ISOLATION AND CHARACTERIZATION OF HUMAN PERIODONTAL LIGAMENT STEM CELLS

by

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ISABEL C GAY

MASTERS IN CLINICAL DENTISTRY

ABSTRACT

The periodontal ligament (PDL) is thought to include progenitor cells capable of forming fibroblasts, osteoblasts and cementoblasts. However, characterization of PDL stem cell populations (SC) remains undetermined due in part, to the diversity of cell types that conform to the periodontal apparatus. Objective: To isolate and characterize PDLSC and assess their pluripotent capabilities to differentiate into bone, cartilage and adipose cell types. Methods: PDL was scraped from human teeth, enzymatically digested, and cells stained for STRO-1 (SC marker) using fluorescent immunohistochemistry. Positive cells were FACS sorted and expanded in culture. Human bone marrow SC (BMSC) served as a positive control. PDLSC and BMSC were cultured using conditions conducive for osteogenic, chondrogenic and adipogenic differentiation. Osteogenic induction was assayed using alizarine red staining, and expression of alkaline phosphatase (ALP) and bone sialoprotein (BSP) by RT-PCR and immunohistochemistry. Adipogenic induction was assayed using Oil Red O staining and expression of PPARγ 2 and LPL (early/late specific markers) by RT-PCR. Chondrogenic induction was immunoassayed by collagen type II expression (cartilage marker) and toluidine blue staining. Results: Of the PDL cells isolated, 27% were positive for STRO-1, with 3% staining strongly. ALP expression was initially observed in PDLSC osteogenic cultures by day-14, where BMSC showed expression by day-7. BSP expression was detectable by day-7; with more intense staining found in PDLSC cultures. Under adipogenic conditions both population showed positive Oil Red O staining by day-
35 with expression of PPARγ 2 and LPL. By day-21 both BMSC and PDLSC chondrogenic cultures expressed collagen type II. Control cultures showed no differentiation. Conclusions: Human PDL tissue contains a high percentage of STRO-1 positive stem cells. These PDLSC remain undifferentiated until challenged with various differentiating conditions having the potential to differentiate into osteoblasts, chondrocytes and adipocytes, comparable to BMSC. Our method for obtaining stem cell populations can be utilized for potential therapy procedures and possibly formation of a periodontal ligament around dental implants.
DEDICATION

I wish to dedicate this thesis to my mother Maria Teresa, my brother, niece and nephew. To my beloved Larry; my colleague and best friend Martha Alicia. Also, I want to dedicate this thesis to Dr Mary MacDougall for her continuous, exemplary support as a friend and role model. Thanks to her wise advice, I am able reach my dream.
ACKNOWLEDGEMENTS

I want to express my grateful acknowledgements to the many faculty and staff members that have helped me along my tenure at UAB. I want to emphasize my appreciation for my mentor, Dr. Mary MacDougall, who is a wonderful friend and a person whom I respect and admire immensely. Drs. Nico Geurs, Firoz Rahemtulla and Amjad Javed served as my committee members and have provided me with careful guidance and patience with my research endeavors. I also want to express my admiration and respect for Dr. Michael Reddy, chair of the Periodontics Department. Drs. Kent Palcanis, Thomas Weatherford, Hung Wen Lee and Alvin Stevens who have provide advice and guidance through my training.

I also want to acknowledge the support from my closest colleagues and friends Drs. Shuo Chen and Juan Dong.

Finally, I wish to also thank to every person that was involved in this work for their support.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PDL</td>
<td>Periodontal Ligament</td>
</tr>
<tr>
<td>PDLSC</td>
<td>Periodontal Ligament Stem Cells</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone Marrow Stem cells</td>
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<tr>
<td>DPSC</td>
<td>Dental Pulp Stem Cells</td>
</tr>
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<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
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<td>SC</td>
<td>Stem Cells</td>
</tr>
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<td>STRO-1</td>
<td>Stromal Cell Antigen</td>
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<td>CFU</td>
<td>Colony Forming Units</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>RT-PCR</td>
<td>Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma 2</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone Sialoprotein</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor Beta 1</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Transforming Growth Factor Beta 3</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
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<td>--------------------------------------------</td>
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<tr>
<td>Runx2</td>
<td>Runt-Related Transcription Factor 2</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error from the Media</td>
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INTRODUCTION

The periodontal ligament (PDL) is a soft, richly vascular and cellular connective tissue which surrounds the roots of the teeth and joins the root cementum with the alveolar bone wall. The alveolar bone surrounds the tooth to a level approximately 1mm apical to the cemento-enamel junction. The PDL space has the shape of an hourglass, narrowest at the mid root level. The presence of a periodontal ligament permits forces elicited through masticatory function and other tooth contacts to be distributed and absorbed by the alveolar process. Tooth mobility is to a large extent determined by the width, height, and quality of the PDL. Other functions include tooth nutrition, homeostasis, and repair of damaged tissue.

The PDL cell population is heterogeneous, consisting of two major lineages, fibroblastic and mineralizing tissues, further divided into osteoblastic and cementoblastic subsets\(^1\text{-}^4\). The fibroblasts are aligned along the principal fibers, while cementoblasts line the surface of cementum and osteoblasts cover the surface of the bone.

During wound healing, PDL cells derive from progenitors located at the paravascular zones\(^5\). This progenitor stem cell population is enriched by precursor cell populations derived from the endosteal spaces of the alveolar bone\(^6\). Cell kinetic studies during the healing process suggest that some of these progenitors migrate toward alveolar bone and cementum where they terminally differentiate into osteoblasts, cementoblasts, and fibroblasts\(^6\text{-}^8\). As a result, repair or regeneration is regulated by a vast array of extracellular matrix molecules and by cytokines that induce both selective and non-selective responses in
the different cell lineages and their precursors. Unfortunately, our understanding concerning the numerous important signaling systems is irretrievably lost after development or repair is complete.3

Cell migration and differentiation pathways are the basis for remodeling and healing in periodontal tissues and it is likely that some form of signaling has evolved to orchestrate, in a temporally and spatially appropriate manner, these repopulation and differentiation responses.9 However, a detailed understanding of the biochemical and molecular events associated with the remodeling process in the PDL has not been determined due to the complex diversity of cells and tissue types.10 Future prospects for improved healing and regeneration of PDL tissue may derive from the identification and isolation of specific PDL cell populations and PDL stem cells (PDLSC) and their informational molecules that are stored in the connective tissue matrices. This knowledge may provide novel approaches to periodontal therapies that facilitate regeneration, and potentially, formation of a true periodontal apparatus around dental implants.

Embryonic and Adult Stem Cells.

By definition, a stem cell has the ability to proliferate in culture for indefinite time periods and give rise to specialized cells.11 Embryonic stem cells, derived from the inner cell mass of the blastocyst are termed pluripotent because they can give rise to all the cells necessary for fetal development.12 These cells possess unique characteristics such as:

1. Capability of undergoing an unlimited number of symmetrical mitotic events without differentiation.
2. Exhibit and maintain a stable diploid karyotype.

3. Give rise to differentiated cell types that are derived from all three primary germ layers of the embryo: endoderm, mesoderm and ectoderm; capable of integrating into fetal tissues during development.

4. Clonogenic capabilities; a single stem cell can give rise to a colony of genetically identical cells or clones that have the same properties as the original cell.

5. Expression of the transcription factor Oct-4 which activates or inhibits target genes and maintains stem cells in a proliferative, non-differentiating state.

6. Lack of the G1 checkpoint in the cell cycle, spending most of their time in the 5th phase of the cycle, during which they synthesize DNA and do not show X chromosome inactivation\textsuperscript{13}.

In contrast, unlike embryonic stem cells, adult stem cells share no such definitive means of characterization. In fact, no one knows the origin of adult stem cells found in mature tissues. Some authors have proposed that stem cells are set aside during fetal development and restrained from differentiation. Most current research pertaining to adult stem cells has been performed in bone marrow tissue which has demonstrated the presence of undifferentiated cell types, termed mesenchymal stem cells (MSC)\textsuperscript{14}. There are 3 proposed mechanisms for MSC arrival to the bone marrow:

1. They can enter the bone marrow along with the vasculature.

2. They can migrate into the bone marrow space after vascularization along the vessel paths; for example the periosteum which has documented multipotentiality.

3. They can arrive via the blood proper, indicating the existence of circulating MSC.
The first two mechanisms differ only with respect to the timing of MSC entrance into the marrow, not by the path of arrival or migration. The third mechanism differs only with respect to which side of the capillary basement membrane the MSC use, the luminal or abluminal. However, any of these mechanisms of MSC migration or circulation have important implications for adult tissue repair due to the possibility to deliver reparative stem cells via the circulation as opposed to only localized applications, especially if MSC docking sites exist on the endothelium lining the vascular network.

The first and second mechanisms of MSC arrival (along with the vasculature) to the bone marrow space are supported by two different observations and experiments with vascular associated cells (pericytes) and on smooth muscle cells from marrow. Pericytes are cells that are closely associated with capillaries which express smooth muscle markers and have the potential to differentiate into osteoblasts and odontoblasts.

The pericytes of the periosteum are one candidate cell type for the origin of marrow MSC because they are in the correct anatomical region for migration into the nascent marrow space and also because pericytes have been shown to have the potential to differentiate into chondrocytes, adipocytes, and odontoblasts. In addition, pericytes express the STRO-1 antigen, a known marker for bone marrow colony forming cells which is expressed in multipotential MSC (Table 1). The antibody STRO-1 has been the most useful antibody for identifying and positively selecting for MSC in bone marrow. This circumstantial evidence suggests a lineage relationship between pericytes and odontoblasts that further, implies a logical source of marrow and dental stem cells.
Stem Cell Markers

Researchers now know that many different types of stem cells exist, but they are found in sparse populations in the human body. Scientists identify these rare type of cells found in different tissues by the use of stem cell markers. Coating the surface of every cell in the body are specialized proteins, called receptors, which have the capability of selectively binding or adhering to “signaling” molecules. There are many different types of receptors that vary in their structure and affinity for the signaling molecules. Normally, cells use these receptors and the molecules that bind to them as a way of communicating with other cells and to carry out their proper functions in the body. These same cell surface receptors are the stem cell markers. Each cell type, for example an odontoblast, has a certain combination of receptors on their surface that makes them distinguishable from other kinds of cells. Scientists have taken advantage of the biological uniqueness of stem cell receptors and chemical properties of certain compounds to tag or mark cells. Much of the success in finding and characterizing stem cells is due to the use of markers.

Stem cell markers are given short-hand names based on the molecules that bind to the stem cell surface receptors. For example, a cell that has the receptor stem cell antigen – 1 on its surface is identified as Sca-1. In many cases, a combination of multiple markers is used to identify a particular stem cell type by a combination of marker names reflecting the presence (+) or absence (–) of them. Researchers use the signaling molecules that selectively adhere to the receptors on the cell surface as a tool that allows them to identify stem cells by attaching to the signaling molecule another molecule (or tag) that has the ability to fluoresce or emit light energy when activated by an energy source such as an ultraviolet light or a laser beam. Table 1 presents a list of some of the commonly used stem cell markers.
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<th>MARKER</th>
<th>CELL TYPE</th>
<th>DESCRIPTION</th>
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<tr>
<td>BMPR</td>
<td>Mesenchymal stem and progenitor cells</td>
<td>Important for the differentiation of committed mesenchymal cell types from mesenchymal stem and progenitor cells; BMPR identifies early mesenchymal lineages (stem and progenitor cells)</td>
</tr>
<tr>
<td>CD4 and CD8</td>
<td>White blood cell (WBC)</td>
<td>Cell-surface protein markers specific for mature T lymphocyte (WBC subtype)</td>
</tr>
<tr>
<td>CD34</td>
<td>Hematopoietic stem cell (HSC), satellite, endothelial progenitor</td>
<td>Cell-surface protein on bone marrow cell, indicative of a HSC and endothelial progenitor; CD34 also identifies muscle satellite, a muscle stem cell</td>
</tr>
<tr>
<td>CD34+ Sca1+ Lin- profile</td>
<td>Mesenchymal stem cell (MSC)</td>
<td>Identifies MSCs, which can differentiate into adipocyte, osteocyte, chondrocyte, and myocyte</td>
</tr>
<tr>
<td>CD38</td>
<td>Absent on HSC Present on WBC lineages</td>
<td>Cell-surface molecule that identifies WBC lineages. Selection of CD34+/CD38- cells allows for purification of HSC populations</td>
</tr>
<tr>
<td>CD44</td>
<td>Mesenchymal</td>
<td>A type of cell-adhesion molecule used to identify specific types of mesenchymal cells</td>
</tr>
<tr>
<td>c-Kit</td>
<td>HSC, MSC</td>
<td>Cell-surface receptor on BM cell types that identifies HSC and MSC; binding by fetal calf serum (FCS) enhances proliferation of ES cells, HSCs, MSCs, and hematopoietic progenitor cells</td>
</tr>
<tr>
<td>Colony-forming unit (CFU)</td>
<td>HSC, MSC progenitor</td>
<td>CFU assay detects the ability of a single stem cell or progenitor cell to give rise to one or more cell lineages, such as red blood cell (RBC) and/or white blood cell (WBC) lineages</td>
</tr>
<tr>
<td>Fibroblast colony-forming unit (CFU-F)</td>
<td>Bone marrow fibroblast</td>
<td>An individual bone marrow cell that has given rise to a colony of multipotent fibroblastic cells; such identified cells are precursors of differentiated mesenchymal lineages</td>
</tr>
<tr>
<td><strong>Hoechst dye</strong></td>
<td>Absent on HSC</td>
<td>Fluorescent dye that binds DNA; HSC extrudes the dye and stains lightly compared with other cell types</td>
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<td>-----------------</td>
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</tr>
<tr>
<td><strong>Leukocyte common antigen (CD45)</strong></td>
<td>WBC</td>
<td>Cell-surface protein on WBC progenitor</td>
</tr>
<tr>
<td><strong>Lineage surface antigen (Lin)</strong></td>
<td>HSC, MSC Differentiated RBC and WBC lineages</td>
<td>Thirteen to 14 different cell-surface proteins that are markers of mature blood cell lineages; detection of Lin-negative cells assists in the purification of HSC and hematopoietic progenitor populations</td>
</tr>
<tr>
<td><strong>Mac-1</strong></td>
<td>WBC</td>
<td>Cell-surface protein specific for mature granulocyte and macrophage (WBC subtypes)</td>
</tr>
<tr>
<td><strong>Muc-18 (CD146)</strong></td>
<td>Bone marrow fibroblasts, endothelial</td>
<td>Cell-surface protein (immunoglobulin superfamily) found on bone marrow fibroblasts, which may be important in hematopoiesis; a subpopulation of Muc-18+ cells are mesenchymal precursors</td>
</tr>
<tr>
<td><strong>Stem cell antigen (Sca-1)</strong></td>
<td>HSC, MSC</td>
<td>Cell-surface protein on bone marrow (BM) cell, indicative of HSC and MSC Bone Marrow and Blood cont.</td>
</tr>
<tr>
<td><strong>Stro-1 antigen</strong></td>
<td>Stromal (mesenchymal) precursor cells, hematopoietic cells</td>
<td>Cell-surface glycoprotein on subsets of bone marrow stromal (mesenchymal) cells; selection of Stro-1+ cells assists in isolating mesenchymal precursor cells, which are multipotent cells that give rise to adipocytes, osteocytes, smooth myocytes, fibroblasts, chondrocytes, and blood cells</td>
</tr>
<tr>
<td><strong>Thy-1</strong></td>
<td>HSC, MSC</td>
<td>Cell-surface protein; negative or low detection is suggestive of HSC</td>
</tr>
<tr>
<td><strong>VCAM-1</strong></td>
<td>HSC, MSC</td>
<td>Vascular cell adhesion molecule-1 found on HSC and MSC</td>
</tr>
<tr>
<td><strong>SH2</strong></td>
<td>HMSC</td>
<td>Binds endoglin, a type III TGF-β receptor found on mesenchymal tissues and on macrophages on endothelial cells, indicating that this antibody is not specific for MSC</td>
</tr>
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SH3 and SH4 | Have been shown to specifically bind an ecto-5’-nucleotidase, CD73

Table 1. Markers commonly used to identify bone marrow stem cells and hematopoietic precursors. From these, STRO-1 sorted cells have been shown to be osteogenic, chondrogenic, adipogenic, and hematopoietic.

History, Characteristics, and Functions of MSC

The presence of mesenchymal progenitor cells within bone marrow has been documented from the late 19th century by the work of Goujon. He was the first to show the osteogenic potential in heterotopic transplants of rabbit marrow, later confirmed in transplantation experiments by Biakow and Danis. They showed that whole marrow itself was osteogenic, and not just an inductive factor or chemoattractant for osteogenic cells, by placing marrow tissue within a diffusion chamber, implanting this in an animal host, and observing the results by histological methods. Similar confirmatory experiments were conducted by other groups wherein the formation of cartilage and bone within the diffusion chamber was observed, demonstrating that bone marrow had at least the potential to form bone and cartilage. Friedenstein showed that the osteogenic potential of bone marrow was a feature of a specific subgroup of cells termed the CFU (colony forming units), which made up a very small percentage of the total marrow cell population. Friedenstein later, showed that CFU were formed from single cells and some of these CFU were able to form both bone and the microenvironment necessary for the formation of hematopoietic elements.

The first formal presentation of the concept of a stem cell residing in the bone marrow was in a publication by Owen in 1978 wherein the marrow stroma was hypothesized to consist of a lineage analogous to the hematopoietic lineage. Owen, in 1985, expanded on this hypothesis and proposed a model for the stromal lineage that contained “stem cell”,

- 8 -
“committed progenitor”, and “maturing cell compartments”. He designed a lineage diagram for “stromal stem cells” that included “reticular”, “fibroblastic”, “adipocytic”, and “osteogenic” cells as end stage phenotypes. In 1991, Caplan\textsuperscript{43} proposed the existence of a MSC having the capacity to differentiate into multiple mesenchymal phenotypes including adipose, tendon, ligament, muscle and dermis\textsuperscript{44}. Years later, in 2002, Gronthos described the presence of undifferentiated cell types in human dental pulp\textsuperscript{45}.

Formal proof of the multipotentiality of MSC or CFU came from experiments with clonal populations. It was demonstrated that approximately 30\% of the isolated CFU colonies were able to form bone alone or bone with marrow in open transplants\textsuperscript{37} and about 40\% of rabbit marrow cells, isolated by limiting dilution or by cloning rings, were able to form bone\textsuperscript{46}. Multipotential mesenchymal cells were also identified in studies using MSC isolated from the marrow of transgenic mice containing a gene for conditional immortality\textsuperscript{47,48}. In that study, multiple colonies were isolated by cloning rings and limiting dilution while under immortalized culture conditions and then tested for differentiation potential under standard culture conditions or \textit{in vivo}. Mouse marrow derived mesenchymal progenitor cells contain a mixture of cells having various differentiation potentials\textsuperscript{47}, ranging from monopotent to quadripotent\textsuperscript{48}. Similar results were reported for MSC isolated from human marrow, wherein three of six isolated and expanded MSC colonies were able to differentiate into bone, adipose and cartilage, while all six were able to differentiate into bone\textsuperscript{49}.

Adult stem cells, like all stem cells, share at least 2 characteristics. First, they possess the ability to clone themselves for long periods of time; this ability to proliferate is referred to as long term self renewal. Second, they can give rise to mature cell types that have tissue
specific morphology and function. Typically, adult stem cells generate an intermediate cell type or types before they reach their fully differentiated state. The intermediate cell type is called the precursor or progenitor cell. Precursor or progenitor cells are partly differentiated cells in fetal or adult tissues. Such cells are usually committed to differentiate under a particular cellular development pathway, although, this characteristic is not as definitive as it was once thought. Primary functions of adult stem cells include maintenance of steady state cell function, or homeostasis, and to replace cells that die due to injury or disease. Adult stem cells are dispersed throughout the tissues of the adult human or animal and behave very differently depending on the local microenvironment. In solid tissues with specialized form, stem cell differentiation is accompanied by progressive remodeling of preexisting extracellular matrix (ECM) scaffolds, and it is the coordination between these processes in both time and space that ensures correct pattern formation. Maintenance of tissue architecture throughout life also requires fine control over both tissue mass and ECM pattern integrity, which are provided through spatially coordinated growth and differentiation of tissue specific stem cells.

The term “plasticity” has been defined in stem cell research as the ability of a cell to transdifferentiate or dedifferentiate and redifferentiate into other cell types and has been found to be more common than previously thought. For example, studies have shown that blood stem cells (derived from mesoderm) may be able to generate both skeletal muscle (also derived from mesoderm) and neurons (derived from the ectoderm). That realization triggered a flurry of papers reporting that stem cells derived from one adult tissue can change their appearance and assume characteristics that resemble those of differentiated cells from other tissues. The term plasticity means that a stem cell from one adult tissue
can generate the differentiated cell types of another tissue. At this time, there is no formally accepted name for this phenomenon in the scientific literature, so it continues to be referred to as plasticity or “transdifferentiation”\textsuperscript{52,54}. To be able to claim that adult stem cells demonstrate plasticity, it is first important to show that such cell populations exists in the primary tissue that has the identifying features of stem cells. Then, it is necessary to show that the adult stem cells give rise to cell types that normally occur in a different tissue\textsuperscript{55}. Neither of these criteria is easily met. Simply proving the existence of an adult stem cell population in a differentiated tissue is a laborious process. It requires that the candidate stem cells are shown to be self-renewing and that they can give rise to the differentiated cell types that are characteristic of that tissue. It is also important to demonstrate that those cells have adopted key structural and biochemical characteristics of the new tissue that they are claimed to have generated. Ultimately—and most importantly—it is necessary to demonstrate that the cells can integrate into their new tissue environment, survive in the tissue, and function like the mature cells of the tissue\textsuperscript{46-59}.

Pluripotent stem cells that can differentiate into various cell types have been isolated and some can spontaneously assemble into structures that resemble mature tissues\textsuperscript{60}. At the same time, ECM molecules have been shown to have potent effects on cell proliferation and differentiation and to direct assembly into three dimensional tissues \textit{in vitro}\textsuperscript{61,62}.

The future challenge in stem cell research is to understand how these cells can spontaneously generate 3D functional tissue architecture. To address this challenge we must answer some fundamental questions. First, what is the nature of the signals conveyed by both soluble and insoluble factors that govern where and when cells are triggered to organize into tissues and for tissues to organize into organs? Second, how do these cells process
multiple simultaneous inputs to produce a single coordinated cell fate response? To answer
these questions we should focus first on how individual cells chemically and physically in-
teract with their local tissue environment, as well as, how cells use regulatory networks to
process information provided by multiple environmental cues.

Extracellular Matrix, Cell Shape and Control of Proliferation

The common view of mitogenic stimulation holds that cooperative stimulation of
cell-surface growth factor receptors is orchestrated by soluble mitogens and transmembrane
adhesion receptors, known as integrins\textsuperscript{63,64}. This dual requirement is the basis for the con-
cept of “anchorage dependence” for growth of nontransformed cells. However, it has been
long suggested that cell shape or mechanical distortion also controls the switch between
quiescence and growth\textsuperscript{65,66}. Thus, cell ECM interactions have two components: a chemical
component, which is mediated by the ligand-induced activation of integrins and associated
biochemical signaling cascades, and a mechanical component, which is mediated by the
integrins role in physically resisting cell generated tractional forces. This later structural
effect of ECM binding to integrins is responsible for the rearrangement of the actin cy-
toskeleton and ensuing cell shape changes that are observed in response to cell adhesion to
the ECM. However, in living tissues, integrins can also modulate cell and cytoskeleton
form based on their ability to transmit external mechanical loads from the ECM to the cy-
toskeleton\textsuperscript{67,68}.

Taken together, the switch between growth, differentiation, and quiescence is gov-
erned by a set of inputs from the cell environment that includes soluble mitogens, insoluble
adhesive cues from ECM, cell-cell adhesion molecules, and mechanical forces that can
produce cytoskeleton distortion and modulate cell shape. Any of these signals alone is not sufficient to decide cell fate; behavioral control is an integrated response that simultaneously takes all of these signals into account.

Differentiation Studies

Evidence of stem cell plasticity comes from analysis of the expression of markers for differentiation of specific cell phenotypes as well as differentiated tissue like characteristics. In this segment we will describe what is known about stem cell qualities, of putative postnatal dental pulp stem cells, understand the basis of research performed with dental related cell lines, and set the framework for our research design.

During development, interactions between epithelial and mesenchymal cells from the dental papilla lead to the differentiation of ameloblasts and odontoblasts. Each deposit unique mineralized matrices, termed enamel and dentin, respectively. Once formed, these matrices do not undergo further remodeling. However, after tooth eruption, dentinal damage caused by mechanical trauma, exposure to chemicals, or disease processes, induces the formation of reparative dentin. It is thought that progenitors are recruited from the dental pulp to develop both supportive connective tissue and differentiated odontoblasts. To determine the existence of such cells in dental pulp, Gronthos et al applied methodology that had been previously developed for the isolation and characterization of bone marrow stem cells (BMSC). By using a colony forming efficiency essay, they demonstrated that a minor population within the adult human dental pulp is clonogenic. Those colonies occurred in higher frequency in comparison to BMSC. Also, they were able to demonstrate...
that dental pulp stem cells (DPSC) exhibited a higher proliferation rate when compared to the well characterized BMSC. These results were further confirmed in subsequent publications\textsuperscript{45, 70, 19}. DPSC differentiation towards a tissue type was examined in cells exposed to ascorbic acid, dexamethasone, and $\beta$-glycerophosphate and evaluated by the expression of alkaline phosphatase activity \textit{in vitro} as well as the presence of calcium deposits, with 2\% alizarine red stain. These results were compared to BMSC under the same circumstances. It is important to highlight that the bone matrix protein, bone sialoprotein, was absent in DPSC cultures and present in low levels in BMSC\textsuperscript{69}.

The potential of DPSC to differentiate into adipocytes and neural cells, in analogy to what has been demonstrated in BMSC, was published by Gronthos et al in 2002\textsuperscript{45}. After 5 weeks of culture with an adipogenic- inductive cocktail, Oil red O positive lipid clusters were identified in DSPC. This correlated with an upregulation in the expression of two adipocyte-specific transcripts, PPAR$\gamma$2 and lipoprotein lipase, detected by RT-PCR.

The chondrogenic potential of DPSC has not been elucidated, whereas an extensive body of literature describes BMSC differentiation towards a cartilage like tissue that expresses collagen type II, a specific marker of cartilage, as well as toluidine blue staining, a metachromatic stain for ECM glycosaminoglycans\textsuperscript{24}.

Progenitor Cells Involved in Periodontal Wound Healing and Regeneration

The functional periodontal attachment apparatus anchors the tooth and consists of periodontal fibers that run between alveolar bone and cementum lining the root surface. Furthermore, gingival connective tissues and epithelium overlay the alveolar bone and form a dentogingival junction at the interface with the tooth. The complex structure of the
periodontium, which consists of the soft tissues of the gingiva and PDL, as well as the mineralized tissues cementum and bone, makes periodontal wound healing a unique process.

Following conventional periodontal therapy involving debridement of the root surface, periodontal tissues heal by repair and migration of the epithelium along the previously contaminated root surface (long junctional epithelium), which prevents connective tissue attachment to the root surface\textsuperscript{71}. Regeneration of this destroyed attachment apparatus has long been the goal of periodontal therapy. Periodontal regeneration requires new connective tissue attachment to the root surface, a process that involves the regeneration of periodontal fibers and the insertion of these fibers into newly formed cementum. Unfortunately, current available regeneration techniques are clinically unpredictable, resulting in only partial regeneration at best\textsuperscript{72}. From a biological perspective, current and future prospects for improved regeneration of periodontal tissues are dependent on the ability to facilitate the repopulation of the periodontal wound by cells capable of promoting regeneration. From this perspective, the periodontal ligament has been shown to be of critical importance in the regenerative process. It has been demonstrated that only the periodontal ligament, but not gingival connective tissue, contains cells capable of establishing new attachment fibers between cementum and bone\textsuperscript{73,74}. It has also been shown that gingival fibroblasts grown \textit{in vitro} failed to contribute to regeneration of the PDL around teeth implanted in dogs, while periodontal ligament fibroblasts were observed to either contribute to regeneration or at least not inhibit regeneration\textsuperscript{75}. The ability of periodontal ligament cell populations to achieve regeneration implies that progenitor cells exist within the PDL.

Although it is clear that cells residing in the PDL can achieve regeneration, this population is heterogeneous\textsuperscript{76} and it is not known which subpopulations are capable of
achieving regeneration. Indeed, cells derived from regenerating defects were found to have specific properties, such as increased proliferation rates, representative of a regenerative phenotype that is different from periodontal ligament cells. Therefore, in order to identify progenitor and/or stem cells from the periodontium, it will be useful to establish where these cells reside in the tissue, as well as identify markers that can be used to distinguish these types of cells.

Periodontal regeneration during wound healing is likely to share many common biological processes with those of periodontal development. Aside from the gingival epithelium, the PDL is almost entirely derived from ectomesenchyme, the embryonic connective tissue derived from the neuroectoderm. MSC are precursors for a diverse group of connective tissue cells including smooth muscle cells, endothelial cells, chondroblasts, pericytes, adipocytes, osteoblasts and fibroblasts. At the initiation of tooth formation in fetal jaws, MSC cells are induced by the overlying oral epithelium towards odontogenic specificity. Initially, this interaction results in the formation of a tooth crown comprising the ectoderm (oral epithelium) derived enamel and the mesenchyme derived dentin. Subsequently, during root formation and the development of the periodontal attachment apparatus, epithelial root sheath cells derived from the enamel organ fragment to allow the attachment of MSC on the exposed root dentine. These MSC proceed to deposit cementum and establish an attachment apparatus. In order to understand the cellular origins of the developing periodontal apparatus, transplanted tooth buds were used to show that the mesenchymal derived dental follicle surrounding the developing tooth root is the source of progenitor cells for cementum, alveolar bone, and PDL.
Further evidence that cells from the dental follicle are precursors to cementoblasts has been shown by direct cell migration studies in rat molars \(^{80,81}\). More recently, the dental follicle associated with third molars has been shown to contain precursor cells which are clonogenic and have the ability to differentiate under \textit{in vitro} conditions to a membrane-like structure containing calcified nodules \(^{82}\).

The source of postnatal progenitor cells which may be capable of regenerating the periodontium has been investigated for a number of years. Cell kinetic experiments in mice and rats \(^{83,84}\) have shown that PDL fibroblast populations represent a steady state renewal system, with the number of new cells generated by mitosis equal to the number of cells lost by apoptosis and migration. This capacity for self renewal, which is further evidenced by the rapid turnover of the PDL, supports the notion of progenitor/stem cell populations. Furthermore, a significant number of periodontal cells do not enter the cell cycle \(^{85}\), suggesting that these cells may act in a similar manner to quiescent, self-renewable, and multipotent stem cells.

The relationship between progenitor cells in regenerating tissues and normally functioning (steady-state) tissues has been investigated in studies performed in normal mouse PDL \(^{10}\), wounded mouse ligament \(^{86}\), normal rat gingival tissues \(^{87}\) and inflamed monkey gingival tissues \(^{88}\). These studies have identified a common paravascular location of fibroblast progenitors which exhibit some of the classical cytological features of stem cells, including small size, responsiveness to stimulating factors, and slow cycle time \(^{86,83,10}\). Furthermore these paravascular cells exhibit spatial clustering which suggests a possible clonal distribution of progenitors and their progeny \(^{10}\). Other possible sources of osteoblasts and
cementoblast precursors are the endosteal spaces of alveolar bone, from which cells have been observed to adopt a paravascular location in the periodontal ligament of mice\textsuperscript{6}.

**Stem Cells in the Periodontal Ligament**

More than any other tissue, the complexity of the periodontium, along with the understanding that only certain components are capable of achieving regeneration, makes identification of stem cells important in the understanding of normal wound healing events. The inherent properties of stem cells, including their ability to form different tissue types and self renewal capabilities make their presence within the healing periodontal defect desirable in order to facilitate periodontal regeneration. Therefore, the ability to identify, characterize and manipulate stem cells within the periodontium is of considerable clinical significance, especially in terms of developing novel mechanisms of achieving periodontal regeneration.

Although it is established that precursor cells might exist within the PDL, stem cell properties such as self-renewal, clonogenicity, and multi-tissue differentiation have only begun to be elucidated. For these properties to be demonstrated, it is necessary to isolate stem cells *in vitro* using available markers for the identification of MSC. However, although the use of stromal cell markers and/or the absence of stromal cell markers has been successful in isolating cells from the bone marrow, additional challenges have been encountered when trying to extract MSC from connective tissues composed largely of fibroblastic cells, such as the PDL.

Single specific markers for MSC and also a combination of markers have been used to obtain enriched MSC cell populations. A single marker that has been extensively used
to isolate dental progenitor cells is STRO-1 antibody; results claim the specificity of this marker to target undifferentiated phenotypes in dental pulp tissues $^{89,19}$. A methodology for isolating MSC from the PDL should be based on techniques shown to successfully isolate BMSC and stem cells in dental pulp tissues $^{69,45,90}$.

MSC from postnatal dental pulp express the stem cell marker STRO-1, as well as CD 44, integrin β1, VCAM-1, CD 146/MUC-18, and alkaline phosphatase. When dental pulp stem cells were transplanted into immunocompromised mice, they generated a dentin like structure lined with odontoblast like cells that surround a pulp like interstitial tissue. These DPSC were also capable of multi-lineage differentiation into adipocytes and neural-like cells $^{69,45}$.

Stem cells have also been isolated from the pulp of human exfoliated deciduous teeth (SHED) $^{70}$. Immunohistochemical analyses of these cells have shown that they are positive for STRO-1 and CD-146/MUC-18. After transplantation into immunocompromised mice, SHED were found to induce bone formation, generate dentin, survive in the mouse brain, and express neural tissue markers. It has been suggested that SHED represent a population of multipotent stem cells that are more immature in the cell hierarchy than previously examined postnatal stem cell populations.

Subsequent research using frozen pulp and bone marrow sections has indicated that MSC are located in the perivascular regions and constitutively express STRO-1, CD 146, and α smooth muscle actin on the cell surface $^{90}$. The perivascular localization of these cells is consistent with previous reports of localization of progenitor cells in the periodontal ligament.
In light of these findings, the identification of stem cells within the periodontal ligament will be a significant development towards a tissue engineering approach to obtain periodontal regeneration. Furthermore, the ready availability of periodontal ligament tissue from redundant teeth, such as third molars, may provide a supply of stem cells that may be utilized for regenerating, not only the masticatory apparatus, but also other body tissues.

Therefore, the objectives of this study are to isolate, identify and characterize PDL stem cells (PDLSC) as well as demonstrate their ability to differentiate into adipose, chondrogenic, and mineralized type tissues. This research is based on the hypothesis that undifferentiated progenitor cells exist within the PDL and demonstrate capabilities to differentiate into chondrogenic, adipogenic and bone like tissues.
ISOLATION AND CHARACTERIZATION OF MULTIPOTENT HUMAN PERIODONTAL LIGAMENT STEM CELLS.

by

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ABSTRACT:

Periodontal ligament (PDL) repair is thought to involve mesenchymal progenitor cells capable of forming fibroblasts, osteoblasts and cementoblasts. However, full characterization of PDL stem cell (SC) populations has not been achieved. Here we isolate and characterize PDLSC and assess their capability to differentiate into bone, cartilage and adipose tissue. Human PDL cells were stained for STRO-1, FACS sorted and expanded in culture. Human bone marrow SC (BMSC) served as a positive control. PDLSC and BMSC were cultured using standard conditions conducive for osteogenic, chondrogenic and adipogenic differentiation. Osteogenic induction was assayed using alizarine red S staining and expression of alkaline phosphatase (ALP) and bone sialoprotein (BSP). Adipogenic induction was assayed using Oil Red O staining and the expression of PPARγ 2 (early) and LPL (late) adipogenic markers. Chondrogenic induction was assayed by collagen type II expression and toluidine blue staining. Human PDL tissue contains about 27% STRO-1 positive cells with 3% strongly positive. In osteogenic cultures ALP was observed by day-7 in BMSC and day-14 in PDLSC. BSP expression was detectable by day-7; with more intense staining in PDLSC cultures. In adipogenic cultures both cell populations showed positive Oil Red O staining by day-25 with PPARγ 2 and LPL expression. By day-21, both BMSC and PDLSC chondrogenic induced cultures expressed collagen type II and glycosaminoglycans. The PDL contains SC that have the potential to
differentiate into osteoblasts, chondrocytes and adipocytes, comparable to previously characterized BMSC. This adult PDLSC population can be utilized for potential therapeutic procedures related to PDL regeneration.
INTRODUCTION

The dental attachment apparatus consists of two mineralized tissues; cementum and alveolar bone, with an interposed fibrous, cellular and vascular soft connective tissue termed the periodontal ligament (PDL). The PDL provides anchorage and support to the functional tooth, dispersing the mechanical forces associated with mastication. The PDL cell population is heterogeneous, consisting of two major mesenchymal lineages, fibroblastic and mineralizing tissues, further divided into osteoblastic and cementoblastic subsets (1-4). In the adult PDL, the progenitors for both cell lineages originate in the paravascular zones (5). This progenitor stem cell (SC) population is enriched by precursors derived from the endosteal spaces of the alveolar bone (6). Cell kinetic studies suggest that some of these progenitors migrate toward alveolar bone and cementum where they terminally differentiate into osteoblasts, cementoblasts and fibroblasts (5,7). Cell migration and differentiation pathways are the basis for remodeling and healing in periodontal tissues, and it is likely that some form of signaling has evolved to orchestrate, in a temporally and spatially appropriate manner, these repopulation and differentiation responses (8). However, a detailed understanding of the biochemical and molecular events associated with the remodeling process in the periodontum has not been determined due to the diversity of cells and tissue types. Methods that can identify specific PDL cell populations and PDL stem cells (PDLSC) are of central importance for our understanding of remodeling and healing in these tissues.
By definition, a stem cell has the ability to proliferate in culture for indefinite periods and give rise to specialized cells. Embryonic stem cells derived from the inner cell mass of the embryo are termed pluripotent because they can give rise to many cell types but not all the cells necessary for fetal development. In addition, multipotent SC have been identified in young and adult tissues, and are capable of further differentiation. Examples are blood and skin SC that play a key role in homeostasis and tissue repair by continually replenishing our blood cells and skin layers throughout life.

Adult SC, once thought able to develop only the same type of tissue, have recently been shown to be able to develop into many types of specialized cells (9). These data suggest that adult SC have outstanding potential for the development of novel therapies such as tissue transplantation and regeneration. It is well documented that adult bone marrow (BM) contain a population of SC that retain the capacity to differentiate along different lineages including reticular, adipogenic, fibroblastic, osteogenic, and chondrogenic (10-13). In addition, BMSC can interact with other cell phenotypes in bone marrow microenvironments to influence and regulate a variety of physiological pathways including bone remodeling (14,15), hematopoiesis (16-18) and angiogenesis (19). Effective techniques have been developed to isolate BMSC using specific antibodies to cell surface markers with fluorescence activated cell (FACS) sorting to enrich cell populations.

Stem cell differentiation processes have been successfully demonstrated *in vitro* by exposing undifferentiated stem cells to a variety of microenvironments and tissue culture conditions (20). Adipocyte differentiation is accompanied not only by gross changes in cellular morphology, but also by the transcriptional activation of many genes. A member of the nuclear receptor superfamily of ligand-activated transcription factors identified as peroxisome proliferator-activated receptor gamma 2 (PPARγ2) has been shown to be expressed early in the adipocyte differentiation program. It acts synergistically with
CCAATT enhancer-binding protein α to coordinate the adipocyte differentiation cascade. Importantly, the peroxisome proliferator response element has been identified in the promoter regions of several enzymes associated with triglyceride metabolism (21). LPL encodes lipoprotein lipase, which is expressed in heart, muscle, and adipose tissue. LPL functions as a homodimer and has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake (22).

Cells isolated from postnatal mammalian BM have also the potential for differentiation into cartilage when implanted in vivo (23). However, attempts to develop in vitro conditions in which mesenchymal stem cells, derived from postnatal mammalian bone marrow, will progress down the chondrogenic lineage has been less successful. A system that cultures cells in aggregates has been described (24, 25) and utilized in studies of terminal differentiation of growth plate chondrocytes (26). This culture system allows cell-cell interactions analogous to the ones that occur in pre-cartilage condensation during embryonic development. However, this cell configuration is not sufficient for the induction of chondrogenesis; it requires defined medium and bioactive factors such as TGF-β (27). Evidence of chondrogenic differentiation includes the appearance of toluidine blue metachromasia and immunohistochemical detection of type II collagen (28).

Recent findings show that mesenchymal progenitors can differentiate into osteoblast-like cells when challenged with dexamethasone, β-glycerophosphate and ascorbic acid in vitro. These cells express terminal phenotype identification markers such as Runx2 promoter activity, alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OP), osteocalcin (OC), and collagen type I, as well as mineral deposits in the extracellular matrix (29). In this study, we report that PDL tissue contains undifferentiated progenitor cells, adult SC, that are capable of differentiation into bone, cartilage and adipose-like tissues comparable with the well-defined BMSC. Also, we have demonstrated PDLSC capability
to express gene products compatible with differentiated cell types. This, therefore, clearly demonstrates the existence of progenitor undifferentiated STRO-1 positive cells that could potentially be used for PDL regenerative therapies as well as the possible formation of a true PDL apparatus associated with a titanium implant surfaces.

MATERIALS AND METHODS

Cell Isolation, FACS Sorting and Cell Culture

Disease free impacted third molars were collected from 10 patients, 18 to 26 years old, at the Oral and Maxillofacial Surgery Department at the University of Texas Health Science Center at San Antonio (UTHSCSA). Individuals were selected on the basis of good general health and elective surgery procedures, following the approved guidelines of the UTHSCSA Institutional Review Board.

PDL cells were scraped from 3rd molars, enzymatically digested for 1 hour at 37°C in a solution of 3 mg/ml collagenase type I (Worthington Biochem, Freehold NJ) and 4 mg/ml of dispase (Worthington Biochem, Freehold NJ). PDL cells from different individuals were pooled and single cell suspensions were obtained through a 70 μm cell strainer (Falcon BD, Franklin Lakes NJ). Samples were centrifuged at 400 g for 10 minutes and resuspended in blocking buffer (HBSS, 20 mM HEPES, 1% normal human serum, 1% bovine serum albumin, 5% FCS) for 20 min. on ice and stained with STRO-1 IgM antibody (Developmental Studies Hybridoma Bank, Iowa City IW) (2 μg/ml) for 1 hour on ice. A goat anti-mouse IgM FITC secondary antibody (Molecular Probes, OR) (2 μg/ml) was incubated for 1 hour. Cell surface-marker STRO-1 positive cells were analyzed and sorted by flow cytometry. This procedure was performed on a FACScan with automated cell deposition unit (Becton Dickinson, Parsippany, NJ) equipped with an argon laser and data ana-
alyzed with CellQuest software (Becton Dickinson, Parsippany, NJ) at the Institutional Flow Cytometer Core Facility (UTHSCSA, San Antonio, TX).

Cells were expanded with Dulbecco's modified Eagle's medium containing 10% FCS and 1% antibiotics. The cultures were incubated at 37°C in a 5% CO₂ atmosphere. Human BMSC kindly provided by Dr. A Caplan (Case Western Reserve University, Cleveland OH) were grown under the same conditions as PDLSC and used as a positive control. Cultures at 2
nd or 3
rd passage were utilized for all experiments, unless otherwise stated.

Cell Growth Rate Assay

To compare cell growth capabilities we determined the growth rates on both cell populations (BMSC and PDLSC). Cells were plated at a density of 5 X 10³ cells per cm² in 12 well plates and cultured with DMEM containing 20% FCS and 1% antibiotics for 0, 48 and 96 hours. At harvest, cells were washed twice with PBS and released from the culture surface by the addition of 200 μl of trypsin-EDTA 1X for 10 min at 37°C. The cell suspension was transferred to a vial containing 9.8 ml of 0.9% NaCl. Cell number was determined with a Coulter Counter (Coulter Electronics Inc, Hialeah, FL). Cells harvested in this manner exhibited greater than 95% viability on the basis of Trypan blue exclusion.

Clonogenic Assays

Colony forming units were evaluated for the BMSC and PDLSC using DMEM (GIBCO/BRL) with 20% FBS (Equitech-Bio, Kerrville, TX) under standard culture conditions at 37°C. At day 7 and 14, cultures were fixed with 10% formalin (Fischer Scientific, Fair Lawn, NJ), stained with 0.1% toluidine blue (Sigma-Aldrich, Milwaukee, WI) and ob-
served under an inverted microscopy. Aggregates of 50 cells or more were scored as colonies, results were recorded and compared between PDLSC and BMSC cultures.

**Differentiation Culture Conditions**

*Osteogenic Induction:* PDLSC and BMSC were plated at $5 \times 10^3 \text{ cm}^2$ in 24 well plates. At 24 hours the media was changed to an osteogenic inducing media (Cambrex Bio Science Walkersville, MD). Control groups were treated with DMEM containing 10% FBS and 1% antibiotics only. Cells were assayed at 7, 14, 21, 28, 35, and 42 days for ALP expression and calcium deposits using alizarine red S staining.

*Chondrogenic Induction:* BMSC and PDLSC populations ($2.5 \times 10^5$ cells) were centrifuged in 15 ml polypropylene tubes (Corning Incorporated Life Sciences, Acton, MA). Cell pellets were treated with chondrogenic differentiation media (Cambrex Bio Science Walkersville, MD) supplemented with 10 ng/ml of TGF-β3 (Cambrex Bio Science Walkersville, MD). Pellets were left free floating for 14 and 21 days and fixed with 10% phosphate buffered formalin.

*Adipogenic Induction:* PDLSC and BMSC were plated at $5 \times 10^3 \text{ cm}^2$ in 6 well plates. At 100% confluence, three alternated cycles of induction/maintenance were performed utilizing Cambrex adipogenic induction medium and Cambrex maintenance medium (Cambrex Bio Science Walkersville, MD). Control cultures were treated with Cambrex maintenance medium. At day 25, after induction, cultures were fixed with 10% formalin (Fischer Scientific, Fair Lawn, NJ) and stained with Oil Red O.

**Culture Histochemical Staining**

Osteogenic cultures were stained with 2% alizarine red S (Sigma-Aldrich, Milwauk ee, WI), (pH 4.2) according to the manufactures instructions. Chondrogenic culture pel-
lets were embedded in 2% agarose followed by paraffin. Serial histological sections were
stained with 0.2% toluidine blue (Sigma-Aldrich, Milwaukee, WI) according to suppliers’
instructions. Adipogenic cultures were stained with 3% Oil Red O (Sigma-Aldrich, Mil-
wakee, WI) for 5 minutes and counterstained with Harris hematoxylin (Sigma-Aldrich,
Milwaukee, WI) for 1 min suing standard techniques.

In situ ALP Staining:

For localization of ALP activity, cultures were washed twice with phosphate-
buffered saline (PBS), fixed with 70% ethanol for 5 min, and washed for 5 min. in buffer
(100mm/l Tris-HCL, ph 9.5; 100 mmol/l NaCl; 50 mmol/l MgCl2). In situ ALP staining
was performed according to the supplier’s instructions (Bio-Rad, Hercules, CA)

Immunohistochemistry:

Chondrogenic culture samples were immunostained for collagen type II expression
using a commercial collagen type II antibody (Santa Cruz Biotechnology, Santa Cruz CA)
at a 1:100 dilution for 1 hour incubation according to the supplier’s instructions. The sec-
ondary antibody used was a rabbit anti goat IgG FITC (Molecular Probes, OR) (2 μg/ml)
incubated for 1 hour. Images were obtained at the optical facility, UTHSCSA under the
same parameters using an Olympus Wide Field microscope.

RT-PCR Analysis:

Total RNA was isolated from osteogenic and chondrogenic cultures (PDSLAA and
BMSC) using RNA STAT-60 (TEL-TEST, Inc. Friendswood, TX.). First-strand cDNA
was produced from 500 ng of total RNA with random hexamers using MultiScribe reverse transcriptase according to manufacturer’s instructions (ABI TaqMan Kit, Applied Biosystems, Foster City, CA). After mRNA conversion to cDNA, 500 ng of target cDNA was amplified with 1 Unit of Thermus aquaticus (Taq) polymerase (Perkin-Elmer Cetus, Boston, MA) using various specific primer sets.

### Osteogenic Marker Primer Sets and PCR Conditions

Specific primer sets (10 pmol) included ALP (accession number NM_000478: antisense 5’-atgcaggctgcaatagc-3’, sense 5”-atctttggtctggccccatg-3”) and BSP (accession number AH_006462: antisense 5’-ttcctctcctcctctcaactg-3’, sense 5’-atggtctgtgctttctaat-3’). PCR amplification for each primer included denaturation at 95°C for 1’, annealing at 58-60°C for 1’ and an elongation step of 72°C for 1’ for a total of 40 cycles. GAPDH (accession number AL_136219: antisense 5’-gcaagttgtcatgg atgacc-3’, sense 5’-ccatggagaaggctgggg-3’) was used as an internal control for cDNA integrity. PCR products were separated by agarose gel electrophoresis (2-4%) in 1 X Tris-borate buffer (0.01M Tris-base, 0.1 M boric acid, and 0.02 M EDTA, pH 8.3).

### Adipogenic Marker Primer Sets and Real-Time PCR Conditions

To verify gene expression in the adipogenic induction cultures amplification reactions were analyzed in real-time on an ABI 7500 (Applied Biosystems Foster City, CA) using SYBR Green chemistry and the threshold values calculated using SDS2 software (Applied Biosystems, Foster City, CA) according to the supplier’s instructions. Thermal
cycling parameters were 95°C for 30 s and 60°C for 1 min for 40 cycles. Reactions were performed in quadruplicate and threshold cycle numbers were averaged. A single melt curve peak was observed for each sample used in data analysis, confirming the purity and specificity of all amplified products. Expression fold was calculated according to the formula $2^{(\text{Rt}-\text{Et})/2^{(\text{Rn}-\text{En})}}$ where Rt is the threshold cycle number for the housekeeping gene (18S) from non-induced PDLSC and Et is the threshold number for the experimental gene observed in non-induced PDLSC. Rn is the threshold value for the housekeeping gene in induced PDLSC (18S) and En is the threshold cycle number for the experimental gene in induced PDLSC (30). Real Time PCR primers were designed to the following human genes: PPARγ2 (accession number D83233: antisense 5’-caggaaagacaacagacaaatca-3’, sense 5’-ggggtgatgtttgaacttg-3’), LPL (accession number NM_000237: antisense 5’-gtggccgagagttagacttggaacttg-3’), and 18S (accession number NM_022551: antisense 5’-agacctggagcgactgaaga-3’, sense 5’agaagtgacgcagccctct-3’).

Statistical Analysis:

Each individual value represents the mean ± SEM of 6 individual sample measurements, performed in triplicate. The data were analyzed by use of Student’s t-test. Significance level was set at p< 0.05 for all tests.

RESULTS

To identify the existence of potential stem cells within the PDL cell population single cell suspension of the PDL was plated on 4 chamber slides and cultured for 24 hours to
promote cell attach. As a positive control, purified BMSC were plated in the same manner. Immunohistochemistry was performed using an antibody directed against a known stem cell marker STRO-1. Experiments were performed in parallel on the PDL and BMSC cultures to avoid possible time-related biological fluctuation. The BMSC, an enriched population, served as a positive control due to their well-characterized stem cell properties. Figure 1 shows the immunohistochemistry of 3rd passage human BMSC from a 31 year old male and primary PDL cells obtained from a pool of patients (18 to 26 year olds). The BMSC stained positive for STRO-1 confirming their stromal stem cell status (Fig. 1, B & C). The PDL cell heterogeneity was confirmed showing a low number of STRO-1 positive cells. Figure 1 panel H shows a single STRO-1 positive cell observed in the field of the PDL mixed cell population, The negative controls lacking the primary antibody for both cell populations shown no staining (Fig. 1, E, F, K & L).

Next, PDL single cell suspensions obtained from the 38 molars were stained with STRO-1 antibody. Surface-marker STRO-1 positive cells were analyzed and sorted by flow cytometry. A total of 27% of the PDL cell population was positive for STRO-1 and of that population 3% were strongly positive. This enriched population had several intensity ratios to the mesenchymal stem cell antibody as demonstrated by various peaks within the M1 region after sorting was performed a second time and the population re-analyzed (Fig. 2, C). The PDL STRO-1 negative cell population analysis demonstrated 99.8% purity as seen in Figure 2, panel D.

To investigate the potential of the enriched PDLSC to undergo mitosis compared with the BMSC, cell suspensions from both cell types were plated and counted at 0, 48, and 96 hours using a Coulter Counter. At 96 hours, PDLSC demonstrated a statistically signifi-
cant (p<.05) nearly double number of cells as compared to the BMSC, whereas at 48 hours there was no significant difference between the two cell populations (Fig. 3).

The ability of PDLSC to form characteristic stem cell adherent clonogenic cell clusters of fibroblast-like cells was compared with BMSC. Both PDLSC and BMSC showed the ability to form multi-cell clusters (Fig. 4, A & B). PDLSC showed the formation of about 50 colonies (Fig. 4, C) generated from single cells cultured at low density (5x10^3 cm^2) for 7 days; whereas BMSC generated 35 colonies. These results showed statistically significant difference with PDLSC having an increased colony forming unit behavior as compared to BMSC. To investigate the potential of PDLSC to differentiate into mineralizing osteoblast or cementoblast-like lineages, we induced the formation of mineralized matrix containing calcium deposits by the addition of culture media containing L-ascorbate-2-phosphate, dexamethasone and β-glycerophosphate (osteogenic media). After 48 days in culture, PDLSC showed calcium deposits, whereas at day 35, BMSC demonstrated calcium deposits as visualized by alizarine red stain (Fig. 5). Staining of the PDLSC cultures was associated with cell clusters whereas staining within the BMSC cultures was more diffuse seen throughout the culture. To confirm the differentiation of PDLSC towards an osteoblast/cementoblast-like tissue, we investigated the presence of ALP involved in the early mineralization events and BSP a gene expression product known as a late mineralized tissue marker (Fig. 6). Immunohistochemical evaluation (Fig. 6, A-C) and RT-PCR analysis (Fig. 6, D) showed that osteogenic induced PDLSC cultures express known mineralized tissue markers: ALP and BSP. The positive control samples BMSC cultures were positive for both proteins. In situ staining for ALP activity was detectable by 14 days of culture in the PDLSC as compared to 7 days for the BMSC cultures. However, by 21 days no detect-
able difference in level of ALP activity was seen between the two cell populations. ALP expression was confirmed by RT-PCR analysis as well as the expression of a late mineralized tissue marker BSP. Expected amplification products (ALP 288 bp and BSP 204 bp) were detectable in the PDLSC and BMSC cultures as early as 7 days.

GAPDH, a housekeeping gene, served as a positive control for the PCR reaction. Control samples were negative for all tested markers at all time points.

We next assessed whether PDLSC, like BMSC, had the potential to differentiate into other cell lineages such as adipocytes. After 25 days of culture with an adipogenic inducing media, PDLSC as well as BMSC developed oil red O positive lipid-laden droplets (Fig. 7, A & C) whereas control cultures grown in control media failed to produce similar results (Fig. 7, B & D). These finding clearly correlate with an up-regulation in the expression of two adipocyte specific early/late differentiation transcripts LPL and PPARγ2, respectively. As shown in Figure 8 panel E quantitative RT-PCR normalized against the pseudogene 18 S (31) showed increase in the two adipocyte specific gene markers in both cell populations. While the two transcripts increased in the PDLSC cultures, the change in the later marker PPARγ2 was greater in the BMSC culture showing a nearly 10 fold increase.

Another well-characterized feature of undifferentiated mesenchymal stem cells such as BMSC is their capability to differentiate into chondrocytes, the process of chondrogenesis. After 14 days of exposure to chondrogenic differentiation media containing 10 ng/ml of TGF-β3, BMSC express metachromasia with toluidine blue stain as well as positive immunohistochemistry for collagen type II a gene marker for chondrocytes. PDLSC failed to express metachromasia at day 14, although they were positive for collagen type II expression. At 21 days both cell populations show chondrocyte-like cell morphology as well as the expression of collagen type II and toluidine blue metachromasia. Staining with
touidine blue demonstrates the accumulation of glycosaminoglycans in the extracellular matrix (Figs 8, 9). Negative controls exposed to control media were not possible to obtain due to the fact that stem cells fail to form aggregates unless they are exposed to differentiation media.

**DISCUSSION**

Our findings clearly demonstrate the successful isolation and characterization of undifferentiated progenitor cells contained in human PDL that can be expanded *in vitro*, providing a unique reservoir of stem cells obtained with minimally invasive procedures. Therefore, the PDL apparatus represents a viable alternative to obtain potentially high numbers of cells for regenerative procedures without the necessity of bone marrow aspiration or more invasive procedures that will result in increased morbidity.

Our experiments show PDLSC multipotential capability to differentiate *in vitro* towards osteogenic, adipogenic and chondrogenic tissues. These results positively correlate with the well characterized BMSC when exposed to appropriate culture conditions. In this study we found that PDLSC are similar to other mesenchymal stem cells such as BMSC (32-34) and dental pulp derived stem cells (35-38) with respect to their expression of the STRO-1 marker. The development of a series of monoclonal antibodies raised towards BMSC surface antigens, along with other antibodies developed to characterize BM stromal cells has been crucial for the immunophenotyping of these cells. Results have shown that the antigenic profile of BMSC is not unique, presenting features of mesenchymal, endothelial, epithelial and muscle cells (39). Several research laboratories have joined efforts to find the potential “gold standard” antibody for stem cell identification. From these, STRO-1 IgM antibody has shown consistent, promising results (40-42). In our laboratory, other
surface antibodies, such as SH2, SH3, SH4, endoglin (CD105) and CD44 (43-46) yielded 100% positive results which would seem to indicate that all PDL cells are multipotent (data not shown). These results are not compatible with heterogeneous PDL cell population that contains fibroblasts, osteoblasts, cementoblasts and progenitor cells. After exhaustive surface marker testing we were able to isolate and purify a PDLSC population derived from the PDL using the STRO-1 antibody.

PDLSC demonstrated by 48 hours the same growth rate as BMSC. At 96 hours PDLSC cell numbers were almost double those of BMSC cells. Similar findings have been reported by other authors for other dental tissue derived stem cells such as those isolated from permanent tooth pulp (35-38). The exact mechanism for this increased cell growth remains obscure. The nature of this difference may arise from several determinants, including the procedure used to harvest the cells, the age difference of the PDLSC donors (18-26 years) versus the BM donor (37 years). Another possibility is that dental tissue derived stem cells exhibit higher mitotic properties that cells derived from BM aspirations, specifically BMSC. As a consequence of faster cell growth, the clonogenic capabilities of PDLSC were superior as compared with BMSC.

The osteogenic potential of the heterogeneous population of PDL cells has been demonstrated earlier with in vitro procedures as well as the ability of such cultures to form a mineralized matrix (47). In our series of experiments PDLSC fail to produce such results at all time points tested. Mineralization was induced after 14 days of exposure to differentiating media. Our data and other published data (35-38,48) shows the potential of PDLSC to form mineralized deposits in vitro as previously has been shown with other mesenchymal cell populations (49). However, PDLSC formed sparse calcified focal nodules that took longer to form as compared with their BMSC counterparts. These results are supported by
the gene expression profiles and protein expression patterns as determined by RT-PCR analyses, *in situ* expression, and immunohistochemistry.

A possible explanation for the delay in differentiation seen in the PDLSC cultures is the presence of terminally differentiated cell types in the enriched population. By FACS sorting techniques, we were able to obtain a 73.3% enrichment of STRO-1 positive cells, thus it is possible that differentiated cell types such as fibroblasts interfered with the mineralization process. In other words, it is possible that PDLSC used in our experiments represent a more heterogeneous SC enriched population mixed with a greater subpopulation of terminally differentiated cell types. Another possible explanation for this finding is that our PDLSC population is at a different stage of commitment as compared to the BMSC.

Recent in vivo data has shown that SC differentiation may be due to cell fusion with other differentiated cell types (50,51). Although it is known that several various cell types reside in PDL tissue, adipocytes have not been reported as a native component. In our series of experiments, we were able to demonstrate that an inductive adipogenic tissue culture media can induce PDLSC and BMSC to form characteristic oil red O positive lipid laden droplets that are comparable to those found in adipocyte cells. This phenotypic conversion was also correlated with the expression of the early adipogenic transcription factor PPARγ2 and the later marker LPL. Similar results for dental pulp SCs and PDLSC have been published (35-38, 48).

A differentiation pathway that has not been reported for PDLSC is their ability to form chondrocyte like tissues when exposed to chondrogenic media with the addition of TGF-β3. We successfully induced not only a shift in cell morphology towards a chondrocyte cell morphology, but also the expression of collagen type II and proteoglycans as indicated by metachromasia with toluidine blue stain seen at 21 days. The resulting differentiated tissue shows characteristics representing a prechondroyd differentiation stage. Carti-
lage is not a normal tissue type associated with the periodontal apparatus or surrounding areas, which further demonstrates the multipotent potential of PDLSC to differentiate into multiple cell types.

The specific cues to initiate the proliferation and differentiation of PDLSC in vivo are not known. The ability to isolate, expand in culture and direct the differentiation of PDLSC in vitro to particular lineages provides the opportunity to study events associated with commitment and differentiation. From the various unique culture conditions required for terminal differentiation, we conclude that basal nutrients, cell density, spatial organization, growth factors and cytokines present in the culture media have a profound influence on PDLSC differentiation. PDLSC cultured in each of the differentiation conditions may also produce autocrine and paracrine factors that are essential for specific lineage progression. The PDLSC described here have the ability to proliferate extensively and maintain their capability to differentiate into multiple cell types in vitro, establishing their SC characteristics. Their cultivation and selective differentiation should provide further understanding of this important progenitor of multiple tissue types.

We are currently evaluating the behavior of these cells in vivo on an osteoconductive scaffold environment as well as their interaction with SLA titanium surfaces for the possible formation of a natural PDL on the surface of dental implants. Therefore, PDLSC can potentially be isolated and expanded in vitro for their use in PDL regenerative therapies, with minimal discomfort due to invasive procedures commonly performed.
REFERENCES


FIGURE LEGENDS

Figure 1. Immunohistochemistry of human BMSC and PDL cells with mesenchymal stem cell marker STRO 1. Panels A-F represent images from BMSC cultures bright field (A & D), fluorescence (B & E) and overlay of both images (C & F). Panel B and C show positive cell staining for STRO-1 of the BMSC population (20X). Panels E, and F represent the negative control results lacking the primary antibody (20X). Panel H and I show the STRO-1 staining with a single positive cell observed in the visible field (10X), in contrast to the negative controls with no staining shown in panels K and L. Images were obtained with an Olympus Wide Field Microscope at the Imaging Core Facility University of Texas Health Science Center at San Antonio (UTHSCSA). Details of the experiments are described under Materials and Methods section.

Figure 2. FACS Sorting of STRO-1 Positive PDL Cells. Primary digested pooled adult human PDL cells were immunostained with STRO-1 antibody and cells analyzed on a FACScan with automated cell deposition unit (Becton Dickinson, Parsippany, NJ) equipped with an argon laser and data analyzed with CellQuest software (Becton Dickinson, Parsippany, NJ) at the UTHSCSA Institutional Flow Cytometry Core Facility. A) The gated cell population. B) The percent of the STRO-1 positive cell population identified (27%). C) A 73.3% enrichment of STRO-1 positive PDL cells at second cell sorting. Strongly positive STRO-1 cells represent around 3% of this total cell population. D) Analysis of the negative STRO-1 cell population with a 99.8% purity.
Figure 3. PDLSC and BMSC Cell Proliferation Rates. PDLSC and BMSC were cultured for 0, 48 and 96 hours in 12 well plates with media changes every other day. The cells were harvested by trypsinization and counted with a Coulter Counter. The data are from 1 of 3 representative experiments each yielding similar results. Values are the mean ± SEM for 6 cultures (P<0.05).

Figure 4. PDLSC and BMSC Colony Forming Units. At day 7 after plating, PDLSC and BMSC were fixed with 10% formalin and stained with toluidine blue. Aggregates of 50 or more cells were considered colonies and counted. A) PDLSC representative colony (10X). B) BMSC representative colony (10X). C) Graph represents a statistically significant difference in total colony number between PDLSC (n=50) and BMSC (n=35) cultures. Values are the mean ± SEM for 6 cultures (P<0.05).

Figure 5. Expression of mineral deposits within PDLSC and BMSC long term cultures. At day 35 BMSC show mineral deposits as is evidenced by alizarine red stain. Those deposits are distributed throughout the tissue culture well. In contrast, PDLSC cultures demonstrate calcium deposits at day 48 and the distribution was seen as dense foci associated with cell clusters (20X).

Figure 6. BSP and ALP gene expression profiles of osteogenic BMSC and PDLSC cultures. BMSC and PDLSC were grown in osteogenic (experimental) or normal (control) media for 7, 14 and 21 days. ALP staining increased temporally in the experimental induced groups whereas the control groups failed to express detectable enzyme activity (A & B). Immunohistochemistry images reveal the presence of BSP positive cells after 7 days of culture (10X) in both cell populations. Staining was more intense in the PDLSC and continued through day 21. Negative controls, lacking primary antibody showed no
staining at all days tested (C). RT-PCR image for ALP, BSP and the housekeeping gene GAPDH on PDLSC cells. At day 7 there is no detectable ALP expression. Starting at day 14, ALP expression increases throughout whereas BSP remains as a constant expression from day 7 through day 21 in culture (D). Lanes were loaded as follows: 1) PDLSC 7 days induced; 2) PDLSC 7 days control; 3) PDLSC 14 days induced; 4) PDLSC 14 days control; 5) PDLSC 21 days induced; and 6) PDLSC 21 days control.

Figure 7. Characterization of adipogenic differentiation of PDLSC and BMSC cultures. Panel A shows BMSC induced cultures at 25 days with visible positive oil red O staining lipid droplets. This is in contrast to the control cultures grown for the same period in normal media (B). Adipogenic Induced cultures of PDLSC also formed positive lipid droplets after 25 days in culture (C), These droplets were not seen in the negative control cultures (D). Magnification 20X. Panel E shows the RT-PCR analysis of adipogenic markers demonstrating statistical significant upregulation of PPARγ2 and LPL in BMSC. Direct comparison between BMSC and PDLSC demonstrates increased production of both gene expression markers and lipid accumulation in BMSC. Values are the mean ± SEM for 6 cultures (P< 0.05).

Figure 8. In vitro chondrogenic differentiation of PDLSC and BMSC. Panels A and C show BMSC cultures after 14 and 21 days, respectively treated with chondrogenic differentiation media (10X). Panels B and D show PDLSC cultures after 14 and 21 days, respectively treated with chondrogenic differentiation media (10X). In both cell populations metachromasia and cell morphology correspond with embryonic stages of cartilage formation.
Figure 9. Immunohistochemistry for collagen type II expression in PDLSC and BMSC chondrogenic induced cultures. BMSC and PDLSC were cultured as aggregates in a differentiation media for 14 days (A & B) and 21 days (C & D). Top rows show images correspond to samples exposed to primary collagen type II antibody; whereas bottom rows show negative control images lacking collagen type II antibody (10X). Both BMSC and PDLSC induced cultures were strongly positive for type II collagen expression at the time points tested.
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Figure 8
Figure 9
FUTURE PROSPECTS FOR PERIODONTAL REGENERATION

Numerous clinical techniques, including bone grafts, root surface conditioning, barrier membranes, and various growth factors, have been utilized over the years in an attempt to achieve PDL regeneration. Unfortunately, current therapeutic measures are unable to obtain predictable regeneration, thus underscoring the importance of restoring or providing the cells and microenvironment capable of initiating and promoting new periodontal tissue formation.

From a biological perspective, in order for periodontal regeneration to occur, the availability of appropriate cell types, together with a favorable local environment promoting cell migration, adhesion, proliferation and differentiation, all need to be precisely coordinated both temporally and spatially. Thus, a tissue engineering strategy for periodontal regeneration that exploits the regenerative capacity of stem cells residing within the PDL, grown in a three-dimensional construct and subsequently implanted into the defect may help to overcome many limitations with current regeneration modalities. In doing so, the need for recruitment of various different cells to the site is negated and the predictability of the outcome may be enhanced.

Mesenchymal stem cells are already being utilized in regenerative medicine, most notably in orthopedic medicine where they have been shown to facilitate repair of bone and cartilage. Furthermore, MSC have also been shown to produce de novo myocardium.
as well as assist in the functional recovery following spinal cord injury. The potential of stem cell-based tissue engineering has also been harnessed in the reconstruction of murine teeth using cultured stem cells which when transferred into renal capsules, resulted in the development of tooth structures and associated bone. These results provide significant advances towards the creation of artificial embryonic tooth primordia from cultured stem cells that can be used to replace missing teeth following transplantation into the adult in order to fulfill the criteria of true biomimetic tissue regeneration.

The plausibility of a stem-cell based tissue engineering approach to achieve periodontal regeneration and formation of a true periodontal ligament around dental implants is supported by animal studies demonstrating that periodontal ligament cells cultured in vitro can be successfully reimplanted into periodontal defects and or around titanium dental implants in order to promote true regeneration.

In light of these findings, the identification of stem cells within the periodontal ligament with the ability to achieve new attachment formation in vivo is a significant development towards a tissue engineering approach to obtain periodontal regeneration. Furthermore, the ready availability of periodontal ligament tissue from redundant teeth, such as third molars, may provide a supply of stem cells that may be utilized for regenerating, not only the masticatory apparatus, but also other body tissues.

SUMMARY AND CONCLUSIONS

Our findings clearly demonstrate the successful isolation and characterization of undifferentiated progenitor cells in the periodontal ligament that can be expanded in vitro, providing a unique reservoir of stem cells obtained with minimally invasive procedures.
Therefore, the periodontal ligament apparatus represents a viable alternative to obtain potentially high numbers of cells for regenerative procedures without the necessity of bone marrow aspiration or more invasive procedures that result in increased morbidity.

Our experiments show PDLSC multipotential capability to differentiate \textit{in vitro} towards osteogenic, adipogenic, and chondrogenic like tissues. These results positively correlate with the well characterized BMSC when exposed to appropriate culture conditions. We have found that PDLSC are similar to other mesenchymal stem cells such as BMSC\textsuperscript{32-34} and dental pulp derived stem cells\textsuperscript{35-38} with respect to their expression of the STRO-1 marker.

The specific cues to initiate the proliferation and differentiation of PDLSC in vivo are not known. The ability to isolate, expand in culture, and direct the differentiation of PDLSC \textit{in vitro} to particular lineages provides the opportunity to study events associated with commitment and differentiation. From the different assay conditions required for terminal differentiation, we conclude that basal nutrients, cell density, spatial organization, growth factors, and cytokines present in the culture media have profound influence on PDLSC differentiation. PDLSC cultured in each of the differentiation conditions may also produce autocrine and paracrine factors that are essential for lineage progression. The PDLSC described here have the ability to proliferate extensively and maintain their capability to differentiate into multiple cell types in vitro, establishing their irrefutable stem cell characteristics and nature. Their cultivation and selective differentiation should provide further understanding of this important progenitor of multiple tissue types.

Future challenges include the molecular engineering of PDLSC to provide potential gene therapy in patients affected with genetic diseases. It would be beneficial to engineer a patient’s own PDLSC to replace a defective gene and restore basic cell genotype and normal
function. To permanently modify PDLSC with retroviral vectors proven to be efficient and neutral with respect to cell function represents a viable research option. In this respect, these tools will provide not only long-term production of a desired protein, but also the opportunity to design constructs able to modulate, or silence the expression of genes associated with genetic diseases of the craniofacial skeleton.

Another possible application of multipotential PDLSC might be the percutaneous delivery of these cells to a localized lesion such as cysts or cleft defects in bony tissues. The use of a semisolid carrier, via injection directly to the defect through the epithelial barrier, represents an alternative approach that will be less invasive than a surgical procedure. A number of injectable carriers are currently available, such as calcium phosphate and calcium sulfate, polylactide and polyglicolide acids, which could be used to deliver PDLCS with combinations of growth factors or cells alone. With further development, direct delivery of cells without open surgery could provide a major clinical advantage.

Current dental implant treatment relies on the ability of bone to interface with the titanium surface in a process known as oseointegration. This interface may be improved by the development of cementum on the implant surface, along with the generation of a true PDL between the newly formed cementum and alveolar bone. This type of ideal connection will provide the implant with loading dispersion capabilities better able to resist occlusal forces.

The ultimate goal of our stem cell research will be to recreate a tooth bud that can be surgically implanted in an edentulous area in order to develop a tooth like structure on its own. A recent study\textsuperscript{99} showed that cells isolated from un-erupted tooth buds have the ability to reorganize into dental like structures when transplanted with a carrier \textit{in vivo}. Further-
more, it can be envisioned that tooth buds can be formed by introducing mesenchyme from
dental or non-dental sources or using different types of stem cells in conjunction with oral
epithelium. One study using a similar approach generated tooth bud like structures in rodents
96.

In conclusion, current science clearly indicates that the use of stem cells for regenera-
tion, reconstruction, or bone repair is feasible. Translation of these advances into clinical
practice will occur. This endeavor is multidisciplinary in nature, with the periodontist and
the researcher playing a substantial role. However, there are still limitations in knowledge
that need to be solved before this “biomimetic dentition” becomes a clinical reality.
GENERAL REFERENCES

cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J. Neurosci. 16,1091-1100.


APPENDIX: IRB APPROVAL FORM

INSTITUTIONAL REVIEW BOARD APPROVAL
MEMORANDUM

TO: Isabel Gay

FROM: Ferdinand Urthaler, M.D.
Chairman, IRB

Sheila Moore, CIP
Director, IRB

RE: “Isolation and Characterization of Multipotent Human Periodontal Ligament Stem Cells” – Graduate Thesis

Date: August 9, 2007

This memorandum should serve as notification that the UAB IRB is in receipt of and has reviewed your Exemption application regarding the above-mentioned research project. The OIRB understands that protocol activities were conducted on this project without approval from the UAB IRB.

As a UAB student all research involving stem cells must be submitted to the UAB IRB Office for approval before the research begins. The UAB IRB cannot retrospectively approve projects; however, in this case, it appears that the UAB IRB approval would have been granted for this project had it been submitted for review.

A copy of this memorandum will be sent to the graduate school and should serve as the final paperwork needed for your dissertation.

Cc: Julie Bryant, Graduate School