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This thesis is dedicated to:

My family, for your endless love and support and for all the sacrifices you have made to help me get to where I am today.
First and foremost I would like to express my sincere gratitude to my research mentor and role model Dr. Giuseppe Intini who has been a guiding light for me during the past four years at both professional and personal levels. You have truly inspired me to pursue a future career in academia and serve as a clinician-scientist. Without your guidance and continuous support, this thesis would not have been possible.

I would like to acknowledge Intini Lab members: Sasan Garakani, Allison Yeh, Zahra Aldawood, and Katarzyna Wilk for their support and friendship. I am very fortunate to be part of this supportive team and working with you talented friends.

I would like to thank the members of my oral qualifying committee (Dr. German Gallucci, Dr. Shigemi Nagai and Dr. Yefu Li), thesis proposal committee (Dr. Shigemi Nagai, Dr. Yefu Li, and Dr. Tatiana Besschetnova), and thesis defense committee (Dr. Dr. T. Howard Howell, Dr. Nadeem Karimbux, and Dr. Yefu Li) for your time, insightful comments, and valuable advices on my pathway towards my degree.

I would like to express my gratitude to my program director, Dr. David Kim, for your guidance and support during my time at HSDM. I am grateful to the full-time faculty members of the division of periodontology: Dr. Roland Baron, Dr. Howard Howell, and Dr. SooWoo Kim for your supports and encouragements. I would like to express my appreciation to our dedicated
clinical instructors specially Dr. Emilio Arguello, Dr. Jacob Pourati, and Dr. Sarah Shih for all that I learnt from you to become a better surgeon and person.

I would like to particularly thank my family for your endless love and unconditional support. I am very grateful to my mother, Simin Teymouri, for all sacrificed that you have made and for your unquestionable support and continuous encouragement you have given me throughout my years of study. I am also thankful to my brother, Ali Bassir, and my sister, Taravat Bassir, for always believing in me, for cheering with me every great moment, and for supporting me whenever I needed it. I would have not been able to stand where I am today without your love and support.
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ABSTRACT

Objectives: The first specific aim of this thesis was to define the presence, assess the distribution, and characterize PRX1 expressing cells within the periodontium. The second specific aim was to assess the contribution of PRX1 expressing cells to the periodontal formation, post-natal periodontal hemostasis, and periodontal regeneration.

Methods: To assess the significance of the expression of PRX1 in human periodontium, we tested the expression of the Prx1 gene in samples of human bone, periodontal ligament (PDL), and periodontal stem cells (PDLSCs). The presence of PRX1 expressing cells (PRX1+ cells) in the mouse periodontium was explored by means of in vivo imaging. The distribution of PRX1+ cells in mouse periodontium was investigated in inducible lineage tracing analysis performed postnatally (Prx1-CreER-EGFP+/−;Rosa26-tdTOMATO+/−). PRX1+ cells also were isolated from periodontal ligament of mouse mandibular incisors and their gene expression pattern was explored. The contribution of PRX1+ cells to the formation of the mouse periodontium was investigated by constitutive lineage tracing analysis performed during embryogenesis (Prx1-cre+/−;Rosa26-tdTOMATO+/−). By means of diphtheria toxin (DTA)-mediated conditional ablation of PRX1+ cells, we also explored their contribution to periodontal homeostasis by assessing the width of PDL in Prx1-CreER-EGFP+/−;Rosa26-DTA+/− mice (ablation mouse model) (n=5) and their non-ablating littermates. The contribution of PRX1+ cells to periodontal regeneration was assessed by creating sub-critical size periodontal fenestration defects by the mandibular incisor and first molar teeth. The healing of defects was compared in ablation mouse model (test group) and non-ablation mouse model (control group) (n=5).

Results: Gene expression analysis of human samples confirmed that PRX1 is significantly expressed in human PDL and PDLSCs. The gene expression signature of PRX1+ cells
demonstrated the expression mesenchymal stem cells markers, up-regulation of Notch signaling, and down-regulation of Wnt signaling. Lineage tracing analyses showed that PRX1+ cell are present in high abundance within the PDL of mouse incisor teeth, and PRX1-lineage cells contribute to the formation of the mouse incisor’s periodontium. Post-natal ablation of PRX1+ cells resulted in a significant enlargement of the periodontal ligament space and lack of periodontal regeneration in sub-critical size periodontal defects in the continuously regenerating periodontal ligament of mouse incisor.

**Conclusions:** PRX1 expressing cells are present in high abundance within the continuously regenerating periodontal ligament of mouse incisor teeth and at such location they contribute to periodontal formation, periodontal homeostasis, and periodontal regeneration. This study, for the first time, reports the presence of PRX1 expressing cells within human periodontal ligament. Thus, studying mouse periodontal PRX1+ cells may provide significant information for the development of novel and more effective therapeutic approaches to periodontal regeneration in humans.

**Keywords:** Endogenous stem cells, Periodontal formation, Periodontal homeostasis, Periodontal regeneration, PRX1, and Lineage tracing
CHAPTER 1
INTRODUCTION AND RESEARCH BACKGROUND

RESEARCH BACKGROUND

Periodontal disease: alternative treatment approaches are needed

Periodontal diseases are characterized by bacterial-induced chronic inflammation that causes destruction of tooth supporting structures, including periodontal ligament (PDL), cementum, and alveolar bone (Pihlstrom et al., 2005, Page, 1991, Socransky and Haffajee, 1992). Periodontal diseases are highly prevalent, and in fact severe periodontitis is the sixth-most prevalent health condition worldwide (Kassebaum et al., 2014). Current scientific evidence highlights the association and possible cause-effect correlation between periodontitis and other high prevalence diseases, such as diabetes, cardiovascular diseases, chronic kidney diseases, and pulmonary infections (Fisher et al., 2008, Friedewald et al., 2009, Scannapieco et al., 2003, Borgnakke et al., 2013). Thus, periodontal disease is an important public health issue and the development of effective therapies to treat periodontal disease should be a major goal of the scientific community.

The goal of periodontal treatment is to stop the progression of the disease and regenerate the structure and function of the damaged tissues. Conventional non-surgical or surgical treatments, such as scaling and root planing, open flap debridement, and osseous surgery can control the progression of periodontal disease (Salvi et al., 2014, Lindhe and Nyman, 1984, Lindhe and
Nyman, 1975). However, achieving complete and functional periodontal regeneration is still challenging (Giannobile, 2014).

Periodontal regeneration has been defined as the regeneration of alveolar bone, PDL, and cementum over a previously diseased root surface (Lindhe et al., 1998). Several treatment modalities have been developed to achieve periodontal regeneration, including guided tissue regeneration, use of bone grafts, application of growth factors and host modulating factors, and the combination of the above methodologies (Reynolds et al., 2015, Murphy and Gunsolley, 2003, Darby and Morris, 2013). Although there is some evidence showing that periodontal regeneration can be achieved by employing these techniques, all regenerative treatment modalities have shown limited success, especially in challenging clinical situations (Figure 1) (Reynolds et al., 2015, Murphy and Gunsolley, 2003). Thus, alternative treatment approaches to achieve predictable periodontal regeneration are still highly desirable (Bassir et al., 2016).

![Figure 1- Examples of challenging clinical situations for periodontal regeneration.](image)

(A) Maxillary canine with one-wall defect; (B) second mandibular molar with large circumferential periodontal defect and furcation involvement; (Images from the author clinical practice).
Recent efforts for development of novel regenerative treatments have focused on cell-based regenerative therapy using stem cells. Adult skeletal stem cells may represent an effective therapeutic tool for periodontal regeneration due to their plasticity and ability to differentiate into different mesodermal cell lineages; thus, providing a cellular source for the regeneration of the different missing periodontal tissues (PDL, cement, and bone) (Intini, 2010). A brief introduction to the biology of stem cells is required in order to fully understand the potentials and the efficacy of stem cell-based therapeutic approaches for periodontal regeneration.

**Stem cells biology: characteristics of stem cells**

Stem cells have two important characteristics: self-renewal and differentiation potential. Self-renewal refers to their ability to renew themselves through mitosis, even after long periods of inactivity. The differentiation potential entails stem cells to differentiate into a different phenotype (Bianco et al., 2010). These two abilities, together, allow stem cells to both divide and repair/regenerate specific tissues.

Based on their differentiation potential, stem cells can be categorized in totipotent cells (able to differentiate into cells of all three germ lines as well as cells of the extraembryonic tissue), pluripotent cells (able to differentiate into cells of all three germ lines but not in cells of the extraembryonic tissue), multipotent (able to differentiate into cells of only one or two germ lines), and unipotent (able to differentiate into only one cell type) (Hynes et al., 2012).

Based on their derivation or methods of generation, stem cells are denominated as:

1) embryonic stem cells, 2) post-natal stem cells, and 3) reprogrammed stem cells.

Here, these three categories are briefly described:
I. Embryonic stem cells:
They are pluripotent cells derived from the inner cell mass of the blastocyst (Thomson et al., 1998). These cells have the ability to form derivatives of all three embryonic germ layers (Evans and Kaufman, 1981, Thomson et al., 1998). However, the therapeutic use of embryonic stem cells has raised major ethical concerns as well as some safety concerns such as the possibility of immunogenicity and tumorigenicity (Jung, 2009, Lu et al., 2009). The same ethical and safety concerns are not present with the use of post-natal stem cells.

II. Post-natal stem cells:
Post-natal stem cells have been isolated from a variety of tissue sources, including bone marrow, epithelium, adipose tissue, liver, nervous system, teeth, and periodontal ligament (Baum et al., 1992, Slack, 2000, Uchida et al., 2000, Castro-Malaspina et al., 1980, Campagnoli et al., 2001, Maldonado-Soto et al., 2014, Barker, 2014, Codega et al., 2014, Smith et al., 2014, Seo et al., 2004, Gronthos et al., 2000, Jo et al., 2007). It is believed that tissue-resident post-natal stem cells play a role in maintaining tissue homeostasis, physiological tissue renewal, and regeneration after tissue damage (Mali et al., 2010). In contrast to embryonic stem cells, post-natal stem cells are multipotent, and therefore they are more limited in their differential potential. However, post-natal stem cells may represent a safer approach to stem cell–based tissue regeneration (Chapman, 2009, Jing et al., 2008, Taghizadeh et al., 2011). Consequently, numerous pre-clinical and clinical studies have investigated the application of these stem cells for cell-based regenerative therapy in a variety of conditions.

III. Reprogrammed stem cells:
Reprogrammed stem cells are cells whose genetic program is modified to induce a switch from one cell phenotype to another (Gurdon and Melton, 2008). Cell reprogramming can be achieved
using the following four different methodologies: a) nuclear transfer from somatic cells to oocytes, b) overexpression of certain genes or modulation of certain signaling pathways, c) lineage switching, and d) direct conversion (Gurdon and Melton, 2008, Intini, 2010). Takahashi and Yamanaka were the first to induce plasticity in mouse fibroblasts by viral transduction of four genes named Oct 3/4, Sox2, c-Myc, and KLF4 (Takahashi and Yamanaka, 2006). The transduced cells, named induced pluripotent stem (iPS) cells, exhibited plasticity similar to that observable in embryonic stem cells; thus, by all means, iPS cells may be considered as pluripotent stem cells. Since their inception, extensive efforts have been made to improve the iPS technology and to develop iPS cell-based therapeutic approaches for regeneration of a wide variety of tissues (Duan et al., 2011; Revilla et al., 2015). However, the application of iPS cells in the clinical setting still appears far from being feasible due to major safety concerns related to these cells such as phenotypic instability as well as the possibility of immunogenicity and tumorigenicity (Bassir et al., 2016, Zhao et al., 2011, Lin et al., 2015).

Skeletal stem cells: promising candidates for cell-based regenerative medicine

Skeletal Stem Cells (SSCs) are post-natal stem populations that are able to form mesenchymal and connective tissues (Pittenger et al., 1999, Bianco and Robey, 2015). Among the large variety of post-natal stem cells that can be found within the human body, SSCs are of interest to periodontists because of their potential to regenerate periodontal tissues (Hynes et al., 2013, Seo et al., 2004). They can differentiate into at least three mesenchymal cell lineages, including osteoblasts, chondrocytes, and adipocytes (Pittenger et al., 1999, Huang et al., 2009). SSCs were initially isolated from bone marrow more than 50 years ago (Becker et al., 1963). Since then, SSC-like cells have been isolated from several tissues using various methods of isolation and
expansion; therefore, in order to standardize the isolation and preparation methods, the International Society for Cellular Therapy proposed the following criteria to identify human SSCs (defined by the Society as adult mesenchymal stem cells): 1) Adherence to plastic when maintained in standard culture conditions; 2) Expression of markers including CD105, CD73 and CD90, and lack expression of hematopoietic cell markers such as CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR; 3) Capacity to differentiate into osteoblasts, adipocytes and chondroblasts in vitro (Dominici et al., 2006). Skeletal stem cells have shown great promise for stem cell-based regenerative medicine due to their accessibility, extensive proliferation capacity, and their multipotential differentiation ability (Prockop, 1997, Di Trapani et al., 2013, Hynes et al., 2012).

Stem cell-based therapy for the regeneration of periodontal tissues has been performed in the animal models with stem cells isolated from several non-dental or –dental sources (Bassir et al., 2016). The most commonly studied non-dental-derived adult skeletal stem cells for periodontal regeneration are bone marrow-derived stem cells and adipose tissue-derived stem cells (Hasegawa et al., 2006, Wei et al., 2010, Lin et al., 2009, Akita et al., 2014, Tobita et al., 2013, Huang et al., 2009). The common sources for dental-derived post-natal skeletal stem cells are periodontal ligament, dental pulp, exfoliated deciduous teeth, dental follicle, dental apical papilla, and extraction sockets (Seo et al., 2004, Hynes et al., 2013, Lin et al., 2009). Dental-derived post-natal skeletal stem cells have recently gained a great deal of attention since they can be isolated from tissues that are often discarded in dental clinics and since their isolation presents with lower morbidity compared to non-dental-derived post-natal stem cells. Here, we focus on potential of post-natal skeletal stem cells isolated from periodontal ligament, as these cells are the most relevant to the present project.
Periodontal ligament stem cells: their isolation and transplantation for periodontal regeneration

It has been proposed that periodontal regeneration is mediated by a heterogeneous cell population present in the PDL that can differentiate into fibroblasts, osteoblasts, and cementoblasts (Melcher, 1976). However, the presence of multipotent stem cells in PDL remained elusive until they were isolated from PDL of extracted human third molars by Seo and colleagues in 2004 (Seo et al., 2004). Seo and colleagues found that human PDL contains a group of cells that express mesenchymal stem cell surface markers such as STRO-1 and CD146. These cells, which were defined as periodontal ligament stem cells (PDLSCs), present with self-renewal ability and have multipotent capacity, being able to differentiate into cementoblasts/osteoblasts, adipocytes, and collagen-forming cells. Furthermore, these cells formed cementum-like and PDL-like structures after ectopic transplantation into the dorsal surface of immunocompromised mice (Seo et al., 2004). This research represents the milestone of all studies investigating the potential of periodontal-derived stem cells for cell-based regenerative periodontal therapy. Subsequent studies focused on the characterization of these PDLSCs; for instance several studies compared the properties of these cells with the properties of other mesenchymal stem cells. These studies showed that PDLSCs present with self-renewal and multilineage differentiation capabilities, expression of mesenchymal stem cell surface markers such as CD44, CD73, CD 90, CD105, CD106 (VCAM-1), CD146 (MUC-18), and Stro-1, and lack of expression of hematopoietic markers such as CD31, CD34, and. CD45 (Fujii et al., 2008, Wada et al., 2009, Huang et al., 2009). In addition, PDLSCs possess unique characteristics that make them distinct from other mesenchymal or skeletal stem cells. In fact, it has been shown that these cells have higher proliferation rate than skeletal stem cells derived
from bone marrow (Seo et al., 2004). Furthermore, PDLSCs express scleraxis, a tendon/ligament-specific transcription factor, at higher levels compared to bone marrow or dental pulp derived stem cells (Seo et al., 2004). It has also been demonstrated that PDLSCs are able to generate PDL attachment in vivo by forming Sharpey's fiber-like collagen bundles that are connected to cementum-like structure (Seo et al., 2004). These unique features of PDLSCs make them a promising cell source for cell-based regenerative periodontal therapy. Hence, several studies have isolated these cells evaluated the potential of transplantation of these cells into periodontal defects to promote periodontal regeneration (Table 1).

A study by Liu et al used autologous PDLSCs extracted from teeth of miniature pigs for periodontal regeneration in a swine periodontitis model (Liu et al., 2008). The periodontal defect was created by surgically removing alveolar bone around teeth and by subsequently inserting a ligature around them. In this study, periodontal defects were treated with a combination of alloplasts (hydroxyapatite and tricalcium phosphate) and cultured autologous PDLSCs. The results showed newly formed bone, cementum, and periodontal ligament in the treated defect, supporting the feasibility of periodontal regeneration therapies based on the use of ex vivo expanded PDLSCs (Liu et al., 2008). The application of autologous PDLSCs for the treatment of periodontal defects is also reported in one clinical case series (Feng et al., 2010). Feng and colleagues treated intrabony periodontal defects in a limited number of patients with autologous PDLSCs from extracted third molars using hydroxyapatite as a carrier. They reported that periodontal parameters were significantly improved in all shown cases, without any adverse event during 32–72 months of follow-up (Feng et al., 2010). Obviously, further well-designed clinical trials with larger patient population and appropriate controls are needed before drawing a conclusion regarding the clinical efficacy and safety of autologous PDLSCs.
Table 1- Animal studies of periodontal regeneration by transplantation of periodontal ligament-derived stem cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Defect type</th>
<th>Animal model</th>
<th>Cell delivery method</th>
<th>Significance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous PDLSCs</td>
<td>Surgical created periodontal defects in 1st molars and insertion of ligature</td>
<td>Miniature pigs</td>
<td>HA/TCP carrier</td>
<td>Formation of new bone, cementum, and PDL was reported. Significant improvements in clinical parameters, and height of regenerated alveolar bone were observed in the cells + HA/TCP group compared to HA/TCP alone and no treatment groups.</td>
<td>(Liu et al., 2008)</td>
</tr>
<tr>
<td>Autologous PDLSCs; DFSCs; and DPSCs</td>
<td>Surgically created apical involvement defects</td>
<td>Beagle dogs</td>
<td>No carrier</td>
<td>PDLSCs had the best regenerative potential. Defects in PDLSCs groups were regenerated by means of new cementum, bone, and PDL fibers. Periodontal regeneration was not achieved in DPSCs group.</td>
<td>(Park et al., 2011)</td>
</tr>
<tr>
<td>Autologous vitamin C-induced PDLSC sheets; UpCell dish PDLSC sheets; Autologous</td>
<td>Same as Liu et al</td>
<td>Miniature pigs</td>
<td>No carrier for cell sheets; Gelfoam for autologous dissociat</td>
<td>Regeneration of defects up to normal levels was observed in Vitamin C-induced PDLSCs sheet group. Formation of Sharpey’s fibers was seen in all groups, but fibers were irregular in dissociated PDLSCs group. Significantly higher percentage</td>
<td>(Wei et al., 2012)</td>
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<tr>
<td>dissociated PDLSCs</td>
<td>ed PDLSCs</td>
<td>of newly formed alveolar bone was evident in Vitamin C-induced PDLSCs sheet group compared to the other groups</td>
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<tr>
<td>Autologous PDLSC sheets; Allogeneic PDLSC sheets; Autologous heterogenic PDL cells (PDLC)</td>
<td>Same as Liu et al</td>
<td>Miniature pigs</td>
<td>HA/TCP carrier</td>
<td>Significantly improved clinical and histological outcomes were observed for allogeneic and autologous PDLSCs sheets compared to PDLCs and control groups. Similar regenerative outcomes were obtained using allogeneic and autologous PDLSCs sheets. No evidence of immunological rejections of allogeneic PDLSCs was found. (Ding et al., 2010)</td>
<td></td>
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<tr>
<td>Allogeneic PDLSCs</td>
<td>Surgically created zero-wall dehiscence defects</td>
<td>Merino sheep</td>
<td>Gelfoam sponge carrier</td>
<td>No significant differences were found in mean area of new bone formation, length of new cementum formation, and Sharpey’s fiber thickness between PLDSCs + Gelfoam group compared to Gelfoam alone group. All regenerative parameters were significantly improved in both group compared to untreated defects. (Mrozik et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>Monolayered PDLSC cell sheets (MCSs); Monolayer PDLSC</td>
<td>Surgically created periodontal defects in the mesial region of the maxillary 1st molars.</td>
<td>Sprague–Dawley rats</td>
<td>None</td>
<td>Perpendicular insertion of fibers into defects was evident in MUCPs and MCPs groups. Higher formation of mineralized tissue was found in MUCPs group compared to MCPs (Guo et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>Pellets (MCPs); Multilayered PDLSC pellets (MUCPs)</td>
<td>Xenogeneic (human) PDLSCs</td>
<td>Surgically created periodontal defects on buccal aspect of the mandibular molars</td>
<td>Immunod efficient rats</td>
<td>HA/TCP carrier</td>
<td>Human PDLSCs integrated into the periodontal tissue in two of six samples</td>
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<tr>
<td>Xenogeneic (human) PDLSCs</td>
<td>Surgically created class II furcation defects</td>
<td>Immunod efficient rats</td>
<td>Amniotic membrane</td>
<td>Enhanced regeneration of periodontium and a higher percentage of bone fill in were observed in defects treated with PDLSCs-amnion compared to the amnion-alone.</td>
<td>(Iwasaki et al., 2014)</td>
</tr>
</tbody>
</table>

DFSCs- Dental Follicle- derived Stem Cells; DPSCs- Dental Pulp- derived Stem Cells; HA/TCP: Hydroxyapatite/Tricalcium Phosphate; PDL- Periodontal Ligament; PDLSCs- Periodontal Ligament-derived Stem Cells.
Delivery of autologous periodontal ligament stem cells for periodontal regeneration: there are some limitations

Although autologous PDLSCs have shown promising results in preclinical studies, sources of autologous PDLSCs are limited for clinical applications as isolation of PDLSCs involves extraction of teeth. Furthermore, availability and function of PDLSCs are influenced by the age and disease status of donors (Mrozik et al., 2013). Indeed, it appears that the proliferative capacity, migratory potential, and multi-lineage differentiation ability of PDLSCs diminishes in older compared with younger individuals (Zhang et al., 2012). Hence, additional studies started to investigate the potential of cryopreserved or allogeneic PDLSCs as a feasible alternative cell source for PDLSCs-based regenerative periodontal therapy.

Two studies have shown that human PDLSCs can be recovered from cryopreserved PDLSCs and that cryopreservation does not affect the growth capacity of these cells (Seo et al., 2005, Vasconcelos et al., 2012). The cryopreserved PDLSCs maintained their stem cell characteristics such as expression of STRO-1, multipotent differentiation capacity, and ability to form cementum/periodontal-ligament-like tissues (Seo et al., 2005, Vasconcelos et al., 2012). These data suggest that utilization of cryopreserved human PDLSCs for cell-based therapy may be a valid clinical approach.

In lack of autologous PDLSCs, allogeneic PDLSCs may represent a valid alternative. A study by Ding and colleagues compared the autologous and allogeneic PDLSCs for the treatment of induced periodontitis in a swine model of periodontal disease. They demonstrated successful periodontal regeneration of the defects with both autologous and allogeneic PDLSCs. Importantly, they reported that there were no significant differences in percentage of T cell-related immunological markers such as CD3, CD4, and CD8 between the autologous or the
allogeneic PDLSCs, suggesting that transplanted allogeneic PDLSCs cause no immunological rejection (Ding et al., 2010). Similar observation was reported by Mrozik and colleagues using allogeneic PDLSCs to reconstruct surgically created periodontal dehiscence in an ovine model of periodontal defects. Although no immunological evaluations were performed in this study, they reported that allogeneic PDLSC implants were in general well tolerated, as no inflammation, infection, or root exposure was observed in any of the animals (Mrozik et al., 2013).

The putative immunoprivilege status of stem cells has formed the basis for the development of allogeneic stem cell-based therapies in various medical fields. There are several clinical trials that have reported that SSCs may be administered to humans without inducing clinically relevant immune reactions (Hare et al., 2012, Hare et al., 2009, Ascheim et al., 2014). However, it should be noted that there are some studies that have raised concerns over the clinical application of allogeneic stem cells. An animal study by Huang and colleagues reported that there is the possibility that allogeneic bone marrow-derived stem cells lose their immnoprivileged status during differentiation (Huang et al., 2010). They demonstrated that levels of expression of the major histocompatibility complex-Ia and –II are very low in undifferentiated cells, while their level of expression increases significantly after differentiation into endothelial or smooth muscle cells. (Huang et al., 2010). Thus, it becomes crucial to determine, by means of additional studies, if the phenomenon translates into the use of dental-derived stem cells, such as allogeneic PDLSCs.

Apart from a case report (Feng et al., 2010), there is no available information on the safety and efficacy of PDLSCs for periodontal regeneration in the clinical setting. If long-term clinical trials confirm the safety and efficacy of these multipotent stem cells, standard clinical protocols may be developed for the effective use of these cells in periodontal regenerative therapy.
If effective clinical therapeutic protocols are established, a challenge remains in terms of tissue
culturing and ex-vivo expansion of the autologous multipotent stem cells. Culturing SSCs or PDLSC is challenging, time-consuming, time-sensitive and, as per today, very costly. Additionally, the FDA has implemented strict regulations for stem cell tissue culturing (Halme and Kessler, 2006). This is because xenogenic products, such as fetal bovine serum, are often used in stem cell-culture medium. Furthermore, the culture conditions that would maintain the cells in an optimal environment without cross-contaminations or infections have neither been fully studied nor fully developed (Karring et al., 1993, Lin et al., 2009). Another technical challenge arises from cell manipulation as instability and gene mutations have been observed after prolonged culturing of stem cells, thus suggesting that freshly prepared stem cell cultures should be used (Jo et al., 2007). Although cryopreservation has been routinely utilized for hematopoietic stem cells and other stem cells (Hunt, 2011), questions remain on whether or not it transmission of infections may occur during direct immersion of cells into liquid nitrogen (Mazzilli et al., 2006, Hawkins et al., 1996).

**Harnessing regenerative potential of endogenous stem cells: a promising alternative to stem-cell delivery**

The current stem-based regenerative treatment modalities are based on ex-vivo expansion of autologous, allogeneic, or xenogeneic stem cells, and their transplantation into the body. In addition to the practical barriers and limitations in the clinical applicability of the stem cell transplantation approaches, there are major concerns associated with the used of ex vivo-manipulated cells specially for non-life threatening conditions such as periodontal disease as the transplantation of ex-vivo manipulated stem cells may have as yet undetermined consequences
Due to these limitations, rather than ex-vivo expansion and transplantation of stem cells, an emerging philosophy in field of stem cell-based regenerative medicine is to develop regenerative treatment modalities that harness regenerative potential of endogenous stem cells (Dawson et al., 2014, Dimmeler et al., 2014, Zhou and van den Beucken, 2016, Shafiq and Kim, 2016).

Our body has the capacity of regeneration and repair through endogenous stem cells residing in the different tissues even without any external therapeutic intervention (Chen et al., 2011). It is well know that stem cell niches are present in many adult tissues (Morrison and Spradling, 2008, Moore and Lemischka, 2006). The stem cell niches function to maintain a constant number of stem cells by balancing the proportions of quiescent and activated cells (Wang et al., 2016, Morrison and Spradling, 2008, Scadden, 2006). Stem cells may remain in the quiescence state in their niche until they are activated as a response to a need for more cells to maintain or repair the tissue. Activated stem cells may exit the niche and proliferate, self-renew, and differentiate in order to regenerate lost structures (Chen et al., 2011). Discovering the endogenous stem cells that contribute to repair and regeneration in each tissue and understanding the endogenous regenerative mechanisms that control activation, proliferation, and differentiation of these stem cells may lead to development of novel therapeutic approaches to harness the regenerative potential of these stem cells.

Harnessing the regenerative potential of the endogenous stem cells has gained increasing attention as a promising simpler, safer, and more applicable alternative to stem cell transplantation especially for the regeneration of the tissue with low regenerative capacity such as cardiac tissue, neural tissue, and tendons (Mohapel and Brundin, 2004, Lee et al., 2015,
Ranganath et al., 2012). However, there is still very limited information available on harnessing the regenerative potential of dental-derived skeletal stem cells in order to enhance the regeneration of periodontal or dental tissues.

**PRX1 expressing cells: a sub-population of skeletal stem cells and a potential target for harnessing endogenous regenerative mechanisms**

Paired-related homeobox protein 1 (PRX1) is a transcription factor that has been identified as the regulator of mesenchymal cell fate (Cserjesi et al., 1992, Lu et al., 1999, ten Berge et al., 1998, Lu et al., 2011, Du et al., 2013). During embryonic development, PRX1 expression is restricted to the mesoderm (Cserjesi et al., 1992, Hu et al., 1998, Lu et al., 1999, Du et al., 2013). It is reported that the PRX1 gene is involved in embryonic skeletal development (Du et al., 2013, ten Berge et al., 1998, Lu et al., 1999, Nohno et al., 1993), and mice lacking PRX1 exhibit perinatal death probably due to severe cleft palate. These mice also showed defective skeletogenesis including malformation of cranial bones, vertebral, and limb skeletal structures, which are considered to be due to impaired formation of osteogenic and chondrogenic progenitors (Martin et al., 1995). The presence of limb and cranial bone abnormalities suggest that PRX1 is involved in both endochondral and membranous bone development (Chesterman et al., 2001). PRX1 also indirectly regulates expression of sonic hedgehog gene (ten Berge et al., 2001), which controls the proliferation of adult stem cells (Warzecha et al., 2006, Yu et al., 2002, Machold et al., 2003).

In adults, PRX1 is expressed in a number of undifferentiated mesenchymal cell populations (Kawanami et al., 2009). It has been demonstrated that PRX1 expressing cells are present in the periosteum of post-natal long bones and calvaria (Kawanami et al., 2009, Ouyang et al., 2014).
These PRX1+ cells have been shown to have characteristics of skeletal stem cells, and they were able to differentiate in osteoblasts and chondroblasts (Kawanami et al., 2009, Ouyang et al., 2014). A recently published study from our laboratory revealed that PRX1 expressing cells are present within the calvarial sutures of adult mice. We also found that these PRX1 expressing cells contribute to intramembranous bone formation (Figure 2). Furthermore, it was found that these cells are required for bone regeneration in a calvarial defect mouse model (Figure 3), suggesting bone regeneration is mediated by an adult population of skeletal stem cells expressing PRX1 (Wilk et al., 2017).

Currently, there is no information on the presence, distribution, or function of PRX1 expressing cells in the periodontium. Based on the role of PRX1 expressing cells in bone formation and bone regeneration, investigating the expression and function of PRX1 in periodontium may lead to important discoveries that enable us to harness the endogenous regenerative potential of periodontal stem cells resulting in development of novel regenerative treatment modalities.
Figure 2- Lineage-tracing analysis of the PRX1-expressing cells of the neural crest-derived frontal (top) and mesoderm-derived parietal (bottom) bones during bone regeneration. Images were acquired at the time of the defect creation (T0), 5 days later (T5), 10 days later (T10), and 30 days later (T30) to show Prx1 progeny (red cells) migrating into the defect and forming new bones as osteocytes.

**Figure 3- PRX1 expressing cells are required for bone regeneration in the calvarial Bones.**

Micro-computed tomography (A and B) and histological sections (C and D) of calvarial bones dissected 28 days after subcritical calvarial defect surgery (0.5 mm in diameter) in the presence (A and C) and absence (B and D) PRX1+ cells. Dashed lines demarcate the calvarial subcritical bone defect.

SIGNIFICANCE AND INNOVATION

Significance

Advanced periodontitis is the sixth-most prevalent health condition worldwide (Kassebaum et al., 2014). Despite recent advancements in regenerative medicine, a complete regeneration of the structures damaged through the progression of the periodontal disease is still challenging and unpredictable (Reynolds et al., 2015, Murphy and Gunsolley, 2003, Giannobile, 2014). The challenge in periodontal regeneration lie in the ability to induce the regeneration of a complex apparatus composed of different tissues, such as bone, cementum, and periodontal ligament. Adult mesenchymal stem cells may represent a more effective therapeutic tool for periodontal regeneration due to their ability to differentiate into different mesodermal cell lineages (Intini, 2010). Stem cell-like population has been previously isolated from periodontal ligament (Seo et al., 2004). However, there is still no information regarding distribution, location, and function of stem cells present in periodontium. Moreover, mechanisms and signals that control or contribute to migration, proliferation, and differentiation of periodontal stem cells are still unknown. Therefore, a better understanding of the biological characteristics and regulatory mechanisms of endogenous periodontal stem cells may lead to the development of promising treatment modalities in that harness the regenerative potential of these stem cells.

Paired-related homeobox protein 1 (PRX1) is a transcription factor that, in adults, expresses in a sub-population of skeletal stem cells (Cserjesi et al., 1992, Lu et al., 1999, Lu et al., 2011, ten Berge et al., 1998, Du et al., 2013). Previous experiments in our laboratory have revealed that PRX1 expressing cells present in calvarial sutures of adult mice and are required for bone regeneration in calvarial defects (Wilk et al., 2017). Based on the role of PRX1 expressing cells in bone regeneration, investigating the presence and function of PRX1 expressing cells in
periodontium may provide us valuable information for better understanding of the endogenous regenerative mechanisms and harnessing these endogenous mechanisms to development novel regenerative approaches.

**Innovation**

The presence, characteristics, or function of PRX1 expressing cells in the periodontium has not been investigated. The present study will be the first to assess the presence, distribution, characteristics, and function of PRX1 expressing cells in the periodontium and their role in periodontal regeneration.

In this project, we used three innovative transgenic mouse models. The first one is Prx1-cre<sup>+/−</sup>;Rosa26-tdTOMATO<sup>+/−</sup> mouse model in which constitutive expression of cre recombinase identifies PRX1-expressing cells and their progeny during embryogenesis. The second mouse model is Prx1<sub>creER-EGFP/−</sub>;Rosa26-tdTOMATO<sup>+/−</sup> mouse model where the inducible expression of cre recombinase identifies PRX1 progeny cells, which allows us to perform postnatal lineage tracing study. The other transgenic mouse model is Prx1<sub>creER-EGFP/−</sub>-Rosa26-DTA<sup>+/−</sup> mouse model that allows us to globally ablate PRX1 expressing cells. These mouse models are discussed in detail in the material and method sections.

Another innovative component of the present study is the use of intravital microscopy. The intravital microscopy developed by Dr. Lin’s laboratory at Massachusetts General Hospital. It is based on a hybrid two-photon/confocal fluorescence microscope that allows for the *in vivo* real-time imaging and quantification of fluorescent cells (Lo Celso et al., 2009). We used intravital microscopy for the *in vivo* detection of fluorescent cells in periodontal ligament.
In addition, in this project we developed a sub-critical size periodontal defect model where periodontal defects in the control animals would regenerate spontaneously. To the best of our knowledge, the sub-critical size periodontal defect model has not been described in the literature previously.

Therefore, we suggest that innovation in this research project is evident in the topic of the research, concept and hypotheses that was tested, and the approach that was used.
HYPOTHESIS AND SPECIFIC AIMS

The global hypothesis in the present research project is that PRX1 expressing cells of the periodontium contribute to formation, homeostasis, and regeneration of the periodontium. We addressed this hypothesis in the following two specific aims:

A. Specific aim 1: To define the presence, assess the distribution, and characterize PRX1 expressing cells within periodontium

In order to address this specific aim we performed four experiments. First, we explored the presence of PRX1 expressing cells in mouse periodontium using in vivo imaging. Second, we investigated the presence of PRX1 expressing cells in human periodontium by assessing the expression of PRX1 gene in human periodontal ligament stem cells as well as in RNA samples collected from human extracted teeth. Third, the distribution of PRX1 expressing cells in mouse periodontium was studied in the inducible lineage tracing study. Lastly, the gene expression signature of these cells was investigated in PRX1 expressing cells isolated from periodontal ligament of mouse mandibular incisor teeth.

B. Specific aim 2: To assess the contribution of PRX1 expressing cells of mouse periodontium to periodontal formation, hemostasis, and regeneration

This aim was addressed in three different experiments. First, the contribution of PRX1 expressing cells to the formation of mouse periodontium was investigated in the cre/loxP- based constitutive lineage tracing study. Second, we assessed the role of PRX1+ cells to post-natal periodontal hemostasis by assessing the effects of post-natal ablation of these cells on the width of periodontal ligament space. Third, we investigated the contribution of PRX1 expressing cells to the periodontal regeneration by exploring the effect of post-natal ablation of PRX1+ cells on the healing of surgically created sub-critical size periodontal fenestration defects.
CHAPTER 2
PRESENCE, DISTRIBUTION, AND CHARACTERISTICS OF PRX1 EXPRESSING CELLS WITHIN PERIODONTIUM

Post-natal expression of PRX1 is limited to a number of undifferentiated mesenchymal cell populations (Kawanami et al., 2009). A recent publication from Intini laboratory revealed that PRX1 expressing cells are present within the calvarial sutures of adult mice (Wilk et al., 2017). In addition, this study demonstrated that these PRX1 expressing cells contribute to intramembranous bone formation and they are required for the bone regeneration in calvarial defects (Wilk et al., 2017).

Currently, there is no information on the presence, distribution, or characteristics of PRX1 expressing cells in the periodontium. The first step in exploring the possibility of harnessing the regenerative potential of these endogenous skeletal stem cells for periodontal regeneration is to investigate their presence and distribution within the periodontium. In addition, characterizing the gene expression pattern of these cells may lead to determining physiological signals that enhance their activation, proliferation, or differentiation. Therefore, the first specific aim of the present research project was to define the presence, assess the distribution, and characterize PRX1 expressing cells within the periodontium. Here, we revealed the presence of PRX1 expressing cells in periodontium in both human and mice. We investigated the distribution of these cells in periodontium of mouse by means of lineage tracing technology. In addition, we isolated PRX1 expressing cells from mice periodontal ligament and reported the gene expression signature of these cells.
MATERIALS AND METHODS

The presence of the PRX1 expressing cells in mouse periodontium was explored by means of in vivo imaging experiment. The presence of the PRX1 expressing cells in human periodontium was investigated by evaluating the expression of PRX1 in human PDLSCs and in RNA samples collected from human extracted teeth. We assessed the distribution of the PRX1 expressing cells in mouse periodontium in the inducible lineage tracing study. Gene expression signature of these cells was investigated in the PRX1 expressing cells isolated from periodontal ligament of mouse mandibular incisors using quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis.

I. In vivo imaging experiment

In vivo imaging experiment was performed by means of Intravital microscopy (Figure 1). Intravital microscopy allows 3D- imaging of cells in live animals (Lo Celso et al., 2009). In this experiment, we used a transgenic mouse model of Prx1-creER-EGFP. In this mouse model, green fluorescent protein (GFP) is expressed in all cells that express the PRX1 gene. Therefore, expression of GFP, as an indicator of the PRX1 expressing cells, was evaluated using intravital microscopy.

Intravital microscopy was performed according to the method previously described (Lo Celso et al., 2009, Wilk et al., 2017). The excitation beam was focused into the sample plane using a 60x objective lens. Fluorescence emission was collected by confocal or two-photon detectors. EGFP signals were detected with a 525/38 band pass filter using either two-photon or confocal acquisition excited by a 491 nm laser (Cobolt Calypso™, Sweden). Bone was visualized using second harmonic generation (SHG) of collagen excited by the Ti: Sapphire laser pulsing between
840 to 900 nm and collected by a 435/40 nm band pass filter. Cell distributions were visualized in stack images at 1-5 μm interval acquired via two-photon acquisition. Two-dimensional sections were processed by the reslice function of Image J software (National Institutes of Health).

**Figure 1- Intravital microscopy.** (A) Confocal two-photon hybrid microscope designed for intravital microscopy; (B) live animal imaging.

**II. Human in vitro assays**

For human in vitro assays, RNAs were obtained from human samples of bone and periodontal ligament (PDL) and from cultured human periodontal ligament stem cells (kindly provided by Dr. Pamela Robey, NIH, USA). We quantified and compared the *PRX1* gene expression levels in RNA obtained from human samples of bone and PDL using qRT-PCR. We also compared the expression of *PRX1* with the expression of Scleraxis and MCAM/CD146, which are considered as PDL stem cell markers (Seo et al., 2004), in human periodontal ligament stem cells (PDLSCs) using qRT-PCR.
Briefly, total RNAs were isolated using miRNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. First-strand cDNAs were synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s protocol. Real-time PCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers used were as follow: Prx1 transcriptional variant 1 (Prx1 V1; Hs01014477_m1), Prx1 transcriptional variant 2 (Prx1 V2; Hs01025547_m1), MCAM/CD146 (Hs00174838_m1), and Scleraxis (Hs03054634_g1). Tubb5 (Hs00742828_s1) and ACTB primers (Hs01060665_g1) were served as endogenous reference genes. Data were analyzed using the comparative threshold cycle method as described in detail previously (Schmittgen and Livak, 2008).

III. Inducible lineage tracing study

To assess the distribution of post-natal PRX1 expressing cells in the mouse periodontium, we performed an inducible lineage tracing analysis. To do so, we used a Prx1creER-EGFP+/-;Rosa26-tdTOMATO+/- transgenic mouse model (Figure 2). This transgenic mouse model was generated by crossing Prx1-creER-EGFP mouse, which expresses green fluorescent protein and tamoxifen-inducible cre recombinase under the control of the Prxl promoter, with a transgenic Rosa26-tdTOMATO mouse, which is engineered to conditionally express tdTOMATO upon cre recombination of a loxP-flanked STOP sequence.
Figure 2- Generation of Prx1creER-EGFP\(^{+/−}\) -Rosa26-tdTOMATO\(^{+/−}\) mouse model

In this transgenic mouse model, the gene encoding TdTOMATO is only expressed upon cre recombination of a loxP-flanked STOP sequence using tamoxifen-inducible cre recombinase. Therefore, TdTOMATO protein, which appears red under fluorescence microscopy, is only produced after administration of Tamoxifen to the animals.

In this experiment, cre recombination was induced in 4 week-old male Prx1creER-EGFP;Rosa26-tdTOMATO mice (n=3) by 7 days injection of tamoxifen (Intraperitoneal, 40mg/kg in sterile oil). Animals were sacrificed after 12 weeks. Mandibles were collected and prepared for cryosectioning and histological analysis.

Mandibles were fixed in 4% paraformaldehyde (PFA) overnight at 4° C and then washed with 1% Dulbecco’s phosphate-buffered saline (DPBS) five times for 5 minutes each on a shaker at the room temperature. Samples were decalcified in 14% EDTA (pH 7.4) for two weeks on a shaker at the room temperature. Subsequently, samples were washed again with 1% DPBS five times for 5 minutes each on a shaker at the room temperature. Next, samples were immersed in 30% sucrose at 4 °C overnight for cryoprotection of fixed tissue. Samples were then embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA) and snap-frozen in
liquid nitrogen. 10-um sections were prepared with a cryostat (Research Cryostat Leica CM3050 S, Leica Microsystems Inc., Buffalo Grove, IL). Sections were visualized and imaged using a fluorescence microscope (Keyence BZ-X700 microscope, Keyence, Osaka).

IV. Gene expression signature study

In this experiment, we isolated the PRX1 expressing cells from the PDL of mouse mandibular incisors and characterized these cells by identifying their gene expression patterns. In this experiment, we used 4-week-old Prx1-creER-EGFP+/− transgenic mice (n = 7). In this mouse model, all cells expressing PRX1 co-express green fluorescent protein.

Mandibular incisors were extracted, and PDL tissues were separated from the root surface of teeth. The obtained PDL tissues were pooled, and digested at 37°C in a shaking water bath in two steps to prepare PDL single-cell suspensions (Seo et al., 2004). First with 3mg/ml of Collagenase II (Worthington Biochemical Corporation, Freehold, NJ, USA) in αMEM (Gibco, Grand Island, NY) and 1% penicillin/streptomycin for 90 minutes; then, with 1.2 unites/ml of Collagenase P (Roche, Mannheim, Germany), 0.67 unites/ml of Dispase (Worthington Biochemical Corporation, Freehold, NJ, USA) in αMEM, and 1% penicillin/streptomycin for 30 minutes.

Then, cells positive for GFP were sorted and isolated from PDL single-cell suspension by means of Fluorescent-Activated Cell Sorting (FACS). GFP+ and GFP- cells were sorted (4,200 cells each). Isolated cells were processed using a Single Cell to CT Kit (Ambion by Life Technologies, Carlsbad, CA) according to manufacturer’s protocols. Briefly, cells lysis was performed in the presence of DNase I and Single Cell Lysis Solution. cDNA was synthesized by reverse transcription in a thermal cycler (25°C for 10 min, 42°C for 60 min, and 85°C for 5 min).
Then, preamplification step was performed by mixing preamplification reaction mix with 0.2 × pooled TaqMan Gene Expression Assays for genes of interest in a thermal cycler (95°C for 10 min, 14 cycles of 95°C for 15 s, 60°C for 4 min, and 99°C for 10 min). The Preamplified cDNAs were used for Real-time PCR reaction (50°C for 2 min, 95°C 10 min, and 40 cycles of 95°C for 5 s and 60°C for 1 min) using Taqman Gene Expression Master Mix and Taqman Gene Expression Assays on an Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). GADPH (Mm99999915_g1) served as endogenous reference gene. The primers used are listed in the Table 1. We investigated the expression of the genes in the following categories: A) progenitor cell and stem cell markers, B) Wnt signaling pathway genes, C) BMP signaling pathway genes, D) Notch signaling pathway genes, E) osteoblast-related markers, F) genes related to adhesion molecules, G) cell-cycle regulatory markers, H) pluripotency-associated genes, and I) other relevant genes.

Comparative threshold cycle method was used to analyze the data (Schmittgen and Livak, 2008).

**V. Statistical analysis**

The comparison of the mean relative expression of *PRX1* gene between human PDL and alveolar bone (experiment II: Human in vitro assays) was performed using Student's t-test. The relative expression Prx1, Scleraxis and MCAM/CD146 in human PDLSCs (experiment II: Human in vitro assays) were compared using ANOVA test. The comparison of the mean relative levels of expression for each gene between PRX1+ cells and PRX1- cells (experiment IV: Gene expression signature study) were performed using Student's t-test. A significance level of \( a = 0.05 \) was used for all comparisons.
Table 1- List of genes investigated in this study and their relevance

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Relevance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd44</td>
<td>CD44 antigen</td>
<td>Early progenitor cells marker</td>
<td>(Itzkovitz et al., 2012)</td>
</tr>
<tr>
<td>Col2a1</td>
<td>Collagen type II alpha chain</td>
<td>Marker of stemness</td>
<td>(Bahney et al., 2014)</td>
</tr>
<tr>
<td>Gli1</td>
<td>Zinc finger protein GLI1</td>
<td>Component of Hedgehog signaling and marker of stem/progenitor cells</td>
<td>(Clement et al., 2007)</td>
</tr>
<tr>
<td>Kit</td>
<td>Proto-oncogene receptor tyrosine kinase (KIT)</td>
<td>Marker of stem/progenitor cells</td>
<td>(da Silva Meirelles et al., 2006)</td>
</tr>
<tr>
<td>Mcam</td>
<td>CD146 / MCAM</td>
<td>Mesenchymal stem cell marker</td>
<td>(Crisan et al., 2008)</td>
</tr>
<tr>
<td>Pdgfra</td>
<td>Platelet derived growth factor receptor, alpha (PDGFRA)</td>
<td>Mesenchymal stem/progenitor cell marker</td>
<td>(Houlihan et al., 2012)</td>
</tr>
<tr>
<td>Sca1</td>
<td>Stem cells antigen-1 (SCA-1)</td>
<td>Hematopoietic stem cells marker</td>
<td>(Okada et al., 1992)</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened (SMO)</td>
<td>Component of Hedgehog signaling and marker of stem/progenitor cells</td>
<td>(Trzaska et al., 2007)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
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<td>-----------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Axin2</td>
<td>Axin-2</td>
<td>Negative regulator of Wnt signaling</td>
<td>(Jho et al., 2002)</td>
</tr>
<tr>
<td>Dkk1</td>
<td>Dickkopf-related protein 1</td>
<td>Inhibitor of Wnt signaling</td>
<td>(Wang et al., 2000)</td>
</tr>
<tr>
<td>Fzd1</td>
<td>Frizzled-1</td>
<td>Receptor for Wnt signaling proteins</td>
<td>(Baron and Kneissel, 2013)</td>
</tr>
<tr>
<td>Fzd2</td>
<td>Frizzled-2</td>
<td>Receptor for Wnt signaling proteins</td>
<td>(Baron and Kneissel, 2013)</td>
</tr>
<tr>
<td>Fzd7</td>
<td>Frizzled-7</td>
<td>Receptor for Wnt signaling proteins</td>
<td>(Baron and Kneissel, 2013)</td>
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<tr>
<td>Fzd9</td>
<td>Frizzled-9</td>
<td>Receptor for Wnt signaling proteins</td>
<td>(Baron and Kneissel, 2013)</td>
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<td>Sost</td>
<td>Sclerostin</td>
<td>Antagonist to Wnt signaling</td>
<td>(Li et al., 2005)</td>
</tr>
<tr>
<td>Twist1</td>
<td>Twist-related protein 1</td>
<td>Up-regulated in response to Wnt signaling</td>
<td>(Willert and Jones, 2006)</td>
</tr>
<tr>
<td>Wif1</td>
<td>Wnt inhibitory factor 1 (WIF1)</td>
<td>Inhibitor of Wnt signaling</td>
<td>(Kawano and Kypta, 2003)</td>
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<td>Wnt3a</td>
<td>Wnt3a</td>
<td>Activator of canonical Wnt signaling</td>
<td>(Gaur et al., 2005)</td>
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<td>Wnt5a</td>
<td>Wnt5a</td>
<td>Ligand for Wnt/b-catenin signaling</td>
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<td>Wnt10b</td>
<td>Wnt10b</td>
<td>Ligand for Wnt/b-catenin signaling</td>
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<td><strong>BMP signaling pathway genes</strong></td>
<td><strong>Bmp2</strong></td>
<td>Bone morphogenetic protein 2</td>
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<tr>
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</tr>
<tr>
<td><strong>Bmp4</strong></td>
<td>Bone morphogenetic protein 4</td>
<td>Member of BMP family</td>
<td>(Rosen and Thies, 1992)</td>
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<tr>
<td><strong>Chrd</strong></td>
<td>Chordin (CHRD)</td>
<td>Antagonist to BMP signaling</td>
<td>(Balemans and Van Hul, 2002)</td>
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<tr>
<td><strong>Id1</strong></td>
<td>DNA-binding protein inhibitor ID-1</td>
<td>BMP target gene</td>
<td>(Miyazono et al., 2005)</td>
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<td><strong>Id2</strong></td>
<td>DNA-binding protein inhibitor ID-1</td>
<td>BMP target gene</td>
<td>(Miyazono et al., 2005)</td>
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<td><strong>Nog</strong></td>
<td>Noggin</td>
<td>Antagonist to BMP signaling</td>
<td>(Lim et al., 2000)</td>
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<td><strong>Dll1</strong></td>
<td>Delta-like protein 1</td>
<td>Activator of Notch pathway</td>
</tr>
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<td></td>
<td><strong>Dll4</strong></td>
<td>Delta-like protein 4</td>
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<td><strong>Jag1</strong></td>
<td>Jagged-1</td>
<td>Activator of Notch pathway</td>
</tr>
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<td>Neurogenic locus notch homolog protein 1</td>
<td>Notch receptor</td>
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<td>Neurogenic locus notch homolog protein 2</td>
<td>Notch receptor</td>
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<td>Osteoblast-related markers</td>
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<td>Protein</td>
<td>Marker</td>
</tr>
<tr>
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</tr>
<tr>
<td><strong>Alpl</strong></td>
<td>Alkaline phosphatase</td>
<td>Early marker of osteoblast differentiation</td>
<td>(Owen et al., 1990)</td>
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<tr>
<td><strong>Bglap3</strong></td>
<td>Osteocalcin-related protein</td>
<td>Late marker of osteoblast differentiation</td>
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<td><strong>Col1a1</strong></td>
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<td>Marker of Preosteoblasts and osteoblasts</td>
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<td><strong>Ibsp</strong></td>
<td>Bone sialoprotein 2</td>
<td>Marker of osteogenic differentiation</td>
<td>(Kalajzic et al., 2005)</td>
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<td><strong>Runx2</strong></td>
<td>Runt-related transcription factor 2</td>
<td>Marker of committed osteoprogenitors</td>
<td>(Aqeilan et al., 2008)</td>
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<td><strong>Sp7</strong></td>
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<td>Regulator of osteoblast differentiation</td>
<td>(Zhu et al., 2012)</td>
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<td>Cadherin-1</td>
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<td>(Konno et al., 2005)</td>
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<td>Cadherin-2</td>
<td>Marker of cell–cell adhesion in mesenchymal tissue</td>
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<td><strong>Itgb1</strong></td>
<td>Integrin beta-1</td>
<td>Adhesion Marker of mesenchymal stem cells</td>
<td>(Deans and Moseley, 2000)</td>
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<td><strong>Itga2</strong></td>
<td>Integrin alpha-2</td>
<td>Marker of cell–cell adhesion</td>
<td>(van der Flier and Sonnenberg, 2001)</td>
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<tr>
<td>Cell-cycle regulatory markers</td>
<td>Marker of cell–cell adhesion and cell migration</td>
<td>Marker of cell adhesion and cell-surface mediated signaling</td>
<td>Anti-apoptotic gene and regulator of mitosis</td>
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<td>Integrin alpha-3</td>
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<td>Itga6</td>
<td>Integrin alpha-6</td>
<td>Marker of cell adhesion and cell-surface mediated signaling</td>
<td>(Messier et al., 2016)</td>
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<td>Birc5</td>
<td>Baculoviral IAP repeat-containing protein 5</td>
<td>Anti-apoptotic gene and regulator of mitosis</td>
<td>(Mita et al., 2008)</td>
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<tr>
<td>Ccnb1</td>
<td>G2/mitotic-specific cyclin-B1</td>
<td>Regulator of mitosis and marker of G2/M phases of cell cycle</td>
<td>(Kurzawa and Morris, 2010)</td>
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<td>Ccnd1</td>
<td>G1/S-specific cyclin-D1</td>
<td>Regulator of G1/S phase transition in cell cycle</td>
<td>(Feng et al., 2011)</td>
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<tr>
<td>Ccnd3</td>
<td>G1/S-specific cyclin-D3</td>
<td>Marker of cell proliferation and regulator of cell cycle G1/S transition</td>
<td>(Huang et al., 2015)</td>
</tr>
<tr>
<td>Ccne2</td>
<td>G1/S-specific cyclin-E2</td>
<td>Regulator of cell cycle G1/S transition</td>
<td>(Aleem et al., 2005)</td>
</tr>
<tr>
<td>Mcm4</td>
<td>DNA replication licensing factor MCM4</td>
<td>Marker of cell proliferation and is required for initiation of DNA replication</td>
<td>(Kikuchi et al., 2011)</td>
</tr>
<tr>
<td>Mki67</td>
<td>Proliferation marker protein Ki-67</td>
<td>Marker of cell proliferation</td>
<td>(Whitfield et al., 2006)</td>
</tr>
<tr>
<td>Pcna</td>
<td>Proliferating cell nuclear</td>
<td>Marker of cell proliferation</td>
<td>(Hall et al., 2005)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
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<tr>
<td>antigen</td>
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<tr>
<td><strong>Kif4</strong></td>
<td>Chromosome-associated kinesin KIF4</td>
<td>Pluripotency-associated gene</td>
<td>(Mali et al., 2010)</td>
</tr>
<tr>
<td><strong>Myc</strong></td>
<td>c-Myc protein</td>
<td>Pluripotency-associated gene</td>
<td>(Mali et al., 2010)</td>
</tr>
<tr>
<td><strong>Rexo1</strong></td>
<td>RNA exonuclease 1</td>
<td>Pluripotency-associated gene</td>
<td>(Shi et al., 2006)</td>
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<td><strong>Ezh1</strong></td>
<td>Histone-lysine N-methyltransferase EZH1</td>
<td>Regulator of stem cell homeostasis</td>
<td>(Cheung and Rando, 2013)</td>
</tr>
<tr>
<td><strong>Foxo3</strong></td>
<td>Forkhead box protein O3</td>
<td>Regulator of stem cell homeostasis</td>
<td>(Renault et al., 2009)</td>
</tr>
<tr>
<td><strong>Pparg</strong></td>
<td>Peroxisome proliferator-activated receptor gamma</td>
<td>Promotes adipogenesis</td>
<td>(Brun et al., 1996)</td>
</tr>
<tr>
<td><strong>Pthlr</strong></td>
<td>Parathyroid hormone/parathyroid hormone-related peptide receptor</td>
<td>Parathyroid hormone signaling</td>
<td>(Calvi et al., 2003)</td>
</tr>
<tr>
<td><strong>Sox9</strong></td>
<td>Transcription factor SOX-9</td>
<td>Promotes chondrogenesis</td>
<td>(Wright et al., 1995)</td>
</tr>
<tr>
<td><strong>Tnfrsf21</strong></td>
<td>Tumor necrosis factor receptor superfamily member 21</td>
<td>Apoptosis-related gene</td>
<td>(Benschop et al., 2009)</td>
</tr>
<tr>
<td><strong>Trp53</strong></td>
<td>Cellular tumor antigen p53</td>
<td>Tumor suppressor gene</td>
<td>(Liu et al., 2004)</td>
</tr>
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</table>
RESULTS

I. In vivo imaging experiment

Prx1-creER-EGFP transgenic mice expressing green fluorescent protein under the control of the Prx1 promoter were live imaged using Intravital microscopy to investigate the presence of PRX1 expressing cells in mouse periodontal ligament. As illustrated in the Figure 3, PRX1+ cells expressing GFP were found within periodontal ligament. Thus, this experiment confirms the presence of post-natal PRX1 expressing cells in mouse periodontal ligament.

![Figure 3- Intravital microscopy image of mouse periodontal ligament. PRX1+ cells expressing GFP (white arrows) within the periodontal ligament (PDL) of 8 week-old mouse. Dentin (D) appears in red (by reflection) and bone (B) appears in blue (by second harmonic generation).](image-url)
II. Human in vitro assays

To assess the expression of PRX1 in human periodontium, *PRX1* gene expression levels in RNA obtained from human samples of bone and periodontal ligament (PDL) were quantified using qRT-PCR.

Gene expression analysis of human samples confirmed *PRX1* gene expression within both PDL and alveolar bone (Figure 4).

![Bar chart comparing PRX1 expression in bone and PDL](image)

**Figure 4- Comparison of relative levels of expression of *PRX1* in human PDL and bone.**

Expression of Prx1 gene was higher in human PDL compared to the alveolar bone.
Expression levels of Prx1 gene were quantified in human periodontal ligament stem cells, and this expression levels were compared with those of Scleraxis and MCAM/CD146, two known PDL stem cell markers (Seo et al., 2004).

We found a significant statistical difference between the expression levels of these three genes ($p < 0.05$). The level of Prx1 expression in human PDLSCs was more than 60 times higher than Scleraxis and MCAM/CD146 (Figure 5).

\[\text{Figure 5- Comparison of relative levels of expression of MCAM/CD146, Scleraxis, and PRX1 in PDLSCs. Expressing of PRX1 gene was significantly higher than MCAM/CD146 and Scleraxis.}\]

Therefore, these in vitro assay studies confirmed the expression of PRX1 in human periodontium as well as human periodontal ligament stem cells.
III. Inducible lineage tracing study

In this experiment, Prx1creER-EGFP;Rosa26-tdTOMATO transgenic mice, which conditionally expressing tdTOMATO after induction of cre recombination, were used. Cre recombination was induced in 4 week-old mice, and distribution of PRX1 linage cells was investigated 12 weeks later. All cells that derived from PRX1+ cells after induction of cre recombination would express tdTOMATO and appear in red; therefore, we would be able to distinguish post-natal PRX1 lineage cells.

We found PRX1 progeny cells (expressing tdTOMATO) to be mainly localized in the periodontal ligament and dental pulp of mandibular incisor (Figure 6 and 7).

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**Prx1creER-EGFP^{+/-};Rosa26-tdTOMATO^{+/-}**

![Phases contrast, DAPI, tdTOMATO](image)

**Figure 6. Inducible lineage tracing analysis of post-natal PRX1 progeny cells by inducing activity of Prx-cre in 4 week-old mice for 7 days.** PRX1 progeny cells were found in periodontal ligament (pdl) and pulp (p) of mandibular incisor in 16 week-old mice. b: bone, d: dentine, p: pulp, and pdl: periodontal ligament.
Figure 7. Inducible lineage tracing analysis of post-natal PRX1 progeny cells by inducing activity of Prx-cre in 4 week-old mice for 7 days. PRX1 progeny cells were localized in high abundance in PDL (pdl) and pulp (p) of mandibular incisor (yellow marks). b: bone, d: dentine, p: pulp, and Pdl: periodontal ligament; yellow marks: incisor, white marks: second molar.

Hence, this experiment demonstrated that post-natal PRX1 lineage cells reside within periodontium and pulp of mandibular incisors, which are regeneration-capable teeth.
IV. Gene expression signature study

In this study, we used Prx1-creER-EGFP transgenic mice expressing green fluorescent protein under the control of the Prx1 promoter. We isolated cells that expressed GFP, as indicator for the PRX1 expressing cells, from the PDL of mouse mandibular incisors and compared gene expression patterns of these cells with those of GFP negative cells isolated from PDL ligament of the same animals.

A) Progenitor and stem cells markers: Progenitor and stem cells markers that were investigated in this experiment were as follow: Cd44, Col2a1, Gli1, Kit, Mcam, Platelet-derived growth factor receptor alpha (Pdgfra), Smoothened (Smo), and Stem cells antigen-1 (Sca1).

The results of this study demonstrated that Gli1 and Kit were expressed in the PRX1+ cells, but not in the PRX- cells. The expression of Sca1 gene, which is a marker of hematopoietic stem cells, was undetectable in both PRX1+ and PRX- cells. The other five genes were expressed in both groups. The levels of expression of Cd44, Col2a1, Pdgfra, and Smo were significantly higher in the PRX1+ cells compared to PRX- cells ($p < 0.001$, $p = 0.006$, $p = 0.031$, and $p = 0.016$, respectively). There was no significant difference in the levels of expression of Mcam between the two groups ($p = 0.348$). Figure 8 illustrates the relative levels of expression of Cd44, Col2a1, Mcam, Pdgfra, and Smo in PRX1+ and PRX- cells isolated from the PDL of mouse mandibular incisors.
Figure 8- Relative levels of expression of *Cd44*, *Col2a1*, *Mcam*, *Pdgfra*, and *Smo* in PRX1+ and PRX1- cells. The relative levels of expression of these stem cell markers were significantly higher in the PRX1 expressing cells.
B) Wnt signaling pathway genes: We evaluated the levels of expression of the following three genes associated with Wnt signaling pathway: *Wnt3a*, *Wnt5a*, and *Wnt10b*. Also, we assessed the expression of *Fzd1, Fzd2, Fzd7, and Fzd9* genes, which encode transmembrane domain protein receptors for Wnt signaling proteins. In addition, the levels of expression of *Axin2* gene, which is a negative regulator of Wnt signaling, as well as WNT Inhibitory Factor 1 (*Wif1*), Dickkopf WNT Signaling Pathway Inhibitor 1 (*Dkk1*), and *Sost* genes, which are Wnt signaling down-regulators, were assessed. Furthermore, the expression *Twist1* gene, which is up regulated in response to Wnt signaling, was investigated.

It was found that the levels of expression *Wnt3a, Wnt10b*, and *Sost* were undetectable in both groups. The expression of *Fzd2* gene was only detected in the PRX1- cells, while *Wnt5a, Dkk1*, and *Twist1* genes were expressed only in the PRX1+ cells. No significant differences between the groups were found for the levels of expression of *Fzd7* (*p* = 0.477) and *Fzd9* (*p* = 0.056). The results exhibited that the levels of expression of *Axin2* (*p* = 0.024), *Fzd1* (*p* = 0.014), and *Wif1* (*p* = 0.002) were significantly greater in the PRX1+ cells compared to PRX1- cells (Figure 9).
Figure 9- Comparison of relative levels of expression of *Axin2, Wif1, Fzd1, Fzd7, and Fzd9* between the PRX1+ and PRX1- cells. Significantly greater levels of expression of *Axin2, Fzd1,* and *Wif1* were detected in the PRX1+ cells compared to the PRX1- cells.
C) BMP signaling pathway genes: The levels of expression of two bone morphogenetic protein (BMP) family members, Bmp2 and Bmp4, were investigated in this study. Furthermore, we evaluated the levels of expression genes that encode BMP antagonists including Noggin (Nog) and Chordin (Chrd) as well as BMP target genes such as Id1 and Id2. Our data showed that the expressions of Bmp4, Chrd, Id1, and Id2 genes were only detected in the PRX1 expressing cells. Furthermore, we found that the levels of expression of Bmp2 was significantly higher in the PRX1+ cells ($p = 0.004$), while Nog (0.003) was found to be significantly expressed at higher levels in the PRX1- cells ($p = 0.003$) (Figure 10).

![Figure 10](image_url)  

**Figure 10- Relative levels of expression of Bmp2 and Nog in the PRX1+ and PRX1- cells.**

The levels of expression of these two genes were significantly different between the PRX+ and PRX1- cells.
D) Notch signaling pathway genes: We investigated the following genes associated with Notch signaling pathway: *Dll1*, *Dll4*, *Jag1*, *Notch1*, and *Notch2*. *Dll1* and *Dll4* encode notch ligands Delta-like protein 1 (DLL1) and Delta-like protein 4 (DLL4), and *Jag1* encode notch ligand Jagged-1. These ligands interact with Notch1 and Notch2 receptors that are encoded by *Notch1* and *Notch2* genes. The result of gene expression analysis demonstrated that the expression of *Dll1* gene was only detectable in the PRX1 expressing cells. The difference in gene expression level of *Notch1* between the groups was not statistically significant (*p* = 0.233). However, the expression levels of *Dll4* (*p* < 0.001), *Jag1* (*p* = 0.010), and *Notch2* (*p* = 0.014) were significantly higher in the PRX1+ cells compared to the PRX1- cells (Figure 11).

![Graphs showing gene expression levels](image)

**Figure 11-** Comparison of relative levels of expression of *Dll4*, *Jag1*, *Notch1*, and *Notch2* between the PRX1+ and PRX1- cells. Levels of expression of *Dll4*, *Jag1*, and *Notch2* were significantly higher in the PRX1+ cells compared to the PRX1- cells.
E) Osteoblast-related markers: The expression levels of the following osteoblast-related markers were investigated: Alpl, Bglap3, Colla1, Ibsp, Runx2, and Sp7. The expression of Bglap3 was undetectable in both groups. In addition, Alpl, Ibsp, Runx2, and Sp7 were expressed in the PRX1+ cells, but not in the PRX1- cells. Colla1 gene was expressed in both PRX1+ and PRX1- cells with a significantly higher level of expression in the PRX1+ cells ($p = 0.010$; Figure 12).

F) Genes related to adhesion molecules: We also explored the expression of six genes related to cell adhesion molecules including Cadherin-1 (Cdh1), Cadherin-2 (Cdh2), Integrin beta-1 (Itgb1), Integrin alpha-2 (Itga2), Integrin alpha-3 (Itga3), and Integrin alpha-6 (Itga6). The expression levels of Cdh1, Itga2, and Itga3 genes were only detected in the PRX1+ cells. Moreover, the expression level of Itga6 gene was found in neither group. Our results also exhibited that Cdh2 and Itgb1 were expressed at greater levels in the PRX1 expressing cells ($p < 0.001$ and $p = 0.075$, respectively; Figure 12).

G) Cell-cycle regulatory markers: Cell-cycle regulatory markers assessed in this study were: Birc5, Ccnb1, Ccnd1, Ccnd3, Ccne2, Mcm4, Mki67, and Pcna. The gene expression data demonstrated that Birc5, Ccnd3, and Ccne2 were solely expressed in the PRX1+ cells, while Ccnb1 was only expressed in the PRX1- cells. Furthermore, we found that the levels of expression of Ccnd1 ($p = 0.013$), Mki67 ($p < 0.001$), and Pcna ($p = 0.037$) were significantly higher in the PRX1+ cells compared to the PRX1- cells (Figure 13). On the other hand, Mcm4 was significantly expressed at higher levels in the PRX1- cells ($p = 0.005$; Figure 13)
Figure 12- Relative levels of expression of \textit{Cdh2}, \textit{Itgb1}, and \textit{Colla1} in the PRX1+ and PRX1- cells. Statistically significant differences between the groups were found for \textit{Cdh2} and \textit{Colla1}. 
Figure 13- Comparison of relative levels of expression of Ccnd1, Mcm4, Mki67, and Pcna between the PRX1+ and PRX1- cells. Significant differences were found between the PRX1+ and PRX1- cells for all four genes.

H) Pluripotency-associated genes: Expressions of Kif4, Myc, and Rexol genes, which are pluripotency-related genes, were investigated in the PRX1+ and PRX1- cells. The data demonstrated that these pluripotency-related genes are expressed in the PRX1+ cells, but they are not expressed in the PRX1- cells.
I) Other relevant genes: The other genes investigated in this study were as follow: Ezh1, Foxo3, Pparg, Pth1r, Sox9, Tnfrsf21, and Trp53. Ezh1 and Foxo3 genes contribute to stem cell homeostasis. We found that expression of Ezh1 was only detectable in the PRX1 expressing cells, but Foxo3 was expressed in both PRX1+ and PRX1- cells with more than 800 folds higher levels of expression in the PRX1+ cells than the PRX1- cells ($p = 0.013$; Figure 14). The results also revealed that Pparg, which promotes adipogenesis, and Sox9, which promotes chondrogenesis, were solely expressed in the PRX1 expressing cells. In addition, we found that Pth1r, which encodes Parathyroid hormone/parathyroid hormone-related peptide receptor and has a key role in Parathyroid hormone signaling, was expressed at extremely higher levels (almost 24600 folds higher) in the PRX1+ cells compared to the PRX1- cells ($p < 0.001$; Figure 14). We also found that the level of expression of Tnfrsf21, which is an apoptosis-related gene, was significantly higher in the PRX1+ cells than PRX1- cells ($p = 0.017$; Figure 14). Moreover, our data demonstrated that Trp53, which is considered as a tumor suppressor gene, was only detected in the PRX1+ cells.
Figure 14- Relative levels of expression of *Pthlr*, *Tnfrsf21*, and *Foxo3* in the PRX1+ and PRX1- cells. Statistically significant differences were found for both genes between the PRX1+ and PRX1- cells.
CHAPTER 3
CONTRIBUTION OF PRX1 EXPRESSING CELLS TO
PERIODONTAL FORMATION, HOMEOSTASIS, AND
REGENERATION

Skeletal stem cells (SSCs) have a promising therapeutic potential in regenerative medicine due to their ability to differentiate into different mesodermal cell lineages (Pittenger et al., 1999, Murphy et al., 2013). SSCs have been isolated from several extra- or -intra oral sources (Huang et al., 2009). It has been shown that periodontal ligament harbors a heterogeneous population of skeletal stem cells (Gay et al., 2007, Seo et al., 2004). Investigating the characteristics and functions of these skeletal stem cells may lead to the development of effective treatment modalities that target a specific population of stem cells to harness their regenerative potential.

A series of experiments in our laboratory have shown that adult PRX1 expressing cells contribute to intramembranous bone formation and these cells are required for bone regeneration in calvarial defects. In the chapter 2, we demonstrated that the PRX1 expressing cells, which are a sub-population of skeletal stem cells, are present in human and mice periodontium. Yet, there is no information available on the function of the PRX1 expressing cells in the periodontium.

Hence, the second specific aim of the present research project was to assess the contribution of the PRX1 expressing cells of the mouse periodontium to periodontal formation, post-natal periodontal hemostasis, and periodontal regeneration. In this study, we demonstrated the contribution of the PRX1 expressing cells to periodontal formation using lineage-tracing analyses performed during embryogenesis. In addition, we showed that the PRX1 expressing
cells contribute to the periodontal homeostasis as the ablation of post-natal PRX1+ cells resulted in the changes in the width of periodontal ligament space, which is an important indicator of the homeostasis in the periodontium (Lim et al., 2014, McCulloch et al., 2000). Moreover, we examined whether or not the PRX1+ cells have a role in the periodontal regeneration by evaluating the effects that ablation of these cells have on the regeneration of periodontal defects.

MATERIALS AND METHODS

The specific aim two was addressed in three different experiments. First, the contribution of PRX1 expressing cells to the formation of mouse periodontium was investigated in the cre/loxP-based constitutive lineage tracing during embryogenesis (post-natal inducible lineage tracing study is described in the chapter 2). Second, we assessed the role of the PRX1+ cells to the post-natal periodontal hemostasis by assessing the effects of post-natal ablation of these cells on the width of the periodontal ligament space. Third, we investigated the contribution of the PRX1 expressing cells to the periodontal regeneration by exploring the effect of post-natal ablation of the PRX1+ cells on the healing of surgically created sub-critical size periodontal fenestration defects.

I. Constitutive lineage tracing study

To investigate the contribution of the PRX1 expressing cells to the formation of mouse periodontium, we conducted a constitutive lineage tracing analysis. In this study, a Prx1-cre<sup>+</sup>;Rosa26-tdTOMATO<sup>+</sup> transgenic mouse model was created (Figure 1).
This mouse model was created by crossing a mouse expressing cre recombinase under the constitutive control of the Prx1 promoter (Prx1-Cre mouse) (Logan et al., 2002) with a transgenic mouse engineered to conditionally express tdTOMATO upon cre recombination of a loxP-flanked STOP sequence (Rosa26-tdTOMATO mouse) (Madisen et al., 2010). In this generated transgenic mouse model, the constitutive expression of cre recombinase identifies the PRX1 expressing cells and their progeny (henceforth, all together indicated as PRX1 lineage cells), and all PRX1 lineage cells appear in red (indicative of the expression of Td-Tomato) under fluorescence microscopy.

In this experiments, Prx1-cre<sup>+/−</sup>;Rosa26-tdTOMATO<sup>+/−</sup> male mice (n=3) were sacrificed at the age of 4 weeks. The mandibles were collected, and samples were processed for the cryosectioning and histological analysis.

The method of preparation of the samples for cryosectioning is described in detail in the chapter 2, experiment III. Briefly, mandibles were fixed in 4% PFA. Decalcification was done using 14% EDTA (pH 7.4) for two weeks on a shaker at the room temperature. Samples were cryoprotected.
with 30% sucrose, and they were then embedded and frozen in Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA). Samples were cryosectioned at 10 um with a cryostat (Research Cryostat Leica CM3050 S, Leica Microsystems Inc., Buffalo Grove, IL) and imaged using a fluorescence microscope (Keyence BZ-X710 microscope, Keyence, Osaka).

**II. Inducible lineage ablation study and post-natal periodontal homeostasis**

To evaluate the contribution of PRX1+ cells to the post-natal periodontal homeostasis, we investigated the effects of the post-natal ablation of these cells on the width of periodontal space, as the maintenance of this space is a key indicator of periodontal homeostasis (McCulloch et al., 2000, Lim et al., 2014, Beertsen et al., 1997).

We created Prx1creER-EGFP+/−-Rosa26-DTA+/− mouse model (Ablation mouse model; Figure 2) in this experiment. To do so, we crossed Prx1creER-EGFP+/− mouse with mouse engineered to conditionally express diphtheria toxin A (DTA) upon cre recombination of a loxP-flanked STOP sequence (Rosa26-DTA mice). This system would allow the in vivo cell-specific activation of the toxin gene, diphtheria toxin A in this case. Diphtheria toxin A protein catalyzes the inactivation of elongation factor 2, which results in cessation of protein synthesis and apoptosis of the target cells (Ivanova et al., 2005). However, in this mouse model, DTA is expressed only conditionally, and its expression requires induction of cre recombination of a loxP-flanked STOP sequence by tamoxifen. Upon induction of recombination by injection of tamoxifen in the ablation mice, all cells that are expressing PRX1 will also express DTA, which leads to apoptosis of PRX1 expressing cells and their global ablation.
In this experiment, we had the following two groups (Figure 3):

Test group (ablation group): Prx1creER-EGFP+/−-Rosa26-DTA+/−, in which the ablation of the PRX1+ cells occurs after injection of tamoxifen due to presence of DTA gene,

Control group (non-ablation group): Prx1creER-EGFP+/−-Rosa26-DTA−/− or Prx1creER-EGFP+/−-Rosa26-DTA+/−, in which the ablation of the PRX1+ cells does not occur after injection of tamoxifen due to the absence of creER-EGFP or DTA gene.

In both groups, transgenic mice (n=5 in each group) at post-natal age 3 days (P3) were treated with tamoxifen (Intraperitoneal, 40mg/kg in sterile oil) everyday for 10 days (Wilk et al., 2017). Animals in both groups were sacrificed at post-natal age 21 days (P21) (Figure 4). Mandibles were collected and fixed in 70% ethanol. All samples underwent micro-CT scanning (SCANCO)
μCT 35, Scanco Medical, Bruttisellen, Switzerland) with similar experimental parameters (source voltage: 70 kVp, power: 8 W, exposure time: 300 ms, and voxel size: 10 micron).

Figure 3- Genotype of animals in the test (ablation) and control (non-ablation) groups

Figure 4- Scheme of the study design for inducible lineage ablation study and post-natal periodontal homeostasis
Width of periodontal ligament spaces was measured in right mandibular incisor as well as mesial and distal roots of right mandibular first molar. Measurements were taken using Avizo software package (version 9.2.0 FEI Visualization Sciences Group, MA) based on methodologies described elsewhere (Niver et al., 2011).

For each sample, the measurements of the width of periodontal ligament space around mesial root of right mandibular first molar tooth were made on three horizontal (transverse) slices (Coronal, Mid-root, and Apical slices). Two reference points were used to select these three slices. The reference points were: the furcation entrance of the tooth (coronal reference) and the apex of the mesial root (apical reference) (orange lines in Figure 5). Similar methodology was used to select the three horizontal slices for the distal root of the tooth with the only difference being that the apex of the distal root was used as the apical reference. The Mid-root slice was generated at the midpoint of the two reference points (Figure 5). The Coronal slice was made at the midpoint of the distance between the coronal reference and the Mid-root slice, and the Apical slice was generated at the midpoint of the distance between the Mid-root slice and the apical reference (Figure 5).

The linear measurement of the width of periodontal ligament space on each slice was performed at 8 points of buccal, mesiobuccal, mesial, mesiolingual, lingual, distolingual, distal, and distobuccal. The average of these eight measurements was calculated and served as the average width of periodontal ligament space on each slice (Figure 6).
Figure 5- Generated slices to measure the width of periodontal ligament space of the mesial root of right mandibular first molar tooth in a test sample. Three transverse slices of Coronal, Mid-root, and Apical (yellow lines) were generated based on the two coronal and apical reference points (orange lines).

Figure 6- Measurements of the width of periodontal ligament space for the mesial root of right mandibular first molar tooth in a test sample. Linear measurements were performed on eight surfaces on each slice.
The measurements of the width of periodontal ligament space around the right mandibular incisor tooth were performed on five coronal slices (Coronal 1, Coronal 2, Mid-root, Apical 1, and Apical 2 slices) (Figure 7). The two reference points for these measurements were the most coronal level of the alveolar bone (coronal reference) and the most apical point of the tooth socket (apical reference). At the mid point distance between these two reference points, the Mid-root slice was generated. Coronal 1 slice was made at the midpoint of the distance between the coronal reference and the Mid-root section. Coronal 2 slice was generated at the midpoint between the Coronal 1 and the Mid-root slices. At the middle of distance between Mid-root slice and the Apical reference, the apical 2 slice was made, and the Apical 1 slice was generated at the midpoint between the Mid-root and the Apical 2 slices (Figure 7).

Figure 7- Generated slices to measure the width of periodontal ligament space of the incisor tooth in a test sample. Five coronal slices of Coronal 1, Coronal 2, Mid-root, Apical 1, and Apical 2 (yellow lines) were made according to the two coronal and apical reference points (orange lines).
The linear measurements of the width of periodontal ligament space were performed at the following 8 points: buccal, mesiobuccal, mesial, mesiolingual, lingual, distolingual, distal, and distobuccal. The measurements at the enamel surface (buccal measurement in the most of slices) were excluded from the analysis. These linear measurements were averaged and served as the average width of periodontal ligament space at each slice (Figure 8).

Figure 8- Measurements of the width of periodontal ligament space for the root of mandibular incisor tooth in a test sample. Linear measurements were performed at eight surfaces on each slice.
III. Inducible lineage ablation study and periodontal regeneration

We studied the role of the PRX1 expressing cells in the periodontal regeneration by investigating the effects that global ablation of these cells have on the regeneration of periodontal defects. To do so, we first created a periodontal defect model of sub-critical size, where periodontal defects in the animals would regenerate spontaneously. Next, we compared the healing of the sub-critical size periodontal defects between the test group (ablation group: PRX1 expressing cells were globally ablated) and the control group (non-ablation group: PRX1 expressing cells were present).

The size and location of sub-critical defects on the buccal surfaces of mandibular incisor and first molar teeth were optimized in a series of pilot experiments. We found that 0.5 mm periodontal fenestration defects would regenerate spontaneously in the control animals (non-critical size defect). Therefore, we decided to use two 0.5 mm sub-critical size periodontal fenestration defects at the buccal aspect of the right mandibular incisor tooth and at the buccal surface of the distal root of the right first mandibular molar (Figures 9-11).

In this experiment, we used the transgenic mouse model of Prx1creER-EGFP+/−-Rosa26-DTA+/− (Ablation mouse model), which is the same mouse model that was generated and used in the experiment II (Figure 2). Similar to the experiment II, after the induction of recombination by tamoxifen in these mice, all cells that are expressing PRX1 will also express DTA, which leads to apoptosis of the PRX1 expressing cells and global ablation of these cells.

The experimental groups for this study were as follow (Figures 3 and 9):

Test group (ablation group): 7-9 week-old male transgenic mice (n = 5) with the genotype of Prx1creER-EGFP+/−-Rosa26-DTA+/−
Control group (non-ablation group): 7-9 week-old male transgenic mice (n = 5) with the genotype of Prx1creER-EGFP^{+/−}-Rosa26-DTA^{−/−} or Prx1creER-EGFP^{−/−}-Rosa26-DTA^{+/−}.

Scheme of the study design for this experiment is illustrated in the Figure 9. 7-9 week-old adult male mice in both groups were treated with tamoxifen (Intraperitoneal, 40mg/kg in sterile oil) for 5 days preoperatively and 5 days post-operatively (Wilk et al., 2017). For each mouse in both groups, two periodontal fenestration sub-critical size defects were created by the right mandibular incisor and first mandibular molar teeth (Figure 9). To do so, an extra-oral incision was made at the lower border of the mandible. After separating superficial fascia, master muscle, and periosteum, the alveolar bone was exposed.

**Figure 9- Scheme of the study design for inducible lineage ablation study and periodontal regeneration**
The location of the molar defect was determined as follow: the distal cuspid of the first molar was considered as the reference point, the defect was created 1 mm below the most coronal aspect of alveolar bone at the level of distal cuspid (Figure 10). Then, hand instruments were utilized to create a defect with 0.5 mm diameter and penetrate into the bone for 0.25 mm, creating a defect in the alveolar bone, PDL, and dentin of the distal root of the first molar.

Figure 10- Location of the sub-critical size fenestration periodontal defect by the mandibular first molar. (A) Distal cuspid of the first molar was used as the reference point to locate the frontal plane in correspondence of which the periodontal defect would be created. (B) Once the reference frontal plane was identified, the exact location of the defect was located by measuring a distance of 1 mm from the most coronal aspect of the alveolar bone.
Location of the incisor defect was determined using the mesiocoronal surface of the first molar as the reference point (Figure 11). The defect was created 1 mm apical to the masseteric ridge at the level of mesiocoronal surface of the first molar. The incisor defect was 0.5 mm in diameter and 0.5 mm in depth to involve the alveolar bone, PDL, and dentin of the mandibular incisor.

**Figure 11- Location of sub-critical size fenestration periodontal defect by the mandibular incisor.** (A) Mesiocoronal surface of the first molar was utilized as the reference point to locate the frontal plane in correspondence of which the periodontal defect would be created; (B) after identifying the reference frontal plane, the periodontal defect was created 1 mm apical to the masseteric ridge.
Both defects in each animal left unfilled to allow spontaneous healing. Extra-oral incision was sutured using continuous suturing technique with absorbable 5-0 Vicryl sutures (Hu-Friedy, Chicago, IL, USA). Following the surgery, animals were given injection of Buprenorphine (subcutaneous injection, 0.05 mg/kg) for pain control at the time of surgery and every 12 hours for the first 48 hours. All protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Harvard University.

After an eight-week healing period, animals in both groups were sacrificed. Mandibles were collected and fixed in 70% ethanol. Micro-CT and histological analyses were performed for the samples.

**Micro-CT analysis:** All samples underwent micro-CT scanning (SCANCO μCT 35, Scanco Medical; source voltage: 70 kVp, power: 8 W, exposure time: 300 ms, and voxel size: 10 micron). The healing of periodontal defects were assessed using Avizo software package (version 9.2.0 FEI Visualization Sciences Group, MA), and the healing was reported as 1) completely healed, 2) partially healed, or 3) not healed. The defects were considered “completely healed” when a bony bridge formed across the defect borders and osseous continuity of mandible was restored. The defects were scored as “partially healed” if a bony bridge was not completely formed in all sections. When no bony bridge formed inside the defect, it was considered as “not healed”.

**Histological analysis:** Samples were prepared for cryosectioning and histological analysis after micro-CT scanning. Briefly, samples were washed with 1% Dulbecco's phosphate-buffered saline (DPBS) and decalcified in with a 22.5% formic acid and 10% sodium citrate solution for two weeks on a shaker at 4 °C. After washing again with 1% DPBS and Cryoprotection with 30% sucrose at 4 °C overnight, samples were embedded and frozen in Tissue-Tek OCT.
compound (Sakura Finetek USA, Inc.). 10-um sections were prepared with a cryostat (Research Cryostat Leica CM3050 S). Sections were stored in -80 °C prior to the staining. Section were stained with Hematoxylin and Eosin (H&E) and prepared for light microscopy. Briefly, frozen sections were thawed at room temperature for 15 minutes. Slides were first washed in 1% Phosphate-buffered saline (PBS) for two minutes and were stained with hematoxylin for 30 seconds. Then, slides were washed in tap water for 10 minutes. Next, slides were stained with eosin for 90 seconds. Subsequently, slides were dehydrated using ethanol serial dilution (70%, 90%, 100%, 100%; each 2 minutes), cleared in xylene (10 minutes), and mounted onto glass slides. Slides were visualized and imaged under microscope (Keyence BZ-X710 microscope, Keyence, Osaka).

IV. Statistical Analysis

The mean width of periodontal space in each section (continuous variable) was compared between the test and control groups using Student's t-test (Experiment II). The type of healing of the periodontal defects (categorical variable) was compared between the groups using Fisher's exact test (Experiment III). Power analysis of data obtained from our preliminary data indicated a sample size of n = 5 to be sufficient for a power of 0.8. A significance level of a = 0.05 was used for all comparisons.
RESULTS

I. Constitutive lineage tracing study

We used a Prx1-cre\textsuperscript{+/−};Rosa26-tdTOMATO\textsuperscript{+/−} transgenic mouse model to study the contribution of PRX1 expressing cells to the formation of mouse periodontium. In this mouse model, the constitutive expression of cre recombinase results in expression of Td-Tomato in PRX1 lineage cells. Therefore, all PRX1 lineage cells in this mouse model appear in red (indicative of the expression of Td-Tomato) under fluorescence microscopy.

The expression of Td-Tomato (red color) was observed in periodontal ligament, bone, cells surrounding the dentin, and dental pulp (Figure 12). Therefore, it was discovered that PRX1 lineage cells were present in periodontal ligament, bone, cells surrounding the dentin, and dental pulp. This experiment showed that PRX1+ lineage cells contribute to the formation of both periodontium and dental pulp.

Figure 12- Constitutive lineage tracing of PRX1 lineage cells. Constitutive expression of Cre recombinase under the PRX1 promoter identifies PRX1 progeny cells in bone (b), periodontal ligament (pdl), cells surrounding the dentine (d), and pulp (p).
II. Inducible lineage ablation study and post-natal periodontal homeostasis

The contribution of PRX1+ cells in post-natal periodontal homeostasis was studied by comparing the width of periodontal ligament (PDL) space around the root of mandibular incisor tooth as well as the mesial and distal roots of the mandibular first molar tooth between the ablation group (test group) and the non-ablation group (control group).

A. Width of periodontal space around the mandibular incisor tooth:

Width of PDL space around the mandibular incisor was assessed in five standardized slices of Coronal 1, Coronal 2, Mid-root, Apical 1, and Apical 2. Eight linear measurements were performed on each slice, and the average of these measurements (after excluding the measurements at the enamel surfaces) served as the width of PDL space on each slice. The comparison of the width of PDL space between the test and control groups is illustrated in the Figure 13.

The average width of PDL space in the Coronal 1 slice was 0.0974 ± 0.0114 mm for the test group and 0.0654 ± 0.0115 mm for the control group. The difference between the test and control groups was found to be statistically significant ($p = 0.002; 95\% \text{ CI} = 0.0152 - 0.0487$).

In the Coronal 2 slice, the width of PDL space in the test group (0.1162 ± 0.0168 mm) was significantly greater than the width of PDL space in the control group (0.0711 ± 0.0126 mm) ($p = 0.001; 95\% \text{ CI} = 0.0235 - 0.0668$).

A significant difference was found for the average width of PDL space between the test and control groups in the Mid-root section ($p < 0.001; 95\% \text{ CI} = 0.0312 - 0.0782$). The average width of PDL space for the test group in the Mid-root section was 0.1336 ± 0.0121 mm, and the average width of PDL space for the control group in the same section was 0.0789 ± 0.0194 mm.
The differences in the width of PDL space between the test and control groups were also highly significant in both the Apical 1 and Apical 2 sections ($p < 0.001; 95\% \text{ CI} = 0.0400 - 0.0755$ and $p < 0.001; 95\% \text{ CI} = 0.0403 - 0.0755$, respectively). The width of PDL space in the test group was $0.1415 \pm 0.0142 \text{ mm}$ for the Apical 1 slice and was $0.1397 \pm 0.0161 \text{ mm}$ for the Apical 2 slice. In the control group, the width of PDL space was $0.0838 \pm 0.0097 \text{ for the Apical 1 section}$ and was $0.0818 \pm 0.0055 \text{ mm}$ for the Apical 2 section.

![Bar chart showing the comparison of PDL space width between test and control groups.](image)

Figure 13- Comparison of the width of PDL space of the mandibular incisor tooth between the test and control groups. The width of PDL space was significantly greater in the test group compared to the control group in all comparisons (*$p < 0.05$, **$p < 0.001$).
B. Width of periodontal space around the mesial and distal roots of the mandibular first molar:

The measurements of the width of PDL spaces around the mesial and distal roots of the mandibular first molar were performed separately for each root. The width of PDL space was measured for each root in three standardized sections of Coronal, Mid-root, and Apical. Similar to the measurements for the incisor tooth, the width of PDL space on each slice was the average of eight linear measurements performed on each section.

**Mesial root:** The average width of PDL spaces around the mesial root for the test group in the Coronal, Mid-root, and Apical sections were 0.0734 ± 0.0127 mm, 0.0871 ± 0.0101 mm, and 0.0995 ± 0.0087 mm, respectively. Corresponding measurements for the control group in the Coronal, Mid-root, and Apical sections were 0.0590 ± 0.0072 mm, 0.0766 ± 0.0082 mm, and 0.0800 ± 0.0115 mm, respectively.

Figure 14 illustrates the comparison of the width of PDL space around the mesial root between the test and control groups. Although the width of PDL space around the mesial root was greater in the test group than the control group in all sections, there were no statistical significant differences between the groups in the Coronal section \( (p = 0.059; 95\% \, CI = -0.0006 - 0.0296) \) and the Mid-root section \( (p = 0.108; 95\% \, CI = -0.0029 - 0.0239) \). A statistically significant difference was found for the width of PDL space around the mesial root between the test and control group in the Apical section \( (p = 0.017; 95\% \, CI = 0.0046 - 0.0343) \).
Figure 14 - Comparison of the width of PDL space around the mesial root of the mandibular first molar between the test and control groups. A statistical significant difference between the groups was found in the apical section (* p < 0.05).

**Distal root:** The average width of the PDL space around the distal root in the Coronal section was 0.0968 ± 0.0105 mm for the test group and 0.0742 ± 0.0163 mm for the control group (Figure 15). The difference between the test and control groups for the measurements in this section was statistically significant (p = 0.032; 95% CI = 0.0026 - 0.0426). In the Mid-root section, the average width of the PDL space for the test group was 0.1120 ± 0.0145 mm and the
The corresponding value for the control group was 0.0901 ± 0.0095 mm. The difference between the test and control groups for the average width of the PDL space around the distal root in the Mid-root section was found to be significant ($p = 0.022$; 95% CI = 0.0041 - 0.0399). In the Apical section, the width of PDL space in the test group (0.1166 ± 0.0198 mm) was significantly higher compared to the corresponding measurement in the control group (0.0914 ± 0.0110 mm) ($p = 0.038$; 95% CI = 0.0018 - 0.0485).

![Graph showing comparison of PDL space width](image)

**Figure 15**- Comparison of the width of PDL space around the distal root of the mandibular first molar between test and control groups. Statistical significant differences were found between the groups in all comparisons (* $p < 0.05$).
III. Inducible lineage ablation study and periodontal regeneration

Healing of the sub-critical size fenestration periodontal defects was compared between the test group, where PRX1 expressing cells were globally ablated, and the control group, where PRX1 expressing cells were present.

We created two defects in each animal, one by the right mandibular first molar and the other one by the mandibular incisor. All animals in the test group (n=5) and control group (n=5) survived after the surgeries, and the healing of the defects was assessed 8 weeks postoperatively.

In the control group, we found that sub-critical periodontal fenestration defects around both incisor and molar teeth healed. Healing was observed clinically and confirmed using micro-CT analysis (Figure 16), and periodontal regeneration was observed in the histological analysis (Figure 17). For the incisor defects, the complete healing was observed in 4 out 5 animals, and the defect in one animal had partial healing (Figure 18). For the molar defects, the defects in all five animals healed completely, but the healing was accompanied by excessive bone formation in two animals (Figure 19).

In the test group, we found that all defects by the incisor tooth did not heal in the animals, while the defects by the molar tooth healed mainly by excessive bone formation (Figure 20 and 21). For the incisor defects, we observed the lack of healing in the 5 out of 5 animals (Figure 22). For the molar defects, we observed the partial healing in two defects, and the complete healing in the other three defects. It should be mentioned that the healing was accompanied by excessive bone formation in four defects (Figure 23).

Fisher's exact test showed a statistical significant difference between the test and control groups in the healing of incisor defects (p = 0.008). There was no significant difference between the two groups in the healing of molar defects (p = 0.44, Fisher's exact test) (Table 1).
Figure 16- Healing of sub-critical defects in the control (non-ablation) group. Two defects were created around the mandibular incisor and first molar teeth (A). Eight weeks post-operatively, healing of both defects was observed clinically (B). Micro-CT analysis confirmed the healing of the incisor (C and D) and the molar (E and F) defects.
Figure 17- Healing of sub-critical defects in the control (non-ablation) group. Regeneration of periodontal defects by formation of new bone, new cemented, new periodontal ligament was observed in the incisor (A) and the molar (B) defects. b: bone, c: cementum, d: dentine, and pdl: periodontal ligament.
Figure 18- Healing of sub-critical defects by the incisor tooth in the control (non-ablation) group (frontal sections). Complete healing was observed in 4 animals (A-D), and the partial healing was noted in one animal (E).
Figure 19- Healing of sub-critical defects by the molar tooth in the control (non-ablation) group (frontal sections). Complete healing was observed in all defects (A-E); healing was accompanied by excessive bone formation in two animals (D and E; red arrows).
Figure 20- Healing of sub-critical defects in the test (ablation) group. Two fenestration defects were made around the mandibular incisor and first molar teeth (A). After 8 weeks, the incisor defect did not heal in this animal (B) while the molar defect was healed by excessive and irregular bone formation (B). Micro-CT analysis confirmed the clinical observation for the incisor (C and D) and the molar (E and F) defects.
Figure 21- Healing of sub-critical defects in the test (ablation) group. Lack of regeneration in the incisor defect was observed (A). Irregular bone formation and excessive proliferation of osteoblasts were observed in the healed molar defect (B).
Figure 22- Healing of sub-critical defects by the incisor tooth in the test (ablation) group (frontal sections). No healing was observed in the incisor defects of the five animals in the test group.
Figure 23- Healing of sub-critical defects by the molar tooth in the test (non-ablation) group (frontal sections). Complete healing was observed in three defects (A, B, E), and the partial healing was occurred in two defects (C and D); healing was accompanied by excessive bone formation in four animals (A, C, D, and E; red arrows).
### Table 1- Distribution of the type of healing of the defects in the test and control groups.

<table>
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<tr>
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<th>Incisor defects</th>
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<th>Molar defects</th>
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<tbody>
<tr>
<td></td>
<td>No healing</td>
<td>Partial healing</td>
<td>Complete healing</td>
<td>No healing</td>
<td>Partial healing</td>
<td>Complete healing</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
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<tr>
<td>Control group</td>
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<td>1</td>
<td>4</td>
<td>0</td>
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A significant difference was observed between test and control groups in the healing of incisor defects.

# Fisher's exact test

* Significant differences between the groups
DISCUSSION

Endogenous stem cell-based regenerative therapies hold great promise for the future of regenerative medicine. Identifying the endogenous stem cells present in the periodontium and studying their characteristics may provide insight to better understand the mechanisms that regulate the regeneration and repair of damaged periodontal structures. With the ultimate goal of promoting the periodontal tissue regeneration through harnessing the regenerative potential of the endogenous stem cells, we studied a sub-population of skeletal stem cells that express PRX1 in the periodontium. In this project, for the first time, we reported the presence of PRX1 expressing cell within the mouse and human periodontal ligament. We demonstrated, in mouse, post-natal PRX1 lineage cells mainly reside within the periodontal ligament and pulp of mandibular incisors, which are regeneration-capable teeth. We also isolated PRX1 expressing cells from the mouse periodontal ligament and characterized the gene expression pattern of these skeletal stem cells. Furthermore, the contributions of PRX1 expressing cells to the periodontal formation, periodontal homeostasis, and periodontal regeneration were studied.

We found that PRX1 is highly expressed in the human PDL stem cells and post-natal PRX1 expressing cells are present in the periodontal ligament in both human and mice. Post-natal PRX1 expressing cells have been previously isolated from the periosteum of post-natal long
bones (Kawanami et al., 2009) and from the post-natal calvaria (Ouyang et al., 2014). It is well documented that these cells have characteristics of skeletal stem cells, and they are able to differentiate into osteoblasts and chondroblasts (Kawanami et al., 2009, Wilk et al., 2017). Recently, we demonstrated that PRX1 expressing cells also reside in the calvarial sutures, and in such location they are required for the bone regeneration of calvarial defects (Wilk et al., 2017). Given the contribution of PRX1 expressing cells in the bone regeneration, the presence of these endogenous skeletal stem cells in human and mice periodontium opens new doors to investigate the endogenous mechanisms that regulate the self-renewal, activation, proliferation, and differentiation of skeletal stem cells and exploit these mechanisms to enhance the regeneration and repair of periodontal structures. Therefore, studying mouse periodontal PRX1 expressing cells may provide significant information for the development of novel and more effective therapeutic approaches to periodontal regeneration in humans.

Our post-natal lineage tracing study of PRX1+ cells in adult mice demonstrated that PRX1 expressing cells are mainly reside in the pulp and periodontal ligament of the incisor teeth. The mouse incisors, unlike the molars, erupt continuously throughout the life of the animal (Harada et al., 2002). This continuous eruption requires the continuous remodeling of the periodontal ligaments in order to adapt to this function (Marks and Schroeder, 1996, Rooker et al., 2010). The periodontal ligament is believed to remodel through a population of multipotent stem cells that have the ability to differentiate into cementoblast, osteoblast, and fibroblasts (Lin et al., 2008). However, the location of the periodontal stem cell niches that contribute to the continuous remodeling of periodontal ligament, and the molecular mechanisms that regulate the self-renewal, proliferation, and differentiation of these cells have not been identified. The presence of PRX1 expressing cells in the periodontal ligament of the incisor teeth is consistent with the fact
that periodontal ligament around the incisor teeth goes through continuous periodontal remodeling, and it suggests that these cells may have key roles in the remodeling of periodontal ligament and maintaining periodontal homeostasis.

We aimed to characterize the PRX1 expressing cells isolated from the periodontal ligament of mouse incisor teeth by identifying their gene expression patterns. We found that these cells highly express markers of mesenchymal progenitor and stem cells such as \textit{Cd44}, \textit{Pdgfra}, \textit{Smo}, \textit{Gli1}, and \textit{Kit}, but the expression of \textit{Sca1} gene, which is a marker of hematopoietic stem cells, was undetectable in these cells. These findings are in accordance with previously published studies that have shown PRX1+ cells are a sub-population of mesenchymal stem cells (Wilk et al., 2017, Kawanami et al., 2009).

The results of the gene expression signature study also demonstrated that Wnt signaling inhibitors are highly expressed in the PRX1+ cells. We found that the expression of Dickkopf Wnt Signaling Pathway Inhibitor 1 (\textit{Dkk1}) was only detected in the PRX1+ cells, and the levels of expression of \textit{Axin2} and WNT Inhibitory Factor 1 (\textit{Wif1}), which are both Wnt signaling inhibitors, were significantly higher in the PRX+ cells compared to the PRX1- cells. The Wnt signaling pathway plays a key role in regulating the self-renewal and differentiation of mesenchymal stem cells (Ling et al., 2009). It has been shown that the low levels of Wnt signaling promote the proliferation and self-renewal of mesenchymal stem cells, while the high levels of Wnt signaling promote osteogenesis and differentiation of mesenchymal stem cells (Ling et al., 2009, De Boer et al., 2004). Hence, the down-regulation of Wnt signaling in the PRX1 expressing cells seems to promote their self-renewal and stemness maintenance.

Notch signaling and BMP signaling are important pathways that contribute to the regulation of
stem cells fate. Notch signaling mediates signals exchanged between neighboring cells to control the cell responds to intrinsic and extrinsic stimuli and to regulate cell fate decisions (Artavanis-Tsakonas et al., 1999, Hilton et al., 2008). The gene expression analysis of the PRX1 expressing cells showed that genes encoding Notch ligands (Dll1, Dll4, and Jagged-1) and Notch receptor (Notch 2) were highly expressed in the PRX1+ cells, which indicates that the Notch signaling is up regulated in the PRX1 expressing cells. It has been demonstrated that up-regulation of the Notch signaling pathway plays a key role in maintaining the pool of mesenchymal progenitor and stem cells by suppressing osteogenic differentiation (Hilton et al., 2008). Thus, up-regulation of Notch signaling system in the PRX1 expressing cells is in accordance with the down-regulation of the Wnt signaling in these cells, both promoting maintenance of stemness in the cells. On the other hand, our gene expression data indicate that BMP signaling pathway is up regulated in the PRX1 expressing cells. The level of expression Bmp2 was significantly greater in the PRX1+ cells than the PRX1- cells, while Noggin (Nog), which is an antagonist for BMP signaling, was found to be expressed at significantly lower levels in the PRX1+ cells compared to the PRX1- cells. Expression of Bmp2 is important for the commitment of mesenchymal stem cells to osteogenesis (Lin and Hankenson, 2011), while Noggin induces adipogenesis in mesenchymal stem cells (Sawant et al., 2012). Although the high level of expression of Bmp2 facilitates osteogenic differentiation, it appears that PRX1 expressing cells maintain their stemness and self-renewal capability due to the down-regulation of Wnt signaling and the up-regulation of Notch signaling.

We also found that Ezh1 and Foxo3 genes, which are involved in stem cell homeostasis (Cheung and Rando, 2013, Renault et al., 2009), are highly expressed in the PRX1 expressing cells. Foxo3 gene encodes Forkhead box protein O3 that is activated in the response to oxidative
stresses in order to diminish the levels of reactive oxygen species, which is necessary for the maintenance of the self-renewal capability of stem cells (Wang et al., 2013). Both Ezh1 and Foxo3 genes have shown to be up regulated in the quiescent stem cells (Cheung and Rando, 2013), suggesting the quiescent state of the PRX1 expressing cells isolated from the PDL of the mouse incisor.

Adhesion molecules including cadherins and integrins regulate cell-to-cell interactions and influence several aspects of cellular function such proliferation, differentiation, and apoptosis (Alimperti and Andreadis, 2015, Giancotti and Ruoslahti, 1999). We found that the genes encoding Cadherin-2 and Integrin beta-1 are expressed in significantly higher levels in the PRX1+ cells compared to the PRX1- cells. Both Cadherin-2 and Integrin beta-1 have key roles in MSC differentiation. High levels of expression of Integrin beta-1 and Cadherin-2 are associated with osteogenic differentiation, while low levels of expression of these adhesion molecules promote adipogenesis (Ferrari et al., 2000, Alimperti and Andreadis, 2015, Marie et al., 2014, Globus et al., 2005). It is has been shown that the level of expression of Cadherin-2 is up regulated by parathyroid hormone (Alimperti and Andreadis, 2015); therefore, administration of parathyroid hormone might be a viable approach to externally manipulate the expression of Cadherin-2 in order to promote differentiation of the PRX1 expressing cells.

The gene encoding parathyroid hormone/parathyroid hormone-related peptide receptor was expressed in extremely high levels in the PRX1 expressing cells. This finding indicates that the PRX1 expressing cells are highly responsive to the parathyroid hormone (PTH) and parathyroid hormone-related peptide. Regulatory function of PTH in modulating calcium and phosphate homeostasis is well established (Brunner et al., 2008). In addition to this regulatory function, it has been reported that PTH mediates the survival and self-renewal of stem cells through the
Notch signaling (Weber et al., 2006, Brunner et al., 2008). It has also been shown that human PDL cells respond to PTH stimuli with changes in the proliferation, survival, and differentiation (Lossdörfer et al., 2005, Lossdörfer et al., 2006). Furthermore, PTH regulates production of Periostin (Fortunati et al., 2010, Bonnet et al., 2012), an extracellular matrix protein found in periodontal ligament, that plays a key role in periodontal hemostasis by remodeling collagen matrix (Rios et al., 2008, Yamada et al., 2014). Hence, manipulating the PRX1 expressing cells using PTH might be a feasible approach to harness the regenerative potential of the endogenous stem cells.

The width of periodontal ligament is strictly regulated in the periodontium, and the maintenance of the width of periodontal space is considered as the main indicator of periodontal homeostasis (Beertsen et al., 1997, McCulloch et al., 2000, Lim et al., 2014). Our study demonstrated that the width of periodontal ligament increased in incisor and molar teeth after the ablation of the PRX1 expressing cells. The magnitude of this increase in the width of PDL was greater around the incisor teeth. This finding indicates that the PRX1 expressing cells have an active role in the maintenance of the periodontal homeostasis. The mechanisms and molecular signaling that regulate the width of periodontal ligament and maintenance of periodontal homeostasis are not known (Lim et al., 2014, Lim et al., 2015). It has been demonstrated that the manipulation of Wnt signaling pathway disrupts periodontal homeostasis by affecting the expression of osteogenic genes (Lim et al., 2015). It has been suggested that the maintenance of the width of periodontal ligament is mediated by a balance between formation and remodeling of PDL fibers by fibroblasts and bone formation and remodeling by osteoblasts and osteoclast in respond to mechanical forces or other external stimuli (McCulloch et al., 2000). Periodontal ligament progenitor/stem cells are believed to undergo extensive turnover in order to maintain of this
balance (McCulloch et al., 2000, Beertsen et al., 1997). We speculate that the increase in the width of periodontal ligament after the ablation of the PRX1 expressing cells in the present study is a direct result of the absence of these skeletal stem cells, which may affect the differentiation of progenitor cells to fibroblasts and osteoblasts as well as the turnover of these cells.

The results of the present study showed that sub-critical fenestration periodontal defects regenerated spontaneously in the presence of PRX1 expressing cells, but these defects did not regenerate by the incisor teeth in the absence of the PRX1 expressing cells. This finding indicates that PRX1 expressing cells are required for regeneration of periodontal defects in the continuously regenerating periodontal ligament of mouse incisor. This finding is in line with the result of our post-natal lineage tracing study that exhibited the PRX1 expressing cells are mainly located in the periodontal ligament of the incisor teeth. We also observed that the sub-critical periodontal defects around the molar teeth healed even after ablation of PRX1 expressing cells; however, this healing was accompanied by excessive and irregular bone formation in 4 out of 5 cases. The difference between the incisor teeth and molar teeth in the healing of the defects after ablation of the PRX1 expressing cells might be attributable to the fact that the need for proliferation and differentiation of PRX1 expressing cells are greater in the mouse incisor due to the continuous regeneration of periodontal ligament (Rooker et al., 2010). It is possible that, after ablation of the PRX1 expressing cells, the remaining progenitor cells that previously derived from the PRX1 expressing cells contribute to the regeneration of the sub-critical periodontal defects around the molar teeth.

Stem cell-like populations have been previously isolated from the periodontal ligament (Seo et al., 2004, Gay et al., 2007). In this project, we demonstrated that PRX1 expressing cells, which are a sub-population of skeletal stem cells, are present in the both human and mice periodontal
ligament, and at such location they contribute to periodontal formation, periodontal homeostasis, and periodontal regeneration. The challenge with mesenchymal stem cells is that they are present in low abundance in the post-natal human tissues, and their self-renewal capacity decreases in respond to the aging and stress (Orford and Scadden, 2008, Rooker et al., 2010). Although there are differences between the continuously regenerating periodontal ligament of mouse incisor and the periodontal ligament in human teeth, the presence of the PRX1 expressing in high abundance in the periodontal ligament of mice incisor may allow us to study and understand the regulatory mechanisms that control self-renewal, proliferation, and differential of these cells. These mechanisms then could be exploited to expand the limited population of adult stem cells in human periodontium and harness their endogenous regenerative potential.
CONCLUSIONS AND FUTURE DIRECTIONS

Presence of PRX1 expressing cells, which are a sub-population of skeletal stem cells, within human periodontal ligament is revealed in this study for the first time. In mouse, our lineage tracing study demonstrated that the PRX1 expressing cells are present in high abundance within the continuously regenerating periodontal ligament of incisor teeth. The gene expression signature study of the PRX1 expressing cells isolated from mouse incisors demonstrated the expression mesenchymal stem cells markers, up-regulation of Notch signaling, and down regulation of Wnt signaling in these cells. We found that the PRX1 expressing cells contribute to the development of periodontal tissue and to the post-natal periodontal hemostasis. In addition, the PRX1 expressing cells are required for the regeneration of periodontal defects in the continuously regenerating periodontal ligament of mouse incisors. This project represents an initial step towards future investigations aimed to identify novel and more effective therapeutic approaches for the regeneration of periodontal structures in humans.

The present model for periodontal regeneration provides an invaluable tool to future drug discovery and molecular mechanism studies. Future studies are needed to investigate the regulatory mechanisms and molecular signals that mediate self-renewal, proliferation, and differential of the PRX1 expressing cells. We envision understanding these mechanisms would allow us to enhance the self-renewal, proliferation, and differential of these cells in human periodontium. Moreover, considering the presence of PRX1 expressing cells in the dental pulp of mouse incisors, studying these cells in such location may lead to new discoveries regarding pulp regeneration. In addition, future studies should investigate the use of external stimuli, such as parathyroid hormone or parathyroid hormone-related peptide, to manipulate the self-renewal,
proliferation, or differential of these cells in order to expand the population of these endogenous stem cells and harness their regenerative potential for periodontal regeneration.
REFERENCES


FODOR, W. L. 2003. Tissue engineering and cell based therapies, from the bench to the clinic: the potential to replace, repair and regenerate. *Reproductive Biology and Endocrinology*, 1, 102.


FRIEDEWALD, V. E., KORNMAN, K. S., BECK, J. D., GENCO, R., GOLDFINE, A., LIBBY, P., OFFENBACHER, S., RIDKER, P. M., VAN DYKE, T. E. & ROBERTS, W. C.


MACHOLD, R., HAYASHI, S., RUTLIN, M., MUZUMDAR, M. D., NERY, S., CORBIN, J. G., GRITLI-LINDE, A., DELLOVADE, T., PORTER, J. A., RUBIN, L. L., DUDEK, H.,


ROSEN, V. 2009. BMP2 signaling in bone development and repair. *Cytokine & growth factor reviews*, 20, 475-480.


