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The efficacy of mesenchymal stem cells to regenerate and repair dental structures

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Structured Abstract

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Objectives – Identification, characterization, and potential application of mesenchymal stem cells (MSC) derived from human dental tissues.

Methods – Dental pulp and periodontal ligament were obtained from normal human impacted third molars. The tissues were digested in collagenase/dispase to generate single cell suspensions. Cells were cultured in α-MEM supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 μM L-ascorbate-2-phosphate. Magnetic and fluorescence activated cell sorting were employed to characterize the phenotype of freshly isolated and *ex vivo* expanded cell populations. The developmental potential of cultured cells was assessed following co-transplantation with hydroxyapetite/tricalcium phosphate (HA/TCP) particles into immunocompromised mice for 8 weeks.

Results – MSC were identified in adult human dental pulp (dental pulp stem cells, DPSC), human primary teeth (stem cells from human exfoliated deciduous teeth, SHED), and periodontal ligament (periodontal ligament stem cells, PDLSC) by their capacity to generate clongenic cell clusters in culture. *Ex vivo* expanded DPSC, SHED, and PDLSC populations expressed a heterogeneous assortment of makers associated with MSC, dentin, bone, smooth muscle, neural tissue, and endothelium. PDLSC were also found to express the tendon specific marker, Scleraxis. Xenogeneic transplants containing HA/TCP with either DPSC or SHED generated donor-derived dentin-pulp-like tissues with distinct odontoblast layers lining the mineralized dentin-matrix. In parallel studies, PDLSC generated cementum-like structures associated with PDL-like connective tissue when transplanted with HA/TCP into immunocompromised mice.

Conclusion – Collectively, these data revealed the presence of distinct MSC populations associated with dental structures with

the potential of stem cells to regenerate living human dental tissues *in vivo*.

Key words: cementum; dental pulp; mesenchymal stem cells; periodontal ligament; teeth

Introduction

Tooth development occurs through mutually inductive signaling between interacting oral epithelial and ectomesenchymal cells (1,2) both originating from migrating neural crest cells (3). These interactions result in the formation of an outer layer of enamel formed by the activity of cells derived from the oral epithelium known as ameloblasts, and an inner layer of mineralized dentin synthesized by odontoblasts, which are derived from the dental papilla. The central chamber of teeth is comprised of a soft fibrous pulp tissue also derived from the dental papilla, infiltrated by a network of blood vessels, and nerve bundles emanating from the apical foraman. The whole tooth structure is held in place in the surrounding bone by a fibrocellular stratum of periodontal ligaments (PDL) derived from the dental follicle, which is also of ectomesenchymal origin. The PDL is secured by connective tissue fibers (Sharpey's Fibers) embedded between the thin mineralized outer layer of cementum and the inner wall of the alveolar bone socket.

Whilst, the complex structural composition of teeth provides hardness and durability, these rigid structures are vulnerable to damage caused by mechanical trauma, chemicals, congenital defects, cancer, and bacterial infections. Unlike other tissues such as bone, which have the capacity to repair and remodel throughout post-natal, the relatively static components of teeth do not readily undergo complete regeneration following insult. However, adult teeth do demonstrate some limited reparative processes such as the formation of reparative (tertiary) dentin, a more poorly organized mineralized matrix to primary, and secondary dentin that serves as a protective barrier to the dental pulp (4). Once the dentin/odontoblast layer has been breached it is thought that pre-odontoblasts are recruited from somewhere within the pulpal tissue (5,6) to the injured site before developing into functional odontoblasts. Similarly, the periodontium (PDL, cementum, and alveolar bone) has a limited capacity for regeneration. New cementum formation, remodeling of the PDL and new bone formation can be observed during orthodontic tooth movement, however, this may be classified more a physiological response rather than true repair or regeneration. While some minor regeneration of the periodontium may be seen in the early phases of periodontal disease, the main cause of tooth loss, once the disease becomes established then spontaneous regeneration does not occur without some form of therapeutic intervention (7). The process of periodontal regeneration is thought to involve the recruitment of locally derived uncommitted cell populations with the capacity to develop into either periodontal ligament forming cells or mineral forming cementoblasts, which combine to secure the connections between the cementum and the adjacent alveolar bone (8).

Despite our extensive knowledge concerning the pathology of diseases of teeth, restoration of damaged or diseased dental tissues, to date, has relied primarily on the use of synthetic implants and structural substitutions comprised of inert compounds. For example, conventional protection of exposed pulp by capping using calcium hydroxide is widely used in clinical practice, which often leads to inflammation and necrosis of the pulp tissue (9). The consequences of this approach includes tooth discoloring, increased susceptibility to dentin fracture and tooth loss. Generally, placement of a root canal filling is the only real alternative to salvaging mature teeth with severe papal destruction. However, several reports have been trialing a range of potential options for pulp capping such as demineralized dentin particles, extracellular matrix components (collagen, fibronectin, and fibrin glue), and biocompatible calcium phosphate based cements (hydroxyapatite, α/β-tricalcium phosphate, tetracalcium, and octacalcium). More sophisticated tissue engineering approaches include the use of cytokines (BMPs, TGF-β), and artificial scaffolds (polyglycolic acid) to help regenerate viable endogenous pulp tissue in order to facilitate reparative dentin formation (10,11).

Attempts to regenerate periodontal tissues have focused almost exclusively on regenerating lost alveolar bone and have included the use of autografts (cortical/cancellous bone, bone marrow, allografts (demineralized freeze-dried/freeze-dried bone) and alloplastic materials (ceramics, hydroxyapatite, polymers, and bioglass). However, the majority of these strategies have been plagued by variability in their safety, effectiveness and stability over time, and thus their effectiveness as true periodontal regenerative agents has been questioned (7). More recently, several novel tissue-engineering approaches have started to emerge as prospective alternatives to conventional treatments including gene therapy and the local administration of biocompatible scaffolds with or without the presence of growth factors such as BMPs, TGF_{β}, bFGF, PDGF, and IGF-1 (11–14). Whilst, still largely experimental in nature, these developments are expected to offer new and improved alternatives to existing therapies to repair and maintain mature teeth.

Most recently, the identification of putative dental stem cell populations capable of regenerating organized tooth structures has stimulated interest into the potential use of post-natal stem cell based therapies to treat the damaged caused by trauma, cancer, caries, and periodontal disease (15–17). The present review summarizes the 'stem cell'-like properties and characteristics of putative post-natal human dental pulp and periodontal stem cells and speculates on the future clinical benefits that may arise from these studies.

Identification of human dental-derived mesenchymal stem cells (MSC)

Bone marrow stromal stem cells (BMSSC) have previously been identified by their capacity to form adherent colonies, morphologically similar to fibroblasts (colony forming unit-fibroblastic, CFU-F), when plated at low-cell densities in the presence of media supplemented with mitogenic growth factors or serum (18–20). Previous studies have shown that each colony is originally derived from the clonal expansion of a single adherent progenitor cell using limiting dilution and chromosomal mixing techniques (19–23).

In analogy, our group has recently identified other mesenchymal stem cell populations derived from adult third molars (dental pulp stem cells, DPSCs), exfoliated deciduous teeth (stem cells from human exfoliated deciduous teeth, SHED), and adult periodontal ligament (periodontal ligament stem cells, PDLSCs), by their ability to generate clonogenic adherent cell clusters when plated under the same growth conditions as described for BMSSCs (15–17,21). However, since dental pulp and periodontal ligament are solid tissues, colony efficiency assays were performed using single cell suspensions prepared by collagenase/dispase digestion followed by filtration through fine mesh strainers. The incidence in the number of CFU-F colonies (aggregates of fifty cells or more) formed at day 10 to 12 of culture was then evaluated for all unfractionated cell preparations. Approximately 14 BMSSC-, 400 DPSC-, 200 SHED- and 170 PDLSC-derived CFU-F generated per 10^5 cells plated (15–17,24). The higher frequency of CFU-F in the dental tissues was most likely due to their fibrous nature in contrast to fluid bone marrow aspirates, which are comprised predominantly of bone marrow haematopoietic cells including varying proportions of contaminating peripheral blood cells.

Growth potential of human dental-derived MSC

Cloning experiments indicated that the majority of individually isolated colonies (>80%) failed to proliferate beyond 20 population doublings for all MSC types tested (15–17,21,24). Consequently, the bulk of *ex vivo* expanded MSC over successive cell passages were representative of only a minor proportion of high proliferating BMSSC-, DPSC-, SHED- or PDLSC-derived CFU-F. Proliferation studies using BrdU labeling of multi-colony-derived DPSCs, SHED, and PDLSC cell cultures exhibited higher rates of proliferation, approximately 30, 50, and 30% when compared to the growth of cultured BMSSCs, respectively (15–17,21,24). Ongoing studies, thus far, indicate that DPSC, SHED, and PDLSC maintain a higher growth potential beyond 100 population doublings, in contrast to BMSSC that begin to undergo cellular senescence at approximately 50 population doublings. While the precise mechanisms for these observed discrepancies are not entirely known, the higher incidence of DPSCs undergoing S-phase over BMSSC, has been correlated with increased levels of the cell

cycle activator, cyclin-dependent kinase six, and the mitogen, insulin-like growth factor-2, both known mediators of cell cycle progression from G1 to the start of DNA synthesis (25–27).

The finite life-span of post-natal mesenchymal stem cell populations has previously been correlated with a decline in their developmental potential as the cells begin to exhaust their growth potential *in vitro*. This is in contrast to embryonic stem cells, which are virtually immortal due to their high expression of telomerase, the enzyme complex responsible for maintaining telomere lengths and chromosomal stability during cellular division (28). Our group has shown that telomerase activity is absent in cultured BMSSC and other mesenchymal stem cell populations and appears to be a critical factor for prolonging cellular senescence by up-regulating cell cycle regulators such as cyclin D3, cyclin E1, E2F-4, DP2, and inhibiting hypophosphorylated pRb to allow progression from G1 to S phase leading to an increased proliferation potential and survival rate (29,30). Furthermore, enforced expression of telomerase activity by cultured BMSSCs resulted not only in an extension of lifespan of BMSSC by almost three fold but also an enhanced (fivefold) capacity to regenerate new bone formation *in vivo* (29–31). Research efforts are continuing to unravel the mechanisms of how telomerase activity regulates the growth of MSC. It is anticipated that these studies will help develop strategies to genetically manipulate *ex vivo* expanded cells, in order to enhance and regulate the growth potential of expanded BMSSC, DPSC, SHED, and PDLSC for potential clinical applications.

Characterization and origin of dental-derived MSC

Given the different anatomical sites resided by DPSCs/SHED/PDLSC/BMSSC, it was intriguing to discover that the putative stem cell marker, STRO-1, used to isolate and purify BMSSC was also expressed by dental-derived stem cells using immunomagnetic activated cell selection. Further analyses revealed that BMSSC, DPSC, SHED, and PDLSC expressed the perivascular cell marker CD146, where a proportion of these cells were also shown to co-express express alpha-smooth muscle actin and/or the pericyte associated antigen, 3G5 (5,15,16,21). These observations were correlated with co-localization studies of these markers to perivascular cells *in situ*, using dual-color immunostaining of frozen human bone marrow and dental pulp tissue sections. In comparative immunophenotypic studies, cultured DPSC, SHED, PDLSC, and BMSSC failed to react with the haematopoietic markers CD14 (monocyte/macrophage), CD45 (common leukocyte antigen) and CD34 (haematopoietic stem/progenitor cells/endothelium). Therefore, despite the diverse ontogeny between BMSSC, PDLSC and pulp-derived stem cells, different mesenchymal stem cell populations appear to reside in a common perivascular niche in their respective tissues.

Previous studies have conducted comprehensive phenotypic analysis of *ex vivo* expanded dental pulp, periodontal, cementum, and bone marrow-derived stromal cell populations demonstrating a common expression pattern profile for a variety of antigens associated with endothelium (CD106, CD146), perivascular tissue (α-smooth muscle actin, CD146, 3G5), bone/dentin/cementum (BMPs, alkaline phosphatase, Type-I collagen, osteonectin, osteopontin, osteocalcin, bone siaoloprotein) and fibroblasts (Type-III collagen) (Table 1) (5,15–17,21,32–34). The expression of common proteins implicates the existence of a common molecular pathway regulating dentin, cementum and bone formation through the activity of important regulatory transcription factors (Runx2, Msx1/2, Dlx1/2, and Pax6/9) and growth factors (BMP, FGF, TGF, and WNT) (35–37).

Importantly, the odontoblast specific protein, dentin sialophosphoprotein (DSPP) (38,39) a precursor to two matrix proteins, dentin sialoprotein (DSP) (40) and dentin phosphoprotein (DPP) (41) was notably absent in primary DPSC/SHED cultures, using *in situ* hybridization and western blot analysis (16,17,24,42). However, immunohistological studies identified the presence of DSPP during early stages of ectopic mineralization by DPSCs or SHEDs in xenogeneic transplants, using a human DSPP specific polyclonal antibody. Conversely, no DPSS staining could be observed in transplants of ectopic bone formed by BMSSC. These data suggest that the clonogenic dental pulp-derived cells represent an undifferentiated pre-odontogenic phenotype *in vitro* under non-inductive conditions, in accord with data observed in animal studies (43,44).

Table 1. **Protein or gene expression profile of different Mesenchymal stem cell populations *in vitro***

Antigen	DPSC	SHED	PDLSC	BMSSC
CD14	–	–	–	–
CD34	–	–	–	–
CD44	++	++	++	++
CD45	–	–	–	–
CD106	+	+/–	+/–	++
CD146	++/+/–	++/+/–	++/+/–	++/+/–
3G5	+/–	+/–	+/–	+/–
STRO-1	++/+/–	++/+/–	++/+/–	++/+/–
α-SM actin	++/–	++/–	++/–	++/+/–
Collagen Type-I	++	++	++	++
Collagen Type-III	++/+	++/+/–	++/+/–	++/+
Alkaline phosphatase	++/+/–	++/+/–	++/+/–	++/+/–
Osteocalcin	++/+	++/+/–	++/–	+/–
Osteonectin	++/+	++/+	++/+	++/+
Osteopontin	+/–	+/–	+/–	+/–
Bone sialoprotein				
Scleraxis	+	+	++	+
Dentin sialophosphoprotein	–	–	–	–

(++) strong expression; (+) weak expression; (–) negative; (/) subpopulation.

A greater challenge confronting the characterization of PDLSC was the lack of known specific markers associated with either PDL or cementum. The characteristic, dense collagen fibers of PDL are somewhat similar to those found in tendon, where both tissues are endowed with the ability to absorb mechanical forces of stress and strain. Given this similarity, we examined the expression levels of Scleraxis, a tendon specific transcription factor, in human cultured PDLSCs (45). These studies showed that PDLSCs expressed measurably higher levels of Scleraxis transcripts when compared with BMSSCs and DPSCs, using semiquantative RT-PCR analysis. These data imply that PDLSCs represent a unique population of post-natal stem cells distinct from dental pulp or bone marrow-derived MSC. Efforts are now under way to compile a more comprehensive analysis of the genotypic and protein expression patterns of BMSSC, DPSC, SHED, and PDLSC using cDNA microarray and proteonomics technologies. It is hoped that this analysis will help facilitate the identification of key molecules that regulate the development of bone, dentin, cementum, and PDL.

Differentiation potential of dental-derived stem cells *in vitro* and *in vivo*

We have previously shown that human-derived BMSSC have the capacity to form mineralized deposits *in vitro*, physiologically similar to hydroxyapatite *in vivo*, when grown in the presence of inductive media containing ascorbic acid, dexamethasone and an excess of inorganic phosphate (46). Other studies have reported the formation of mineralized nodules following the induction of human dental pulp-derived stromal cultures (47). In analogy, human-derived DPSC, SHED, and PDLSC all demonstrated a capacity to form Alizarin Red-positive mineralized nodules *in vitro* to varying degrees, under the similar conditions (15–17). Furthermore, BMSSC, DPSC, SHED, and PDLSC were also shown to have multi-potential by their ability to form oil-red O-positive lipid containing clusters of fat cells, when cultured in the presence of adipogenic inductive medium (15,16,24). These studies facilitated experiments to address an important property of stem cells, that is, the capacity of a given stem cell population to regenerate an organized, functional tissue following implantation *in vivo*. Previous reports have shown that, unlike rodent-derived bone marrow and dental pulp stromal cell human equivalents require a suitable inductive carrier such as hydroxyapatite/tricalcium phosphate (HA/TCP) to induce the formation of bone, cementum and dentin *in vivo*, respectively (17,24,48,49). Subsequent experiments were performed to determined the developmental capacity of *ex vivo* expanded SHED and PDLSC *in vivo*, using a HA/TCP carrier in an established, xenogeneic transplantation system as described above (15,16).

Cultured adult DPSC and SHED demonstrated the ability to generate a dentin/pulp-like complex *in vivo* when co-transplanted with HA/TCP particles subcutaneously into immunocompromized mice (16,17). Typical DPSC and SHED transplants developed areas of vascularized pulp tissue surrounded by a well-defined layer of odontoblast-like cells, aligned around mineralized dentin with their processes extending into tubular structures. In addition, orientation of the collagen fibers within the dentin was characteristic of ordered primary dentin, perpendicular to the odontoblast layer. Backscatter EM analysis demonstrated that

the dentin-like material formed in the transplants had a mineralized globular appearance consistent with the structure of dentin in human teeth. Importantly, human DSPP was detected at the odontoblast/dentin interface by immunohistochemical analysis, and confirmed by RT-PCR and *in situ* hybridization. Furthermore, the pulp and odontoblast-like cells were found to be donor in origin due to their reactivity with the human alu specific DNA probe.

Parallel experiments demonstrated that *ex vivo* expanded PDLSCs were capable of forming typical cementum/PDL-like structures when co-transplanted with HA/TCP particles subcutaneously into immunocompromised mice (15). Moreover, transplanted human PDLSCs formed Type-I collagen-positive PDL-like tissue within the transplants that connected with the newly formed cementum that seemed to mimic physiological attachment of Sharpey's fibers. Human cells were shown to be responsible for the formation of the collagen fibers and cementum layers within the transplants as demonstrated by their reactivity with the human-specific anti-mitochondria antibody. Collectively, these data clearly demonstrated the differential tissue regeneration capacity between bone marrow pulp and PDL-derived mesenchymal stem cell populations *in vivo*.

Interestingly, cloning studies have shown that individual *ex vivo* expanded BMSSC, DPSC, SHED, and PDLSC CFU-F-derived clones, demonstrate great variability in their proliferation capacity and differentiation potential, irrespective of the tissue source used. These observations support the proposal that the CFU-F compartment contains a mixed population of stromal progenitor cells at various stages of development, maintained by a minor population of multipotential, stromal stem cells with the capacity for self-renewal, as first proposed by Friedenstein and Owen (50) (Fig. 1).

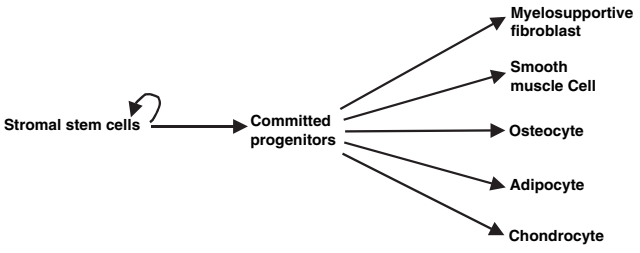

Fig. 1. The proposed bone marrow stromal system modified from Owen and Friedenstien (50).

Potential clinical applications for human-derived dental stem cells

The routine use of post-natal stem cells in clinical applications has been successfully demonstrated by hematopoietic bone marrow reconstitution using mobilized peripheral blood $CD34^+$ stem cells, in cancer patients undergoing myeloablative therapy. These results have subsequently led to further investigations assessing the clinical potential of other stem cell populations such as BMSSCs, as potential novel cellular-based therapies, using a range of pre-clinical animal models representative of various human diseases and congenital defects involving neural, bone, cartilage and muscle tissues (51–59). Preliminary human clinical trials have reported improved levels of bone formation in children with osteogenesis imperfecta, following systemic infusion of BMSSC or bone marrow cells (60,61). In another study, rapid hematological recovery was achieved in breast cancer patients that received co-transplantations of autologous *ex vivo* expanded BMSSC and mobilized peripheral blood stem cells, after high-dose chemotherapy (62). One potential application of interest to our group is assessing the utility of BMSSC to regenerate and repair cranial defects as demonstrated in several animal models (63–65). These studies utilized whole bone marrow aspirates or *ex vivo* expanded BMSSC in combination with biocompatible materials and growth factors to try and correct craniofacial critical sized defects. It is envisaged that stem cell based therapies may help alleviate the complications of craniofacial-related surgical procedures requiring allogeneic tissue grafts or extraction of autologous bone from secondary sites. This approach may alleviate donor site morbidity and allow a virtual unlimited source of cellular material, particularly if the complications of using allogeneic MSC can be adequately addressed. Collectively, these studies demonstrate the clinical potential of human BMSSC for different tissue engineering strategies and could theoretically include the repair of alveolar bone destruction caused by trauma, periodontal disease or cancer.

The recent identification of different MSC residing in dental or craniofacial tissues expands the scope of potential clinical benefits of MSC to help regenerate other connective tissues such as dentin, cementum, and PDL. In accord with the regenerative capacity of

bone marrow-derived MSC described above, we recently examined whether human PDLSCs were capable of contributing to periodontal tissue repair *in vivo*. In these studies cultured human PDLSCs were implanted into surgical defects at the periodontal region of mandibular molars in immunocompromised nude rats (15). Histological examination of harvested transplants demonstrated attachment of human-derived PDL-like tissue to the surfaces of alveolar bone and teeth. These preliminary data imply a potential functional role of human PDLSCs for periodontal tissue regeneration. Comparative studies are also being conducted to assess the dentin/pulp reparative capacity of human DPSC and SHED using a Nude rat model.

Recent work, has identified the ovine counterparts of human BMSSC, DPSC, and PDLSC, which demonstrate similar functional properties when co-transplanted into immunocompromised mice with the HA/TCP carrier particles. We are currently using a tissue engineering approach to try and regenerate critical sized defects created in aveolar bone, PDL, cementum, and dentin by selectively implanting autologous BMSSC, PDLSCs, or DPSC in combination with different biocompatible materials/scaffolds using an established ovine preclinical model as a prelude to human trials.

Concluding remarks

The studies discussed in this review provide describe the first characterization of different adult human stem cell populations residing in pulp and PDL tissues, establishing the foundation for further studies to determine the efficacy of *ex vivo* expanded stem cells to repair dental structures and periodontal defects. What is clear, is the that a tremendous amount of work is still required to identify and maintain multipotential MSC *in vitro*, in order to complement the recent advances in tissue engineering and gene manipulation technologies. Some important hurdles that need to be overcome include: the identification of multi-potential cells following *ex vivo* expansion; establishment of growth and differentiation conditions that induce lineage specific commitment; the development of suitable carriers and inductive factors able to help implants integrate into the surrounding environment for the reconstruction of functional complex organ systems. It is expected that such a multilevel cellular/biomaterial/cytokine approach is essential for developing practical and viable-mediated therapeutic alternatives in the not so distant future.

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