Stem Cells and Development

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Periodontal Ligament Stem Cells: current knowledge and future perspectives.

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Abstract (218 words)

Teeth represent a fascinating area of study in regenerative medicine, because of their unique and complex developmental origin. Several types of mesenchymal stem cells (MSC) have been characterized in the oral cavity, and those derived from the periodontal ligament (PDL) first isolated by our group in 2005, can be expanded in a xeno-free medium preserving morphological features and markers associated with pluripotency. These postnatal MSC can be easily recovered by non invasive procedures and cultured. This could facilitate the use of adult stem cells in human clinical regeneration therapy. In this review we summarize the results of our studies describing morphofunctional features, surface markers and multilineage differentiation capacity in vitro of PDL MSC obtained in our laboratories. In vivo characterization of PDLSC location and heterogeneity are still lacking. However we describe studies exploring the potential use of PDLSC to treat both periodontal diseases and regeneration of other tissues. These MSC may have an advantage in possessing also angiogenetic, immunoregulatory as well as anti-inflammatory properties. The secretome of such cells contains several interesting molecules mimicking the effects of the producer cells. We describe some recent studies from our group on the use of conditioned medium from PDL MSCs, and purified extracellular vescicles therein contained, in animal models of experimental autoimmune encephalomyelitis (EAE) and their potential application to human disease.

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Introduction

"All cells come from cells" represent the paradigm trough which Rudolf Virchow (1821-1902) postulates that all cells of the human body derive from a single cell and so starting regenerative medicine studies. Actually, the modern medicine, and in particular the regenerative medicine, focused its attention on adult stem cells, which are located in specific niches; they avoid the ethical and legal problems of earlier stem cells and they also can differentiate not only into their original source tissue, but also into cells of unrelated tissue.

Stem cells derived from oral cavity are particularly interesting. It is well known that tooth development occurs through mutually inductive signalling between oral epithelium and ectomesenchyme. Cells originating from migrating neural crest cells represent a multipotent cell population derived from the lateral ridges of the neural plate during craniofacial development [1].

Neural crest cells possess stemness features and multipotency, play a strategic role in tooth organ development, contributing to craniofacial bone formation, actually today they can be considered as the fourth germ layer [2]. To date, six different human dental stem cells, derived from oral cavity tissues, have been reported in literature: dental pulp stem cells (DPSCs), exfoliated deciduous teeth stem cells (SHED), periodontal ligament stem cells (PDLSCs), apical papilla stem cells, dental follicle stem cells (DFSCs), and gingiva stem cells (hGSCs) [3-7].

Among others, in the periodontal ligament a peculiar population of multipotent postnatal stem cells is present. PDLSCs were isolated with different scientifically sound techniques and described for the first time by Seo BM, (2004) [8] and Trubiani (2005) [9]. PDLSCs can be expanded in a xeno-free medium preserving morphological features, markers associated with pluripotency and a normal karyotype. The novel xeno-free culture method could facilitate the use of adult stem cells in human clinical regeneration therapy representing the basis for Good Manufacturing Procedure culture of autologous stem cells. PDLSCs are easily accessible from periodontal tissue with non invasive procedure during standard dental scaling and root planning [10,11].

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The periodontal ligament (PDL) is a soft connective tissue insert between the cementum and the inner wall of the alveolar bone socket, with a specific role: sustain and support the teeth within the jaw. PDL contributes to tooth nutrition, homoeostasis, and repair of damaged tissue and contains an heterogeneous cell populations, including progenitors cells other than Malassez residual epithelial sheet [8].

PDL can be expanded ex vivo, providing an exceptional reservoir of autologous stem cells with phenotypic profile similar to bone marrow derived stem cells (BMMSCs), but with a higher cell growth [12] and the possibility to undergo toward several differentiated phenotypes (Figure 1). PDLSCs cultured until 15 passages did not show signs of senescence [2].

Morphology and Stem Cell-Related Marker Expression of Periodontal Ligament Stem Cells.

Many methodological approaches have been used to obtain stem cells from periodontal ligament. Primary cultures of PDLSCs are colonies of bipolar fibroblastoid cells with oval nuclei containing two or three nucleoli. Ultrastructural analysis of PDLSCs showed cells with a large cytoplasm, extensive rough endoplasmic reticulum profiles, abundant mitochondria, some residual lysosomal bodies containing electron-dense material and bundles of filaments. In nuclei, the dispersed chromatin indicated an active gene transcription status. Cytoplasmic membrane take contacts with neighbouring cells and numerous filopodia, sometimes desmosome-like junctions are detected. Flow cytometry analysis put in evidence the homogeneous expression of the mesenchymal-related antigens CD13, CD29, CD44, CD73, CD90, CD105, CD146, CD166, other than OCT3/4, Sox2, and SSEA4 intracellular antigens (Figure 2), p75, Nestin, CD49, SLACS, SOX10 neural crest related markers [13,14]. PDLSCs are negative for the hematopoietic markers, essential for defining mesenchymal cells. The PDLSCs express also the following proteins: CLPP, NQO1, SCOT1, a new isoform of TBB5 and DDAH1 that are not present in BMMSCs, implicated in the cell cycle regulation and stress reaction, homing, and detoxification [15].

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Multipotency of PDLSCs.

PDLSCs have the ability to differentiate into several cells under defined culture conditions (Figure 3). In particular, PDLSCs derived spheres showed a cell population able to differentiate into neural and mesodermal tissue [16,17]. Previously, it has been demonstrated that PDLSCs can differentiate into osteoblast/cementoblast-like cells, adipocytes and chondrogenic cells [8,10,18].

Recently, our research group reported that xeno-free hPDLSCs cultured in basal medium spontaneously express neural protein markers as Nestin and GAP-43 according with their neural crest origin. GAP-43 is expressed in the nervous system, in glial cells and is also involved in mechanisms which control pathfinding and branching during development and regeneration [19,20]. This protein present in the periodontal Ruffini endings has an important role in nerve regeneration/development processes [21]. PDLSCs can differentiate toward neurogenic lineage and the PKCα/GAP-43 nuclear signalling pathway controls the neuronal progression. It has been also evidenced that during PDLSCs neurogenic differentiation occur a cytoskeleton rearrangement, the cytoskeleton actin, is localized at the periphery with a typical epithelial arrangement and cells showed a very small and rounded cell body with thin neurite-like processes [22,23].

In addition, PDLSCs could differentiate into Schwann cells via the ERK1/2 signalling pathway [24]. Okubo reported the differentiation ability of PDLSCs toward endothelial cells [25] and Pizzicannella demonstrated that LPS-G stimulus in endothelial cells obtained from PDLSCs causes a slowdown of cell growth and the release of IL6, IL8 and MCP1 molecules with the involvement of TLR4/NFkB/ERK1/2/p-ERK1/2 signalling [26-28]. PDLSCs can be induced to differentiate into cardiac myocytes expressing cardiac cell markers as sarcomeric actin and cardiac troponin T [29]. Other authors reported the possibility to generate islet-like cells from PDLSCs expressing endoderm- and pancreas-related genes [30]. Also the differentiation into retinal ganglion-like cells has been demonstrated in PDLSCs [31]. The in vivo and in vitro experiments have been demonstrated that the PDLSCs are the master regulator of osteogenic differentiation. In fact, PDLSCs used in combination with different biomaterials were able to promote a bone-regeneration process for the

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treatment of bone and ossification defects caused by senescence, accidental or surgery trauma [32]. Interestingly, the upregulation of Mir-2861 and Mir-210 offers a novel regulatory pathway in the early steps of the bone-regeneration process related to the angiogenesis and osteogenesis processes [33,34].

PDLSCs in regenerative medicine.

Mesenchymal stem cells (MSCs) in teeth share some properties with MSCs found in other tissues/ organs, and most derive basically from the defining features of MSCs (generic multipotent differentiation in vitro into osteoblasts, chondrocytes and adipocytes, expression of a set of surface markers) modelled on the bone marrow (BM)- derived MSCs [35-37]. However, recent in vivo research clearly shows that mesenchymal stem cells have different origins, properties and functions in different tissues [38]. The multilineage differentiation potential of PDLSCs and their ability to be a producer of large amounts of a vast array of cytokines [12] and extracellular microvescicles with high content of anti-inflammatory mediators [39], renders these cells and/or their products a very interesting application for disease treatment. In particular inflammatory disorders and autoimmune diseases have attracted the attention of researchers, given the immunosuppressive and proresolving properties of PDLSCs [40]. We provided recent evidence that PDLSCs regulate neutrophil leukocyte functions regarding bacterial killing and production of bioactive mediators including the D-series resolvins. Moreover, lipoxin A4 was found to stimulate PDLSCs proliferation and migration, functions relevant to wound healing as well as regeneration of periodontium.

Up to eight unique populations of dental tissue-derived MSCs have been isolated and characterized until now [41]. MSCs in teeth have been exploited as seed cells for stem cell-based dental medicine, and both odontogenic and non-odontogenic sources of MSCs have been identified in dental tissues [42].

MSCs, as stem cells therapies, have shown exceptional therapeutic abilities in oro-facial, neurologic, corneal, cardiovascular, hepatic, diabetic, renal, muscular dystrophy and autoimmune conditions [35,38,41]. This has been shown for some (i.e. from dental pulp)

but not for all types of DSCs, which are becoming extremely relevant in tissue engineering and regenerative medicine [38,41,43].

Periodontal ligament stem cells, originated from dental follicle [38,43,44], were first cultured from periodontal ligament where their majority is located: they are thought to play a role in repair, and may also be involved in homeostatic turnover of this tissue [43-47].

In vivo characterization studies of PDLSCs are scarce and several questions regarding their origin, location and heterogeneity as well as their in vivo differentiation potential remain unanswered [42-44]. Studies exploring the potential use of PDLSCs to treat periodontal diseases in animal models are ongoing. Cell proliferation rates in the periodontal ligament increase following injury and during orthodontic treatment [45,48-50], but resting rates in adults are low and decrease with age [51], suggesting that PDLSCs, similar to other MSCs of different origin, are sensitive to specific stimuli [51]. One of such stimuli is provided in vitro for the expression of immunomodulatory properties, by coculture with peripheral blood mononuclear cells or with cytokines produced by them, e.g. interferon-γ [52-54]. The expression of TGF-beta1, hepatocyte growth factor (HGF) and indoleamine 2, 3-dioxygenase (IDO) was upregulated while IDO expression was upregulated following stimulation with interferon-g [54]. Also exosomes and extracellular vescicles release from MSCs has been proposed to mediate the immunomodulatory activity of these cells [55,56]. This finding has been confirmed also for MSCs of dental origin [52]. The presence of an abundance of active molecules secreted by MSCs has undoubtedly captured the attention of several groups for their potential to substitute for the cells in mediating their effects [40,57-59]. The comparison among differentiation capacity and surface phenotypes in MSCs of various origin has therefore been extended to the analysis of their secretome [60,61] and extracellular vescicles [62,63].

As for the immunomodulatory action, several mechanisms and molecules have been proposed [52,53] involving both cell contact and soluble mediators. However, waiting for the results of several ongoing clinical trials [64] we can only observe at this point that few

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of these compare MSCs from different tissue sources, and none at present is being conducted with dental MSCs[65].

The in vitro immunosuppressive effects of MSCs are mostly assessed by in vitro assays on T cell proliferation [66]; this may not reflect the ability to suppress ongoing inflammatory or autoimmune processes in general, and are most likely a reflection of their in vivo behaviour, where they may suppress inflammation occurring because of trauma and during tissue repair. Most clinical applications of this property have utilized BMMSCs, but perhaps other sources will prove to be equally or even more potent, both in vitro and in vivo [41,54].

The architectural reconstruction of efficient bone remains an important goal in orthopedic and dental conditions, such as bone trauma, osteoporosis, arthritis, osteonecrosis, and periodontitis. An acute inflammatory response is crucial at the onset of bone repair, while an adaptive immune response has additional functions during later bone remodeling. The direct or cell-free interactions between MSCs and inflammatory cells (or molecules derived thereof) seem to give an advantage in bone regeneration to cells originating from similar niches. MSCs have demonstrated regulatory functions, such as suppressing the differentiation of monocytes/hematopoietic precursors and the secretion or activity of pro-inflammatory cytokines. In this context the therapeutic application of MSCs in bone regeneration offers not only the osteogenic preferential differentiation, but also the modulation of the immune/inflammatory milieu in situ. This may be important in view of the difficulty of engraftment of large numbers of stem cells in regenerative applications.

However, a recent study reported that PDLSCs isolated from inflamed periodontium showed significantly diminished inhibitory effects on the proliferation of T cells compared to those of healthy cells, aminly due to reduced induction of Tregs [67]. This finding should have directed the attention of therapeutic attempts for periodontitis towards immunomodulation. Erythropoietin has been shown to be able to reverse this impaired function, enhancing osteogenic ability of PDLSCs via the p38 MAPK pathway [48]. Aspirin incubation modulates the osteogenic potential of PDLSCs through upregulated epression of several growth factor genes [68]. MSCs derived from inflamed periodontal tissue have

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increased proliferative capacity, together with higher collagen content but diminished osteogenic differentiation [50]. Dental tissue-derived MSCs have been used to engineer bone for orofacial bone regeneration in model systems [41].

The perivascular region has been recognized as the origin of many types of MSCs, and this has suggested the in vivo angiogenic potential of PDLSCs [69]. PDLSCs could be a potential source of perivascular cells, which could contribute to in vivo angiogenesis a prerequisite for the trophic effect needed in regenerative medicine [70]. Both production of VEGF and other angiogenic factors and their trophic action on endothelial cells were responsible for increased and better vascularization when PDLSCs were co-transplanted with endothelial cells [70]. The angiogenic potential of PDLSCs seems to rely on the functionality of the CXC12/CXCR4 axis [71] which we first described in these cells [49] along with the IL7/IL7R system, possibly related to the PDGF-BB molecule which stimulates proliferation, stemness maintenance and differentiative ability of PDLSCs in vitro [72]. Perivascular characteristics of PDLSCs and their contribution to in vivo angiogenesis might imply potential use of PDL-MSCs in tissue engineering and also in vascular diseases.

The musculoskeletal applications of MSCs are the area of greatest interest, despite some doubts on the feasibility of successful regeneration at the present technological state [61]. The suppressive activities are much more promising, as demonstrated by the use of MSCs in hematopoietic transplants, for prevention and treatment of graft -versus-host disease [73]. MSCs of dental origin have shown important neurotrophic effects in peripheral nerve injury [74] through expression of several neural growth and differentiation factors. The secretome of MSCs acts as master regulator of the so-called neurogenic niche [70] a reservoir of regenerating neuronal cells with a great therapeutic potential. Experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS), has focused the attention of our group, since injection of PDLSCs at the beginning of the disease (i.e. 14 days after immunization with myelin peptides) decreased signs of inflammation and demyelination in the spinal cord of the animals, both through the increased production of neurotrophic factors and the suppression of inflammatory mediators [75]. The conditioned medium of the same cells were able to reduce inflammatory damage in the same model [76] and purified extracellular vescicles from

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PDLSCs obtained a similar effect. The vescicles were found to contain anti-inflammatory cytokines IL-10 and TGF-β, stained positive for surface mesenchymal antigens CD90 and CD29, and were a mixed population of exosomes and shedding vescicles. The same result was produced by injecting conditioned medium or vescicles obtained from patients suffering from relapsing-remitting MS [76]. This was proof of a pathology-independent functional niche of PDLSCs, which may be of importance for several other neurodegenerative and inflammatory diseases. Production of culture medium of PDLSCs from MS patients under hypoxic conditions induced a potent modulation of oxidative stress, autophagic and apoptotic markers when injected in mice where EAE was induced [77], with reduction of IL-17 and Interferon-γ expression, and increased expression of beclin-1 and LC3, principal markers of autophagy. This was correlated with a strong increase in the content of the anti-inflammatory cytokine IL-37, a member of the IL-1 family [77]. PDLSC conditioned medium increased the functionality of the PI3K/Akt/mTOR axis, and also reduced inflammation and oxidative stress in injured NSC-34 neurons, restoring BDNF production [78]. Moreover, CM was revealed to contain NT3, and IL-10, suggesting a neuroprotective effect as the result of the presence of growth factors and immunomodulatory cytokines. Since MS is one of the prominent disorders where clinical trials with MSCs are being conducted [64] these novel findings may indicate the possible beneficial effects of treatment with PDLSCs or their secretome, which may represent a great advancement for safety and availability of such therapeutics.

Concluding remarks

The PDL harbors specialized SC which have important homeostatic functions in vivo and can be easily recovered to be cultured ex vivo. In vitro they display angiogenic, immunomodulatory and multilineage differentiative capacity, which makes them a very attractive source of MSC for regenerative medicine. The observation that different molecules in their conditioned medium as well as in extracellular vescicles therein can substitute for the cells in most functions has paved the way for the investigation of their possible use in vivo. Recent findings seem to indicate that autoimmune and inflammatory conditions such as MS may be ideal candidates for therapeutic applications.

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Figure legends

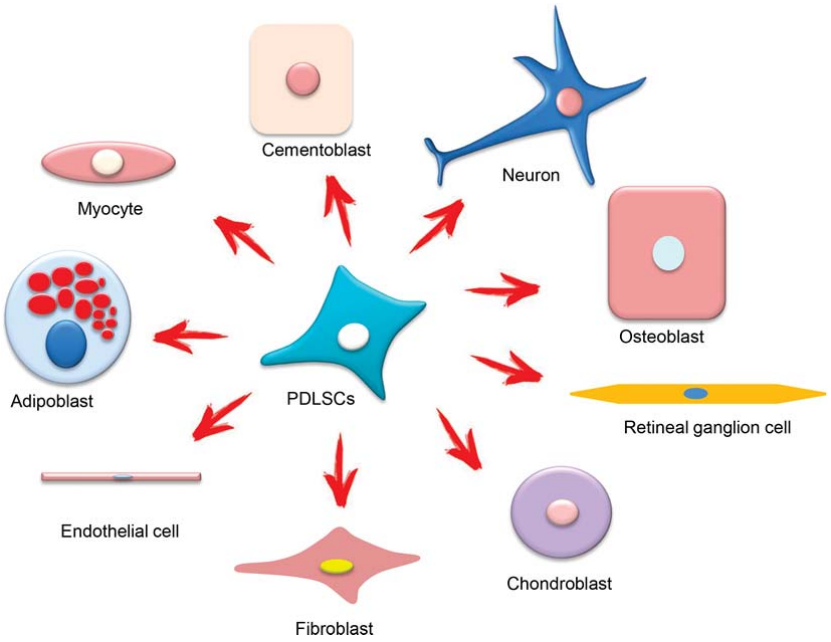

Figure 1. PDLSCs multipotency. PDLSCs have demonstrated the capacity to differentiate into several cytotypes shown in figure.

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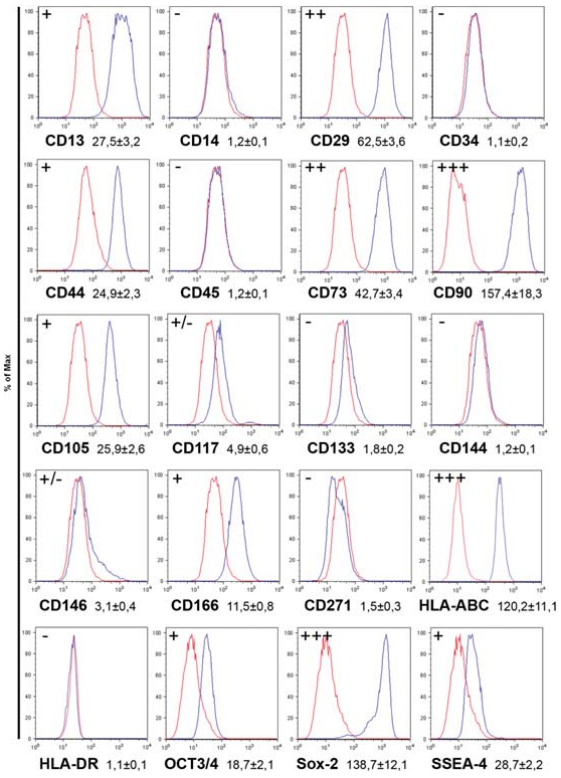

Figure 2. Flow cytometric analyses of PDLSCs. Flow cytometric analysis of surface (CD13, CD14, CD29, CD34, CD44, CD45, CD73, CD90, CD105, CD117 CD133, CD144, CD146, CD166, CD271, HLA-ABC and HLA-DR) and intracellular (OCT3/4, Sox-2 and SSEA4) antigens expression profile. Blue histograms represent cells stained with the expression markers; red histograms show the respective IgG isotype control. These data are representative of five separate biological samples. **-** negative expression; **+/-** low expression; **+** moderate expression; **++** positive; **+++** high expression; MFI Ratio is the average of five different biological samples ± standard deviation; Cutoff Ratio positivity MFI Ratio >2.0.

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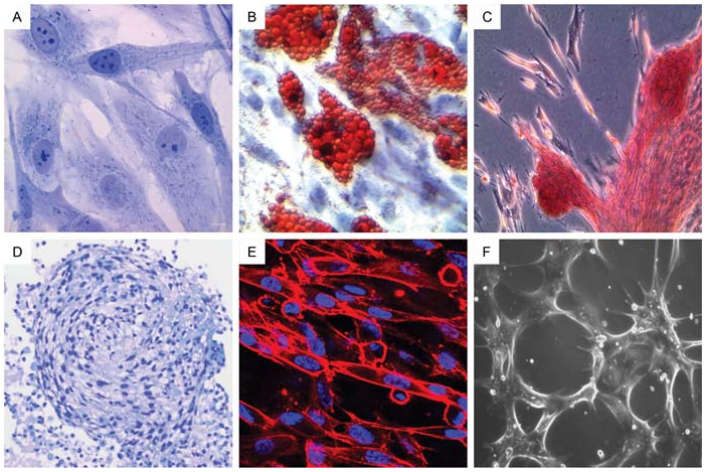

Figure 3. Differentiation ability of PDLSCs. (A) PDLSCs stained with toluidine blue solution. (B) PDLSCs after adipogenic differentiation showed a positive staining to adipo oil red. (C) Osteogenic differentiated PDLSCs showed a positive staining to alizarin red. (D) Chondrogenic differentiated PDLSCs showed a positivity to alcian blue staining. (E) Neurogenic differentiated PDLSCs showed a rearrangement of cytoskeleton actin. (F) Endothelial differentiated PDLSCs showed a tube like formation when cultured on Cultrex®.

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