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Periodontal tissue stem cells and mesenchymal stem cells in the periodontal ligament☆

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ABSTRACT

Periodontal tissue stem cells, which play a crucial role in maintaining the homeostasis of periodontal tissues, are found in the periodontal ligament (PDL). These cells have long been referred to as mesenchymal stem/stromal cells (MSCs), and their clinical applications have been extensively studied. However, tissue stem cells in the PDL have not been thoroughly investigated, and they may be different from MSCs. Recent advances in stem cell biology, such as genetic lineage tracing, identification of label-retaining cells, and single-cell transcriptome analysis, have made it possible to analyze tissue stem cells in the PDL *in vivo*. In this review, we summarize recent findings on these stem cell populations in PDL and discuss future research directions toward developing periodontal regenerative therapy.

Contents

1. Introduction

Tissue stem cells (also known as adult stem cells or somatic stem cells) are cell populations that give rise to mature cell types that constitute tissues; they are responsible for tissue turnover by repeatedly proliferating and differentiating, as needed [1]. The rate of cell turnover varies considerably among tissues. The intestinal

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epithelium, skin epithelium, and hematopoietic cells, which have more active cell turnover than other tissues, have been studied extensively [2], and marker molecules have been used to define and elucidate the signaling pathways that control their activation [3–5]. Periodontal tissue consists of the gingiva, periodontal ligament (PDL), cementum, and alveolar bone, and tissue homeostasis is maintained by periodontal tissue stem cells. Studies using human and animal models have suggested that periodontal tissue stem cells reside in the PDL [6–8], and they are often referred to as periodontal ligament stem cells (PDLSCs) [9–11]. Current periodontal tissue regeneration therapies aim to utilize these cells [12,13]. On the other hand, mesenchymal stem/stromal cells (MSCs) have been the focus of attention for a long time, especially for regenerative medicine [14–16]. The differences between tissue stem cells and MSCs in various tissues are often ambiguous and have been the subject of debate [17]. Recently, tissue stem cells in the bone have been defined as skeletal stem cells (SSCs) in mice [18] and humans [19]. Additionally, comprehensive profiling of SSCs and other cell types in the tissue has been conducted using single-cell RNA sequencing (scRNA-seq), and their cellular hierarchy has been clarified [20]. However, the periodontal tissue stem cells in the PDL remain largely unknown. In this review, we summarize the recent discussions of stem cell populations in periodontal tissues and introduce recent research advances using cell transplantation, genetic lineage tracing, scRNA-seq, and label-retaining cells, for PDLSCs. In addition, we discuss the fate of transplanted MSCs and issues to be addressed in the future.

2. Stem cell populations in periodontal tissue

2.1. Definition of stem cells

The existence of cells isolated from the bone marrow that are capable of forming colony-forming unit-fibroblasts (CFU-F) and bone through ectopic grafting has been known for a long time, and these cells are referred to as bone marrow-derived MSCs (BMSCs) [21]. MSCs were proposed by Caplan in 1991 based on the concept that they can form or regenerate multiple mesenchymal tissues [22]. Since then, numerous studies have shown that MSCs can be cultured, and MSCs can differentiate beyond mesenchymal lineages, such as bone, cartilage, and fat, to mesodermal tissues, including muscle, *in vitro* and *ex vivo*. Several preclinical studies and clinical trials have also shown the efficacy of MSC transplantation [23–25]. However, unlike hematopoietic stem cells, which are more strictly defined and can form the entire hematopoietic system by single cells, MSCs are heterogeneous, and single cells do not form entire tissues. The verification of such clonality and *in vivo* differentiation is necessary for the proof of *bona fide* stemness. There is also mounting evidence that transplanted MSCs do not form or regenerate tissues in a cell-autonomous manner[26,27]. Therefore, the term mesenchymal stem/stromal cells are debatable. Mao et al. advocated calling these cells “connective tissue stem cells” [28]. Caplan insisted on not calling them “stem cells” [29], and mesenchymal “stromal” cell is the most widely used [30]. Recently, MSCs have been used to represent cells that produce extracellular vesicles (EVs), such as exosomes, and they are now used by basic researchers beyond the field of clinical medicine [31]. Importantly, each study should refer to an appropriate name for the cells, according to what level of stemness has been proven. MSCs are phenotypically diverse based on their tissue origins and preparation, which should be explicitly stated. In this review, we refer to periodontal tissue stem cells in the PDL that are proved their stemness by rigorous *in vivo* or *ex vivo* assays as PDLSCs; the cells that were isolated from the PDL, expanded *in vitro*, and showed multi-differentiation potential are referred to as PDL-MSCs.

2.2. Stem cell populations in the periodontal ligament

The existence of multi-differentiating PDL-MSCs isolated from PDLs was first shown in a seminal study by Seo et al. [6]. These cells were isolated from human PDL and expressed STRO-1. *In vitro* and transplantation experiments have shown that these cells are capable of differentiating into multiple lineages. Furthermore, in a detailed study of the periodontal tissue regeneration process using a beagle dog model of periodontal disease, it was shown that cells migrated and proliferated from the PDL to the bone defect and adhered to the root surface [7]. These findings suggest that stem cells that form and regenerate periodontal tissues exist within PDL and differentiate into three lineages: fibroblasts, cementoblasts, and osteoblasts; this has been a long-standing dogma in periodontology (Fig. 1). In order to understand the cell populations in PDL at the single-cell level, Ueda et al. performed single-cell cloning of cells migrated from the mouse PDL and established single cell-derived cell lines with various degrees of osteogenic differentiation potential [32]. When each

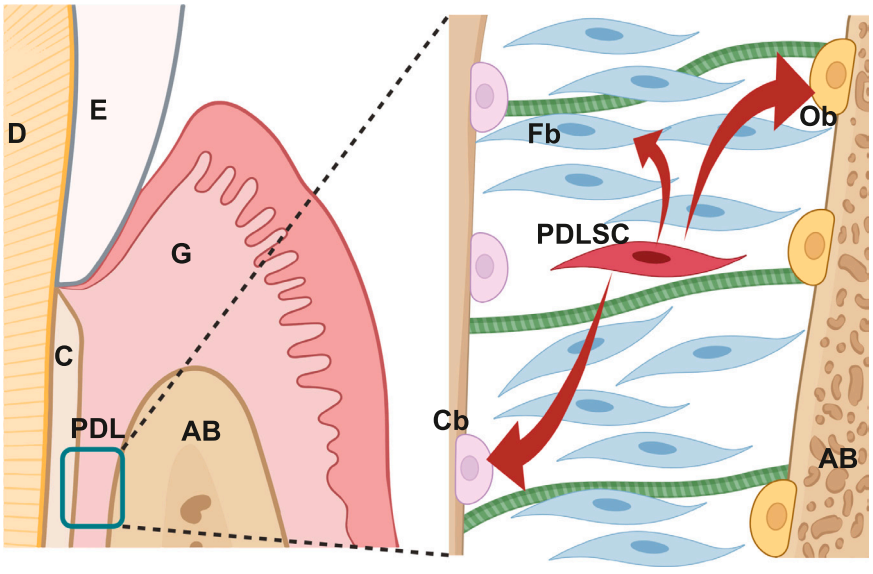

Fig. 1. Three lineages differentiated from PDLSC. E: Enamel, D: Dentin, C: Cementum, G: Gingiva, AB: Alveolar bone, PDL: Periodontal ligament, Fb: Fibroblast, Ob: Osteoblast, Cb: Cementoblast, PDLSC: Periodontal ligament stem cell.

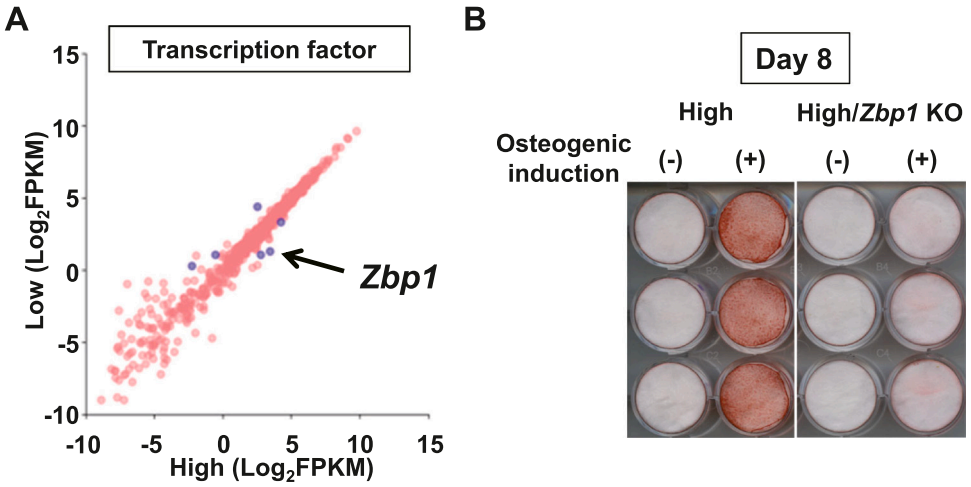

Fig. 2. High and low osteogenic progenitors in PDL. A. Scatter plots of the mean FPKM values of transcription factors in high and low osteogenic clones calculated by RNA-seq analysis. The differentially expressed genes showing > 2-fold statistically significant differences are shown in purple. FPKM: fragments per kilobase of exon per million reads mapped. B. High osteogenic clones and *Zbp-1* knockout clones were cultured in osteogenic medium and stained with Alizarin Red S on day 8 of differentiation induction. These images were adapted from [32].

clone was characterized, there were no significant differences in their morphology, surface antigens, or proliferative capacity, despite their apparently different osteogenic potential. The study identified *Zbp1* as one of the genes highly expressed in the osteogenic clones by RNA sequencing (RNA-seq, Fig. 2 A). Deletion of *Zbp1* from high osteogenic clones by genome editing abolished differentiation potential, while over-expression of *Zbp1* in low osteogenic clones using viral vectors promoted osteogenic differentiation (Fig. 2B). Together with other studies [6,33,34], PDL contains heterogeneous cell populations, including PDLSCs and progenitors. However, characterization and functional analysis of these stem cells have been biased toward *in vitro* and transplantation experiments, and a few studies, until recently, have evaluated the direct proof of stemness described later. Additionally, there are several difficulties in characterizing and defining PDLSCs. First, each cell type in the PDL is not strictly defined; thus, the differentiation pathways from stem cells to differentiated cells are unclear. In particular, the differences between cementoblasts, which form cementum, and osteoblasts, which form alveolar bone, have not been clarified. It has been questioned whether they are of the same cell type but reside in different anatomical locations [35,36]. No definitive marker molecules or specific transcription factors have been identified, although candidates such as cementum protein 1 (CEMP1) have been suggested [37]. For this reason, many studies in this field use cells without distinguishing between osteoblasts and cementoblasts, but collectively refer to hard tissue-forming cells or merely osteoblasts. There is no cementogenic induction regimen *in vitro* available to examine cementogenic potential. The next difficulty specific to PDL is that they are fibrous connective tissues, and the largest portion of the volume is consisted of fibroblasts. However, fibroblasts are very heterogeneous [38], and it is difficult to determine whether they are stem cells that have proliferated or differentiated *in vivo*. Thus, it is common to verify the osteogenic potential of stem cells by *in vitro* or transplantation assays, which is to form alizarin red S-stained calcified nodules or bone-like tissue by ectopic transplantation. In addition, whether these cells have adipogenic potential has not been established, although adipose is not originally present in the periodontal tissue. Strategies for examining, defining, and directly demonstrating stem cell potential have become available recently and will be discussed later.

Other stem-like cell populations present in the connective tissues include pericytes. All pericytes are not MSCs, but it has been proposed that at least some of MSCs are derived from pericytes [39]. In particular, green fluorescent protein (GFP)-positive cells isolated from bone marrow or adipose tissue of nestin-GFP transgenic mice are pericytes and have been shown to have high self-renewal and multi-lineage potential in multiple serial transplantation experiments [40,41], suggesting that they are *bona fide* MSCs. It has been suggested that pericytes may exist in the PDL, too [42].

3. Research strategies for PDLSCs

Although histological and *in vitro* analyses using human tissue and cultured cells are essential, *in vivo* analysis to genetically trace cell fates and *ex vivo* analysis using freshly isolated cells are the driving forces of current stem cell research. We illustrate recent research approaches for solving the above-mentioned issues regarding PDLSCs.

3.1. Cell transplantation assay

One strategy that has been used for regenerative therapy is to isolate and transplant stem or progenitor cell populations. A pioneering work showed that hematopoietic stem cells could be transplanted into lethally irradiated mice to reconstitute all blood lineage cells [43]. However, in connective tissues, it is impossible to deplete the original tissue, and cell transplantation is usually performed ectopically, which is different from transplantation in native tissues [28]. Isolated cells from periodontal tissue can ectopically form bone-like tissues, indicating that the transplanted cell population contains progenitor and stem cells. However, transplanted cells have been shown to exhibit different dynamics from the endogenous cells [44], and this technique is no longer considered the most rigorous method for demonstrating stemness in animal model [28]. In contrast, regenerative therapy using cell transplantation has been extensively examined, and transplantation assay remains one of the most important predictors for the clinical efficiency.

3.2. Genetic lineage tracing using selective markers

One of the most direct approaches to revealing stem cell lineages is genetic lineage tracing using mouse genetics. This method provides evidence of stem cell differentiation *in vivo* since the cells can be permanently labeled and thus traced their cellular fate even if they differentiate and lose their original cell characteristics. It is achieved by crossing a lineage-specific Cre driver mouse and a Cre

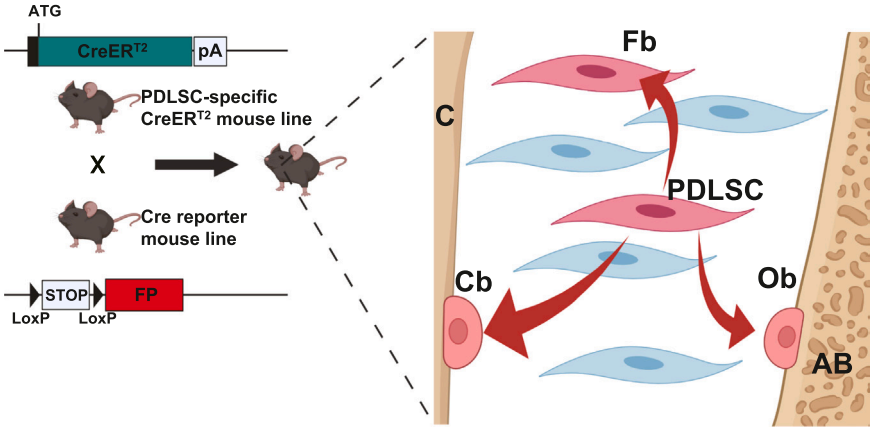

Fig. 3. Lineage tracing strategy of the periodontal ligament. Genetic lineage tracing of the PDLSC requires double transgenic mice generated by crossing PDLSC-specific CreER^{T2} and Cre reporter mouse lines. In the system, a fusion protein of a mutated estrogen receptor and Cre recombinase is expressed in PDLSC, and the two loxP sites in the reporter allele will undergo recombination once tamoxifen is administrated. Upon removal of the STOP sequence, the PDLSC expresses the fluorescent protein (*e.g.*, tdTomato). Once PDLSC is marked, the mark is heritable and permanent and thus transmitted to all the descendants of PDLSC. PDLSCs: periodontal ligament stem cells, ATG: start codon; pA: polyadenylation signal sequence, STOP: stop cassette, FP: fluorescent protein, C: Cementum, AB: Alveolar bone, Fb: fibroblast, Ob: osteoblasts, Cb: cementoblasts.

reporter mouse. For applying this technique to periodontal tissue stem cells, it is essential to use Cre driver mice that expressed Cre only in the target cells (periodontal stem cells) but not in the cells after differentiation (Fig. 3). In addition, periodontal tissue should be analyzed after PDL development is completed. For this reason, it is desirable to use CreER^{T2} mice in which Cre is active in a tamoxifen-induced manner, although it is difficult to achieve 100% recombination efficiency with tamoxifen. Thus, meticulous validation is necessary to determine whether the targeted cells are labeled. In periodontal tissues, the use of CreER^{T2}-expressing mice under the control of a PDLSC-specific promoter, which is expressed exclusively in PDLSCs and not in other differentiated lineages such as fibroblasts, osteoblasts, and cementoblasts, would provide evidence that PDLSCs differentiate to other terminally differentiated cells. To date, several lineage-tracing studies on periodontal tissues have been published. First, Roguljic et al. used αSMA-CreER^{T2} mice to show that αSMA-positive cells in the perivascular area differentiate to Col2.3-GFP-positive osteoblasts/cementoblasts and scleraxis-GFP-positive PDL fibroblasts [45]. They were also shown to differentiate to mature cell types in an injury model. The other two studies showed that Axin2-positive cells, or Wnt-responsive cells, are sparsely present in the PDL, and lineage tracing analysis was performed using Axin2-CreER^{T2} knock-in mice [46,47]. Although the cells were in a quiescent state and did not proliferate extensively, they contributed to bone formation after tooth extraction and during cementum formation. Men et al. recently traced the fate of Gli1-positive cells, which were suggested to be stem/progenitor cells in other tissues, using Gli1-CreER^{T2} knock-in mice, and showed that Gli1-positive cells in periodontal tissues actively differentiate to periodontal tissue components [48]. Gli1-positive cells in the PDL have been further studied during cementogenesis [49]. Leptin receptor-positive cells, the other stem cell population in the bone marrow, in the PDL contributed to the healing of extraction sockets [50], and paired related homeobox 1 (Prrx1)-positive cells contributed to cell-autonomous PDL reconstruction after tooth transplantation according to a recent study [51]. These results are significant because they provide *in vivo* evidence that a group of cells in the PDL can differentiate into the osteoblast and cementoblast lineages. However, these molecules also tend to be expressed in other cell types, including osteoblasts and cementoblasts. Therefore, it would be desirable to develop mice that express CreER ^{T2} under the control of a PDLSC-specific gene. Alternatively, specific gene that strictly labels only periodontal ligament cells (PDLCs), including PDLSC and fibroblast lineage but not osteoblast and cementoblast lineages, in the central zone of PDL [52] but not expressed in the osteoblast or cementoblast lineages in the enthesis zone of PDL [53] can be used. Periostin [54], Periodontal ligament associated protein-1/Asporin [55,56], Scleraxis [57] are candidate genes specific to periodontal ligament.

The lineage tracing approach has been effective in analyzing the fate and dynamics of stem cells in many tissues; however, there are some caveats. First, Cre may not be expressed in the target tissue as designed. Cre must be inserted downstream of the promoter to be expressed, and it is primarily the choice of the promoter that determines the specificity of Cre expression. There are multiple methods to generate Cre driver mice, including transgenics through pronuclear injection, bacterial artificial chromosome transgenics, and knock-ins through gene targeting by homologous recombination or recently genome editing technologies. Knock-ins usually carry a single copy of Cre under the endogenous promoter, thus faithfully recapitulating target gene expression. For PDL lineage tracing experiments, Cre driver mice should be validated meticulously in periodontal tissue. Secondly, some Cre strains exhibit cellular toxicity, unreported recombinase activities, mosaicism, inconsistent activity, and parent-of-origin effects [58]. Thirdly, the recombination efficacy by tamoxifen is not always 100%, and some inducible CreER^{T2} lines have leaky recombinase activities [59]. Therefore, it is necessary to ensure accurate Cre expression in the targeted tissue after tamoxifen treatment.

Recent advances in the lineage tracing approach enable us to analyze stem cell dynamics further in detail. Multicolor lineage tracing can reveal clonal dynamics of the target stem cells by using small amounts of tamoxifen to deliberately lower recombination efficiency and stochastically label cells with multicolor fluorescent proteins [60]. The use of other site-specific recombinases such as Flp/Frt or Rox/Dre, in addition to the most commonly used Cre/LoxP, enables us to trace the cells at the intersection of the two molecules [61,62]. Applying these methods to periodontal tissues will reveal the dynamics of PDLSCs in detail. The Cre/LoxP system can also be applied to conditional gene knockout studies by crossing floxed mice.

3.3. Identification of label-retaining cells using non-selective markers

These lineage tracing approaches are based on the use of specific markers to trace the dynamics of stem cells; however, it is necessary to use marker molecules that are expressed exclusively in PDLCs or PDLSCs. However, these markers are not currently available. An alternative marker-independent method for identifying stem cells involves the use of label-retaining cells. This technique utilizes the property that

stem cells rarely proliferate [63]. In other words, all cells are labeled at a given point in time and analyzed after a sufficient turnover period to identify quiescent stem cells as those that retain the label. In periodontal tissues, it has been reported that there are cells around blood vessels that retain the same label after long-term observation following the administration of a thymidine analog [64]. However, thymidine analogs can only enter the nuclei of cells during the proliferative phase at the time of administration and cannot label stem cells that are already in the quiescent phase. To overcome this disadvantage, a method has been developed to express a histone 2B-GFP fusion protein in the target cell population and stop the new expression using a tet-on/off system that can regulate the entry and exit of expression in a doxycycline-inducible manner [65]. It has already been applied in stem cell research in the hematopoietic system, intestinal epithelium, and incisor [66–68]. As described above, the strategy using non-selective markers is superior; it does not introduce bias; however, it should be noted that it is impossible to trace the further dynamics of the cells, such as how they respond to wound healing. The cell populations identified using this method should be further defined using specific markers, and a lineage tracing approach is being used.

3.4. Single-cell transcriptome analysis

Single-cell transcriptome analysis is useful for the comprehensive identification of cell types, including tissue stem cells and other cellular components. This strategy has been successfully applied to many other tissues, including bone [69,70]. Several analyses of dental tissues have been reported. Three single-cell analyses of mouse incisors have been summarized in a recent review [71]. There have also been single-cell RNA sequencing (scRNA-seq) analyses of periodontal tissues from human and mouse molars. Krivanek et al. performed scRNA-seq of human adult molars, and the resulting atlas was mainly derived from pulp and apical papillae and contained few PDLCs [72]. Caetano et al. isolated cells from the gingiva of periodontal patients and healthy controls and performed scRNA-seq analysis [73]. Pagella et al. performed a scRNA-seq analysis of PDL tissue scraped from the root surfaces of extracted human wisdom teeth. More than half of the cells analyzed were epithelial and endothelial cells; the mesenchymal cells were divided into MSCs, fibroblasts, and Schwann cells [74]. Nagata et al. isolated cells from molars from 25-day-old Col1a1-GFP mice and performed a scRNA-seq analysis, which yielded an Scleraxis-positive PDL cell population and identified molecules specific to cementoblast or osteoblast; *Pthlh*, *Tubb3*, and *Wif1* as cementoblast-specific molecules and *Phex*, *Nfib*, and *Pthr1r* as osteoblast-specific molecules [75]. However, the development of PDL is usually not complete for PDL at 25 days old, and whether these results can be adapted for adult PDL, cementoblasts, and osteoblasts remains to be investigated.

In single-cell analysis, the most important factor is the type of cell population used as input. Therefore, it is necessary to isolate PDL cells efficiently and comprehensively without contamination of other tissue. Mesenchymal cells of the cementum, alveolar bone, and PDLs also express several common extracellular matrices in PDLCs. In contrast, cells in the PDL express master regulators of osteoblasts, such as *Runx2* and *Sp7* [57,76,77]. To isolate PDL, it is critical to identify PDL-specific molecules and monitor their expression for cell isolation. Another new technology, spatial transcriptomics, does not require cell isolation and retains spatial information, it can be fascinating if it can be applied to periodontal tissue.

4. Fate of transplanted MSCs

Several clinical studies have been conducted to evaluate the efficacy and safety of stem/progenitor cell transplantation for periodontal tissue regeneration (recently reviewed in [78]). Transplanted stem cells are considered PDL-MSCs because they are expanded *in vitro* before transplantation, and no rigorous *in vivo* analysis has been conducted to prove their stemness and clonality. There has been a recent advancement in our understanding of the fate of transplanted cells. Initially, it was thought that the transplanted cells differentiate to mature cells and regenerate the tissue through a cell-autonomous mechanism. However, the transplanted cells were often undetectable within a few days, regardless of their administration [26,44]. It has been reported that transplanted cells do not differentiate during their survival, but they secrete various cytokines; in particular, the paracrine factor is important [27,44]. For example, Ozasa et al. demonstrated that autologous transplantation of adipose tissue-derived multi-lineage progenitor cells (ADMPCs) into alveolar bone defects enhances periodontal tissue regeneration[79]. Interestingly, ADPMCs release trophic factors, including insulin-like growth factor binding protein 6, and stimulates the differentiation of PDLSCs into osteoblasts and cementoblasts [80]. Other secreted factors from MSCs are extracellular vesicles, such as exosomes, which are used for cell communication [81], and MSC-derived exosomes have been studied extensively [31]. Recently, it has become clear that transplanted MSCs undergo apoptosis and are phagocytosed by macrophages, thereby activating anti-inflammatory and other pathways and inducing appropriate tissue regeneration [23,82,83]. Thus, MSCs have been shown to have therapeutic effects following transplantation, but their function may be different from that of endogenous PDLSCs. In bone tissue, human SSCs have recently been classified as PDPN+CD146-CD73 +CD164 + cells [19], and their stemness has been proven in serial transplantation experiments. SSCs, like hematopoietic stem cells, withstand long-term follow-up of donor cells after transplantation. These features were not observed in MSCs [84].

5. Future perspectives & conclusion

Recent studies have suggested that PDL-MSCs and PDLSCs are fundamentally different cell types, although there may be some overlap. PDLSCs have not yet been rigorously analyzed, and *in vivo* analysis using modern techniques is required. As in other tissues, genetic lineage tracing, identification of label-retaining cells, and single-cell transcriptome analysis will be useful, but reliable evidence will not be established without the use of PDL-specific mice and efficient isolation methods. Combining the results of these analyses with the accumulated results of MSC research will lead to the identification of stem cell population that sustains periodontal tissue and contributes to its regeneration. Elucidating the molecular mechanism of stem cell differentiation can lead to the development of new periodontal tissue regeneration therapies.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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