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REVIEW

Bone marrow-derived products: A classification proposal – bone marrow aspirate, bone marrow aspirate concentrate or hybrid?

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Author contributions: Purita J and Kolber M wrote the manuscript; Lana JFSD and Rodrigues BL designed the ACH classification concepts; Huber SC and Caliari-Oliveira C created Tables 1 and 2 and compiled the reference list; Santos GS proposed the schematic representation of the ACH classification illustrated in Figure 1 and Mosaner T reviewed the literature to validate information.

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Abstract

Degenerative musculoskeletal disorders are one of the top causes of pain and disability in the adult population. Current available alternatives to mitigate symptoms include conservative treatments such as the administration of pharmacological agents and an educative approach towards lifestyle modification. The use of certain analgesics, such as opiates and corticosteroids, delivers short term results but do not address the etiological source of pain and disability. Also, prolonged use of such medications may cause additional complications. Therefore, the demand for musculoskeletal tissue regeneration has led to an alternative approach referred to as "orthobiologics". This alternative is based on cellular and molecular components capable of inducing and promoting tissue repair. Bone marrow (BM) aspirate (BMA) and concentrate are well-known orthobiologics used to treat musculoskeletal conditions. Orthobiologics derived from the BM have been discussed in the literature; however, the lack of standardization regarding collection and processing protocols presents a challenge for generalization of study outcomes and determination of efficacy. Since BM-derived orthobiologics have not yet been classified, to our knowledge, this manuscript proposes the ACH classification system, which speaks to BMA (A), BMA and concentrate (C) and hybrid (H), which combines A and C. This classification proposes and describes 8 parameters that are relevant for the quality of biological products. The more parameters used would imply greater characterization and complexity of the evaluation of the biological product used.

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The ACH classification envisages a necessary contribution to the comprehension of both clinical procedures and research outcomes, ultimately ushering in a standardization of best practice.

Key words: Bone-marrow classification; Bone-marrow aspirate concentrate; Regenerative medicine; Stem cells; Musculoskeletal disorders; Orthobiologics

Core tip: Degenerative musculoskeletal disorders are one of the top causes of pain and disability in the adult population. The use of certain analgesics delivers short term results but do not address the etiological source of pain and disability. The demand for musculoskeletal tissue regeneration has led to an alternative approach referred to as orthobiologics, which is based on cellular and molecular components capable of promoting tissue repair. Bone marrow aspirate and concentrate are well-known orthobiologics used to treat musculoskeletal conditions. Since bone marrow-derived orthobiologics have not yet been classified, to our knowledge, this manuscript proposes the ACH classification system.

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INTRODUCTION

The increasing incidence of degenerative diseases affecting the musculoskeletal system is the main cause of pain and disability among adults. Current options for the management of these conditions mainly focus on conservative care such as activity modification and pharmacological therapies. While pharmacological therapies such as opiates and non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids offer short term efficacy, they are associated with well-known side effects if used on a long-term basis[1,2]. Moreover, few options exist outside of surgical solutions for those individuals recalcitrant to conservative care.

The need for musculoskeletal tissue regeneration has led to an alternative approach referred to as orthobiologics, which is based on cellular and molecular components responsible for inducing and promoting tissue repair[3]. Orthobiologics, which comprise platelet rich plasma (PRP), bone marrow (BM) aspirate (BMA) and concentrate (BMAC), fat grafting (Bio fat), and expanded mesenchymal stem cells (MSCs), have shown promising results for the care of musculoskeletal disorders[4-7].

Orthobiologics have been discussed in the literature with promising results, however, the lack of standardization regarding the methods of obtaining and processing the cells and associated components, have led to uncertain conclusions in terms of efficacy and ability to generalize outcomes[8]. Specifically, the main components of orthobiologics (platelet concentrations, growth factors, and cytokines) may vary based on the processing method, which might affect anabolic and anti-inflammatory properties, and consequently lead to inconsistent outcomes[8]. Thus, the need for standardization and classification of orthobiologics is imperative for understanding procedures and dissemination of research outcomes. A classification system has been developed for PRP[9]; however, no such classification exists for BM-derived orthobiologics. Thus, the purpose of this paper is to present a proposal for a classification system for BM derived orthobiologics.

BM

The main function of BM is to provide circulating blood with an optimal supply of erythrocytes, leukocytes, and platelets. In addition to this, BM supplies hematopoietic stem cells (HSCs), endothelial cells, MSCs and other precursor cells. The human

skeleton possesses red BM which is hematopoietically active, and yellow, which is hematopoietically inactive[10].

Red and yellow BMs have different cellular and molecular content: Yellow BM comprises 95% fat cells, whereas the red BM comprises 60% hematopoietic cells. The whole skeleton is filled with red BM at birth, however, during childhood a physiological conversion of red BM into yellow BM occurs. The conversion of red to yellow marrow and progresses to the axial skeleton, and this entire process may be completed by the age of 25 years[10].

BM is a potent source of stem and progenitor cells, and this characteristic has gained attention for cell-based therapies in orthopedics[11-13]. Given the diversity in stem cell lineages and phenotypes in the marrow, BM represents a functional organ in which distinct types of cells function cooperatively. Specifically, HSCs play a critical role in the formation of the hematopoietic microenvironment, whereas MSCs support hematopoiesis and both MSCs and/or skeletal stem cells are responsible for the development and maintenance of skeletal tissues[14,15].

Cellular content

MSCs are non-hematopoietic stromal cells that are composed of a small fraction (0.001%–0.01%) of the stem cell content in BM[16]. MSCs are found in other tissues, such as adipose tissue, placenta, and umbilical cord, and although they differ in their differentiation potential, they possess common features associated with those from the BM, which might imply that MSC-like populations share a similar ontogeny[17,18].

MSCs exhibit the potential ability to differentiate into mesodermal linage cells (*e.g.*, cartilage, bone, fat, muscle, meniscus and tendon)[19], which is fundamental for the regeneration process. Moreover, these cells have paracrine effects, thus are able to alter their local microenvironment[20].

Given the varying MSC markers that laboratories may use to characterize these cells, there is a lack in standard phenotypic criteria. This heterogeneity is also due to the fact that MSCs are able to express a range of cell-lineage specific antigens that may differ depending on the culture preparation, culture duration, or plating density[21,22]. However, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have proposed minimal criteria to characterize MSCs, which comprise the following attributes: Must be plastic-adherent when maintained in culture; must be able to differentiate in vitro into chondroblasts, adipocytes and osteoblasts; and must express CD105, CD73 and CD90, and lack expression of CD45, CD11b, CD34 or CD14, CD79α or CD19 and HLA-DR surface molecules[23].

MSCs lack significant immunogenicity and can be easily isolated, which allows allogenic transplantation. In allogenic circumstances these cells should be considered immune evasive. However, the effects of MSCs in cellular-based therapies depends on the ability of these cells to home and engraft (long-term) into the target tissue[24]. One theory suggests that MSCs have a rather short life span and are phagocytized by monocytes and subsequently stimulate the production of T-reg cells which may very well contribute to the overall clinical improvement[25].

Cells from injured tissue release chemokines responsible for MSC recruitment. Once in the target tissue, MSCs are able to modulate wound-healing responses by reducing apoptosis and fibrosis, attenuate the inflammatory process and stimulate cell proliferation and differentiation via paracrine and autocrine pathways[26]. These properties are attributed to the ability of MSCs to release key agents, such as vasculoendothelial growth factor, transforming growth factor beta (TGF-β), stromal-derived factor 1, and stem cell factor, among others. Also, they induce a downregulation of pro-inflammatory cytokines, including interleukin 1 (IL-1), IL-6, interferon-γ, and tumor necrosis factor α[16,27,28]. MSCs also possess immunomodulatory properties as they are able to inhibit the activation of type 1 macrophages, natural killer cells, and both B and T lymphocytes[29].

HSCs also known for expressing CD34+, are located at the top of the hematopoietic hierarchy. They are responsible for the daily supply of more than 100 billion mature blood cells, including erythrocytes, leukocytes, and platelets[30]. This process, called hematopoiesis, is of extreme importance in the maintenance and regulation of the immune system, especially for the cells from myeloid lineage, such as granulocytes, monocytes and dendritic cells, due to their short half-life[31,32].

Past studies have reported that the hematopoietic and stromal environments are related and overlapped. For example, Simons *et al*[33] observed generations of fibroblasts colony-forming unit (CFU-F) from CD34+ human BM cells. Also, it has been reported that the number of osteoblast progenitor cells is higher in sorted CD34+ cells (1/5000 approximately) than in CD34 - populations (1/33000), and when these sorted cells were cultured in a long-term marrow system, the generation of a heterogeneous population that included smooth muscle cells, adipocytes, fibroblast and macrophages was observed[34]. This possible relation was then supported by

Mehrotra *et al*[35] who reported that HSC give rise to osteocytes and chondrocytes in an experimental study.

Immune cells – Leukocytes have a common origin from the hematopoietic stem cell and develop along distinct differentiation pathways in response to external and internal stimuli. In order to promote regeneration, leukocytes circulate through the blood and lymphatic system and are recruited to specific regions of the body when damage occurs[32].

The mononuclear phagocyte system represents a subset of leukocytes that was originally described as BM-derived myeloid cells[32]. Monocytes are immune effector cells that, although they circulate in the blood, BM and spleen, they do not proliferate in a steady state[36,37]. They are equipped with chemokine receptors that mediate migration from blood to the injured sites and produce inflammatory cytokines. During inflammation, the monocytes differentiate into dendritic cells (DC) or macrophages, and this process is likely determined by the inflammatory environment and pathogen-associated pattern-recognition receptors[38].

Macrophages are phagocytic cells that reside in lymphoid and nonlymphoid tissues[32]. Given the broad range of pathogen-recognition receptors that macrophages possess, they are known as an efficient tool at maintaining tissue homeostasis as they provide clearance of apoptotic cells and remodeling of the extracellular matrix[39,40]. Macrophages play a key role in recruiting and inducing the proliferation of osteoblasts, stem and progenitor cells as they secrete bone morphogenetic proteins, IL-1β, TGF-β, platelet derived growth factor and insulin-like growth factors, in areas of infection or injury in different tissues in the body[41]. Extrinsic stimuli that induce an inflammatory process, such as infection or injury, promote changes in gene transcription that classify macrophages as type 1 (M-1) and type 2 (M-2). The M-2 type offers a healing function, while M-1 promotes the host defense. After injury, M2 can switch into M1, and this change is modulated by the cytokines such as interferon-γ, and M2 type by IL-4[42].

Neutrophils belong to a polymorphonuclear family and are known for being the main cell type response to bacterial infections. It was reported that neutrophils are highly plastic cells influenced by environmental cues that result in a site-specific neutrophil transcriptome as they migrate from BM to sites of inflammation[43]. As a granulocyte, which includes eosinophils and basophils, neutrophils are able to secrete a variety of cytokines, such as TGF-β, vasculoendothelial growth factor and platelet derived growth factor, playing an important role in angiogenesis and vasculogenesis[44]. Neutrophils undergo spontaneous apoptosis to regulate the resolution of inflammation[45].

BM-DERIVED PRODUCTS

BMA

The main goal in treating orthopedic injuries, especially joint disease, is cartilage regeneration. One approach to achieve this outcome is by using BM-derived MSC (BM-MSC), which has been supported in the literature[46,47]. However, its clinical utility is limited by complexity, such as the need for a specialized laboratory and procedural cost. In this sense, the use of BMA has emerged as a novel regenerative tool for degenerative joint diseases as a non-fractioned product that retains potentially supportive chondrogenic components[48].

Even though different harvest sources for BM have been described in the literature the main harvest site (either for BMA or BMAC use) is the posterior iliac crest, which allows a considerable amount of BM and about 1.6-fold more osteoblastic connective tissue progenitor cells than other sites[49,50]. However, evidence suggests the quality of the product is technique-dependent[51].

There are a few studies that have used this approach in the literature; however, most of them are related to nonunion fractures. The first to describe the use of unprocessed marrow was Lindholm and Urist[52] that reported the replacement of bone matrix by new bone in composite grafts *in vivo* (non-human study). Almost a decade later, Connolly *et al*[53] observed callus formation sufficient to unite tibial nonunions in humans after injection of autologous BMA.

In 2013, Hauser and Orlofsky published a case series describing their experience with BMA in combination with hyperosmotic dextrose, also known as prolotherapy, in the treatment of knee, hip, and ankle osteoarthritis. After two to seven treatments over twelve months, all patients reported improvement in pain, joint function, and quality of life. Also, three out of seven patients had achieved complete symptomatic relief[48].

Butala *et al*[54] reported the efficacy of BMA in bone union as they injected

unprocessed BM at fracture sites in 10 patients with tibia, humerus, femur, and forearm delayed union fractures. After 12 wk, nine of these patients had signs of union, such as decreased tenderness at fracture site, pain-free joint mobilization and ability to ambulate without assistance[54].

A study performed in 2017 by Lal[55], evaluated the use of percutaneous autologous BM injections in 56 patients with delayed and 37 patients with nonunion of long bone. Twelve weeks after the injections, it was observed that all fractures were united, and the minimum period for union was 8-weeks. Although a significant correlation (P = 0.081) was not present, it was reported that the time to observe bone union after the injection of autologous BM was longer in patients who were smokers. Women, however, were observed to have a reduced time for bone union than the male patients (P = 0.041)[55].

Although the number of studies with BMA are limited and of lower quality, they show a promising efficacy and safety profile with regards to adverse events.

BMAC

In an attempt to increase the proportion of MSCs, the aspirate of BM may be processed to produce BMAC, which has been widely investigated in orthopedics, especially for nonunions, surgical augmentation, osteonecrosis, as well as osseous and cartilage defects[11-13].

Although the exact mechanism of action has not been fully elucidated, the effects of BMAC may rely on the recovery of nucleated cells from BM, which possesses a paracrine effect by delivering cytokines into the injured site in order to stimulate endogenous tissue repair[56]. *In vitro* studies have shown that the platelets present in BMAC release growth factors that induce stem cells migration to the injured area. Moreover, a concentrated number of HSCs may provide vascular support and drive MSC into osteogenic differentiation pathways[57].

Current clinical studies have reported the efficacy and safety of BMAC for the treatment of small lesions. Centeno *et al*[6] studied the effects of BMAC on 115 shoulders of 102 patients who had rotated cuff injuries and shoulder osteoarthritis. In the aforementioned study, a 52.6% improvement in joint function and disability and 44.2% decrease in pain was reported with both outcomes reaching statistical significance (P = 0.001). The mean improvement reported by the patients was 48.8%. The reduction of disability and pain was observed from the first month after treatment and was maintained for up to 2 years after the treatment, based on this time being the terminal point of data collection. No side effects or adverse events were reported with BMAC in these 2 years of study[6].

BMAC has also been studied with various surgical scaffolds. Gobbi *et al*[58] evaluated 15 patients with grade IV cartilage lesions who underwent injections of BMAC on a collagen matrix. Two years after the injections improvements in pain, joint functionality and quality of life were identified. Biopsy of these lesions showed hyaline-like tissue at repeat arthroscopy 2- years later[58]. Enea *et al*[59] evaluated patients who underwent microfracture covered with a resorbable composite of natural hyaluronan matrix and synthetic polyglycolic acid with BMAC. It was observed that, 12 mo after the injection, the lesions were macroscopically normal, presenting production of hyaline-like tissue. The defect filling was confirmed by magnetic resonance imaging[59].

The use of BMAC has also been studied in combination with other regenerative medicine approaches. Sampson *et al*[60] evaluated the injection of BMAC followed by PRP in 125 patients who presented moderate/severe ankle, knee, spine and/or shoulder osteoarthritis, eight weeks after the injection, The authors observed a median of 5 points in pain relief, based on a visual analogic scale (VAS), and the patients reported 9.0/10 satisfaction with the treatment. Kim *et al*[61] studied the association of BMAC with adipose tissue (fat graft) in 75 osteoarthritic knees (41 patients). Twelve months after the injections, a decrease in pain, improved joint function, and an increase in quality of life was reported. The authors also suggest that BMAC would present a more effective result in early to moderate phases of osteoarthritis.

Some studies evaluated the optimal volume of BM needed to achieve clinical response: The quality of the product decreases with higher volume of BM withdrawn, and it was observed that small volume of marrow aspirated in a 10 mL syringe would be an ideal volume to concentrate MSC and progenitor cells. Larger volume syringes may cause blood dilution[62,63]. The components of BM aspirated are concentrated following centrifugation steps. Although there are some protocols of BMAC preparation in the literature[64,65] there is no study regarding the optimal centrifuge force and time to achieve an increased cellular concentration.

Although BMAC presents a well-established cellular and molecular content, only few studies evaluating its efficacy and safety have performed quantitative and qualitative assessment[8].

PROPOSAL OF A NEW CLASSIFICATION FOR BM-DERIVED PRODUCTS: THE ACH CLASSIFICATION

The lack of standardization of the BM-derived products for regenerative medicine has emerged, thus the need to classify the processing methods according to quality and procedural details has been established[11]. Classification of such factors would allow for procedural standardization and interpretation of both clinical results and research findings.

The ACH (aspirate, concentrate, hybrid) classification system comprises the two main techniques involving bone marrow-derived products: BMA, which represents the letter A (for aspirate), BMAC, which represents the letter C (for concentrated), and the letter H (for hybrid) is used when BMA is combined with BMAC.

The ACH classification is focused on whether the cellular and molecular content present in the product was evaluated and described increasing the complexity of description/characterization. For each classification (A, C and H) sub grouping would occur as follows: (1) Product would only be collected and injected with no additional analysis; (2) Description of harvesting – BM site of harvesting (posterior/anterior iliac crest, axial skeleton), type of needle, multiple insertions, single insertion, type of syringe, type of anticoagulant, volume harvested; (3) The cellular content would be assessed by a cell count machine, which would enable to quantify mono- and polymorphonuclear cells, giving the number of total nucleated cells; (4) Dosage of molecular content, such as interleukins and/or growth factors is made by multiplex platform or ELISA technique; (5) Indirect quantification of MSC number measured through CFU in culture; (6) Phenotyping of MSC and HSC for characterization through flow cytometry – it is wise to use a full panel for the clusters of differentiation, especially of the MSC since there are a lot of markers for positive and negative evaluation; (7) For the complete characterization of MSC the differentiation in three cell types in culture is necessary, including the induction of chondrocytes, adipocytes and osteocytes; and (8) To finalize, the most complex level of evaluation of MSC is the evaluation of its function, which includes assays like wound healing (proliferation and migration), lymphocytes proliferation (immunossupressor potential), and population doubling time. The representation of the ACH classification is shown in Table 1.

The idea of this classification is that for each type of BM used (BMA, BMAC or hybrid) the increase of the number indicates an improvement in the characterization and complexity of the evaluation of this biological product. When a study or procedure with BMAC reports that only BM was collected and injected, it would be classified as C1, according to the ACH classification. On the other hand, if the BMAC presents the description of harvesting procedure (site, syringe, volume, and anticoagulant use) it will be classified as C1-2. If the total nuclear cells were counted by a cell counter, which would include leukocytes, MSC and HSC, using the description of technique for harvesting it would be classified as C1-3. In this BMAC if the harvesting technique was described, cell count was made and evaluation of molecular content, it will be classified as C1-4. If the HSC and/or MSC are quantified and characterized by flow cytometry in the same BMAC, has the description of harvesting, dosage of cytokines and CFU it would be classified as C1-6 product. The last level of description is the C1-8 which encompass the description of harvesting, cell count, evaluation of cytokines and growth factors, MSC and HSC phenotyping, CFU, evaluation of differentiation and functional assays, being classified as C1-8.

In the case where this is not a progression of steps in numeric order the omitted step number would not be used. For example, if a procedure with BMA harvesting had a description, cell count, and CFU, without the quantification of the molecular content, this study will be classified as A1-3;5, as demonstrated in Table 2.

For a general view of ACH, we described a schematic illustration of the ACH classification exemplified by Figure 1.

CONCLUSION

Although studies using both BMA and BMAC for the treatment of various musculoskeletal disorders have shown promising clinical results, inconsistent preparation methods with deficient reporting has led to questionable outcomes with

Table 1 ACH classification

Letter	Relates to	Classification
A	BMA	1 – Collection and injection
		2 – Description of harvesting
		3 – Cell count
		4 – Dosage of cytokines (GF and/or IL)
		5 – CFU
		6 - MSC and HSC phenotyping
		7- Differentiation evaluation
		8 – Functional assays
C	BMAC	1 – Collection and injection
		2 – Description of harvesting
		3 – Cell count
		4 – Dosage of cytokines (GF and/or IL)
		5 - CFU
		6 – HSC and/or MSC phenotyping
		7 – Differentiation evaluation
		8 – Functional Assays
H	BMA + BMAC used together	1 – Collection and injection
		2 – Description of harvesting
		3 – Cell count
		4 – Dosage of cytokines (GF and/or IL)
		5 - CFU
		6 – HSC and/or MSC phenotyping
		7 – Differentiation evaluation
		8 – Functional assays

BMA: Bone marrow aspirate; BMAC: Bone marrow aspirate concentrate; CFU: colony-forming unit; MSC: Mesenchymal stem cell; HSC: Hematopoietic stem cells.

respect to generalization and reproducibility. In order to optimize the efficacy and safety of BM-derived products, and to allow validation and standardization of such products, studies should report stepwise descriptions of the preparation protocol and additional information to further classify the product used. The ACH classification focuses on describing parameters that are relevant for the quality of biological products, such as the collection technique, cell count and its nature (whether stromal or hematopoietic), and molecular content dose. The ACH classification would contribute to a greater understanding of both clinical procedures and research outcomes and, over time, lead to a standardization of best practice. Together, we believe that the ACH Classification proposal is an easily recalled and useful method for the classification of BM-derived products in order to provide a comparative between product composition and clinical outcomes.

It should also be emphasized that this classification is pertaining only to BMA products. There are other aspects of bone marrow preparation such as photobiomodulation of the aspirate or the concentrate that have not been discussed. Unfortunately, there is not much literature supporting this concept. Thus, this is mentioned as a matter of anecdotal interest.

Table 2 Example of ACH classification

1	2	3	4	5	6	7	8	Example
X	X	X		X	X	X		1-3; 5-7
X		X	X	X	X			1; 3-6
	X	X	X			X	X	2-4; 7-8
X	X	X					X	1-3; 8

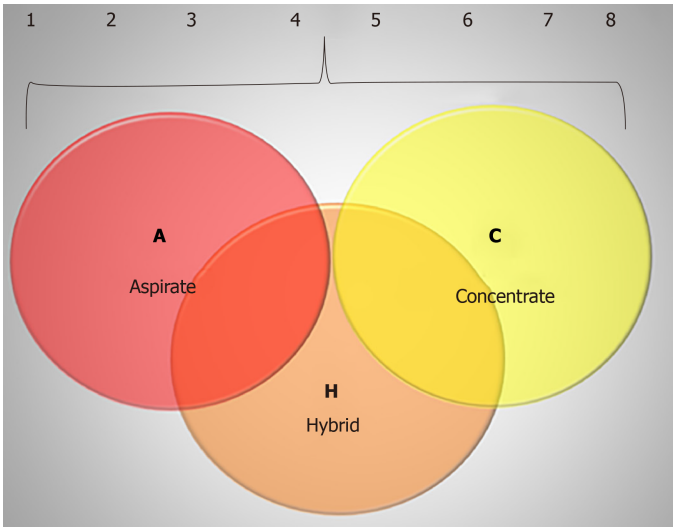

Figure 1 Schematic example of ACH classification proposal.

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REVIEW

Overview of noncoding RNAs involved in the osteogenic differentiation of periodontal ligament stem cells

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Abstract

Periodontal diseases are infectious diseases that are characterized by progressive damage to dental support tissue. The major goal of periodontal therapy is to regenerate the periodontium destroyed by periodontal diseases. Human periodontal ligament (PDL) tissue possesses periodontal regenerative properties, and periodontal ligament stem cells (PDLSCs) with the capacity for osteogenic differentiation show strong potential in clinical application for periodontium repair and regeneration. Noncoding RNAs (ncRNAs), which include a substantial portion of poly-A tail mature RNAs, are considered "transcriptional noise." Recent studies show that ncRNAs play a major role in PDLSC differentiation; therefore, exploring how ncRNAs participate in the osteogenic differentiation of PDLSCs may help to elucidate the underlying mechanism of the osteogenic differentiation of PDLSCs and further shed light on the potential of stem cell transplantation for periodontium regeneration. In this review paper, we discuss the history of PDLSC research and highlight the regulatory mechanism of ncRNAs in the osteogenic differentiation of PDLSCs.

Key words: Noncoding RNAs; Periodontal regeneration; Periodontal ligament stem cells; Osteogenic differentiation

Core tip: Periodontal ligament stem cells (PDLSCs) are widely utilized in therapeutic applications for periodontium repair and regeneration in periodontal disease treatment. However, more evidence is required to elucidate what determines and regulates the multilineage differentiation potential of PDLSCs. Noncoding RNAs (ncRNAs) are essential elements in gene expression and signal transduction, being involved in diverse cellular processes and diseases. Concerning ncRNAs that may collectively or individually alter the osteogenic differentiation of PDLSCs, this review is based on current studies and aims to summarize the most significant ncRNAs identified in the osteogenic differentiation of PDLSCs.

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INTRODUCTION

Periodontal diseases are infectious diseases characterized by progressive destruction of the periodontium (tooth-supporting tissue), which includes the periodontal ligament (PDL), cementum, alveolar bone, and gingiva[1]. Tooth loss mainly results from periodontal diseases in adults, which adds a substantial burden to public health worldwide[2,3]. Periodontal treatment is not as easy as only controlling inflammation and preventing disease development; the reconstruction of a healthy periodontium destroyed by diseases deserves equal attention[4,5]. Current therapies for periodontal diseases in the clinic, including conservative approaches, radicular conditioning, bioactive bone grafting/substitution, and guided tissue regeneration (GTR), encounter difficulty in regenerating the periodontium completely[6]. Therefore, the stem cell-based tissue regeneration approach involving transplantation of stem cells to enhance periodontal tissue regeneration has gradually taken the place of guided bone/tissue regeneration[7-9].

PDL is a specialized soft connective tissue that connects the cementum and alveolar bone; it shows the function of maintaining and supporting teeth *in situ*, preserving tissue homoeostasis and repairing damaged periodontal tissue[1]. In the 1980s, Bordin *et al*[10] reported that PDL tissue possessed periodontal regenerative properties due to its resident cells, which were considered to be seed cells and a reliable source for periodontium regeneration. In 2004, Seo *et al*[11] first identified and characterized multipotent stem cells in human PDL and termed them periodontal ligament stem cells (PDLSCs). PDLSCs show similar features to other postnatal mesenchymal stem cells (MSCs): Multilineage differentiation potential and potent self-renewal ability. PDLSCs can further differentiate into cementoblasts/osteoblasts, chondrocytes and adipocytes *in vitro* and regenerate cementum/PDL-like tissues *in vivo*[12]. As a consequence, PDLSC-mediated periodontium tissue regeneration is likely to be a practical cellular-based treatment for periodontal diseases[13]. However, what determines and regulates the multilineage differentiation potential of PDLSCs warrants further research.

Instead of the potential to encode proteins or peptides, noncoding RNAs (ncRNAs) are a category of unique RNAs that are widely present in eukaryotic cells[14-16]. Following the development of this field, scientists have determined that ncRNAs play a significant role in the regulation of gene expression by controlling the expression levels of protein-coding RNAs and are involved in diverse cellular processes, including cell proliferation, cell differentiation, and ontogenesis, and are thus closely related to embryonic development and disease pathogenesis[17-19]. However, there are currently no uniform criteria for ncRNA classification. ncRNAs can be divided into cytoplasmic and nuclear ncRNAs based on their subcellular localization. In addition, ncRNAs are generally categorized into structural and regulatory ncRNAs, as well as regarding their function in cellular processes[20]. Structural ncRNAs include ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), whereas regulatory RNAs can be further divided into categories based on their length, such as long noncoding RNAs (lncRNAs) (size from > 200 nt to 100 kb) and several types of small RNAs, which include small interfering siRNAs (18–30 nt), piwiRNAs (24–30 nt) and microRNAs (miRNAs, 20–24 nt)[21]. Circular RNAs (circRNAs) are covalently linked to the end of RNA molecules, in which the 3′ and 5′ ends are connected in a non-collinear way through the back-splicing process[22]. CircRNAs, which are a type of competing endogenous RNA (ceRNA), can act as miRNA sponges. Recently, growing research has indicated that circRNAs are involved in embryonic development, cellular activities, and many other human diseases[23-25]. In summary, the cell differentiation of PDLSCs is collectively or individually regulated by ncRNAs. This review focuses on the three most important ncRNAs, namely, miRNAs, lncRNAs and circRNAs, currently identified to play a role in osteogenic differentiation (Tables 1 and 2).

Table 1 Expression profile of ncRNAs involved in the osteogenic differentiation of periodontal ligament stem cells

ncRNAs	Method	Subjects	Results	Ref.
mi-RNA	Microarray	3 hPDLSCs from normal healthy premolars without periodontitis or caries cultured in mineralized medium and 3 in non-mineralized medium	30 upregulated and 86 downregulated miRNAs (P < 0.05, FC ≥ 2)	Hao *et al*[37] 2017
mi-RNA	Microarray	6 samples from untreated hPDLSCs and 6 hPDLSCs under osteogenic induction	miR-24-3p with the minimum fold change was significantly downregulated (P < 0.05, FC ≥ 2)	Li *et al*[38] 2019
mi-RNA	Microarray	3 hPDLSCs cultured with 5.5 mmol/L glucose or 3 cultured with 25 mmol/L glucose mineralized medium	Analyzed 700 miRNAs and found miR-31 was the most upregulated in hPDLSCs cultured with 25 mmol/L glucose (P < 0.01, FC ≥ 2)	Zhen *et al*[48] 2017
mi-RNA	Microarray	3 hPDLSCs from 3 volunteers not subjected to stretch and 3 hPDLSCs from 3 volunteers subjected to mechanical stretch	26 miRNAs were up-regulated while 27 miRNAs were down-regulated with stretching (P < 0.01, FC ≥ 2)	Wei *et al*[55] 2014
lncRNA	RNA sequencing	3 PDLSCs every group cultured in an osteogenic medium for 0, 3, 7, or 14 d	lncRNAs showed stage-specific expression, and 17 lncRNAs were up-regulated while31 were down-regulated in PDLSCs in an osteogenic medium for 3, 7, or 14 d (P < 0.05, FC ≥ 2)	Zheng *et al*[68] 2018
lncRNA	Microarray	3 hPDLSCs both in osteoblast-induced group and non-induced group	994 lncRNAs were up-regulated and 1177 lncRNAs were down-regulated during osteogenic differentiation in PDLSCs at 14 d (P < 0.05, FC ≥ 2)	Qu *et al*[69] 2016
lncRNA	RNA sequencing	3 hPDLSCs subjected to static compressive stress (2 g/cm^2) for 12 h and 3 normal hPDLSCs	72 lncRNAs were upregulated and 18 downregulated by compressive stress (P < 0.05, FC ≥ 1.5)	Huang *et al*[70] 2019
lncRNA	Microarray	3 noninduced and 3 osteogenically induced hPDLSCs	12 upregulated and 8 downregulated lncRNAs and MEG3 belonging to significant downregulation genes in induced cells (P < 0.05, FC ≥ 2)	Liu *et al*[73] 2019
lncRNA	Microarray	3 hPDLSCs from 3 normal persons and 3 pPDLSCs from 3 periodontitis patients with osteogenic differentiation	89 lncRNAs were differentially expressed between the two groups of cells and lncRNA-POIR was the most significantly altered between the non-induced group and osteogenic-induced group (P < 0.05, FC > 2)	Wang *et al*[77] 2016
lncRNA and circRNA	RNA sequencing	3 samples from untreated hPDLSCs and 3 hPDLSCs under osteogenic induction	A total of 960 lncRNAs and 1456 circRNAs were found to be differentially expressed (P < 0.05, FC ≥ 2)	Gu *et al*[67] 2017
circRNA	RNA sequencing	3 hPDLSCs were subjected to mechanical force and 3 hPDLSCs were not subjected to force	identified 2970 and 2788 circRNAs, respectively, in the control group and the force group, and 1191 circRNAs were significantly upregulated and 1,487 were downregulated in the force group (P < 0.05, FC > 2)	Wang *et al*[89] 2019

miRNAs: MicroRNAs; lncRNAs: Long non-coding RNAs; circRNAs: Circular RNAs; hPDLSCs: Human periodontal ligament stem cells; PDLSCs: Periodontal ligament stem cells; MEG3: Maternally expressed gene 3; POIR: Osteogenesis impairment-related lncRNA of PDLSCs.

Table 2 Overview of ncRNAs involved in the osteogenic differentiation of periodontal ligament stem cells

ncRNAs	Regulatory levels	Modes of action	Associated signaling pathways or biomarkers	Ref.
miR-24-3p	Posttranscriptional regulation	Interacting with Smad5 3′-UTR	Inhibits Smad5 and Runx2, BMP2, OCN biomarkers	Li *et al*[38] 2019
miR-21	Posttranscriptional regulation	Interacting with Smad5 3′-UTR	Inhibits Smad5 and Runx2, ALP, BSP, OSX biomarkers	Wei *et al*[39] 2017
		Interacting with Spry1 3′-UTR	Inhibits Spry1 and Runx2, OSX biomarkers	Yang *et al*[40] 2017
		Interacting with ACVR2B 3′-UTR	Inhibits ACVR2B and enhances Runx2, OCN, ALP biomarkers	Wei *et al*[56] 2015
miR-203	Posttranscriptional regulation	Interacting with Runx2 3′-UTR	Inhibits Runx2 and ALP, OCN, OPN biomarkers	Feng *et al*[41] 2019
miR-1305	Posttranscriptional regulation	Interacting with Runx2 3′-UTR	Inhibits Runx2 and ALP, OCN, OPN biomarkers	Chen *et al*[42] 2017
miR-218	Posttranscriptional regulation	Interacting with Runx2 3′-UTR	Inhibits Runx2 and OCT4, NANOG cytokines	Gay *et al*[43] 2014
miR-214	Posttranscriptional regulation	Interacting with ATF4 3′-UTR	Inhibits ATF4 and Runx2, ALP, OCN biomarkers	Yao *et al*[44] 2017
		Interacting with CTNNB1 3′-UTR	Activates Wnt/β-catenin signaling pathway and inhibits ALP, OCN, BSP biomarkers	Cao *et al*[45] 2017
miR-17	Posttranscriptional regulation	Interacting with TCF3 3′-UTR	Inhibits Runx2, ALP biomarkers	Liu *et al*[46] 2013
		Interacting with Smurf1 3′-UTR	Activates Smad family proteins and Runx2, ALP, OCN biomarkers	Liu *et al*[47] 2011
miR-31	Posttranscriptional regulation	Interacting with Stab2 3′-UTR	Inhibits Stab2 and Runx2, OSX, OCN biomarkers	Zhen *et al*[48] 2017
miR-200c	Posttranscriptional regulation	Interacting with IL-6, IL-1β and CCL-5 3′-UTRs	Inhibits IL-6, IL-1β, CCL-5 and enhances Runx2, ALP, OCN, OPG biomarkers	Hong *et al*[49] 2016
miR-543	Posttranscriptional regulation	Interacting with TOB2 3′-UTR	Inhibits TOB2 and enhances Runx2, ALP, BSP, COL1A1 biomarkers	Ge *et al*[50] 2018
miR-22	Posttranscriptional regulation	Interacting with HDAC6 3′-UTR	Inhibits HDAC6 and enhances Runx2, OCN biomarkers	Yan *et al*[51] 2017
lncRNA TUG1	Transcriptional regulation	Binding with Lin28A protein	Promotes Lin28A and Runx2, ALP, OCN biomarkers	He *et al*[72] 2018
lncRNA MEG3	Transcriptional regulation	Suppresses BMP2 through binding with hnRNPI protein	Inhibits BMP2 and Runx2, ALP, OCN biomarkers	Liu *et al*[73] 2019
lncRNA ANCR	Transcriptional regulation	Activates Wnt/β-catenin signaling pathway indirectly	Activates Wnt/β-catenin signaling pathway and inhibits ALP, BSP, DSPP, OCN, Runx2, Gsk3-β biomarkers	Jia *et al*[74] 2015
	Posttranscriptional regulation	lncRNA ANCR/miR-758/Notch2 axis "ceRNA"	Activates Notch2-Wnt/β-catenin signaling pathway and inhibits ALP, Runx2, OSX biomarkers	Peng *et al*[78] 2018
	Epigenetic regulation	Interacting with EZH2 and catalysis H3K27me3 of Runx2	Inhibits Runx2 and ALP, OCN biomarkers	Zhu *et al*[82] 2013
lncRNA POIR	Posttranscriptional regulation	LncRNA POIR/miR 182/FoxO1 axis "ceRNA"	Inhibits TCF-4-Wnt/β-catenin signaling pathway and promotes ALP, Runx2, COL1 biomarkers	Wang *et al*[77] 2016
lncRNA PCAT1	Posttranscriptional regulation	A feed-forward regulatory loop of lncPCAT1/miR-106a-5p/E2F5/ BMP2 axis "ceRNA"	Activates BMP2 and ALP, Runx2, OSX biomarkers	Jia *et al*[79] 2019
lncRNA HIF1AAS2	Posttranscriptional regulation	Base complementary pairing with the mRNA of HIF-1α	Inhibits HIF-1α and ALP, Runx2 biomarkers	Chen *et al*[80] 2017

circRNA CDR1as	Posttranscriptional regulation	circRNA CDR1as /miR-7/GDF5 axis "ceRNA"	Enhances Smad1/5/8 and p38 MAPK phosphorylation and promotes ALP, BMP2, Runx2, OCN biomarkers	Li *et al*[88] 2018
circRNA3140	Posttranscriptional regulation	CircRNA3140/miR-21/ACVR2B axis "ceRNA"	Promotes ACVR2B and inhibits Runx2, OCN, SP7 biomarkers	Wang *et al*[89] 2019

miRNAs: MicroRNAs; lncRNAs: Long non-coding RNAs; circRNAs: Circular RNAs; hPDLSCs: Human periodontal ligament stem cells; PDLSCs: Periodontal ligament stem cells; Smad5/: SMAD family member 5; Runx2: Runt-relatedtranscriptionfactor 2; BMP2: Bone morphogenetic protein-2; OCN: Osteocalcin; ALP: Alkaline phosphatase; BSP: Bone Sialoprotein; OSX: Osterix; Spry1: Palmitate phosphoprotein Sprouty1; ACVR2B: Activin A receptor type 2B; OPN: Osteopontin; OCT4: Octamer-binding transcription factor-4; NANOG: Homeobox transcription factor nanog; ATF4: Activated transcription factor 4; CTNNB1: Catenin beta 1; TCF3: Transcriptional factor 3; Smurf1: Smad ubiquitin regulatory factor 1; Satb2: Special AT-rich sequence-binding protein 2; IL: Interleukin; CCL-5: Chemokines-5; OPG: Osteoprotegerin; TOB2: Transducer of ERBB2; COL1A1: Collagen type I alpha 1 chain; HDAC6: Histone deacetylase 6; Lin28A: Lin-28 homolog A; hnRNP I: Heterogeneous nuclear ribonucleoprotein I; DSPP: Dentin sialophosphoprotein; GSK3β: Glycogen synthase kinase 3β; ANCR: Anti-differentiation noncoding RNA; Notch2: Neurogenic locus notch homolog protein 2; ceRNA: Competing endogenous RNAs; EZH2: Enhancer of zeste homolog 2; H3K27me3: Histone H3 trimethylated at lysine 27; POIR: Osteogenesis impairment-related lncRNA of PDLSCs; FoxO1: Forkhead box O1; TCF4: Transcription factor 4; COL1: Collagen type I; PCAT1: Prostate cancer-associated ncRNA transcript-1; HIF1AAS1/2: HIF1A antisense RNA 1/2; HIF-1α: Hypoxia-inducible factor-1α; CDR1as: Antisense to the cerebellar degeneration-related protein 1 transcript; GDF5: Growth differentiation factor 5; MAPK: Mitogen-activated protein kinase; ACVR2B: Activin A receptor type 2B; SP7: Transcription Factor Sp7.

HISTORY OF PDLSCS

The progenitor cells residing within the PDL (periodontal ligament progenitor, PDLPs) were first described in seminal studies by Melcher in 1994[26]. Seo *et al*[11] described the identification and characterization of multipotent stem cells in human PDL in 2004, although these cells had been suspected to be present in the PDL for a long time. Nevertheless, there is no uniform standard for defining the features of PDLSCs. Often, reports suggest that the isolation of particular subsets of cells from bulk explant cultures is far less rigorous and was too liberal for the use of the term PDLSC[27]. Prateeptongkum *et al*[28] reported that the isolation methods of PDLPs and PDLSCs from PDL tissues are different and demonstrated that PDLPs could be isolated using outgrowth methods, while PDLSCs need single-cell isolation methods for isolation. PDLSCs can be further characterized by their cell surface expression of CD29, CD44, STRO-1, STRO-4, CD146, CD73, CD90, CD105 and CD166 and the lack of expression of endothelial (CD31), haematopoietic (CD14, CD34, CD45, and CD79a), and helper immune antigens (HLA-DR, CD40, CD54, CD80, and CD86)[10,29]. Functionally, PDLSCs have been determined to fulfil all of the criteria of identifiable MSC-like properties, including self-renewal capacity, multipotency *in vitro*, tissue regenerative capacity *in vivo*, and immunomodulation[30,31]. These processes are illustrated in Figure 1.

MICRORNAS IN PDLSCS: ORCHESTRATING CELLULAR OSTEOGENIC DIFFERENTIATION

miRNAs are endogenous, single-stranded noncoding RNAs derived from genomic sequences[32]. The lengths of mature miRNAs are typically 20~24 nucleotides, 8 of which are identified as the "seed sequences" (with nt positions 2 to 7 that were 99% conserved)[33,34]. miRNAs have been extensively investigated in the past two decades, and the underlying mechanism is relatively clear. Mature miRNAs are targeted to a sequence in the 3′ UTR (untranslatedregion) of mRNAs matching the seed sequence and further influence the stability of mRNAs or inhibit their translation to eventually downregulate protein expression[35]. miRNAs are a leading representative of small ncRNAs, and they are closely associated with diverse biological and pathological processes.

miRNA microarrays are a widely accepted high-throughput method and are very effective in analysing miRNA expression levels during osteogenic differentiation of PDLSCs[36]. Our team used a miRNA microarray to detect the different expression profiles of miRNAs in PDLSCs during the osteogenic differentiation process *in vitro*[37]. The results showed a significant change in the expression level of 116 miRNAs, 30 of which were increased, while 86 miRNAs were downregulated in PDLSCs after 14 d of osteogenic induction. The results probably suggested an important regulatory role that miRNAs might play in the osteogenic differentiation of PDLSCs.

Similarly, a microarray was used in the study of Li *et al*[38] to detect the expression level of miRNAs in differentiated and undifferentiated PDLSCs and demonstrated that the expression level of miR-24-3p was significantly downregulated in

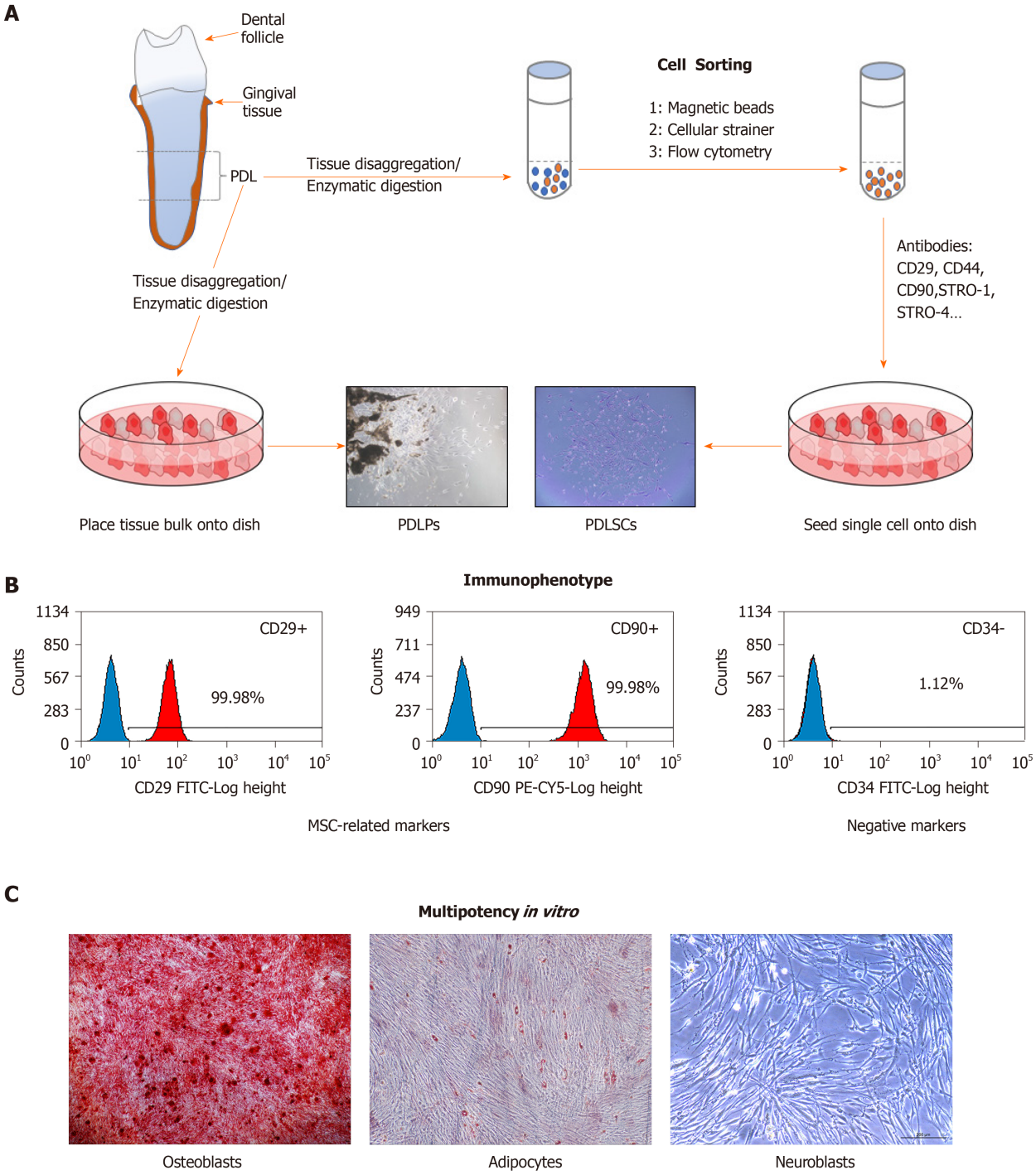

Figure 1 Isolation and characterization of periodontal ligament stem cells. A: Diagram of the isolation of periodontal ligament stem cells (PDLPs) and PDLSCs from human PDL tissue; B: Flow cytometric analysis to assess the immunophenotype of PDLSCs. Markers of mesenchymal stem cells (CD29, CD90) and non-mesenchymal stem cells (CD34); C: Assessment of the differentiation potential of PDLSCs *in vitro*. PDL: Periodontal ligament; PDLPs: Periodontal ligament progenitor; PDLSCs: Periodontal ligament stem cells.

osteogenically differentiated PDLSCs. Furthermore, double luciferase reporter assays and genetic engineering experiments demonstrated that miR-24-3p directly bound to the 3′-UTR of transduction protein 5 (SMAD family member 5, Smad5) and inhibited the transcription of the target gene.

The inhibition of the osteogenic differentiation of PDLSCs was the result of miR-21 downregulating the expression of Smad5 in the research of Wei *et al*[39]. Yang *et al*[40] found that the inhibition was attributed to the regulation of the miR-21/palmitate phosphoprotein Sprouty1 axis by tissue tumour necrosis factor-α. In addition, miR-203, miR-1305, and miR-218 have all been confirmed to target runt-related transcriptionfactor 2 (Runx2) and play important inhibitory roles in the osteogenic differentiation of PDLSCs[41-43]. miR-214 not only targeted activated transcription factor

4[44] but also bound with catenin beta 1 to modulate the Wnt/β-catenin signalling pathway, which is involved in osteogenic differentiation of PDLSCs[45]. Liu *et al*[46] found that miR-17 regulated the osteogenic differentiation of PDLSCs by reducing the expression of transcriptional factor 3 and inhibiting the Wnt signalling pathway. In contrast, Liu *et al*[47] demonstrated that miR-17 promoted differentiation by binding to the Smad ubiquitin regulatory factor one 3′-UTR in PDLSCs isolated from PDL tissue from periodontitis patients. miR-31 plays a regulatory role by targeting special AT-rich sequence-binding protein 2 in osteogenic differentiation mediated by a high dose of glucose in PDLSCs[48]. All of the abovementioned miRNAs exerted inhibitory effects on osteogenic differentiation by targeting osteogenesis-related transcription factors through the classic miRNA regulatory mechanism.

Although several miRNAs suppress the osteogenic differentiation of PDLSCs, recent research has revealed that miRNAs promote the osteogenic differentiation of PDLSCs, including miR-200c, miR-543 and miR-22. Hong *et al*[49] demonstrated that miR-200c decreased the levels of interleukin-6, interleukin-8 and chemokines-5 and increased the osteogenic differentiation of PDLSCs and BMSCs. miR-200c is a potentially effective means of preventing periodontitis-associated bone loss by arresting inflammation and osteoclast/osteogenesis and regenerating bone tissue. Previous research by our team found that miR-543 directly interacted with the 3′-UTR of transducer of ERBB2 and promoted osteogenesis in PDLSCs[50]. Yan *et al*[51] claimed that miR-22 promoted the osteogenic differentiation of PDLSCs by inhibiting the expression of histone deacetylase 6.

According to previous work, one of the factors affecting osteogenic differentiation of PDLSCs is mechanical stretch[52-54]. To investigate miRNA expression specifically in stretched PDLSCs, a microarray assay was utilized by Wei *et al*[55] to describe the differential expression of miRNAs in normal and stretched PDLSCs by using a tension system to achieve external mechanical stimulation. The results showed that 53 miRNAs were differentially expressed in stretched PDLSCs, and 26 of the miRNAs were upregulated, while 27 were downregulated. Noticeably, miR-21 directly targeted the 3′-UTR of activin A receptor type 2B (ACVR2B), thereby reducing the expression of ACVR2B and repressing the osteogenic differentiation of stretched PDLSCs[56].

The main regulatory mechanism of microRNAs is the posttranscriptional repression of target genes. However, several studies have reported that miRNAs function in other unconventional ways, including pri-miRNAs coding for short peptides and miRNAs interacting with non-AGO proteins, activating toll-like receptors, upregulating protein expression, targeting mitochondrial transcripts, directly activating transcription, and targeting nuclear ncRNAs[57-59]. To date, research has mainly focused on the classic regulatory mechanism of miRNAs in PDLSCs, and other regulatory mechanisms require further in-depth exploration.

LNCRNAS INVOLVED IN OSTEOGENIC DIFFERENTIATION OF PDLSCs

LncRNAs are a family of RNA molecules with transcript lengths of 200 nt to 20000 nt. These RNAs are unable to encode proteins or are only translated into small peptides at a very low level. Initially, lncRNAs were identified as a by-product of RNA polymerase II transcription and thought to be the "noise" of genomic transcription (referred to as "the dark matter" of the genome) with no biological function[36,60,61]. However, research on lncRNAs has rapidly developed in recent years. According to their position relative to protein-coding genes in the genome, lncRNAs can be divided into five types: Sense, antisense, bidirectional, intronic, and intergenic lncRNAs[62]. Recent studies show that lncRNAs act as novel and important regulators of numerous biological, developmental, and numerous cellular processes, including chromatin modification, X-chromosome silencing, genomic imprinting, transcriptional activation or interference, and intranuclear transport[63], and act through such mechanisms as transcriptional regulation, posttranscriptional regulation, and epigenetic regulation[64-66].

Gu *et al*[67] compared the lncRNA profiles of PDLSCs on the 7th day with or without osteogenic differentiation medium using RNA sequencing. The results showed that 17 lncRNAs were upregulated and 31 were downregulated during osteogenic differentiation in PDLSCs. Zhang *et al*[68] also used RNA sequencing to detect the different expression profiles of lncRNAs in PDLSCs at different time points during osteogenic differentiation. These results indicated that 48 lncRNAs had significant changes on days 3, 7 and 14, of which 17 lncRNAs were upregulated and 31 were downregulated in PDLSCs. Our team used a lncRNA microarray to determine the expression levels of lncRNAs in osteoblast-induced and noninduced PDLSCs, and the

results showed that 994 lncRNAs were upregulated and 1177 lncRNAs were downregulated at 14 d of osteogenic differentiation in PDLSCs. Further GO analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that a total of 83 signalling pathways were involved in the osteogenic differentiation of PDLSCs, including the mitogen-activated protein kinase (MAPK) and transforming growthfactor-β signalling pathways. In addition, coding-noncodinggenecoexpression analysis indicated a potential regulatory relationship between the differentially expressed lncRNAs and the osteogenesis-related mRNAs, of which 131 pairs of lncRNAs and mRNAs had negative correlations and 262 pairs had positive correlations[69]. Huang *et al*[70] used RNA sequencing to describe the lncRNA landscape during osteogenic differentiation in PDLSCs subjected to compressive force. The results indicated that 90 lncRNAs and 519 mRNAs were differentially expressed in PDLSCs under compressive stress. Of the lncRNAs, 72 were upregulated, and 18 were downregulated. To summarize, investigations of the regulatory mechanisms of lncRNAs in the osteogenic differentiation of PDLSCs are mainly focused on transcriptional regulation and posttranscriptional regulation.

Transcriptional regulation by lncRNAs in PDLSCs

Studies of the underlying mechanisms indicated that lncRNAs can specifically bind with a specific family of proteins called RNA binding proteins (RBPs) to regulate the biological functions of these proteins and then affect the transcriptional activation of downstream genes[71]. As a novel protein type, RBPs have been demonstrated to interact with lncRNAs and have attracted increasing attention from researchers. He *et al*[72] identified the novel lncRNA- taurine upregulated gene 1 (TUG1), which was significantly upregulated in osteogenically induced PDLSCs. Gain/loss-of-function experiments showed that lncRNA-TUG1 was positively correlated with the osteogenic differentiation of PDLSCs following induction. Meanwhile, bioinformatic analysis demonstrated that lin-28 homologue A (Lin28A, a member of the RBP family) containing multiple binding sites of lncRNA-TUG1 was a potential target in the process of osteogenic differentiation of PDLSCs. Further investigation demonstrated that suppression of Lin28A inhibited the expression of several osteogenic-related gene markers, such as alkaline phosphatase, osteocalcin, and Runx2, in PDLSCs. These results suggested that lncRNA-TUG1 might interact with Lin28A to form a positive regulatory network of osteogenic-related genes to promote osteogenic differentiation of PDLSCs. However, there is insufficient evidence about the direct binding between lncRNA-TUG1 and Lin28A in this study. In a study by Liu *et al*[73], lncRNA maternally expressed gene 3 (MEG3) was markedly downregulated in osteogenically differentiated human PDLSCs compared with undifferentiated cells, as determined by microarray analysis. Overexpression of lncRNA MEG3 inhibited the activation of bone morphogenetic protein-2 (BMP2) and reversed osteogenic differentiation induced by mineralizing solution in PDLSCs. Furthermore, RNA-binding protein immunoprecipitation (RIP) assays verified that lncRNA MEG3 suppressed BMP2 through direct interaction with heterogeneous nuclear ribonucleoprotein I (hnRNPI) during osteogenic differentiation in hPDLCs. These results indicated that lncRNA MEG3 could modulate the osteogenic differentiation of hPDLCs by interacting with hnRNPI and thus inhibiting the transcriptional activity of BMP2. Moreover, lncRNAs can also interfere with the transcriptional activation of mRNAs or other ncRNAs. Jia *et al*[74] found an inhibitory effect of ANCR (anti-differentiation noncoding RNA) on the gene expression of glycogen synthase kinase 3β and Runx2 and the classic Wnt/β-catenin signalling pathway, thereby suppressing osteogenic differentiation of PDLSCs.

Posttranscriptional regulation of lncRNAs in PDLSCs

Recent studies revealed that lncRNAs might act as miRNA sponges to compete with target genes for miRNA binding sites, thereby affecting the activity of the targeted genes in various biological processes[75]. These regulatory mechanisms involving lncRNAs, miRNAs and mRNAs are one type of ceRNA[76] mechanism. Wang *et al*[77] successfully identified the novel lncRNA-POIR (osteogenesis impairment-related lncRNA of PDLSCs), which is an osteogenesis impairment-related lncRNA of PDLSCs, and the gradual reduction in the expression of this lncRNA in PDLSCs was recorded among periodontitis patients, as demonstrated by lncRNA microarray analysis. Overexpression or knockdown of lncRNA-POIR was performed, and lncRNA-POIR was shown to positively regulate osteogenic differentiation of PDLSCs both *in vitro* and *in vivo*. Further luciferase reporter assays and quantitative real-time PCRs demonstrated that lncRNA-POIR was likely to act as a ceRNA for miR-182, thereby leading to derepression of the target gene Forkhead box O1 (FoxO1). Activated FoxO1 could compete with transcription factor 4 for β-catenin binding and inhibit the classic Wnt signalling pathway, thereby promoting osteogenic

differentiation of inflammatory PDLSCs. On the other hand, Peng *et al*[78] suggested that lncRNA-ANCR targeted miR-758 directly as a molecular sponge *via* RNA immunoprecipitation, and a dual luciferase reporter assay was also performed to demonstrate that miR-758 modulates the transcript expression of neurogenic locus notch homolog protein 2 (Notch2) by targeting the 3′-UTR of Notch2. These findings suggest that the lncRNA-ANCR/miR-758/Notch2 axis plays an essential role in regulating the osteogenic differentiation of PDLSCs. In addition, prostate cancer-associated ncRNA transcript-1 (lncPCAT1) was significantly increased in osteogenically induced PDLSCs and could positively regulate osteogenic differentiation both *in vitro* and *in vivo* according to the study of Jia *et al*[79]. Thereafter, these researchers inferred a predicted interaction and then confirmed the direct binding sites of miR-106a-5p on lncPCAT1. In conclusion, lncRNA-PCAT1 promoted osteogenic differentiation of PDLSCs by sponging miR-106a-5p to upregulate the miR-106a-5p-targeted gene BMP2. Interestingly, the authors also found that another target of miR-106a-5p, E2F5, could bind to the promoter of lncPCAT1 and then form a feed-forward regulatory network targeting BMP2. Chen *et al*[80] studied two lncRNAs, HIF1A antisense RNA 1 and HIF1A antisense RNA 2, that regulated the mRNA expression of HIF1α. The results showed that HIF1A-AS2 exerted a remarkable negative regulatory function on hypoxia-inducible factor-1α (HIF-1α) through complementary base pairing with HIF-1α mRNA in PDLCs under hypoxia.

Epigenetic regulation of lncRNAs in PDLSCs

Epigenetics plays a central role in regulating many critical cellular processes. From the perspective of epigenetics, several lncRNAs can interact with protein complexes and modulate the levels of DNA methylation as coordinators of chromatin modification, thereby regulating the expression of related genes at the epigenetic level[81]. Studies have claimed that lncRNAs affect the osteogenic differentiation of PDLSCs by epigenetic regulation. Zhu *et al*[82] found that lncRNA ANCR posed a physical interaction with enhancer of zeste homologue 2, and Runx2 expression was suppressed due to the catalysis of H3K27me3 in the Runx2 gene promoter, leading to the inhibiting effect on osteoblast differentiation of hFOB1.19 cells. At present, further exploration is needed to explore the function of lncRNAs in regulating the osteogenic differentiation of PDLSCs at the epigenetic level.

The discovery of lncRNAs greatly broadened the understanding of molecular regulatory mechanisms. LncRNAs regulate the expression of related genes via three different regulatory mechanisms at the levels of transcription, posttranscription, and epigenetics and participate in many important cellular processes. Current studies on lncRNAs regulating the osteogenic differentiation of PDLSCs have mainly focused on the transcriptional and posttranscriptional levels. However, most studies have not determined the subcellular localization of lncRNAs. Generally, the localization of an lncRNA in the cytoplasm is important when considering its functions, especially as a ceRNA sponge[83]. Due to the numerous lncRNAs and their complexity, the mechanisms of lncRNAs regulating osteogenic differentiation in PDLSCs need to be explored further.

CIRCRNAS ASSOCIATED WITH OSTEOGENIC DIFFERENTIATION OF PDLSCS

circRNAs are a novel class of endogenous lncRNAs that are characterized by a structure of covalently closed continuous loops lacking 5′ or 3′ polarities and are widely present in eukaryotic cells[84]. The circRNAs are more stable than linear RNAs because of their covalently circular structure, making them more resistant to RNase R digestion. The majority of circRNAs are conserved across species and often exhibit cell type-specific, tissue-specific or developmental stage-specific expression[85]. Evidence is increasing that circRNAs might act as miRNA sponges and play critical roles in signal transduction in a posttranscriptional manner. The circRNA-miRNA axis is involved in several cellular processes, such as proliferation, differentiation and apoptosis[86].

Gu *et al*[67] conducted high-throughput sequencing to detect the different expression profiles of circRNAs in PDLSCs after 7 d of osteogenic differentiation. These researchers found that 766 circRNAs were significantly upregulated and 690 circRNAs were downregulated in PDLSCs. Furthermore, the authors predicted the potential functions of circRNAs as ceRNAs based on miRanda analysis and further investigated them using GO and KEGG analysis. The results showed that a total of 1382 circRNAs (including circRNA PTPRG, EXOC4, PRKCA, and SETBP1) were predicted to be able to interact with 148 miRNAs and compete for miRNA binding sites with 744 mRNAs, which were predicted to be significantly associated with

osteoblast differentiation and the MAPK and Wnt signalling pathways regulating pluripotency of mesenchymal stem cells. Among these circRNAs, one circRNA could bind with multiple miRNAs, and the same miRNA could also interact with multiple circRNAs. Li *et al*[87] revealed that circRNA antisense to the cerebellar degeneration-related protein 1 transcript (CDR1as) could act as a miR-7 adsorption sponge and then induce the upregulation of growth differentiation factor 5 and activate the Smad1/5/8 and p38MAPK signalling pathways, thereby promoting osteogenesis in PDLSCs. Wang *et al*[88] found that circRNA expression patterns were responsive to mechanical force in PDLSCs. Bioinformatic analysis showed that one circRNA could modulate one or several miRNA/miRNAs and vice versa. Importantly, the authors found that circRNA3140 was widely and highly related to microRNA-21, which played a key role in mechanical force-induced osteogenic differentiation of PDLSCs. These findings revealed that mechanical force induced the differential expression of circRNAs in PDLSCs, which might regulate the orthodontic tooth movement process and alveolar bone remodelling.

Overall, as a novel class of endogenous lncRNAs, circRNAs may modulate many pathophysiological processes, serve as diagnostic or predictive biomarkers for several diseases, and represent a novel and useful therapeutic method[89]. Compared with research on miRNAs and lncRNAs, research on circRNAs is in its infancy, and the potential functions and regulatory mechanisms of circRNAs are diverse. Investigating the regulatory mechanisms and functions of circRNAs in the osteogenic differentiation of PDLSCs may provide exciting potential therapies in periodontal regeneration.

CONCLUSION

Numerous ncRNAs are associated with the osteogenic differentiation of PDLSCs (Figure 2). ncRNAs offer an additional and promising possibility of osteogenesis-related gene regulation that has not been fully elucidated to date. With increasing numbers of miRNAs, lncRNAs and circRNAs discovered in this process, it has become possible to use these ncRNA-related therapeutic methods in the field of periodontium repair and regeneration.

To date, ncRNA-related research on the osteogenic differentiation of PDLSCs has mainly focused on miRNAs. The demonstrated regulatory mechanism of miRNAs (miR-24-3p, miR-21, miR-203, miR-1305, miR-218, miR-214, miR-17, miR-31, miR-200c, miR-543 and miR-22) is to inhibit the mRNA levels or protein expression of targets. Other unconventional mechanisms could impact osteogenic differentiation. Currently, miRNAs are considered to be potential therapeutic targets based on their defined regulatory mechanism and clear functioning mode. miRNA-based therapeutic methods could become valuable in promoting periodontium repair and regeneration. Similarly, there are several studies on the role of lncRNAs during this process. Among these lncRNAs, lncRNA TUG1, MEG3 and ANCR regulate the osteogenic differentiation of PDLSCs in a transcriptional manner. The lncRNAs POIR, PACT1, HIF1A-AS2 and ANCR act as miRNA sponges and play critical roles during osteogenic differentiation of PDLSCs in a posttranscriptional manner (Figure 3). However, lncRNA-ANCR has been demonstrated to suppress osteogenic differentiation of PDLSCs in an epigenetic regulatory manner. To date, few studies have investigated circRNAs during osteogenic differentiation in PDLSCs. The demonstrated mechanism of circRNAs is to act as miRNA sponges to inhibit the mRNA levels of target genes (circRNA CDR1as and circRNA3140). Other types of ncRNAs involved in the osteogenic differentiation of PDLSCs warrant further exploration.

With the development of sequencing and microarray technologies, numerous novel ncRNAs have been screened out and identified in the past few years, and their regulatory mechanisms have also been predicted and explored, benefiting from the advancement of related bioinformatics databases. Subsequently, standard molecular biology experiments and genetic engineering methods, such as quantitative real-time PCR, western blotting, dual luciferase reporter assays, RNAi and overexpression plasmid transfections, have been used to characterize ncRNA functions and explore their regulatory mechanisms. In addition, some new experimental methods have emerged, such as RIP, RNA pull-down, chromatin isolation by RNA purification, cross-linking immunoprecipitation, cross-linking, ligation, and sequencing of hybrids, and capture hybridization analysis of RNA targets, which provide an ideal research platform for elucidating the signalling transduction mechanisms of ncRNAs. In addition, the regulatory mechanisms of ncRNAs, especially lncRNAs and circRNAs, in cellular processes and diseases are highly complex. However, there are few studies

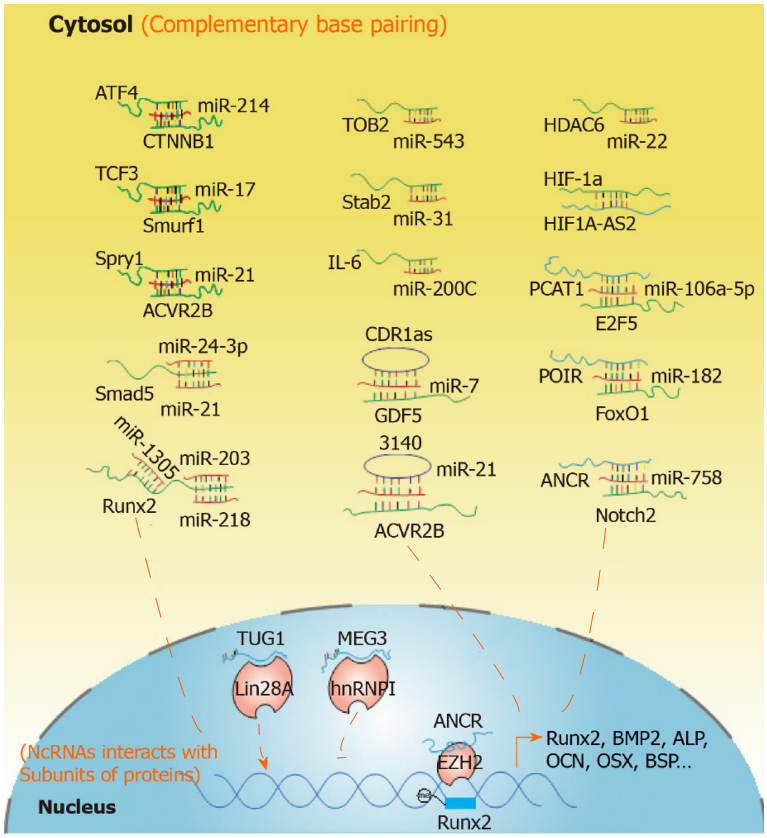

Figure 2 Regulatory mechanisms of noncoding RNAs associated with the osteogenic differentiation of periodontal ligament stem cells. Red indicates miRNAs, green indicates mRNAs, blue indicates long noncoding RNAs and purple indicates circular RNAs. Collectively, the regulatory mechanism of miRNAs is to directly bind to the 3'-UTR of target genes and inhibit the mRNA levels or protein expression. Long noncoding RNAs regulate the osteogenic differentiation of periodontal ligament stem cells at the transcriptional, posttranscriptional or epigenetic level. And the demonstrated mechanism of circular RNAs is to act as miRNA sponges to inhibit the mRNA levels of target genes. ATF4: Activated transcription factor 4; CTNNB1: Catenin beta 1; TCF3: Transcriptional factor 3; Smurf1: Smad ubiquitin regulatory factor 1; Spry1: Palmitate phosphoprotein Sprouty1; ACVR2B: Activin A receptor type 2B; Smad5: SMAD family member 5; Runx2: Runt-relatedtranscriptionfactor 2; TOB2: Transducer of ERBB2; Satb2: Special AT-rich sequence-binding protein 2; IL-6: Interleukin-6; CDR1as: Antisense to the cerebellar degeneration-related protein 1 transcript; GDF5: Growth differentiation factor 5; HDAC6: Histone deacetylase 6; HIF-1α: Hypoxia-inducible factor-1α; HIF1AAS1/2: HIF1A antisense RNA 1/2; PCAT1: Prostate cancer-associated ncRNA transcript-1; E2F5: E2F transcription factor 5; POIR: Osteogenesis impairment-related long noncoding RNA of periodontal ligament stem cells; FoxO1: Forkhead box O1; ANCR: Anti-differentiation noncoding RNA; Notch2: Neurogenic locus notch homolog protein 2; TUG1: Taurine upregulated gene 1; Lin28A: Lin-28 homolog A; MEG3: Maternally expressed gene 3; hnRNP I: Heterogeneous nuclear ribonucleoprotein I; EZH2: Enhancer of zeste homolog 2; BMP2: Bone morphogenetic protein-2; ALP: Alkaline phosphatase, OCN: Osteocalcin; OSX: Osterix; BSP: Bone sialoprotein; miR: MicroRNA; ncRNAs: Non-coding RNAs.

concerning the nonconventional mechanisms of ncRNAs during the osteogenic differentiation of PDLSCs.

Due to their osteogenic differentiation capability, PDLSCs show effective potential in the clinical application of periodontium repair and regeneration (Figure 4). However, reports have appeared that are less rigorous in the isolation and identification of PDLSCs. In addition, most current studies of ncRNAs involved in osteogenic differentiation in PDLSCs have focused on the cell level *in vitro*; therefore, *in vivo* experiments in this field warrant further in-depth exploration. Therefore, whereas interest and investigation in the contribution of ncRNAs to the osteogenesis of PDLSCs have increased considerably, the field is still a long way from understanding the full extent of the contribution of ncRNAs and the mechanisms by which ncRNAs exert their potential effects in this field.

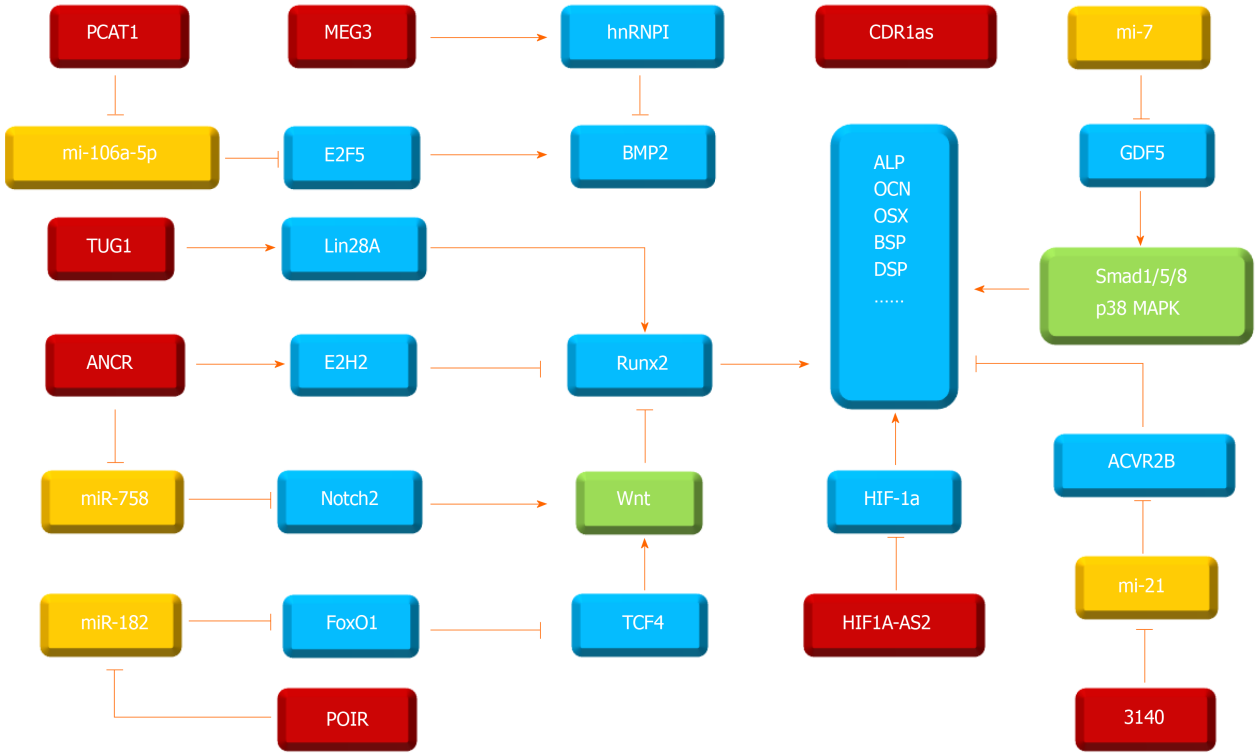

Figure 3 Overview of the role of long noncoding RNAs and circular RNAs during osteogenic differentiation of periodontal ligament stem cells. Red frame indicates long noncoding RNAs (lncRNAs) or circular RNAs (circRNAs), yellow frame indicates miRNAs interacted by lncRNAs and circRNAs, blue frame indicates target mRNAs or osteogenesis-related biomarkers, green frame indicates signaling pathways associated with osteogenic differentiation. These lncRNAs and circRNAs affect downstream factors to trigger related biomarkers or signaling pathways and then regulate the osteogenic differentiation of periodontal ligament stem cells. PCAT1: Prostate cancer-associated ncRNA transcript-1; E2F5: E2F transcription factor 5; MEG3: Maternally expressed gene 3; hnRNP I: Heterogeneous nuclear ribonucleoprotein I; BMP2: Bone morphogenetic protein-2; TUG1: Taurine upregulated gene 1; Lin28A: Lin-28 homolog A; Runx2: Runt-relatedtranscriptionfactor 2; ANCR: Anti-differentiation noncoding RNA; EZH2: Enhancer of zeste homolog 2; Notch2: Neurogenic locus notch homolog protein 2; FoxO1: Forkhead box O1; POIR: Osteogenesis impairment-related lncRNA of periodontal ligament stem cells; TCF4: Transcription factor 4; CDR1as: Antisense to the cerebellar degeneration-related protein 1 transcript; GDF5: Growth differentiation factor 5; Smad1/5/8: SMAD family member 1/5/8; MAPK: Mitogen-activated protein kinase; HIF-1α: Hypoxia-inducible factor-1α; HIF1AAS1/2: HIF1A antisense RNA 1/2; ACVR2B: Activin A receptor type 2B; ALP: Alkaline phosphatase, OCN: Osteocalcin; OSX: Osterix; BSP: Bone Sialoprotein; DSP: Desmoplakin; miR: MicroRNA.

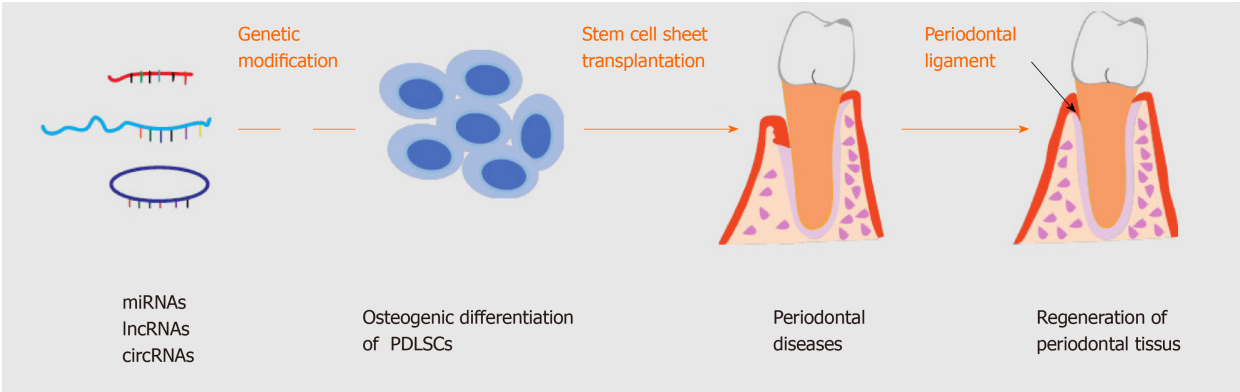

Figure 4 Schematic diagram of noncoding RNAs genetic modification-based periodontal ligament stem cells transplantation therapy applications for periodontium regeneration of periodontal disease. Genetic modification of noncoding RNAs in periodontal ligament stem cells can regulate the capability of osteogenic differentiation. periodontal ligament stem cells sheet with powerful osteogenic differentiation capability be injected or transplanted into the location of bone defects to regenerate the periodontium in periodontal diseases. miRNAs: MicroRNAs; lncRNAs: Long non-coding RNAs; circRNAs: Circular RNAs; PDLSCs: Periodontal ligament stem cells.

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REVIEW

Insights of stem cell-based endogenous repair of intervertebral disc degeneration

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Abstract

Low back pain has become more prevalent in recent years, causing enormous economic burden for society and government. Common therapies used in clinics including conservative treatment and surgery can only relieve pain. Subsequent cell-based treatment such as mesenchymal stem cell transplantation poses problems such as short duration of therapeutic effect and tumorigenesis. Recently, the discovery and identification of stem cell niche and stem/progenitor cells in intervertebral disc bring increased attention to endogenous repair strategy. Therefore, we review the studies involving endogenous repair strategy and present the characteristics and current status of this treatment. Meanwhile, we also discuss the strategy and perspective of endogenous repair strategy in future.

Key words: Low back pain; Intervertebral disc degeneration; Stem cell niche; Stem/progenitor cell; Endogenous repair strategy; Stem cell treatment

Core tip: Low back pain has become more prevalent and brought enormous economic burden in recent years. However, therapies including conservative treatment, surgery, and cell-based treatment still have several defects. Endogenous repair is a novel therapeutic strategy for intervertebral disc degenerative disease that draws increased attention. We review the research regarding endogenous repair strategy using

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stem/progenitor cells as main cell resource, concluding and analyzing the status at present and perspective of endogenous repair strategy in future.

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INTRODUCTION

Low back pain (LBP) has become one of the most frequent causes for hospital visits and the leading reason of disability with population aging in the worldwide today[1]. In addition, about 80% of adults will suffer from LBP at some point in their lives, which brings frequent sick leave and enormous economic burden for society and government[2]. Especially in the United States, the estimated total expenses including direct and indirect costs of LBP exceed $100 billion per year[3]. Despite the complex and dim pathogeny and pathology of LBP, it is widely reported that 40% of LBP cases are associated with intervertebral disc (IVD) degeneration[4-6].

The IVD is fibrocartilaginous tissue located between the vertebral bodies and composed of outer annulus fibrosus (AF), inner nucleus pulposus (NP), and cartilaginous endplates (CEP)[7]. The structure of AF is formed by types I and II collagen fibers and elastin fibers arranged in concentric circles, which can withstand the tension produced by vertebral motion to maintain the position of NP[8]. Meanwhile, the NP tissue is constituted by the extracellular matrix (ECM) including collagen type II and aggrecan synthesized and secreted by NP cells[9]. Therefore, NP cells are so vitally important in IVD degeneration that we should pay more attention to them.

However, clinical therapies such as pharmacological treatments and surgeries are mainly focused on removing NP tissue and thus can only deal with relief of symptoms rather than restoration[10]. Although many studies based on mesenchymal stem cell (MSC) transplantation have obtained positive results in attenuating or even reversing IVD degeneration in pre-clinical and clinical studies[11-13], there are still some obstacles such as survival and differentiation of transplanted MSC caused by a specifically harsh environment in the degenerated IVD[14,15]. Moreover, various complications including oncogenicity, ectopic ossification, and immune reactions indicated that more therapies should be explored[16,17]. Thus, searching a new candidate for degenerative IVD treatment is especially important.

Niche, first proposed by Schofield[18], is a specific anatomic localization composed of ECM and other noncellular components[18,19]. Since this concept was introduced, many niches were found in a variety of tissues and organs including the skin, bone marrow, and the neural and digestive systems. The niche in the IVD defined as the perichondrium region adjacent to the epiphyseal plate (EP) and outer zone of the AF (AFo), raises a growing interest in boosting endogenous repair strategy (ERS) in degenerative IVD[20-24]. And the novel ERS focuses on improving proliferation and promoting differentiation of stem cells derived from the IVD niche.

Thus, this review concentrates on the retrospect and assessment of research regarding the conception of endogenous repair through activating and mobilizing reparative MSCs located in specific anatomical niches of the IVD. Besides that, the review will also analyse the obstacles and difficulties needed to be conquered, which may help to accelerate the process of endogenous repair.

ENDOGENOUS REPAIR STRATEGY

Cell resource of ERS

It has been well accepted that NP cells consist of notochordal cells (NTC) and nucleopulpocytes (NPCy; another known name is chondrocyte-like cells). Among them, NTC are responsible for maintaining tissue homeostasis and promoting growth while NPCy play a vital role in ECM synthesis[25]. However, the NTC usually coexist with NPCy in the young and health IVD and decline with aging, thus other endogenous stem/progenitor cells may help to boost the progress of endogenous

repair in IVD degeneration by differentiating into various damaged IVD cells or excreting intercellular signaling molecules such as exosomes.

The cells first isolated from degenerative human NP and AF cells were characteristic of marrow MSCs and showed capacity of osteogenic, adipogenic, and chondrogenic differentiation[26]. The following research studies illustrated that MSCs derived from NP can better withstand the terrible environment in degenerative IVD with improved proliferation and vitality compared with other MSCs[27-29]. And the results of our previous study showed that the vitality and characteristics of such cells would be affected with aggravative degeneration[30]. Besides that, previous studies have not only proved the presence of stem/progenitor cells in the NP, but also isolated stem/progenitor cells from the AF and CEP[31-33]. Above all, these results show that stem/progenitor cells may migrate from the IVD niche to NP, AF, and CEP tissues. Thus, the key point of endogenous repair strategy is to increase the vitality of stem/progenitor cells in NP tissue or motivate their migration from the niche (Figure 1).

Characteristics of stem/progenitor cells in the NP, AF, and CEP

Stem/progenitor cell immunophenotypes: Those kinds of cells, also called MSCs derived from the NP, AF, and CEP, grow adherently in spindle shape after passage[30] and mostly express MSC-like marker including CD73, CD90, and CD105 but not CD11b, CD14, CD19, CD34, CD45, or HLA-DR according to criteria of International Society for Cellular Therapy (ISCT) (Table 1)[35]. Various research studies also found that NP-MSCs can be isolated from human[40], rat[39,56,58], rabbit[54], and dog samples, which were positive (> 95%) for marker proteins CD29 and CD44 except in dogs. Furthermore, CD13 expressed frequently in granulocyte and CD24 related to proliferation and differentiation of B cells are membrane glycoprotein and detected in NP-MSCs from degenerative and normal IVD, respectively[50,57]. Interestingly, a study by Jia *et al*[54] indicated that NP-MSCs derived from rabbits are negative not only for CD14 but also for CD4 and CD8, which has not been proposed before.

Stem/progenitor genes: Besides cellular markers, stem genes are considered as another criterion for identification of MSCs derived from the IVD, especially in NP tissue. Among them, Nanog (homeobox-containing), Oct4 (the POU domain-containing), and Sox2 (the HMG domain-containing) are transcription factors that play an essential role in the development and maintenance of normal pluripotent cells and are often used to assess the stemness of NP-MSCs (Table 1)[28,30,59]. In addition, Notches and their ligand named Jagged show a crucial effect on the function and differentiation of human bone marrow MSCs (hBMMSC) and NP-MSCs[35,60,61]. Moreover, our previous study provided further evidence that PCNA, CD166, and C-KIT can be chosen as stem/progenitor markers for NP-MSCs and decline with aging[30,62]. Notably, a recent study by Tekari *et al*[63] demonstrated that $Tie2^+$ NP-derived progenitor cells could be maintained in subsequent monolayer culture for up to 7 d by addition of fibroblast growth factor 2 or hypoxic conditions. Thus, it may be better to isolate NP-MSCs by Tie sorting method rather than just by plastic-adherent method[36,63].

Multi-differentiation: Many studies confirmed that MSCs derived from the NP, AF, and CEP have the ability to differentiate into osteogenic, adipogenic, and chondrogenic lineages[35-44]. Liu *et al*[49] compared the characteristics of three types of MSCs derived from human IVD including NP-MSCs, AF-MSCs, and CEP-MSCs and found that they showed similar multilineage differentiation capacities, whereas CEP-MSCs showed the best migration and invasion potency. Moreover, a stronger capacity of osteogenic and chondrogenic differentiation was confirmed by Wang *et al*[41] in CEP-MSCs. Above all, CEP-MSCs may be a new useful candidate for cell-based therapy and ERS.

NP-MSCs, another essential cell resource, show different advantages and drawbacks in differentiated capacity compared with other MSCs. Several reports have shown that NP-MSCs have the regeneration ability similar to BM-MSCs and adipose tissue-derived MSCs with same or superior capacity of chondrogenesis[27,36,45,69]. But in the studies by Blanco *et al*[27] and Wang *et al*[41], NP-MSCs displayed weaker multilineage differentiation potentials and were even not able to differentiate into adipocytes. Besides the multilineage differentiation potential, such stem/progenitor cells are also capable of differentiation along neurogenic lineages both *in vitro* and *in vivo*, which needs to be further compared with other MSCs[47,50]. Furthermore, Wu *et al*[40] discovered that MSCs derived from degenerative NP tissue show lower differentiation potentials compared with umbilical cord derived MSCs (UC-MSCs). These results demonstrated the differentiation potentials of NP-MSCs may be affected and impaired by the degeneration status of the IVD.

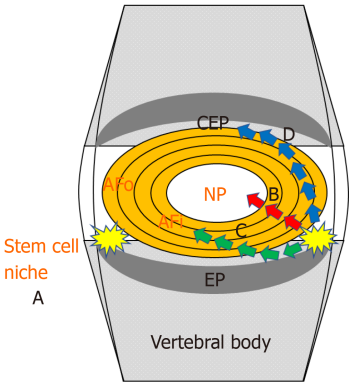

Figure 1 Cell resources of endogenous repair strategy. A: Stem cell niche, defined as the perichondrium region adjacent to the epiphyseal plate and outer zone of the annulus fibrosus (AF); B-D: Stem/progenitor cells could be isolated from the nucleus pulposus, AF, and cartilaginous endplates or migrate from the stem cell niche toward the nucleus pulposus (B), AF (C), and cartilaginous endplates (D). NP: Nucleus pulposus; EP: Epiphyseal plate; AF: Annulus fibrosus; AFo: Outer zone of the AF; AFi: Inner zone of the AF; CEP: Cartilaginous endplates.

Obstacles of endogenous repair

Since the existence of stem cell niche and stem/progenitor cells in the IVD has been proved, there may be three reasons for the degeneration of the IVD and failure of restoration still occurring. First, with increasing age and degeneration of the IVD, the stem/progenitor cells in the IVD or stem cell niche are possible to exhaust with aging, which cannot support the requirement of endogenous repair. Second, the degeneration of the IVD is prone to destroy the potential cellular migration pathways from the specific location of stem cell niche defined as the perichondrium region adjacent to the EP and AFo[24]. Lastly, the harsh environment such as low pH condition[35], inflammation[37,56], compression loading[42], high glucose[57,68], oxidative stress[64], hypoxia[45], and hyperosmolarity[55] may impair the biological function and arrest the proliferation of stem/progenitor cells in the IVD. Thus, searching solutions to resolve these questions may be our first priority to overcome the obstacles of endogenous repair.

Strategies and outcomes of endogenous repair

One simply effective strategy is to reduce the apoptosis and senescence of stem/progenitor cells in the IVD during IVD degeneration induced by various factors or increase the vitality and differentiation of stem/progenitor cells directly. It is well accepted that a normal pH is necessary to maintain normal cell function, whereas an excessively acidic environment induces increased cell apoptosis, reduced cell proliferation, and disordered matrix metabolism in the degenerated IVD[67]. Amiloride, an acid-sensing ion channel, may meliorate IVD degeneration by improving the biological characteristics of NP-MSCs[35]. Besides acid condition, inflammation is also a vitally important factor to induce IVD degeneration *via* some cytokines such as tumor necrosis factor (TNF)-α[37]. Cheng *et al*[56] found that TNF-α at low concentrations (0.1-10 ng/mL) promote the proliferation and migration ability of NP-MSCs, but inhibit their differentiation toward NP cells, indicating that the function of inflammatory cytokines may be a double-edged sword. In addition, pure/leukocyte-containing platelet-rich plasma (P/L-PRP) and modified notochordal cell-rich NP explants were confirmed to attenuate cell apoptosis and dysfunction of NP-MSCs induced by inflammation in the IVD[37,54]. Moreover, oxidative stress caused by mitochondrial dysfunction plays a vitally important role in IVD degeneration[68]. Our pervious research and other studies illustrated that some medicines such as cyclosporine and naringin are capable of alleviating mitochondrial dysfunction and oxidative stress[64,52]. Tao *et al*[53] found that synergy between transforming growth factor beta 3 and insulin-like growth factor 1 could enhance NP-MSC viability, ECM biosynthesis, and differentiation towards NPCs by the MAPK/ERK signaling pathway.

The other strategy of endogenous repair is to replenish the stem/progenitor cells straightly. Various pre-clinical and clinical studies claimed that injection of MSC or MSC-like cells with or without biomaterial could significantly relieve degeneration of the IVD[25,65]. The results of our recent study demonstrated that injectable hydrogel loaded NP-MSCs transplantation could delay the degeneration of the IVD and promote IVD regeneration in a rat model[46]. This kind of strategy involved the expansion and reservation of NP-MSCs *in vitro*, which is the foundation of

Table 1 Expression of stem/progenitor immunophenotypes and genes of the intervertebral disc

Ref.	Type of stem cells	Expression of stem cell/progenitor immunophenotypes and genes
Human		
Liu *et al*[35], 2016	NP-MSCs	CD73+, CD90+, CD105+, Oct4+, Nanog+, Jagged+ and Notch1+, CD34-, CD45-,HLA-DR-
Li *et al*[36], 2017	NP-SCs	GD2+, Tie2+
Li *et al*[37], 2018	NP-MSCs	CD73+, CD90+, CD105+, CD34-, CD45-, HLA-DR-
Jia *et al*[38], 2017	D-NP-MSCs/ND-NP-MSCs	CD73+, CD90+, CD105+, Oct4+ and Nanog+, CD34-, CD45-, HLA-DR-
Wu *et al*[40], 2017	NP-SCs/NPPCs	CD29+, CD44+, CD 73+, CD90+, CD105+, Oct4+ and Nanog+, CD11b-, CD14-, CD34-, CD45-, HLA-DR-
Wang *et al*[41], 2016	NP-SCs/AF-SCs/CEP-SCs	CD73+, CD90+, CD105+, CD19-, CD34-, CD45-, HLA-DR-
Liang *et al*[42], 2018	NP-MSCs	CD73+, CD90+, CD105+, Sox2+ and Oct4+, CD14-, CD19-, CD34-, HLA-DR-
Daisuke *et al*[43], 2012	NPPCs	Tie2+, GD2+
Chen *et al*[44], 2018	NP-MSCs	CD73+, CD90+, CD105+, CD34-, HLA-DR-
Quan *et al*[48], 2015	NP-MSCs	CD29+, CD44+, CD105+, CD14-, CD34-, CD45-, HLA-DR-
Liu *et al*[49], 2017	AF-MSCs, NP-MSCs, CEP-MSCs	CD73+, CD90+, CD105+, CD34-, CD45-, HLA-DR-
Blanco *et al*[27], 2010	NP-MSCs	CD73+, CD90+, CD105+, CD106+, CD166+, CD19-, CD34-, CD45-, HLA-DR-
Lazzarini *et al*[50], 2018	NP-MSCs	CD13+, CD73+, CD90+, CD105+, CD11b-, CD14-, CD19-, CD45-, HLA-DR-
Pereira *et al*[51], 2016	CEP-MSCs	Not shown
Qi *et al*[57], 2018	NP-MSCs	CD24+, CD73+, CD90+, CD105+, CD29-, CD45-
Rat		
Zhao *et al*[34], 2017	NP-MSCs	CD73+, CD90+, CD105+, CD34-, CD45-
Li *et al*[39], 2019	NP-MSCs	CD44+, CD73+, CD90+, CD105+, Oct4+, Nanog+ and Sox2+, CD34-, HLA-DR-
Li *et al*[45], 2013	NP-MSCs	CD73+, CD90+, CD105+, CD34-, CD45-
Wang *et al*[46], 2019	NP-MSCs	CD73+, CD90+, CD105, Tie2+, CD34-, CD45-
Nan *et al*[52], 2019	NP-MSCs	CD73+, CD90+, CD105+, CD34-, CD45-
Han *et al*[29], 2014	NP-MSCs	CD73+, CD90+, CD105+, CD34-, CD45-
Tao *et al*[28], 2013	NP-MSCs	CD73+, CD90+, CD105+, Nanog+, Sox2+, Rex1+ and Oct4+, CD34-, CD45-
Tao *et al*[53], 2015	NP-MSCs	CD73+, CD90+, CD105+, CD34-, CD45-
Liu *et al*[30], 2019	N-NP-MSCs/D-NP-MSCs	CD73+, CD90+, CD105+, CD166+, Sox2+, Nanog+, Oct4+, LIF+, PCNA+ and C-KIT+, CD34-, CD45-
Li *et al*[55], 2018	NP-MSCs	CD73+, CD90+, CD105+, CD34-, CD45-
Cheng *et al*[56], 2019	NP-MSCs	CD29+, CD44+, CD90+, CD34-, CD45-
Lin *et al*[58], 2017	NP-MSCs/NPPCs	CD29+, CD44+, CD90+, Nanog+, Oct4+ and Sox2+, CD34-, CD45-
Liu *et al*[68], 2019	NP-MSCs	CD73+, CD90+, CD105+, Sox2+, Nanog+ and Oct4+, CD34-, CD45-
Zhang *et al*[69], 2015	NP-MSCs	CD44+, CD73+, CD90+, CD105+, Sox2+, Nanog+ and Oct4+, CD14-, CD34-, CD45-, HLA-DR-
Dog		
Erwin *et al*[47], 2013	NPPCs	Oct3/4+, Sox2+, CD133+, Nanog+ and Nestin+
Bovine		
Tekari *et al*[63], 2016	NPPCs	Tie2+
Rabbit		
Jia *et al*[54], 2018	NP-MSCs	CD29+, CD44+, CD166+, CD4-, CD8-, CD14-

MSC: Mesenchymal stem cell; NP: Nucleus pulposus; AF: Annulus fibrosus; CEP: Cartilaginous endplates; NPPC: NP-derived progenitor cell.

endogenous repair. Therefore, searching techniques that can facilitate culturing and

preservation seems to be especially crucial. A study by Lin *et al*[58] indicated that NP-MSCs at a low plated density (L-PD) (5 cells/cm^2) show better biological characteristics, stronger multilineage differentiation, and higher expression of stem cell biomarkers compared with those at an M-PD (100 cells/cm^2) and H-PD (10000 cells/cm^2), suggesting that the limiting dilution method is a better method to isolate NP-MSCs[58]. Moreover, the importance of cryopreservation cannot be ignored as it could prolong the application of NP-MSCs. The conventional cryopreservation methods were classified into slow freezing and vitrification (rapid freezing). The most often used dimethyl sulfoxide is regarded as the standard cryoprotectant which may cause cytotoxicity to MSCs[69,70]. A recent study by Chen *et al*[44] showed that the addition of Icariin known as antioxidant to the conventional freezing medium could improve the viability and function of cryopreserved human NP-MSCs, which may be a new method of preserving stem/progenitor cells in the IVD for ERS (Table 2).

CONCLUSION

Although IVD cell-based therapies have achieved some accomplishment in pre-clinical and clinical studies, there are still some defects such as short duration and tumorigenicity. The discovery of stem cell niche and stem/progenitor cells in the IVD inspired a novel treatment for degenerative IVD. These stem/progenitor cells, isolated from the NP, AF, and CEP, express MSC markers proposed by the ISCT. In addition, stemness related genes including *Nanog*, *Oct4*, and *Sox2* are proved to be expressed in these stem/progenitor cells. Moreover, such cells are similar to other MSCs as not only be capable of osteogenic, chondrogenic, and adipogenic differentiation, but also differentiate into the neurogenic lineages.

However, the ERS still faces some challenges such as exhaustion of stem/progenitor cells, broken migration pathway, and harsh microenvironment such as acid condition, hypoxia, compression loading, hyperosmolarity, high glucose, inflammation, and oxidative stress in degenerative IVD. Therefore, it is essential to look for methods which are able to overcome these obstacles and boost the process of ERS. These methods including reduced apoptosis and senescence caused by degenerative microenvironment or supplying stem/progenitor cells directly have been confirmed to be beneficial to treat degenerative IVD. Nevertheless, the ERS is still in pre-clinical studies and needs to be further investigated in future. In addition, seeking factors or medicines that are able to promote mobilization and migration of stem/progenitor cells in stem cell niche may become another novel direction of ERS.

Table 2 Highlights and strategy of endogenous repair

Ref.	Cells, biomaterial, and medicine	Highlights and strategy
Human		
Liu *et al*[35], 2016	Amiloride	The biological behavior of NP-MSCs could be inhibited by acidic conditions, and amiloride may meliorate IVD degeneration by improving the activities of NP-MSCs.
Li *et al*[36], 2017	NP-SCs	NP-SCs keep the regeneration ability similar to BMSCs with superior capacity in chondrogenesis.
Li *et al*[37], 2018	Modified notochordal cell-rich NP explants	Modified notochordal cell-rich NP explants can attenuate degeneration and senescence of NP-MSC induced by TNF-α.
Jia *et al*[38], 2017	D-NP-MSCs/ND-NP-MSCs	D-NP-MSCs displayed decreased biological characteristics compared with NP-MSCs.
Wu *et al*[40], 2017	D-NP-MSCs/UCMSCs	D-NP-MSCs had lower expression of phenotype markers and exhibited reduced proliferation capability and differentiation potentials compared with UCMSCs.
Wang *et al*[41], 2016	NPSCs/AFSCs/CESCs	A comparison of the osteogenic capacities: CESCs > AFSCs > BM-MSCs > NPSCs; for adipogenesis: BM-MSCs > NPSCs > CESCs > AFSCs; in chondrogenesis: CESCs > AFSCs > BMSCs > NPSCs.
Liang *et al*[42], 2018	NP-MSCs	The biological behavior of NP-MSCs could be inhibited by compression loading.
Daisuke *et al*[43], 2012	NPPCs	The frequency of Tie2+ cells decreases markedly in tissue with age and degeneration of the IVD, suggesting exhaustion of their capacity for regeneration.
Chen *et al*[44], 2018	ICA	The addition of ICA to the conventional freezing medium could improve the viability and function of cryopreserved human NP-MSCs.
Quan *et al*[48], 2015	MSC-like cells from NP	NP tissue contains MSC-like cells which could be isolated and proliferate *in vitro*.
Liu *et al*[49], 2017	AF-MSCs, NP-MSCs, CEP-MSCs	AF-MSCs, NP-MSCs, and CEP-MSCs showed similar multilineage differentiation abilities; CEP-MSCs have the most powerful properties of migration and invasion when compared with AF-MSCs and NP-MSCs.
Blanco *et al*[27], 2010	NP-MSCs	NP-MSCs were quite similar to BM-MSCs, with the exception that NP-MSCs are not able to differentiate into adipocytes.
Lazzarini *et al*[50], 2018	NP-MSCs	NP-MSCs have the capacity of neuronal differentiation and could express neural markers without any electric functional properties.
Pereira *et al*[51], 2016	CEP-MSCs	MSCs from CEP promote IVD regeneration by remodeling ECM.
Qi *et al*[57], 2018	MSC-CM	MSC-CM has potential to alleviate HG induced cell cycle arrest and ECM degradation of NP-MSCs *via* p38 MAPK pathway.
Li *et al*[64], 2018	CsA	CsA efficiently inhibited compression-induced NP-MSCs apoptosis by alleviating mitochondrial dysfunction and oxidative stress.
Rat		
Zhao *et al*[34], 2017	NP-MSCs	The efficacy of NP-MSCs is compromised by age, and old NP-MSCs displayed senescent features.
Li *et al*[39], 2019	NP-MSCs	The MSC-CM + CC method (MSC complete medium culture + cloning cylinder) is a more reliable and efficient way for isolating and purifying NP-MSCs.
Li *et al*[45], 2013	NP-MSCs	Compared to AD-MSCs, NP-MSCs showed greater viability, proliferation, and chondrocytic differentiation under hypoxia.
Wang *et al*[46], 2019	Injectable hydrogel-loaded NP-MSCs	Injectable hydrogel-loaded NP-MSCs transplantation can delay the level of IVD degeneration and promote the regeneration of the degenerative IVD in a rat model.

Nan *et al*[52], 2019	Nar	Nar efficiently attenuated H_2O_2-induced NP-MSCs apoptosis and mitochondrial dysfunction through PI3/AKT pathway.
Han *et al*[29], 2014	NP-MSCs	An acidic environment is a major obstacle for IVD regeneration by AD-MSCs or NP-MSCs; NP-MSCs appeared less sensitive to inhibition by acidic PH.
Tao *et al*[28], 2013	NPCs-NP-MSCs co-culture	NP-MSCs could tolerate IVD-like high osmolarity and NPCs-NP-MSCs co-culture increased cell proliferation and the expression of SOX-9, aggrecan, and collagen-II.
Tao *et al*[53], 2015	TGF-β3/IGF-1	The synergy between TGF-β3 and IGF-1 enhanced NP-MSCs viability, ECM biosynthesis, and differentiation towards NPCs by activating the MAPK/ERK signaling pathway.
Liu *et al*[30], 2019	N-NP-MSCs/D-NP-MSCs	N-NP-MSCs showed a significantly higher proliferation rate, better stemness maintenance ability, but reduced cell apoptosis rate compared with D-NP-MSCs.
Li *et al*[55], 2018	NP-MSCs	Hyperosmolarity of the IVD significantly inhibited the proliferation and chondrogenic differentiation of NP-MSCs by activating the ERK pathway.
Cheng *et al*[56], 2019	TNF-α	Treatment with a high concentration of TNF-α (50-200 ng/mL) could induce apoptosis of NP-MSCs, whereas a relatively low TNF-α concentration (0.1-10 ng/mL) promoted the proliferation and migration of NP-MSCs, but inhibited their differentiation toward NP cells.
Lin *et al*[58], 2017	L-PD of NP-MSCs	NP-MSCs at a L-PD (5 cells/cm^2) have better biological characteristic, stronger multilineage differentiation, and higher expression of stem cell biomarkers compared with those at an M-PD (100 cells/cm^2) and H-PD (10000 cells/cm^2).
Yang *et al*[65], 2009	BMSCs	BMSCs could arrest the degeneration of the murine notochordal NP and contribute to the augmentation of the ECM in the NP by both autonomous differentiation and stimulatory action on endogenous cells.
Liu *et al*[68], 2019	NP-MSCs	High glucose concentration significantly decrease vitality, migration, and stemness of NP-MSCs.
Zhang *et al*[69], 2015	NP-MSCs	The chondrogenic ability of NP-MSCs and BM-MSCs was similar under induction *in vitro*.
Dog		
Erwin *et al*[47], 2013	NPPCs	NPPCs have higher expression of the *Nanog* gene compared to MSCs and are capable of differentiation along chondrogenic, adipogenic, and neurogenic lineages *in vitro* and into oligodendrocyte, neuron, and astroglial specific precursor cells *in vivo* in the myelin-deficient shiverer mouse.
Bovine		
Tekari *et al*[63], 2016	NPPCs	The Tie2+ cells (NPPC) were spheroid in shape with capacity of multi-differentiation and may decline fast, which was partially reversed by FGF2 and hypoxic conditions.
Rabbit		
Jia *et al*[54], 2018	P-PRP/L-PRP	Both P-PRP and L-PRP could induce the proliferation and NP-differentiation of NP-MSCs; P-PRP could reduce the inflammatory and catabolic responses by avoiding the activation of the NF-κB pathway.
Rhesus macaque		
Huang *et al*[66], 2013	DPCs, SLRP	SLRP could reduce the susceptibility of DPCs to hypoxia-induced apoptosis *via* promoting the activation/stabilization of HIF-1α and HIF-2α.

MSC: Mesenchymal stem cell; IVD: Intervertebral disc; NP: Nucleus pulposus; AF: Annulus fibrosus; CEP: Cartilaginous endplates; UCMSCs: Umbilical cord MSCs; AD: Adipose tissue; ECM: Extracellular matrix; ICA: Icariin; CM: Conditioned medium; HG: High glucose; CsA: Cyclosporine; Nar: Naringin; TGF-β3: Transforming growth factor beta 3; IGF: Insulin-like growth factor; TNF: Tumor necrosis factor; L-PD: Low plating density; NPPC: NP-derived progenitor cell; BM-MSC: Bone marrow mesenchymal stem cell; P-PRP: Pure platelet-rich plasma; L-PRP: Leukocyte-containing platelet-rich plasma; DPCs: Intervertebral disc progenitor cells; SLRP: Leucine-rich proteoglycans.

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ORIGINAL ARTICLE

Basic Study

Ability of human umbilical cord mesenchymal stem cells to repair chemotherapy-induced premature ovarian failure

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Abstract

BACKGROUND

Premature ovarian insufficiency (POI) and premature ovarian failure (POF) have become one of the major problems threatening women of childbearing age. Studies have shown that stem cells transplanted from bone marrow, umbilical cord, peripheral blood and amniotic fluid can migrate and proliferate to the ovary, promote ovarian function repair, increase the number of follicles and granulosa cells at all levels of ovary, improve endocrine function, and can differentiate into oocytes in specific ovarian environment to restore fertility to some extent.

AIM

To study the ability of human umbilical cord mesenchymal stem cells (hUCMSCs) to repair ovarian injury after chemotherapy.

METHODS

A total of 110 female BALB/c mice (aged 7-8 wk old) with body masses of 16.0-20.0 g were selected. The mice were fed until 12 wk of age, and cyclophosphamide was administered by intraperitoneal injection for 14 consecutive days to induce premature ovarian failure in mice. Seventy-five mice with estrous cycle disorder were screened and randomly divided into 3 groups according to their body weight: model group, positive control group and hUCMSC group, and each group had 25 mice. Another 25 mice were used as negative controls. The mice in the hUCMSC group were injected with hUCMSCs in the tail vein, and the mice in the positive control group were given an oestradiol valerate solution and a medroxyprogesterone acetate solution in the tail vein. On the 1st, 15th, 30th, 45th, and 60th days after intravenous administration, vaginal smears were made to monitor the estrous cycles of the mice. The ovaries were weighed, and pathological sections were made to observe the morphology of the follicles; blood samples were collected to monitor the concentration of sex hormones (oestradiol and follicle-stimulating hormone).

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RESULTS

The estrous cycles of the model group mice were disrupted throughout the experiment. Mice in the hUCMSC group and the positive control group resumed normal estrous cycles. The ovarian weight of the model group mice continued to decline. The ovarian weight of the hUCMSC group mice and the positive control group mice decreased first and then gradually increased, and the ovarian weight of the hUCMSC group mice was heavier than that of the positive control group mice. The difference was statistically significant ($P < 0.05$). Compared with the negative control group, the model group experienced a decrease in oestradiol and an increase in follicle-stimulating hormone, and the difference was statistically significant ($P < 0.05$). Compared with the model group, the hUCMSC and positive control groups experienced a slight increase in oestradiol and a decrease in follicle-stimulating hormone; the difference was statistically significant ($P < 0.05$). The pathological examination revealed that the mouse ovaries from the model group were atrophied, the volume was reduced, the cortical and medullary structures were disordered, the number of follicles at all stages was significantly reduced, the number of atretic follicles increased, the number of primordial follicles and corpus luteum significantly decreased, and the corpus luteum had an irregular shape. Compared with those of the model group, the lesions of the hUCMSC and positive control groups significantly improved.

CONCLUSION

hUCMSCs can repair ovarian tissue damaged by chemotherapy to a certain extent, can improve the degree of apoptosis in ovarian tissue, and can improve the endocrine function of mouse ovaries.

Key words: Umbilical cord mesenchymal stem cells; Premature ovarian failure; Chemotherapy; Repair; Ovarian injury; Endocrine function

Core tip: Human umbilical cord mesenchymal stem cells (hUCMSCs) can repair ovarian tissue damaged by chemotherapy to a certain extent, can improve the degree of apoptosis in ovarian tissue, and can improve the endocrine function of mouse ovaries. The effect of hUCMSCs on chemotherapy-induced premature ovarian failure mice was observed by closely monitoring the changes in mouse ovarian structure and endocrine function to further verify whether hUCMSCs can be used in the treatment of chemotherapy-induced premature ovarian failure.

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INTRODUCTION

Premature ovarian insufficiency (POI) and premature ovarian failure (POF) have become one of the major problems threatening women of childbearing age. These disorders can not only cause female reproductive endocrine dysfunction[1,2], but also seriously affect the physical and mental health and the quality of life of women of reproductive age[3]. At present, the treatment widely used for premature ovarian failure is mainly hormone replacement therapy (HRT). However, HRT mainly improves clinical symptoms, and it is impossible to fundamentally treat or repair damaged ovarian tissue[4], and long-term application of HRT can easily lead to side effects such as cardiovascular and cerebrovascular diseases, so HRT has not yet achieved satisfactory therapeutic effects. In recent years, with the deepening of research on mesenchymal stem cells (MSCs), MSC therapy has been considered as a treatment for repairing damaged tissues and reconstructing normal tissue function. Some of the research results have been applied in clinical trials, such as malignant

tumours, autoimmune diseases, and cardiovascular diseases[5,6]. Related experimental studies have shown that MSCs can be colonized in ovarian tissue and can migrate towards the damaged tissue, thereby partially repairing the drug-induced ovarian damage caused by chemotherapy[7,8]. Therefore, the treatment of premature ovarian failure has also made some progress. According to animal experiments, human umbilical cord mesenchymal stem cells (hUCMSCs) have many advantages, such as easy collection, low immunogenicity, no tumourigenicity and no ethical restrictions[9-11], thus bringing hope to improve ovarian function and restore fertility in patients with POI/POF. In this study, an animal model of chemotherapy-induced premature ovarian failure was constructed. The effect of hUCMSCs on chemotherapy-induced premature ovarian failure mice was observed by closely monitoring the changes in mouse ovarian structure and endocrine function to further verify whether hUCMSCs can be used in the treatment of chemotherapy-induced premature ovarian failure.

MATERIALS AND METHODS

Experimental cells and animals

hUCMSCs were provided by Shenyang Cell Therapy Engineering Technology Research and Development Center Co., Ltd.

A total of 110 SPL-level BALB/c female mice, aged 7-8 wk old with a body mass of 16.0-20.0 g, were purchased from Liaoning Changsheng Biotechnology Co., Ltd., Animal Quality Certificate No. 211002300048121, Animal License No. SCXK (Liao) 2015-0001. The mice were housed in the SPF-class animal room of the Animal Experimental Center at the Heping Campus of Northern War Zone General Hospital. The mice were grouped in 5 mice per cage, were free to eat and drink and were exposed to a day/night discontinuous illumination (12 h:12 h). The food, litter, water and cages used for feeding were strictly disinfected and sterilized.

Main reagents

Cyclophosphamide for injection (Jiangsu Disheng); oestradiol valerate tablets (Guangzhou Xianling); medroxyprogesterone acetate tablets (Zhejiang Xianju Pharma); sodium chloride injection (Huaren Pharmaceutical); mouse oestrogen 2 (E2) kit (Shanghai Guang Rui Biological Technology Co., Ltd., LOT 201905, RET ER2619); mouse follicle-stimulating hormone (FSH) kit (Shanghai Guang Rui Biological Technology Co., Ltd., LOT 201905, RET ER2936).

Equipment

OLYMPUS Optical Microscope (OLYMPUS, Japan); Microscopic Image Analysis Software (Image-Pro, USA); rotary slicer, automatic dewatering machine, biological tissue embedding machine, automatic dyeing machine, spreading film drying machine (LEICA, Germany); Z236K Centrifuge (HERMLE); Nu-437-400E Biosafety Cabinet (Tianmei Technology Co., Ltd., China); BS224S Electronic Analytical Balance (Sartorius, Germany).

Sources of hUCMSCs

hUCMSCs were provided by Shenyang Cell Therapy Engineering Technology Research and Development Center Co., Ltd., and the samples in the public library are selected using strict and accurate detection methods. For the hUCMSCs used in this experiment, from the original sample collection, cell separation, stem cell (line) establishment, to the final cell preparation and release, all processes are in line with the "Good Manufacturing Practices" requirements, including personnel, materials, instruments and equipment, and environment management and control.

Experimental grouping and processing of animals

After raising SPF-class BALB/c mice to 12 wk of age, the mice were subjected to vaginal smear observation for 7 consecutive days. We observed under an optical microscope and determined the animal's estrous cycle based on cell classification. A total of 110 mice with a normal estrous cycle were randomly divided into a negative control group (25 mice) and a model group (85 mice). A model of chemotherapy-induced POF was established for the mice in the model group by intraperitoneal injection of cyclophosphamide for a first-time loading of 50 mg/kg, followed by a continuous intraperitoneal injection of 8 mg/(kg·d) for 14 d[12]. A vaginal smear was prepared and observed every day from day 4 to day 10 after administration of cyclophosphamide. Seventy-five mice with estrous cycle disorder were screened and randomly divided into the following three groups according to their body weight with 25 mice in each group: model group, hUCMSC group and positive control

group. In the negative control group and the model group, the mice were injected with sodium chloride in the tail vein, 0.2 mL each; the hUCMSC group was injected with hUCMSCs at a concentration of 1×10^6 cells/mL in the tail vein, and the injection volume was 0.2 mL each. The injection was performed once a week for 4 wk. The positive control group was injected with 0.012 mg/mL oestradiol valerate solution in the tail vein in a volume of 10 mL/kg body weight. On day 4, the mice were given a tail vein injection of 0.096 mg/mL solution of medroxyprogesterone acetate in a volume of 10 mL/kg body weight (this is considered as a course of treatment). Subsequently, the two drugs were discontinued for 1 d, and then the next course of treatment was repeated for 4 wk.

Observation of experimental indicators and pathological examination

Mouse vaginal smear and estrous cycle: The mice were observed once a day at 8:00 am, and the cotton swabs were taken lightly from the vaginal secretions to make a smear. After air drying, an appropriate amount of methylene blue dye solution was added dropwise. After 1 to 2 min, the mixture was rinsed with water, dried naturally and placed under a microscope.

Ovarian weighing and pathological section observation of follicular morphology: Five mice were sacrificed on the 1st, 15th, 30th, 45th and 60th days after intravenous administration, ovarian specimens were taken, and the ovaries were weighed. Paraffin-embedded sections were routinely prepared and stained with haematoxylin-eosin, and the ovarian structure was observed under a light microscope.

Determination of sex hormone (E2 and FSH) concentration: On the 1st, 15th, 30th, 45th and 60th day after intravenous administration, blood was collected from the eyelids, with heparin as an anticoagulant. After centrifugation for 15 min, the upper serum was taken and stored in a refrigerator at -80 °C. The E2 and FSH concentrations were determined at the elective stage.

Statistical analysis

Statistical processing was performed using SPSS 17.0 software. For those who conform to the normal distribution, the measurement data are expressed as the mean ± SD using one-way analysis of variance; data that do not conform to the normal distribution are statistically analysed using nonparametric tests. $P < 0.05$ indicates that the difference was statistically significant.

RESULTS

Mouse estrous cycle changes

The estrous cycle of normal mice is approximately 3 to 5 d and includes the proestrus, oestrus, late oestrus and anestrous stages (Figure 1). The estrous cycle can be identified by changes in the cells in the vagina: The early stage of oestrus is characterized by small, round, squamous epithelial cells (nucleated epithelial cells); oestrus is characterized by irregular (keratinized) squamous epithelial cells; the late stage of oestrus is characterized by white blood cells and keratinocytes; and the anestrous stage is characterized by the appearance of white blood cells and round epithelial cells. In the experiment, the oestrus cycle of the normal control group did not change significantly and was the normal estrous cycle; the estrous cycle of the model group mice continued to be disordered. Within 1 to 15 d after cell transplantation, the estrous cycle of the hUCMSC group and the positive control group remained unresolved, and they were all in a disordered state. After 16 to 30 d, the hUCMSC group and the positive control group began to gradually return to the normal estrous cycle. Although the number of mice in the hUCMSC group that returned to the normal estrous cycle was greater than that in the positive control group, the difference was not statistically significant, as shown in Table 1.

Changes in ovarian weight

Compared with that of the negative control group, the ovarian weights of the model, hUCMSC and positive control groups were significantly decreased on the first day and the 15th day after transplantation, and the difference was statistically significant ($P < 0.05$). There was no significant difference between the 3 groups ($P > 0.05$). On the 30th, 45th and 60th days after transplantation, the ovarian weight of the model group continued to decrease, and the ovarian weight of the hUCMSC group and the positive control group continued to increase. The difference was statistically significant ($P < 0.05$). There was no significant difference between the hUCMSC group and the positive control group ($P > 0.05$, Table 2).

Table 1 The number of mice in the normal oestrus cycle at each time point in each group

Groups	1-15 d	16-30 d	31-45 d	46-60 d
Negative control group	20	15	10	5
Model group	0	0	0	0
hUCMSC group	0	7	4	3
Positive control group	0	6	4	4

hUCMSC: Human umbilical cord mesenchymal stem cell.

Sex hormone changes

Before the model group was established, there was no significant difference in the basic concentrations of the sex hormones E2 and FSH between the groups ($P > 0.05$). After the model group was established, the E2 concentration of the model group, the hUCMSC group and the positive control group decreased, and the FSH concentration increased, while the negative control group E2 and FSH concentrations fluctuated within the normal range. The difference between the 3 groups (model group, hUCMSC group, and the positive control group) and the negative control group was statistically significant ($P < 0.05$) and continued until the end of the experiment. On the 1st day after transplantation, the E2 concentration in the model group, hUCMSC group and positive control group was significantly different from that in the negative control group ($P < 0.05$). There was no significant difference between the 3 groups ($P > 0.05$). On the 15th day after transplantation, the E2 and FSH concentrations in the hUCMSC group and the positive control group were significantly different from those in the model group ($P < 0.05$). There was no significant difference between the hUCMSC group and the positive control group ($P > 0.05$). On the 30th, 45th, and 60th days after transplantation, the E2 and FSH concentrations in the hUCMSC group and the positive control group were significantly different from those in the model group ($P < 0.05$). There was no significant difference between the hUCMSC group and the positive control group ($P < 0.05$, Table 3 and Table 4).

Observation of ovarian pathological sections

In the negative control group, the ovary volume was larger, and the ovarian cortex medulla was clear. It was found that the follicles at all levels were active, arranged regularly, and developed normally. The corpus luteum was well developed, and the luteal cells were abundant. The model group had obvious ovarian atrophy, decreased volumes, and disordered cortical and medullary structures. The number of follicles at all levels was significantly reduced, and the number of atresia follicles increased. Only a small number of primordial follicles, ovarian oocytes and zona pellucida were abnormal. The corpus luteum number was significantly reduced, and the volume was reduced. The shape was not regular. The ovarian volume of the positive control group did not decrease significantly. The medullary structure of the ovarian cortex was slightly better than that of the model control group. The number of follicles at each level decreased, and the atresia follicles remained larger. The corpus luteum number decreased, the volume decreased, and the morphology was irregular. The ovarian volume of the hUCMSC group was slightly lower than that of the negative control group and the cortical medulla was structurally disordered but superior to that of the model group. The number of follicles at each level was reduced, the number of atresia follicles was significantly reduced, and the number of corpus luteum was slightly reduced, but the morphology was not regular (Figure 2).

DISCUSSION

With the development of modern science and technology, cancer treatment methods are increasing, and chemotherapy and radiotherapy treatments are widely used. Although the therapeutic effect of the disease is improved and the lifetime limit of patients is prolonged, the adverse consequences of chemotherapy radiotherapy for women of childbearing age also make patients troubled. According to some surveys, approximately 5% of women of childbearing age in the world are diagnosed with cancer[13-16]. After active treatment, they will face serious complications, such as ovarian insufficiency and even POF. Coupled with the fast pace of life and high work pressure, the incidence of POI has become younger, affecting more than 10% of women[17]. POI is a clinical syndrome in which women's ovarian activity declines

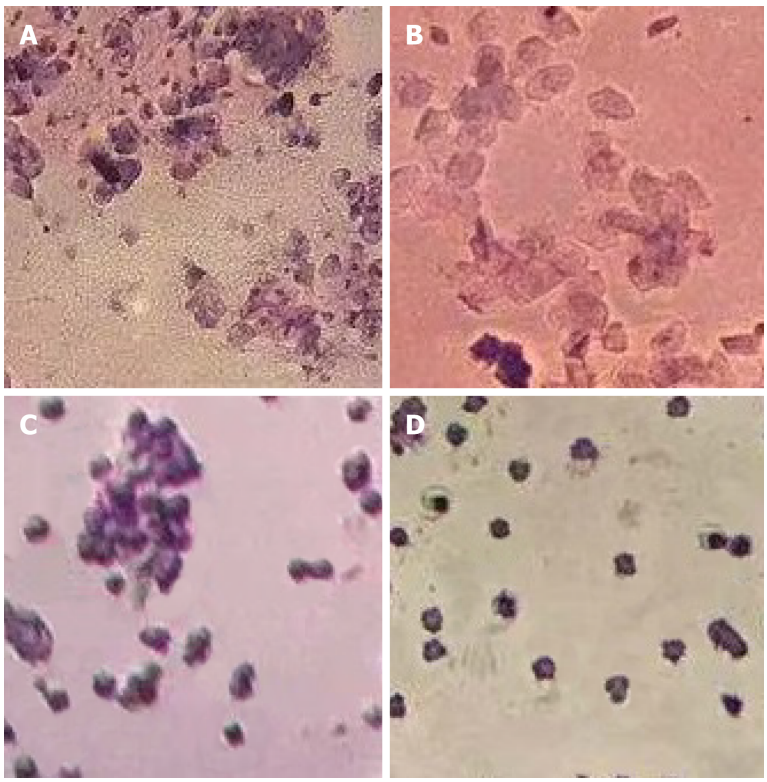

Figure 1 Mouse oestrus cycle. A: Proestrus; B: Oestrus; C: Late oestrus; D: Anestrous. Methylene blue staining 100×.

before the age of 40, with menstrual disorders (such as menopause or thin menstruation), ovarian atrophy, low levels of oestrogen in the body, and gonadotropin up to the level of menopause[18,19]; if progression continues, ovarian failure before the age of 40 can lead to POF. POI and POF are caused by a variety of reasons, and their aetiology and pathogenesis are more complicated. The pathogenesis of this is not fully understood. Commonly known causes include genetic factors (number or structural abnormalities of X chromosomes), iatrogenic factors (such as radiotherapy and chemotherapy, surgery and other tumour treatments), infection factors, immune factors, *etc*.[20,21].

Stem cells are a class of early undifferentiated cells with self-renewal ability and multi-directional differentiation potential, which not only have tissue regeneration and repair functions but also have low immunogenicity, immunomodulatory effects and no toxicity[22-25]. At the same time, after entering the human body, the stem cells have the characteristics of automatic migration and homing to the tissues of the damaged organs, thereby having the function of participating in repairing the damaged tissue structure[26]. hUCMSCs are MSCs, which are mainly isolated from the Wharton's jelly of the umbilical cord after the foetus is born. hUCMSCs have many advantages, such as convenient material extraction, a wide range of sources, isolation and culture in vitro, stable biological properties, no tumourigenicity and low immunogenicity[27]. There is no ethical problem in culturing MSCs with umbilical cord, and it does not cause extra pain to patients, and it is not easy to cause disease spread among people[27-30]. Due to the above characteristics, hUCMSCs have gradually become a new research hotspot of MSCs[31-36].

In patients with POF, ovarian failure and a decreased ovary reserve causes the amount of E2 production to be reduced in sinusoid follicles, so the negative feedback effect on the gonadal axis is weakened, resulting in increased levels of FSH and LH hormones secreted by the pituitary. This experiment examined the changes in serum E2 and FSH in each group of mice to verify whether hUCMSCs can improve the endocrine function of the ovary. The results showed that compared with the negative control group, the E2 of the model group decreased, and the FSH increased, and the difference was statistically significant ($P < 0.05$ or 0.01), indicating that the cyclophosphamide-induced OPF model was established. Compared with the model group, the hUCMSC group and the positive control group had an increase in E2 to a certain extent, and FSH decreased; the difference was statistically significant ($P < 0.05$ or 0.01), indicating that both hUCMSCs and HRT can improve the ovary and that the

Table 2 Comparison of ovarian weight in each group at each time point

Items	After transplantation				
	1 d	15 d	30 d	45 d	60 d
Negative control group	36.67 ± 0.68	36.97 ± 0.32	37.45 ± 0.35	34.86 ± 0.43	38.13 ± 0.35
Model group	23.19 ± 0.98	20.23 ± 0.49	18.30 ± 0.28	18.02 ± 0.41	17.25 ± 0.28
hUCMSC group	21.19 ± 0.65	19.54 ± 0.27	21.77 ± 0.48	23.11 ± 0.38	24.28 ± 0.48
Positive control group	22.50 ± 0.62	19.30 ± 0.37	24.53 ± 0.93	27.64 ± 0.34	28.06 ± 0.93
P value	$P < 0.05$				

hUCMSC: Human umbilical cord mesenchymal stem cell.

OPI can be restored to a certain extent. In the pathological examination of mouse ovarian tissue, the model group had obvious ovarian atrophy, decreased volume, a disordered cortical and medullary structure, the number of follicles at all levels was significantly reduced, and the number of atresia follicles increased; only a small amount of primordial follicles, the number of corpus luteum decreased significantly and the shape was not regular. The lesions of the hUCMSC group and the positive control group were significantly improved compared with those of the model group. The oestrus cycle of the model group mice continued to be disordered during the experiment. The hUCMSC group and the positive control group mice resumed the normal estrous cycle. The total number of follicles and the number of follicles at all stages in the mouse ovaries were lower in the model group than in the normal group, and the ovarian weight continued to decrease. The total number of follicles and the number of follicles at all levels in the hUCMSC group and the positive control group were higher than those in the model group, and the ovarian weight continued to increase; the difference was statistically significant ($P < 0.05$). The results indicated that hUCMSCs can repair damaged ovarian tissue to a certain extent, improve the degree of apoptosis in ovarian tissue, and improve the endocrine function of mouse ovaries.

In summary, hUCMSCs have a certain repair effect on damaged ovarian tissue after cyclophosphamide-induced chemotherapy damage and regulate endocrine secretion of hormones, thereby further slowing ovarian failure. It can provide new treatments for chemotherapy patients and provide a corresponding experimental basis for more clinical experimental research. However, the specific molecular mechanism requires further study.

Table 3 E2 concentration of each group at each time point

Items	Before transplantation	After transplantation				
		1 d	15 d	30 d	45 d	60 d
Negative control group	56.94 ± 4.43	51.42 ± 2.35	53.99 ± 2.73	53.43 ± 3.26	52.04 ± 1.65	55.43 ± 1.57
Model group	55.23 ± 4.35 (P = 0.147)	35.41 ± 2.25	28.32 ± 1.51	24.86 ± 0.90	22.16 ± 1.25	18.99 ± 1.40
hUCMSC group	55.83 ± 3.35 (P = 0.325)	37.84 ± 1.69	38.33 ± 1.79	35.04 ± 2.31	35.12 ± 1.47	34.10 ± 1.42
Positive control group	55.03 ± 3.43 (P = 0.357)	37.34 ± 1.86	37.80 ± 1.83	36.86 ± 2.09	36.86 ± 1.11	37.08 ± 1.47
P value	P > 0. 05					

hUCMSC: Human umbilical cord mesenchymal stem cell.

Table 4 Follicle-stimulating hormone concentration of each group at each time point

Items	Before transplantation	After transplantation				
		1 d	15 d	30 d	45 d	60 d
Negative control group	8.61 ± 0.03	9.25 ± 0.35	8.64 ± 0.56	9.27 ± 0.36	8.93 ± 0.26	9.11 ± 0.10
Model group	8.76 ± 0.17 (P = 0.19)	18.33 ± 0.79	20.36 ± 0.74	22.38 ± 1.05	24.26 ± 1.15	25.19 ± 0.96
hUCMSC group	8.70 ± 0.38 (P = 0.93)	19.44 ± 1.23	14.07 ± 0.53	15.52 ± 0.57	18.22 ± 1.14	18.55 ± 0.66
Positive control group	8.66 ± 0.45 (P = 0.10)	18.5 ± 0.31	12.50 ± 0.63	12.39 ± 0.03	13.50 ± 1.04	14.28 ± 0.64
P value	P > 0. 05					

hUCMSC: Human umbilical cord mesenchymal stem cell.

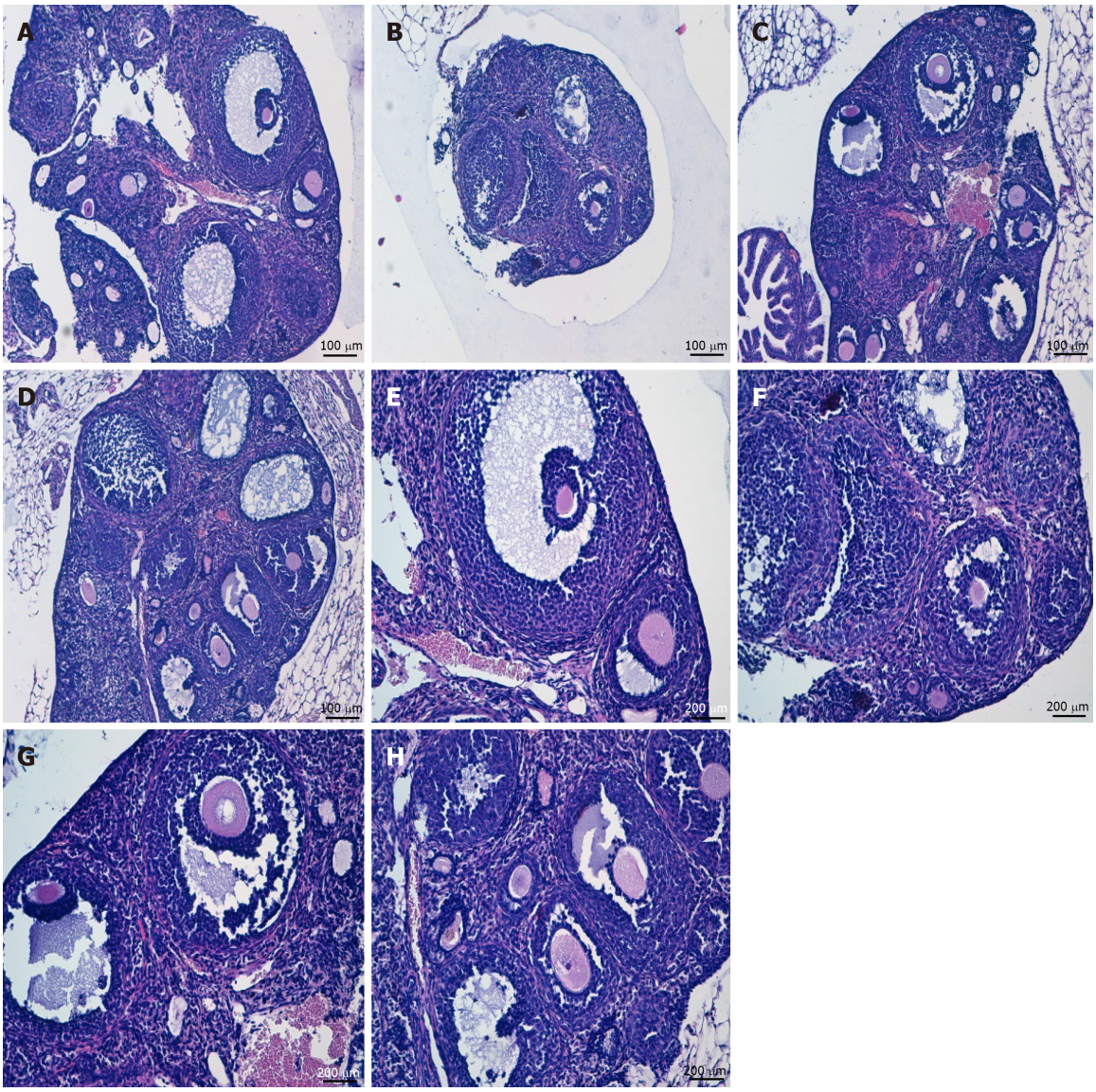

Figure 2 Results of ovarian tissue staining (Haematoxylin-eosin staining). A: Negative control group; B: Model group; C: Human umbilical cord mesenchymal stem cells group; D: Positive control group (100×); E: Negative control group; F: Model group; G: Human umbilical cord mesenchymal stem cells group; H: Positive control group (200×).

ARTICLE HIGHLIGHTS

Research background

Premature ovarian insufficiency (POI) and premature ovarian failure (POF) have become one of the major problems threatening women of childbearing age. Studies have shown that stem cells transplanted from bone marrow, umbilical cord, peripheral blood and amniotic fluid can migrate and proliferate to the ovary, promote ovarian function repair, increase the number of follicles and granulosa cells at all levels of ovary, improve endocrine function, and can differentiate into oocytes in specific ovarian environment to restore fertility to some extent.

Research motivation

According to animal experiments, human umbilical cord mesenchymal stem cells (hUCMSCs) have many advantages, such as easy collection, low immunogenicity, no tumourigenicity and no ethical restrictions, it bringing hope to improve ovarian function and restore fertility in patients with POI/POF.

Research objectives

In this study, the authors aimed to study the ability of hUCMSCs to repair ovarian injury after chemotherapy.

Research methods

A total of 110 female BALB/c mice were fed until 12 wk of age, and cyclophosphamide was

administered by intraperitoneal injection for 14 consecutive days to induce premature ovarian failure in mice. The mice in the hUCMSC group were injected with hUCMSCs in the tail vein, and the mice in the positive control group were given an oestradiol valerate solution and a medroxyprogesterone acetate solution in the tail vein.

Research results

Mice in the hUCMSC group and the positive control group resumed normal estrous cycles. The ovarian weight of the model group mice continued to decline. The ovarian weight of the hUCMSC group mice and the positive control group mice decreased first and then gradually increased, and the ovarian weight of the hUCMSC group mice was heavier than that of the positive control group mice. The model group experienced a decrease in oestradiol and an increase in follicle-stimulating hormone. The hUCMSC and positive control groups experienced a slight increase in oestradiol and a decrease in follicle-stimulating hormone. The pathological examination revealed that the mouse ovaries from the model group were atrophied, the volume was reduced, the cortical and medullary structures were disordered, the number of follicles at all stages was significantly reduced, the number of atretic follicles increased, the number of primordial follicles and corpus luteum significantly decreased, and the corpus luteum had an irregular shape. The lesions of the hUCMSC and positive control groups significantly improved.

Research conclusions

hUCMSCs can repair ovarian tissue damaged by chemotherapy to a certain extent. And it can improve the degree of apoptosis in ovarian tissue, and improve the endocrine function of mouse ovaries.

Research perspectives

The specific molecular mechanism requires further study.

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SYSTEMATIC REVIEWS

Human umbilical cord derived mesenchymal stem cells in peripheral nerve regeneration

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Abstract

BACKGROUND

Peripheral nerve injury can occur as a result of trauma or disease and carries significant morbidity including sensory and motor loss. The body has limited ability for nerve regeneration and functional recovery. Left untreated, nerve lesions can cause lifelong disability. Traditional treatment options such as neurorrhaphy and neurolysis have high failure rates. Surgical reconstruction with autograft carries donor site morbidity and often provide suboptimal results. Mesenchymal stem cells (MSCs) are known to have promising regenerative potential and have gained attention as a treatment option for nerve lesions. It is however, unclear whether it can be effectively used for nerve regeneration.

AIM

To evaluate the evidence for the use of human umbilical cord derived MSCs (UCMSCs) in peripheral nerve regeneration.

METHODS

We carried out a systematic literature review in accordance with the PRISMA protocol. A literature search was performed from conception to September 2019 using PubMed, EMBASE and Web of Science. The results of eligible studies were appraised. A risk of bias analysis was carried out using Cochrane's RoB 2.0 tool.

RESULTS

Fourteen studies were included in this review. A total of 279 subjects, including both human and animal were treated with UCMSCs. Four studies obtained UCMSCs from a third-party source and the remainder were harvested by the investigators. Out of the 14 studies, thirteen conducted xenogenic transplantation

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into nerve injury models. All studies reported significant improvement in nerve regeneration in the UCMSC treated groups compared with the various different controls and untreated groups.

CONCLUSION
The evidence summarised in this PRISMA systematic review of i*n vivo* studies supports the notion that human UCMSC transplantation is an effective treatment option for peripheral nerve injury.

Key words: Umbilical cord; Mesenchymal stem cells; Transplantation; Peripheral nerve regeneration

Core tip: While human umbilical cord derived mesenchymal stem cells hold promise as a treatment option for peripheral nerve lesions, robust *in vivo* models are required in order to determine the best method of delivering mesenchymal stem cells to sites of injury.

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INTRODUCTION

Peripheral nerve injuries can occur as a result of trauma or disease and can lead to significant morbidity including sensory loss, motor loss and chronic pain[1]. These injuries cause life-long disability in up to 2.8% of all trauma patients[2]. Damage to peripheral nerves most commonly occurs as a result of laceration, compression, ischaemia or traction[1]. As classified by Seddon in 1943, nerve injury can range from focal demyelination termed neurapraxia, to total nerve transection termed neurotmesis[3,4]. The mechanism of recovery post-injury occurs by either branching of collateral axons or by regeneration of the damaged neuron[4,5]. In order for full neuronal recovery to occur, Wallerian degeneration, axonal regeneration and end-organ reinnervation must take place. This is driven by an array of neurotropic factors[4]. However, recovery in function following peripheral nerve injury is hindered by complex pathological mechanisms such as poor nerve regeneration, neuromuscular atrophy, and end-plate degeneration which can lead to suboptimal neuron function[6-9].

Traditionally, peripheral nerve injury can be managed conservatively or surgically with neurolysis, neural suturing, end-to-side neurorrhaphy and nerve autograft[10-12]. Even with optimum surgical repair, most methods will attain partial but not full return of nerve function[10]. Certain peripheral nerve injuries, such as severe brachial plexus or long traction injuries remain inoperable[10]. Autografts have several disadvantages, including donor site morbidity, mismatch in nerve and graft size resulting in poor engraftment, and the potential for development of painful neuromas[11,13,14]. Alternative methods of treating peripheral nerve injuries may be through cell-based regenerative therapies[15].

Transplantation of mesenchymal stem cells (MSCs), given their regenerative properties and highly proliferative capacity, has been proposed as a promising therapeutic option for peripheral nerve regeneration[16,17]. MSCs are plastic-adherent, undifferentiated, multipotent cells that can be harvested from numerous sites of the body including bone marrow, adipose tissue, dental pulp, amniotic fluid and umbilical cord[17-19]. MSCs from different tissue origins can have distinct cytokine expression profiles, and thus may enable different MSCs to be particularly suited to certain clinical applications[20,21]. Owing to low immunogenicity, MSCs may be transplanted allogenically with minimal consequence[22]. The particular mechanisms through which MSCs aid nerve repair have not yet been fully characterised. MSCs from various sources such as adipose tissue and bone marrow are able to differentiate into Schwann cells[23,24]. While some *in vitro* experiments suggest that transplanted

MSCs may be stimulated by peripheral nerves to differentiate into Schwann cells[25], alternative findings have instead shown that transplanted MSCs encourage endogenous cells to express regenerative phenotypes[26]. Increasingly, MCSs are believed to mediate their regenerative properties predominantly through paracrine effects[27,28]. Aside from acting through soluble factors[29], MSCs have also demonstrated the ability to secrete extracellular vesicles that contain bioactive components such as miRNA and cytokines[30]. Indeed, native Schwann cells have been shown to facilitate axonal regeneration following injury through secretion of exosomes that decrease GTPase RhoA activity[31]. Similarly, human MSCs may act to achieve the same result through exosomes by upregulation of the PI3 kinase and Akt signalling cascades[32].

MSCs from umbilical cord are convenient to harvest from post-natal tissue in a non-invasive manner and possess a high capacity to expand *ex vivo*[33]. They express low levels of HLA-DR compared to MSCs from other cell sources and therefore pose low risk of immunogenic complications following allogenic transplantation[34]. Through sequential treatment with β-mercaptoethanol and various cytokines, umbilical cord derived MSCs (UCMSCs) can adopt a Schwann-like phenotype[35]. In addition, UCMSCs have been shown to possess greater paracrine effects than those of bone marrow-derived MSCs (BMMSC) and adipose-derived MSCs[17,29], and are able to potentiate axonal regeneration and peripheral nerve functional regeneration through these effects[11,17,29,36]. UCMSCs have been proposed to exert neuroprotective effects through secretion of Brain Derived Neurotrophic Factor (BDNF)[37], angiopoietin-2 and CXCL-16[38,39]. Other studies have suggested that they indirectly promote neurogenesis[40,41]. UCMSCs are also able to indirectly enhance expression of neurotransmitters such as BDNF and neurotrophin-3 (NTF3) which are postulated to aid neuro-regeneration[42,43].

To date, there have been over 400 clinical trials that explore the use of MSCs in transplantation; UCMSCs follow BMMSCs as the second most commonly used cell source[44]. In this PRISMA systematic review, we analyse the evidence for the use of human UCMSCs in peripheral nerve regeneration by examining *in vivo* studies.

MATERIALS AND METHODS

A literature search was performed from conception to September 2019 using PubMed, EMBASE and Web of Science. The following search terms were used: (((((((Mesenchymal stem cells) OR mesenchymal stem cell) OR MSC) OR MSCs) OR Mesenchymal stromal cell) OR Mesenchymal cell)) AND (((((Nerve) OR Peripheral nerve) OR Peripheral nerve injury) OR damaged nerve) OR nerve injury)) AND ((((((repair) OR regeneration) OR regrowth) OR regenerate) OR renew) OR restore). We adhered to the recommendations as stipulated by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[45].

We included case series, case control, cohort studies and randomised controlled trials. We enrolled studies that examined peripheral nerve lesions treated with human UCMSCs in *in vivo* human and animal subjects. Studies that only conducted *in vitro* experiments were excluded. Studies that investigated central nervous system regeneration using UCMSCs were excluded. All included studies were published in the English language. We excluded all unpublished and retracted literature.

CB and KT carried out the search independently. RoB2 tool was used by CM and BZ to assess the risk of bias in the studies, all discrepancy in results were resolved by discussion.

RESULTS

A total of 210 studies were screened for title, abstract and the inclusion/exclusion criteria were applied (Figure 1). One retracted study was excluded. Fourteen studies were reviewed in full text. The overall bias of studies is shown in Figure 2. The summary of results is shown in Figure 3. All 14 studies were of a case control design (Table 1). Four studies obtained UCMSCs from a third-party source and the remainder were harvested directly from human subjects. Out of the 14 studies, ten involved xenogenic transplantation into sciatic nerve injury specimens that were either crushed or transected. The studies were grouped according to Seddon's seminal nerve injury classification system, which includes axonotmesis (injury to nerve sheath alone) and neurotmesis (injury to the entire nerve)[3]. A total of 279 subjects were treated with UCMSCs. All studies reported significant improvement in UCMSC treated groups compared with the various different controls and untreated groups.

The studies did not report any significant complications.

UCMSCs in peripheral nerve axonotmesis

Four studies that included a total of 90 treated subjects assessed the use of UCMSCs in peripheral nerve axonotmesis models of sciatic nerve crush injury (Table 1). All four studies harvested UCMSCs from human subjects and transplanted the UCMSCs into murine subjects. The methods of UCMSC delivery to the crush injury varied among studies.

Studies, by Sung *et al*[46] (2012) and Hei *et al*[47] (2016) examined the effect of direct intralesional UCMSC injections on murine subjects with sciatic nerve crush injuries. Both studies monitored subjects up to 4 wk post-intervention. Sung *et al*[46] (2012) found that expression of brain-derived neurotrophic factor (BDNF) and tyrosine kinase receptor B mRNA increased at 4 wk following UCMSC injection. Functional recovery was measured in terms of the sciatic function index (SFI), which showed a dramatic improvement at 4 wk in UCMSC treated groups compared to untreated groups. Retrograde axonal transport was estimated through fluoro Gold-labelled neuron counts and the UCMSC group was found to have a significantly higher neuron count. It was found that axon density was significantly greater in the UCMSC group. Hei *et al*[47] (2016) transfected UCMSCs with a BDNF-adenovirus vector. The authors found that both UCMSC and BDNF-UCMSC groups had significant improvements in SFI, axon count and axon density at 4 wk after treatment. The BDNF-UCMSC group displayed increased peripheral nerve regeneration compared with UCMSC alone.

Gartner *et al*[48,49] conducted two studies published in the same year. In one study, Chitosan type III membrane was used to aid UCMSC infiltration in murine sciatic crush models[48]. The authors evaluated motor and sensory functional recovery up to 12 wk following transplantation with and without Chitosan type III wrapping. Both treatment groups showed improvement in SFI, extensor postural thrust (EPT) and withdrawal reflex latency (WRL). The control group with Chitosan type III membrane alone showed a significant improvement in post-traumatic axonal regrowth compared to the untreated control group. In a separate study, the same group examined the effect of using poly (DL-lactide-e-caprolactone) (PLC) membranes to deliver UCMSCs into sciatic nerve crush injuries[49]. Peripheral nerve regeneration was assessed in terms of SFI, EPT, and WRL at 12 wk. Undifferentiated and differentiated UCMSCs were used in different groups. Both groups showed an increase in myelin sheath thickness compared to control groups. The SFI was severely affected at week-2 post-crush injury in all experimental groups and improved gradually up to week 12 when values were indistinguishable from controls.

Studies of UCMSCs in peripheral nerve neurotmesis

Nine of the fourteen studies assessed the use of UCMSCs in peripheral nerve neurotmesis models (Table 2). All nine were case control studies. Five studies had murine subjects, two had rabbit subjects, one had canine subjects, and one had human subjects. Six studies transplanted UCMSCs into a sciatic nerve gap model. Two studies transplanted UCMSCs into tibial nerve and recurrent laryngeal nerve crush models. One study conducted allogenic transplantation in humans. A total of 151 subjects were treated. Methods of MSC delivery and transplantation varied among studies.

Several groups sought to improve nerve regeneration with UCMSCs combined with longitudinal scaffolds. Zarbakhsh *et al*[11] (2015) loaded UCMSCs on a silicone tube and interposed it into a murine sciatic nerve gap model. The authors attempted to compare the histological outcomes of human UCMSCs and rat BMMSCs in regenerating sciatic nerve gap in rats. While the author showed favourable results in nerve regeneration for both UCMSCs and BMMSC, the latter was found to produce superior results at the end point of 12 wk. The BMMSC group showed greater axon number and thicker myelin sheath diameter than the UCMSC group.

Ma *et al*[17] (2019) injected UCMSC-derived extracellular vesicles (EVs) into the tail veins of rats and sutured a silicone rubber tube into the sciatic nerve gaps of 24 rats. The authors found that UCMSC-EVs promoted motor function recovery and regeneration of axons and attenuated muscle atrophy. SFI analysis was used to assess the functional improvements. At 8 wk, UCMSC-EV group had similar SFI values to normal rats.

Matsuse *et al*[35] (2010) combined UCMSCs and Matrigel into transpermeable tubes and transplanted it into transected murine sciatic nerve tissue specimens. The authors induced UCMSCs into cells with Schwann cell properties by using β-mercaptoethanol, all-trans-retinoic acid and various cytokines. Subsequently, Matsuse *et al*[35] examined the effect of these induced UCMSCs and used two control groups; a positive control of human Schwann cells and a negative control of Matrigel alone. The

Table 1 Studies of umbilical cord derived mesenchymal stem cells in peripheral nerve axonotmesis and diabetic neuropathy *in vivo*

Ref.	Study design	Cell source	Subject	Treatment group	Control group	Extraction method	Cell treatment	Delivery method	Follow-up duration (wk)	Results
Hei *et al*[47], 2016	Case Control	Human	Murine	20 BDNF-transfected UCMSCs; 20 UCMSCs only	20 PBS	Human umbilical vein obtained immediately after delivery	UCMSCs were expanded in keratinocyte-serum free medium with various growth factors. Passage 5 UCMSCs were transfected with adenovirus vector containing BDNF	Xenogenic transplantation into crushed left sciatic nerve	4	Significant improvement in SFI, axon count, axon density, and nerve regeneration in both treated groups. BDNF-loaded UCMSCs showed greater improvements in the above metrics than the UCMSC group
Sung *et al*[46], 2012	Case Control	Human	Murine	18 UCMSCs	18 PBS	Human umbilical vein obtained immediately after delivery	UCMSCs were culture-expanded in growth factors. Passage 5 UCMSCs were labelled with PKH26 fluorescent cell linker	Xenogenic transplantation into crushed sciatic nerve	4	Significant improvement in SFI, axon density and axon regeneration in UCMSCs group compared to control. Increased BDNF and tyrosine kinase receptor B mRNA compared to control
Gartner *et al*[48], 2012	Case Control	Human	Murine	6 undifferentiated UCMSCs + PLC; 7 differentiated UCMSCs + PLC; 7 UCMSCs only	6 injury only; 7 injury repaired with PLC only; 6 without injury	UCMSCs from human umbilical cord Wharton's jelly matrix purchased from third-party source (PromoCell GmbH)	Passage 5 UCMSCs were supplemented with bovine foetal serum. UCMSCs were treated with neurogenic media and differentiated into neuroglial-like cells	Xenogenic transplantation into right sciatic nerve lesion (3 mm) crushed with non-serrated clamp	12	Significant improvement in both undifferentiated and differentiated UCMSCs groups in terms of SFI, EPT, WRL as well as myelin sheath thickness compared to all controls

Gartner *et al*[49], 2012	Case Control	Human	Murine	6 UCMSCs only; 6 undiffe-rentiated USMSCs + Chitosan type III	6 negative control; 6 wrapped in Chitosan type III	UCMSCs from human umbilical cord Wharton's jelly matrix purchased from third-party source (PromoCell GmbH)	Passage 5 UCMSCs were loaded on Chitosan type III biomaterial scaffold	Xenogenic transplan-tation into crushed right sciatic nerve lesion	12	Significant impro-vement in muscle force deficit and axonal regrowth in UCMSC Chitosan type III group compared to controls
Xia *et al*[54], 2015	Case Control	Human	Murine	40 UCMSCs	40 saline solution; 40 untreated rats	Human umbilical cord blood plasma obtained from different individuals with identical blood type	UCMSCs were culture-expanded in normal MSC media. Number of passage was not specified	Intra-vascular injection into left femoral artery of rat with streptozotoc in induced diabetic foot ulcer	2	Significant impro-vement in restoring femoral nerve conduction in UCMSCs group compared to control groups at 3 days, 1 wk and 2 wk

UCMSCs: Umbilical cord derived mesenchymal stem cells; BDNF: Brain-derived neurotrophic factor; SFI: Sciatic function index; PBS: Phosphate buffered saline; PLC: Poly (DL-lactide-ε-caprolactone); WRL: Withdrawal reflex latency; EPT: Extensor postural thrust.

group assessed SFI values and compared immunoelectron micrographs. They concluded that the treatment group with Schwann Cell-UCMSCs group was equivalent to treatment with Schwann cells based on histological criteria and functional recovery.

Cui *et al*[14] (2018) and Pan *et al*[50] (2017) delivered UCMSCs using a collagen conduit. Cui *et al*[14] (2018) transplanted human UCMSCs into canine sciatic nerve gap models via a longitudinally orientated collagen conduit embedded with UCMSCs. Compound muscle action potential (CMAP) was found to be statistically greater in the UCMSC treated group compared with the collagen conduit only group. Pan *et al*[50] (2017) appraised the use of UCMSCs with a heparinised collagen conduits in transected rabbit recurrent laryngeal nerves. The authors assessed the effectiveness of passage-4 UCMSCs loaded on heparinised scaffold that released Nerve growth factor (NGF). Electromyograms at 8 wk revealed that treated lesions recovered normal nerve function. Biological markers of neurogenesis, including calcium-binding protein S100, neurofilament and AChE, were expressed at a greater level following treatment. Xiao *et al*[51] (2015) undertook a study exploring the effect of UCMSCs in a chitosan conduit interposed into the tibial nerve of a rabbit model. Xiao *et al*[51] found that nerve conduction velocity was significantly higher in the treatment group. The myelin sheath thickness and the growth of axis bud were both increased in the UCMSC group. Pereira *et al*[52] (2014) used PLC as a conduit for UCMSCs in murine sciatic nerve crush models. The group compared differentiated and undifferentiated UCMSCs. They established no difference in the degree of nerve regeneration between UCMSC that were differentiated into neural-glial-like cells and undifferentiated UCMSC groups. Both UCMSC groups showed increased myelin sheath thickness and enhanced recovery in motor and sensory function.

Two groups sought to investigate the use of UCMSCs embedded on a human amniotic membrane scaffold[5,53]. Li *et al*[53] (2012) found significant improvements in SFI, CMAP and gastrocnemius muscle diameter in UCMSC-loaded scaffolds group compared to cell-free scaffolds. Li *et al*[6] (2013) analysed how UCMSCs loaded on a human amniotic membrane scaffold affected the repair of a transected radial nerve in human subjects. Thirty-two patients with radial nerve injuries from radial shaft fractures were included in the study; twelve patients received neurolysis to remove neural scar tissue, and transplantation of UCMSCs on an amniotic membrane. The remainder 18 patients received neurolysis only. At 12 wk, the electrophysiological function of the UCMSC-treated group had improved electromyography readings. The muscular power, touch sensation and pain sensation were also significantly improved as compared to the neurolysis group.

Studies of UCMSCs in diabetic neuropathy

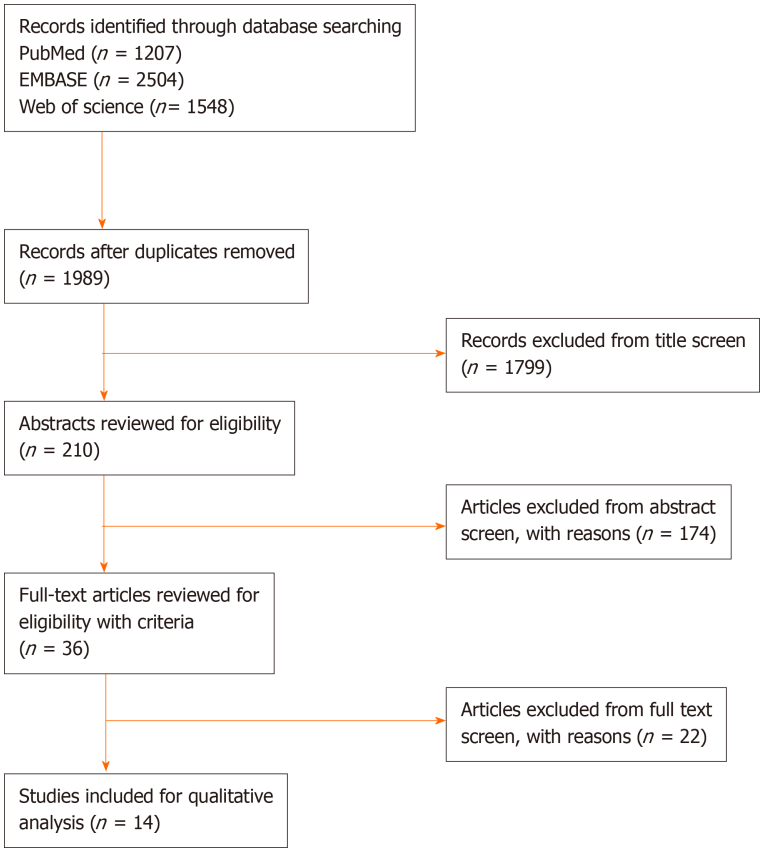

Figure 1 PRISMA Flow diagram.

One study explored the role of UCMSCs in femoral nerve neuropathy[54] (Table 1). The authors modelled diabetic neuropathy in murine subjects by inducing diabetes with streptozotocin and created a dorsal hind foot ulcer through empyrosis. UCMSCs were delivered intravascularly through the femoral artery in the treatment group. Saline injections were used in the control group. Serum NGF and neurofilament 200 (NF-200) were measured by Enzyme Linked Immunosorbent Assay (ELISA). The results demonstrated that serum NGF and NF-200 increased in the UCMSC treated rats. Additionally, functional studies using electroneurogram showed that femoral nerve conduction was improved in the UCMSC subjects.

DISCUSSION

The studies in this review reported compelling positive outcomes for the use of human UCMSC to repair peripheral nerve lesions. None of the studies reported immunogenic nor significant complications. While the source cell utilised was consistent among the studies, there were significant variability in cell treatment and methods of transplantation with variable effectiveness as determined by several different outcome measures. There was also moderate heterogeneity in the *in vivo* models used. It is therefore difficult to draw conclusions on the optimal method of cell delivery to nerve lesions. Nevertheless, it does imply that UCMSCs are a useful cell source.

The process of cell harvest did not vary greatly between the studies. Human umbilical cord and umbilical cord blood are generally considered medical waste and so there are minimal ethical barriers to tissue sampling[55]. This provides a practical advantage for the use of UCMSCs and may explain why it is commonly used in tissue engineering experiments. The biochemical properties of UCMSCs may also be of advantage as studies comparing different source cells for MSCs have found UCMSCs to possess a greater ability to proliferate *ex vivo* and express a higher level of Vascular Endothelial Growth Factor (VEG-F) and Human Growth Factor (HGF) at late passages[56,57]. One study in this review compared UCMSCs to BMMSCs in sciatic nerve regeneration and found BMMSCs to produce superior results. The authors however, evaluated cell architecture on microscopy but did not carry out nerve conduction studies or functional analysis which may better inform clinical

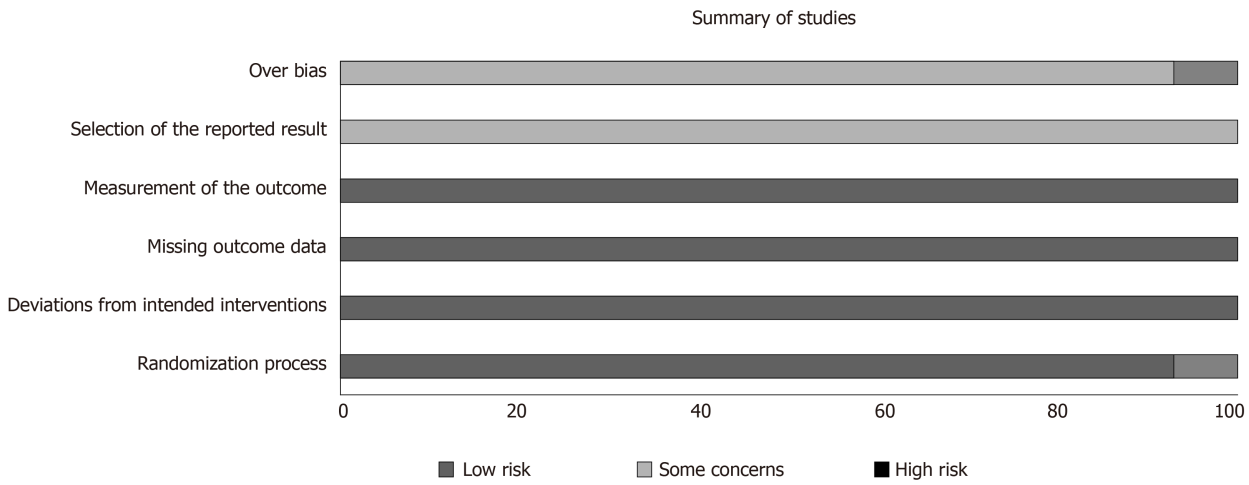

Figure 2 Summary of overall bias.

relevance[11]. UCMSCs also appear to have a different multilineage differentiation profiles to other MSC, and is able to be induced into neuron-like cells *in vitro*, which may favour applications in nerve regeneration[58]. There is some evidence to suggest that characteristics of the donor affect the ability of UCMSCs to differentiate. For example, undifferentiated UCMSCs obtained from patients with pre-eclampsia may produce greater levels of neuronal markers[59]. Therefore, exploration of different patient and gestational characteristics, such as age could help determine optimal source conditions.

There is no set protocol for the *ex vivo* expansion of UCMSCs in the literature. In our article, UCMSCs were generally transplanted between the third and fifth passage. Indeed, the gene expression profile of UCMSC is known to vary according to the number of passages, with some studies showing that UCMSCs do not express CD105, a defining marker for MSCs, until passage-5[60]. In view of this, it would be meaningful to identify and investigate the expression of important neurogenic markers as a function of stages of passage in future experiments. Some of the studies in this review pre-treated UCMSCs in order to induce them into particular cellular phenotypes prior to transplantation. Pereira *et al*[52] and Gartner *et al*[49] utilised a similar culture protocol and pre-treated UCMSCs with neurogenic media. They observed a neuroglial-like morphology on microscopy and a transcriptomic profile showing upregulation of neuroglial genes including Glial Fibrillary Acidic Protein (GFAP), Growth Associated Protein 43 (GAP43) and Neuronal Specific Nuclear Protein (NeuN). Both studies found differentiated MSCs to be more effective than undifferentiated UCMSCs. Aside from gene expression, it would be important to clarify the exact mechanism through which these differentiated cells act to promote nerve regeneration. The optimal protocol for differentiation is also ill-defined as Matsuse *et al*[35] used a different set of culture condition to induce UCMSCs into Schwann-like cells and found the latter to be more effective. Furthermore, there is a lack of agreement on the primary mode of action of MSCs in promoting nerve regeneration. It is unclear whether transplanted cells directly replicate and replace cells in the lesion. Some experiments of optic nerve lesions suggest that transplanted MSCs remain local and replicate[61]. Emerging *in vitro* evidence points towards paracrine effects as the predominant mechanism of action. It appears that pre-treatment of Schwann cells with UCMSC conditioned media increases BDNF and NGF expression which are surrogate measures of neurogenic potential[29]. In our review, Mak *et al*[17] examined the effectiveness of UCMSC-derived exosomes in nerve repair. Through peripheral intravenous injection of UCMSC EVs, they demonstrated that it could act systemically to encourage nerve regeneration at a nerve gap without off-site complications. As the use of EVs in this endeavour gains attention, further studies would be required to establish a dose-response relationship and the best method for delivering EVs to lesions.

It is difficult to determine the best UCMSC implantation method. Our review has captured studies that directly implanted UCMSCs and reported good outcomes. Studies investigating the use of conduits to guide nerve regeneration suggest that this is superior to direct implantation of MSCs alone[14,50]. The use of conduit that elude growth factors such as NGF along with UCMSC implantation appear to confer additional benefit[50]. Additionally, intravenous injection of UCMSC-EVs at a peripheral site also produce positive outcomes[17]. Interestingly, a comparison of local

Figure 3 Risk of bias in individual studies.

and intravenous BMMSC administration in sciatic nerve injury models suggest that systemic treatment provides a more significant improvement in nerve conduction, whereas local treatment improved neuronal fibre counts[62]. Other experiments have shown that peripherally injected MSCs localise to nerve lesions in murine models of sciatic nerve injury[63]. It could be inferred from these findings that there are differing mechanisms and sites of action for the two methods of implantation, suggesting that a treatment regime including both delivery methods concomitantly may produce the best outcome.

There are several issues pertaining to translating the findings derived from *in vivo* animal models for therapeutic application in humans. The majority of studies in our review employed a murine surgical sciatic nerve defect model to assess nerve regeneration. The critical nerve gap length, defined as a gap across which regeneration would not occur without nerve grafting or bridging is considered to be greater in humans than murine subjects[64,65]. Therefore, studies assessing murine nerve gaps may overestimate the therapeutic potential of treatments. Furthermore, it may be difficult to scale-up effective concentrations of transplanted UCMSCs to humans. In a study of rat nerve defects treated with tacrolimus, functional recovery tapers off at 9 wk following treatment and becomes indistinguishable from untreated rats at 10 wk[66]. Therefore, at later time points, which are more relevant to clinical presentations of nerve injury, the regenerative biology of murine nerve appears to differ from that of humans. Our interpretation from the *in vivo* animal studies in this review is complicated by the use of the sciatic nerve, which possesses a sensory and motor component, and thus renders functional analysis difficult. It is conceivable for sensory loss to mask a post-surgical motor defect on gait analysis, similarly, it may be possible for loss in motor function to cause underestimation of sensory recovery. Owing to the heterogeneity in starting points for different functional measures, a pooled analysis of quantitative outcomes could not be performed in this review. Therefore, clinically relevant and robust quantifiable outcome measures remain a significant barrier to the reliability of animal studies. One study in this review assessed UCMSC transplantation in human radial nerve defects and reported improved motor and sensory function and electrophysiological measures[53]. The group however, delivered the MSCs through a scaffold, and did not compare outcomes with a control group of the scaffold alone.

According to the results of our risk of bias analysis, 13 of 14 studies had a moderate risk of bias, and one study had a high risk of bias (Figure 1). The reporting of outcome measures contributed to an increased risk of bias in all studies, as most of the studies reported improvement in some but not all outcomes yet concluded that UCMSCs were effective overall. This could be owing to the significant heterogeneity in cell treatment and delivery methods which as the literature suggests, could contribute to different aspects of nerve regeneration.

Table 2 Studies of umbilical cord derived mesenchymal stem cells in Peripheral Nerve Neurotmesis *in vivo*

Ref.	Study Design	Cell Source	Subject	Treatment Group	Control Group	Extraction Method	Cell Treatment	Delivery Method	Follow-up length (wk)	Results
Ma *et al*[17], 2019	Case Control	Human	Murine	24 UCMSC-extracellular vesicles injections	24 PBS	Human umbilical cords obtained from full-term deliveries	UCMSCs were expanded *ex vivo*. Passage 3 UCMSCs were used	UCMSC-EV were injected into the tail veins	8	Significant improvement in SFI, axon regeneration, recovery of motor function and reduced muscle atrophy. Regenerated nerve fibre diameter was larger in USMSC-EV injection groups compared to control
Zarbakhsh *et al*[11], 2015	Case Control	Human	Murine	8 silicone tubes filled with fibrin glue seeded with 500000 UCMSCs	8 silicone tubes filled with fibrin glue seeded with 500000 rat BMMSCs; 8 control rats with nerve gaps filled with fibrin glue	Human umbilical cords obtained from full-term deliveries	Passage 3 UCMSCs were loaded on a 12 mm silicone tube interposed into a 10 mm nerve gap	Xenogenic transplantation into sciatic nerve gap specimens	12	Significant improvement in nerve histomorphology in UCMSC and BMMSC groups compared to controls. BMMSC showed the greater improvement
Cui *et al*[14], 2018	Case Control	Human	Canine	5 LOCC with UCMSCs	5 negative control; 5 positive control (autografted nerve segment reversed); 5 LOCC only	Human umbilical cords obtained from full-term deliveries.	UCMSCs were expanded. Passage 3 UCMSCs were cultured and embedded into a LOCC	Xenogenic transplantation into transected sciatic nerve of 15 months adult Beagles	39	Significant improvement in CMAP and conduction latency in LOCC embedded with UCMSC compared to LOCC alone
Pan *et al*[50], 2017	Case Control	Human	Rabbit	12 NGF loaded HC-scaffold with UCMSCs; 12 HC-scaffold with UCMSCs	12 negative control (no grafting into nerve gap); 12 HC-scaffold with PBS; 12 collagen (C)-scaffold	Human UCMSCs obtained from third party source (Stem Cell Bank of Guangdong Province)	Passage 4 UCMSCs were embedded into NGF-loaded HC-scaffold or C-scaffold	Xenogenic transplantation into transected recurrent laryngeal nerve tissue specimens with daily penicillin injection until day 5 post-intervention	8	Significant improvement in transected nerve repair in UCMSC NGF-loaded HC-scaffold as compared to all other groups

Li *et al*[53], 2012	Case Control	Human	Murine	40 amnion tube with UCMSCs	40 amnion tube with saline implant	Human umbilical cords obtained from full-term deliveries	Passage 3-4 UCMSCs were cultured and loaded on an amniotic scaffold	Xenogenic transplantation into transected sciatic nerve tissue specimens	20	Significant improvement in SFI and CMAP in UCMSC group compared to control. Gradual improvement in threshold stimulus and maximum stimulus intensity in UCMSC group compared to control
Li *et al*[6], 2013	Case Control	Human	Human	12 neurolysis followed by 10 mL UCMSCs injection of 1.75×10^7 cells	20 neurolysis only	Human umbilical cords obtained from full-term deliveries	Passage 2 UCMSCs were loaded on an amniotic membrane scaffold. Both groups received 3 days of oral cephalosporin	Allogenic transplantation into radial nerve injury following radial shaft fracture	12	Significant improvement in muscular strength, touch and pain sensations in UCMSC group compared to control. Improved electrophysiological function in UCMSC group as compared to control
Matsuse *et al*[35], 2010	Case Control	Human	Murine	6 UCMSCs; 10 Induced UCMSC	6 negative control; 5 induced UCMSC	Wharton's Jelly extracted from umbilical cords of full-term caesarean deliveries	Passage 3 UCMSCs were induced into Schwann-like cells	Xenogenic transplantation into transected sciatic nerve tissue specimens	3	Significant improvement in SFI in all treated as compared to control with the greatest improvement in UCMSC group
Xiao *et al*[51], 2015	Case Control	Human	Rabbit	10 chitosan conduit anastomosis bridge filled with UCMSCs	10 chitosan conduit anastomosis only; 10 untreated	Not specified	UCMSCs were loaded into a chitosan conduit	Xenogenic transplantation into tibial-common peroneal nerve end-to-side anastomosis	12	Significant improvement in myelin sheath thickness, Schwann cell growth, growth of axis bud and growth velocity of regenerated fibre in UCMSC group compared to controls. No significant difference observed between either control groups

Pereira *et al*[52], 2014	Case Control	Human	Murine	6 undifferentiated UCMSCs + PLC; 6 differentiated UCMSCs into neural-glial-like cells + PLC	6 untreated; 6 treated with suture; 6 without nerve gap	Human Wharton's Jelly UCMSCs obtained from third-party source (PromoCell GmbH)	Passage 5 UCMSCs were fixed onto PLC scaffold	Xenogenic transplantation into sciatic nerve gap specimens	20	Both UCMSC treated groups showed increased myelin sheath thickness, enhanced recovery in motor and sensory function. No significant difference was noted between differentiated and undifferentiated groups. PLC use did not significantly improve nerve regeneration

UCMSCs: Umbilical cord derived mesenchymal stem cells; LOCC: Longitudinally orientated collagen conduit; SFI: Sciatic function index; NGF: Nerve growth factor; PBS: Phosphate buffered saline; HC: Heparinized collagen; PLC: Poly (DL-lactide-ε-caprolactone); EV: Extracellular vesicle.

In conclusion, while there are homeostatic responses that promote nerve regeneration following injury, the body's natural capacity is inadequate for the recovery of satisfactory nerve function. The evidence summarised in this systematic review supports the notion that UCMSC transplantation is an effective treatment option for nerve injury. Several barriers must be overcome before these findings can be translated into the clinical setting. Importantly, development of a reliable *in vivo* animal model, and a standardised method of assessing nerve regeneration would allow the optimal method of cell transplantation to be determined.

ARTICLE HIGHLIGHTS

Research background

Peripheral nerve injury can be a debilitating condition. Traditional treatment options are often ineffective. There is an urgent need for new treatment modalities. Mesenchymal stem cell (MSC) transplantation holds promise as a cell-based regenerative approach in treating nerve lesions. MSCs can be sourced from various tissues, and this may affect their regenerative capacity. Here, we appraise the *in vivo* evidence for the use of human umbilical cord-derived MSCs (UCMSCs) in peripheral nerve regeneration.

Research motivation

There is contention regarding the optimal cell-source for the harvest of MSCs. Some evidence suggests that MSCs from certain tissue types have superior neurogenic capacity. It is critical that we determine the best cell-source for nerve repair, in order to facilitate an efficient production protocol and maximise clinical benefit.

Research objectives

To investigate whether UCMSCs are effective in nerve regeneration in *in vivo* models of nerve injury.

Research methods

We performed a systematic literature review according to the PRISMA statement. A search was conducted on three databases (PubMed, EMBASE and Web of Science) by two independent investigators from inception to September 2019 for studies examining the use of UCMSCs in *in vivo* models of nerve injury. The evidence was appraised using Cochrane's RoB 2.0 Tool.

Research results

A total of 14 studies were included in the review, with a total of 279 subjects. The studies reported that transplantation of human umbilical cord MSCs were effective in regenerating nerve lesions. There were general improvements in histological and functional outcomes. The studies did not report significant complications.

Research conclusions

Human umbilical cord-derived MSCs were effective in repairing nerve lesions in both animal and human models of nerve injury. Additional studies are required to correlate histological outcomes with functional improvements, as not all studies assessed both. More human studies are necessary to inform the efficacy in humans. High quality randomized controlled trials would be instructive in this case. Long-term follow up in these types of study will help inform the safety of MSC transplantation.

Research perspectives

There is limited evidence examining the use of MSCs derived from other tissues in their capacity to regenerate nerve lesions. Further studies comparing different tissue cell-source directly would be highly informative. *In vitro* studies of MSC-biomaterial scaffolds may aid the development of more efficient MSC delivery methods. As the nature of nerve injury can vary significantly, the approach to transplantation, such as dose delivery may need to be catered to the individual lesion. Studies comparing the effect of MSCs on different *in vivo* models could help delineate this.

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