Biomaterials* 2019; 206: 87-100 [PMID: 30927715 DOI: 10.1016/j.biomaterials.2019.03.022]
- 158 **Jin Z**, Ren J, Qi S. Human bone mesenchymal stem cells-derived exosomes overexpressing microRNA-26a-5p alleviate osteoarthritis via down-regulation of PTGS2. *Int Immunopharmacol* 2020; 78: 105946 [PMID: 31784400 DOI: 10.1016/j.intimp.2019.105946]
- 159 **Qi X**, Zhang J, Yuan H, Xu Z, Li Q, Niu X, Hu B, Wang Y, Li X. Exosomes Secreted by Human-Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells Repair Critical-Sized Bone Defects through Enhanced Angiogenesis and Osteogenesis in Osteoporotic Rats. *Int J Biol Sci* 2016; 12: 836-849 [PMID: 27313497 DOI: 10.7150/ijbs.14809]
- 160 **Zhao P**, Xiao L, Peng J, Qian YQ, Huang CC. Exosomes derived from bone marrow mesenchymal stem cells improve osteoporosis through promoting osteoblast proliferation via MAPK pathway. *Eur Rev Med Pharmacol Sci* 2018; 22: 3962-3970 [PMID: 29949171 DOI: 10.26355/eurrev\_201806\_15280]
- 161 **Yang X**, Yang J, Lei P, Wen T. LncRNA MALAT1 shuttled by bone marrow-derived mesenchymal stem cells-secreted exosomes alleviates osteoporosis through mediating microRNA-34c/SATB2 axis. *Aging (Albany NY)* 2019; 11: 8777-8791 [PMID: 31659145 DOI: 10.18632/aging.102264]
- 162 **Zuo R**, Liu M, Wang Y, Li J, Wang W, Wu J, Sun C, Li B, Wang Z, Lan W, Zhang C, Shi C, Zhou Y. BM-MSC-derived exosomes alleviate radiation-induced bone loss by restoring the function of recipient BM-MSCs and activating Wnt/ $\beta$ -catenin signaling. *Stem Cell Res Ther* 2019; 10: 30 [PMID: 30646958 DOI: 10.1186/s13287-018-1121-9]
- 163 **Zhang J**, Guan J, Niu X, Hu G, Guo S, Li Q, Xie Z, Zhang C, Wang Y. Exosomes released from human induced pluripotent stem cells-derived MSCs facilitate cutaneous wound healing by promoting collagen synthesis and angiogenesis. *J Transl Med* 2015; 13: 49 [PMID: 25638205 DOI: 10.1186/s12967-015-0417-0]
- 164 **Zhang B**, Wang M, Gong A, Zhang X, Wu X, Zhu Y, Shi H, Wu L, Zhu W, Qian H, Xu W. HucMSC-Exosome Mediated-Wnt4 Signaling Is Required for Cutaneous Wound Healing. *Stem Cells* 2015; 33: 2158-2168 [PMID: 24964196 DOI: 10.1002/stem.1771]
- 165 **Hu L**, Wang J, Zhou X, Xiong Z, Zhao J, Yu R, Huang F, Zhang H, Chen L. Exosomes derived from human adipose mesenchymal stem cells accelerates cutaneous wound healing via optimizing the characteristics of fibroblasts. *Sci Rep* 2016; 6: 32993 [PMID: 27615560 DOI: 10.1038/srep32993]
- 166 **Ma T**, Fu B, Yang X, Xiao Y, Pan M. Adipose mesenchymal stem cell-derived exosomes promote cell proliferation, migration, and inhibit cell apoptosis via Wnt/ $\beta$ -catenin signaling in cutaneous wound healing. *J Cell Biochem* 2019; 120: 10847-10854 [PMID: 30681184 DOI: 10.1002/jcb.28376]
- 167 **He X**, Dong Z, Cao Y, Wang H, Liu S, Liao L, Jin Y, Yuan L, Li B. MSC-Derived Exosome Promotes M2 Polarization and Enhances Cutaneous Wound Healing. *Stem Cells Int* 2019; 2019: 7132708 [PMID: 31582986 DOI: 10.1155/2019/7132708]
- 168 **Dalirfardouei R**, Jamialahmadi K, Jafarian AH, Mahdipour E. Promising effects of exosomes isolated from

- menstrual blood-derived mesenchymal stem cell on wound-healing process in diabetic mouse model. *J Tissue Eng Regen Med* 2019; **13**: 555-568 [PMID: 30656863 DOI: 10.1002/term.2799]
- 169 **Li B**, Luan S, Chen J, Zhou Y, Wang T, Li Z, Fu Y, Zhai A, Bi C. The MSC-Derived Exosomal lncRNA H19 Promotes Wound Healing in Diabetic Foot Ulcers by Upregulating PTEN via MicroRNA-152-3p. *Mol Ther Nucleic Acids* 2020; **19**: 814-826 [PMID: 31958697 DOI: 10.1016/j.omtn.2019.11.034]
- 170 **Yin K**, Wang S, Zhao RC. Exosomes from mesenchymal stem/stromal cells: a new therapeutic paradigm. *Biomark Res* 2019; **7**: 8 [PMID: 30992990 DOI: 10.1186/s40364-019-0159-x]
- 171 **Kordelas L**, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, Epple M, Horn PA, Beelen DW, Giebel B. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 2014; **28**: 970-973 [PMID: 24445866 DOI: 10.1038/leu.2014.41]
- 172 **Petersen KE**, Manangon E, Hood JL, Wickline SA, Fernandez DP, Johnson WP, Gale BK. A review of exosome separation techniques and characterization of B16-F10 mouse melanoma exosomes with AF4-UV-MALS-DLS-TEM. *Anal Bioanal Chem* 2014; **406**: 7855-7866 [PMID: 25084738 DOI: 10.1007/s00216-014-8040-0]
- 173 **Vizoso FJ**, Eiro N, Cid S, Schneider J, Perez-Fernandez R. Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. *Int J Mol Sci* 2017; **18** [PMID: 28841158 DOI: 10.3390/ijms18091852]

## Basic Study

# Assessment of tobacco heating system 2.4 on osteogenic differentiation of mesenchymal stem cells and primary human osteoblasts compared to conventional cigarettes

Romina H Aspera-Werz, Sabrina Ehnert, Monja Müller, Sheng Zhu, Tao Chen, Weidong Weng, Johann Jacoby, Andreas K Nussler

**ORCID number:** Romina H Aspera-Werz 0000-0002-5160-2111; Sabrina Ehnert 0000-0003-4347-1702; Monja Müller 0000-0002-4265-653X; Sheng Zhu 0000-0001-7078-4371; Tao Chen 0000-0002-1156-2899; Weidong Weng 0000-0002-4258-453X; Johann Jacoby 0000-0002-7017-9638; Andreas K Nussler 0000-0002-6666-6791.

**Author contributions:** Aspera-Werz RH, Ehnert S, and Nussler AK designed and coordinated the study; Müller M, and Aspera-Werz RH performed the experiments, acquired and analyzed data; Aspera-Werz RH, Ehnert S, Müller M, Jacoby J, and Nussler AK interpreted the data; Aspera-Werz RH wrote the manuscript; Ehnert S, Müller M, Zhu S, Chen T, Weng W, Jacoby J, and Nussler AK made critical revisions to the manuscript; all authors approved the final version of the article.

**Institutional review board**

**statement:** This study was conducted under ethical approval by committee at the Medical Faculty of Eberhard-Karls-University Tübingen, No. 538/2014BO2.

**Informed consent statement:** All

**Romina H Aspera-Werz**, Department of Traumatology, BG Trauma Clinic, Siegfried Weller Institute for Trauma Research, Eberhard Karls Universität Tübingen, Tübingen 72076, Germany

**Sabrina Ehnert, Monja Müller, Sheng Zhu, Tao Chen, Weidong Weng, Andreas K Nussler**, Department of Traumatology, BG Trauma Clinic, Siegfried Weller Institute for Trauma Research, Eberhard Karls Universität Tübingen, Tübingen 71076, Germany

**Johann Jacoby**, Institute for Clinical Epidemiology and Applied Biometry, Eberhard Karls Universität Tübingen, Tübingen 71076, Germany

**Corresponding author:** Andreas K Nussler, Dr. rer.nat., Professor, Department of Traumatology, BG Trauma Clinic, Siegfried Weller Institute for Trauma Research, Eberhard Karls Universität Tübingen, Schnarrenbergstraße 95, Tübingen 71076, Germany. [andreas.nuessler@gmail.com](mailto:andreas.nuessler@gmail.com)

## Abstract

### BACKGROUND

Cigarette smoking (CS) is the most common method of consuming tobacco. Deleterious effects on bone integrity, increased incidence of fractures, and delayed fracture healing are all associated with CS. Over 150 of the 6500 molecular species contained in cigarette smoke and identified as toxic compounds are inhaled by CS and, *via* the bloodstream, reach the skeletal system. New technologies designed to develop a reduced-risk alternative for smokers are based on electronic nicotine delivery systems, such as e-cigarettes and tobacco heating systems (THS). THS are designed to heat tobacco instead of burning it, thereby reducing the levels of harmful toxic compounds released.

### AIM

To examine the effects of THS on osteoprogenitor cell viability and function compared to conventional CS.

### METHODS

Human immortalized mesenchymal stem cells ( $n = 3$ ) and primary human pre-osteoblasts isolated from cancellous bone samples from BG Unfall Klinik Tübingen ( $n = 5$ ) were osteogenically differentiated *in vitro* with aqueous extracts



patients gave their written informed consent to participate in the study.

**Conflict-of-interest statement:** The authors have not conflict of interest.

**Data sharing statement:** The data used to support the findings of this study are available from the corresponding author upon reasonable request.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Invited manuscript

**Received:** February 25, 2020

**Peer-review started:** February 25, 2020

**First decision:** May 26, 2020

**Revised:** July 17, 2020

**Accepted:** August 1, 2020

**Article in press:** August 1, 2020

**Published online:** August 26, 2020

**P-Reviewer:** Durán Alonso MB, Hassan A, Kode JA, Park JB

**S-Editor:** Ma YJ

**L-Editor:** A

**P-Editor:** Xing YX



generated from either the THS 2.4 “IQOS” or conventional “Marlboro” cigarettes for up to 21 d. Cell viability was analyzed using resazurin conversion assay (mitochondrial activity) and calcein-AM staining (esterase activity). Osteogenic differentiation and bone cell function were evaluated using alkaline phosphatase (AP) activity, while matrix formation was analyzed through alizarin red staining. Primary cilia structure was examined by acetylated  $\alpha$ -tubulin immunofluorescent staining. Free radical production was evaluated with 2',7'-dichlorofluorescein-diacetate assay.

## RESULTS

Our data clearly show that THS is significantly less toxic to bone cells than CS when analyzed by mitochondrial and esterase activity ( $P < 0.001$ ). No significant differences in cytotoxicity between the diverse flavors of THS were observed. Harmful effects from THS on bone cell function were observed only at very high, non-physiological concentrations. In contrast, extracts from conventional cigarettes significantly reduced the AP activity (by two-fold) and matrix mineralization (four-fold) at low concentrations. Additionally, morphologic analysis of primary cilia revealed no significant changes in the length of the organelle involved in osteogenesis of osteoprogenitor cells, nor in the number of ciliated cells following THS treatment. Assessment of free radical production demonstrated that THS induced significantly less oxidative stress than conventional CS in osteoprogenitor cells.

## CONCLUSION

THS was significantly less harmful to osteoprogenitor cells during osteogenesis than conventional CS. Additional studies are required to confirm whether THS is a better alternative for smokers to improve delays in bone healing following fracture.

**Key words:** Primary human osteoblast; Cigarette smoke; Tobacco heating system; Mesenchymal stem cells; Electronic nicotine delivery systems; Bone

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Aqueous extracts (AE) generated with tobacco heating systems (THS) showed no differences in suspended particles compared to the cell culture medium. This finding supports previous studies demonstrating reduced levels of harmful constituents reported in THS AE in comparison to conventional cigarettes AE. The time to consume one unit (stick/cigarette) was longer for THS than for a conventional cigarette. The pH from both AE fractions was similar to the cell culture medium. Following a single exposure, THS AE was significantly less toxic to bone-forming cells and osteoprogenitor cells than conventional cigarettes AE. The cytotoxicity observed following THS exposure was associated with very high, non-physiological concentrations. No significant differences in cytotoxicity were observed between different flavors of THS AE. Moreover, THS AE displayed less impact on osteogenic differentiation of osteoprogenitor cells and the function of bone-forming cells when compared to conventional cigarettes AE. Finally, compared to conventional cigarettes AE, THS AE induced lower levels of oxidative stress due to the reduced level of harmful constituents, resulting in less damage to primary cilia structure and reduced impact on osteogenic differentiation.

**Citation:** Aspera-Werz RH, Ehnert S, Müller M, Zhu S, Chen T, Weng W, Jacoby J, Nussler AK. Assessment of tobacco heating system 2.4 on osteogenic differentiation of mesenchymal stem cells and primary human osteoblasts compared to conventional cigarettes. *World J Stem Cells* 2020; 12(8): 841-856

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/841.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.841>

## INTRODUCTION

Cigarette smoking (CS) is the most popular way to consume tobacco, and is one of the leading causes of preventable death worldwide<sup>[1]</sup>. Of the current estimated one billion smokers, 6 million die per year due to harmful substances that arise when tobacco is burned and become distributed throughout the body *via* the bloodstream, thereby affecting several organs<sup>[2,3]</sup>.

Detrimental effects of CS also manifest in the musculoskeletal system<sup>[4,5]</sup>. Recent evidence demonstrated that CS could lead to an imbalance in bone turnover mechanisms, leading to osteoporosis, osteoarthritis, and fracture<sup>[6-8]</sup>. Moreover, CS increases the risk of delayed fracture healing<sup>[9]</sup>, non-union<sup>[10]</sup>, complication rate<sup>[11]</sup>, and leads to more extended hospital stays<sup>[12-14]</sup>.

Tobacco combusted at about 800 °C generates approximately 6500 molecular species, more than 150 of which have been identified as toxic compounds<sup>[3,15-17]</sup>. However, it remains unknown which of these compounds are involved in the impaired bone homeostasis observed in smokers. Our previous results, as well as other publications, have demonstrated that the most pharmacologically active component, nicotine, and its first metabolite, cotinine are not the main factors responsible for the adverse effects observed in bone-forming cells<sup>[18-20]</sup>.

Interestingly, it has been demonstrated that oxidative stress induced by compounds produced during conventional cigarette combustion may be one of the factors responsible for the impaired osteogenic differentiation of bone-forming cells and osteogenic precursors cells<sup>[18,21-25]</sup>.

Quitting conventional CS is the most efficient way to significantly reduce the harmful effects of cigarette smoke on human health<sup>[11]</sup>; unfortunately, quitting smoking is not always a viable alternative for many smokers (*i.e.*, those which cannot, wish not, or fail to quit)<sup>[26]</sup>. Many attempts have been made to replace cigarettes with smoke-free nicotine replacement therapies (*e.g.*, nicotine patches, sprays, or chewing gums). However, these replacement products tend to fail in smokers because, although they deliver nicotine, the smoking ritual is absent. Therefore, new approaches have been on developing reduced-risk alternatives for smokers that maintain the smoking ritual, while providing the same levels of nicotine as conventional cigarettes with less harmful constituents.

For this purpose, electronic nicotine delivery systems (ENDS), including e-cigarettes or tobacco heating systems (THS), focus on heating rather than combustion to reduce the generation of harmful constituents. E-cigarettes heat liquids based on propylene glycol, glycerin, flavor, and selectively nicotine into an aerosol that is inhaled. Instead of burning tobacco, THS heat tobacco rolled up in a stick form up to 350 °C (avoiding combustion and formation of ashes). In contrast to E-cigarettes, THS contain tobacco and convey the feeling of smoking a conventional cigarette.

Several studies have demonstrated reduced levels of toxic and harmful compounds from ENDS<sup>[27-29]</sup>. However, effects on cell toxicity and function have shown controversial results<sup>[30-34]</sup>. To our knowledge, the effects of THS compared to conventional cigarettes on skeletal tissue and bone-forming cells has not previously been explored.

Therefore, the present study aimed to evaluate the effect of THS on osteogenic differentiation of mesenchymal stem cells and primary human osteoblasts, as well as to directly compare THS and conventional cigarette combustion on bone cells.

## MATERIALS AND METHODS

Cell Culture Medium and supplements were purchased from Life Technologies (Darmstadt, Germany). Chemicals were obtained from Sigma (Munich, Germany). Tobacco heating system 2.4 "IQOS®" and sticks (three commercially available flavors; bronze, amber and yellow) were provided by Philip Morris (Germany).

### *Isolation of human pre-osteoblasts and osteogenic differentiation*

Human osteoblasts (hOBs) were isolated from cancellous bone samples from BG Unfallklinik Tübingen. A consent form was obtained from all participants included in the study. hOBs isolation as well as all following experiments were performed in accordance with the 1964 Declaration of Helsinki and accordance with the ethical vote (538/2016BO2) approved by the ethics committee of the medical faculty of the Eberhard-Karls-Universität and University clinic Tübingen. The donors' average age was 73.2 ± 4.3 years (1 male and 4 female). Bone fragments were collected from

cancellous bone by mechanical disruption and washed with PBS to remove residual blood. Cancellous bone fragments were digested with 0.07%<sub>w/v</sub> Collagenase II (Serva, Heidelberg, Germany) in PBS at 37 °C for one hour. Following washing with PBS, released hOB were cultured in MEM/Ham's F12, 5%<sub>v/v</sub> FCS, 100 U/mL penicillin and 100 mg/mL streptomycin, 50 µmol/L L-ascorbate-2-phosphate, 50 µmol/L β-glycerol-phosphate in a water-saturated atmosphere of 5% CO<sub>2</sub> at 37 °C. Medium change was performed every 5 d. To induce osteogenic differentiation, cells in passage 2 were seeded at a density  $1.3 \times 10^5$  cells/cm<sup>2</sup> and treated with MEM/Ham's F12, 1%<sub>v/v</sub> FCS, 2 mmol/L L-glutamine, 200 µmol/L L-ascorbate-2-phosphate, 10 mmol/L β-glycerol-phosphate, 25 mmol/L HEPES, 1.5 mmol/L CaCl<sub>2</sub>, 100 nmol/L dexamethasone. For experiments, several concentrations of AE of conventional cigarettes or THS were added to the differentiation medium. Twice a week, the medium was changed during osteogenic differentiation, which was sustained for 21 d<sup>[35]</sup>.

### **MSCs culture and osteogenic differentiation**

Human immortalized bone marrow mesenchymal stem cells (SCP-1 cells, provided by Dr. Matthias Schieker<sup>[36]</sup>) were cultured in Minimum Essential Medium Eagle alpha (MEM α) supplemented with 5%<sub>v/v</sub> fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin, in a water-saturated atmosphere of 5% CO<sub>2</sub> at 37 °C. Medium change was performed every 5 d. Osteogenic differentiation of SCP-1 cells seeded at a density  $1 \times 10^5$  cells/cm<sup>2</sup> was induced with MEM α medium containing 1%<sub>v/v</sub> FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, 200 µmol/L L-ascorbate-2-phosphate, 10 mmol/L β-glycerol-phosphate, 25 mmol/L HEPES, 1.5 mmol/L CaCl<sub>2</sub>, and 100 nmol/L dexamethasone. For experiments, several concentrations of AE of conventional cigarettes or THS were added to the differentiation medium. The medium was changed twice a week during osteogenic differentiation, which was sustained for 21 d<sup>[18]</sup>.

### **Conventional cigarette and THS AE generation**

AE were generated according to the standard of Health Canada smoking regime which better represent the human smoking behavior<sup>[37]</sup>. For conventional cigarettes (Marlboro, Philip Morris, New York City, NY, United States) AE preparation, a blocked filter cigarette was placed in a standard gas washing bottle, subjected to a negative pressure by using a peristaltic pump. One cigarette was bubbled through 25 mL of cell culture medium at a rate of 2 puff/min, each puff lasting for 2 s and puff volume  $58.89 \pm 4.54$  mL. For THS AE preparation, a stick connected with the device THS 2.4 "IQOS®" was placed in a standard gas washing bottle and proceeded as described before. The freshly prepared AE was sterile filtered (0.22 µm filter) before use and freshly prepared for every exposure. The concentration of AE was determined and standardized by its optical density at 320 nm (OD<sub>320</sub>). An OD<sub>320</sub> of  $0.61 \pm 0.08$  or  $0.25 \pm 0.01$  was considered  $4 \times 10^{-1}$  puff/mL AE of conventional cigarettes or THS, respectively.

### **Experimental setup**

SCP-1 cells ( $n = 3$ ) and hOBs ( $n = 5$ ) were seeded in culture medium at a concentration of  $1 \times 10^5$  or  $1.3 \times 10^5$  cells/cm<sup>2</sup>, respectively. After attachment, cells were washed with PBS and stimulated with AE from conventional cigarettes or THS in concentrations between  $4 \times 10^{-1}$ – $4 \times 10^{-5}$  puffs/mL in differentiation medium. Untreated cells were considered as control. The medium was changed twice a week with fresh AE during osteogenic differentiation, which was sustained for 21 d.

### **Mitochondrial activity – resazurin conversion assay**

Cell viability was indirectly measured by resazurin conversion assay (mitochondrial activity). Briefly, cells were incubated with 0.0025%<sub>w/v</sub> resazurin in PBS for 30 min at 37 °C. The resulting Resorufin fluorescence was measured (excitation = 544 nm/emission = 590 nm) with a plate reader and corrected to the background. Changes in resazurin conversion are displayed relative to untreated cells<sup>[18,25]</sup>. The EC<sub>50</sub> was calculated using the EC<sub>50</sub> calculator tool of the AAT Bioquest webpage ([www.aatbio.com/tools/ec50-calculator](http://www.aatbio.com/tools/ec50-calculator)).

### **Live staining – calcein-AM staining**

Cell viability was determined by intracellular esterase activity with Calcein-AM staining (permeable non-fluorescent dye which is converted to a green fluorescent dye by esterases). Cells stimulated with AE according to the experimental setup were washed with PBS and were incubated with calcein-AM (2 µmol/L), and Hoechst 33342

(1:1000 in PBS) at 37 °C for 30 min. Cell images were taken (Epifluorescence: EVOS FL, life technologies, Darmstadt, Germany) after washing with PBS<sup>[25,38]</sup>.

### **Osteoblast function - AP activity assay**

Osteoblast function was evaluated by AP activity (early osteogenic marker). Cells were incubated with AP reaction buffer (0.2%<sub>w/v</sub> 4-nitrophenyl-phosphate, 50 mmol/L glycine, 1 mmol/L MgCl<sub>2</sub>, 100 mmol/L TRIS, pH = 10.5) for 40 min at 37 °C. Formed 4-nitrophenol was determined photometrically ( $\lambda$  = 405 nm) with a plate reader, corrected to the background and normalized to relative cell numbers. Changes in AP activity are displayed relative to untreated cells<sup>[18,25]</sup>.

### **Total protein content - sulforhodamine B staining**

The cell number was determined by sulforhodamine B (SRB) staining (total protein content). Cells were fixed with ice-cold ethanol for one hour at -20 °C. After washing with tap water, cells were stained with 0.4%<sub>w/v</sub> SRB (in 1% acetic acid) for 30 min at ambient temperature. Unbound SRB was removed by washing with 1% acetic acid. Bound SRB was resolved with 10 mmol/L unbuffered TRIS solution (pH = 10.5). Resulting SRB staining was quantified photometrically ( $\lambda$  = 565 nm) with a plate reader and corrected to the background<sup>[18]</sup>.

### **Calcium deposition - alizarin red staining**

Calcium deposition was measured by Alizarin red staining (late osteogenic marker). Cells were fixed with ice-cold ethanol for one hour at -20 °C. After washing with tap water, cells were stained with 0.5%<sub>w/v</sub> Alizarin Red solution (pH = 4.0) for 30 min at ambient temperature. Unbound Alizarin red was removed by washing with tap water. The resulting staining (red) was assessed microscopically. Bound Alizarin red was resolved with 10%<sub>w/v</sub> cetylpyridinium chloride. Resolved Alizarin Red was quantified photometrically ( $\lambda$  = 562 nm) with a plate reader and corrected to the background. Changes in matrix mineralization are displayed relative to untreated cells<sup>[18,25]</sup>.

### **Free radical production – 2',7'-dichlorofluorescein-diacetate assay**

To measure the formation of reactive oxygen species (ROS), 2',7'-dichlorofluorescein-diacetate (DCFH-DA) fluorescent probe was used. Briefly, cells were washed with PBS and incubated with 10  $\mu$ mol/L DCFH-DA in serum-free culture medium for 25 min at 37 °C. After washing twice with PBS, cells were stimulated with AE according to the experimental setup. As a positive control, 0.01%<sub>v/v</sub> (882  $\mu$ mol/L) H<sub>2</sub>O<sub>2</sub> was used. The fluorescence intensity, representing levels of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, HO, and ONOO<sup>-</sup>, was quantified after 0, 5, 10, and 15 min of incubation using a plate reader (excitation = 485 nm/emission = 520 nm). The slope of the linear part of the curve, resembling the product formation rate, was calculated. Cellular localization of the fluorescence was confirmed by fluorescence microscopy<sup>[18,25]</sup>.

### **Primary cilia length - Immunofluorescent staining**

Primary cilia length was determined by acetylated  $\alpha$ -tubulin immunofluorescence staining. After washing with PBS, cells were fixed with 4%<sub>w/v</sub> paraformaldehyde for 10 min at room temperature. Briefly, cells were washed and permeabilised with 0.2%<sub>v/v</sub> Triton-X-100 solution for 20 min at room temperature, followed by treatment with 2%<sub>v/v</sub> paraformaldehyde for 10 min at room temperature. Unspecific binding sites were blocked (5%<sub>w/v</sub> BSA in PBS) for one hour at room temperature, followed by incubation with anti-acetylated  $\alpha$ -tubulin antibody SC-23,950 (1:100 in PBS, Santa Cruz, Heidelberg, Germany) overnight at 4 °C. After washing, cells were incubated with ALEXA-488 labelled secondary antibody (1:2000 in DPBS, Life Technologies, Darmstadt, Germany) for 2 h at room temperature. Nuclei were stained with Hoechst 33342 (1:1000). Images were taken with an epifluorescence microscope (EVOS FL, life technologies, Darmstadt, Germany), and primary cilia length was analyzed using the ImageJ software (Version 1.5, NIH, Bethesda, MD, United States) (line tool) based on the maximum intensity projection method<sup>[39,40]</sup>.

### **Statistical analysis**

Results are presented as mean  $\pm$  SE. Each experiment was performed three independent times for SCP-1 cells or with five donors for hOBs (biological replicates,  $n$  = 3;  $n$  = 5 respectively) measured as triplicate or more (technical replicates,  $n \geq 3$ ). Statistical analyses were performed using the GraphPad Prism Software (Version 5, El Camino Real, United States). Data sets were compared by the nonparametric Mann Whitney test  $U$ -test (two single groups) or the Kruskal-Wallis  $H$  test (multiple groups)



followed by Dunn's multiple comparison test.  $\alpha = 0.05$  was set as the maximum type I error rate. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$  are used to classify  $P$  values in comparisons with untreated cells within AE concentration; <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$ , <sup>g</sup> $P < 0.001$  are used to classify  $P$  values for comparisons between AE from conventional cigarette and AE from THS system within AE concentration. The statistical methods of this study were reviewed by Johann Jacoby from Institute for Clinical Epidemiology and Applied Biometry at the University of Tübingen.

## RESULTS

### ***THS and conventional cigarette AE characterization***

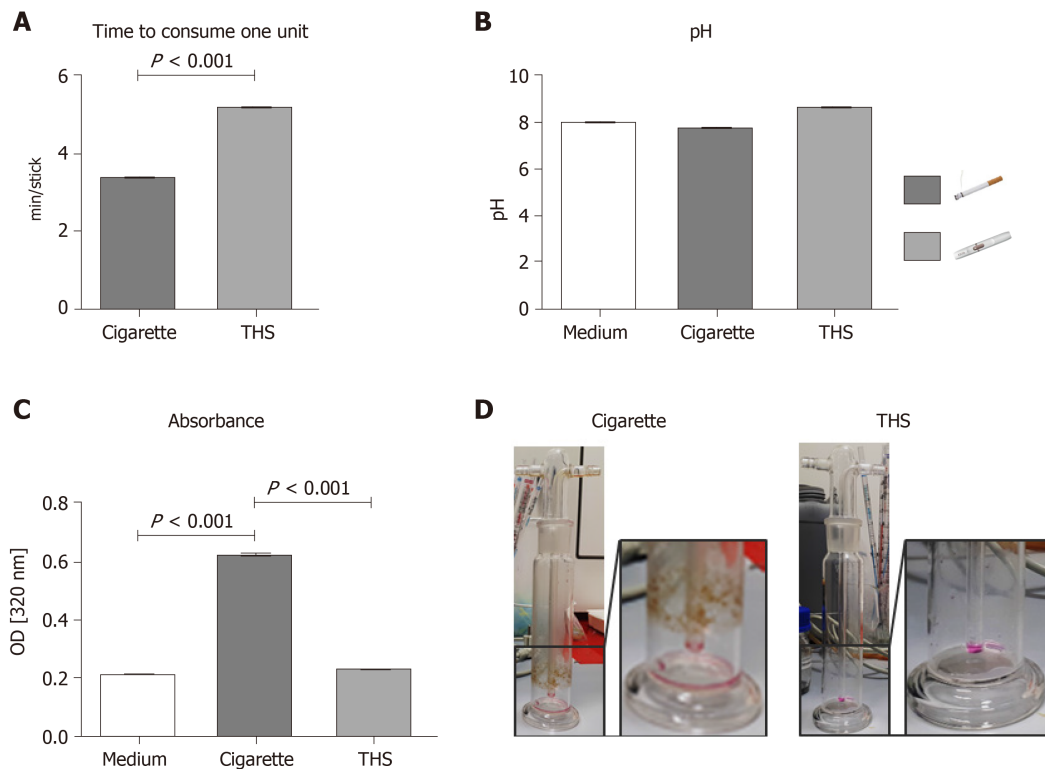
In order to compare the AE generated with THS and conventional cigarettes, the time to consume one unit, the pH, and the absorbance at 320 nm were measured after AE generation. The time to consume one unit was 1.6 minutes longer for THS than for a conventional cigarette, a difference that was significant (Figure 1A). The pH of the AE varied: Kruskal-Wallis  $\chi^2_{df=2} = 8.9027$ ,  $P = 0.0116$ . In particular, the pH of the AE generated from the THS (Median = 8.17) was higher than that generated from conventional cigarettes (Median = 7.70, Figure 1B). Interestingly, the absorbance at 320 nm of the AE produced from conventional cigarettes was two-fold higher than the AE produced from THS (Figure 1C), suggesting that the AE generated with regular cigarettes possessed significantly increased turbidity (presence of suspended particles) compared to AE generated using THS. AE generated with THS showed no significant differences in suspended particulates compared to the cell culture medium. This result was corroborated by increases in the amount of particles found in the gas washing bottle after conventional cigarette AE generation compared to THS AE generation (Figure 1D).

### ***THS has less effect on cell viability than conventional cigarettes following a single exposure***

In order to evaluate the impact of THS on MSCs and the viability of bone-forming cells, SCP-1 cells and hOBs were exposed to fresh AE generated using either conventional cigarettes or THS. After 48 h, cell viability was evaluated by resazurin conversion and esterase activity. The use of THS produced a significant reduction in SCP-1 cells' metabolic activity with concentrations of  $2 \times 10^{-1}$  and  $4 \times 10^{-1}$  puff/mL when compared to control cells (Figure 2A). However, for conventional cigarettes AE, similar adverse effects on SCP-1 cell viability were observed with concentrations of  $2 \times 10^{-1}$ ,  $4 \times 10^{-1}$  and  $4 \times 10^{-2}$  puff/mL (Figure 2A). We detected a significant decrease of SCP-1 cell viability by approximately 30% for THS and conventional cigarettes compared to control, however concentrations from THS were five times higher than the conventional cigarettes (Figure 2A). Esterase activity (measured with calcein-AM) confirmed the previous results, as  $4 \times 10^{-2}$  puff/mL AE from conventional cigarettes produced detrimental effects on SCP-1 cell viability compared to AE generated from THS (Figure 2C). Cytotoxicity from THS was decreased in hOBs when compared to MSCs. Interestingly, AE from conventional cigarettes produced a stronger effect on the viability of hOBs compared to MSCs (Figure 2A, B). A significant reduction to 35% viable cells was observed with AE from conventional cigarettes compared to untreated hOBs (Figure 2B). THS only showed a significant decrease to 67% viable cells for the highest concentration evaluated ( $4 \times 10^{-1}$  puff/mL) (Figure 2B). From these results, we concluded that THS AE was significantly less toxic to bone-forming cells and osteoprogenitor cells than AE from conventional cigarettes following a single exposure.

### ***THS is less toxic to bone cells than conventional cigarettes following chronic exposure***

Chronic smokers consume cigarettes on a regular basis over long periods of time. Therefore, smokers are in continuous and repeated contact with the toxic compounds present in cigarette smoke. The toxic components of cigarette smoke are distributed throughout the bloodstream to the entire body, producing adverse effects on several different tissues and cells, including the bone-forming cells. To better understand the cytotoxicity of tobacco alternative products on MSCs and hOBs, we evaluated the effects of chronic exposure. SCP-1 cells and hOBs were osteogenically differentiated with AE from conventional cigarettes and THS. The concentrations of AE ranged from  $4 \times 10^{-5}$  puff/mL to  $4 \times 10^{-2}$  puff/mL. These concentrations were chosen based on

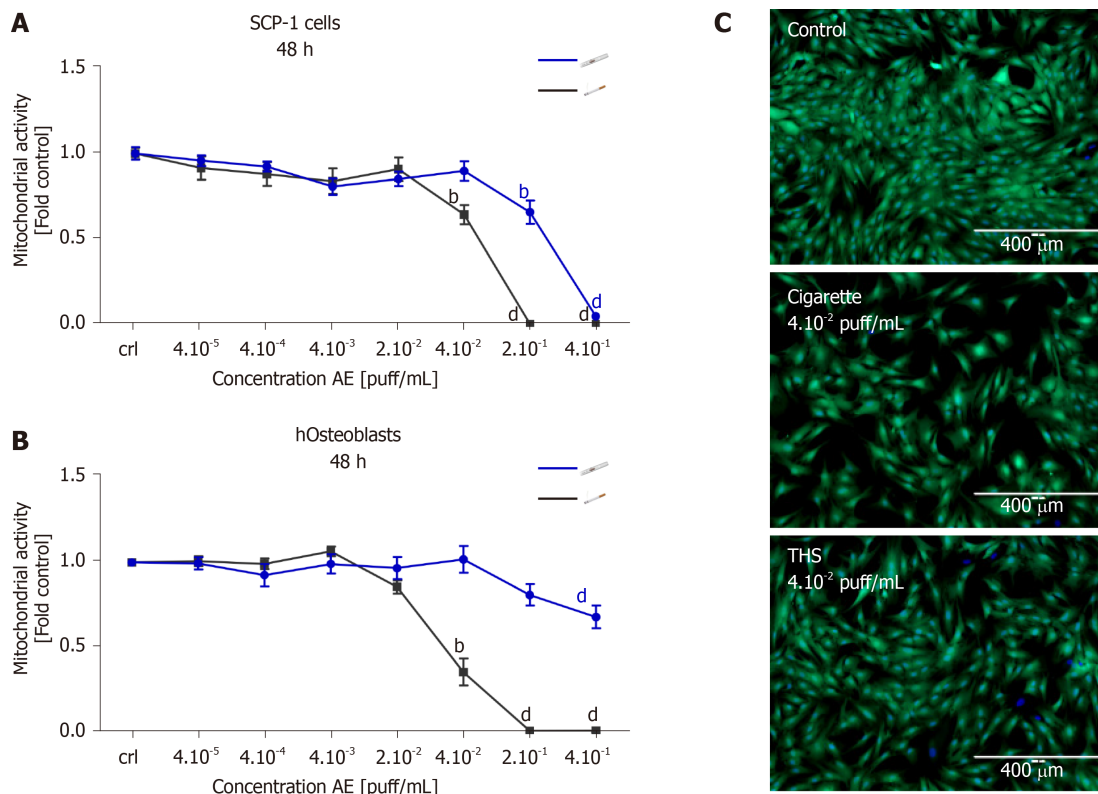


**Figure 1 Tobacco heating systems and conventional cigarette aqueous extract characterization.** A: Time to consume one unit [cigarette or tobacco heating systems (THS) stick] measured in minutes; B: pH from the aqueous extract (AE) generated with conventional cigarettes or THS; C: Absorbance at 320 nm from the different fractions produced from conventional cigarette or THS; D: Representative picture of gas washing bottle after conventional cigarette or THS AE generation, respectively. Each measure was conducted with six independent AE for each condition in triplicates. Data were analyzed using the nonparametric Mann Whitney test or the Kruskal-Wallis *H* test followed by Dunn's post-hoc tests. Data are presented as mean  $\pm$  SE, and  $P < 0.001$  for the comparison. THS: Tobacco heating systems.

previous results obtained from the single exposure experiment (Figure 2). The concentrations  $2 \times 10^{-1}$  puff/mL and  $4 \times 10^{-1}$  puff/mL were not used for further experiments due to the cytotoxic effects observed from the single exposure (Figure 2). Following either 14 or 21 d of osteogenic differentiation, the metabolic status of the cells was evaluated using resazurin conversion assay (Figure 3). Chronic exposure to THS AE resulted in no significant changes in SCP-1 cell viability after either 14 or 21 days of osteogenic differentiation when compared to untreated cells (Figure 3A, B). As expected, conventional cigarette AE significantly reduced cell viability by 50% using a concentration of  $2 \times 10^{-2}$  puff/mL after 14 and 21 d of exposure compared to control cells (Figure 3A, B). Conventional cigarette AE significantly increased cytotoxicity using concentrations of  $4 \times 10^{-2}$  puff/mL and  $2 \times 10^{-2}$  puff/mL on SCP-1 cells after 14 and 21 d of chronic exposure, respectively (Figure 3A, B). Similarly, THS AE did not show cytotoxic effects in hOBs exposed to concentrations up to  $4 \times 10^{-2}$  puff/mL after either 14 or 21 d of chronic exposure (Figure 3C, D). However, conventional cigarette AE significantly decreased hOBs viability following exposure to a concentration of  $4 \times 10^{-2}$  puff/mL (Figure 3C, D). Chronic exposure to  $4 \times 10^{-2}$  puff/mL conventional cigarette AE resulted in a significant decrease in hOBs mitochondrial activity in comparison to THS exposed to the same concentration (Figure 3C, D). These results were confirmed by the  $EC_{50}$  calculated for THS and conventional cigarette AE. In the case of THS AE, an  $EC_{50}$  of 0.19 puff/mL and  $> 0.04$  puff/mL were calculated for SCP-1 cells and hOBs, respectively. The  $EC_{50}$  of conventional cigarettes was lower in SCP-1 cells (0.02 puff/mL) and hOBs (0.03 puff/mL). Thus, THS showed less cytotoxic effects than conventional cigarettes on bone cells chronically exposed to AE. Additionally, MSCs were more sensitive to AE adverse effects than hOBs.

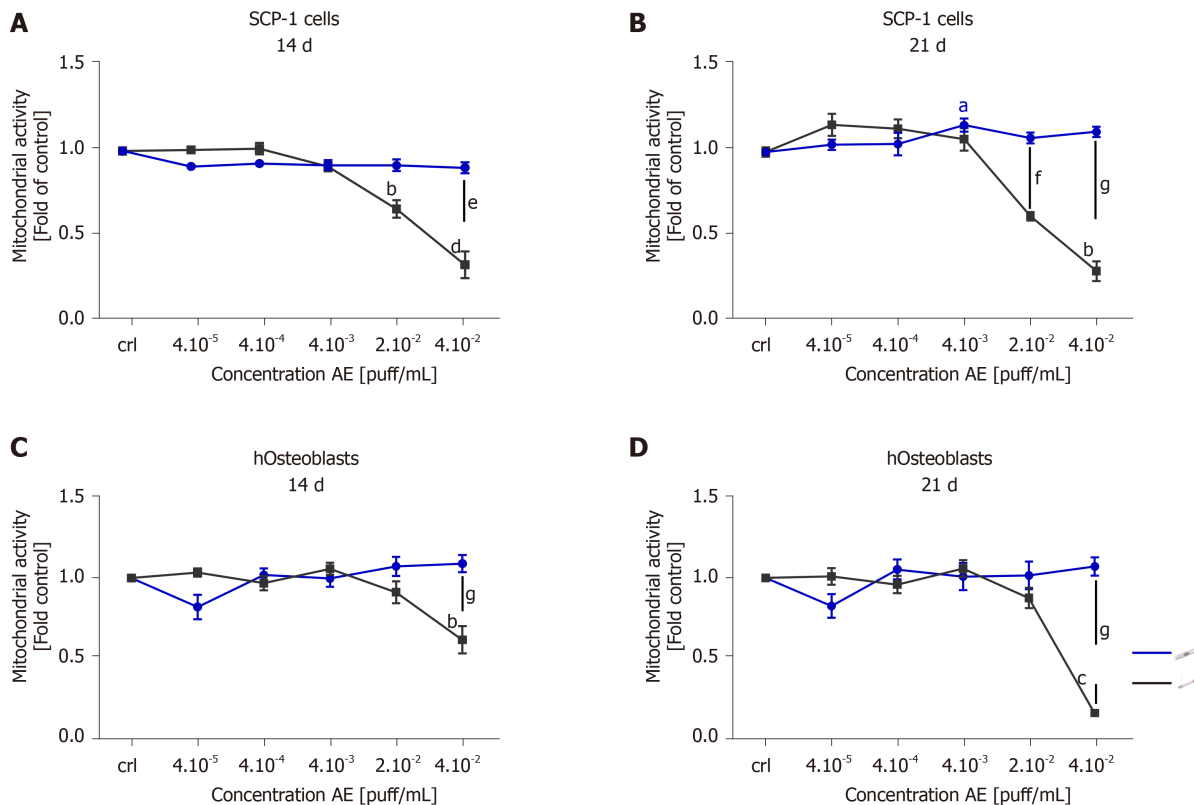
#### **THS has a lower impact on MSCs and human osteoblast function after chronic exposure**

Since AE from THS produced less cytotoxic effects than AE from conventional cigarettes, we were interested in whether THS would affect osteogenic differentiation of MSCs and hOBs function. The potential of SCP-1 cells to differentiate into



**Figure 2** High concentrations of aqueous extract from tobacco heating systems has minor effects on bone cells viability after a single exposure than aqueous extract from conventional cigarettes. SCP-1 cells and primary human osteoblasts were treated with increasing concentrations of aqueous extract (AE) from conventional cigarettes and tobacco heating systems. Cell viability was evaluated by resazurin conversion (mitochondrial activity) in SCP-1 cells (A) and primary osteoblast (B) after 48 h of treatment; C: Representative live staining pictures from SCP-1 cells using calcein-AM (green) and nuclear staining using Hoechst 33342 (blue) was shown after 48 h of exposure to AE (scale bar 400 μm). Each measure was conducted at least three independent times in triplicates. Data were analyzed using the Kruskal-Wallis H test followed by Dunn's post-hoc tests. Data are presented as mean ± SE, and *P* values indicated as <sup>b</sup>*P* < 0.01 and <sup>d</sup>*P* < 0.001 for comparisons with untreated cells within the same AE type. AE: Aqueous extract.

osteoblasts and hOBs function were evaluated using AP activity (an early marker of osteogenic differentiation<sup>[41]</sup>) and calcium deposition (a late marker of osteogenic differentiation<sup>[41]</sup>) after 14 and 21 d, respectively. As expected,  $2 \times 10^{-2}$  puff/mL conventional cigarette AE significantly decreased AP activity three-fold in SCP-1 cells when compared to untreated cells following 14 d (Figure 4A). In contrast, increased AP activity was observed in SCP-1 cells treated with  $4 \times 10^{-2}$  puff/mL AE from conventional cigarettes (Figure 4A). This increase in AP activity could be due to the detrimental effects on SCP-1 cell viability observed after treatment with  $4 \times 10^{-2}$  puff/mL AE from conventional cigarettes (Figure 2A). Nevertheless, THS AE produced significant reductions in the AP activity of SCP-1 cells by 1.6 times that of untreated cells, however the concentration of THS AE was twice as high as that of conventional cigarette AE (Figure 4A). Regarding matrix production, THS decreased calcium deposition in a dose-independent manner up to 20% (Figure 4B). However, conventional cigarette AE significantly decreased calcium deposition at concentrations of  $2 \times 10^{-2}$  and  $4 \times 10^{-2}$  puff/mL (Figure 4B). Due to the role of AP and matrix formation as early and late markers of osteogenic differentiation respectively, these results indicate that THS had less impact on osteogenic differentiation in SCP-1 cells compared to conventional cigarettes. Repeated exposure of hOBs to THS AE did not affect AP activity (Figure 4C). Conventional cigarettes AE significantly decreased AP activity in hOBs in a dose-dependent manner using concentrations higher than  $4 \times 10^{-3}$  puff/mL (Figure 4C). THS did not significantly affect calcium deposition in hOBs using any of the concentrations evaluated (Figure 4D). Only conventional cigarette AE significantly decreased calcium deposition in hOBs using concentrations of  $2 \times 10^{-2}$  and  $4 \times 10^{-2}$  puff/mL in comparison to THS (Figure 4D), suggesting that THS has less impact on hOBs function compared to conventional cigarettes.



**Figure 3 Tobacco heating systems is less toxic than conventional cigarettes after chronic exposure.** SCP-1 cells and primary human osteoblasts were osteogenically differentiated with increasing concentrations of aqueous extract (AE) from conventional cigarette and tobacco heating systems (THS) for 21 d. Cell viability was evaluated by resazurin conversion (mitochondrial activity) in SCP-1 cells (A, B) and primary human osteoblast (C, D) after 14 (A, C) and 21 d (B, D). Each measurement was conducted at least three independent times in triplicates. Data were analyzed using the Kruskal-Wallis *H* test followed by Dunn's post-hoc test. Data are presented as mean  $\pm$  SE. *P* values are classified as <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 for comparisons with untreated cells within AE type and as <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 for comparisons of conventional cigarette with THS within the same concentration. AE: Aqueous extract.

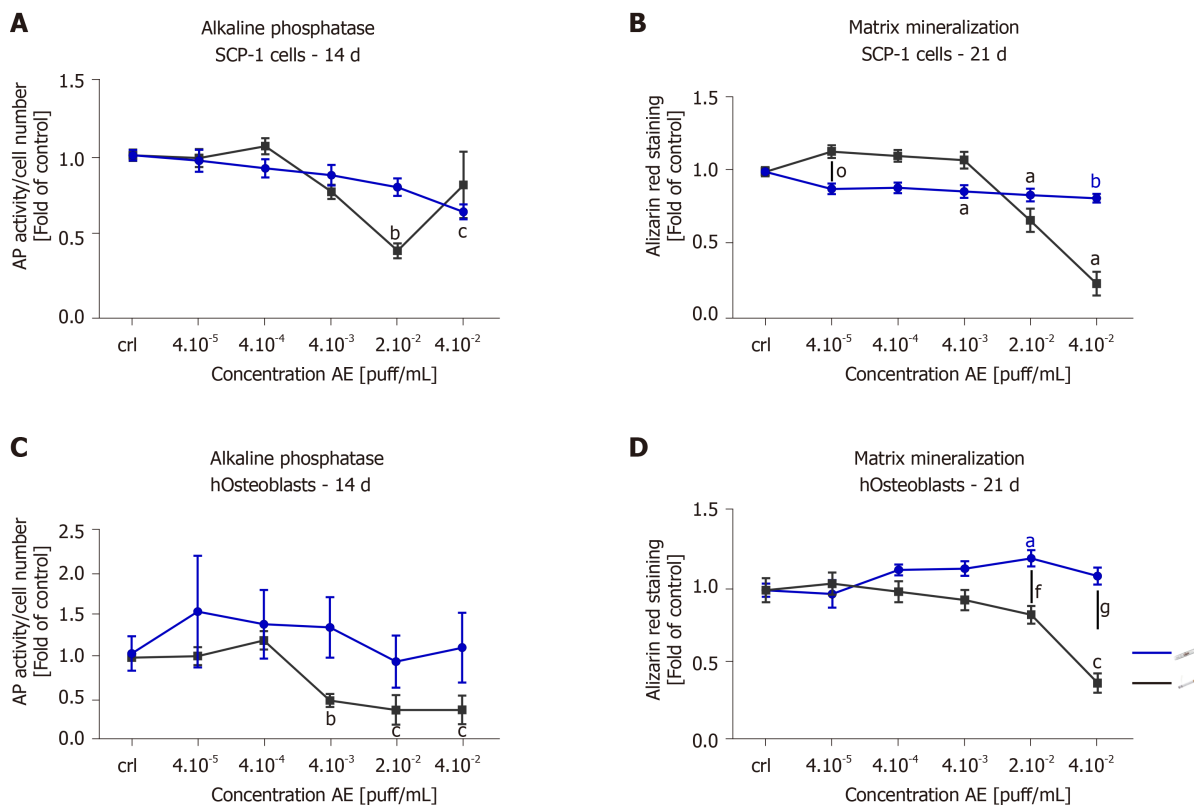
### **THS induces less oxidative stress and causes less damage to primary cilia structures than conventional cigarettes in MSCs.**

Since it has previously been demonstrated that oxidative stress induced by the compounds contained in CS causes primary cilia structure disruption, which in turn impairs osteogenic differentiation of SCP-1 cells<sup>[18,25]</sup>, we were interested in evaluating the levels of ROS produced by SCP-1 cells as well as primary cilia structure integrity following THS exposure. As expected, conventional cigarettes AE significantly increased ROS levels by ten-fold in SCP-1 cells using a concentration of  $2 \times 10^{-1}$  puff/mL (Figure 5A). This increase in oxidative stress seemed to be dose-dependent. Surprisingly, THS showed no significant increase in ROS levels in SCP-1 cells (Figure 5A). Only a slight (not significant) increase in ROS levels was observed in SCP-1 cells treated with the highest concentration of THS evaluated (Figure 5A). Additionally, THS did not affect the length of the microtubule-based organelles, primary cilia (involved in the initiation and maintenance of MSCs osteogenic differentiation<sup>[42,43]</sup>) in SCP-1 cells after 14 d of chronic exposure for any of the concentrations evaluated (Figure 5B, C). However, primary cilia structure of SCP-1 cells exposed to conventional cigarettes showed significant reductions in length by 50% using a concentration of  $4 \times 10^{-1}$  puff/mL (Figure 5B, C). Representative immunofluorescence staining pictures of acetylated  $\alpha$ -tubulin in SCP-1 cells differentiated with either conventional cigarette AE or THS AE at a concentration of  $4 \times 10^{-1}$  puff/mL, or untreated, are shown in Figure 5C. In summary, THS induced less oxidative stress due to reduced levels of harmful constituents, and did not impair primary cilia structure, thereby producing less impact on MSCs osteogenic differentiation than conventional cigarettes.

### **No differential variation in cytotoxicity was observed between different THS flavors in MSCs**

Our previous results showed that THS AE was less harmful than conventional



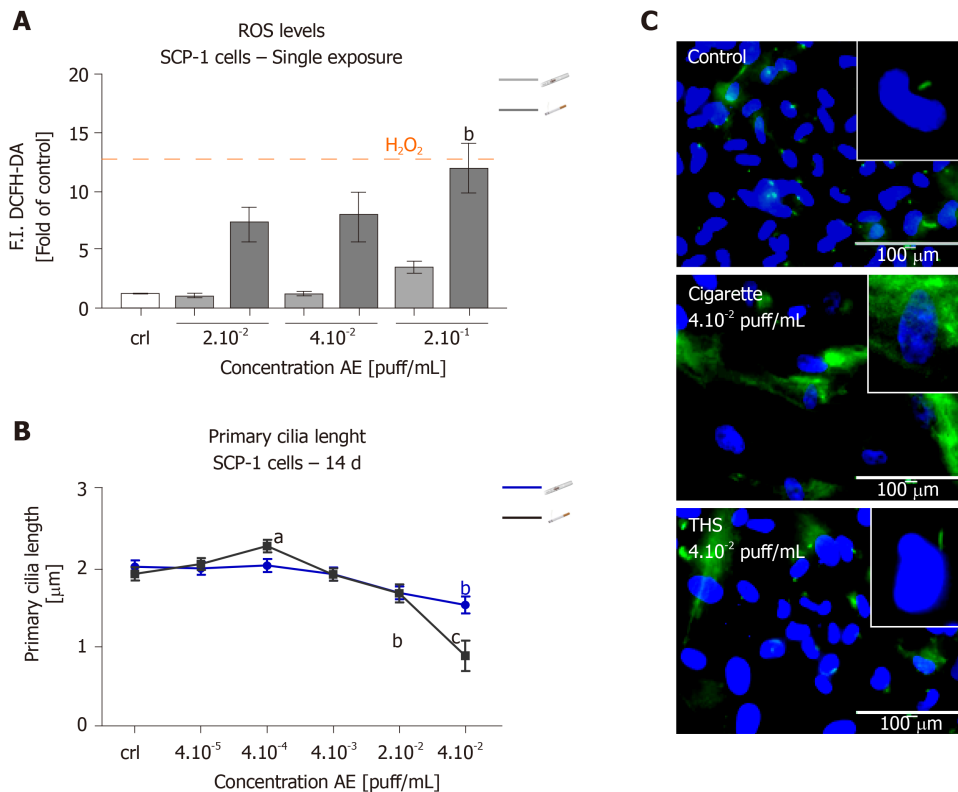


**Figure 4 Tobacco heating systems has a lower impact on bone cells function than conventional cigarettes.** SCP-1 cells and primary human osteoblasts were osteogenically differentiated with increasing concentrations of aqueous extract (AE) from conventional cigarettes and tobacco heating systems (THS) for 21 d. Cell function was evaluated by alkaline phosphatase activity (early differentiation marker) (A, C) after 14 d. Calcium deposition (late marker of differentiation) was evaluated by Alizarin red staining (B, D) after 21 d (B, D). Each measurement was conducted at least three independent times in triplicates. Data were analyzed using the Kruskal-Wallis *H* test followed by Dunn's post-hoc test. Data are represented as mean  $\pm$  SE, and *P* values are classified as <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 for comparisons with untreated cells within the same AE type and <sup>f</sup>*P* < 0.01, <sup>g</sup>*P* < 0.001 for comparisons conventional cigarette with THS within the same concentration. AE: Aqueous extract.

cigarettes on MSCs osteogenic differentiation, as well as hOBs function. Three THS-flavored sticks (bronze - mocha and dried fruit flavor, amber - wood and nut flavor, and yellow - herbal flavor) were selected for further cytotoxicity screening. Dose-responses of mitochondrial activity in SCP-1 cells exposed to selected THS-flavored AE are shown in Figure 6. No significant differences in cell viability were detected after a 48 h exposure to all AE tested with a concentration up to  $4 \times 10^{-2}$  puff/mL compared to untreated cells. It is essential to highlight that a significant decrease in cell viability was observed with concentrations higher or equal to  $2 \times 10^{-1}$  puff/mL with all flavored THS AE evaluated (Figure 6), thus confirming the results shown in Figure 2A. These results suggest that the cytotoxicity observed following THS treatment may be associated with exposure to very high, non-physiological concentrations, and was not associated with flavor.

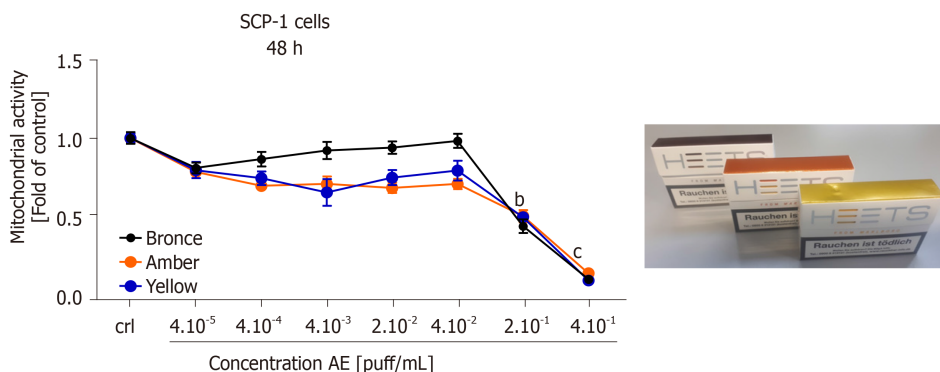
## DISCUSSION

While the detrimental effects of conventional cigarettes on the skeletal system have been extensively reported<sup>[4,5]</sup>, the effects of THS on bone health remain unknown. Smoking conventional cigarettes disturbs bone homeostasis, resulting in an imbalance of bone-forming and bone-resorbing cells, resulting in loss of bone mass and an increased risk of osteoporosis and fracture<sup>[6,7,9-11]</sup>. The detrimental impact of conventional cigarettes on bone function raises the need to develop new alternatives for smokers. ENDS, including e-cigarettes or THS, focus on heating rather than combustion, in order to reduce the generation of harmful constituents. Therefore, ENDS provide the same levels of nicotine as conventional cigarettes and maintain the smoking ritual. E-cigarettes heat liquids based on propylene glycol, glycerin, flavor and (optionally) nicotine into an aerosol that is then inhaled. THS, in contrast, heat tobacco rolled up in a stick form up to 350 °C instead of burning it.



**Figure 5 Tobacco heating systems induces less oxidative stress and causes less damage to primary cilia structures on bone cells precursor than conventional cigarettes.**

SCP-1 cells were treated with increasing concentrations of aqueous extract (AE) from conventional cigarettes and tobacco heating systems (THS). ROS production was evaluated by DCFH-DA assay in SCP-1 cells. 0.01%  $\text{H}_2\text{O}_2$  was used as a positive control (A). Primary cilium length was quantified on day 14 by acetylated  $\alpha$ -tubulin (green), and nuclei (blue) immunostaining (B). Representative immunostaining images of SCP-1 cells primary cilia after 14 days of chronic exposure to THS or conventional cigarettes AE (C) (scale bar 100  $\mu\text{m}$ ). Each measurement was conducted at least three independent times in triplicates. Data were analyzed using the Kruskal-Wallis H test followed by Dunn's post-hoc tests. Data are represented as mean  $\pm$  SE, and *P* values are classified as <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 for comparisons with untreated cells within AE type. AE: Aqueous extract.



**Figure 6 No differential variation in cytotoxicity between different tobacco heating systems flavors on bone cells precursor.**

SCP-1 cells were treated with increasing concentrations of aqueous extract (AE) from tobacco heating systems bronze, amber and yellow flavor. Cell cytotoxicity was evaluated by resazurin conversion (mitochondrial activity) in SCP-1 cells after 48 h of exposure. Each measurement was conducted at least three independent times in triplicates. Data were analyzed using the Kruskal-Wallis H test followed by Dunn's post-hoc tests. Data are presented as mean  $\pm$  SE, and *P* values are classified as <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 for comparisons with untreated cells within AE type. AE: Aqueous extract.

A better understanding of the effects of THS on the skeletal system is required. Previous *in vitro* studies demonstrated that the detrimental effects of cigarette smoke on bone homeostasis could take place independently of nicotine<sup>[18-20]</sup>. The effects appear to be dependent on oxidative stress induced by constituents present in the cigarette smoke<sup>[18,21,23,25,44]</sup>.

The results presented in this study have demonstrated that the time to consume one unit was significantly longer for THS than conventional cigarettes, thus the smoking experience was longer for THS. Additionally, pH did not significantly change between

the different AE fractions and the cell culture medium, validating that this was not the reason for the stronger cytotoxicity observed from conventional cigarette AE. Interestingly, AE produced from THS showed no substantial differences in suspended particulates compared to the cell culture medium. Conversely, AE generated with conventional cigarettes had a significantly increased turbidity. This result was in line with previous studies that have demonstrated that THS delivers less harmful constituents than conventional cigarettes<sup>[27,28]</sup>.

Our analysis demonstrated a reduced impact on MSCs and hOBs viability following treatment with THS AE compared to conventional cigarettes after a single exposure. Moreover, different THS flavors were not related to the cytotoxic effects on bone cells. More sustained adverse effects were observed in response to chronic exposure for 21 d to conventional cigarette AE than to THS AE. This result was supported by reduced cell viability, reduced AP activity, and less mineralized matrix formation measured in bone-forming cells and bone precursor cells. This demonstrated a significantly lower influence by THS AE on the differentiation of MSCs and hOBs function, supporting previous publications regarding decreases in detrimental effects by THS in lung tissue and the cardiovascular system evaluated *in vivo* and *in vitro* when compared to 3R4F cigarette combustion<sup>[30,31,45,46]</sup>.

It was demonstrated that oxidative stress induced by the compounds contained in conventional cigarette combustion could be one of the responsible factors for the impaired osteogenic differentiation of bone-forming cells and precursors cells, due to an imbalance in the anti-oxidative system that negatively affects the function of the anti-oxidative enzymes<sup>[18,21-25]</sup>. Interesting, Munakata *et al.*<sup>[32]</sup> demonstrated that THS induced less oxidative stress than conventional cigarettes in bronchial epithelial cells. Our results also show that THS AE did not increase the level of ROS production in MSCs. Nevertheless, previous results demonstrated that nicotine and cotinine inhibit catalase and glutathione reductase enzymatic activity, affecting the function of the cells' antioxidant system<sup>[18]</sup>. This fact alludes that using ENDS may be a less harmful alternative for smokers that are orthopedic patients with increased oxidative stress levels (due to the trauma and associated surgery).

In our study, we observed no detrimental effect of THS AE on MSCs primary cilia structure. Since primary cilia play an essential role in the initiation of osteogenic differentiation in MSCs, as well as in the maintenance of the differentiated status of the cells<sup>[42,47]</sup>, we conclude that the minor effect of THS on bone-forming cells and progenitor cells is due to decreased oxidative stress, which conserved the primary cilia structure, in contrast to the previous results reported with conventional cigarettes<sup>[18,25,39]</sup>.

A major limitation in this study that could be addressed in future research is that this study focused on the effect of THS AE only in MSCs and hOBs. As several cell types are involved in bone homeostasis, THS could potentially influence the function of other cells, such as immune cells, osteoclasts, or osteocytes. Co-culture systems may provide a better alternative for screening the effects of ENDS on bone metabolism and predicting cytotoxicity in bone tissue. Furthermore, the addition of cytokines, normally increased after fracture, to a co-culture system could better represent the fracture healing process. By combining a more complex setup with different cells types and an inflammatory environment expected after fracture, disturbance in factors that influence the healing could be analyzed under ENDS exposure (*e.g.*, TGF- $\beta$ 1, MSCs chemoattractant affected conventional cigarette AE<sup>[39]</sup>).

Additionally, our system could be improved by including other cell types, *e.g.*, endothelial cells or immune cells, to represent better the molecular species that finally cross the bloodstream. Additionally, multiple interconnected cell culture systems representing functionalities of different organs, could represent exposure of bone-forming cells closer to humans. In this system, the paracrine interaction among different organs could be instigated as well as the potential toxicity of or side effects of the additional metabolites of ENDS coming from different tissues (*e.g.*, lung, liver, *etc.*) could be evaluated. However, it should be consider that increasing the complexity of the model also decreases the analytical methods available for characterization.

This research serves as one of the first *in vitro* studies to demonstrate reductions in the harmful effects on bone-forming cells and bone progenitor cells treated with THS compared to conventional cigarettes. THS could be a potential alternative for smokers to maintain appropriate bone homeostasis and delay development of secondary osteoporosis, which consequently would reduce health system costs. However, more studies are required to confirm if THS could be a viable alternative for smokers to maintain appropriate bone homeostasis and improve delays in bone healing.

## ARTICLE HIGHLIGHTS

### Research background

Cigarette smoking (CS) is the most common method of consuming tobacco. Deleterious effects on bone integrity, increased incidence of fractures, and delayed fracture healing are all associated with CS. Tobacco combusted at about 800 °C generates approximately 6500 molecular species, more than 150 of which have been identified as toxic compounds. New approaches have been on developing reduced-risk alternatives for smokers that maintain the smoking ritual, while providing the same levels of nicotine as conventional cigarettes with less harmful constituents.

### Research motivation

New technologies designed to develop a reduced-risk alternative for smokers are based on electronic nicotine delivery systems, such as e-cigarettes and tobacco heating systems (THS). Instead of burning tobacco, THS heat tobacco rolled up in a stick form up to 350 °C (avoiding combustion and formation of ashes). THS contain tobacco and convey the feeling of smoking a conventional cigarette. Several studies have demonstrated reduced levels of toxic and harmful compounds from electronic nicotine delivery systems.

### Research objectives

The present study aim to examine the effects of THS on osteoprogenitor cell viability and function compared to conventional CS.

### Research methods

Human immortalized mesenchymal stem cells and primary human pre-osteoblasts isolated from cancellous bone samples were osteogenically differentiated with aqueous extracts generated from either the THS 2.4 "IQOS" or conventional "Marlboro" cigarettes for up to 21 d. Cell viability was analyzed using resazurin conversion assay (mitochondrial activity) and calcein-AM staining (esterase activity). Osteogenic differentiation and bone cell function were evaluated using alkaline phosphatase (AP) activity, while matrix formation was analyzed through alizarin red staining. Primary cilia structure was examined by acetylated  $\alpha$ -tubulin immunofluorescent staining. Free radical production was evaluated with 2',7'-dichlorofluorescein-diacetate assay.

### Research results

THS is significantly less toxic to bone cells than CS when analyzed by mitochondrial and esterase activity ( $P < 0.001$ ). No significant differences in cytotoxicity between the diverse flavors of THS were observed. Harmful effects from THS on bone cell function were observed only at non-physiological concentrations. In contrast, conventional cigarettes significantly reduced the AP activity (by two-fold) and matrix mineralization (four-fold) at low concentrations. Moreover, morphologic analysis of primary cilia revealed no significant changes in the length of the organelle involved in osteogenesis of osteoprogenitor cells, nor in the number of ciliated cells following THS treatment. Assessment of free radical production demonstrated that THS induced significantly less oxidative stress than conventional CS in osteoprogenitor cells.

### Research conclusions

The present study demonstrate reductions in the harmful effects on bone-forming cells and bone progenitor cells treated with THS compared to conventional cigarettes.

### Research perspectives

THS could be a potential alternative for smokers to maintain appropriate bone homeostasis and delay development of secondary osteoporosis, which consequently would reduce health system costs.

## ACKNOWLEDGEMENTS

We would like to Dr. Alexander Nussbaum for providing the THS 2.4 system "IQOS" device and sticks, Bianca Braun for their excellent technical assistance, Dr. Julia Hoeng, Dr. Bjorn Titz, Dr. Anita Iskandar and Shoaib Majeed (M.Eng.) for the interesting



discussions.

## REFERENCES

- Kahnert S, Pötschke-Langer M, Kahnert S, Viariso V, Heidt C, Schunk S, Mons U, Fode K. Tabakatlas deutschland 2015. Pabst Science Publishers. 2015
- GBD 2015 Tobacco Collaborators. Smoking prevalence and attributable disease burden in 195 countries and territories, 1990-2015: a systematic analysis from the Global Burden of Disease Study 2015. *Lancet* 2017; **389**: 1885-1906 [PMID: 28390697 DOI: 10.1016/S0140-6736(17)30819-X]
- Cooke M. The chemical components of tobacco and tobacco smoke. *Chromatographia* 2010; **71**: 977-977 [DOI: 10.1365/s10337-010-1556-3]
- Cusano NE. Skeletal Effects of Smoking. *Curr Osteoporos Rep* 2015; **13**: 302-309 [PMID: 26205852 DOI: 10.1007/s11914-015-0278-8]
- Wong PK, Christie JJ, Wark JD. The effects of smoking on bone health. *Clin Sci (Lond)* 2007; **113**: 233-241 [PMID: 17663660 DOI: 10.1042/cs20060173]
- Ward KD, Klesges RC. A meta-analysis of the effects of cigarette smoking on bone mineral density. *Calcif Tissue Int* 2001; **68**: 259-270 [PMID: 11683532 DOI: 10.1007/bf02390832]
- Kanis JA, Johnell O, Oden A, Johansson H, De Laet C, Eisman JA, Fujiwara S, Kroger H, McCloskey EV, Mellstrom D, Melton LJ, Pols H, Reeve J, Silman A, Tenenhouse A. Smoking and fracture risk: a meta-analysis. *Osteoporos Int* 2005; **16**: 155-162 [PMID: 15175845 DOI: 10.1007/s00198-004-1640-3]
- Amin S, Niu J, Guermazi A, Grigoryan M, Hunter DJ, Clancy M, LaValley MP, Genant HK, Felson DT. Cigarette smoking and the risk for cartilage loss and knee pain in men with knee osteoarthritis. *Ann Rheum Dis* 2007; **66**: 18-22 [PMID: 17158140 DOI: 10.1136/ard.2006.056697]
- Adams CI, Keating JF, Court-Brown CM. Cigarette smoking and open tibial fractures. *Injury* 2001; **32**: 61-65 [PMID: 11164405 DOI: 10.1016/s0020-1383(00)00121-2]
- Scolaro JA, Schenker ML, Yannascoli S, Baldwin K, Mehta S, Ahn J. Cigarette smoking increases complications following fracture: a systematic review. *J Bone Joint Surg Am* 2014; **96**: 674-681 [PMID: 24740664 DOI: 10.2106/JBJS.M.00081]
- Mills E, Eyawo O, Lockhart I, Kelly S, Wu P, Ebbert JO. Smoking cessation reduces postoperative complications: a systematic review and meta-analysis. *Am J Med* 2011; **124**: 144-154.e8 [PMID: 21295194 DOI: 10.1016/j.amjmed.2010.09.013]
- Abate M, Vanni D, Pantalone A, Salini V. Cigarette smoking and musculoskeletal disorders. *Muscles Ligaments Tendons J* 2013; **3**: 63-69 [PMID: 23888288 DOI: 10.11138/mltj/2013.3.2.063]
- Singh JA, Schleck C, Harmsen WS, Jacob AK, Warner DO, Lewallen DG. Current tobacco use is associated with higher rates of implant revision and deep infection after total hip or knee arthroplasty: A prospective cohort study. *BMC Med* 2015; **13**: 283 [DOI: 10.1186/s12916-015-0523-0]
- Ehnert S, Aspera-Werz RH, Ihle C, Trost M, Zirn B, Flesch I, Schroter S, Relja B, Nussler AK. Smoking dependent alterations in bone formation and inflammation represent major risk factors for complications following total joint arthroplasty. *J Clin Med* 2019; **8**: 406 [DOI: 10.3390/jcm8030406]
- Rothem DE, Rothem L, Soudry M, Dahan A, Eliakim R. Nicotine modulates bone metabolism-associated gene expression in osteoblast cells. *J Bone Miner Metab* 2009; **27**: 555-561 [PMID: 19436947 DOI: 10.1007/s00774-009-0075-5]
- Pappas RS. Toxic elements in tobacco and in cigarette smoke: inflammation and sensitization. *Metallomics* 2011; **3**: 1181-1198 [PMID: 21799956 DOI: 10.1039/c1mt00066g]
- U.S. Food and Drug Administration. Harmful and potentially harmful constituents in tobacco products and tobacco smoke: Established list. 2012. Available from: <https://www.fda.gov/tobacco-products>
- Aspera-Werz RH, Ehnert S, Heid D, Zhu S, Chen T, Braun B, Sreekumar V, Arnscheidt C, Nussler AK. Nicotine and Cotinine Inhibit Catalase and Glutathione Reductase Activity Contributing to the Impaired Osteogenesis of SCP-1 Cells Exposed to Cigarette Smoke. *Oxid Med Cell Longev* 2018; **2018**: 3172480 [PMID: 30533170 DOI: 10.1155/2018/3172480]
- Daffner SD, Waugh S, Norman TL, Mukherjee N, France JC. Nicotine Increases Osteoblast Activity of Induced Bone Marrow Stromal Cells in a Dose-Dependent Manner: An in vitro Cell Culture Experiment. *Global Spine J* 2012; **2**: 153-158 [PMID: 24353962 DOI: 10.1055/s-0032-1326946]
- Kim BS, Kim SJ, Kim HJ, Lee SJ, Park YJ, Lee J, You HK. Effects of nicotine on proliferation and osteoblast differentiation in human alveolar bone marrow-derived mesenchymal stem cells. *Life Sci* 2012; **90**: 109-115 [PMID: 22115820 DOI: 10.1016/j.lfs.2011.10.019]
- Braun KF, Ehnert S, Freude T, Egaña JT, Schenck TL, Buchholz A, Schmitt A, Siebenlist S, Schyschka L, Neumaier M, Stöckle U, Nussler AK. Quercetin protects primary human osteoblasts exposed to cigarette smoke through activation of the antioxidative enzymes HO-1 and SOD-1. *ScientificWorldJournal* 2011; **11**: 2348-2357 [PMID: 22203790 DOI: 10.1100/2011/471426]
- Ehnert S, Braun KF, Buchholz A, Freude T, Egaña JT, Schenck TL, Schyschka L, Neumaier M, Döbele S, Stöckle U, Nussler AK. Diallyl-disulphide is the effective ingredient of garlic oil that protects primary human osteoblasts from damage due to cigarette smoke. *Food Chem* 2012; **132**: 724-729 [DOI: 10.1016/j.foodchem.2011.11.008]
- Ehnert S, Stefan D, Friedrich BK, Britta B, Valeska H, Mario H, Tomas EJ, Ulrich S, Thomas F, Klaus NA. N-acetylcysteine and flavonoid rich diet: The protective effect of 15 different antioxidants on cigarette smoke-damaged primary human osteoblasts. *Adv Biosci Biotechnol* 2012; **3**: 1129-1139 [DOI: 10.4236/abb.2012.38139]
- Holzer N, Braun KF, Ehnert S, Egaña JT, Schenck TL, Buchholz A, Schyschka L, Neumaier M, Benzing S, Stöckle U, Freude T, Nussler AK. Green tea protects human osteoblasts from cigarette smoke-induced injury: possible clinical implication. *Langenbecks Arch Surg* 2012; **397**: 467-474 [PMID: 22160325 DOI: 10.1007/s00423-011-0882-8]

- 25 **Sreekumar V**, Aspera-Werz R, Ehnert S, Strobel J, Tendulkar G, Heid D, Schreiner A, Arnscheidt C, Nussler AK. Resveratrol protects primary cilia integrity of human mesenchymal stem cells from cigarette smoke to improve osteogenic differentiation in vitro. *Arch Toxicol* 2018; **92**: 1525-1538 [PMID: [29264620](#) DOI: [10.1007/s00204-017-2149-9](#)]
- 26 **Centers for Disease Control and Prevention (CDC)**. Quitting smoking among adults--United States, 2001-2010. *MMWR Morb Mortal Wkly Rep* 2011; **60**: 1513-1519 [PMID: [22071589](#)]
- 27 **Haziza C**, de La Bourdonnaye G, Donelli A, Poux V, Skiada D, Weitkunat R, Baker G, Picavet P, Lüdicke F. Reduction in Exposure to Selected Harmful and Potentially Harmful Constituents Approaching Those Observed Upon Smoking Abstinence in Smokers Switching to the Menthol Tobacco Heating System 2.2 for 3 Months (Part 1). *Nicotine Tob Res* 2020; **22**: 539-548 [PMID: [30722062](#) DOI: [10.1093/ntr/ntz013](#)]
- 28 **Li X**, Luo Y, Jiang X, Zhang H, Zhu F, Hu S, Hou H, Hu Q, Pang Y. Chemical Analysis and Simulated Pyrolysis of Tobacco Heating System 2.2 Compared to Conventional Cigarettes. *Nicotine Tob Res* 2019; **21**: 111-118 [PMID: [29319815](#) DOI: [10.1093/ntr/nty005](#)]
- 29 **Margham J**, McAdam K, Forster M, Liu C, Wright C, Mariner D, Proctor C. Chemical Composition of Aerosol from an E-Cigarette: A Quantitative Comparison with Cigarette Smoke. *Chem Res Toxicol* 2016; **29**: 1662-1678 [PMID: [27641760](#) DOI: [10.1021/acs.chemrestox.6b00188](#)]
- 30 **Davis B**, To V, Talbot P. Comparison of cytotoxicity of IQOS aerosols to smoke from Marlboro Red and 3R4F reference cigarettes. *Toxicol In Vitro* 2019; **61**: 104652 [PMID: [31526836](#) DOI: [10.1016/j.tiv.2019.104652](#)]
- 31 **Phillips B**, Szostak J, Titz B, Schlage WK, Guedj E, Leroy P, Vuillaume G, Martin F, Buettner A, Elamin A, Sewer A, Sierro N, Choukrallah MA, Schneider T, Ivanov NV, Teng C, Tung CK, Lim WT, Yeo YS, Vanscheeuwijck P, Peitsch MC, Hoeng J. A six-month systems toxicology inhalation/cessation study in ApoE<sup>-/-</sup> mice to investigate cardiovascular and respiratory exposure effects of modified risk tobacco products, CHTP 1.2 and THS 2.2, compared with conventional cigarettes. *Food Chem Toxicol* 2019; **126**: 113-141 [PMID: [30763686](#) DOI: [10.1016/j.fct.2019.02.008](#)]
- 32 **Munakata S**, Ishimori K, Kitamura N, Ishikawa S, Takanami Y, Ito S. Oxidative stress responses in human bronchial epithelial cells exposed to cigarette smoke and vapor from tobacco- and nicotine-containing products. *Regul Toxicol Pharmacol* 2018; **99**: 122-128 [PMID: [30227175](#) DOI: [10.1016/j.yrtph.2018.09.009](#)]
- 33 **Bozier J**, Zakarya R, Chapman DG, Oliver BGG. How harmless are E-cigarettes? Effects in the pulmonary system. *Curr Opin Pulm Med* 2020; **26**: 97-102 [PMID: [31652155](#) DOI: [10.1097/MCP.0000000000000645](#)]
- 34 **Gotts JE**, Jordt SE, McConnell R, Tarran R. What are the respiratory effects of e-cigarettes? *BMJ* 2019; **366**: l5275 [PMID: [31570493](#) DOI: [10.1136/bmj.l5275](#)]
- 35 **Ehnert S**, Linnemann C, Aspera-Werz RH, Bykova D, Biermann S, Fecht L, De Zwart PM, Nussler AK, Stuby F. Immune Cell Induced Migration of Osteoprogenitor Cells Is Mediated by TGF- $\beta$  Dependent Upregulation of NOX4 and Activation of Focal Adhesion Kinase. *Int J Mol Sci* 2018; **19** [PMID: [30065198](#) DOI: [10.3390/ijms19082239](#)]
- 36 **Böcker W**, Yin Z, Drosse I, Haasters F, Rossmann O, Wierer M, Popov C, Locher M, Mutschler W, Docheva D, Schieker M. Introducing a single-cell-derived human mesenchymal stem cell line expressing hTERT after lentiviral gene transfer. *J Cell Mol Med* 2008; **12**: 1347-1359 [PMID: [18318690](#) DOI: [10.1111/j.1582-4934.2008.00299.x](#)]
- 37 ISO and health canada intense smoking parameters - Part 2: Examination of factors contributing to variability in the routine measurement of TPM, water and NFDPM smoke yields of cigarettes. ISO, 2015. Available from: <https://www.iso.org/obp/ui/#iso:std:iso:tr:19478:-2:ed-1:v1:en>
- 38 **Häussling V**, Deninger S, Vidoni L, Rinderknecht H, Ruoff M, Arnscheidt C, Athanasopulu K, Kemkemer R, Nussler AK, Ehnert S. Impact of Four Protein Additives in Cryogels on Osteogenic Differentiation of Adipose-Derived Mesenchymal Stem Cells. *Bioengineering (Basel)* 2019; **6** [PMID: [31394780](#) DOI: [10.3390/bioengineering6030067](#)]
- 39 **Aspera-Werz RH**, Chen T, Ehnert S, Zhu S, Fröhlich T, Nussler AK. Cigarette Smoke Induces the Risk of Metabolic Bone Diseases: Transforming Growth Factor Beta Signaling Impairment via Dysfunctional Primary Cilia Affects Migration, Proliferation, and Differentiation of Human Mesenchymal Stem Cells. *Int J Mol Sci* 2019; **20** [PMID: [31207955](#) DOI: [10.3390/ijms20122915](#)]
- 40 **Ehnert S**, Sreekumar V, Aspera-Werz RH, Sajadian SO, Wintermeyer E, Sandmann GH, Bahrs C, Hengstler JG, Godoy P, Nussler AK. TGF- $\beta_1$  impairs mechanosensation of human osteoblasts via HDAC6-mediated shortening and distortion of primary cilia. *J Mol Med (Berl)* 2017; **95**: 653-663 [PMID: [28271209](#) DOI: [10.1007/s00109-017-1526-4](#)]
- 41 **Huang W**, Yang S, Shao J, Li YP. Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Front Biosci* 2007; **12**: 3068-3092 [PMID: [17485283](#) DOI: [10.2741/2296](#)]
- 42 **Tummala P**, Arnsdorf EJ, Jacobs CR. The Role of Primary Cilia in Mesenchymal Stem Cell Differentiation: A Pivotal Switch in Guiding Lineage Commitment. *Cell Mol Bioeng* 2010; **3**: 207-212 [PMID: [20823950](#) DOI: [10.1007/s12195-010-0127-x](#)]
- 43 **Anderson CT**, Castillo AB, Brugmann SA, Helms JA, Jacobs CR, Stearns T. Primary cilia: cellular sensors for the skeleton. *Anat Rec (Hoboken)* 2008; **291**: 1074-1078 [PMID: [18727074](#) DOI: [10.1002/ar.20754](#)]
- 44 **Kode A**, Rajendrasozhan S, Caito S, Yang SR, Megson IL, Rahman I. Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2008; **294**: L478-L488 [PMID: [18162601](#) DOI: [10.1152/ajplung.00361.2007](#)]
- 45 **Sewer A**, Kogel U, Talikka M, Wong ET, Martin F, Xiang Y, Guedj E, Ivanov NV, Hoeng J, Peitsch MC. Evaluation of the Tobacco Heating System 2.2 (THS2.2). Part 5: microRNA expression from a 90-day rat inhalation study indicates that exposure to THS2.2 aerosol causes reduced effects on lung tissue compared with cigarette smoke. *Regul Toxicol Pharmacol* 2016; **81** Suppl 2: S82-S92 [PMID: [27866933](#) DOI: [10.1016/j.yrtph.2016.11.018](#)]
- 46 **Haswell LE**, Corke S, Verrastro I, Baxter A, Banerjee A, Adamson J, Jaunky T, Proctor C, Gaça M, Minet E. In vitro RNA-seq-based toxicogenomics assessment shows reduced biological effect of tobacco heating products when compared to cigarette smoke. *Sci Rep* 2018; **8**: 1145 [PMID: [29402904](#) DOI: [10.1038/s41598-018-19627-0](#)]

- 47 **James AW.** Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. *Scientifica (Cairo)* 2013; **2013**: 684736 [PMID: [24416618](#) DOI: [10.1155/2013/684736](#)]

## Basic Study

## Human embryonic stem cell-derived mesenchymal stem cells improved premature ovarian failure

Khadijeh Bahrehbar, Mojtaba Rezazadeh Valojerdi, Fereshteh Esfandiari, Rouhollah Fathi, Seyedeh-Nafiseh Hassani, Hossein Baharvand

**ORCID number:** Khadijeh Bahrehbar 0000-0003-0694-7396; Mojtaba Rezazadeh Valojerdi 0000-0002-6592-8243; Fereshteh Esfandiari 0000-0003-3785-3149; Rouhollah Fathi 0000-0001-7328-2027; Seyedeh-Nafiseh Hassani 0000-0001-5047-8406; Hossein Baharvand 0000-0001-6528-3687.

**Author contributions:** Bahrehbar K and Esfandiari F were involved in collection and/or assembly of the data and manuscript writing; Bahrehbar K, Rezazadeh Valojerdi M, Baharvand H, Fathi R and Hassani SN were involved in data analysis and interpretation; Esfandiari F, Fathi R and Hassani SN were involved in conception and design; Rezazadeh Valojerdi M and Baharvand H were co-corresponding authors and were involved in conducting the experiments, manuscript proof, administrative and financial support.

**Supported by** Royan Institute, the National Institute for Medical Research Development, No. 963255; and the Ministry of Health and Medical Education, No. 700/147.

**Institutional review board statement:** This study was reviewed and approved by the

Khadijeh Bahrehbar, Fereshteh Esfandiari, Seyedeh-Nafiseh Hassani, Hossein Baharvand, Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, Tehran 1665659911, Iran

Khadijeh Bahrehbar, Hossein Baharvand, Department of Developmental Biology, University of Science and Culture, Tehran 1665659911, Iran

Mojtaba Rezazadeh Valojerdi, Rouhollah Fathi, Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, Tehran 1665659911, Iran

Mojtaba Rezazadeh Valojerdi, Department of Anatomy, Faculty of Medical Science, Tarbiat Modares University, Tehran 1665659911, Iran

**Corresponding author:** Hossein Baharvand, PhD, Full Professor, Department of Stem Cells and Developmental Biology, Royan Institute for Stem Cell Biology and Technology, Banihashem St., Tehran 1665659911, Iran. [baharvand@royaninstitute.org](mailto:baharvand@royaninstitute.org)

## Abstract

## BACKGROUND

Premature ovarian failure (POF) affects many adult women less than 40 years of age and leads to infertility. According to previous reports, various tissue-specific stem cells can restore ovarian function and folliculogenesis in mice with chemotherapy-induced POF. Human embryonic stem cells (ES) provide an alternative source for mesenchymal stem cells (MSCs) because of their similarities in phenotype and immunomodulatory and anti-inflammatory characteristics. Embryonic stem cell-derived mesenchymal stem cells (ES-MSCs) are attractive candidates for regenerative medicine because of their high proliferation and lack of barriers for harvesting tissue-specific MSCs. However, possible therapeutic effects and underlying mechanisms of transplanted ES-MSCs on cyclophosphamide and busulfan-induced mouse ovarian damage have not been evaluated.

## AIM

To evaluate ES-MSCs *vs* bone marrow-derived mesenchymal stem cells (BM-MSCs) in restoring ovarian function in a mouse model of chemotherapy-induced premature ovarian failure.



Institutional Review Board at Royan Institute.

**Institutional animal care and use committee statement:** All animal experiments of the study were reviewed and approved by the Institutional Ethical Committee at Royan Institute.

**Conflict-of-interest statement:** The authors declare that they have no competing interests.

**Data sharing statement:** No additional data are available.

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared according to these guidelines.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Invited manuscript

**Received:** February 22, 2020

**Peer-review started:** February 22, 2020

**First decision:** April 29, 2020

**Revised:** June 1, 2020

**Accepted:** July 18, 2020

**Article in press:** July 18, 2020

**Published online:** August 26, 2020

**P-Reviewer:** Tanabe S

**S-Editor:** Gong ZM

**L-Editor:** Filipodia

**P-Editor:** Wang LL



## METHODS

Female mice received intraperitoneal injections of different doses of cyclophosphamide and busulfan to induce POF. Either human ES-MSCs or BM-MSCs were transplanted into these mice. Ten days after the mice were injected with cyclophosphamide and busulfan and 4 wk after transplantation of the ES-MSCs and/or BM-MSCs, we evaluated body weight, estrous cyclicity, follicle-stimulating hormone and estradiol hormone concentrations and follicle count were used to evaluate the POF model and cell transplantation. Moreover, terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labeling, real-time PCR, Western blot analysis and immunohistochemistry and mating was used to evaluate cell transplantation. Enzyme-linked immunosorbent assay was used to analyze vascular endothelial growth factor, insulin-like growth factor 2 and hepatocyte growth factor levels in ES-MSC condition medium in order to investigate the mechanisms that underlie their function.

## RESULTS

The human ES-MSCs significantly restored hormone secretion, survival rate and reproductive function in POF mice, which was similar to the results obtained with BM-MSCs. Gene expression analysis and the terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labeling assay results indicated that the ES-MSCs and/or BM-MSCs reduced apoptosis in the follicles. Notably, the transplanted mice generated new offspring. The results of different analyses showed increases in antiapoptotic and trophic proteins and genes.

## CONCLUSION

These results suggested that transplantation of human ES-MSCs were similar to BM-MSCs in that they could restore the structure of the injured ovarian tissue and its function in chemotherapy-induced damaged POF mice and rescue fertility. The possible mechanisms of human ES-MSC were related to promotion of follicular development, ovarian secretion, fertility *via* a paracrine effect and ovarian cell survival.

**Key words:** Premature ovarian failure; Human embryonic stem cells; Chemotherapy drugs; Mesenchymal stem cell; Bone marrow; Apoptosis

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Transplanted human embryonic stem cells are similar to bone marrow-derived mesenchymal stem cells. They can restore injured ovarian tissue structure and function in chemotherapy-induced premature ovarian failure mice and rescue fertility through the paracrine effect and ovarian cell survival.

**Citation:** Bahrehbar K, Rezazadeh Valojerdi M, Esfandiari F, Fathi R, Hassani SN, Baharvand H. Human embryonic stem cell-derived mesenchymal stem cells improved premature ovarian failure. *World J Stem Cells* 2020; 12(8): 857-878

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/857.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.857>

## INTRODUCTION

Premature ovarian failure (POF) disease has similar characteristics such as hypoestrogenism, elevated gonadotropin levels and infertility in animal models and in human. Some women also have symptoms such as hot flashes, night sweats, vaginal dryness, chronic anxiety, sadness and depression<sup>[1]</sup>. POF affects 1%-3% of women < 40 years of age<sup>[2]</sup>. Hot flashes, depression, anxiety, osteoporosis and sexual dysfunction are the consequences of this disease<sup>[3,4]</sup>. Although the cause of POF is often idiopathic, possible causes include autoimmune disorders, smoking, toxic chemicals, drugs and genetic defects<sup>[5-7]</sup>. Chemotherapeutics such as cyclophosphamide (Cy) and busulfan

(Bu) are the most gonadotoxic agents that lead to POF in the majority of patients<sup>[8]</sup>. Currently, ovarian protection methods, oocyte or ovarian tissue cryopreservation and embryo freezing are strategies used for fertility preservation in women diagnosed with cancer. However, these methods have serious disadvantages such as the risk of reintroducing the cancer cells, delays in cancer treatment and low success rate. Therefore, it is necessary to develop advanced therapies for women with POF<sup>[9]</sup>.

Emerging evidence suggests that mesenchymal stem cells (MSCs) derived from bone marrow (BM) and other adult tissues (adipose, skin, amniotic membrane, placenta) and menstrual blood could restore ovarian function in animal models of POF<sup>[10-14]</sup>. A meta-analysis from 16 preclinical studies of animal models was conducted to assess the efficacy of stem cell transplantation. The results indicated that MSC therapy significantly improved ovarian function in cases with POF<sup>[15]</sup>. In two case studies, MSC transplantation also improved POF<sup>[15-16]</sup>. However, despite the promising results, the numbers of harvested MSCs and their *in vitro* expansion was a challenge<sup>[17]</sup>. Moreover, obtaining MSCs from bone marrow requires suitable donors and invasive procedures. The number of bone marrow-derived mesenchymal stem cells (BM-MSCs) is very limited, which greatly restricts use of BM-MSCs for clinical application<sup>[18]</sup>. The immunomodulating feature of MSCs seems to be different between species<sup>[19]</sup>. Human MSCs decrease the secretion of interferon gamma, interleukin 12 and tumor necrosis factor alpha and increase interleukin 10 secretion<sup>[20-22]</sup>. Moreover, human MSC-mediated inhibition of the T cell response could not be reversed by nitric oxide synthase inhibitor compared with mice MSCs<sup>[23]</sup>. Integrin  $\beta 1$  expression is important for mice MSCs migration, while C-X-C chemokine receptor type 4 expression is involved for human MSC migration to sites of tissue injury<sup>[24-25]</sup>. It has been demonstrated that 92% of MSC protein expression is similar in humans and mice<sup>[26]</sup>. MSCs represent only a small proportion of the cells in bone marrow, and their proliferation and differentiation capacity correlates inversely with age<sup>[20]</sup>.

In addition to adult tissue specific MSCs, human embryonic stem cells (ES-MSCs) are an alternative source of MSCs because of their similar phenotypic characteristics that make them attractive candidates for regenerative cellular therapy<sup>[27-28]</sup>. Recently, it has been reported that ES-MSCs have higher capabilities for cell proliferation and suppression of leukocyte growth compared to MSCs from other sources<sup>[29-30]</sup>. ES-MSCs exhibited more potent anti-inflammatory properties than BM-MSCs<sup>[17,27,31-33]</sup>. The therapeutic potential of ES-MSCs has been reported in numerous animal models. When compared with BM-MSCs, these cells showed a significantly greater improvement in models of thioacetamide-induced chronic liver injury and experimental autoimmune encephalitis<sup>[30,34]</sup>. This evidence indicates that ES-MSCs may serve as better sources for clinical applications.

Human ES-MSCs can overcome the obstacles seen with harvesting MSCs from adult tissues, including lack of appropriate donors, limited numbers of cells obtained during the harvesting process, restricted *in vitro* expansion capacity and the invasiveness of the procedures. Thus, we hypothesized that ES-MSCs might restore ovarian structure and function through the paracrine mechanisms of cytokines in a POF model. To address this issue, we used a POF mouse model to evaluate the potential for transplanted ES-MSCs to restore fertility.

## MATERIALS AND METHODS

### **Derivation of MSCs from human ES cells and BM**

We isolated and cultured ES-MSCs according to our previously published protocols<sup>[17,33]</sup>. Briefly, we obtained MSCs from human ES cells by culturing these cells in basic fibroblast growth factor-free ES medium to enable embryoid body formation. The resultant embryoid bodies were plated in gelatin-coated plates and cultured in MSC medium. Spontaneous differentiation of the embryoid bodies resulted in an outgrowth of ES-MSCs. These cells were further passaged to obtain a homogenous population with spindle-shaped morphology. Passage-2 human BM-MSCs were prepared from Royan Stem Cell Bank (Tehran, Iran) and cultured in low-glucose Dulbecco's Modified Eagle Medium (Life Technologies, United States) supplemented with 10% fetal bovine serum (FBS, Life Technologies, United States) for further expansion. The medium was changed every 3 d.

### **Cell proliferation analysis**

We cultured  $1 \times 10^6$  cells/cm<sup>2</sup> in T25 cm<sup>2</sup> tissue culture flasks (TPP, Germany) to assess their proliferative ability. The population doubling time was calculated according to

the following formula:

Population doubling time = duration  $\times$  log (2)/log (final concentration) – log (initial concentration)

### **Karyotype analysis**

The cells were treated with 0.66 mmol/L thymidine (Sigma-Aldrich) and incubated at 37 °C for 16 h. After the cells were washed with phosphate buffered saline (PBS), they were left for 5 h and then treated with 0.15 mg/mL colcemid (Invitrogen) for 30 min. Then, the cells were exposed to 0.075 mol/L potassium chloride (Merck) and allowed to incubate at 37 °C for 16 min. After the cells were centrifuged, we removed the supernatant and resuspended the pellet in Carnoy's fixative (3:1 ratio of methanol:glacial acetic acid). The cells were dropped onto precleaned, chilled slides and standard G-band staining was performed for chromosome visualization. We screened at least 20 well-spread metaphase cells of which 10 were evaluated for chromosomal rearrangements.

### **Flow cytometry analysis**

We sought to determine the immunophenotypes of the cultured ES-MSCs and BM-MSCs. Surface-marker expression was analyzed by flow cytometry using the following antibodies: Fluorescein isothiocyanate-conjugated human monoclonal antibodies against protein tyrosine phosphatase receptor type C and cluster of differentiation (CD) 90 (CD90); and phycoerythrin-conjugated human monoclonal antibodies against homing cell adhesion molecule, CD73, endoglin, CD11b and CD34. For flow cytometric analysis, the adherent cells were detached by using 0.25% trypsin-ethylenediaminetetraacetic acid, neutralized by FBS-containing culture medium and disaggregated into single cells by pipetting. The cells were incubated with antibodies for 30 min at 4 °C, washed twice with PBS, resuspended in 0.5 mL PBS and immediately analyzed by fluorescence-activated cell sorting Calibur flow cytometer (Becton Dickinson, United States). Analyses were performed on three independent biological samples. Data were analyzed using the FlowJo software (version 7.6.1). **Supplementary Table 1** lists the antibodies used in this study.

### **Multilineage differentiation**

Osteogenic, adipogenic and chondrogenic differentiation were verified by alizarin red, oil red O, and alcian blue staining, respectively to confirm the multipotent properties of the ES-MSCs and BM-MSCs. For osteogenesis, the cells were seeded onto 6-well plates at a density of  $1 \times 10^5$  cells/cm<sup>2</sup>. After 24 h, the medium was replaced by osteogenic differentiation medium, alpha minimum essential medium (Life Technologies, United States) supplemented with 10% FBS (Gibco, United States), 0.1 mmol/L dexamethasone (Sigma-Aldrich, United States), 10 mmol/L  $\beta$ -glycerophosphate (Sigma-Aldrich, United States) and 50 mmol/L ascorbic acid (Sigma-Aldrich, United States) for 2 wk. To induce adipogenesis, the cells were incubated with adipogenic differentiation medium in alpha minimum essential medium supplemented with 10% FBS, 10 mg/mL insulin (Sigma-Aldrich, United States), 1 mmol/L dexamethasone (Sigma-Aldrich, United States), 0.5 mmol/L isobutyl-methylxanthine (Sigma-Aldrich, United States) and 100 mmol/L indomethacin (Sigma-Aldrich, United States) for 3 wk. For chondrogenic differentiation,  $2.5 \times 10^5$  cells were collected in a 15 mL tube and centrifuged at 350 g for 5 min. The cell pellet was subsequently cultured for 3 wk using chondrogenic induction medium (chondrogenesis differentiation kit, Gibco, United States) according to the manufacturer's instructions. Then, the pellets were fixed in 4% paraformaldehyde (Sigma Aldrich, United States) for 30 min, dehydrated in ethanol, cleared in xylene and embedded in paraffin. The paraffin-embedded cells were sectioned into 6  $\mu$ m sections by using a microtome. The sections were stained with alcian blue.

### **Measurement of cytokine secretion**

We analyzed cytokines secreted by the MSCs. Both ES-MSCs and BM-MSCs were cultured in dishes at densities of  $2 \times 10^5$  cells/cm<sup>2</sup> each. After a 24 h culture in serum-free media, the culture media was collected and centrifuged at 2000 g for 5 min. The amount of cytokine expression was measured using a vascular endothelial growth factor (VEGF) Human ELISA kit (Invitrogen, United States), insulin-like growth factor 2 (IGF-2) Human ELISA kit (R&D Systems, United States) and hepatocyte growth factor (HGF) Human ELISA kit (R&D Systems, United States).

### Experimental animals

All animal experiments were approved by the Institutional Ethical Committee of Royan Institute. Adult female C57BL/6 mice (6-8 wk old) were used in our study. The mice were housed under a 14-10 h light-dark cycle and had free access to food and water.

### Estrous cyclicity

Vaginal smears were obtained daily. The four stages of the estrous cycle were determined as follows: Proestrus (100% intact live epithelial cells); estrus (100% cornified epithelial cells); metestrus (about 50% cornified epithelial cells and about 50% leukocytes); and diestrus (80%-100% leukocytes). The mouse estrous cycle lasts for approximately 4 d and includes the proestrus, estrus, metestrus and diestrus stages. Animals with at least two consecutive normal 4-d vaginal estrous cycles were included in the experiments. In order to validate reproductive function, we assessed the animals over 10 consecutive days of the experiment. The number of estrous cycles were checked at 8:00 am daily with a vaginal smear assay starting at 10 d after the animals were injected with Cy and Bu and 4 wk after transplantation of ES-MSCs or BM-MSCs.

### Establishment of the POF model

In this study, we used chemotherapy to create the mouse model because chemotherapy is one of the major causes of POF<sup>[35]</sup>. Various reports of POF models generated in mice used from 8-30 mg/kg of Bu plus 50-200 mg/kg of Cy<sup>[36-40]</sup>, or only Cy<sup>[41-44]</sup>. However, none of the previous studies showed any significant decrease in follicle numbers during the developmental stages. Therefore, we assessed different doses of these drugs to create a POF model in our laboratory setting. Female mice were randomly divided into four treatment groups and one intact group. The treatment groups received intraperitoneal injections of different doses of Cy (Endoxan<sup>TM</sup>, Germany) and Bu (Sigma-Aldrich, United States) as follows: Group 1 (POF1): 50 mg/kg Bu and 100 mg/kg Cy; group 2 (POF2): 100 mg/kg Cy for 10 consecutive days; group 3 (POF3): 200 mg/kg Cy and 50 mg/kg Bu on the 1<sup>st</sup> day followed by 50 mg/kg Cy and 5 mg/kg Bu for 9 consecutive days; and group 4 (POF4): A single injection of 20 mg/kg Bu and 200 mg/kg Cy. In order to confirm successful establishment of POF in the mouse model, we checked their body weights, estrous cyclicity, concentrations of follicle-stimulating hormone (FSH) and estradiol (E2) hormones and follicle counts. In addition, for further confirmation of POF, we also used the terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay and real-time PCR assessments, and the mice were allowed to mate 10 d after the injections.

### Cell transplantation

Once the POF model was established, we randomly divided the mice into three groups. Vehicle POF mice received medium but no cell transplantation. In the ES-MSC group, POF mice were injected with  $1 \times 10^6$  ES-MSCs. In the BM-MSC group, POF mice were injected intravenously with  $1 \times 10^6$  BM-MSCs in 0.1 mL Dulbecco's Modified Eagle Medium. In order to evaluate the effects of the transplanted ES-MSCs and BM-MSCs, we assessed the body weights, estrous cyclicity, concentrations of FSH and E2 hormones and follicle counts in the POF mice at 4 wk after the transplantations. In addition, the mice were allowed to mate. The TUNEL assay, Western blot, immunohistochemistry and real-time PCR assessments were also performed.

### Hormone assay

Blood samples were obtained from hearts of the anesthetized mice to determine serum levels of E2 and FSH. The blood samples were incubated at room temperature for 1 h, and supernatant was collected after centrifugation at 3000 rpm for 20 min. Hormone levels were determined by ELISA kits (Biotech, Shanghai, China).

### Detection of apoptosis by the TUNEL assay

Cell apoptosis in the ovarian tissue was detected by the TUNEL assay. Briefly, 5  $\mu$ m ovarian sections were washed twice in PBS for 5 min after deparaffinization. These sections were permeabilized by incubation in 0.1% Triton X-100 solution and 0.1% sodium citrate for 8 min. Then, the TUNEL assay was performed with an *in situ* cell death detection kit (Roche, Germany) according to the manufacturer's instructions. Counterstaining with DAPI (Sigma-Aldrich) was used to visualize the nuclei. We observed the cells under a fluorescence microscope (Olympus, Japan) for the presence



of apoptosis (green fluorescent color).

### **Hematoxylin and eosin staining and data quantification**

The ovaries were removed and fixed in 4% paraformaldehyde (Sigma-Aldrich) for at least 24 h. The fixed ovaries were dehydrated, embedded in paraffin, serially sectioned into 6 µm sections and mounted on glass microscope slides. Routine hematoxylin and eosin staining was performed for histologic examination under a light microscope.

### **Follicle counting**

Primordial, primary, secondary and antral follicles were counted in each of the five sections based on the method reported by Tilly<sup>[45]</sup>. Only the follicle with a nucleus was counted to avoid duplicate counting of a follicle. The follicles were classified as: Primordial (oocyte surrounded by a single layer of squamous granulosa cells); primary (intact enlarged oocyte with a visible nucleus and one layer of cuboidal granulosa cells); secondary (two or three layers of cuboidal granulosa cells without an antral space); early antral (emerging antral spaces); and preovulatory (the largest follicular types with a defined cumulus granulosa cell layer). [Supplementary Tables 3 and 4](#) present the data for follicle counting both after chemotherapy and cell transplantation. The significance of the changes in follicle numbers in the different study groups were analyzed by two-way analysis of variance.

### **Gene expression analysis**

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed for the apoptosis genes, anti-apoptosis gene [B-cell lymphoma 2 (*Bcl2*)], apoptosis gene [cysteine-aspartic proteases 3 (*caspase 3*)], angiogenesis gene (*Vegf*), proliferation gene (*Igf-2*), granulosa marker anti-Müllerian hormone (*Amh*) and oocyte marker [growth/differentiation factor 9 (*Gdf9*)] in the intact group, POF group and both cell transplantation groups. Total RNA was isolated and purified with TRIzol reagent (Invitrogen) according to the manufacturer's protocol followed by cDNA synthesis with a cDNA synthesis kit (Fermentas). qRT-PCR reactions were performed using SYBR Green Master Mix (Applied Biosystems) and a real-time PCR system (Corbett Life Science; Rotor-Gene 6000 instrument). The samples were collected from three independent biological replicates. [Supplementary Table 2](#) lists the primer sequences used for qRT-PCR.

### **Western blot analysis**

The protein expression of caspase 3 in the ovaries was measured by Western blot. The mice were anesthetized, and we removed their ovaries. The proteins from the ovaries were isolated by the Q Proteome Mammalian Protein Prep kit (Merck, Germany). The total protein concentrations were measured using a standard BCA protein assay kit. The protein from each group was separated on 12% SDS-PAGE and transferred onto PVDF membranes. The blots were then incubated in blocking buffer [2% (w/v) skim milk powder in TBST] for 1 h at room temperature. Then, the membranes were incubated overnight at 4 °C with the primary antibody, anti-caspase 3 (1:2000<sup>[46]</sup>). The membranes were washed three times with TBST and incubated at room temperature for 1 h with anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:1000).

### **Immunohistochemistry**

Expression of the granulosa cell marker (*Amh*) was detected by immunohistochemical staining. The ovaries were fixed in formalin and sectioned into 5 µm sections. The sections were incubated at 60 °C for 1 h, deparaffinized in xylene and rehydrated in a graded ethanol series. Then, antigen retrieval was performed by heating the sections in citrate buffer in an oven for 30 min. The sections were washed in H<sub>2</sub>O<sub>2</sub> for 30 min to eliminate endogenous peroxidase activity and blocked with goat serum for 1 h at room temperature. After that, the sections were incubated overnight at 4 °C with the primary antibody, anti-*Amh*. After three washes with PBS for 10 min each time, the secondary antibody, streptavidin, and DAB were used for immunostaining according to the protocol from an immunostaining kit (Merck, Germany). Finally, the sections were counterstained with hematoxylin, dehydrated and mounted.

### **Mating trial**

The mating trial was initiated 10 d after the mice were injected with Cy or 4 wk after transplantation of the ES-MSCs or BM-MSCs and continued for six weeks. Female mice were housed in the same cages with male mice for natural mating. The presence

of a copulatory plug indicated successful mating. Males were randomly rotated among the cages after each pregnancy and the numbers of offspring per litter were recorded.

### Statistical analysis

All experiments were conducted in at least three independent repeats. All data are shown as mean  $\pm$  standard error of the mean. One-way analysis of variance was used to determine significant differences among groups with Tukey's post-hoc test. Viability was analyzed by the *t* test.  $P < 0.05$  were considered significant.

## RESULTS

### Derivation and characterization of ES-MSCs and BM-MSCs

Figure 1A shows the procedure used to derive MSCs from the ES cells. MSCs derived from both human ES and BM formed a homogeneous cell population with spindle-shaped morphology and a normal karyotype during long-term culture (Figure 1B and 1C). The MSCs successfully differentiated into osteogenic, adipogenic and chondrogenic lineages (Figure 1D). Flow cytometry analysis confirmed the expression of MSC-specific markers homing cell adhesion molecule, CD73, CD90 and endoglin by both human ES-MSCs and BM-MSCs; there were no detectable levels of the hematopoietic and endothelial cell markers (CD11b, CD34 and protein tyrosine phosphatase receptor type C) (Figure 1E and 1F, Supplementary Figure 1). The population doubling time assay showed significant increases in ES-MSC proliferation compared to BM-MSCs (Figure 1G;  $P < 0.05$ ).

### Establishment of a mouse model of chemotherapy-induced POF

We examined various concentrations of four different combinations of two chemotherapy drugs, Cy and Bu, in order to establish a POF model that showed the significant decreases in follicle numbers for all of the developmental stages (Figure 2A). In the intact group, the mice had regular 4-d estrous cycles; however, irregular estrous cycles were observed in the POF1, POF2, POF3 and POF4 mice. On day 5 of the treatment, the POF1, POF2 and POF3 mice were eating less and moved slowly (data not shown). The ovaries of the mice in the intact group were more reddish in color, whereas the ovaries of the mice that survived in the POF groups were pale. All of the animals were weighed before and after modeling, and we found significantly reduced body weights in the POF1 and POF2 groups (Figure 2B, Supplementary Figure 2A;  $P < 0.05$ ). Furthermore, the size of ovaries in mice treated with the chemotherapy drugs in the POF1, POF2, POF3 and POF4 groups were smaller than ovaries from the intact mice (Supplementary Figure 2B).

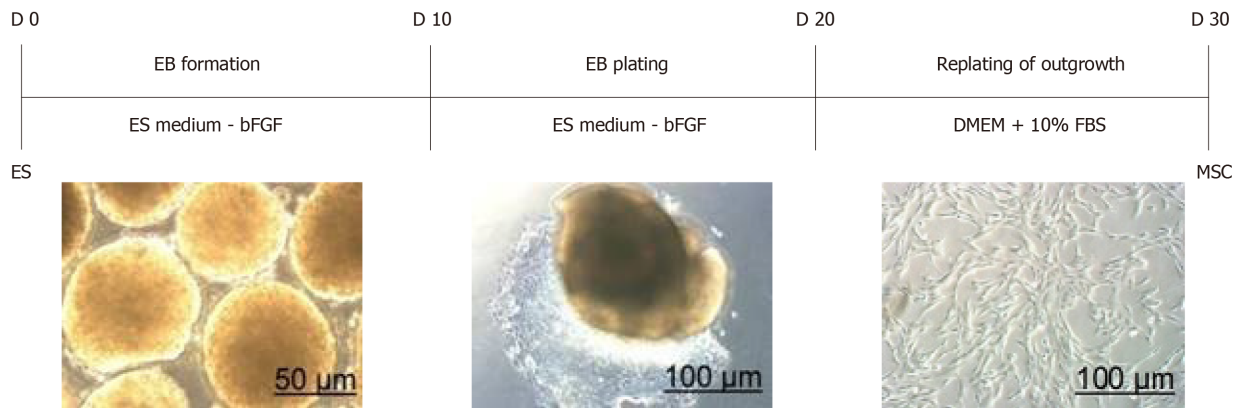
We performed hematoxylin and eosin staining to evaluate the structures of the ovaries following chemotherapy. Quantification of the follicles showed significant decreases in all of the developmental stages in the POF1 and POF2 groups (Figure 2C; Supplementary Figure 3;  $P < 0.05$ ), while the POF3 and POF4 groups did not show significant decreases in the number of follicles in the various developmental stages.

Hormonal analysis demonstrated significant increases in serum levels of FSH and significant decreases in E2 levels in the POF1 and POF2 groups (Figure 2D and 2E;  $P < 0.05$ ). TUNEL assay results to evaluate apoptosis in the ovaries following chemotherapy (Supplementary Figure 4A) showed a significantly increased percentage of TUNEL-positive cells in the POF1 and POF2 groups compared to the intact group (Figure 2F;  $P < 0.05$ ). Next, we sought to determine the optimum POF model by evaluating the survival rate of the mice and the pregnancy rate following chemotherapy. We found significantly higher survival rates in the POF1 group compared to the POF2 group (Figure 2G;  $P < 0.01$ ). However, none of the POF mice became pregnant (Supplementary Figure 4B). Therefore, we selected the POF2 model as the most appropriate model for induction of POF.

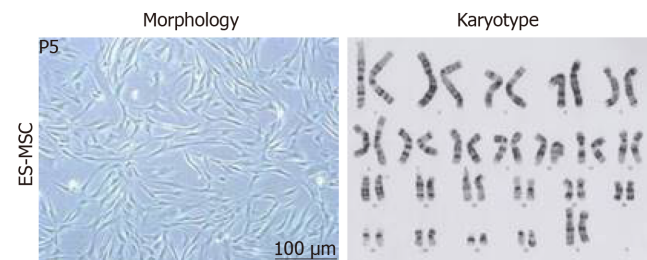
### ES-MSCs and BM-MSCs improved the POF model

We explored the possibility that the MSCs could improve the POF mouse model. There were more regular estrous cycles following transplantation of both human ES-MSCs and BM-MSCs compared to the vehicle group. Moreover, the ovaries of the mice had an increased red color and were larger in size following transplantation of both ES-MSCs and BM-MSCs in comparison with the vehicle group, but they were less than the intact ovaries (Supplementary Figure 5). Body weight significantly increased 4 wk after transplantation of both ES-MSCs and/or BM-MSCs compared to the vehicle

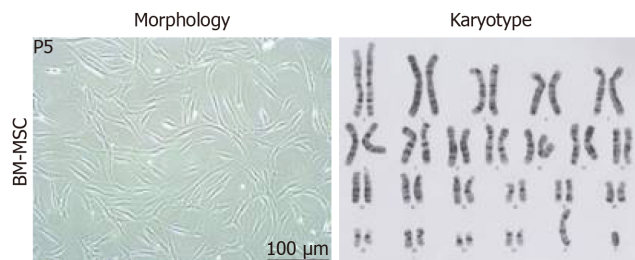
**A**



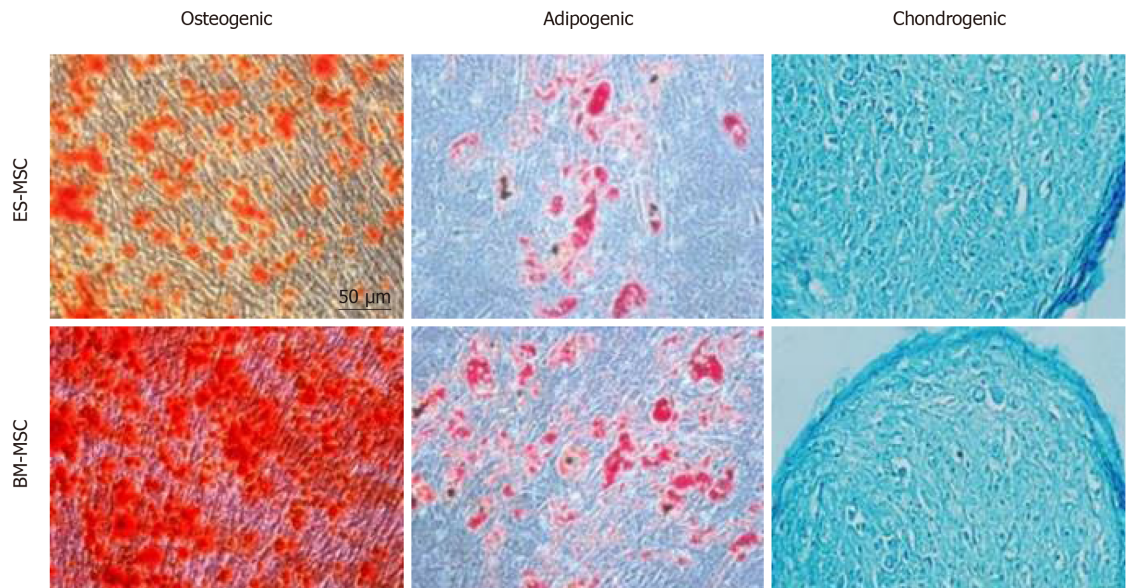
**B**

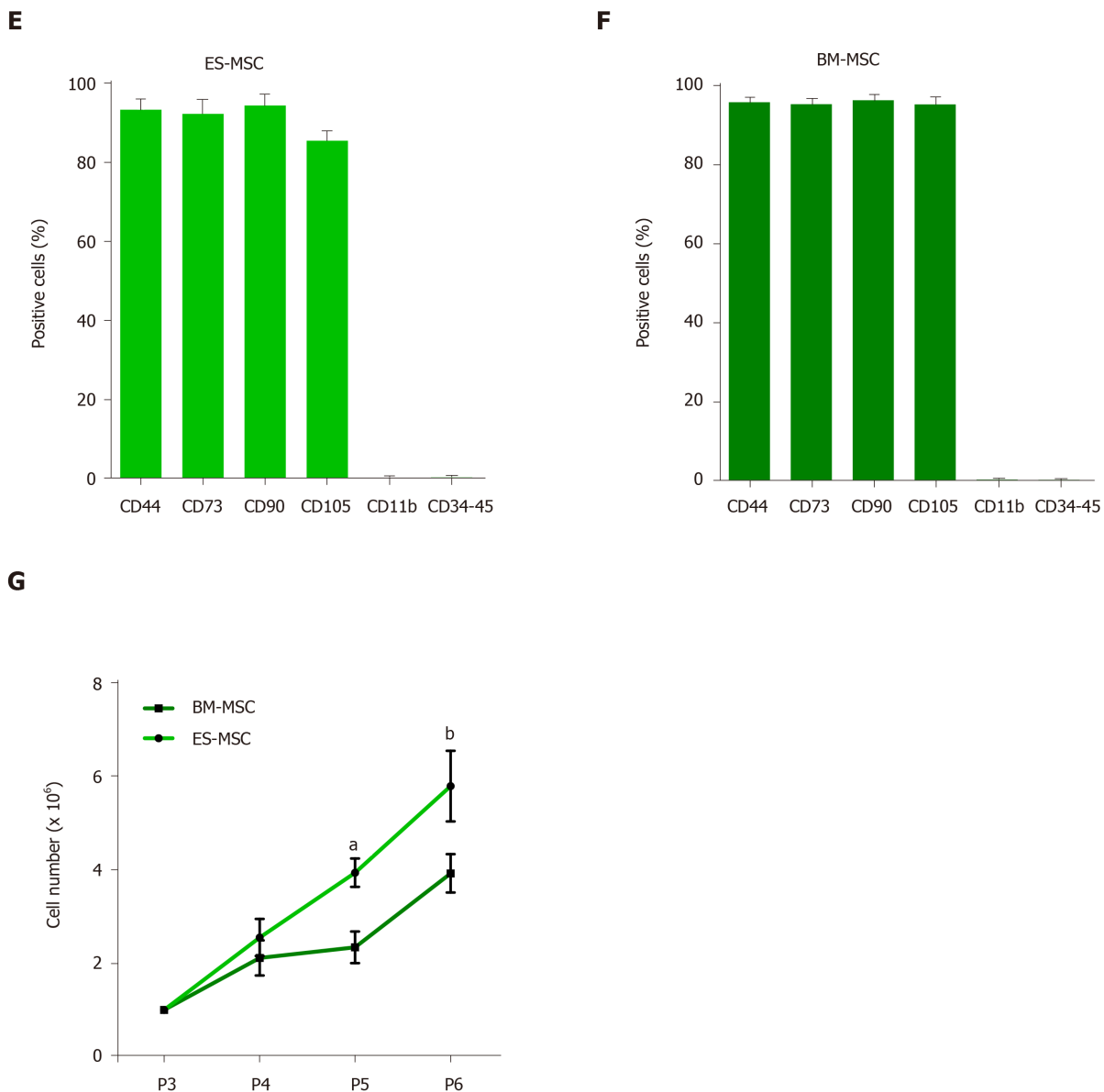


**C**



**D**





**Figure 1 Derivation and identification of human embryonic stem cell-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells.**

A: Schematic presentation of the procedure used to derive human mesenchymal stem cells (MSCs) from embryonic stem (ES) cells. Colonies of ES cells were enzymatically detached and cultured for 10 d in suspension to form embryoid bodies, which were then plated onto gelatin-coated tissue culture plates. After 10 d, outgrowths of the cells that sprouted from embryoid bodies were mechanically isolated by a cell scraper and subsequently expanded in mesenchymal stem cell culture medium; B and C: Morphology and karyotype of ES-MSCs and BM-MSCs. Passage-5 ES-MSCs and BM-MSCs showed a fibroblastic morphology and normal karyotype; D: Alizarin red staining after 14 d of culture in osteogenic medium indicated the osteogenic differentiation potential of ES-MSCs and BM-MSCs (P4). Oil red staining after 21 d of culture in adipogenic medium showed the adipogenic differentiation potential of ES-MSCs and BM-MSCs (P4). Alcian blue staining after 21 d of culture in chondrogenic medium showed chondrogenic differentiation potential of ES-MSCs and BM-MSCs; E, F: Flow cytometric analysis indicated that cultured ES-MSCs and BM-MSCs expressed CD44, CD90, CD73 and endoglin (CD105), but not hematopoietic lineage markers CD11b, CD34 and protein tyrosine phosphatase receptor type C (CD45); G: ES-MSCs proliferated more rapidly than BM-MSCs. Results are expressed as mean  $\pm$  standard error, <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ ;  $n = 3-5$ . ES-MSCs: Embryonic stem cell-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; EBs: Embryoid bodies; bFGF: Basic fibroblast growth factor; P: Passage.

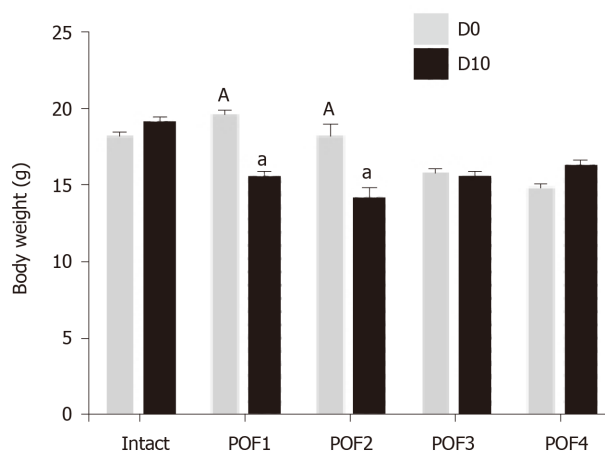
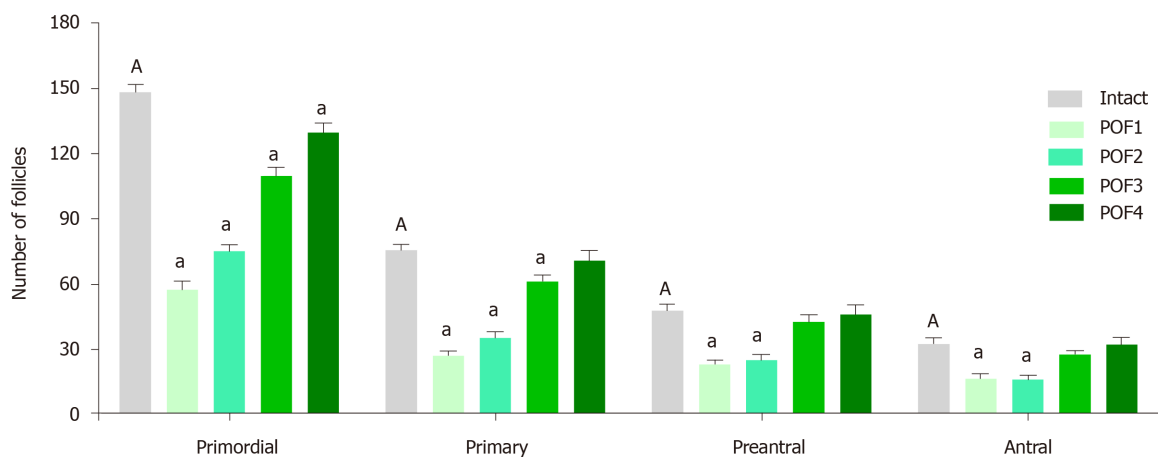
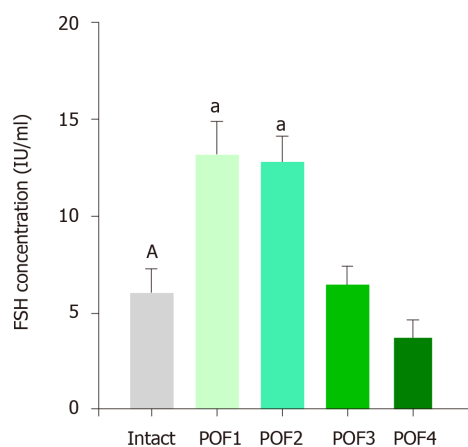
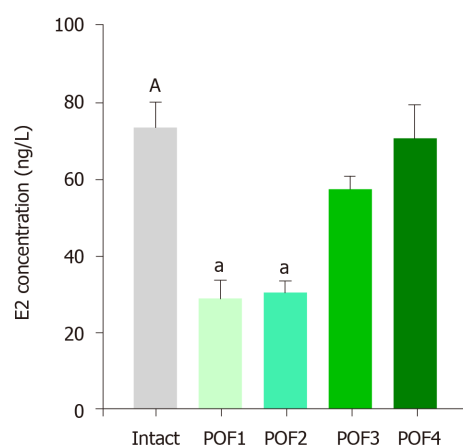
group (Figure 3A;  $P < 0.05$ ). The survival rate significantly increased following transplantation of ES-MSCs and/or BM-MSCs (more than 60%) compared to the vehicle group (20%) (Figure 3B;  $P < 0.01$ ).

Notably, we observed significant increases in the number of follicles at all stages of development following transplantation of both ES-MSCs and BM-MSCs compared with the vehicle group (Figure 3C;  $P < 0.05$ ). Transplantation of both ES-MSCs and BM-MSCs significantly decreased the FSH levels and increased the E2 levels compared with the vehicle group (Figure 3D and 3E;  $P < 0.05$ ).

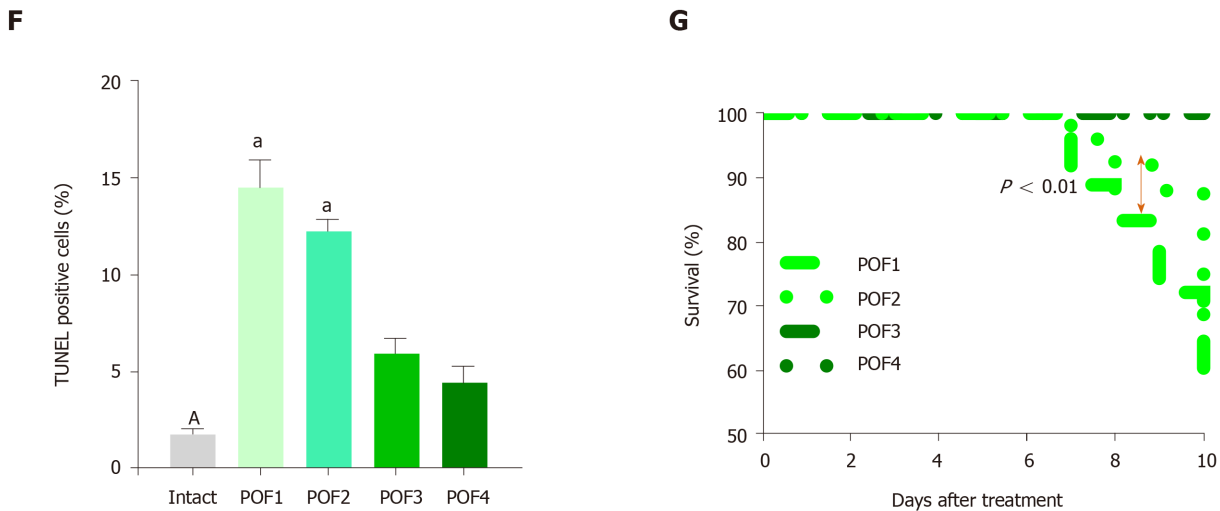
The results of the TUNEL assay confirmed significant decreases in apoptosis in ovaries that received the cell transplantations (Figure 4A and 4B, Supplementary Figure 6;  $P < 0.05$ ). qRT-PCR was conducted in order to gain further insight into the

**A**

Group	Dose of drugs
POF1	Cy (100 mg/kg) and Bu (50 mg/kg)
POF2	Cy (100 mg/kg)
POF3	Cy (200 mg/kg) and Bu (50 mg/kg) at day 1 plus Cy (50 mg/kg) and Bu (5 mg/kg) for 9 d
POF4	Cy (200 mg/kg) and Bu (20 mg/kg)

**B****C****D****E**





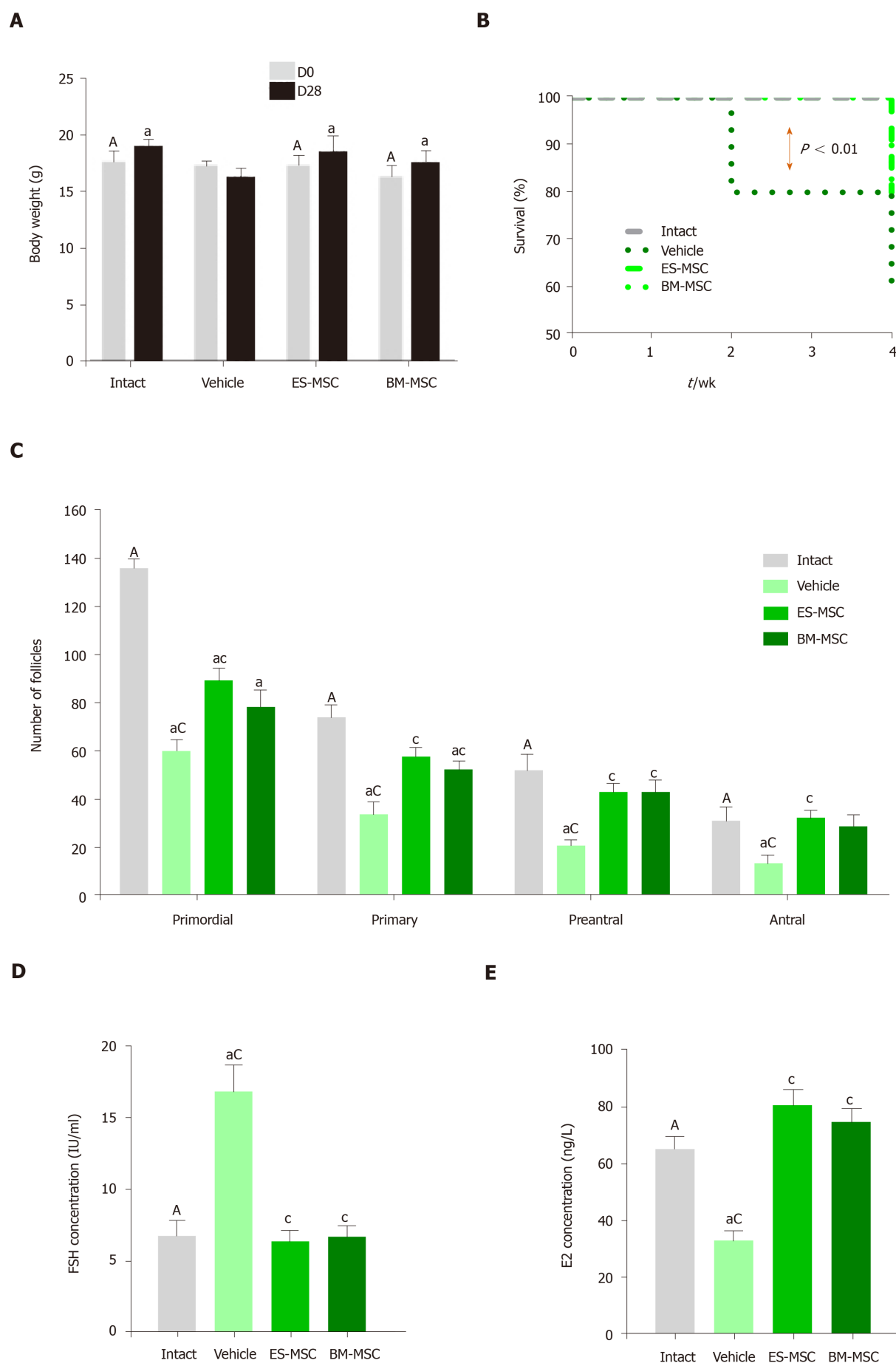
**Figure 2 Establishment of a mouse model of premature ovarian failure.** A: Premature ovarian failure (POF) groups were treated with different dosages of cyclophosphamide and busulfan; B: Body weight changes in the intact and POF groups after 10 d showed that the POF1 and POF2 groups had significant decreases in body weights; C: Ovarian pathology of the intact and POF groups 10 d after injection of cyclophosphamide and busulfan. Follicle count revealed that there were fewer normal follicles in the POF groups than in the intact mice. The ovaries of the intact group contained large numbers of follicles at all developmental stages, whereas the atrophic ovaries of the POF groups had fewer follicles at each stage; D, E: Serum levels of follicle stimulating hormone and estradiol 10 d after injection of cyclophosphamide and busulfan. Serum levels of follicle stimulating hormone were significantly increased in the POF1 and POF2 groups compared to those of the intact group. Serum levels of estradiol were significantly decreased in the POF1 and POF2 groups compared with the intact group; F: Apoptosis rate in the ovary. Green fluorescence indicated the presence of apoptotic cells in the POF1, POF2, POF3 and POF4 groups; G: Survival rate in the POF groups after 10 d. The survival percent showed a significant decrease in the POF1 group compared with the POF2 group. All data are presented as mean  $\pm$  standard error. Small letters (a) indicate the significance ( $P < 0.05$ ) compared to groups labeled by similar capital letters (A); <sup>a</sup> $P < 0.05$  significance of experimental groups vs the intact group;  $n = 3-5$ . POF: Premature ovarian failure; Cy: Cyclophosphamide; Bu: Busulfan; FSH: Follicle stimulating hormone; E2: Estradiol.

effect of transplantation on the ovaries. The results showed significant downregulation of the apoptosis gene, *caspase 3*, while the anti-apoptotic gene, *Bcl2*, was significantly upregulated following cell transplantation compared with the vehicle group. In particular, the level of the angiogenesis gene (*Vegf*), proliferation gene (*Igf-2*) and granulosa marker (*Amh*) significantly increased following cell transplantation. In contrast, we observed no significant differences in the oocyte marker, *Gdf9*, following transplantation (Figure 4C;  $P < 0.05$ ).

Cleaved-caspase 3 acts as a functional enzyme<sup>[47,48]</sup>; therefore, to further validate these results, we performed Western blot assessment of cleaved-caspase 3 protein expression. Our results showed a significant increase in the cleaved-caspase 3 protein expression level in ovaries from the vehicle group compared with the control group, whereas the cleaved-caspase 3 protein expression level decreased significantly in the ovaries after transplantation of ES-MSCs and/or BM-MSCs compared with the vehicle group (Figure 4D and 4E;  $P < 0.05$ ). Previous studies suggested that MSCs secrete cytokines that are important for anti-apoptosis, angiogenesis, anti-inflammation, anti-fibrosis and immunoregulation, which would improve the microenvironment for promoting regeneration of injured tissues in numerous diseases<sup>[49-53]</sup>. In order to investigate the mechanism that underlies the function of these MSCs, we also analyzed VEGF, IGF-2 and HGF levels in ES-MSCs and BM-MSCs condition media by using ELISA. The results showed that in a similar manner ES-MSCs and BM-MSCs secreted VEGF, IGF-2 and HGF *in vitro* (Figure 4F). However, there were only a few GFP-labelled cells after 4 wk in the ovaries (data not shown).

Immunohistochemistry staining for *Amh* to confirm the changes in the granulosa cells showed decreased *Amh* expression in ovaries from the vehicle group compared to the intact group and increased *Amh* expression in ovaries from both the ES-MSCs and BM-MSCs transplantation groups compared with the vehicle group (Figure 5A).

We assessed the ability of mice that received the transplantations to conceive and give birth to offspring. The successful mating rate was investigated over 6 wk, and the presence of a copulatory plug indicated successful mating. The mice that received transplantations of both ES-MSCs (3 out of 5 mice) and/or BM-MSCs (2 out of 5 mice) became pregnant and produced live offspring, 9 pups in mice transplanted with BM-MSCs and 16 pups in mice that received ES-MSCs. None of the vehicle mice became pregnant. These results showed that ovarian functions in mice with POF were partially restored by transplantation with either ES-MSCs or BM-MSCs (Figure 5B and 5C;  $P < 0.05$ ).



**Figure 3** Effects of human embryonic stem cell-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells

**transplantation in mice with premature ovarian failure.** A: Transplantation of embryonic stem cell-derived mesenchymal stem cells (ES-MSCs) and/or bone marrow-derived mesenchymal stem cells (BM-MSCs) improved body weights in mice with premature ovarian failure after 4 wk; B: Survival rate 4 wk after ES-MSCs and/or BM-MSCs transplantation. Survival rate significantly increased in both the ES-MSCs and/or BM-MSCs transplanted mice (more than 60%) compared with the vehicle group (20%); C: The follicle number increased after transplantation. The number of follicles at all stages of development in both cell transplanted groups was significantly higher than that of the vehicle mice, while it was lower than the intact mice; D, E: Both cell transplantations rescued hormone secretion in premature ovarian failure mice. Serum follicle stimulating hormone levels decreased significantly in both cell transplanted groups compared to the vehicle group. The serum estradiol level significantly recovered after both cell transplantations compared to the vehicle group. All data are presented as mean  $\pm$  standard error. Small letters (a, c) indicate the significance ( $P < 0.05$ ) compared to groups labeled by similar capital letters (A, C); <sup>a</sup> $P < 0.05$  significance of experimental groups vs the intact group; <sup>c</sup> $P < 0.05$  significance of ES-MSC and BM-MSC groups vs the vehicle group;  $n = 3-5$ . ES-MSCs: Embryonic stem cell-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; POF: Premature ovarian failure; FSH: Follicle stimulating hormone; E2: Estradiol.

## DISCUSSION

Understanding the pathogenesis of POF plays an important role in the development of effective therapeutic options for this disease. Therefore, elucidation of the mechanism for POF development is critical for the clinical treatment of POF disease<sup>[54]</sup>. The estrous cycle of female mice is similar to that of humans, although the estrous cycle of mice is shorter than that of humans<sup>[55]</sup>.

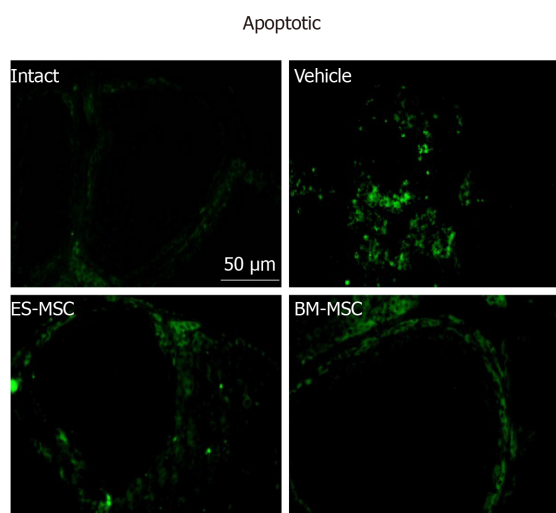
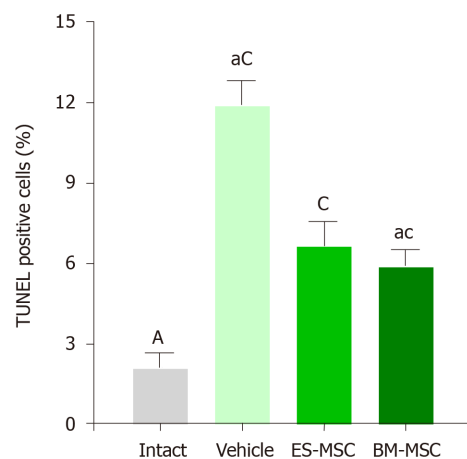
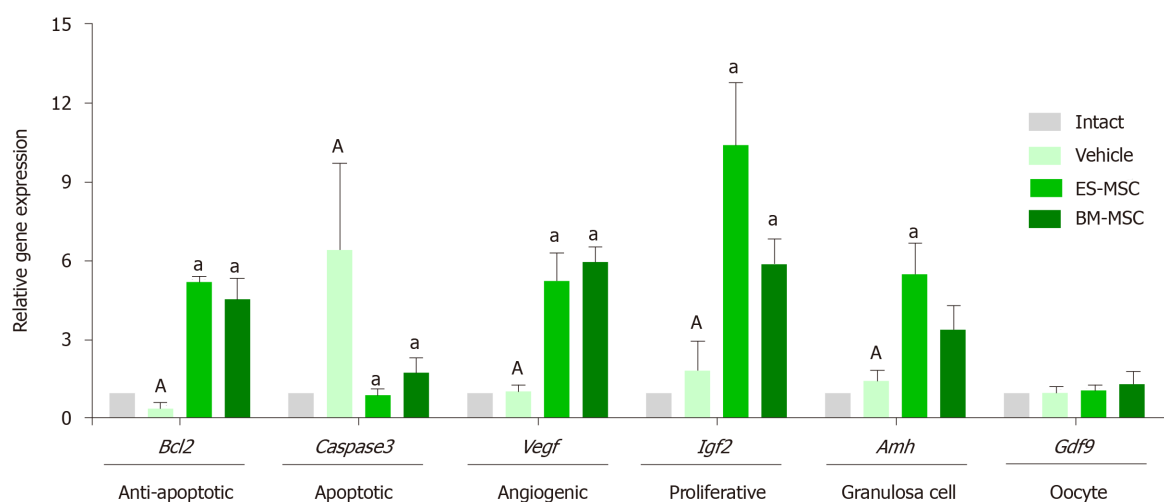
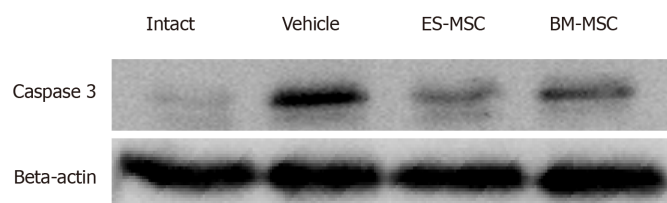
In this study, we initially established a mouse POF model by administration of Cy and Bu as the most effective chemotherapeutic drugs. The results indicated that Cy plus Bu in our established model (POF2) significantly decreased the number of follicles at various stages of development and significantly decreased ovarian size and body weight. In line with previous studies, chemotherapy increased primordial follicle recruitment, which led to significant decreases in the number of follicles at different developmental stages<sup>[35,56]</sup>. Apoptotic cells significantly increased in our established POF model, which was consistent with previous findings where chemotherapeutic drugs destroyed highly proliferating cells by activation of apoptosis<sup>[57]</sup>. We observed increased FSH levels and decreased E2 levels, which supported results of studies that showed similar patterns of hormonal changes in POF<sup>[15]</sup>. Previous studies demonstrated that chemotherapeutic drugs can cause POF in various species such as mouse, rat, rabbit and human<sup>[16,58-61]</sup>. Our results were consistent with previous reports as we showed a decrease in the number of follicles, decreased serum E2 levels, increased serum FSH levels and infertility.

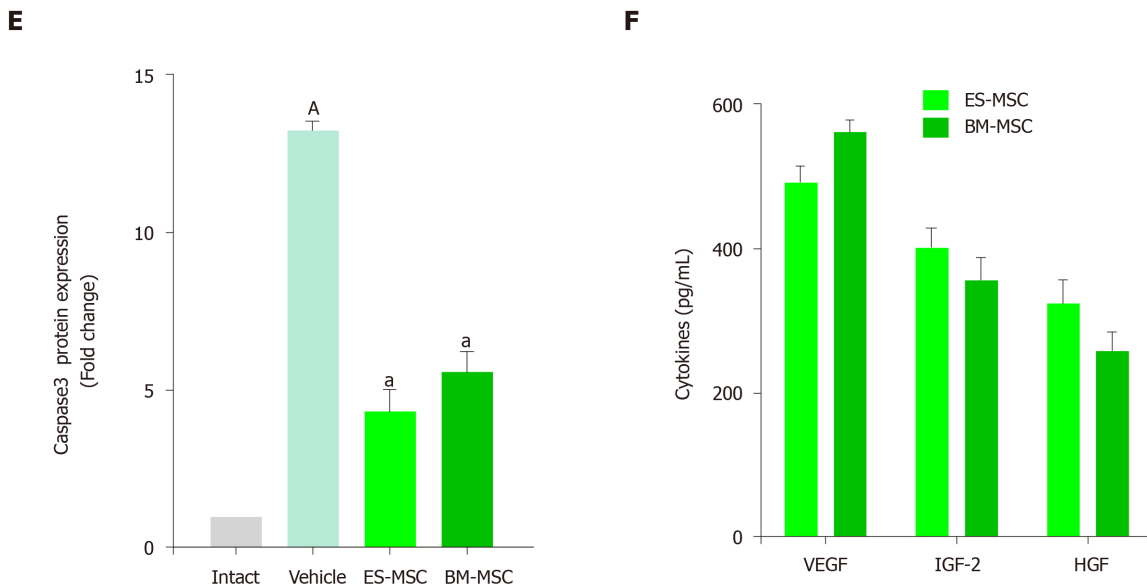
MSCs features depend on both the tissue source from which they were obtained and the species. Previous studies indicated that MSCs obtained from various species and sources differ in their biological characteristics such as surface marker expression, proliferative capacity, multilineage differentiation potential and immunomodulation feature<sup>[62,63]</sup>. In this study, we investigated biological properties of ES-MSCs and BM-MSCs. We have found that ES-MSCs and BM-MSCs both expressed homing cell adhesion molecule, CD73, CD90 and endoglin, but they showed no expression of CD34, protein tyrosine phosphatase receptor type C and CD 11b, which is consistent with a previous study<sup>[31]</sup>. We indicated that ES-MSCs showed enhanced proliferation capacity compared to the BM-MSCs. On the fourth and fifth passages, there were significant differences between ES-MSCs and BM-MSCs. Previous studies have similarly reported that ES-MSCs are more proliferative compared to BM-MSCs<sup>[31,33]</sup>.

In addition, we demonstrated that multilineage differentiation potential of BM-MSCs was greater than ES-MSCs. This finding was consistent with previous studies<sup>[31,33]</sup>. MSCs from different species and sources produce different cytokines. Our results were consistent with previous studies that cytokines secreted from MSCs could influence cell proliferation, differentiation, survival and tissue repair<sup>[64,65]</sup>. We observed no significant difference between ES-MSCs and BM-MSCs secreted cytokines in culture medium.

We transplanted human ES-MSCs into a mouse animal model and showed their capability in restoring ovarian function in POF. In support of transplantation of human derived MSCs to another species, previous studies have demonstrated that the transplantation of MSCs derived from various human tissues including menstrual blood, umbilical cord and amniotic fluid into animal models of POF restore ovarian function<sup>[66]</sup>.

In this study, we transplanted ES-MSCs into a mouse model of POF to investigate the role of these cells and mechanisms of action for improvement of POF. Our results indicated that both ES-MSCs and BM-MSCs showed a similar trend for improvement of POF in this animal model. ES-MSCs improved ovarian structure and function in these mice as evidenced by the increased number of follicles, decreased granulosa cell apoptosis and restored FSH and E2 to near normal levels. E2 is mainly secreted by

**A**

**B**

**C**

**D**




**Figure 4 Human embryonic stem cell-derived mesenchymal stem cells and/or bone marrow-derived mesenchymal stem cells transplantation improved premature ovarian failure conditions.** A and B: Apoptosis was reduced after both cell transplantations. The green stain color indicates terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labelling-positive cells. Data at 4 wk showed decreased levels of apoptosis following transplantation of embryonic stem cell-derived mesenchymal stem cells (ES-MSCs) and/or bone marrow-derived mesenchymal stem cells (BM-MSCs); C: Gene expression analysis showed that the expressions of B-cell lymphoma 2, vascular endothelial growth factor, insulin-like growth factor 2, anti-Müllerian hormone and growth/differentiation factor 9 significantly increased in both cell transplanted groups compared with the vehicle group, whereas cysteine-aspartic proteases 3 (*caspase 3*) significantly decreased in both cell transplanted groups compared to the vehicle group; D, E: Western blot analysis for cleaved-caspase 3 expression in the ovarian tissue. The results showed that cleaved-caspase 3 in ovarian tissue of the vehicle group significantly increased compared to the intact group. Cleaved-caspase 3 protein expression levels decreased in the ovaries from the ES-MSCs and BM-MSCs transplantation groups compared with the vehicle group; F: ELISA assessment of conditioned media of ES-MSCs and BM-MSCs for vascular endothelial growth factor, insulin-like growth factor 2 and hepatocyte growth factor. The results showed that ES-MSCs and BM-MSCs secreted vascular endothelial growth factor, insulin-like growth factor 2, and hepatocyte growth factor *in vitro*. All data are presented as mean  $\pm$  standard error. Small letters (a, c) indicate the significance ( $P < 0.05$ ) compared to groups labeled by similar capital letters (A, C),  $^aP < 0.05$ , significance of experimental groups vs the intact group.  $^cP < 0.05$ , significance of ES-MSC and BM-MSC groups vs the vehicle group. For

Figure 4C:  $^aP < 0.05$  significance of ES-MSC and BM-MSC groups vs the vehicle group,  $n = 3$ . ES-MSCs: Embryonic stem cell-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; POF: Premature ovarian failure; Bcl2: B-cell lymphoma 2; Caspase 3: Cysteine-aspartic proteases 3; Vegf: Vascular endothelial growth factor; Igf-2: Insulin-like growth factor 2; Amh: Anti-Müllerian hormone; Gdf9: Growth/differentiation factor 9.

granulosa cells, which inhibit FSH secretion. Increased FSH levels could accelerate recruitment of follicles and deplete the follicular pool<sup>[14,67,68]</sup>. Increased apoptosis in the POF group might result in decreased E2 and FSH levels. Transplantation of ES-MSCs inhibited granulosa cell apoptosis and increased E2 secretion, which led to decreases in FSH; therefore, the decreased level of FSH in the ES-MSCs transplantation group resulted in an increased number of follicles.

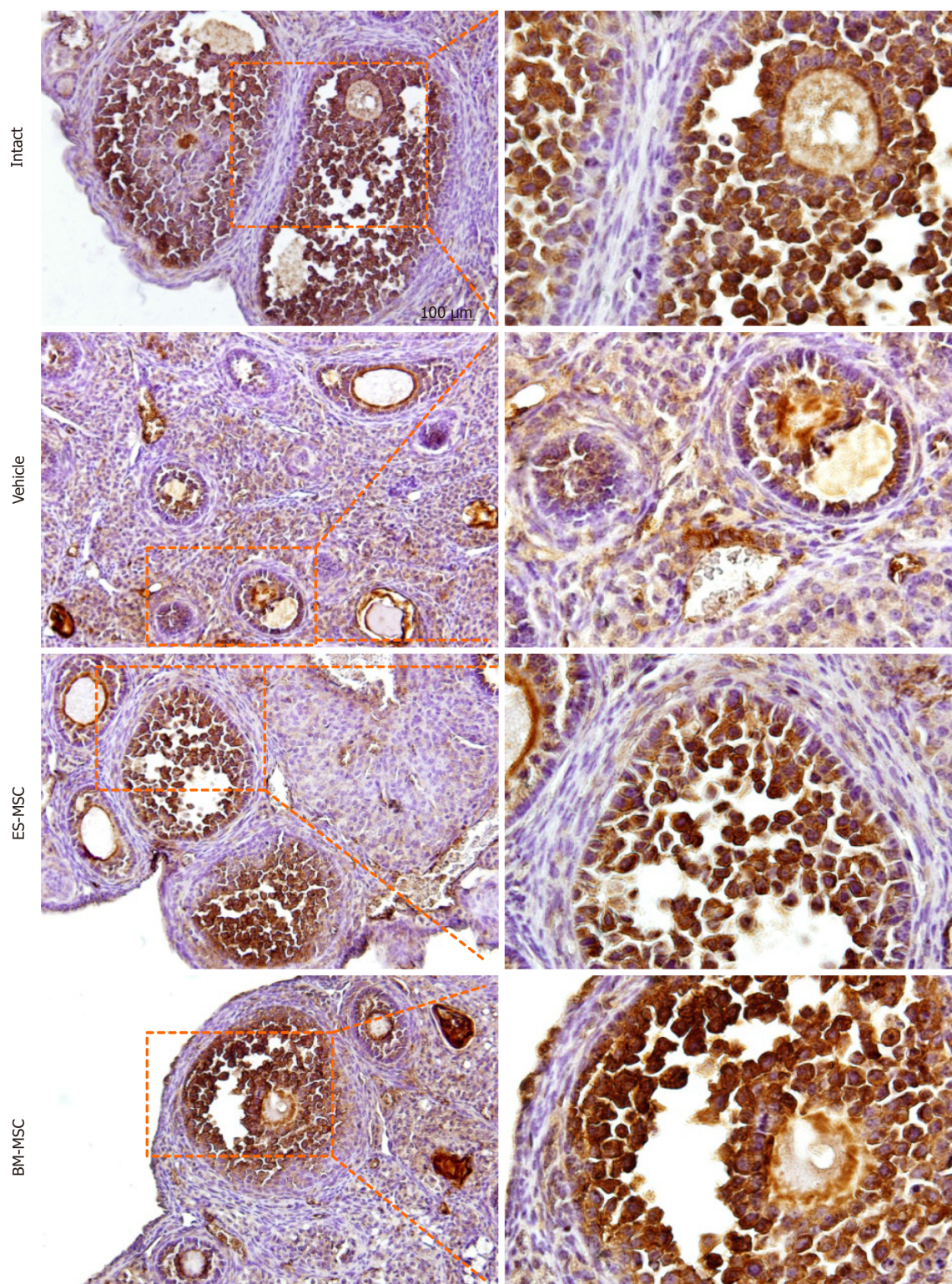
Gene expression and Western blot results reinforced our hypothesis. There were significant increases in *Amh* expression (granulosa cell marker) and no difference in *Gdf9* expression (oocyte marker). Expression of *Bcl2* was upregulated in both of the MSC transplantation groups compared with the POF group, whereas *caspase 3* expression was downregulated in both of these groups compared with the POF group.

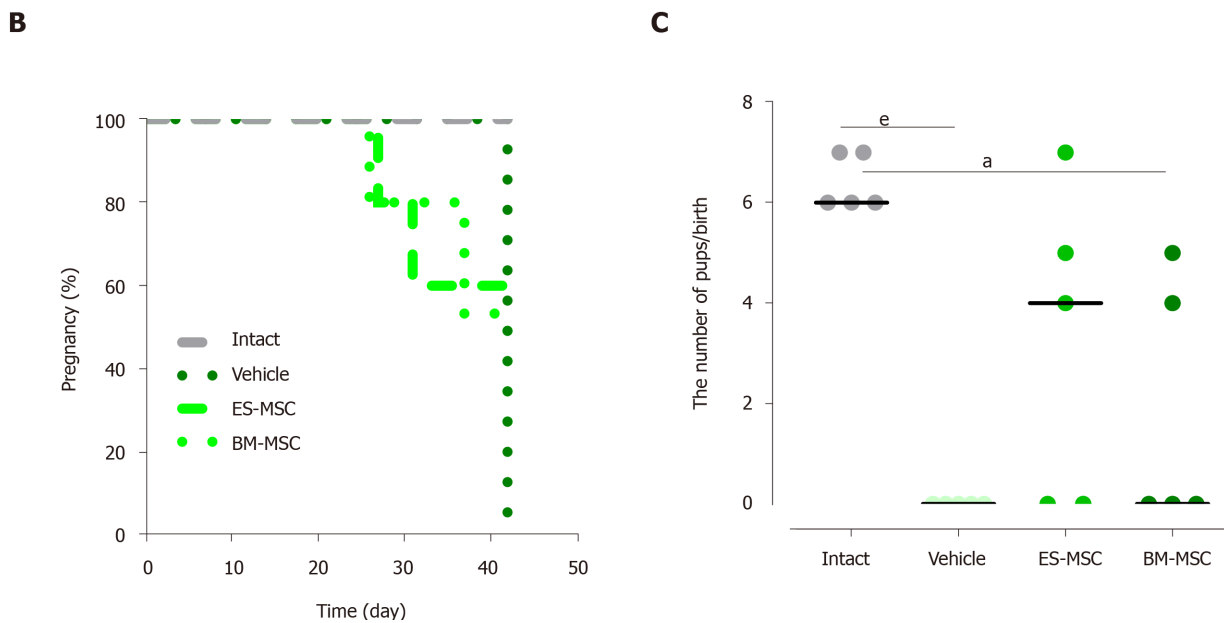
Immunohistochemistry results agreed with the real-time PCR results and indicated that MSCs could increase *Amh* expression compared with the POF mouse group. Folliculogenesis is mainly affected by interactions between the oocyte and granulosa cells<sup>[69]</sup>. *Amh* is expressed by granulosa cells and plays an important role in follicle growth<sup>[70,71]</sup>. We observed decreased apoptosis of granulosa cells after transplantation of the MSCs; therefore, ES-MSCs maintained the follicular niche by inhibiting apoptosis of granulosa cells. Previous findings of granulosa cell function in supporting oocytes also confirmed our results<sup>[72]</sup>. Therefore, granulosa cells support oocytes during development from the primordial state to maturation.

We observed that the ES-MSCs secreted VEGF, IGF-2 and HGF *in vitro*. VEGF, IGF-2 and HGF have an important role in inhibiting granulosa cell apoptosis, stimulating granulosa cell proliferation, inducing angiogenesis and follicle growth. VEGF promotes granulosa and endothelial cell proliferation. IGF-2 and HGF play an important role in suppressing apoptosis of granulosa cells that promote follicle maturation<sup>[73-77]</sup>. We observed decreased mRNA and protein expression of caspase 3 in both of the MSC transplantation groups compared with the POF group. The caspase



**A**





**Figure 5 Human embryonic stem cell-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells transplantation prevented follicular atresia and restored fertility in premature ovarian failure mice.** A: Immunohistochemical staining showed increased anti-Müllerian hormone expression in the ovaries from the embryonic stem cell-derived mesenchymal stem cells (ES-MSCs) and bone marrow-derived mesenchymal stem cells (BM-MSCs) transplantation groups compared with the vehicle group; B, C: The percentage of pregnancies and the numbers of pups six weeks after both cell transplantations. The intact mice had a 100% (5 of 5) pregnancy rate, producing 32 live offspring. The embryonic stem cell-derived MSCs transplanted mice had a 60% (3 of 5) pregnancy rate, producing 16 live offspring. The bone marrow-derived MSCs transplanted mice had a 40% (2 of 5) pregnancy rate, producing nine live offspring. None of the premature ovarian failure mice became pregnant. Results are expressed as mean  $\pm$  standard error, <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.001$ ,  $n = 3-5$ . ES-MSCs: Embryonic stem cell-derived mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells.

family members are important regulators of apoptosis. Caspase 3 is an effector caspase; its activation results in the final phase of cellular death<sup>[78]</sup>. Our study results suggested that ES-MSCs may have decreased caspase 3 expression in the ES-MSC transplantation group by releasing VEGF, HGF and IGF-2; therefore, these cytokines may inhibit apoptosis in granulosa cells by upregulation of *Bcl2* and downregulation of *caspase 3* in the ovaries of POF mice. Therefore, there was increased expression of the granulosa cell marker (*Amh*) after transplantation of the ES-MSCs. Our results might indicate that cytokines secreted by ES-MSCs reduce granulosa cell apoptosis and increase follicles by increasing E2 secretion.

We observed no significant difference in *Gdf9* expression in both MSC transplantation groups compared with POF. *Gdf9* is an adult oocyte-specific marker<sup>[79,80]</sup>, and this finding might indicate that transplantation of ES-MSCs restored ovarian function in POF mice *via* an indirect effect due to cytokine secretion rather than direct differentiation to oocytes.

These findings suggested that a possible mechanism by ES-MSCs and BM-MSCs restored the injured ovary by cytokine suppression of granulosa cell apoptosis and increased follicular growth. In line with our findings, the results from previous studies suggest that MSCs have an effect on restoring ovarian function by the paracrine mechanism of cytokines<sup>[81-85]</sup>, which plays an important role in increased granulosa cell resistance to chemotherapeutic drugs and improves the ovarian microenvironment and follicle growth<sup>[58]</sup>.

In conclusion, our results indicated that human ES-MSCs could restore ovarian structure and function in chemotherapy-induced POF mice and improve fertility. Transplantation of ES-MSCs improved the disturbed endocrine secretion system, reduced apoptosis rate in the ovaries, and improved folliculogenesis possibly through a paracrine effect and ovarian cell survival. Therefore, ES-MSCs could be a promising source for stem cell therapy in individuals with POF.



## ARTICLE HIGHLIGHTS

**Research background**

Premature ovarian failure (POF) is characterized by amenorrhea, hypogonadism, high gonadotropins and infertility in women under 40-years-old. Previous reports demonstrated that various tissue-specific stem cells could restore ovarian function and folliculogenesis in chemotherapy-induced POF mice.

**Research motivation**

Human embryonic stem cell-derived MSC (ES-MSC) have advantages, such as higher proliferation, more potent anti-inflammatory properties and lack of obstacles of harvesting tissue-specific MSCs that make them attractive candidates for restoring fertility in patients with POF.

**Research objectives**

The aim of this study was to evaluate the therapeutic efficacy of ES-MSCs in a model of chemotherapy-induced POF.

**Research methods**

In this study, we initially established a mouse POF model by administration of cyclophosphamide and busulfan, then we transplanted ES-MSCs and bone marrow-derived MSC (BM-MSC) into a mouse model of POF to investigate the role of these cells and mechanisms of action for improvement of POF.

**Research results**

The POF model established by the 100 mg/kg dose of cyclophosphamide showed significant decreases in body weight, follicle count and estradiol level but had an increased follicle-stimulating hormone level. ES-MSC and/or BM-MSC transplantation significantly improved body weight, follicle count, hormone secretion, survival rate and reproductive function in POF mice. Gene expression and Western blot analysis, terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate nick end labelling assay and immunohistochemistry indicated that the ES-MSCs or BM-MSCs reduced apoptosis in the follicles and restored fertility in chemotherapy-induced POF mice. The results of this study indicated that the effects of ES-MSCs and BM-MSCs in restoring ovarian function appear *via* the paracrine mechanisms of cytokines.

**Research conclusions**

Our findings demonstrated that human ES-MSCs, similar to BM-MSCs, improved ovarian function and restored fertility in a mouse POF model.

**Research perspectives**

Our present study results suggest that human ES-MSCs could be a promising source for stem cell therapy in individuals with POF.

## REFERENCES

- 1 Maclaran K, Panay N. Premature ovarian failure. *J Fam Plann Reprod Health Care* 2011; **37**: 35-42 [PMID: 21367702 DOI: 10.1136/jfprhc.2010.0015]
- 2 Woad KJ, Watkins WJ, Prendergast D, Shelling AN. The genetic basis of premature ovarian failure. *Aust N Z J Obstet Gynaecol* 2006; **46**: 242-244 [PMID: 16704481 DOI: 10.1111/j.1479-828X.2006.00585.x]
- 3 Stearns V, Schneider B, Henry NL, Hayes DF, Flockhart DA. Breast cancer treatment and ovarian failure: risk factors and emerging genetic determinants. *Nat Rev Cancer* 2006; **6**: 886-893 [PMID: 17036039 DOI: 10.1038/nrc1992]
- 4 Manger K, Wildt L, Kalden JR, Manger B. Prevention of gonadal toxicity and preservation of gonadal function and fertility in young women with systemic lupus erythematosus treated by cyclophosphamide: the PREGO-Study. *Autoimmun Rev* 2006; **5**: 269-272 [PMID: 16697968 DOI: 10.1016/j.autrev.2005.10.001]
- 5 Laven JS. Primary Ovarian Insufficiency. *Semin Reprod Med* 2016; **34**: 230-234 [PMID: 27513024 DOI: 10.1055/s-0036-1585402]
- 6 Fenton AJ. Premature ovarian insufficiency: Pathogenesis and management. *J Midlife Health* 2015; **6**: 147-153 [PMID: 26903753 DOI: 10.4103/0976-7800.172292]
- 7 Santoro N. Mechanisms of premature ovarian failure. *Ann Endocrinol (Paris)* 2003; **64**: 87-92 [PMID: 12773939 DOI: AE-04-2003-64-2-0003-4266-101019-ART06]
- 8 Stroud JS, Mutch D, Rader J, Powell M, Thaker PH, Grigsby PW. Effects of cancer treatment on ovarian function. *Fertil Steril* 2009; **92**: 417-427 [PMID: 18774559 DOI: 10.1016/j.fertnstert.2008.07.1714]
- 9 Salama M, Winkler K, Murach KF, Seeber B, Ziehr SC, Wildt L. Female fertility loss and preservation:

- threats and opportunities. *Ann Oncol* 2013; **24**: 598-608 [PMID: [23129121](#) DOI: [10.1093/annonc/mds514](#)]
- 10 **Liu T**, Huang Y, Zhang J, Qin W, Chi H, Chen J, Yu Z, Chen C. Transplantation of human menstrual blood stem cells to treat premature ovarian failure in mouse model. *Stem Cells Dev* 2014; **23**: 1548-1557 [PMID: [24593672](#) DOI: [10.1089/scd.2013.0371](#)]
  - 11 **Song D**, Zhong Y, Qian C, Zou Q, Ou J, Shi Y, Gao L, Wang G, Liu Z, Li H, Ding H, Wu H, Wang F, Wang J, Li H. Human Umbilical Cord Mesenchymal Stem Cells Therapy in Cyclophosphamide-Induced Premature Ovarian Failure Rat Model. *Biomed Res Int* 2016; **2016**: 2517514 [PMID: [27047962](#) DOI: [10.1155/2016/2517514](#)]
  - 12 **Su J**, Ding L, Cheng J, Yang J, Li X, Yan G, Sun H, Dai J, Hu Y. Transplantation of adipose-derived stem cells combined with collagen scaffolds restores ovarian function in a rat model of premature ovarian insufficiency. *Hum Reprod* 2016; **31**: 1075-1086 [PMID: [26965432](#) DOI: [10.1093/humrep/dew041](#)]
  - 13 **Lai D**, Wang F, Yao X, Zhang Q, Wu X, Xiang C. Human endometrial mesenchymal stem cells restore ovarian function through improving the renewal of germline stem cells in a mouse model of premature ovarian failure. *J Transl Med* 2015; **13**: 155 [PMID: [25964118](#) DOI: [10.1186/s12967-015-0516-y](#)]
  - 14 **Liu R**, Zhang X, Fan Z, Wang Y, Yao G, Wan X, Liu Z, Yang B, Yu L. Human amniotic mesenchymal stem cells improve the follicular microenvironment to recover ovarian function in premature ovarian failure mice. *Stem Cell Res Ther* 2019; **10**: 299 [PMID: [31578152](#) DOI: [10.1186/s13287-019-1315-9](#)]
  - 15 **Chen L**, Guo S, Wei C, Li H, Wang H, Xu Y. Effect of stem cell transplantation of premature ovarian failure in animal models and patients: A meta-analysis and case report. *Exp Ther Med* 2018; **15**: 4105-4118 [PMID: [29755593](#) DOI: [10.3892/etm.2018.5970](#)]
  - 16 **Edessy M**, Hosni HN, Shady Y, Waf Y, Bakr S, Kamel M. Autologous stem cells therapy, the first baby of idiopathic premature ovarian failure. *Acta Med Int* 2016; **3**: 19-23 [DOI: [10.5530/ami.2016.1.7](#)]
  - 17 **Lotfinia M**, Kadivar M, Piryaei A, Pournasr B, Sardari S, Sodeifi N, Sayahpour FA, Baharvand H. Effect of Secreted Molecules of Human Embryonic Stem Cell-Derived Mesenchymal Stem Cells on Acute Hepatic Failure Model. *Stem Cells Dev* 2016; **25**: 1898-1908 [PMID: [27676103](#) DOI: [10.1089/scd.2016.0244](#)]
  - 18 **Bak XY**, Lam DH, Yang J, Ye K, Wei EL, Lim SK, Wang S. Human embryonic stem cell-derived mesenchymal stem cells as cellular delivery vehicles for prodrug gene therapy of glioblastoma. *Hum Gene Ther* 2011; **22**: 1365-1377 [PMID: [21425958](#) DOI: [10.1089/hum.2010.212](#)]
  - 19 **Le Blanc K**, Ringdén O. Immunomodulation by mesenchymal stem cells and clinical experience. *J Intern Med* 2007; **262**: 509-525 [PMID: [17949362](#) DOI: [10.1111/j.1365-2796.2007.01844.x](#)]
  - 20 **Aggarwal S**, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; **105**: 1815-1822 [PMID: [15494428](#) DOI: [10.1182/blood-2004-04-1559](#)]
  - 21 **Jiang XX**, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, Mao N. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005; **105**: 4120-4126 [PMID: [15692068](#) DOI: [10.1182/blood-2004-02-0586](#)]
  - 22 **Beyth S**, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, Galun E, Rachmilewitz J. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005; **105**: 2214-2219 [PMID: [15514012](#) DOI: [10.1182/blood-2004-07-2921](#)]
  - 23 **Ren G**, Su J, Zhang L, Zhao X, Ling W, L'huillie A, Zhang J, Lu Y, Roberts AI, Ji W, Zhang H, Rabson AB, Shi Y. Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. *Stem Cells* 2009; **27**: 1954-1962 [PMID: [19544427](#) DOI: [10.1002/stem.118](#)]
  - 24 **Wynn RF**, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE, Fairbairn LJ, Bellantuono I. A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* 2004; **104**: 2643-2645 [PMID: [15251986](#) DOI: [10.1182/blood-2004-02-0526](#)]
  - 25 **Ip JE**, Wu Y, Huang J, Zhang L, Pratt RE, Dzau VJ. Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. *Mol Biol Cell* 2007; **18**: 2873-2882 [PMID: [17507648](#) DOI: [10.1091/mbc.e07-02-0166](#)]
  - 26 **Nahar S**, Nakashima Y, Miyagi-Shiohira C, Kinjo T, Kobayashi N, Saitoh I, Watanabe M, Noguchi H, Fujita J. A Comparison of Proteins Expressed between Human and Mouse Adipose-Derived Mesenchymal Stem Cells by a Proteome Analysis through Liquid Chromatography with Tandem Mass Spectrometry. *Int J Mol Sci* 2018; **19** [PMID: [30404232](#) DOI: [10.3390/ijms19113497](#)]
  - 27 **Gadkari R**, Zhao L, Teklemariam T, Hantash BM. Human embryonic stem cell derived-mesenchymal stem cells: an alternative mesenchymal stem cell source for regenerative medicine therapy. *Regen Med* 2014; **9**: 453-465 [PMID: [25159063](#) DOI: [10.2217/rme.14.13](#)]
  - 28 **Wang Q**, Yang Q, Wang Z, Tong H, Ma L, Zhang Y, Shan F, Meng Y, Yuan Z. Comparative analysis of human mesenchymal stem cells from fetal-bone marrow, adipose tissue, and Warton's jelly as sources of cell immunomodulatory therapy. *Hum Vaccin Immunother* 2016; **12**: 85-96 [PMID: [26186552](#) DOI: [10.1080/21645515.2015.1030549](#)]
  - 29 **Karlsson C**, Emanuelsson K, Wessberg F, Kajic K, Axell MZ, Eriksson PS, Lindahl A, Hyllner J, Strehl R. Human embryonic stem cell-derived mesenchymal progenitors--potential in regenerative medicine. *Stem Cell Res* 2009; **3**: 39-50 [PMID: [19515621](#) DOI: [10.1016/j.scr.2009.05.002](#)]
  - 30 **Mardpour S**, Hassani SN, Mardpour S, Sayahpour F, Vosough M, Ai J, Aghdani N, Hamidieh AA, Baharvand H. Extracellular vesicles derived from human embryonic stem cell-MSCs ameliorate cirrhosis in thioacetamide-induced chronic liver injury. *J Cell Physiol* 2018; **233**: 9330-9344 [PMID: [29266258](#) DOI: [10.1002/jcp.26413](#)]
  - 31 **Brown PT**, Squire MW, Li WJ. Characterization and evaluation of mesenchymal stem cells derived from human embryonic stem cells and bone marrow. *Cell Tissue Res* 2014; **358**: 149-164 [PMID: [24927918](#) DOI: [10.1007/s00441-014-1926-5](#)]
  - 32 **Fu X**, Chen Y, Xie FN, Dong P, Liu WB, Cao Y, Zhang WJ, Xiao R. Comparison of immunological characteristics of mesenchymal stem cells derived from human embryonic stem cells and bone marrow. *Tissue Eng Part A* 2015; **21**: 616-626 [PMID: [25256849](#) DOI: [10.1089/ten.TEA.2013.0651](#)]
  - 33 **Krylova TA**, Kol'tsova AM, Zenin VV, Musorina AS, Iakovleva TK, Polianskaia GG. [Comparative characteristics of new mesenchymal stem cell lines derived from human embryonic stem cells, bone marrow and foreskin]. *Tsitologiya* 2012; **54**: 5-16 [PMID: [22567895](#)]

- 34 **Wang X**, Kimbrel EA, Ijichi K, Paul D, Lazorchak AS, Chu J, Kouris NA, Yavanian GJ, Lu SJ, Pachter JS, Crocker SJ, Lanza R, Xu RH. Human ESC-derived MSCs outperform bone marrow MSCs in the treatment of an EAE model of multiple sclerosis. *Stem Cell Reports* 2014; **3**: 115-130 [PMID: [25068126](#) DOI: [10.1016/j.stemcr.2014.04.020](#)]
- 35 **Rosendahl M**, Andersen CY, la Cour Freiesleben N, Juul A, Løssl K, Andersen AN. Dynamics and mechanisms of chemotherapy-induced ovarian follicular depletion in women of fertile age. *Fertil Steril* 2010; **94**: 156-166 [PMID: [19342041](#) DOI: [10.1016/j.fertnstert.2009.02.043](#)]
- 36 **Mohamed SA**, Shalaby SM, Abdelaziz M, Brakta S, Hill WD, Ismail N, Al-Hendy A. Human Mesenchymal Stem Cells Partially Reverse Infertility in Chemotherapy-Induced Ovarian Failure. *Reprod Sci* 2018; **25**: 51-63 [PMID: [28460567](#) DOI: [10.1177/1933719117699705](#)]
- 37 **Xiao GY**, Liu IH, Cheng CC, Chang CC, Lee YH, Cheng WT, Wu SC. Amniotic fluid stem cells prevent follicle atresia and rescue fertility of mice with premature ovarian failure induced by chemotherapy. *PLoS One* 2014; **9**: e106538 [PMID: [25198549](#) DOI: [10.1371/journal.pone.0106538](#)]
- 38 **Luo Q**, Yin N, Zhang L, Yuan W, Zhao W, Luan X, Zhang H. Role of SDF-1/CXCR4 and cytokines in the development of ovary injury in chemotherapy drug induced premature ovarian failure mice. *Life Sci* 2017; **179**: 103-109 [PMID: [28478265](#) DOI: [10.1016/j.lfs.2017.05.001](#)]
- 39 **Lai D**, Wang F, Dong Z, Zhang Q. Skin-derived mesenchymal stem cells help restore function to ovaries in a premature ovarian failure mouse model. *PLoS One* 2014; **9**: e98749 [PMID: [24879098](#) DOI: [10.1371/journal.pone.0098749](#)]
- 40 **Zhang Q**, Xu M, Yao X, Li T, Wang Q, Lai D. Human amniotic epithelial cells inhibit granulosa cell apoptosis induced by chemotherapy and restore the fertility. *Stem Cell Res Ther* 2015; **6**: 152 [PMID: [26303743](#) DOI: [10.1186/s13287-015-0148-4](#)]
- 41 **Lai D**, Wang F, Chen Y, Wang L, Wang Y, Cheng W. Human amniotic fluid stem cells have a potential to recover ovarian function in mice with chemotherapy-induced sterility. *BMC Dev Biol* 2013; **13**: 34 [PMID: [24006896](#) DOI: [10.1186/1471-213X-13-34](#)]
- 42 **Sun M**, Wang S, Li Y, Yu L, Gu F, Wang C, Yao Y. Adipose-derived stem cells improved mouse ovary function after chemotherapy-induced ovary failure. *Stem Cell Res Ther* 2013; **4**: 80 [PMID: [23838374](#) DOI: [10.1186/scrt231](#)]
- 43 **Liu T**, Huang Y, Guo L, Cheng W, Zou G. CD44+/CD105+ human amniotic fluid mesenchymal stem cells survive and proliferate in the ovary long-term in a mouse model of chemotherapy-induced premature ovarian failure. *Int J Med Sci* 2012; **9**: 592-602 [PMID: [23028242](#) DOI: [10.7150/ijms.4841](#)]
- 44 **Pascuali N**, Scotti L, Di Pietro M, Oubiña G, Bas D, May M, Gómez Muñoz A, Cuasnicú PS, Cohen DJ, Tesone M, Abramovich D, Parborell F. Ceramide-1-phosphate has protective properties against cyclophosphamide-induced ovarian damage in a mice model of premature ovarian failure. *Hum Reprod* 2018; **33**: 844-859 [PMID: [29534229](#) DOI: [10.1093/humrep/dey045](#)]
- 45 **Tilly JL**. Ovarian follicle counts--not as simple as 1, 2, 3. *Reprod Biol Endocrinol* 2003; **1**: 11 [PMID: [12646064](#) DOI: [10.1186/1477-7827-1-11](#)]
- 46 **Neishabouri SH**, Hutson SM, Davoodi J. Chronic activation of mTOR complex 1 by branched chain amino acids and organ hypertrophy. *Amino Acids* 2015; **47**: 1167-1182 [PMID: [25721400](#) DOI: [10.1007/s00726-015-1944-y](#)]
- 47 **Jänicke RU**, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 1998; **273**: 9357-9360 [PMID: [9545256](#) DOI: [10.1074/jbc.273.16.9357](#)]
- 48 **Yacobi K**, Wojtowicz A, Tsafiri A, Gross A. Gonadotropins enhance caspase-3 and -7 activity and apoptosis in the theca-interstitial cells of rat preovulatory follicles in culture. *Endocrinology* 2004; **145**: 1943-1951 [PMID: [14726442](#) DOI: [10.1210/en.2003-1395](#)]
- 49 **Kupcova Skalníková H**. Proteomic techniques for characterisation of mesenchymal stem cell secretome. *Biochimie* 2013; **95**: 2196-2211 [PMID: [23880644](#) DOI: [10.1016/j.biochi.2013.07.015](#)]
- 50 **Squillaro T**, Peluso G, Galderisi U. Clinical Trials With Mesenchymal Stem Cells: An Update. *Cell Transplant* 2016; **25**: 829-848 [PMID: [26423725](#) DOI: [10.3727/096368915X689622](#)]
- 51 **He Y**, Chen D, Yang L, Hou Q, Ma H, Xu X. The therapeutic potential of bone marrow mesenchymal stem cells in premature ovarian failure. *Stem Cell Res Ther* 2018; **9**: 263 [PMID: [30286808](#) DOI: [10.1186/s13287-018-1008-9](#)]
- 52 **Fu Y**, Karbaat L, Wu L, Leijten J, Both SK, Karperien M. Trophic Effects of Mesenchymal Stem Cells in Tissue Regeneration. *Tissue Eng Part B Rev* 2017; **23**: 515-528 [PMID: [28490258](#) DOI: [10.1089/ten.TEB.2016.0365](#)]
- 53 **Kusuma GD**, Carthew J, Lim R, Frith JE. Effect of the Microenvironment on Mesenchymal Stem Cell Paracrine Signaling: Opportunities to Engineer the Therapeutic Effect. *Stem Cells Dev* 2017; **26**: 617-631 [PMID: [28186467](#) DOI: [10.1089/scd.2016.0349](#)]
- 54 **Torrealdy S**, Kodaman P, Pal L. Premature Ovarian Insufficiency - an update on recent advances in understanding and management. *F1000Res* 2017; **6**: 2069 [PMID: [29225794](#) DOI: [10.12688/f1000research.11948.1](#)]
- 55 **Zhang J**, Fang L, Shi L, Lai Z, Lu Z, Xiong J, Wu M, Luo A, Wang S. Protective effects and mechanisms investigation of Kuntai capsule on the ovarian function of a novel model with accelerated aging ovaries. *J Ethnopharmacol* 2017; **195**: 173-181 [PMID: [27845267](#) DOI: [10.1016/j.jep.2016.11.014](#)]
- 56 **Goswami D**, Conway GS. Premature ovarian failure. *Hum Reprod Update* 2005; **11**: 391-410 [PMID: [15919682](#) DOI: [10.1093/humupd/dmi012](#)]
- 57 **Zhao XJ**, Huang YH, Yu YC, Xin XY. GnRH antagonist cetrorelix inhibits mitochondria-dependent apoptosis triggered by chemotherapy in granulosa cells of rats. *Gynecol Oncol* 2010; **118**: 69-75 [PMID: [20417958](#) DOI: [10.1016/j.ygyno.2010.03.021](#)]
- 58 **Fu X**, He Y, Xie C, Liu W. Bone marrow mesenchymal stem cell transplantation improves ovarian function and structure in rats with chemotherapy-induced ovarian damage. *Cytotherapy* 2008; **10**: 353-363 [PMID: [18574768](#) DOI: [10.1080/14653240802035926](#)]
- 59 **Abd-Allah SH**, Shalaby SM, Pasha HF, El-Shal AS, Raafat N, Shabrawy SM, Awad HA, Amer MG, Gharib MA, El Gendy EA, Raslan AA, El-Kelawy HM. Mechanistic action of mesenchymal stem cell injection in



- the treatment of chemically induced ovarian failure in rabbits. *Cytotherapy* 2013; **15**: 64-75 [PMID: 23260087 DOI: 10.1016/j.jcyt.2012.08.001]
- 60 **Fleischer RT**, Vollenhoven BJ, Weston GC. The effects of chemotherapy and radiotherapy on fertility in premenopausal women. *Obstet Gynecol Surv* 2011; **66**: 248-254 [PMID: 21756407 DOI: 10.1097/OGX.0b013e318224e97b]
  - 61 **Zhang T**, Yan D, Yang Y, Ma A, Li L, Wang Z, Pan Q, Sun Z. The comparison of animal models for premature ovarian failure established by several different source of inducers. *Regul Toxicol Pharmacol* 2016; **81**: 223-232 [PMID: 27612992 DOI: 10.1016/j.yrtph.2016.09.002]
  - 62 **Uder C**, Brückner S, Winkler S, Tautenhahn HM, Christ B. Mammalian MSC from selected species: Features and applications. *Cytometry A* 2018; **93**: 32-49 [PMID: 28906582 DOI: 10.1002/cyto.a.23239]
  - 63 **Kozłowska U**, Krawczyński A, Futoma K, Jurek T, Rorat M, Patrzalek D, Klimczak A. Similarities and differences between mesenchymal stem/progenitor cells derived from various human tissues. *World J Stem Cells* 2019; **11**: 347-374 [PMID: 31293717 DOI: 10.4252/wjsc.v11.i6.347]
  - 64 **Majumdar MK**, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL. Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. *J Hematother Stem Cell Res* 2000; **9**: 841-848 [PMID: 11177595 DOI: 10.1089/152581600750062264]
  - 65 **Guan YT**, Xie Y, Li DS, Zhu YY, Zhang XL, Feng YL, Chen YP, Xu LJ, Liao PF, Wang G. Comparison of biological characteristics of mesenchymal stem cells derived from the human umbilical cord and decidua parietalis. *Mol Med Rep* 2019; **20**: 633-639 [PMID: 31180542 DOI: 10.3892/mmr.2019.10286]
  - 66 **Yoon SY**. Mesenchymal stem cells for restoration of ovarian function. *Clin Exp Reprod Med* 2019; **46**: 1-7 [PMID: 30827071 DOI: 10.5653/ceerm.2019.46.1.1]
  - 67 **Dewailly D**, Robin G, Peigne M, Decanter C, Pigny P, Cateau-Jonard S. Interactions between androgens, FSH, anti-Müllerian hormone and estradiol during folliculogenesis in the human normal and polycystic ovary. *Hum Reprod Update* 2016; **22**: 709-724 [PMID: 27566840 DOI: 10.1093/humupd/dmw027]
  - 68 **Busch AS**, Hagen CP, Almstrup K, Main KM, Juul A. Genetic variations altering FSH action affect circulating hormone levels as well as follicle growth in healthy peripubertal girls. *Hum Reprod* 2016; **31**: 897-904 [PMID: 26905078 DOI: 10.1093/humrep/dew022]
  - 69 **Cecconi S**, Ciccarelli C, Barberi M, Macchiarelli G, Canipari R. Granulosa cell-oocyte interactions. *Eur J Obstet Gynecol Reprod Biol* 2004; **115** Suppl 1: S19-S22 [PMID: 15196711 DOI: 10.1016/j.ejogrb.2004.01.010]
  - 70 **Kevenaar ME**, Meerasahib MF, Kramer P, van de Lang-Born BM, de Jong FH, Groome NP, Themmen AP, Visser JA. Serum anti-müllerian hormone levels reflect the size of the primordial follicle pool in mice. *Endocrinology* 2006; **147**: 3228-3234 [PMID: 16556768 DOI: 10.1210/en.2005-1588]
  - 71 **La Marca A**, Volpe A. Anti-Müllerian hormone (AMH) in female reproduction: is measurement of circulating AMH a useful tool? *Clin Endocrinol (Oxf)* 2006; **64**: 603-610 [PMID: 16712660 DOI: 10.1111/j.1365-2265.2006.02533.x]
  - 72 **Matzuk MM**, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 2002; **296**: 2178-2180 [PMID: 12077402 DOI: 10.1126/science.1071965]
  - 73 **Kosaka N**, Sudo N, Miyamoto A, Shimizu T. Vascular endothelial growth factor (VEGF) suppresses ovarian granulosa cell apoptosis in vitro. *Biochem Biophys Res Commun* 2007; **363**: 733-737 [PMID: 17904528 DOI: 10.1016/j.bbrc.2007.09.061]
  - 74 **Quintana R**, Kopcow L, Marconi G, Sueldo C, Speranza G, Barañao RI. Relationship of ovarian stimulation response with vascular endothelial growth factor and degree of granulosa cell apoptosis. *Hum Reprod* 2001; **16**: 1814-1818 [PMID: 11527881 DOI: 10.1093/humrep/16.9.1814]
  - 75 **Uzumcu M**, Pan Z, Chu Y, Kuhn PE, Zachow R. Immunolocalization of the hepatocyte growth factor (HGF) system in the rat ovary and the anti-apoptotic effect of HGF in rat ovarian granulosa cells in vitro. *Reproduction* 2006; **132**: 291-299 [PMID: 16885537 DOI: 10.1530/rep.1.00989]
  - 76 **Zachow R**, Uzumcu M. The hepatocyte growth factor system as a regulator of female and male gonadal function. *J Endocrinol* 2007; **195**: 359-371 [PMID: 18000299 DOI: 10.1677/JOE-07-0466]
  - 77 **Martínez-Chequer JC**, Stouffer RL, Hazzard TM, Patton PE, Molskness TA. Insulin-like growth factors-1 and -2, but not hypoxia, synergize with gonadotropin hormone to promote vascular endothelial growth factor-A secretion by monkey granulosa cells from preovulatory follicles. *Biol Reprod* 2003; **68**: 1112-1118 [PMID: 12606472 DOI: 10.1095/biolreprod.102.011155]
  - 78 **Nicholson DW**. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* 1999; **6**: 1028-1042 [PMID: 10578171 DOI: 10.1038/sj.cdd.4400598]
  - 79 **Hayashi M**, McGee EA, Min G, Klein C, Rose UM, van Duin M, Hsueh AJ. Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. *Endocrinology* 1999; **140**: 1236-1244 [PMID: 10067849 DOI: 10.1210/endo.140.3.6548]
  - 80 **Gupta SK**, Jethanandani P, Afzalpurkar A, Kaul R, Santhanam R. Prospects of zona pellucida glycoproteins as immunogens for contraceptive vaccine. *Hum Reprod Update* 1997; **3**: 311-324 [PMID: 9459277 DOI: 10.1093/humupd/3.4.311]
  - 81 **Wang Z**, Wang Y, Yang T, Li J, Yang X. Study of the reparative effects of menstrual-derived stem cells on premature ovarian failure in mice. *Stem Cell Res Ther* 2017; **8**: 11 [PMID: 28114977 DOI: 10.1186/s13287-016-0458-1]
  - 82 **Liu J**, Zhang H, Zhang Y, Li N, Wen Y, Cao F, Ai H, Xue X. Homing and restorative effects of bone marrow-derived mesenchymal stem cells on cisplatin injured ovaries in rats. *Mol Cells* 2014; **37**: 865-872 [PMID: 25410907 DOI: 10.14348/molcells.2014.0145]
  - 83 **Yao X**, Guo Y, Wang Q, Xu M, Zhang Q, Li T, Lai D. The Paracrine Effect of Transplanted Human Amniotic Epithelial Cells on Ovarian Function Improvement in a Mouse Model of Chemotherapy-Induced Primary Ovarian Insufficiency. *Stem Cells Int* 2016; **2016**: 4148923 [PMID: 26664408 DOI: 10.1155/2016/4148923]
  - 84 **Takehara Y**, Yabuuchi A, Ezoe K, Kuroda T, Yamadera R, Sano C, Murata N, Aida T, Nakama K, Aono F, Aoyama N, Kato K, Kato O. The restorative effects of adipose-derived mesenchymal stem cells on damaged

- ovarian function. *Lab Invest* 2013; **93**: 181-193 [PMID: [23212100](#) DOI: [10.1038/labinvest.2012.167](#)]
- 85 **Zhang Q**, Bu S, Sun J, Xu M, Yao X, He K, Lai D. Paracrine effects of human amniotic epithelial cells protect against chemotherapy-induced ovarian damage. *Stem Cell Res Ther* 2017; **8**: 270 [PMID: [29179771](#) DOI: [10.1186/s13287-017-0721-0](#)]

# Role of mesenchymal stem cell derived extracellular vesicles in autoimmunity: A systematic review

Jing-Hua Wang, Xiao-Ling Liu, Jian-Mei Sun, Jing-Han Yang, Dong-Hua Xu, Shu-Shan Yan

**ORCID number:** Jian-Hua Wang 0000-0002-8310-8003; Xiao-Ling Liu 0000-0002-7172-575X; Jian-Mei Sun 0000-0001-7305-5588; Jing-Han Yang 0000-0001-7577-2695; Dong-Hua Xu 0000-0002-9146-7858; Shu-Shan Yan 0000-0002-8194-6409.

**Author contributions:** All authors equally contributed to this work with regard to conception and design of the study, literature analysis, manuscript drafting, critical revision, and editing, and approval of the final version.

**Supported by** the Shandong Natural Science Foundation, No. ZR2019QH012.

**Conflict-of-interest statement:** The authors have no potential conflicts of interests to declare.

**PRISMA 2009 Checklist statement:** The guidelines of the PRISMA 2009 statement have been adopted.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works

**Jing-Hua Wang, Jing-Han Yang,** Clinical Medicine College, Weifang Medical University, Weifang 261000, Shandong Province, China

**Xiao-Ling Liu,** Department of Emergency Medicine, Yantai Shan Hospital, Yantai 264001, Shandong Province, China

**Jian-Mei Sun,** Department of Chemistry, School of Applied Chemistry, Food and Drug, Weifang Engineering Vocational College, Qingzhou 262500, Shandong Province, China

**Dong-Hua Xu,** Department of Rheumatology of the First Affiliated Hospital, Weifang Medical University, Central Laboratory of the First Affiliated Hospital, Weifang 261000, Shandong Province, China

**Shu-Shan Yan,** Department of Gastrointestinal and Anal Diseases Surgery of the Affiliated Hospital, Weifang Medical University, Weifang 261000, Shandong Province, China

**Corresponding author:** Shu-Shan Yan, MD, Doctor, Department of Gastrointestinal and Anal Diseases Surgery, The Affiliated Hospital, Weifang Medical University, Weifang 261000, Shandong Province, China. [yanshushan@163.com](mailto:yanshushan@163.com)

## Abstract

### BACKGROUND

Mesenchymal stem cells (MSCs) have been reported to possess immune regulatory effects in innate and adaptive immune reactions. MSCs can mediate intercellular communications by releasing extracellular vesicles (EVs), which deliver functional molecules to targeted cells. MSC derived EVs (MSC-EVs) confer altering effects on many immune cells, including T lymphocytes, B lymphocytes, natural killer cells, dendritic cells, and macrophages. A large number of studies have suggested that MSC-EVs participate in regulating autoimmunity related diseases. This characteristic of MSC-EVs makes them be potential biomarkers for the diagnosis and treatment of autoimmunity related diseases.

### AIM

To verify the potential of MSC-EVs for molecular targeted therapy of autoimmunity related diseases.

### METHODS

Literature search was conducted in PubMed to retrieve the articles published between 2010 and 2020 in the English language. The keywords, such as “MSCs,”

on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Invited manuscript

**Received:** March 4, 2020

**Peer-review started:** March 4, 2020

**First decision:** April 25, 2020

**Revised:** July 2, 2020

**Accepted:** July 19, 2020

**Article in press:** July 19, 2020

**Published online:** August 26, 2020

**P-Reviewer:** Pelagalli A, Khan I, Kuo FC

**S-Editor:** Zhang L

**L-Editor:** Wang TQ

**P-Editor:** Li X



“EVs,” “exosome,” “autoimmunity,” “tumor immunity,” and “transplantation immunity,” and Boolean operator “AND” and “NOT” coalesced admirably to be used for searching studies on the specific molecular mechanisms of MSC-EVs in many immune cell types and many autoimmunity related diseases. Studies that did not investigate the molecular mechanisms of MSC-EVs in the occurrence and development of autoimmune diseases were excluded.

## RESULTS

A total of 96 articles were chosen for final reference lists. After analyzing those publications, we found that it had been well documented that MSC-EVs have the ability to induce multiple immune cells, like T lymphocytes, B lymphocytes, natural killer cells, dendritic cells, and macrophages, to regulate immune responses in innate immunity and adaptive immunity. Many validated EVs-delivered molecules have been identified as key biomarkers, such as proteins, lipids, and nucleotides. Some EVs-encapsulated functional molecules can serve as promising therapeutic targets particularly for autoimmune disease.

## CONCLUSION

MSC-EVs play an equally important part in the differentiation, activation, and proliferation of immune cells, and they may become potential biomarkers for diagnosis and treatment of autoimmunity related diseases.

**Key words:** Mesenchymal stem cells; Extracellular vesicles; Exosome; Autoimmunity; Tumor immunity; Transplantation immunity

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Mesenchymal stem cells (MSCs) have been reported to possess immunomodulatory effects on autoimmune responses. MSCs can mediate intercellular communications by releasing extracellular vesicles (EVs), which deliver functional molecules to targeted cells. MSC derived EVs (MSC-EVs) exert immunomodulatory effects on many immune cells. A large number of studies have suggested that MSC-EVs and the encapsulated bioactive molecules are potential targets for autoimmune disease, cancer, and other diseases. However, there is still a long way for investigating the molecular mechanism of MSC-EVs in autoimmunity. This review will focus on the immunomodulatory function and underlying mechanism of MSC-EVs in autoimmunity related diseases.

**Citation:** Wang JH, Liu XL, Sun JM, Yang JH, Xu DH, Yan SS. Role of mesenchymal stem cell derived extracellular vesicles in autoimmunity: A systematic review. *World J Stem Cells* 2020; 12(8): 879-896

**URL:** <https://www.wjgnet.com/1948-0210/full/v12/i8/879.htm>

**DOI:** <https://dx.doi.org/10.4252/wjsc.v12.i8.879>

## INTRODUCTION

Mesenchymal stem cells (MSCs) are a group of common multipotent progenitor cells, which can be found in bone marrow<sup>[1,2]</sup>, synovium<sup>[3,4]</sup>, umbilical cord<sup>[5]</sup>, and adipose tissue<sup>[1,6]</sup>. They are characterized by a multilineage differentiation potential and paracrine function<sup>[7]</sup>. There is growing evidence that MSCs exert immunomodulatory effects through their paracrine function<sup>[8]</sup>, in which multiple small molecules, including extracellular vesicles (EVs), cytokines, chemokines, growth factors, and interleukin (IL), are secreted to the extracellular microenvironment in animal models<sup>[9]</sup>. Recently, numerous studies demonstrated that MSCs can be used in clinical therapy for immunomodulation and regenerative medicine *in vivo* and *in vitro*<sup>[10-12]</sup>. Despite great improvements in the MSC therapeutic strategies for autoimmune diseases, treatment failures are still common and there is no doubt that it is imperative to carry out more studies to investigate the specific molecular mechanisms. EVs are key components of the paracrine process that play a vital role in intercellular communication by transmitting biological molecules in pathological and physiological



conditions.

EV is newly identified small vesicle wrapped in lipid membranes, which is widely produced by many cells and secreted into the extracellular microenvironment. In 1967, Wolf first discovered EVs and described them as function-free platelet wastes<sup>[13]</sup>. EVs can be isolated from various extracellular fluids, like blood, urine, saliva, tear, cerebrospinal fluid, milk, and so on, and various cells, including stem cells<sup>[14-18]</sup>, primary cells of the immune and nervous system<sup>[19-22]</sup>, and multiple cancer cell types<sup>[23-25]</sup>. Their encapsulated functional molecules can be novel biomarkers and therapeutic targets for many kinds of diseases, for instance, cancer, autoimmune diseases, and neurodegenerative disorders. The role of EVs in immunity and inflammation regulations has been attracting attention during the past few decades. According to diameter, EVs can be divided into three types, including apoptotic bodies, microparticles, and exosomes (Figure 1)<sup>[26,27]</sup>. Exosomes are the most common EVs with a diameter of 50-100 nm<sup>[28]</sup>. Exosomes were first discovered in sheep reticulocytes, by electron microscopy<sup>[29]</sup>. Microparticles, also called microvesicles, are submicronic vesicles with a diameter of 100-1000 nm, which are formed by budding of the cellular membrane after cell stimulation or stress, such as cell activation, apoptosis, and hypoxia. Apoptotic bodies also belong to EVs with a diameter of 50-4000 nm. They are usually released during the stage of cell apoptosis. EVs participate in the intercellular communication by delivering numerous proteins and nucleotides with biological activity, and nucleotides include microRNAs (miRNAs), long non-coding RNAs (lncRNAs), mRNA, and even extra-chromosomal DNA<sup>[30,31]</sup>. They play vital roles in regulating inflammation, immune response, vascular reactivity, and tissue repair<sup>[32,33]</sup>. During the past few decades, MSC derived EVs (MSC-EVs) have been implicated in regulating inflammation and autoimmunity<sup>[7]</sup>. It has been well established that EVs are involved in regulating autoimmune disorders by delivering a large number of bioactive molecules, including cytokines, enzymes, transcription factors, cytokines receptor antagonists, miRNAs, lncRNAs, and circRNAs<sup>[34]</sup>. MSCs, as a specific group of cells, are multipotent stem cells characterized by immunomodulatory and self-renew properties<sup>[35,36]</sup>. Cosenza *et al*<sup>[37]</sup> have reported the important pathogenic or therapeutic role of MSC-EVs in rheumatic diseases. Therefore, we proposed that MSC-EVs can become potential biotargets for the development of novel molecular targeted drugs in autoimmune related diseases based on the above conclusions. This systematic review will provide in-depth knowledge of biogenesis and functional roles of MSC-EVs, especially exosomes, in autoimmunity.

## MATERIALS AND METHODS

### Literature search

The key words “MSCs,” “EVs,” “exosome,” “autoimmunity,” “tumor immunity,” and “transplantation immunity” were used to retrieve relevant articles published in English from 2010 to 2020 in PubMed database. Besides, Boolean operator “AND” and “NOT” were combined admirably with those keywords to search the related articles. Reference lists from those articles were reviewed to exclude irrelevant articles. Manuscripts available were reviewed and recognized by using document management tool. All available information was obtained by skimming the abstracts of searched articles. Data were analyzed using descriptive statistics.

All repetitive documents were excluded, and the remainder needed to be restored for reading. Nevertheless, full text retrieval was performed due to many documents with unavailable abstract.

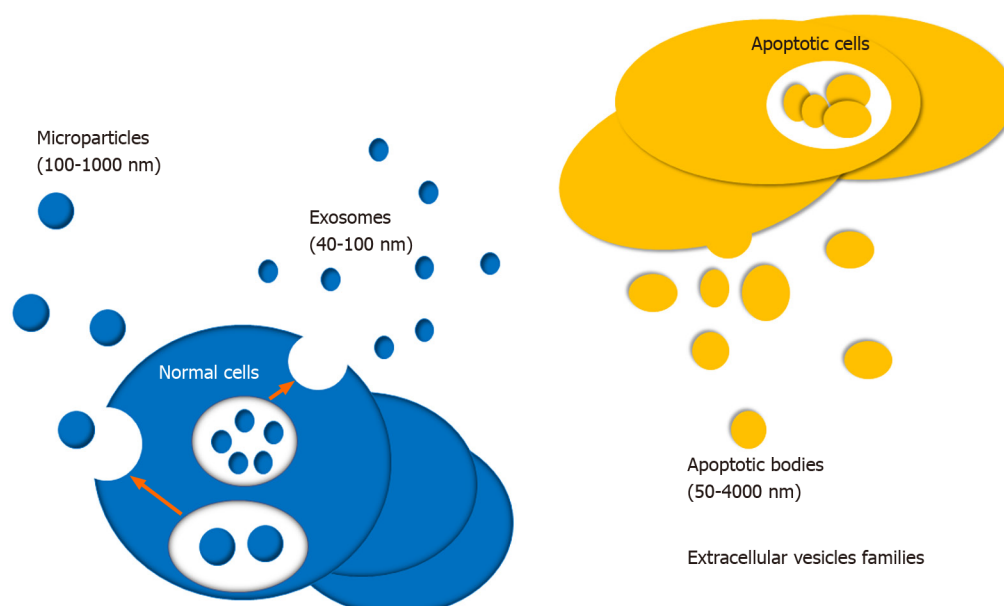
### Statistical analysis

This article was a systematic review and no statistical method was used in this article.

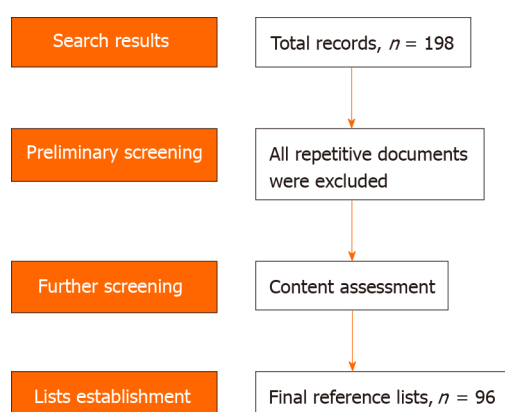
## RESULTS

Initially, we retrieved 198 records for this review. Then, repetitive and irrelevant documents were excluded, and we retained ultimately 96 high-quality papers with innovative viewpoints for reference lists. The screening process of those documents is showed in Figure 2.

EVs can regulate many immune and inflammatory responses by mediating intercellular communication. Moreover, MSC-EVs have been well documented to



**Figure 1 Various kinds of extracellular vesicles.** Extracellular vesicles primarily consist of exosomes, apoptotic bodies, and microparticles derived for normal cells or apoptotic cells.



**Figure 2 Flowchart for literature retrieval and screening.** Flowchart shows that the repeated siftings have brought 198 search records to the 96 articles for final reference lists.

induce multiple immune cells to mediate immune responses in innate immunity and adaptive immunity, namely, they modulate the differentiation, activation, and proliferation of immune cells, like T lymphocytes, B lymphocytes, natural killer cells (NKs), dendritic cells (DCs), and macrophages in the autoimmune system (Figure 3)<sup>[38-41]</sup>.

There is growing evidence that MSC-EVs serving as a type of signal molecules play major biological roles in the initiation, maintenance, and progression of multiple autoimmune related diseases, such as autoimmune diseases, cancer, and graft-versus-host disease. The features of MSC-EVs immunomodulation and their therapeutic potential in autoimmune related diseases are summarized in Tables 1 and 2.

## DISCUSSION

### MSC-EVs and T lymphocytes

T lymphocytes are important immune cells in adaptive immunity and play a significant role in the occurrence and development of many autoimmune and inflammatory diseases. MSC derived exosomes and microparticles down-regulate T cell proliferation, and CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets decrease significantly in quantity<sup>[7]</sup>. Adipose mesenchymal stem cell (AMSC) derived exosomes depress the

Table 1 Immune modulation of extracellular vesicles in autoimmune related diseases

Disease	EVs	Expression	MSC-EVs source/ Target molecules	Target immune cells	Pathway(s) involved	Ref.
RA	MSC derived exosomal miR-150-5p	Down	Bone marrow derived MSC-EVs/MMP 14 and VEGF	Macrophages	TGF- $\beta$ pathway	Chen <i>et al</i> <sup>[80]</sup>
	Exosome-encapsulated miR-548a-3p	Down	TLR4	Macrophages	MiR-548a-3p/TLR4/NF- $\kappa$ B axis	Wang <i>et al</i> <sup>[81]</sup>
	Exosome-encapsulated miR-6089	Down	TLR4	Macrophages	TLR4/NF- $\kappa$ B signaling pathway	Xu <i>et al</i> <sup>[82]</sup>
	Exosome-derived lncRNA Hotair	Up	MMP-2 and MMP-13	Macrophages	-	Song <i>et al</i> <sup>[83]</sup>
	Exosomal miR-17	Up	TGFBR II	T cells	-	Wang <i>et al</i> <sup>[84]</sup>
	MicroRNA-155	Up	SHIP-1	Macrophages	-	Kurowska-Stolarska <i>et al</i> <sup>[85]</sup>
	MicroRNA-146	Up	-	Macrophages, T cells, B cells	-	Nakasa <i>et al</i> <sup>[86]</sup>
SLE	Exosomal miR-26a	Up	Podocyte proteins, actin family members, and intermediate filaments	Podocytes	-	Ichii <i>et al</i> <sup>[99]</sup>
	Exosomal miRNA-146a	Up	-	-	Interferon- $\gamma$ pathway	Perez-Hernandez <i>et al</i> <sup>[100]</sup>
pSS	EV derived LCN2	Up	TNF- $\alpha$	B cells	TNF- $\alpha$ signaling	Aqrawi <i>et al</i> <sup>[107]</sup>
	EV derived APMAP	Up	TNF- $\alpha$	B cells	TNF- $\alpha$ signaling	Aqrawi <i>et al</i> <sup>[107]</sup>
	EV derived CPNE1	Up	TNF- $\alpha$	B cells	TNF- $\alpha$ signaling	Aqrawi <i>et al</i> <sup>[107]</sup>
IBD	MSC-EVs	Up	Bone marrow derived MSC-EVs	Macrophages	JAK1/STAT1/STAT6 signaling pathway	Cao <i>et al</i> <sup>[113]</sup>
Breast cancer	Exosomal PD-L1	Down	PD-1	T cells	PD-L1/ PD-1 pathway	Yang <i>et al</i> <sup>[120]</sup>
Lung cancer	EV derived miR-103a	Up	Lung cancer cell derived EVs/PTEN	Macrophages	PI3K/ AKT and STAT3 axis	Hsu <i>et al</i> <sup>[121]</sup>
Pancreatic cancer	Exosomal miR-301a-3p	Up	PTEN	Macrophages	PI3K $\gamma$ signaling pathway	Wang <i>et al</i> <sup>[122]</sup>
GVHD	MSC-EVs	Up	T cell derived EVs	-	-	Park <i>et al</i> <sup>[126]</sup>

RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; pSS: Primary Sjgren's syndrome; IBD: Inflammatory bowel diseases; GVHD: Graft-versus-host disease; MSC: mesenchymal stem cell; EV: Extracellular vesicle; MSC-EV: Mesenchymal stem cell derived extracellular vesicle; MMP: Matrix metalloproteinase; VEGF: Vascular endothelial growth factor; TGFBR II : Transforming growth factor beta receptor II; SHIP-1: Src homology 2-containing inositol phosphatase-1; PD-1: Programmed death-1; PD-L1: PD-1 ligand; LCN2: Neutrophil gelatinase-associated lipocalin; APMAP: Adipocyte plasma membrane-associated protein.

activity of T cells, and up-regulate IL-4, IL-10, and transforming growth factor- $\beta$  and down-regulate IL-17 and interferon- $\gamma$  in streptozotocin induced type-1 diabetes mellitus mice, thus deadening the progression of diseases<sup>[42]</sup>. MSCs have been extensively reported to decorate the activation of CD4<sup>+</sup> T cells by some specific T cell effector cytokines or direct contact, down-regulating their immune activity and converting them to a regulatory phenotype (Treg)<sup>[43,44]</sup>. Programmed death-1 (PD-1) is a valuable cytokine inducing T cell activity. Research shows that MSCs express and secrete PD-1 ligands (PD-L1 and PD-L2) to regulate T cell dependent immune responses by binding with PD-1<sup>[45]</sup>, suggesting that MSCs possess immunosuppressive properties *via* the modulation of T cells. AMSCs under stimulation with IFN- $\gamma$  can secrete a big body of exosomes to the conditioned medium, and importantly, T cells isolated from that medium are significantly inhibited in activity and proliferation<sup>[46]</sup>. In a word, MSC-EVs down-regulate the activity and proliferation of T cells to inhibit T dependent autoimmune responses.

Table 2 Therapeutic potential of extracellular vesicles

Disease	EVs	Experimental sample	Therapeutic potential	Ref.
RA	MSC derived exosomal miR-150-5p	Collagen induced arthritis mouse model	MiR-150-5p could reduce joint destruction by inhibiting synoviocyte hyperplasia and angiogenesis	Chen <i>et al</i> <sup>[80]</sup>
	Exosome-encapsulated miR-548a-3p	Macrophage-like cells	MiR-548a-3p could inhibit the proliferation and activation of pTHP-1 cells <i>via</i> the TLR4/NF-κB signaling pathway	Wang <i>et al</i> <sup>[81]</sup>
	Exosome-encapsulated miR-6089	Macrophage-like cells	MiR-6089 could regulate LPS/TLR4-mediated inflammatory response	Xu <i>et al</i> <sup>[82]</sup>
	Exosome-derived lncRNA Hotair	Blood mononuclear cells	Hotair may contribute to the dissolution of bone and cartilage matrix through activation of MMP-2 and MMP-13 in osteoclasts and RA synoviocytes. Hotair is more stable and easily detected in body fluid	Song <i>et al</i> <sup>[83]</sup>
	Exosomal miR-17	Blood mononuclear cells	MiR-17 can suppress regulatory T cell differentiation by inhibiting the expression of TGFBR II	Wang <i>et al</i> <sup>[84]</sup>
	MicroRNA-155	MiR-155-deficient mice	MiR-155-deficient mice are resistant to collagen-induced arthritis, and antigen-specific Th17 cell and autoantibody responses are suppressed markedly to reduce articular inflammation	Kurowska-Stolarska <i>et al</i> <sup>[85]</sup>
	MicroRNA-146	Human RA synovial fibroblasts	MiR-146a is expressed in the superficial and sublining layers of synovial tissue, like synovial fibroblasts, macrophages, T cells, and B cells	Nakasa <i>et al</i> <sup>[86]</sup>
SLE	Exosomal miR-26a	Female B6.MRLc1 and C57BL/6 mice; C57BL/6 (9 mo of age)	Podocytes mainly express miR-26a in mouse kidneys. Glomerular miR-26a expression in B6.MRLc1 mice correlates negatively with the urinary albumin levels and podocyte specific gene expression	Ichii <i>et al</i> <sup>[89]</sup>
	Exosomal miRNA-146a	Urine sample of SLE patients	Up-regulated exosomal miRNA-146a is found in the presence of active lupus nephritis	Perez-Hernandez <i>et al</i> <sup>[100]</sup>
pSS	EV derived LCN2	Saliva and tear samples from pSS patients and healthy controls	EV derived LCN2 is over-expressed in pSS patients	Aqrabi <i>et al</i> <sup>[107]</sup>
	EV derived APMAP	Saliva and tear samples from pSS patients and healthy controls	EV derived APMAP is over-expressed in pSS patients	Aqrabi <i>et al</i> <sup>[107]</sup>
	EV derived CPNE1	Saliva and tear samples from pSS patients and healthy controls	EV derived CPNE1 is over-expressed in pSS patients	Aqrabi <i>et al</i> <sup>[107]</sup>
IBD	MSC-EVs	LPS treated macrophages and an <i>in vivo</i> DSS induced mouse model	EVs promote the up-regulation of pro-inflammatory factors (TNF-α, IL-6, and IL-12) and down-regulation of the anti-inflammatory factor IL-10 in LPS-induced macrophages. EVs promote polarization of M1-like macrophages to an M2-like state	Cao <i>et al</i> <sup>[113]</sup>
Breast cancer	Exosomal PD-L1	MDA-MB-231 (231) human breast cancer cells and 4T1 mouse mammary tumor cells with PD-L1 expression or PD-L1KO	Exosomal PD-L1 bind to PD-1 on T cells to inhibit T cell activation and killing activities	Yang <i>et al</i> <sup>[120]</sup>
Lung cancer	EV derived miR-103a	Human adenocarcinoma cell lines NCI-H1437, NCI-H1792, and NCI-H2087 and human embryonic kidney HEK293 cells	miRNA inhibitor could inhibit effectively miR-103a mediated M2-type polarization, improving the cytokine profile of tumor infiltration macrophages	Hsu <i>et al</i> <sup>[121]</sup>



Pancreatic cancer	Exosomal miR-301a-3p	Pancreatic cancer blood samples, Pancreatic cancer cell lines PANC-1, BxPC-3 and monocytic cell line THP-1	Pancreatic cells generate miR-301a-3p-rich exosomes in a hypoxic microenvironment, which polarize macrophages to promote malignant behaviors of cancer cells	Wang <i>et al.</i> <sup>[122]</sup>
GVHD	MSC-EVs	Kidney samples from acute cellular rejection	iKEA (integrated kidney exosome analysis) shows a high level of CD3-positive EVs in kidney rejection patients and achieved high detection accuracy (91.1%)	Park <i>et al.</i> <sup>[126]</sup>

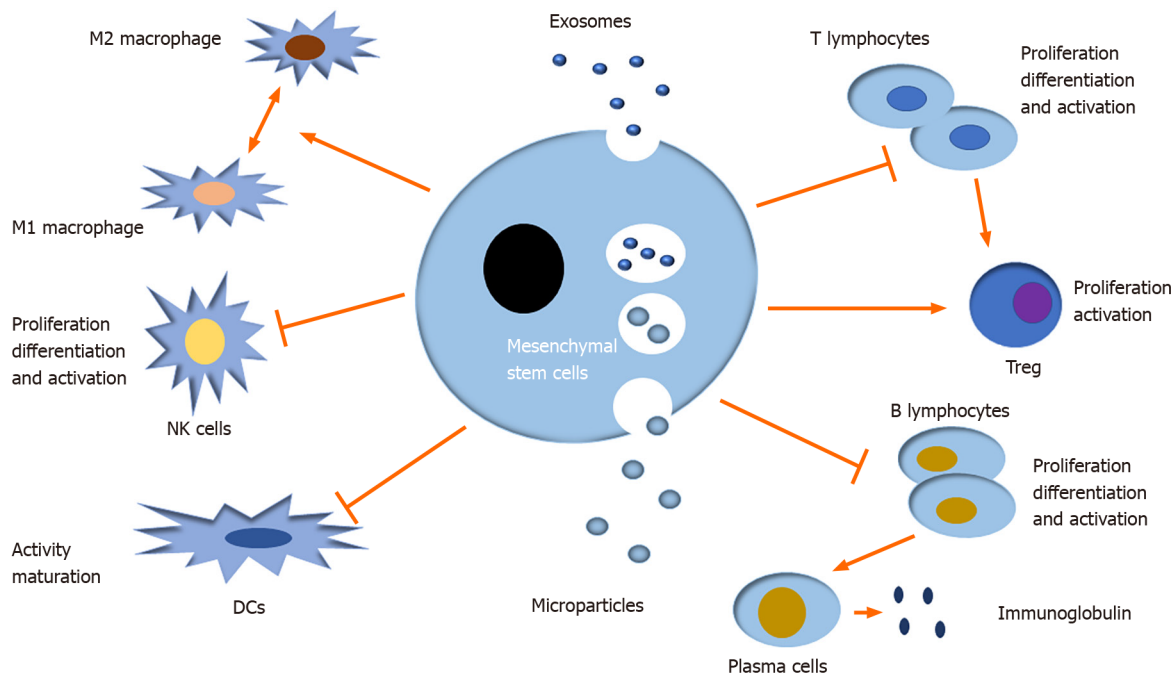
RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; pSS: Primary Sjögren's syndrome; IBD: Inflammatory bowel diseases; GVHD: Graft-versus-host disease; MSC: mesenchymal stem cell; EV: Extracellular vesicle; MSC-EV: Mesenchymal stem cell derived extracellular vesicle; MMP: Matrix metalloproteinase; VEGF: Vascular endothelial growth factor; TGFBR II: Transforming growth factor beta receptor II; SHIP-1: Src homology 2-containing inositol phosphatase-1; PD-1: Programmed death-1; PD-L1: PD-1 ligand; PD-L1KO: PD-L1 knockout; LCN2: Neutrophil gelatinase-associated lipocalin; APMAP: Adipocyte plasma membrane-associated protein; CPNE1: Copine.

### MSC-EVs and B lymphocytes

B lymphocytes are also vital immune cells in adaptive immunity. A growing number of studies suggest that MSCs possess an immunomodulatory effect on B cells, but the molecular mechanisms involved are still mysterious<sup>[47]</sup>. Nevertheless, there is little research on the role of MSC-EVs in mediating the regulatory effect of B cells on inflammatory and immune responses. Membrane vesicles derived from MSCs inhibit both B cell proliferation and differentiation in a dose-dependent fashion<sup>[48]</sup>. Traggiai E and his colleagues found that MSCs positively influence the proliferation and differentiation of B cells into plasma cells secreting more immunoglobulins<sup>[47]</sup>. Thus, MSCs promote downstream immune responses by mediating the conversion of B cells. Systemic lupus erythematosus (SLE) is a typical autoimmune disease characterized by constantly producing various antibodies to counter autologous cells. It is well established that B cells play a critical role in autoimmune responses *via* autoantibodies dependent mechanisms. Therefore, we infer that MSCs mediated cell conversion can boost the inflammatory progression. Therefore, MSCs can serve as a potential therapeutic tool in autoimmune diseases.

### MSC-EVs and monocytes

Monocytes are secreted from bone marrow into the circulatory system and transported to target tissue, where they differentiate into mature macrophages<sup>[49]</sup>. Macrophages are critical effectors and regulators of the immune system and play a central role in inflammation<sup>[50]</sup>. It has been well documented that macrophages can be divided into two subpopulations: The classic M1 and the alternative M2 macrophages under microenvironmental factors. The classical M1 macrophages are induced by TLR ligands and IFN- $\gamma$  and alternative M2 macrophages are induced by the immune complex IL-4/IL-13<sup>[51,52]</sup>. M1 macrophages are characterized by strong microbicidal and tumoricidal activity, which can promote Th1 related inflammatory responses by releasing a range of proinflammatory cytokines, such as IL-6, IL-12, and TNF- $\alpha$ <sup>[53]</sup>, whereas M2 macrophages with anti-inflammatory function produce less proinflammatory cytokines and more IL-10 and other anti-inflammatory factors<sup>[54]</sup>. In short, both M1 and M2 macrophages contribute to the balance between destruction and repair of tissue in pathological conditions. A study suggested that after coculture



**Figure 3 Immunomodulatory effects of mesenchymal stem cell derived extracellular vesicles.** Mesenchymal stem cell derived extracellular vesicles (MSC-EVs) exert immunomodulatory effect on innate and adaptive immune reactions mediated by many immune cells, primarily including T lymphocytes, B lymphocytes, natural killer cells, dendritic cells, and macrophages. In brief, MSC-EVs can inhibit the proliferation, differentiation, and activation of T, B, and natural killer cells and the pathogen-presenting function of dendritic cells and macrophages. In addition, macrophage polarization can be shifted under different microenvironments in accompany with MSC-EVs.

of AMSCs with inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ , a higher level of exosomes can be detected in the medium supernatant, which induce M1 differentiate to anti-inflammatory M2 phenotype<sup>[55]</sup>. Adipose tissue accumulating constantly in the body leads to obesity and inflammatory responses, which increase the risk of incidence of many chronic diseases, including type 2 diabetes, cardiovascular events, and part of cancers<sup>[56-58]</sup>. Previous studies have revealed that the invasion of macrophages and T cells promote the formation of chronic inflammation in white adipose tissues<sup>[59,60]</sup>. In high fat diet fed mice, AMSC derived exosomes promote white adipose tissue hypertrophy by inducing M2 macrophage polarization<sup>[61]</sup>. A study by Németh *et al*<sup>[62]</sup> showed that endotoxin stimulated MSCs induce M2 macrophage polarization to release IL-10 and attenuate sepsis *via* the NF- $\kappa$ B signal pathway in a mouse model<sup>[62]</sup>. MSC-EVs induce the production of M2 macrophages with anti-inflammatory properties to restrain many relevant immune responses.

#### MSC-EVs and NK cells

NK cells, a vital cell type in the innate immune system, mediate cytotoxic activity and produce certain cytokines and chemokines to mediate antigen presentation, antiviral responses, autoimmune responses, and the occurrence of various autoimmune diseases<sup>[63]</sup>. A previous result showed that MSC-EVs injected into periocular tissue depress the transfer of CD161<sup>+</sup> NK cells, delay the progression of disease, and restore damaged tissue in autoimmune uveitis rat models<sup>[64,65]</sup>. Decidua parietalis MSCs release IL-2 to CD69 (NK cell receptor) to stimulate IL-2 dependent NK cells and thus promote the proliferation of activated NK cells<sup>[66]</sup>. Thus, decidua parietalis MSCs induce directly the activity of NK cells through IL-2 and CD69. Recent research suggested that fetal liver MSC derived exosomes carrying LAP, TGF $\beta$ , and TSP1 restrain the proliferation and activation of NK cells *via* TGF $\beta$ /Smad2/3 signaling<sup>[67]</sup>. Although available data show that MSC-EVs depress the activation and proliferation of NK cells, the research on that is limited in quantity and more studies need to be carried out in the future.

#### MSC-EVs and DCs

DCs, important bone marrow derived APCs, present multiple antigenic peptides (major histocompatibility complex - peptide complexes) to other immune cells, like T cells, and play a key role in bridging innate to adaptive immune systems. Coculture of

DCs with MSC-EVs led to down-regulated cellular surfactants and IL-10, IL-6, and IL-17 and up-regulated the number of regulatory T cells<sup>[68]</sup>, and the activity and maturation of DCs are apparently restrained. Many studies suggest that these MSC-EVs stimulate immature DCs to release TGF- $\beta$  and PGE2, and regulate the immunocompetence of T cells in DC and T cell culture medium. Those small molecules mediate autoimmune responses with unclear mechanism. MSCs induce mature DCs to immature status with low immunogenicity and immunoregulatory property. The immature DCs express less immunomodulatory factors Ia, CD11c, CD80, CD86, and CD40, except for increased CD11<sup>[39]</sup>. Overall, MSC-EVs down-regulate the immune activity of DCs and T cell dependent adaptive immune responses indirectly. Nevertheless, the research on the interaction between DCs and MSC-EVs is limited, and the exact molecular mechanisms warrant further studies.

### **MSC-EVs and autoimmune disease**

MSC-EVs have been suggested in many kinds of diseases, which can serve as promising strategies for autoimmune disease diagnosis and treatment, such as rheumatoid arthritis (RA), SLE, primary Sjgren's syndrome (pSS), systemic sclerosis, and inflammatory bowel diseases (IBD) due to their vital role in intercellular communications. Nevertheless, the precise molecular mechanism underlying EV regulation in autoimmunity warrants in-depth investigation.

Epidemiological survey and analysis suggest that the incidence of autoimmune diseases has been increasing year by year over the past several decades<sup>[69]</sup>. Autoimmune diseases usually influence multiple organs and systems, such as the motor system, respiratory system, digestive system, and circulatory system<sup>[70]</sup>. They lead to a heavy burden to public health. It is well known that some autoimmune diseases are genetically susceptible<sup>[71]</sup>. Women tend to be affected by some autoimmune diseases, and approximately 90% of patients with autoimmune disease are female<sup>[72]</sup>. Currently, glucocorticoids and immunosuppressive drugs are still the most frequently used non-specific therapeutic agents. That traditional therapeutic strategy causes many adverse reactions, such as opportunistic infections and metabolic abnormalities, and the development of biological molecular targeted drugs to cause slower disease progression is a priority. Accumulating data reveal the biological features of MSCs in relieving immune cell-driven systemic inflammatory responses to down-regulate immune responses, such as autoimmune diseases<sup>[73]</sup>, and MSC-EVs are a significant regulator<sup>[74]</sup>. The current knowledge of EVs in autoimmune diseases will be discussed in detail in the following text.

### **MSC-EVs and RA**

RA is one of the most common chronic and systemic autoimmune diseases involving multiple systems, which is characterized by the destruction of synovial joints. The representative clinical manifestations are redness, swelling, and pain of distal joints, especially small joints of hands and feet<sup>[75]</sup>. Many researchers have suggested that the occurrence of RA is caused by many complex factors, such as genetic factors and environmental factors<sup>[76,77]</sup>. Dysregulation of immune responses occupies a necessary position in RA.

Increasing data have revealed EVs as critical regulators in the pathogenesis of RA by delivering specific functional molecules to targeted cells. Previously, the effectiveness of MSC therapies has been elucidated in cartilage repair in both animal studies<sup>[78]</sup> and human clinical trials<sup>[79]</sup>. Previous studies have revealed that EVs generated by MSCs play a critical role in protecting against cartilage destruction and enhancing cartilage regeneration. Particularly, exosomal noncoding RNAs (ncRNAs), including miRNAs and lncRNAs, have been implicated in regulating inflammation and immune response. MSC derived exosomal miR-150-5p down-regulated inflammatory responses and reduced joint destruction and vasculitis by targeting matrix metalloproteinase 14 (MMP14) and vascular endothelial growth factor in a collagen-induced arthritis mouse model, which is considered as a potential therapeutic biomarker for RA<sup>[80]</sup>. We have previously demonstrated the important role of exosome-encapsulated miR-6089 and miR-548a-3p in affecting macrophage-mediated inflammatory response in RA<sup>[81,82]</sup>. Exosome-derived lncRNA Hotair affected the migration of activated macrophages and significantly decreased the levels of MMP-2 and MMP-13, suggesting that it is a potential biomarker for RA<sup>[83]</sup>. A study by Wang *et al.*<sup>[84]</sup> has shown that exosomal miR-17 inhibits regulatory T cells by targeting TGFBR II in RA<sup>[84]</sup>. Besides, exosomes-encapsulated miR-155 and miR-146a produced by DCs can serve as important regulators in immune response and inflammatory response in RA<sup>[85-87]</sup>. It has been shown that the expression of exosomal amyloid A is positively correlated with anti-CCP antibody and CRP, suggesting a vital role of exosomal

protein in predicating the disease activity of RA patients<sup>[88]</sup>. Taken together, exosomal ncRNAs play critical roles in regulating immune and inflammatory cells and thus participate in the occurrence and development of RA. Nevertheless, more studies are warranted to explore the molecular mechanisms of those exosomes harboring ncRNAs in the pathogenesis of RA.

### **MSC-EVs and SLE**

SLE is a systemic autoimmune disease with various autoantibodies, which usually affects multi-organ systems due to enhanced inflammation and complex autoimmune disorders<sup>[89,90]</sup>. It has been well established that SLE is caused by the abundant activation of T and B lymphocytes, elevated pro-inflammatory cytokines, sedimentation of immune complex substance, and finally multiple organ damage, while the kidney is the most commonly involved organ in SLE and lupus nephritis (LN) is often caused<sup>[91]</sup>. EVs are significant regulators in mediating cell-to-cell communications involved in inflammation and immune regulations. Mounting evidence has suggested that EV delivered nucleic acids, proteins, autoantigens, cytokines, and surface receptors can serve as significant regulators in SLE<sup>[92,93]</sup>.

Microvesicles purified from SLE patients have been identified to contain higher concentrations of immunoglobulins and complements<sup>[94,95]</sup>. Circulating exosomes from patients with SLE have been shown to induce a proinflammatory immune response, which is characterized by high levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and other inflammatory mediators<sup>[93]</sup>. The study by Asami *et al*<sup>[96]</sup> supports that MSCs may confer immunosuppressive effects in SLE<sup>[96]</sup>. Previously published studies have elucidated that the EVs produced from MSCs, can also contribute to immunosuppressive function in SLE<sup>[97]</sup>. Accordingly, EVs can be used as drug carriers because they are less immunogenic. Umbilical cord derived MSCs have been used in the treatment of SLE patients, which shows good tolerance and few adverse events associated with transplantation<sup>[98]</sup>. Therefore, MSCs and MSC-EVs can effectively control the active SLE and be used as a therapeutic strategy, particularly for the treatment of refractory SLE. Ichii and the colleagues have found that exosomal miR-26a is positively associated with urinary protein level, which suggests that exosomal miR-26a in urine of LN patients can be used as a potential biomarker for predicting podocyte injury<sup>[99]</sup>. In addition, Perez-Hernandez *et al*<sup>[100]</sup> have shown that urinary exosomal miRNA-146a is significantly up-regulated in active LN patients<sup>[100]</sup>. Therefore, testing urinary exosomal miRNA can be a non-invasive method for the detection and monitoring of LN. Nevertheless, the specific molecular mechanism of EVs in regulating autoimmunity in SLE is still unclear, which warrants further investigation by more future studies.

### **MSC-EVs and pSS**

pSS is a systemic autoimmune disease that is characterized by chronic lymphocyte infiltration in the exocrine glands, primarily the lacrimal and salivary glands<sup>[101,102]</sup>. The primary target organs are the lacrimal and salivary glands, and dry eyes and dry mouth are often caused<sup>[103]</sup>. EVs purified from saliva<sup>[104,105]</sup> and tear fluid<sup>[106,107]</sup> have been identified to be potential biomarkers for the diagnosis and treatment of pSS in previous studies. Those differentially expressed proteins isolated from EVs of saliva and tear fluid from patients with pSS can contribute to pSS by regulating TNF- $\alpha$  signaling and B cell survival, including neutrophil gelatinase-associated lipocalin, adipocyte plasma membrane-associated protein, and copine<sup>[107]</sup>. The increase of platelet-derived microvesicles, soluble CD40 ligand (sCD40L), and soluble P-selectin (sCD62P) in pSS patients reflects platelet activation, which can serve as disease biomarkers<sup>[108]</sup>. Currently, studies on MSC-EV mediated immune responses in pSS are rare. More studies are needed to elucidate the role and underlying mechanisms of EVs in pSS.

### **MSC-EVs and IBD**

IBD is a common digestive disease characterized by chronic, relapsing gastrointestinal tract inflammatory reactions, including two main forms, Crohn's disease and ulcerative colitis<sup>[109,110]</sup>. To the best of our knowledge, the pathogenic mechanisms and pathogenesis of IBD are complicated, and many factors contribute to the occurrence of this disease, like autoimmune disorder, genetics, and environment<sup>[111]</sup>. Macrophages have been seen as important immune cells inducing IBD<sup>[112]</sup>. Experimental studies showed that inflammatory responses are significantly restrained by inducing the production of M2 macrophages in the dextran sulphate sodium induced mouse model of colitis<sup>[113]</sup>. Moreover, higher levels of immunosuppressive factors (IL-10 and TGF- $\beta$ )

were observed in mice treated with MSC-EVs, promoting repair and regeneration of damaged epithelial cells<sup>[113]</sup>. Studies have confirmed that MSC-EVs down-regulate the production of IL-1 $\beta$ , NO, and IL-18 by depressing NF- $\kappa$ B and iNOS-driven signaling in 2,4,6-trinitrobenzene sulfonic acid induced colitis<sup>[114,115]</sup>. Therefore, MSC-EVs, as an important regulator, can suppress inflammatory responses and promote injured tissue repair. That delineates the potential of MSC-EVs as biomarkers for IBD treatment.

### **MSC-EVs and tumor immunity**

Tumor immunity is critical in the processes of immune response, immune escape, and immune surveillance in cancer<sup>[116,117]</sup>. Previous research findings show that EVs play an critical role in anti-tumor immune reaction and inflammatory response during carcinogenesis and cancer progression<sup>[118]</sup>. In the last decade, exosomes have attracted more and more attention in cancer immunity, particularly as tumor suppressors<sup>[119]</sup>. Some bioactive factors encapsulated in EVs promote immune and inflammatory responses and thus lead to tumorigenesis, while some exert immune suppressive effects by inducing Tregs and M1 polarization.

A previous study has demonstrated the specific binding capacity of exosomal PD-L1 to its receptor PD-1 to depress the anti-tumor effect of T cells in breast cancer<sup>[120]</sup>. MSCs also express and release PD-L1 to regulate T cell activity, and thus both MSCs and exosomes possess immunosuppressive effect<sup>[45]</sup>. Besides, it has been documented that EVs play a critical in anti-tumor immune response by regulating macrophages polarization. It has been found that EVs-delivering miR-103a contributes to lung cancer by targeting PTEN and inducing M2 polarization<sup>[121]</sup>. Similarly, exosomal miR-301a-3p purified from pancreatic cancer cells was found to induce M2 macrophage polarization *via* the PTEN/PI3K $\gamma$  signaling pathway<sup>[122]</sup>. Taken together, EVs, particularly MSC-EVs, exert immunomodulatory effects on cancer and mediate intercellular communications between cancer cells and immune cells through EVs harboring bioactive molecules, including proteins and ncRNAs.

### **MSC-EVs and transplantation immunity**

Kidney transplantation is the current preferred treatment for end stage renal disease. However, the long-term survival rate of the transplanted kidney is still low because the transplanted recipients often suffer from acute or chronic rejection for a long period of time<sup>[123]</sup>, which finally leads to graft-versus-host disease. Biopsy is still the gold standard for the diagnosis of rejection of kidney transplantation<sup>[124,125]</sup>, but it is risky and traumatic. EVs in urine can be a potential biomarker for monitoring kidney transplant rejection<sup>[126]</sup>. T cells infiltrate the renal tubule during acute inflammatory response, which is a major cause for transplanted renal damage. MSC-EVs possess potential of inhibition of T cell activity and proliferation and thus EVs tend to gather in damaged renal tissues and are more likely to enter the urine. Consequently, using urine for detecting rejection of kidney transplantation is more likely to operate and promising. In addition, a previous report has showed that MSC derived exosomes provide a novel and effective clinical treatment for graft-versus-host disease<sup>[127]</sup>. Nonetheless, the role of MSC-EVs in transplantation immunity needs to be further investigated in the future.

### **Conclusions and prospects**

MSC-EVs are a hot topic in current molecular biology. Accumulated data have implicated their immunomodulatory effects on many immune cells, including T cells, B cells, macrophages, NK cells, and DCs. Increasing studies have confirmed that MSC-EVs can serve as regulators in the pathogenesis of autoimmune related diseases. In particular, MSC-EVs and the encapsulated bioactive molecules are potential targets for the diagnosis and treatment of autoimmune disease, cancer, and other diseases. MSC-EVs can serve as new medicines in the suppression of inflammatory responses. Increasing experimental results show that application of MSC-EVs can effectively inhibit immune reactions and promote the survival and regeneration of injured cells. However, there is still a long way for investigating the therapeutic strategy for autoimmunity related diseases based on MSC-EVs. More in-depth research is warranted in the future, particularly regarding the molecular mechanism of MSC-EVs in autoimmunity.



## ARTICLE HIGHLIGHTS

**Research background**

Mesenchymal stem cells (MSCs) have been reported to possess immune regulatory effects in innate and adaptive immune reactions. MSCs can mediate intercellular communications by releasing extracellular vesicles (EVs), which deliver functional molecules to targeted cells. MSC derived EVs (MSC-EVs) confer altering effects on many immune cells, including T lymphocytes, B lymphocytes, natural killer cells, dendritic cells, and macrophages. A large number of studies have suggested that MSC-EVs participate in regulating autoimmunity related diseases. This characteristic of MSC-EVs makes them be potential biomarkers for the diagnosis and treatment of autoimmunity related diseases.

**Research motivation**

This article describes and focuses on the identification, characteristics, immunomodulatory function, and underlying mechanism of MSC-EVs in autoimmunity related diseases. Understanding the immunomodulation effects of MSC-EVs better will help us to investigate the pathogenesis of diseases and develop novel targeted medicines.

**Research objectives**

The immune modulation of MSC-EVs play a key role in disease initiation, maintenance, and progression. This article provides a new direction for us to understand the precise mechanisms of action of autoimmunity related diseases, which will promote the improvement of therapeutic regimen.

**Research methods**

Literature search was conducted in PubMed to retrieve articles published between 2010 and 2020 in the English language. The keywords, such as "MSCs," "EVs," "autoimmune responses," "immune cells," and "autoimmunity related diseases," and Boolean operator "AND" and "NOT" coalesced admirably to be used for searching *in vitro* studies on the specific molecular mechanisms of MSC-EVs in many immune cell types and many autoimmunity related diseases. Studies that did not investigate the molecular mechanisms of MSC-EVs in the occurrence and development of autoimmune diseases were excluded.

**Research results**

A large number of articles were retrieved and their abstracts were skimmed. When analyzing the publications, we found that it has been well documented that MSC-EVs have the ability to induce multiple immune cells, like T lymphocytes, B lymphocytes, natural killer cells, dendritic cells, and macrophages, to regulate immune responses in innate immunity and adaptive immunity. Many validated EVs-delivered molecules have been identified as key biomarkers, such as proteins, lipids, and nucleotides. Some EVs-encapsulated functional molecules can serve as promising therapeutic targets particularly for autoimmune disease.

**Research conclusions**

MSC-EVs play an important part in the differentiation, activation, and proliferation of immune cells, and they may become potential biomarkers for the diagnosis and treatment of autoimmunity related diseases.

**Research perspectives**

MSC-EVs can serve as regulators in the pathogenesis of autoimmune related diseases. In particular, MSC-EVs and the encapsulated bioactive molecules are potential targets for the diagnosis and treatment of autoimmune disease, cancer, and other diseases. However, there is still a long way for investigating the therapeutic strategy for autoimmunity related diseases based on MSC-EVs. More in-depth research is warranted in the future, particularly regarding the molecular mechanism of MSC-EVs in autoimmunity

## REFERENCES

- 1 **da Silva Meirelles L**, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006; **119**: 2204-2213 [PMID: [16684817](#) DOI: [10.1242/jcs.02932](#)]
- 2 **Pittenger MF**, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147 [PMID: [10102814](#) DOI: [10.1126/science.284.5411.143](#)]
- 3 **De Bari C**, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* 2001; **44**: 1928-1942 [PMID: [11508446](#) DOI: [10.1002/1529-0131\(200108\)44:8<1928::AID-ART331>3.0.CO;2-P](#)]
- 4 **De Bari C**, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymakers JM, Luyten FP. Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *J Cell Biol* 2003; **160**: 909-918 [PMID: [12629053](#) DOI: [10.1083/jcb.200212064](#)]
- 5 **Ding DC**, Chang YH, Shyu WC, Lin SZ. Human umbilical cord mesenchymal stem cells: a new era for stem cell therapy. *Cell Transplant* 2015; **24**: 339-347 [PMID: [25622293](#) DOI: [10.3727/096368915X686841](#)]
- 6 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13**: 4279-4295 [PMID: [12475952](#) DOI: [10.1091/mbc.e02-02-0105](#)]
- 7 **Cosenza S**, Toupet K, Maumus M, Luz-Crawford P, Blanc-Brude O, Jorgensen C, Noël D. Mesenchymal stem cells-derived exosomes are more immunosuppressive than microparticles in inflammatory arthritis. *Theranostics* 2018; **8**: 1399-1410 [PMID: [29507629](#) DOI: [10.7150/thno.21072](#)]
- 8 **Liang X**, Ding Y, Zhang Y, Tse HF, Lian Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant* 2014; **23**: 1045-1059 [PMID: [23676629](#) DOI: [10.3727/096368913X667709](#)]
- 9 **Prockop DJ**, Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. *Mol Ther* 2012; **20**: 14-20 [PMID: [22008910](#) DOI: [10.1038/mt.2011.211](#)]
- 10 **Galipeau J**, Sensébé L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell* 2018; **22**: 824-833 [PMID: [29859173](#) DOI: [10.1016/j.stem.2018.05.004](#)]
- 11 **Squillaro T**, Peluso G, Galderisi U. Clinical Trials With Mesenchymal Stem Cells: An Update. *Cell Transplant* 2016; **25**: 829-848 [PMID: [26423725](#) DOI: [10.3727/096368915X689622](#)]
- 12 **Trento C**, Bernardo ME, Nagler A, Kuçi S, Bornhäuser M, Köhl U, Strunk D, Galleu A, Sanchez-Guijo F, Gaipa G, Intronà M, Buksauskas A, Le Blanc K, Apperley J, Roelofs H, Van Campenhout A, Beguin Y, Kuball J, Lazzari L, Avanzini MA, Fibbe W, Chabannon C, Bonini C, Dazzi F. Manufacturing Mesenchymal Stromal Cells for the Treatment of Graft-versus-Host Disease: A Survey among Centers Affiliated with the European Society for Blood and Marrow Transplantation. *Biol Blood Marrow Transplant* 2018; **24**: 2365-2370 [PMID: [30031938](#) DOI: [10.1016/j.bbmt.2018.07.015](#)]
- 13 **Wolf P**. The nature and significance of platelet products in human plasma. *Br J Haematol* 1967; **13**: 269-288 [PMID: [6025241](#) DOI: [10.1111/j.1365-2141.1967.tb08741.x](#)]
- 14 **Ratajczak J**, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 2006; **20**: 847-856 [PMID: [16453000](#) DOI: [10.1038/sj.leu.2404132](#)]
- 15 **Camussi G**, Deregibus MC, Bruno S, Grange C, Fonsato V, Tetta C. Exosome/microvesicle-mediated epigenetic reprogramming of cells. *Am J Cancer Res* 2011; **1**: 98-110 [PMID: [21969178](#)]
- 16 **Lai RC**, Chen TS, Lim SK. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med* 2011; **6**: 481-492 [PMID: [21749206](#) DOI: [10.2217/rme.11.35](#)]
- 17 **Timmers L**, Lim SK, Hoefer IE, Arslan F, Lai RC, van Oorschot AA, Goumans MJ, Strijder C, Sze SK, Choo A, Piek JJ, Doevendans PA, Pasterkamp G, de Kleijn DP. Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem Cell Res* 2011; **6**: 206-214 [PMID: [21419744](#) DOI: [10.1016/j.scr.2011.01.001](#)]
- 18 **Timmers L**, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA, Piek JJ, El Oakley RM, Choo A, Lee CN, Pasterkamp G, de Kleijn DP. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res* 2007; **1**: 129-137 [PMID: [19383393](#) DOI: [10.1016/j.scr.2008.02.002](#)]
- 19 **Chavez-Muñoz C**, Morse J, Kilani R, Ghahary A. Primary human keratinocytes externalize stratifin protein via exosomes. *J Cell Biochem* 2008; **104**: 2165-2173 [PMID: [18452139](#) DOI: [10.1002/jcb.21774](#)]
- 20 **Fauré J**, Lachenal G, Court M, Hirrlinger J, Chatellard-Causse C, Blot B, Grange J, Schoehn G, Goldberg Y, Boyer V, Kirchhoff F, Raposo G, Garin J, Sadoul R. Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* 2006; **31**: 642-648 [PMID: [16446100](#) DOI: [10.1016/j.mcn.2005.12.003](#)]
- 21 **Guescini M**, Genedani S, Stocchi V, Agnati LF. Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. *J Neural Transm (Vienna)* 2010; **117**: 1-4 [PMID: [19680595](#) DOI: [10.1007/s00702-009-0288-8](#)]
- 22 **Potolichio I**, Carven GJ, Xu X, Stipp C, Riese RJ, Stern LJ, Santambrogio L. Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. *J Immunol* 2005; **175**: 2237-2243 [PMID: [16081791](#) DOI: [10.4049/jimmunol.175.4.2237](#)]
- 23 **Al-Nedawi K**, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol* 2008; **10**: 619-624 [PMID: [18425114](#) DOI: [10.1038/ncb1725](#)]
- 24 **Skog J**, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008; **10**: 1470-1476 [PMID: [19011622](#) DOI: [10.1038/ncb1800](#)]
- 25 **Al-Nedawi K**, Meehan B, Kerbel RS, Allison AC, Rak J. Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc Natl Acad Sci USA* 2009; **106**:

- 3794-3799 [PMID: [19234131](#) DOI: [10.1073/pnas.0804543106](#)]
- 26 **Raposo G**, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013; **200**: 373-383 [PMID: [23420871](#) DOI: [10.1083/jcb.201211138](#)]
  - 27 **Dignat-George F**, Boulanger CM. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol* 2011; **31**: 27-33 [PMID: [21160065](#) DOI: [10.1161/ATVBAHA.110.218123](#)]
  - 28 **Choi DS**, Kim DK, Kim YK, Gho YS. Proteomics of extracellular vesicles: Exosomes and ectosomes. *Mass Spectrom Rev* 2015; **34**: 474-490 [PMID: [24421117](#) DOI: [10.1002/mas.21420](#)]
  - 29 **Pan BT**, Teng K, Wu C, Adam M, Johnstone RM. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J Cell Biol* 1985; **101**: 942-948 [PMID: [2993317](#) DOI: [10.1083/jcb.101.3.942](#)]
  - 30 **Yáñez-Mó M**, Siljander PR, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM, Ghoobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Krämer-Albers EM, Laitinen S, Lässer C, Lener T, Ligeti E, Liné A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-t Hoen EN, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pällinger É, Del Portillo HA, Reventós J, Rigau M, Rohde E, Sammar M, Sánchez-Madrid F, Santarém N, Schallmoser K, Ostfeld MS, Stoorvogel W, Stukelj R, Van der Grein SG, Vasconcelos MH, Wauben MH, De Wever O. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 2015; **4**: 27066 [PMID: [25979354](#) DOI: [10.3402/jev.v4.27066](#)]
  - 31 **Robbins PD**, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* 2014; **14**: 195-208 [PMID: [24566916](#) DOI: [10.1038/nri3622](#)]
  - 32 **Distler JH**, Huber LC, Gay S, Distler O, Pisetsky DS. Microparticles as mediators of cellular cross-talk in inflammatory disease. *Autoimmunity* 2006; **39**: 683-690 [PMID: [17178565](#) DOI: [10.1080/08916930601061538](#)]
  - 33 **Turpin D**, Truchetet ME, Faustin B, Augusto JF, Contin-Bordes C, Brisson A, Blanco P, Duffau P. Role of extracellular vesicles in autoimmune diseases. *Autoimmun Rev* 2016; **15**: 174-183 [PMID: [26554931](#) DOI: [10.1016/j.autrev.2015.11.004](#)]
  - 34 **Tofiño-Vian M**, Guillén MI, Alcaraz MJ. Extracellular vesicles: A new therapeutic strategy for joint conditions. *Biochem Pharmacol* 2018; **153**: 134-146 [PMID: [29427625](#) DOI: [10.1016/j.bcp.2018.02.004](#)]
  - 35 **Maumus M**, Jorgensen C, Noël D. Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: role of secretome and exosomes. *Biochimie* 2013; **95**: 2229-2234 [PMID: [23685070](#) DOI: [10.1016/j.biochi.2013.04.017](#)]
  - 36 **Ansboro S**, Roelofs AJ, De Bari C. Mesenchymal stem cells for the management of rheumatoid arthritis: immune modulation, repair or both? *Curr Opin Rheumatol* 2017; **29**: 201-207 [PMID: [27941390](#) DOI: [10.1097/BOR.0000000000000370](#)]
  - 37 **Cosenza S**, Ruiz M, Maumus M, Jorgensen C, Noël D. Pathogenic or Therapeutic Extracellular Vesicles in Rheumatic Diseases: Role of Mesenchymal Stem Cell-Derived Vesicles. *Int J Mol Sci* 2017; **18** [PMID: [28441721](#) DOI: [10.3390/ijms18040889](#)]
  - 38 **Asari S**, Itakura S, Ferreri K, Liu CP, Kuroda Y, Kandeel F, Mullen Y. Mesenchymal stem cells suppress B-cell terminal differentiation. *Exp Hematol* 2009; **37**: 604-615 [PMID: [19375651](#) DOI: [10.1016/j.exphem.2009.01.005](#)]
  - 39 **Zhang B**, Liu R, Shi D, Liu X, Chen Y, Dou X, Zhu X, Lu C, Liang W, Liao L, Zenke M, Zhao RC. Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2-dependent regulatory dendritic cell population. *Blood* 2009; **113**: 46-57 [PMID: [18832657](#) DOI: [10.1182/blood-2008-04-154138](#)]
  - 40 **Prigione I**, Benvenuto F, Bocca P, Battistini L, Uccelli A, Pistoia V. Reciprocal interactions between human mesenchymal stem cells and gammadelta T cells or invariant natural killer T cells. *Stem Cells* 2009; **27**: 693-702 [PMID: [19096038](#) DOI: [10.1634/stemcells.2008-0687](#)]
  - 41 **Ren G**, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008; **2**: 141-150 [PMID: [18371435](#) DOI: [10.1016/j.stem.2007.11.014](#)]
  - 42 **Nojehdehi S**, Soudi S, Hesampour A, Rasouli S, Soleimani M, Hashemi SM. Immunomodulatory effects of mesenchymal stem cell-derived exosomes on experimental type-1 autoimmune diabetes. *J Cell Biochem* 2018; **119**: 9433-9443 [PMID: [30074271](#) DOI: [10.1002/jcb.27260](#)]
  - 43 **Melief SM**, Schrama E, Brugman MH, Tiemessen MM, Hoogduijn MJ, Fibbe WE, Roelofs H. Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. *Stem Cells* 2013; **31**: 1980-1991 [PMID: [23712682](#) DOI: [10.1002/stem.1432](#)]
  - 44 **Mougiakakos D**, Jitschin R, Johansson CC, Okita R, Kiessling R, Le Blanc K. The impact of inflammatory licensing on heme oxygenase-1-mediated induction of regulatory T cells by human mesenchymal stem cells. *Blood* 2011; **117**: 4826-4835 [PMID: [21389316](#) DOI: [10.1182/blood-2010-12-324038](#)]
  - 45 **Davies LC**, Heldring N, Kadri N, Le Blanc K. Mesenchymal Stromal Cell Secretion of Programmed Death-1 Ligands Regulates T Cell Mediated Immunosuppression. *Stem Cells* 2017; **35**: 766-776 [PMID: [27671847](#) DOI: [10.1002/stem.2509](#)]
  - 46 **Serejo TRT**, Silva-Carvalho AE, Braga LDCF, Neves FAR, Pereira RW, Carvalho JL, Saldanha-Araujo F. Assessment of the Immunosuppressive Potential of INF- $\gamma$  Licensed Adipose Mesenchymal Stem Cells, Their Secretome and Extracellular Vesicles. *Cells* 2019; **8** [PMID: [30621275](#) DOI: [10.3390/cells8010022](#)]
  - 47 **Traggiai E**, Volpi S, Schena F, Gattorno M, Ferlito F, Moretta L, Martini A. Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. *Stem Cells* 2008; **26**: 562-569 [PMID: [18024418](#) DOI: [10.1634/stemcells.2007-0528](#)]
  - 48 **Budoni M**, Fierabracci A, Luciano R, Petrini S, Di Ciommo V, Muraca M. The immunosuppressive effect of mesenchymal stromal cells on B lymphocytes is mediated by membrane vesicles. *Cell Transplant* 2013; **22**: 369-379 [PMID: [23433427](#) DOI: [10.3727/096368911X582769](#)]
  - 49 **Bolego C**, Cignarella A, Staels B, Chinetti-Gbaguidi G. Macrophage function and polarization in cardiovascular disease: a role of estrogen signaling? *Arterioscler Thromb Vasc Biol* 2013; **33**: 1127-1134

- [PMID: 23640494 DOI: 10.1161/ATVBAHA.113.301328]
- 50 **Gordon S**, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity* 2010; **32**: 593-604 [PMID: 20510870 DOI: 10.1016/j.immuni.2010.05.007]
  - 51 **Zhou D**, Huang C, Lin Z, Zhan S, Kong L, Fang C, Li J. Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways. *Cell Signal* 2014; **26**: 192-197 [PMID: 24219909 DOI: 10.1016/j.cellsig.2013.11.004]
  - 52 **Sica A**, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest* 2012; **122**: 787-795 [PMID: 22378047 DOI: 10.1172/JCI59643]
  - 53 **Mantovani A**, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004; **25**: 677-686 [PMID: 15530839 DOI: 10.1016/j.it.2004.09.015]
  - 54 **Martinez FO**. Regulators of macrophage activation. *Eur J Immunol* 2011; **41**: 1531-1534 [PMID: 21607943 DOI: 10.1002/eji.201141670]
  - 55 **Domenis R**, Cifù A, Quaglia S, Pistis C, Moretti M, Vicario A, Parodi PC, Fabris M, Niazi KR, Soon-Shiong P, Curcio F. Pro inflammatory stimuli enhance the immunosuppressive functions of adipose mesenchymal stem cells-derived exosomes. *Sci Rep* 2018; **8**: 13325 [PMID: 30190615 DOI: 10.1038/s41598-018-31707-9]
  - 56 **Hossain P**, Kavar B, El Nahas M. Obesity and diabetes in the developing world--a growing challenge. *N Engl J Med* 2007; **356**: 213-215 [PMID: 17229948 DOI: 10.1056/NEJMp068177]
  - 57 **Hotamisligil GS**. Inflammation and metabolic disorders. *Nature* 2006; **444**: 860-867 [PMID: 17167474 DOI: 10.1038/nature05485]
  - 58 **Xu H**, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003; **112**: 1821-1830 [PMID: 14679177 DOI: 10.1172/JCI19451]
  - 59 **Weisberg SP**, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003; **112**: 1796-1808 [PMID: 14679176 DOI: 10.1172/JCI19246]
  - 60 **Wu H**, Ghosh S, Perrard XD, Feng L, Garcia GE, Perrard JL, Sweeney JF, Peterson LE, Chan L, Smith CW, Ballantyne CM. T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity. *Circulation* 2007; **115**: 1029-1038 [PMID: 17296858 DOI: 10.1161/CIRCULATIONAHA.106.638379]
  - 61 **Zhao H**, Shang Q, Pan Z, Bai Y, Li Z, Zhang H, Zhang Q, Guo C, Zhang L, Wang Q. Exosomes From Adipose-Derived Stem Cells Attenuate Adipose Inflammation and Obesity Through Polarizing M2 Macrophages and Beiging in White Adipose Tissue. *Diabetes* 2018; **67**: 235-247 [PMID: 29133512 DOI: 10.2337/db17-0356]
  - 62 **Németh K**, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009; **15**: 42-49 [PMID: 19098906 DOI: 10.1038/nm.1905]
  - 63 **Biron CA**, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 1999; **17**: 189-220 [PMID: 10358757 DOI: 10.1146/annurev.immunol.17.1.189]
  - 64 **Bai L**, Shao H, Wang H, Zhang Z, Su C, Dong L, Yu B, Chen X, Li X, Zhang X. Effects of Mesenchymal Stem Cell-Derived Exosomes on Experimental Autoimmune Uveitis. *Sci Rep* 2017; **7**: 4323 [PMID: 28659587 DOI: 10.1038/s41598-017-04559-y]
  - 65 **Shigemoto-Kuroda T**, Oh JY, Kim DK, Jeong HJ, Park SY, Lee HJ, Park JW, Kim TW, An SY, Prockop DJ, Lee RH. MSC-derived Extracellular Vesicles Attenuate Immune Responses in Two Autoimmune Murine Models: Type 1 Diabetes and Uveoretinitis. *Stem Cell Reports* 2017; **8**: 1214-1225 [PMID: 28494937 DOI: 10.1016/j.stemcr.2017.04.008]
  - 66 **Abumaree MH**, Bahattab E, Alsadoun A, Al Dosaimani A, Abomaray FM, Khatlani T, Kalionis B, El-Muzaini MF, Alawad AO, AlAskar AS. Characterization of the interaction between human decidua parietalis mesenchymal stem/stromal cells and natural killer cells. *Stem Cell Res Ther* 2018; **9**: 102 [PMID: 29650045 DOI: 10.1186/s13287-018-0844-y]
  - 67 **Fan Y**, Herr F, Vernochet A, Mennesson B, Oberlin E, Durrbach A. Human Fetal Liver Mesenchymal Stem Cell-Derived Exosomes Impair Natural Killer Cell Function. *Stem Cells Dev* 2019; **28**: 44-55 [PMID: 30328799 DOI: 10.1089/scd.2018.0015]
  - 68 **Favaro E**, Carpanetto A, Caorsi C, Giovarelli M, Angelini C, Cavallo-Perin P, Tetta C, Camussi G, Zanone MM. Human mesenchymal stem cells and derived extracellular vesicles induce regulatory dendritic cells in type 1 diabetic patients. *Diabetologia* 2016; **59**: 325-333 [PMID: 26592240 DOI: 10.1007/s00125-015-3808-0]
  - 69 **Ji J**, Sundquist J, Sundquist K. Gender-specific incidence of autoimmune diseases from national registers. *J Autoimmun* 2016; **69**: 102-106 [PMID: 26994904 DOI: 10.1016/j.jaut.2016.03.003]
  - 70 **Cooper GS**, Bynum ML, Somers EC. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J Autoimmun* 2009; **33**: 197-207 [PMID: 19819109 DOI: 10.1016/j.jaut.2009.09.008]
  - 71 **Gershwin LJ**. Current and Newly Emerging Autoimmune Diseases. *Vet Clin North Am Small Anim Pract* 2018; **48**: 323-338 [PMID: 29248206 DOI: 10.1016/j.cvsm.2017.10.010]
  - 72 **Autoimmune disease and the environment**. *Environ Health Perspect* 1998; **106**: A592-A593 [PMID: 10048952 DOI: 10.1289/ehp.106-1533238]
  - 73 **Wang Y**, Chen X, Cao W, Shi Y. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol* 2014; **15**: 1009-1016 [PMID: 25329189 DOI: 10.1038/ni.3002]
  - 74 **Xu H**, Jia S, Xu H. Potential therapeutic applications of exosomes in different autoimmune diseases. *Clin Immunol* 2019; **205**: 116-124 [PMID: 31228581 DOI: 10.1016/j.clim.2019.06.006]
  - 75 **Lee DM**, Weinblatt ME. Rheumatoid arthritis. *Lancet* 2001; **358**: 903-911 [PMID: 11567728 DOI: 10.1016/S0140-6736(01)06075-5]



- 76 **MacGregor AJ**, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, Aho K, Silman AJ. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum* 2000; **43**: 30-37 [PMID: 10643697 DOI: 10.1002/1529-0131(200001)43:1<30::AID-ANR5>3.0.CO;2-B]
- 77 **Padyukov L**, Silva C, Stolt P, Alfredsson L, Klareskog L. A gene-environment interaction between smoking and shared epitope genes in HLA-DR provides a high risk of seropositive rheumatoid arthritis. *Arthritis Rheum* 2004; **50**: 3085-3092 [PMID: 15476204 DOI: 10.1002/art.20553]
- 78 **Guo W**, Zheng X, Zhang W, Chen M, Wang Z, Hao C, Huang J, Yuan Z, Zhang Y, Wang M, Peng J, Wang A, Wang Y, Sui X, Xu W, Liu S, Lu S, Guo Q. Mesenchymal Stem Cells in Oriented PLGA/ACECM Composite Scaffolds Enhance Structure-Specific Regeneration of Hyaline Cartilage in a Rabbit Model. *Stem Cells Int* 2018; **2018**: 6542198 [PMID: 29666653 DOI: 10.1155/2018/6542198]
- 79 **Kamei N**, Ochi M, Adachi N, Ishikawa M, Yanada S, Levin LS, Kamei G, Kobayashi T. The safety and efficacy of magnetic targeting using autologous mesenchymal stem cells for cartilage repair. *Knee Surg Sports Traumatol Arthrosc* 2018; **26**: 3626-3635 [PMID: 29549388 DOI: 10.1007/s00167-018-4898-2]
- 80 **Chen Z**, Wang H, Xia Y, Yan F, Lu Y. Therapeutic Potential of Mesenchymal Cell-Derived miRNA-150-5p-Expressing Exosomes in Rheumatoid Arthritis Mediated by the Modulation of MMP14 and VEGF. *J Immunol* 2018; **201**: 2472-2482 [PMID: 30224512 DOI: 10.4049/jimmunol.1800304]
- 81 **Wang Y**, Zheng F, Gao G, Yan S, Zhang L, Wang L, Cai X, Wang X, Xu D, Wang J. MiR-548a-3p regulates inflammatory response via TLR4/NF- $\kappa$ B signaling pathway in rheumatoid arthritis. *J Cell Biochem* 2018 [PMID: 29315763 DOI: 10.1002/jcb.26659]
- 82 **Xu D**, Song M, Chai C, Wang J, Jin C, Wang X, Cheng M, Yan S. Exosome-encapsulated miR-6089 regulates inflammatory response via targeting TLR4. *J Cell Physiol* 2019; **234**: 1502-1511 [PMID: 30132861 DOI: 10.1002/jcp.27014]
- 83 **Song J**, Kim D, Han J, Kim Y, Lee M, Jin EJ. PBMC and exosome-derived Hotair is a critical regulator and potent marker for rheumatoid arthritis. *Clin Exp Med* 2015; **15**: 121-126 [PMID: 24722995 DOI: 10.1007/s10238-013-0271-4]
- 84 **Wang L**, Wang C, Jia X, Yu J. Circulating Exosomal miR-17 Inhibits the Induction of Regulatory T Cells via Suppressing TGFBR II Expression in Rheumatoid Arthritis. *Cell Physiol Biochem* 2018; **50**: 1754-1763 [PMID: 30384383 DOI: 10.1159/000494793]
- 85 **Kurowska-Stolarska M**, Alivernini S, Ballantine LE, Asquith DL, Millar NL, Gilchrist DS, Reilly J, Ierna M, Fraser AR, Stolarski B, McSharry C, Hueber AJ, Baxter D, Hunter J, Gay S, Liew FY, McInnes IB. MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis. *Proc Natl Acad Sci* 2011; **108**: 11193-11198 [PMID: 21690378 DOI: 10.1073/pnas.1019536108]
- 86 **Nakasa T**, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, Asahara H. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis Rheum* 2008; **58**: 1284-1292 [PMID: 18438844 DOI: 10.1002/art.23429]
- 87 **Taganov KD**, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA* 2006; **103**: 12481-12486 [PMID: 16885212 DOI: 10.1073/pnas.0605298103]
- 88 **Yoo J**, Lee SK, Lim M, Sheen D, Choi EH, Kim SA. Exosomal amyloid A and lymphatic vessel endothelial hyaluronic acid receptor-1 proteins are associated with disease activity in rheumatoid arthritis. *Arthritis Res Ther* 2017; **19**: 119 [PMID: 28569211 DOI: 10.1186/s13075-017-1334-9]
- 89 **Al-Shobaili HA**, Al Robaee AA, Alzolibani AA, Rasheed Z. Antibodies against 4-hydroxy-2-nonenal modified epitopes recognized chromatin and its oxidized forms: role of chromatin, oxidized forms of chromatin and 4-hydroxy-2-nonenal modified epitopes in the etiopathogenesis of SLE. *Dis Markers* 2012; **33**: 19-34 [PMID: 22710866 DOI: 10.3233/DMA-2012-0900]
- 90 **Colasanti T**, Maselli A, Conti F, Sanchez M, Alessandri C, Barbati C, Vacirca D, Tinari A, Chiarotti F, Giovannetti A, Franconi F, Valesini G, Malorni W, Pierdominici M, Ortona E. Autoantibodies to estrogen receptor  $\alpha$  interfere with T lymphocyte homeostasis and are associated with disease activity in systemic lupus erythematosus. *Arthritis Rheum* 2012; **64**: 778-787 [PMID: 21968947 DOI: 10.1002/art.33400]
- 91 **Rahman A**, Isenberg DA. Systemic lupus erythematosus. *N Engl J Med* 2008; **358**: 929-939 [PMID: 18305268 DOI: 10.1056/NEJMra071297]
- 92 **Perez-Hernandez J**, Redon J, Cortes R. Extracellular Vesicles as Therapeutic Agents in Systemic Lupus Erythematosus. *Int J Mol Sci* 2017; **18** [PMID: 28350323 DOI: 10.3390/ijms18040717]
- 93 **Lee JY**, Park JK, Lee EY, Lee EB, Song YW. Circulating exosomes from patients with systemic lupus erythematosus induce a proinflammatory immune response. *Arthritis Res Ther* 2016; **18**: 264 [PMID: 27852323 DOI: 10.1186/s13075-016-1159-y]
- 94 **Nielsen CT**, Østergaard O, Stener L, Iversen LV, Truedsson L, Gullstrand B, Jacobsen S, Heegaard NH. Increased IgG on cell-derived plasma microparticles in systemic lupus erythematosus is associated with autoantibodies and complement activation. *Arthritis Rheum* 2012; **64**: 1227-1236 [PMID: 22238051 DOI: 10.1002/art.34381]
- 95 **Østergaard O**, Nielsen CT, Iversen LV, Tanassi JT, Knudsen S, Jacobsen S, Heegaard NH. Unique protein signature of circulating microparticles in systemic lupus erythematosus. *Arthritis Rheum* 2013; **65**: 2680-2690 [PMID: 23817959 DOI: 10.1002/art.38065]
- 96 **Asami T**, Ishii M, Fujii H, Namkoong H, Tasaka S, Matsushita K, Ishii K, Yagi K, Fujiwara H, Funatsu Y, Hasegawa N, Betsuyaku T. Modulation of murine macrophage TLR7/8-mediated cytokine expression by mesenchymal stem cell-conditioned medium. *Mediators Inflamm* 2013; **2013**: 264260 [PMID: 24191131 DOI: 10.1155/2013/264260]
- 97 **Lai RC**, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010; **4**: 214-222 [PMID: 20138817 DOI: 10.1016/j.scr.2009.12.003]
- 98 **Wang D**, Li J, Zhang Y, Zhang M, Chen J, Li X, Hu X, Jiang S, Shi S, Sun L. Umbilical cord mesenchymal stem cell transplantation in active and refractory systemic lupus erythematosus: a multicenter clinical study. *Arthritis Res Ther* 2014; **16**: R79 [PMID: 24661633 DOI: 10.1186/ar4520]



- 99 **Ichii O**, Otsuka-Kanazawa S, Horino T, Kimura J, Nakamura T, Matsumoto M, Toi M, Kon Y. Decreased miR-26a expression correlates with the progression of podocyte injury in autoimmune glomerulonephritis. *PLoS One* 2014; **9**: e110383 [PMID: 25329154 DOI: 10.1371/journal.pone.0110383]
- 100 **Perez-Hernandez J**, Forner MJ, Pinto C, Chaves FJ, Cortes R, Redon J. Increased Urinary Exosomal MicroRNAs in Patients with Systemic Lupus Erythematosus. *PLoS One* 2015; **10**: e0138618 [PMID: 26390437 DOI: 10.1371/journal.pone.0138618]
- 101 **Jonsson R**, Bolstad AI, Brokstad KA, Brun JG. Sjögren's syndrome--a plethora of clinical and immunological phenotypes with a complex genetic background. *Ann N Y Acad Sci* 2007; **1108**: 433-447 [PMID: 17894008 DOI: 10.1196/annals.1422.046]
- 102 **Jonsson R**, Vogelsang P, Volchenkov R, Espinosa A, Wahren-Herlenius M, Appel S. The complexity of Sjögren's syndrome: novel aspects on pathogenesis. *Immunol Lett* 2011; **141**: 1-9 [PMID: 21777618 DOI: 10.1016/j.imlet.2011.06.007]
- 103 **Ramos-Casals M**, Brito-Zerón P, Sisó-Almirall A, Bosch X, Tzioufas AG. Topical and systemic medications for the treatment of primary Sjögren's syndrome. *Nat Rev Rheumatol* 2012; **8**: 399-411 [PMID: 22549247 DOI: 10.1038/nrrheum.2012.53]
- 104 **Hu S**, Vissink A, Arellano M, Roozendaal C, Zhou H, Kallenberg CG, Wong DT. Identification of autoantibody biomarkers for primary Sjögren's syndrome using protein microarrays. *Proteomics* 2011; **11**: 1499-1507 [PMID: 21413148 DOI: 10.1002/pmic.201000206]
- 105 **Delaleu N**, Mydel P, Kwee I, Brun JG, Jonsson MV, Jonsson R. High fidelity between saliva proteomics and the biologic state of salivary glands defines biomarker signatures for primary Sjögren's syndrome. *Arthritis Rheumatol* 2015; **67**: 1084-1095 [PMID: 25545990 DOI: 10.1002/art.39015]
- 106 **Tomosugi N**, Kitagawa K, Takahashi N, Sugai S, Ishikawa I. Diagnostic potential of tear proteomic patterns in Sjögren's syndrome. *J Proteome Res* 2005; **4**: 820-825 [PMID: 15952728 DOI: 10.1021/pr0497576]
- 107 **Agrawi LA**, Galtung HK, Vestad B, Øvstebø R, Thiede B, Rusthen S, Young A, Guerreiro EM, Utheim TP, Chen X, Utheim ØA, Palm Ø, Jensen JL. Identification of potential saliva and tear biomarkers in primary Sjögren's syndrome, utilising the extraction of extracellular vesicles and proteomics analysis. *Arthritis Res Ther* 2017; **19**: 14 [PMID: 28122643 DOI: 10.1186/s13075-017-1228-x]
- 108 **Sellam J**, Proulle V, Jüngel A, Ittah M, Miceli Richard C, Gottenberg JE, Toti F, Benessiano J, Gay S, Freyssinet JM, Mariette X. Increased levels of circulating microparticles in primary Sjögren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity. *Arthritis Res Ther* 2009; **11**: R156 [PMID: 19832990 DOI: 10.1186/ar2833]
- 109 **Corridoni D**, Arseneau KO, Cominelli F. Inflammatory bowel disease. *Immunol Lett* 2014; **161**: 231-235 [PMID: 24938525 DOI: 10.1016/j.imlet.2014.04.004]
- 110 **Hodson R**. Inflammatory bowel disease. *Nature* 2016; **540**: S97 [PMID: 28002398 DOI: 10.1038/540S97a]
- 111 **Baumgart DC**, Carding SR. Inflammatory bowel disease: cause and immunobiology. *Lancet* 2007; **369**: 1627-1640 [PMID: 17499605 DOI: 10.1016/S0140-6736(07)60750-8]
- 112 **Lee SH**, Kwon JE, Cho ML. Immunological pathogenesis of inflammatory bowel disease. *Intest Res* 2018; **16**: 26-42 [PMID: 29422795 DOI: 10.5217/ir.2018.16.1.26]
- 113 **Cao L**, Xu H, Wang G, Liu M, Tian D, Yuan Z. Extracellular vesicles derived from bone marrow mesenchymal stem cells attenuate dextran sodium sulfate-induced ulcerative colitis by promoting M2 macrophage polarization. *Int Immunopharmacol* 2019; **72**: 264-274 [PMID: 31005036 DOI: 10.1016/j.intimp.2019.04.020]
- 114 **Yang J**, Liu XX, Fan H, Tang Q, Shou ZX, Zuo DM, Zou Z, Xu M, Chen QY, Peng Y, Deng SJ, Liu YJ. Extracellular Vesicles Derived from Bone Marrow Mesenchymal Stem Cells Protect against Experimental Colitis via Attenuating Colon Inflammation, Oxidative Stress and Apoptosis. *PLoS One* 2015; **10**: e0140551 [PMID: 26469068 DOI: 10.1371/journal.pone.0140551]
- 115 **Wu H**, Fan H, Shou Z, Xu M, Chen Q, Ai C, Dong Y, Liu Y, Nan Z, Wang Y, Yu T, Liu X. Extracellular vesicles containing miR-146a attenuate experimental colitis by targeting TRAF6 and IRAK1. *Int Immunopharmacol* 2019; **68**: 204-212 [PMID: 30654310 DOI: 10.1016/j.intimp.2018.12.043]
- 116 **de Visser KE**, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* 2006; **6**: 24-37 [PMID: 16397525 DOI: 10.1038/nrc1782]
- 117 **Ostrand-Rosenberg S**. Immune surveillance: a balance between protumor and antitumor immunity. *Curr Opin Genet Dev* 2008; **18**: 11-18 [PMID: 18308558 DOI: 10.1016/j.gde.2007.12.007]
- 118 **Greening DW**, Gopal SK, Xu R, Simpson RJ, Chen W. Exosomes and their roles in immune regulation and cancer. *Semin Cell Dev Biol* 2015; **40**: 72-81 [PMID: 25724562 DOI: 10.1016/j.semedb.2015.02.009]
- 119 **Zhang HG**, Grizzle WE. Exosomes and cancer: a newly described pathway of immune suppression. *Clin Cancer Res* 2011; **17**: 959-964 [PMID: 21224375 DOI: 10.1158/1078-0432.CCR-10-1489]
- 120 **Yang Y**, Li CW, Chan LC, Wei Y, Hsu JM, Xia W, Cha JH, Hou J, Hsu JL, Sun L, Hung MC. Exosomal PD-L1 harbors active defense function to suppress T cell killing of breast cancer cells and promote tumor growth. *Cell Res* 2018; **28**: 862-864 [PMID: 29959401 DOI: 10.1038/s41422-018-0060-4]
- 121 **Hsu YL**, Hung JY, Chang WA, Jian SF, Lin YS, Pan YC, Wu CY, Kuo PL. Hypoxic Lung-Cancer-Derived Extracellular Vesicle MicroRNA-103a Increases the Oncogenic Effects of Macrophages by Targeting PTEN. *Mol Ther* 2018; **26**: 568-581 [PMID: 29292163 DOI: 10.1016/j.ymthe.2017.11.016]
- 122 **Wang X**, Luo G, Zhang K, Cao J, Huang C, Jiang T, Liu B, Su L, Qiu Z. Hypoxic Tumor-Derived Exosomal miR-301a Mediates M2 Macrophage Polarization via PTEN/PI3Kγ to Promote Pancreatic Cancer Metastasis. *Cancer Res* 2018; **78**: 4586-4598 [PMID: 29880482 DOI: 10.1158/0008-5472.CAN-17-3841]
- 123 **Srinivas TR**, Meier-Kriesche HU. Minimizing immunosuppression, an alternative approach to reducing side effects: objectives and interim result. *Clin J Am Soc Nephrol* 2008; **3** Suppl 2: S101-S116 [PMID: 18308998 DOI: 10.2215/CJN.03510807]
- 124 **Halawa A**. The early diagnosis of acute renal graft dysfunction: a challenge we face. The role of novel biomarkers. *Ann Transplant* 2011; **16**: 90-98 [PMID: 21436782]
- 125 **Williams WW**, Taheri D, Tolkoff-Rubin N, Colvin RB. Clinical role of the renal transplant biopsy. *Nat Rev Nephrol* 2012; **8**: 110-121 [PMID: 22231130 DOI: 10.1038/nrneph.2011.213]

- 126 **Park J**, Lin HY, Assaker JP, Jeong S, Huang CH, Kurdi T, Lee K, Fraser K, Min C, Eskandari S, Routray S, Tannous B, Abdi R, Riella L, Chandraker A, Castro CM, Weissleder R, Lee H, Azzi JR. Integrated Kidney Exosome Analysis for the Detection of Kidney Transplant Rejection. *ACS Nano* 2017; **11**: 11041-11046 [PMID: [29053921](#) DOI: [10.1021/acsnano.7b05083](#)]
- 127 **Kordelas L**, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, Epple M, Horn PA, Beelen DW, Giebel B. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 2014; **28**: 970-973 [PMID: [24445866](#) DOI: [10.1038/leu.2014.41](#)]



Published by **Baishideng Publishing Group Inc**  
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA

**Telephone:** +1-925-3991568

**E-mail:** [bpgoffice@wjgnet.com](mailto:bpgoffice@wjgnet.com)

**Help Desk:** <https://www.f6publishing.com/helpdesk>

<https://www.wjgnet.com>

