Perivascular Niche and Self-renewal of Dental Pulp Stem Cells

by

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Dedication

“We are troubled on every side, yet not distressed; we are perplexed, but not in despair;

Persecuted, but not forsaken; cast down, but not destroyed.” 2 Corinthians 4:8-9.

To my parents, Tae-Ju Oh and Jooyouen Kim, for their unwavering support and love.

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Abstract

Interactions with the microenvironment modulate the fate of stem cells in perivascular niches in tissues (*e.g.* bone) and organs (*e.g.* liver). However, the functional relevance of the molecular crosstalk between endothelial cells and stem cells within the perivascular niche in dental pulps is unclear. Here, we tested the hypothesis that endothelial cell-initiated signaling is necessary to maintain self-renewal of dental pulp stem cells. First, we show preferential localization of cells that express high levels of self-renewal stem cell markers (*i.e.* ALDH1 and Bmi-1) in physiological human dental pulps. Then, through an *in vitro* functional assay for self-renewal (*i.e.* secondary orosphere assay), we show that endothelial cell-derived factors promote self-renewal of dental pulp stem cells. Mechanistic studies demonstrated that endothelial cell-derived Interleukin (IL)-6 induces expression of Bmi-1 in dental pulp stem cells through the STAT3 signaling pathway. STAT3-silenced dental pulp stem cells (stably transduced with shRNA-STAT3) seeded in biodegradable scaffolds and transplanted into immunodeficient mice generated fewer perivascular niches comprised of endothelial cells and stem cells exhibiting features of self-renewal. *In vitro* capillary sprouting assays revealed that inhibition of IL-6 or STAT3 signaling decreases the vasculogenic potential of dental pulp stem cells, suggesting its importance in the establishment of the perivascular niche. Collectively, these data demonstrate that endothelial cell-derived factors mediate self-renewal of dental pulp stem cells through STAT3 signaling and induction of Bmi-1. Overall, these data suggest that a

crosstalk between endothelial cells and stem cells within the perivascular niche is required for the maintenance of stem cell pools in dental pulp.

Chapter I

Introduction

Postnatal stem cells are typically found in niches that provide signaling cues to maintain their self-renewal and multipotency. While stem cell populations may serve distinct purposes within their tissue of origin, understanding the conserved biology of stem cells and their respective niches provides insights to the behavior of these cells during homeostasis and tissue repair. Here, we discuss perivascular niches of two distinct stem cell populations (*i.e.,* hematopoietic stem cells, mesenchymal stem cells) and explore mechanisms that sustain these stem cells postnatally. The cellular crosstalk between stem cells and other cells of the niche appear to play important functions, particularly in regard to maintenance of stem cell self-renewal. We highlight work that demonstrates the impact of cellular crosstalk to stem cell self-renewal and maintenance of functional perivascular niches. We also discuss the importance of the crosstalk within the perivascular niche to the biology of stem cells and describe the regenerative potential of perivascular cells. We postulate that signaling events that establish and/or stabilize the perivascular niche, particularly through the modulation of self-renewing factors, are key to the long-term success of regenerated tissues.

Physiological stem cells enable tissue regeneration and repair. It was postulated that knowledge generated through research guided towards the regeneration of living tissues could lead to the cure of certain congenital and hereditary disorders, as well as to

the development of strategies for tissue engineering that could address the shortage of donor tissues/organs (Vacanti et al. 1999). Successful regeneration of living tissues and/or organs that integrate functionally and properly within the host could also improve or extend the patients' quality of life. Here, we highlight the role of postnatal stem cell populations in tissue repair and regeneration, with focus on their microenvironment (*i.e.,* their niche). Stem cells are maintained in specialized niches, where they are relatively quiescent until external signals (*e.g.,* wound) disrupt this equilibrium and drive their fate through downstream lineages that result in fully differentiated cells. Understanding the biology of these stem cell niches and mechanisms that drive stem cell fate can ultimately provide insights into potential signaling targets that can be exploited to regenerate functional tissues.

Hematopoietic Stem Cells

Seminal work led to the identification of hematopoietic progenitor populations that are multipotent and self-renewing (Thomas et al. 1957; Till et al. 1961; Morrison et al. 1997). Thomas and colleagues introduced the concept of utilizing hematopoietic stem cells (HSCs) for regenerative medicine when they intravenously infused cell suspensions of bone marrow into patients that underwent radiation and chemotherapy (Thomas et al. 1957; Thomas et al. 1975). These initial findings led to the development of bone marrow transplantation that is now commonly utilized to repopulate the bone marrow and the several cell types that comprise the blood (Thomas et al. 1957; Thomas et al. 1975). In association with the discovery of HSCs within the bone marrow, these findings contributed significantly to the development of utilizing multipotent and self-renewing cell populations for regenerative medicine (Till et al. 1961). The long-term survival rates of patients, and

the existence of donor cells within the bone marrow of long-term survivors, provided compelling evidence that self-renewing cells resided within the bone marrow (Storb et al. 1969; Thomas et al. 1975). Furthermore, evidence of spleen colony-forming cells, derived from stem cell populations, suggested that stem cells reside in “niches” (Becker et al. 1963; Siminovitch et al. 1963; Schofield 1978).

Several studies exhibited evidence of a perivascular niche for the maintenance of HSCs *in vitro* and *in vivo* (Cardier and Barberá-Guillem 1997; Ohneda et al. 1998; Li et al. 2004; Kiel et al. 2005; Yao et al. 2005; Ding et al. 2012; Corselli et al. 2013). Kiel and colleagues concluded that HSCs within the spleen and bone marrow were associated with the sinusoidal endothelium, suggesting a perivascular niche (Kiel et al. 2005). Others demonstrated that HSCs localize to heterogeneous vascular niches in the bone marrow including arteries and arterioles and suggested that these niches regulate HSC quiescence (Bourke et al. 2009; Kunisaki et al. 2013; Nombela-Arrieta et al. 2013). Indeed, the evidence illustrated that these HSC perivascular niches are comprised of various cell types, each possessing a distinct function to contribute HSC maintenance. For instance, within the bone marrow, the HSC perivascular niche receives significant contributions from mesenchymal stromal cells via secreted factors, including stem cell factor (SCF) and C-X-C motif chemokine 12 (CXCL12) (Sugiyama et al. 2006; Méndez-Ferrer et al. 2010; Greenbaum et al. 2013). Notably, the evidence demonstrated that endothelial cells which make up these perivascular niches are crucial for the maintenance of HSC.

Endothelial cell-derived factors enabled HSC to produce a significantly higher number of spleen colony forming units (CFU-S8) counts when compared to controls,

suggesting a higher HSC subpopulation when exposed to endothelial cell-secreted factors (Li et al. 2004). Further investigation into adult HSC maintenance utilizing conditional knockouts confirmed the existence of a perivascular niche for HSC (Ding et al. 2012). When Ding and colleagues utilized a tamoxifen-inducible conditional knockout system for stem cell factor (SCF) (Ubc-cre^{ER}; Scf$^{fl/fl}$), the HSC population (CD150^{+}CD48^{-} Lin^{-}Sca1^{+}c-Kit^{+}) was depleted within the bone marrow and spleen postnatally (Ding et al. 2012). As SCF is a ligand for c-Kit, and inhibiting c-Kit resulted in the loss of hematopoietic progenitor cells, these results suggested that SCF is required for postnatal HSC maintenance (Ding et al. 2012; Zsebo et al. 1990; Ogawa et al. 1991). Furthermore, when they conditionally deleted SCF from endothelial cells (Tie2-Cre; Scf$^{fl/-}$), the HSC frequency decreased significantly (Ding et al. 2012). Yao and colleagues demonstrated that when they selectively delete gp130 expression in endothelial cells, utilizing Cre/loxP-mediated recombination (Tie2-Cre;gp130$^{flox/flox}$), progenitor cells did not produce HSC cells in long-term cultures (Yao et al. 2005). Notably, emerging studies showed that endothelial cells are necessary for hematopoietic homeostasis and regeneration, suggesting that signaling events involving crosstalk with endothelial cells play a major role in the maintenance of postnatal hematopoietic stem cells (Hooper et al. 2009; Butler et al. 2010; Kobayashi et al. 2010; Poulos et al. 2013).

Mesenchymal Stem Cells

Friedenstein and colleagues discovered non-hematopoietic stem cells adherent to tissue culture conditions capable of forming fibroblastic colony forming units (CFU-F) (Friedenstein et al. 1970). Later coined as “mesenchymal stem cells” (MSCs), these cell populations were self-renewing and were capable to give rise to multiple lineages (Caplan

1991; Prockop et al. 1997; Pittenger et al. 1999; Bianco 2007; Sacchetti et al. 2007). However, inconsistencies in defining MSCs presented various challenges to investigators within the field (Dominici et al. 2006). New arising evidence showed that perivascular cells within the bone marrow exhibited characteristics of MSCs, suggesting the existence of a perivascular niche (Sacchetti et al. 2007; Méndez-Ferrer et al. 2010).

Perivascular cells were further investigated in various fetal and postnatal human tissues, and characterizing studies identified these populations to be MSC-like (da Silva Meirelles et al. 2006; Zannettino et al. 2008; Crisan et al. 2008; Paul et al. 2012). Utilizing flow cell sorting, perivascular cells expressed MSC markers (*e.g.,* CD10, CD13, CD44, CD73, CD90, CD105) and did not express several markers for other cell types (*e.g.,* CD56, CD106, CD133) (Crisan et al. 2008). Several studies proposed that pericytes exhibit the potential to commit to osteogenic, chondrogenic, and/or adipogenic lineages (Doherty et al. 1998; Farrington-Rock et al. 2004). Notably, long-term cultured perivascular cells possessed the ability to differentiate into MSC lineages, including chondrocytes, multilocular adipocytes, and osteocytes (da Silva Meirelles et al. 2006; Crisan et al. 2008). Isolated pericytes formed mineralized nodules and structures resembling chondrocytes and adipocytes both *in vitro* and *in vivo* (Doherty et al. 1998; Farrington-Rock et al. 2004). Furthermore, mRNA analysis of pericytes cultured in either chondrogenic or adipogenic conditions showed an upregulation of chondrogenic (*i.e.,* Type II collagen, Sox9, aggrecan) and adipogenic (*i.e.,* peroxisome proliferator-activated receptor gamma [PPAR-γ]) genes, respectively (Farrington-Rock et al. 2004). Further investigation into MSC-like subpopulations in various tissues led to its identification and characterization within dental tissues.

A perivascular niche was identified in postnatal MSC populations within dental tissues, particularly the dental pulp (Shi et al. 2003; Machado et al. 2016). Indeed, cells residing near the endothelium exhibited multipotency and self-renewal (Figure 1.1). Seminal work by Shi and colleagues utilized the putative MSC marker Stro-1 to isolate MSC subpopulations within the bone marrow and dental pulp and to verify the potential existence of perivascular niches in these two tissues (Shi et al. 2003). When Stro-1 positive bone marrow stem cells (BMSC) and dental pulp stem cells (DPSC) were analyzed, they showed expression of pericyte markers (α-smooth muscle actin, CD146) but not von Willebrand factor, a marker for platelets and endothelial cells (Shi et al. 2003). These findings suggested that these stem cell populations may reside in a perivascular niche and/or have the capacity to differentiate into other cell populations (Shi et al. 2003).

As the origin and behavior of MSC become better understood, more specific cellular markers can be identified and utilized to isolate MSC for their investigation and/or clinical application. Interestingly, recent studies explored potential markers that are more specific to MSC populations. Lineage tracing studies on $Gli1^+$ cells within a murine incisor pulp suggested that Gli1 may be a possible marker for mesenchymal stem cells (Zhao et al. 2014). Utilizing an inducible tagging construct (Gli1-CE;Zsgreen) to follow $Gli1^+$ cells and their progenitors, $ZsGreen^+$ cells were expressed near the cervical loop of mouse incisors within 72 hours. After 4-weeks, $ZsGreen^+$ cells populated the entire pulp mesenchyme up to the tip of the incisor suggesting that $Gli1^+$ cells were responsible for populating the dental pulp. Indeed, $ZsGreen^+$ cells were still detected when following $Gli1^+$ derivatives for up to 17.5 months, suggesting self-renewal of $Gli1^+$ mesenchymal cells (Zhao et al. 2014). Furthermore, $Gli1^+$ cells are expressed in networks around the

vasculature, and $Gli1^{+}PDGFR\beta^{+}$ (platelet derived growth factor receptor-β) cells represent MSC-like perivascular cells (Zhao et al. 2014; Kramann et al. 2015). The surface marker profile of these MSC-like perivascular cells was maintained both in culture and *in vivo* and exhibited multipotential capacity through several MSC lineages (Kramann et al. 2015). Interestingly, as murine incisors develop continuously, dentinal development was severely stunted after vascular damage to the dental pulp, indicating the importance of the perivascular niche (Zhao et al. 2014). These findings further suggested that MSC are housed and maintained near the host vasculature within perivascular niches.

Recent evidence suggested that DPSC reside in close proximity to blood vessels and nerves in the dental pulp tissue, as determined by expression of the putative stem cell markers aldehyde dehydrogenase (ALDH)-1, CD90 and Stro-1 (Machado et al. 2016). Notably, DPSC formed spheroid bodies, suggesting the existence of a self-renewing subpopulation within these DPSC cells (Xiao et al. 2014). As self-renewing cells form and sustain growth of spheroid bodies in a three-dimensional culture system, evidence from other fields suggested that endothelial cell-derived factors may promote the growth of self-renewing cells, including DPSC (Reynolds et al. 1996; Weiss et al. 1996; Krishnamurthy et al. 2010). In addition, studies involving cancer stem cells inferred that the expression Bmi-1, a marker of self-renewal, might be induced when stem cells are exposed to endothelial cell-secreted factors. Collectively, these data demonstrated that endothelial cells serve as a source of factors that stimulate self-renewal of mesenchymal stem cells in the dental pulp, thus playing a critical role on the maintenance of the mesenchymal stem cell pool within perivascular niches.

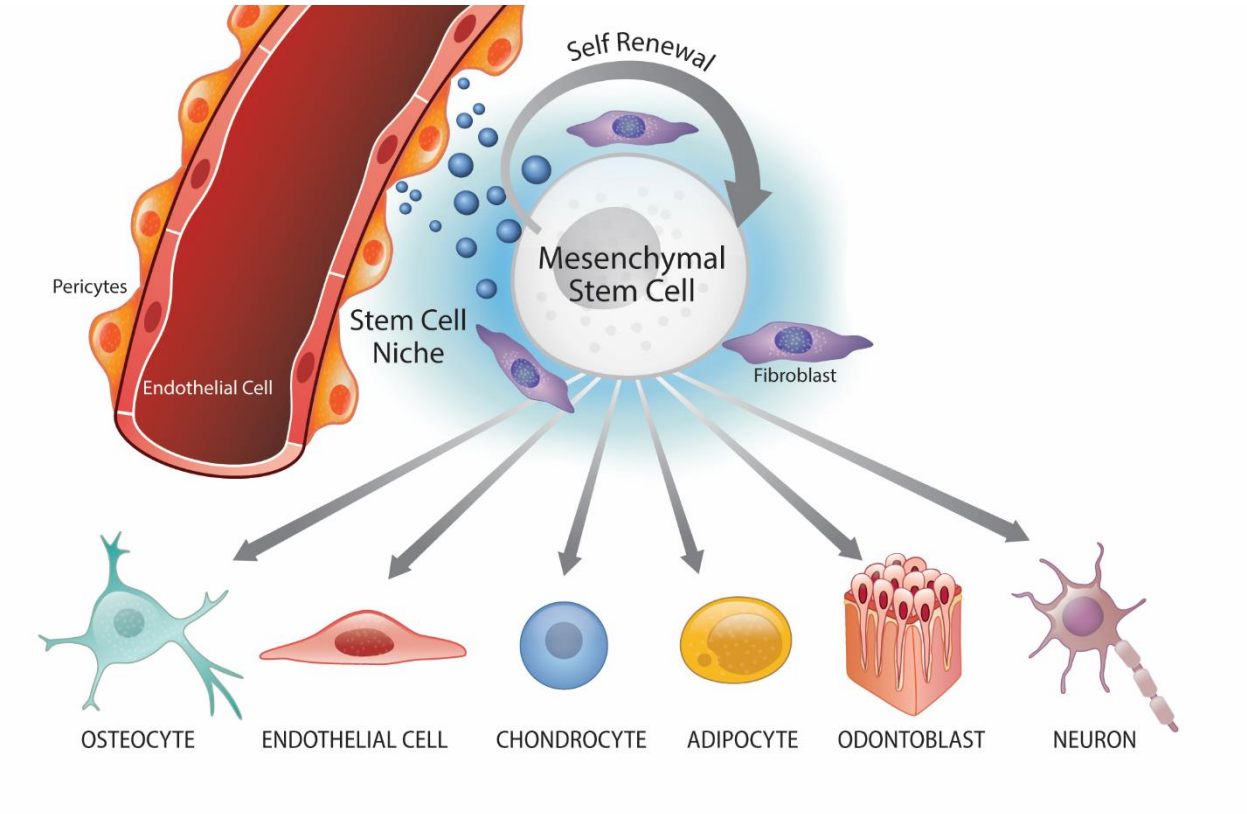

Figure 1.1. Perivascular niche and multipotency of mesenchymal stem cells (MSC). Mesenchymal stem cells reside in perivascular niches where they undergo self-renewal and maintain the surrounding cells/tissue. Under specific signaling conditions, MSC can undergo differentiation into the osteoblastic (osteocytic), endothelial, chondrocytic, adipocytic, odontoblastic, and neural lineages (Oh and Nör, 2015).

Stem Cell Niches and Dental Tissue Regeneration

While the presence of differentiated cells is critical for the function of tissues that have been regenerated, the ability to reconstitute the microenvironment that sustains stem cells is likely critical for the successful long-term outcome of the tissue. In fact, it is possible that creation (or regeneration) of the stem cell niche might be sufficient for effective tissue regeneration. Targeting the perivascular niche via regeneration of the vasculature

exhibited promising results in the context of dental pulp tissue engineering. A dentin/pulp-like complex was regenerated *in vivo* utilizing a tooth slice/scaffold model of dental pulp regeneration, where tooth slice/scaffolds from human third molars were seeded with (stem cells from exfoliated deciduous teeth (SHED) and human dermal microvascular endothelial cells (HDMEC) and transplanted into the subcutaneous space of severe combined immunodeficient (SCID) mice (Cordeiro et al. 2008; Sakai et al. 2010; Bento et al. 2013). Interestingly, when SHED cells were treated with recombinant human vascular endothelial growth factor ($rhVEGF_{165}$) within tooth slice/scaffolds *in vitro*, they showed increased angiogenic potential and expressed higher levels of endothelial cell markers (*e.g.*, VEGFR2, PECAM1) (Sakai et al. 2010). These data suggested that these cells had the potential to differentiate into vascular endothelial cells in addition to the expected differentiation into odontoblasts. Notably, we observed that SHED cells have the potential to differentiate into endothelial cells *in vivo*, forming functional blood vessels that anastomosed with the host vasculature becoming functional (blood-carrying) vessels (Cordeiro et al. 2008; Sakai et al. 2010; Bento et al. 2013). Such data suggested that, under the appropriate conditions, a sub-population of the dental pulp stem cells can differentiate into tissue-specific odontoblasts while other sub-population may differentiate into vascular endothelial cells recreating the perivascular niche for stem cells in the regenerated tissue.

Recent evidence on the effects of endothelial cell-derived factors on head and neck squamous cell carcinoma (HNSCC) cells provided valuable insights on the cellular crosstalk within the perivascular niche. Endothelial cell-derived epidermal growth factor (EGF) promoted epithelial-mesenchymal transition (EMT) of HNSCC cells, endowing

them with cancer stem cell characteristics (Zhang et al. 2014). As EMT has been linked to the generation of cells with stem cell properties, it is without surprise that endothelial cell-derived epidermal growth factor (EGF) induced self-renewal via upregulation of Bmi-1 expression (Zhang et al. 2014; Mani et al. 2008). Interestingly, endothelial cells stimulated the self-renewal of neural stem cells (Shen et al. 2004). Neural stem cells co-cultured with endothelial cells exhibited delayed differentiation, shown by expression of neural progenitor markers ($Nestin^+$ and LeX^+) and an enhanced neural productive potential (Shen et al. 2004). These data further highlighted the significance of signaling within the perivascular niche for the biology of stem cells. It suggested that endothelial cell-secreted factors might play a critical role in the genesis of stem cells.

As stem cells are both, multipotent and self-renewing, a putative approach for tissue regeneration is based on the targeting of self-renewal factors to maintain "stemness" of a sub-population of cells. Emulating the niche via controlled regulation of self-renewal pathways might allow stem cells to continue undergoing some level of asymmetric division, where one daughter cell would remain undifferentiated (*i.e.,* self-renewal) while the other daughter cell would undergo differentiation (*i.e.,* multipotency). Emerging evidence demonstrated the important role of self-renewal factors in dental tissue formation. For example, as Bmi-1 was shown to be a key regulator of neural stem cell self-renewal (Molofsky et al. 2003; Park et al. 2003; Molofsky et al. 2005), $Bmi\text{-}1^{-/-}$ mice incisors exhibited thinner dentinal and enamel layers (Biehs et al. 2013). These data illustrated that self-renewal is essential for odontogenesis and suggested that this process is likely very important within the context of dental tissue engineering. We are currently designing experiments that will test this hypothesis.

In summary, the interaction of stem cells with other cellular components of their niche is critical for self-renewal and the maintenance of the stem cell pool, and for the determination of their differentiation fate through multipotency. Mesenchymal stem cells play a vital role in the maintenance of various tissues (da Silva Meirelles et al. 2006; Crisan et al. 2008) and we strongly believe that mesenchymal stem cells and their niche are critically important in the context of dental tissue regeneration. The perivascular niche has been shown to provide key molecular cues for the maintenance of diverse stem cells populations, including physiological and pathological (*e.g.,* cancer) stem cells. These findings suggested the existence of conserved mechanisms of stem cell maintenance. While new therapeutic strategies are being developed to target the interaction of the perivascular niche with cancer stem cells as a possible approach for cancer treatment, purposeful efforts to regenerate the stem cell niche might be important for tissue engineering. Likewise, as anti-cancer therapies attempt to inhibit self-renewal of cancer stem cells (*e.g.,* therapeutic use of Bmi-1 inhibitors), it might be beneficial to induce self-renewal pathways to expand physiological stem cell populations for tissue engineering. Thus, studies focused on the understanding of conserved mechanisms regulating the biology of stem cell niches will provide valuable insights on the function and maintenance of stem cells and may have a positive impact on the development of strategies that enhance the long-term outcomes of regenerated tissues and organs.

The **hypothesis** underlying this research project is: *Endothelial cell-derived IL-6 regulates the self-renewal of dental pulp stem cells via the STAT3 pathway*

To test this hypothesis, we proposed the following **Specific Aims**:

- **Specific Aim #1:** To understand the functional impact of endothelial cell-derived IL-6 on the regulation of dental pulp stem cell self-renewal
- **Specific Aim #2:** To define the role of STAT3 signaling on the self-renewal of dental pulp stem cells
- **Specific Aim #3:** To define the role of STAT3 signaling on the establishment of perivascular niches

Chapter II

Endothelial Cell-initiated Crosstalk Regulate Dental Pulp Stem Cell Self-renewal

Introduction

Dental tissue engineering is a biologically inspired approach for replacement of tooth tissues lost to caries, infection, trauma, or developmental defects (Nakashima and Reddi, 2003). The discovery and characterization of dental pulp stem cells (DPSC) in permanent teeth and stem cells from human exfoliated deciduous teeth (SHED) inspired the development of cell-based therapies for dental pulp tissue regeneration (Gronthos et al. 2000; Miura et al. 2003; Rosa et al. 2013). These discoveries enabled clinical studies that provided initial evidence of the efficacy and safety of dental pulp stem cell transplantation for treatment of necrotic teeth (Nakashima et al. 2017; Xuan et al. 2018). Emerging evidence suggest that the long-term viability of an engineered dental pulp requires the regeneration of perivascular niches and maintenance of a stem cell pool (Oh and Nör, 2015). Indeed, to enable tissue repair of a tissue engineered with stem cells, it is necessary that some of these cells remain undifferentiated, while others differentiating into tissue-forming cells. As such, understanding mechanisms underlying the

maintenance of a dental pulp stem cell pool is critical for the translation and long-term outcome of cell-based Regenerative Endodontics.

Emerging evidence has suggested the importance of stem cell niches that maintain the self-renewing capacity and multipotency of stem cell populations postnatally (Sacchetti et al. 2007; Sugiyama et al. 2006; Zhao et al. 2018). Studies of perivascular niches in bone suggest that endothelial factors are necessary to promote self-renewal of hematopoietic stem cells (Ding et al. 2012), however their role in the maintenance of dental pulp stem cells is not understood. Thus, the objective of this study was to define the functional impact and regulatory mechanisms that mediate the crosstalk between endothelial cells and dental pulp stem cells.

Bmi-1 has been studied extensively in the context of self-renewal in numerous stem cell populations. Seminal studies have identified this highly conserved nuclear protein that encodes 324 amino acids within the zinc-finger family, critical for proper neurological, hematopoietic, and development in Bmi-1$^{-/-}$ mice (Haupt et al. 1991; van der Lugt et al. 1994). Molofsky and colleagues showed that a significant decrease in the formation of neurospheres in neural stem cells (NSC) derived from Bmi-1$^{-/-}$ mice at various developmental stages (*i.e.,* E14, P0, and P30), suggesting that Bmi-1 plays a functional role in the regulation of self-renewal (Molofsky et al. 2003). Park and colleagues showed that Bmi-1$^{-/-}$ mice possess lower frequencies of hematopoietic stem cells (HSC) and multipotent progenitors postnatally (Park et al. 2003). These results showed the conserved role of Bmi-1 in the functional regulation of stem cell self-renewal and suggested similar roles in other stem cell populations.

Further investigations in Bmi-1$^{-/-}$ mice suggest that it plays a significant role during tooth development (Biehs et al. 2013). Through the utilization of genetic lineage tracing approaches within the tooth incisor model, they showed that Bmi-1$^{-/-}$ mice have a significant tissue volume loss (28%) of the Labial Cervical Loop (LaCL), which house stem cells that give rise to ameloblasts (Biehs et al. 2013). Interestingly, they showed defective enamel and dentin development in Bmi-1$^{-/-}$ mice, suggesting a role in sourcing self-renewing progenitors that terminally differentiate into ectodermal and mesodermal tissues (Biehs et al. 2013). These results suggested the existence of a similar function of Bmi-1 in the regulation of self-renewal in human dental pulp stem cells. Through mechanistic and functional assays, we confirmed the functional role of Bmi-1 and established its role as a self-renewal marker in dental pulp stem cells.

Studies performed in different stem cell populations suggest that the Signal transducer and activator of transcription 3 (STAT3) pathway is also involved in the regulation of self-renewal. Matsuda and colleagues showed that STAT3 activation enables formation of compact colonies of mouse embryonic stem cells (Matsuda et al. 1999). In presence of leukemia inhibitory factor (LIF), a member of the Interleukin-6 family, they observed an increase in the number of colonies. In addition, these colonies were maintained in an undifferentiated state for 15 passages, suggesting their sustained self-renewal (Matsuda et al. 1999). The undifferentiated state of these colonies was confirmed through alkaline phosphatase activity and expression of stage-specific embryonic antigen-1 (SSEA-1), a marker of embryonic cell stemness (Matsuda et al. 1999; Solter and Knowles 1978). In addition, Matsuda and colleagues injected embryonic stem cells expressing albino color coat markers cultured in presence of LIF for 7-10

passages into C57BL/6 blastocysts (Matsuda et al. 1999). The resulting mice were chimeric in their coat colors, suggesting multipotentiality for the differentiation into three functional and viable germ layers (Matsuda et al. 1999). Niwa and colleagues showed that STAT3 docking sites in gp130 are required for self-renewal of mouse embryonic stem (mES) cells *in vitro* and *in vivo* (Niwa et al. 1998). Self-renewal, measured through β-galactosidase activity for *Oct-4* and alkaline phosphatase-positive colonies, was ablated *in vitro* when mutating all four tyrosine residues or two tyrosine sites (Y265 & Y275) *in vivo* (Niwa et al. 1998). The results of these studies indicate that the STAT3 pathway is necessary and sufficient for self-renewal of stem cells (Matsuda et al. 1999; Niwa et al. 1998).

Further studies investigate the mechanistic role of the STAT3 pathway in self-renewal. Bourillot and colleagues showed that downstream targets (23 of 24 studied) of STAT3 signaling play a critical role in maintaining the undifferentiated state of mES cells (Bourillot et al. 2009). Yang and colleagues showed that the STAT3 pathway is involved in the reprogramming of mES and mouse somatic stem cells (*i.e.,* neural stem cells) to an undifferentiated state (Yang et al. 2010). They showed that, through the induction of the STAT3 pathway via LIF treatment, there is a significant increase in undifferentiated colonies and expression of embryonic stem cell markers (*i.e.,* Nanog, Rex1, Klf2). Further studies from van Oosten and colleagues showed that specific induction of the STAT3 pathway via G-CSF (granulocyte colony-stimulating factor)-inducible GY118F chimeric GY118F receptor transgene resulted in Oct4-GFP-positive colonies after transduction with retroviral Oct4, Klf4, and c-Myc (van Oosten et al. 2012). Further analyses of these colonies showed activation of pluripotency genes and loss of H3K27me3, suggesting that

STAT3 signaling can be sufficient to enable reprogramming in stem cells (van Oosten et al. 2012). Withdrawing G-CSF, thus abolishing STAT3 induction, resulted in the loss of Oct4-GFP reporter activity and caused differentiation, further confirming the role of STAT3 signaling in maintaining stemness (van Oosten et al. 2012). Together, these results suggest that STAT3 is critical for the maintenance of self-renewal and pluripotency of stem cell populations.

Various intrinsic and extrinsic factors lead to the malformation and destruction of tooth structures provoking interest in the regeneration of dental structures and the dental pulp complex (Goldberg et al. 2005; Goldberg et al. 2006; Sreenath et al. 2003; Ye et al. 2004). The recent discovery and characterization of dental pulp stem cells (DPSC) and stem cells from exfoliated deciduous teeth (SHED) have enabled the exploration of their use in cell-based therapies for dental pulp regeneration (Arthur et al. 2009; Gronthos et al. 2000; Kaukua et al. 2014; Miura et al. 2003; Sasaki et al. 2011). The natural exfoliation of primary teeth, the high prevalence of premolar and third molar extractions for orthodontic needs, and the ease of access for the harvest, storage, and expansion of dental pulp stem cells enhance the feasibility of using these cells for dental tissue engineering. Thus, understanding the postnatal maintenance of dental pulp stem cells may enable optimization of approaches for cell-based Regenerative Dentistry.

Here, we tested the hypothesis that endothelial cell-derived interleukin-6 (IL-6) regulates the self-renewal of dental pulp stem cells via the STAT3 pathway. We observed that IL-6 signaling through IL-6 receptor (IL-6R) and STAT3 induce Bmi-1 and enhance the self-renewal of dental pulp stem cells. These data suggested that the endothelial-

stem cell crosstalk might enable the maintenance of stem cell pools throughout the life of a tooth.

Materials and Methods

Cell Culture

Human Dental Pulp Stem Cells (DPSC) were purchased from Lonza and Stem Cells from Human Exfoliated Deciduous Teeth (SHED) were obtained from Dr. Songtao Shi (University of Pennsylvania). DPSC and SHED were cultured in alpha-minimum essential medium (α-MEM; Invitrogen) supplemented with 20% fetal bovine serum (FBS; Invitrogen), 1% antibiotic-antimycotic (Invitrogen), and 20 μg/mL Plasmocin (InVivogen). Human dermal microvascular endothelial cells (HDMEC; Lonza) were cultured in endothelial cell growth medium (EGM-2MV; Lonza). HDMEC-conditioned medium was obtained by culturing HDMEC (80-90% confluency) in serum-free endothelial basal medium (EBM-2; Lonza) for 24 hours. The resulting solution underwent centrifugation at 3,000 rpm (10 min) and the supernatant was collected. The following compounds were utilized in mechanistic experiments: PTC-209 (Cat. #5191; Tocris); Tocilizumab (Actemra; Genentech); LY294002 (phosphoinositide 3-kinase [PI3K] inhibitor; Cat. #L-1023; A.G. Scientific); U0126 (MEK1/2 inhibitor; Cat. #9903; Cell Signaling Technologies); Stattic V (STAT3 inhibitor; Cat. #573099; Calbiochem); rhIL-6 (Cat. #PHC0061; Biosource).

Western Blots

Cells were starved overnight, pre-treated with inhibitors for 1 hour, and then induced with cytokines (or conditioned medium) after 15-minutes. DPSC/SHED were lysed in 1% Nonidet P-40 (NP-40) lysis buffer (50 mmol/L Tris-HCL, pH 7.4, 10% glycerol, 200 mmol/L NaCl, and 2 mmol/L $MgCl_2$) containing protease inhibitors. Resulting lysates were loaded onto a 9-12% SDS-PAGE gel. The transferred membranes were blocked with 5% nonfat

milk in Tris-buffered saline with Tween (TBST) for 30-minutes and incubated overnight at 4°C with the following antibodies from Cell Signaling Technologies: mouse anti-human phosphorylated STAT3 (Tyr 705; Cat. #9138), rabbit anti-human STAT3 (Cat. #9132; CST), rabbit anti-human Bmi-1 (Cat. #5856), rabbit anti-human phosphorylated Akt (Ser 473; Cat. #9271), rabbit anti-human Akt (Cat. #9272), rabbit anti-human phosphorylated ERK (Thr 202/Tyr 204; Cat. #4376), mouse anti-human ERK (Cat. #4696), or mouse anti-human GAPDH (Cat. #MAB374) from Chemicon. HRP-conjugated secondary antibodies that were affinity-purified (Jackson Laboratories) and SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) was used to visualize protein expression. Quantification of Western Blot analyses were normalized to GAPDH within the same gel lane and performed in Fiji (ImageJ) Software (Schindelin et al. 2012).

Secondary Orosphere Assay for Self-renewal

Reynolds and Weiss have established an *in vitro* assay to measure self-renewal through culturing stem cells in low attachment conditions (Reynolds and Weiss 1996). They show that neural stem cells are able to form spheres that can be passaged and expanded through multiple passages (Reynolds and Weiss 1996). This assay has been further developed and confirmed in multiple stem cell populations, including mammary stem cells and cancer stem cells, as a viable assay for self-renewal (Dontu et al. 2003; Krishnamurthy et al. 2013).

DPSC or SHED (750,000-1,000,000 cells) were cultured on ultralow attachment 75 cm^2 flasks (ULA; Cat. #3814; Corning) for 7-days with serum-free Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Life Technologies) supplemented with

10 ng/mL basic fibroblast growth factor (bFGF; Cat. #GF003AP-MG; Millipore), 10 ng/mL epidermal growth factor (EGF; Cat. #236-EG-200; R&D Systems) and 2% FBS. Media was added to flasks every 2 days. After 7 days, cells were treated with 0.05% Trypsin-EDTA (Life Technologies) and gently agitated mechanically for a single cell suspension. Cells were then plated and treated onto 6-well ULA plates (Cat. #3471; Corning) at a cell density of 10,000 cells/well. Orospheres were defined as non-adherent spheres greater than 25 cells in diameter (Krishnamurthy and Nör 2013), counted over the span of 7-10 days, and photographed under phase-contrast light microscopy.

Sulforhodamine B (SRB) Assay for Cytotoxicity

DPSC or SHED were plated on 96-well culture plates (Surface area: 0.32 cm^2) at a density of 2,000 cells/well and were incubated at 37°C overnight to allow attachment. Cells were then treated with varying doses of PTC-209 (Bmi-1 inhibitor) and Stattic V (STAT3 inhibitor) and were cultured for 72 hours. Plates were then treated with 10% Trichloroacetic acid (Cat. #T0699; Sigma) and incubated at 4°C for 1 hour. Plates were washed with deionized (DI) water and placed in a heated chamber to dry. Resulting plates were treated with 0.4% sulforhodamine B dye (Cat. #S9012; Sigma) at room temperature (RT) for 30 minutes. Plates were washed with 1% acetic acid (Cat. #A491; Fisher) and then dried in a heated chamber. Bound SRB dye was solubilized through 10 mM Trizma Base (Cat. #BP-152; Fisher) and visualized on a microplate spectrophotometer at 565 nm. Spectrophotometric readings were normalized to untreated cells at each timepoint. Results underwent two-way ANOVA grouped analyses with multiple comparisons and significance was set at $p<0.05$.

Lentiviral-mediated Gene Silencing

DPSC or SHED underwent lentiviral-mediated gene silencing via small hairpin RNA (shRNA). Briefly, HEK-293T cells were co-transfected transiently with packaging vectors psPAX2, pMD2.G, and the following constructs: pGIPZ-shRNA-Control (Vector Core, University of Michigan), pGIPZ-shRNA-STAT3 (Vector Core, University of Michigan) using the calcium phosphate method. The resulting supernatants were used to infect DPSC or SHED overnight. Cells were selected with 1 μg/mL puromycin (InVivogen) in 15% FBS-supplemented α-MEM for at least 1 week. The resulting silenced cells were cultured thereon with puromycin-supplemented media to maintain selection pressure.

Functional Sprouting Assay for Endothelial Differentiation

DPSC or SHED cells ($5x10^4$ cells/well) were seeded on standard 12-well plates coated with growth factor reduced Matrigel (BD Biosciences) and cultured with EGM2-MV medium (Lonza). The number of capillary sprouts was counted in triplicate wells per condition. Representative phase contrast microscopy images were taken at 100x magnification.

In Vivo Model of Vasculogenic Differentiation of DPSC

Highly porous, biodegradable poly-L-lactic acid (PLLA) (Boehringer Ingelheim) scaffolds were prepared, as described (Nör et al., 2001). DPSC or SHED stably transduced with shRNA-Control (n=6) or shRNA-STAT3 (n=6) (10^6 cells/scaffold) were mixed in a 1:1 ratio with Matrigel (BD Biosciences) and seeded onto PLLA scaffolds (6x6x1 mm). Resulting scaffolds were implanted into female 2-3 months-old CB.17 severe combined immunodeficient mice (SCID; Charles River) housed under specific pathogen-free (SPF)

conditions. Specimens were harvested after 28 days and fixed in 10% buffered formalin for 24 hours at 4°C and then processed by the University of Michigan School of Dentistry Histology Core. All animal experiments followed ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Kilkenny et al. 2010) and a strict protocol that was approved by the Institutional Animal Care and Use Committee of University of Michigan (approval number: PRO00007065).

Imaging and Quantification

For immunofluorescence, tissue sections were deparaffinized, rehydrated and antigen retrieval was performed in Citrate Buffer (Cat. #AP-9003-500; Thermo Scientific) with a Decloaking Chamber (Biocare Medical). Sections were incubated overnight at 4ºC with one of the following primary antibodies: rabbit anti-human CD31 (Bethyl Laboratories), rabbit anti-human Bmi-1 (Cat. #5856; Cell Signaling Technologies), or mouse anti-ALDH (Cat. # 611195; BD Transduction Laboratories). The secondary antibodies from Thermo Fisher were, as follows: Alexa Flour 488 goat anti-rabbit (Cat. #A11034); Alexa Flour 488 goat anti-mouse (Cat. #A11001); Alexa Fluor 594 goat-anti mouse (Cat. #A11032); Alexa Fluor 594 goat anti-rabbit IgG (Cat. #11037). Isotype-matched non-specific IgG was used as a negative control. Confocal images were obtained using an inverted Leica SP5 Confocal Microscope (Leica). For excitation, the 405 nm laser and the tunable white light laser were used. The detectors were set to 415-478 nm (blue), 498-550 nm (green), and 571-727 nm (red) for the spectral ranges. Channels were acquired sequentially between lines. Scanning speed was set to 50 Hz in bidirectional mode with line average set to 1 or 2 depending on the experiment. Pinhole size was set to 67 μm and images were recorded in 1024 x 1024p format. Zoom factor varied according to the area analyzed.

The distance between dental pulp stem cells and blood vessels was determined by measuring the distances between ALDH1^{high} or Bmi-1^{high} cells and nearest CD31^{+} blood vessel. The area of CD31^{+} or ALDH1^{high}Bmi-1^{high} (pixels/field) was calculated using standardized confocal microscopy images of identical zoom factor, pinhole size, excitation/detector range/intensity, and image resolution. Five random images were obtained per sample and identical threshold parameters were set to determine positivity between shRNA-Control versus shRNA-STAT3 samples in FIJI ImageJ software. The surface area of positive pixels was then measured among images. The upper/lower outlier values (3 each) were omitted prior to statistical analyses via Prism 8 software.

Statistical Analysis

Data were evaluated by t-test, one-way ANOVA, or two-way ANOVA followed by appropriate post-hoc tests using Prism v8.1.2 software (GraphPad Software). Statistical significance was determined at $p<0.05$.

Results

Dental pulp stem cells are located in perivascular niches and exhibit self-renewal

Immunofluorescence analyses of healthy human pulps demonstrated the presence of perivascular stem cell niches, with ALDH1^{high} (aldehyde dehydrogenase-1) and Bmi-1^{high} cells preferentially located in close proximity to blood vessels (Figure 2.1). Frequency distribution analyses revealed that 52.9% of all ALDH1^{high} cells are located within 15 μm of blood vessels (Figure 2.2). Indeed, ALDH1^{high} cells are located on average 14.0+/-0.6 μm and Bmi-1^{high} cells 16.3+/-1.1 μm from blood vessels (Figure 2.2, 2.3).

Self-renewing stem cells are able to form secondary spheres when cultured in suspension (Krishnamurthy et al. 2013). We observed that stem cells from human dental pulps (DPSC or SHED) are capable of forming secondary orospheres when cultured in sphere culture media in ultralow attachment conditions, demonstrating that these cells are endowed with self-renewing capacity (Figure 2.4). Western Blot analyses show increased expression of Bmi-1 (a marker of self-renewing stem cells) in dental pulp stem cells cultured in low attachment conditions, when compared to cells cultured in standard conditions (Figure 2.5). Treatment of dental pulp stem cells with nonlethal doses of the Bmi-1 inhibitor PTC-209 (Figure 2.6) results in a decrease in the number of secondary orospheres in a dose-dependent manner (Figure 2.7). These data confirmed the functional role of Bmi-1 in dental pulp stem cells as an inducer of self-renewal.

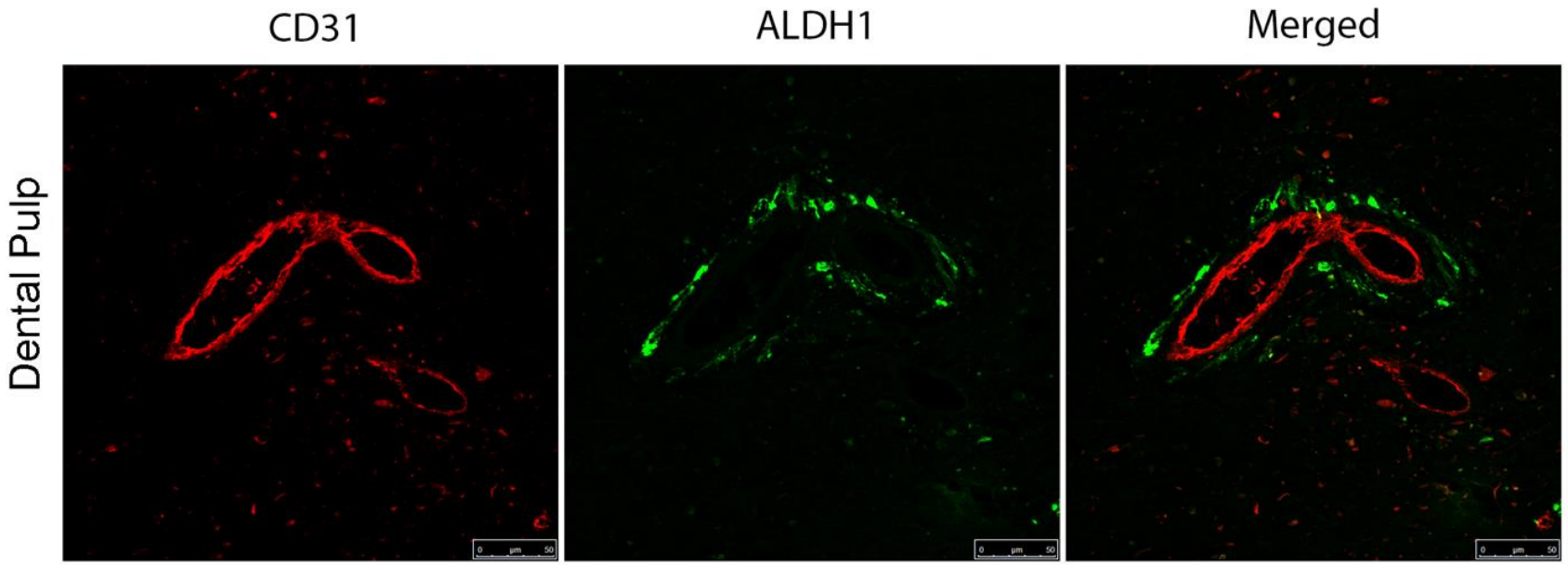

Figure 2.1. Dental pulp stem cells reside near host vasculature in human dental pulps. Confocal microscopy of human dental pulp for aldehyde dehydrogenase-1 (ALDH1) in green (488 nm) and CD31 in red (594 nm) in a heathy human dental pulp.

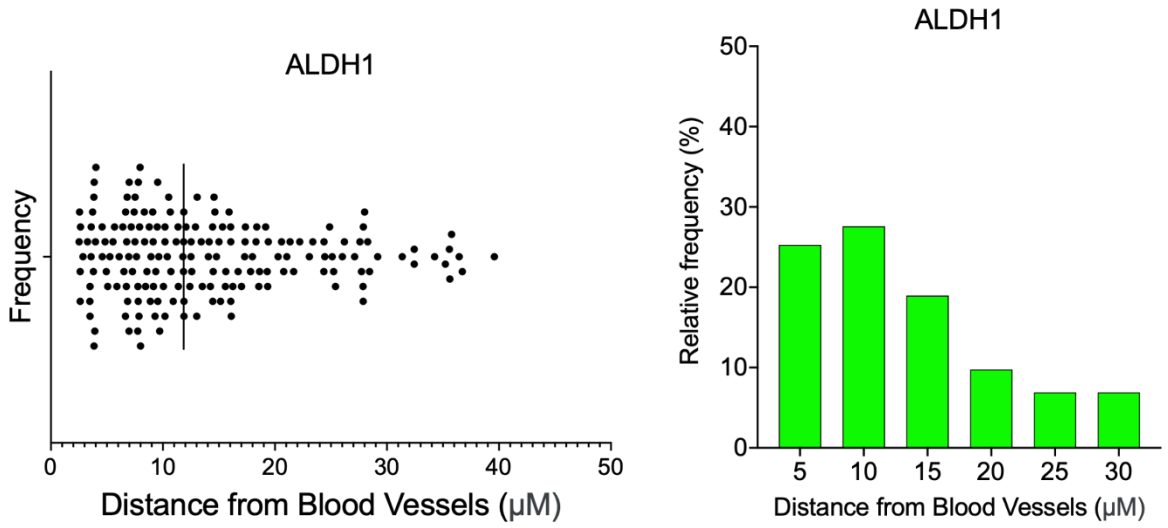

Figure 2.2. ALDH1^{high} cells reside near host vasculature in human dental pulps. [Left] Frequency distribution dot plot of the distance of ALDH1^{high} cells to nearest CD31^{+} blood vessel. Each individual point represents an ALDH1^{high} cell (n = 173); [Right] Frequency distribution (histogram up to 30μm) analysis on the distance of ALDH1^{high} cells near CD31^{+} blood vessels (n = 166).

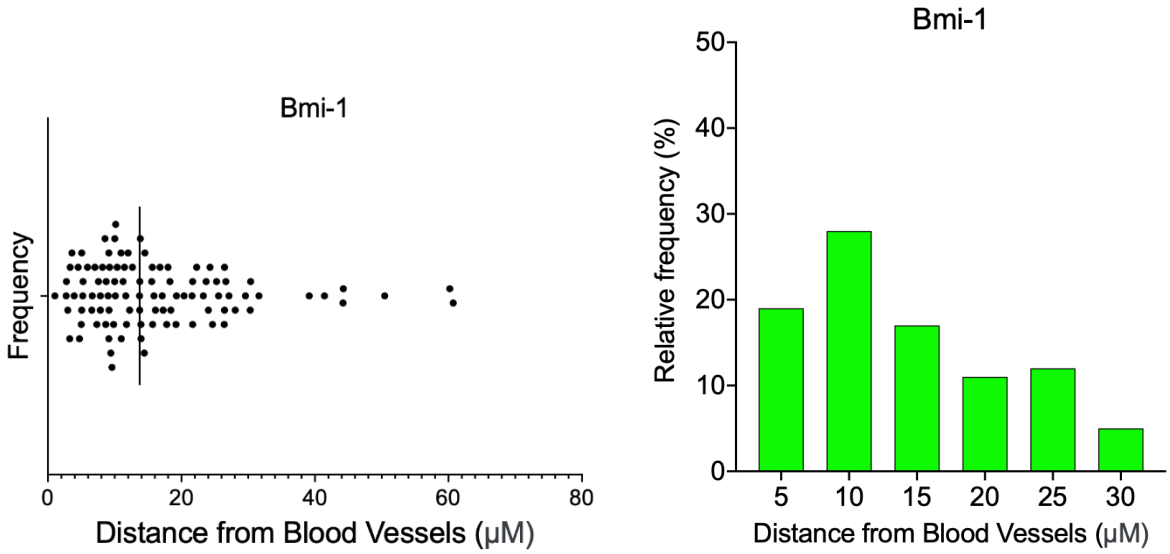

Figure 2.3. Bmi-1^{high} cells reside near host vasculature in human dental pulps. [Left] Frequency distribution dot plot of the distance of Bmi-1^{high} cells to nearest CD31^{+} blood vessel. Each individual point represents a Bmi-1^{high} cell (n = 100); [Right] Frequency distribution (histogram up to 30μm) analysis on the distance of ALDH1^{high} cells near CD31^{+} blood vessels (n = 92).

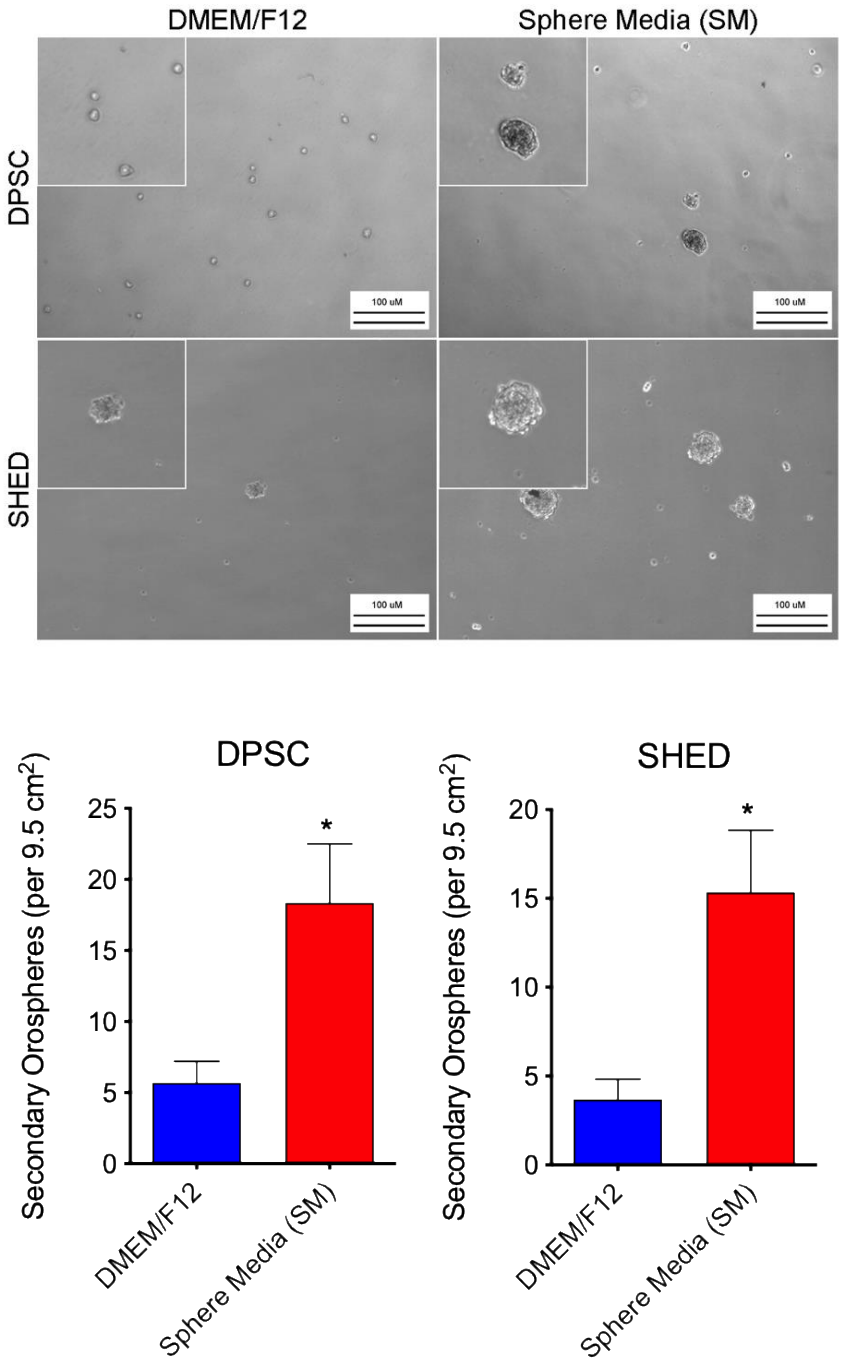

Figure 2.4. Dental pulp stem cells possess self-renewing characteristics *in vitro*. [Top] Phase contrast microscopy of DPSC/SHED secondary orospheres cultured in ultralow attachment conditions (ULA) in DMEM/F12 or Sphere Media [SM] (DMEM/F12 supplemented with 10ng/mL EGF, 10ng/mL bFGF, 2% FBS) at 7 days. [Bottom] DPSC/SHED secondary orosphere counts in 6-well ULA plates ($1x10^5$ cells/well) after 7 days. Asterisk depicts $p<0.05$.

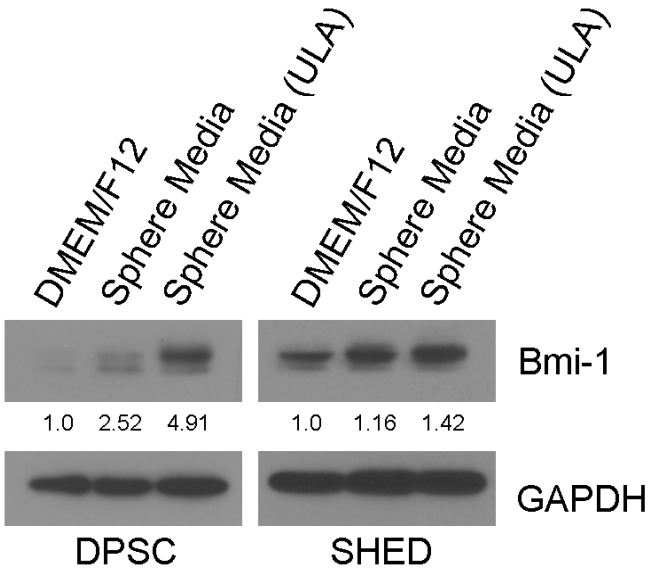

Figure 2.5. Bmi-1 expression is elevated in secondary orospheres. Western blot analysis of Bmi-1 expression (quantification normalized to GAPDH) in DPSC cultured in DMEM/F12 (regular culture conditions), SM (regular culture conditions), SM (ULA conditions) for 7 days.

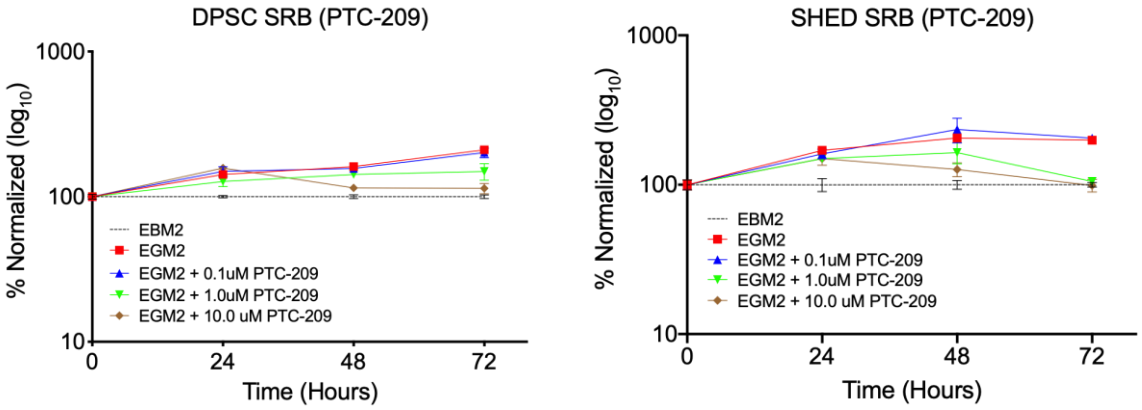

Figure 2.6. Cytotoxicity Assay for PTC-209 (Bmi-1 inhibitor) for DPSC/SHED. Sulforhodamine B colorimetric (SRB) assay for cytotoxicity on DPSC/SHED with 0.1, 1.0, or 10.0μM PTC-209 (Bmi-1 inhibitor) up to 72 hours ($p<0.05$).

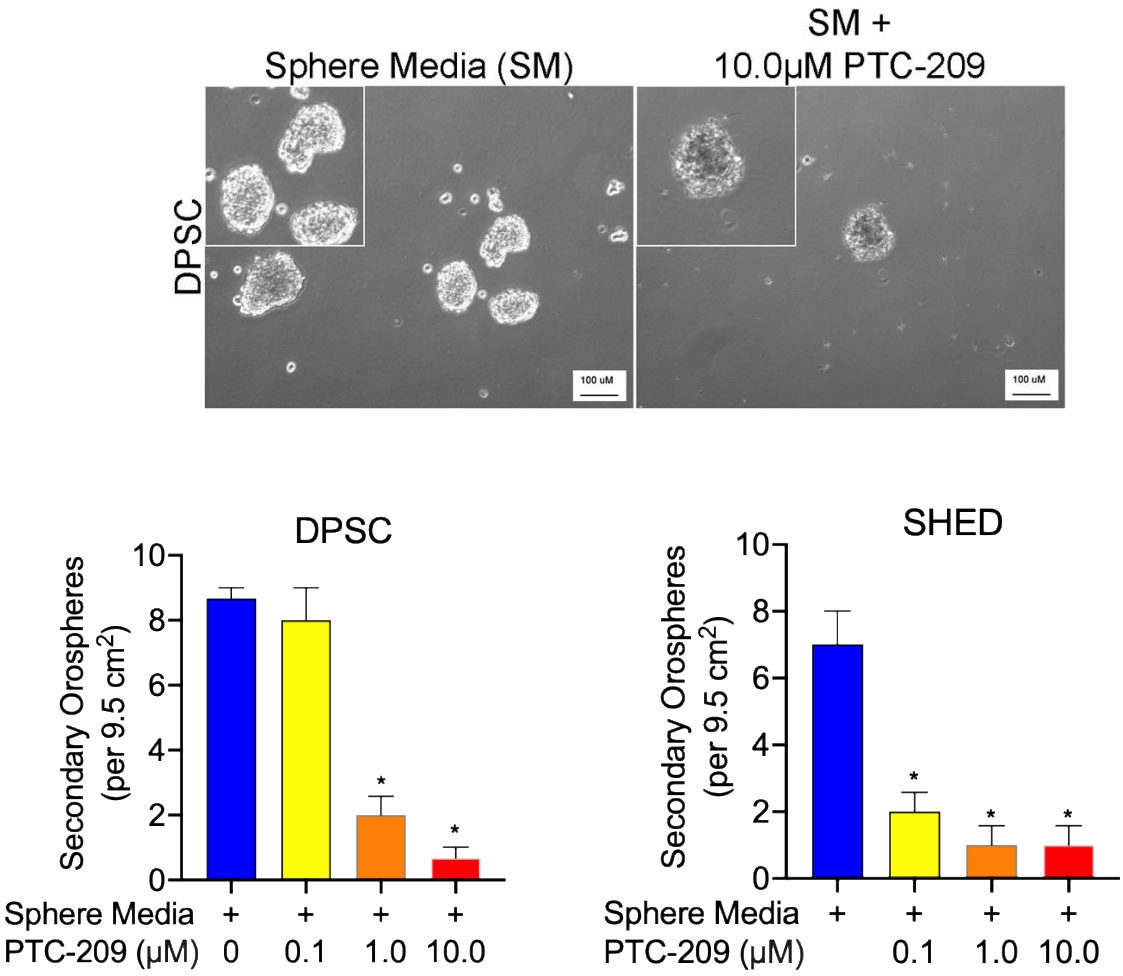

Figure 2.7. Bmi-1 inhibition suppresses secondary orosphere formation. [Top] Phase contrast microscopy of DPSC/SHED secondary orospheres cultured in Sphere Media [SM] or SM containing 10.0μM PTC-209 (Bmi-1 inhibitor); [Bottom] Secondary DPSC/SHED orosphere counts in Sphere Media [SM] containing 0.1, 1.0, or 10.0μM PTC-209 (Bmi-1 inhibitor) at 7 days. Asterisk depicts $p<0.05$.

Endothelial cell-derived IL-6 promotes dental pulp stem cell self-renewal

To understand the impact of endothelial cell-secreted factors on self-renewal of dental pulp stem cells, we exposed these cells to conditioned medium from primary human endothelial cells. We observed that the endothelial growth factor milieu causes a major increase in the number of secondary orospheres, when compared to unconditioned medium (Figure 2.8). Western blots demonstrated an upregulation of Bmi-1 expression and induction of STAT3 phosphorylation in dental pulp stem cells (DPSC or SHED) cultured in endothelial conditioned medium, showing that endothelial cell-derived factors promote activation of pathways regulating self-renewal (Figure 2.9).

To begin to understand mechanisms responsible for endothelial cell-regulated self-renewal of dental pulp stem cells, we used chemical inhibitors of major signaling pathways (*i.e.,* STAT3, ERK, PI3K-Akt) (Figure 2.10). We observed a marked decrease in Bmi-1 expression when dental pulp stem cells exposed to endothelial conditioned medium were treated with Stattic V (STAT3 inhibitor) but not when ERK or PI3K-Akt was inhibited (Figure 2.10). These data suggested a role for STAT3 signaling in the regulation of self-renewal of dental pulp stem cells. Knowing that endothelial cells secrete high levels of Interleukin-6 (IL-6) and that IL-6 is a potent inducer of STAT3 (Krishnamurthy et al., 2014), we anticipated that Bmi-1 expression would be decreased in dental pulp stem cells exposed to endothelial cell conditioned medium containing sub-lethal doses of Tocilizumab (anti-IL-6R antibody) (Figure 2.11, 2.12). To confirm these data, we exposed dental pulp stem cells to recombinant human IL-6 (rhIL-6) in presence of increasing concentrations of Tocilizumab and observed that Tocilizumab caused a dose-dependent inhibition of IL-6-induced pSTAT3 and Bmi-1 expression in dental pulp stem cells (Figure

2.13). To determine the functional impact of endothelial cell-derived IL-6 on orospheres generated by dental pulp stem cells, we cultured these cells with endothelial conditioned medium in presence of increasing concentrations of Tocilizumab. We observed a decrease ($p<0.05$) in secondary orosphere formation in the Tocilizumab-treated group when compared to controls, suggesting that endothelial-derived IL-6 mediates self-renewal of dental pulp stem cells (Figure 2.14).

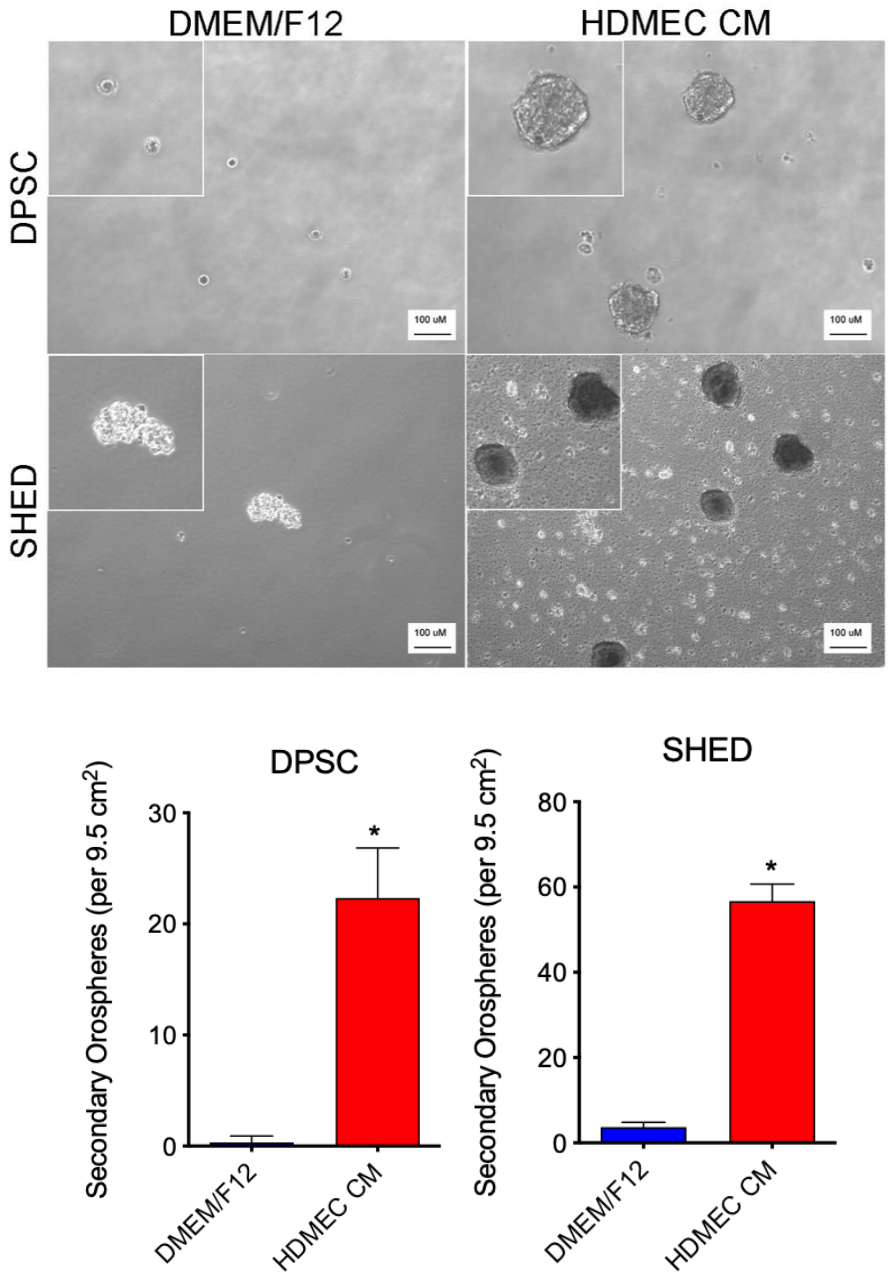

Figure 2.8. Endothelial cell-derived factors promote dental pulp stem cell self-renewal. [Top] Phase contrast microscopy of DPSC/SHED secondary orospheres cultured in DMEM/F12 or HDMEC CM (endothelial cell-conditioned media from human dermal microvascular endothelial cells); [Bottom] DPSC/SHED secondary orosphere counts in 6-well ULA plates ($1x10^5$ cells/well) after treatment with HDMEC CM for 7 days. Asterisk depicts $p<0.05$.

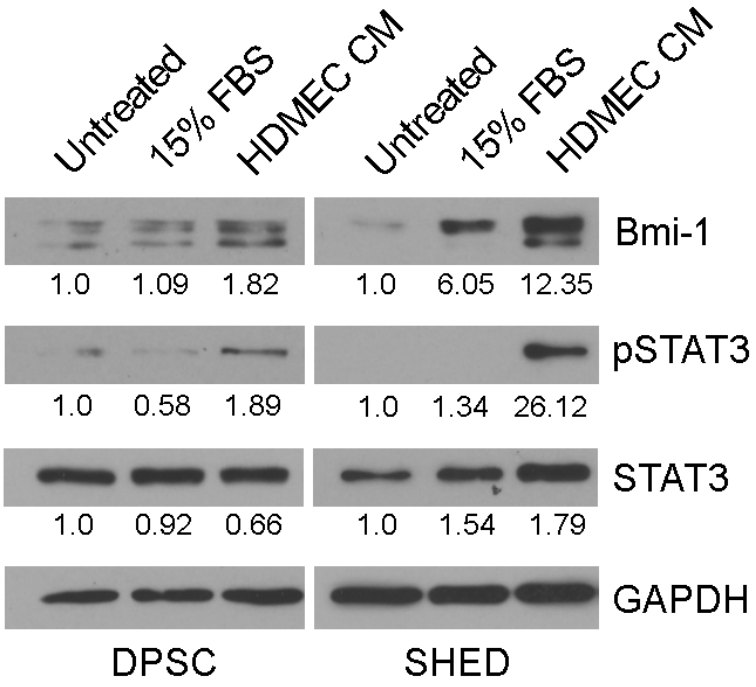

Figure 2.9. Endothelial cell-derived factors promote Bmi-1 expression. Western blot analysis of DPSC or SHED cells treated with basal medium containing 0 or 15% fetal bovine serum (FBS), or endothelial conditioned media (HDMEC CM). Bmi-1 and STAT3 band density was normalized to respective GAPDH lanes in Western blots.

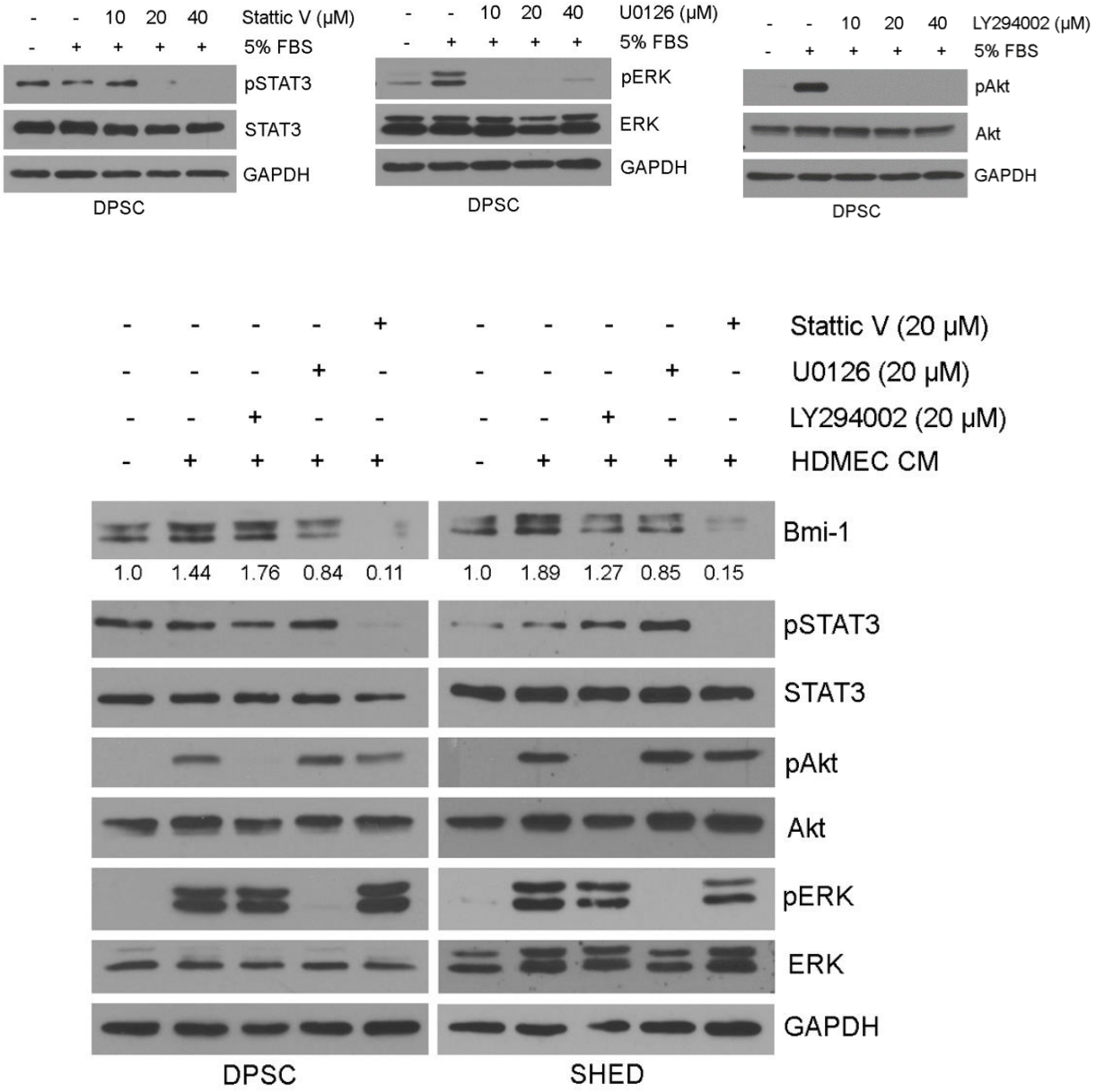

Figure 2.10. Endothelial cells induce Bmi-1 expression through STAT3 signaling. Western blot analysis of DPSC or SHED cells after a 15-minute exposure to HDMEC CM or HDMEC CM containing 20 μM Stattic V (STAT3 inhibitor), 20 μM U0126 (ERK/MAPK inhibitor), or 20 μM LY294002 (Akt/PI3K inhibitor). Bmi-1 band density was normalized to respective GAPDH lanes in Western blots.

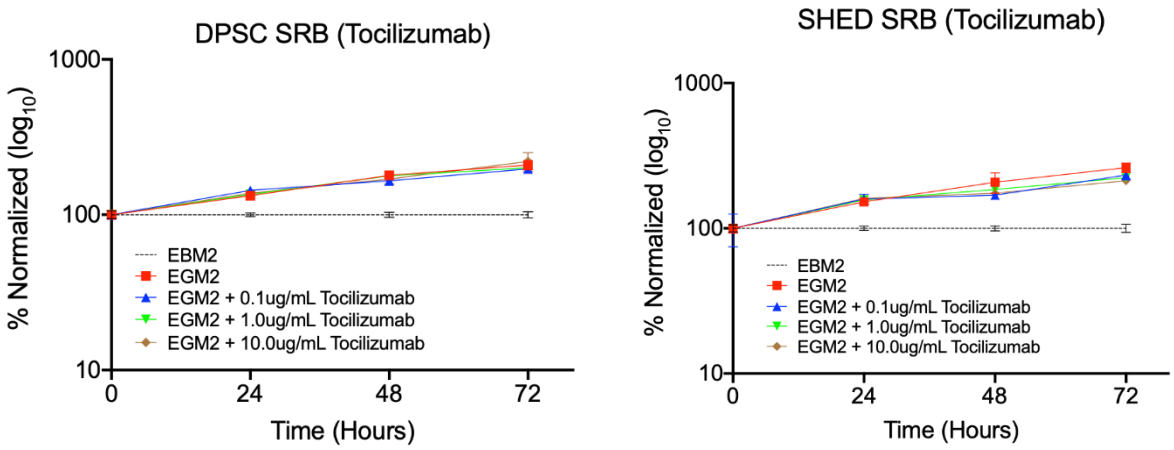

Figure 2.11. Cytotoxicity Assay for Tocilizumab (IL-6R inhibitor) for DPSC/SHED. Sulforhodamine B colorimetric (SRB) assay for cytotoxicity on DPSC/SHED with 0.1, 1.0, or 10.0μM Tocilizumab (IL-6R inhibitor) up to 72 hours ($p<0.05$).

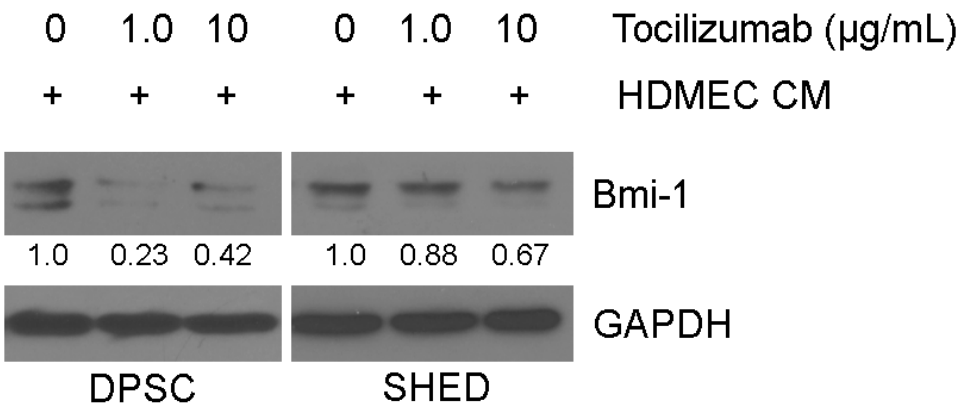

Figure 2.12. Endothelial cell-derived IL-6 regulates Bmi-1 expression. Western blots of DPSC or SHED cells after 15-minute exposure to HDMEC CM containing 0, 1 or 10 μM Tocilizumab (IL-6R inhibitor). Bmi-1 band density was normalized to respective GAPDH lanes in Western blots.

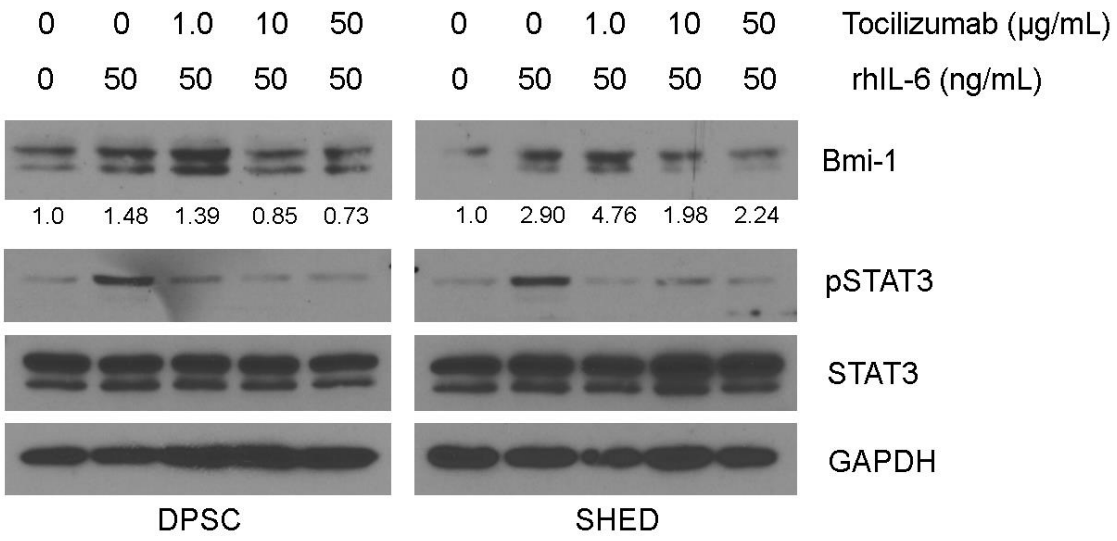

Figure 2.13. IL-6 regulates Bmi-1 expression via STAT3 signaling. Western blots of DPSC or SHED cells exposed to 0 or 50 ng/mL recombinant human interleukin-6 (rhIL-6) in presence of 0, 1, 10, or 50 μM Tocilizumab for 15 minutes. Bmi-1 band density was normalized to respective GAPDH lanes in Western blots.

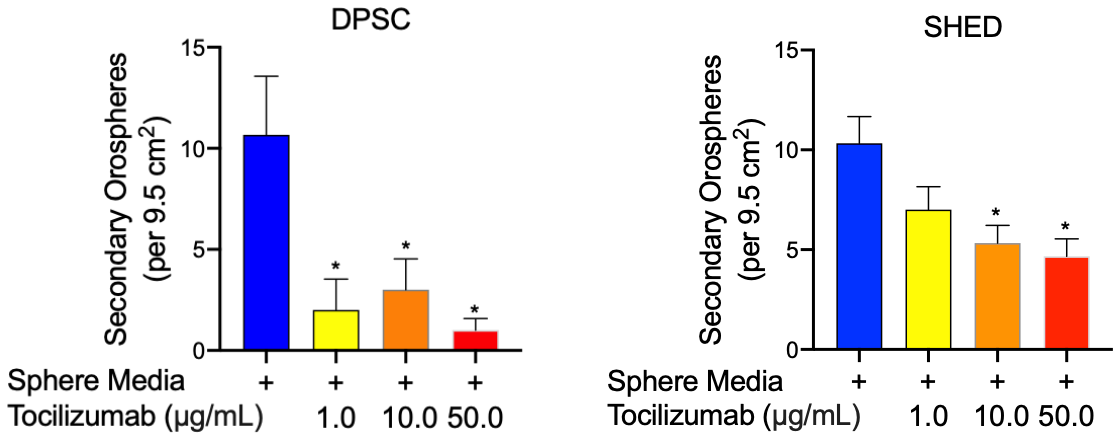

Figure 2.14. The IL-6 pathway mediates self-renewal of dental pulp stem cells. Graph depicting the number of secondary orospheres generated by DPSC or SHED cells cultured in Sphere Media (SM) containing 0, 1, 10 or 50 μg/mL Tocilizumab for 7 days. Asterisk depicts $p<0.05$.

Endothelial cell-derived platelet-derived growth factor-BB (PDGF-BB) does not induce dental pulp stem cell self-renewal

Evidence suggests platelet derived growth factor-BB (PDGF-BB) is implicated in the regulation of self-renewal within various stem cell populations. Bögler and colleagues initially showed that, in bipotential oligodendrocyte-type-2 astrocyte progenitor cells (O-2A), the presence of PDGF prevents the differentiation of O-2A cells (Bögler et al. 1990). Furthermore, they showed that PDGF along with basic fibroblast growth factor (bFGF) are both necessary for O-2A self-renewal (Bögler et al. 1990). Interestingly, they showed a disruption in self-renewal when culturing O-2A cells individually with either PDGF or bFGF, suggesting distinct and overlapping functions that are essential for these cells to maintain self-renewal (Bögler 1990). This observation that PDGF plays a role in the regulation of self-renewal is also illustrated in mesenchymal stem cells (MSCs) by Gharibi and colleagues (Gharibi et al. 2012). Collectively, data from other cell types suggested that PDGF-BB may play a role in the regulation of self-renewal of dental pulp stem cells.

To test if PDGF-BB induces Bmi-1 expression, DPSC were exposed to recombinant human PDGF-BB (rhPDGF-BB) in the presence of chemical inhibitors of key signaling pathways and Bmi-1 expression was evaluated by Western blots (Figure 2.15). Treatment with rhPDGF-BB resulted in a marginal increase in Bmi-1 expression. In addition, there was only a modest decrease in Bmi-1 expression when DPSC cells were treated with 20 μM LY294002 (Akt/PI3K inhibitor) (Figure 2.15), suggesting that PDGF-BB-induced PI3K/Akt activity does not regulate Bmi-1 expression. As previously shown, treatment of DPSC with Stattic V (STAT3 inhibitor) causes a significant inhibition in Bmi-1 expression, suggesting that STAT3 signaling is involved in the regulation of Bmi-1

expression in dental pulp stem cells. To determine if endothelial cell-derived PDGF-BB induces Bmi-1 expression, DPSC were treated with HDMEC CM or HDMEC CM with a neutralizing antibody (Nab) against PDGF-BB (Figure 2.15). We observed a modest induction of Bmi-1 when treating DPSC endothelial cell conditioned medium. Treatment with 1 µg/ml PDGF-BB neutralizing antibody reduced Bmi-1 expression to levels comparable to baseline (Figure 2.15). To explore if PDGF-BB induces self-renewal in dental pulp stem cells, secondary orospheres were treated with varying doses (0.5, 5.0, and 50.0 ng/mL) of rhPDGF-BB (Figure 2.16). We did not observe a significant difference in secondary orosphere formation when DPSC were treated with rhPDGF-BB (Figure 2.16). These results showed that endothelial cell-derived PDGF-BB does not have a major effect in the regulation of Bmi-1 expression and does not promote self-renewal in dental pulp stem cells *in vitro.*

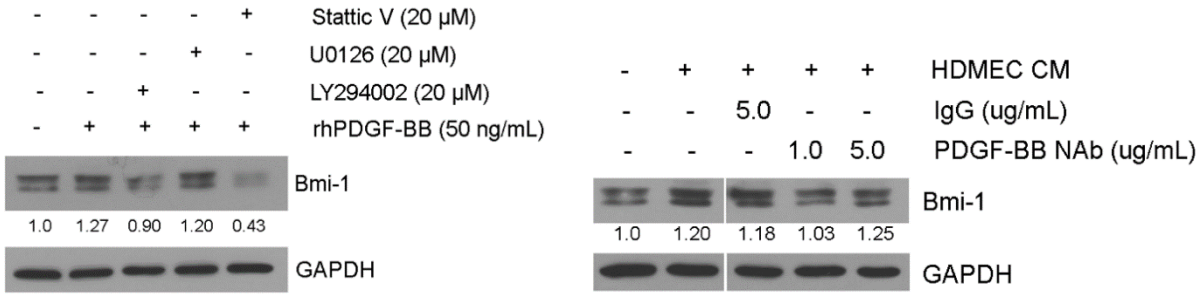

Figure 2.15. PDGF-BB does not regulate Bmi-1 expression. [Left] Western blot analysis of DPSC cells after a 15-minute exposure to 50ng/mL rhPDGF-BB, or 50ng/mL rhPDGF-BB containing 20 µM Stattic V (STAT3 inhibitor), 20 µM U0126 (ERK/MAPK inhibitor), or 20 µM LY294002 (Akt/PI3K inhibitor); [Right] Western blots of DPSC cells after 15-minute exposure to HDMEC CM containing 0, 1 or 5 µg/mL PDGF-BB Neutralizing Antibody (PDGF-BB NAb). Bmi-1 band density was normalized to respective GAPDH lanes in Western blots.

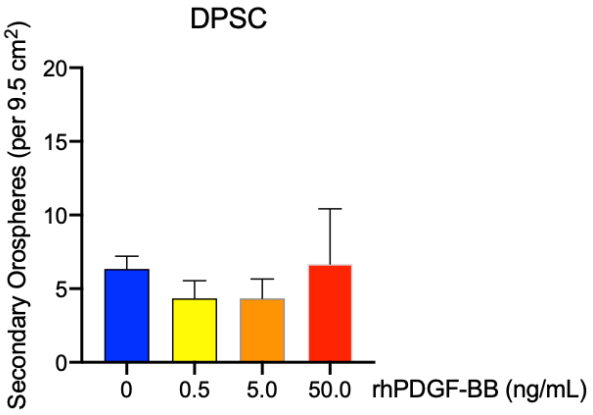

Figure 2.16. PDGF-BB does not induce dental pulp stem cell self-renewal. Graph depicting the number of secondary orospheres generated by DPSC cells cultured in DMEM/F12 containing 0, 0.5, 5 or 50 ng/mL rhPDGF-BB for 7 days. Asterisk depicts $p<0.05$.

STAT3 is a critical regulator of dental pulp stem cell self-renewal

Western Blots showed a marked increase in STAT3 phosphorylation and Bmi-1 expression when dental pulp stem cells are exposed to endothelial conditioned medium or rhIL-6 (Figure 2.9, 2.10, 2.13) and blockade of STAT3 signaling with sub-lethal Stattic V causes a dose-dependent inhibition of Bmi-1 (Figure 2.17, 2.19). To confirm these data, we stably silenced STAT3 in both DPSC and SHED cells using small hairpin RNA (shRNA). Western Blots confirmed the silencing of STAT3 expression and demonstrated a significant decrease in Bmi-1 expression, when compared to cells stably transduced with scramble vector control shRNAs (Figure 2.18).

To determine the role of STAT3 signaling on self-renewal, dental pulp stem cells were cultured in low attachment conditions and treated with sub-lethal doses of Stattic V (Figure 2.19, 2.20). Inhibition of STAT3 signaling decreased the number of secondary orospheres in a dose dependent manner (Figure 2.20). To verify these data, we cultured

stem cells in low attachment conditions and observed that dental pulp stem cells silenced for STAT3 generated fewer orospheres than cells transduced with the control vector (Figure 2.21, 2.22). Together, these results demonstrate a functional role for STAT3 signaling in the regulation of dental pulp stem cell self-renewal, as demonstrated by impacts on Bmi-1 expression and number of orospheres.

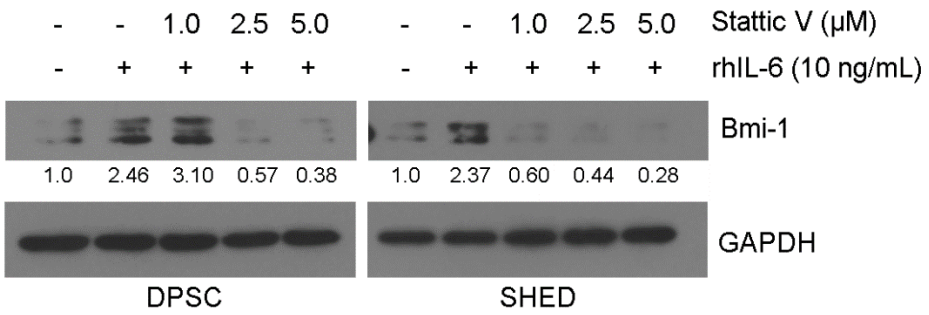

Figure 2.17. IL-6 promotes Bmi-1 expression via STAT3 signaling. Western blot analysis of DPSC or SHED cells after treatment with 10 ng/mL rhIL-6 in presence of 0, 1, 2.5, or 5 μM Stattic V for 15 minutes. Bmi-1 band density was normalized to respective GAPDH lanes in Western blots.

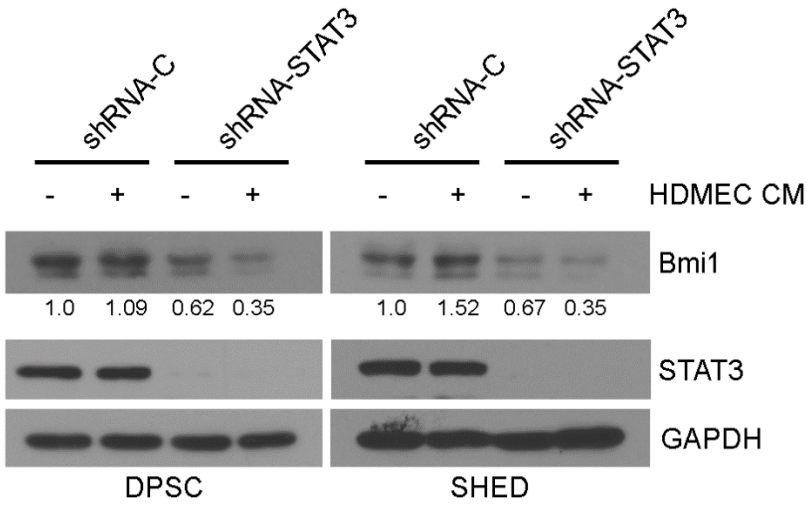

Figure 2.18. STAT3 silencing reduces Bmi-1 expression and HDMEC CM-initiated Bmi-1 induction. Western blot analysis of DPSC or SHED stably transduced with shRNA-Control (scrambled sequence) or shRNA-STAT3 with or without exposure to endothelial conditioned media (HDMEC CM) for 15 minutes.

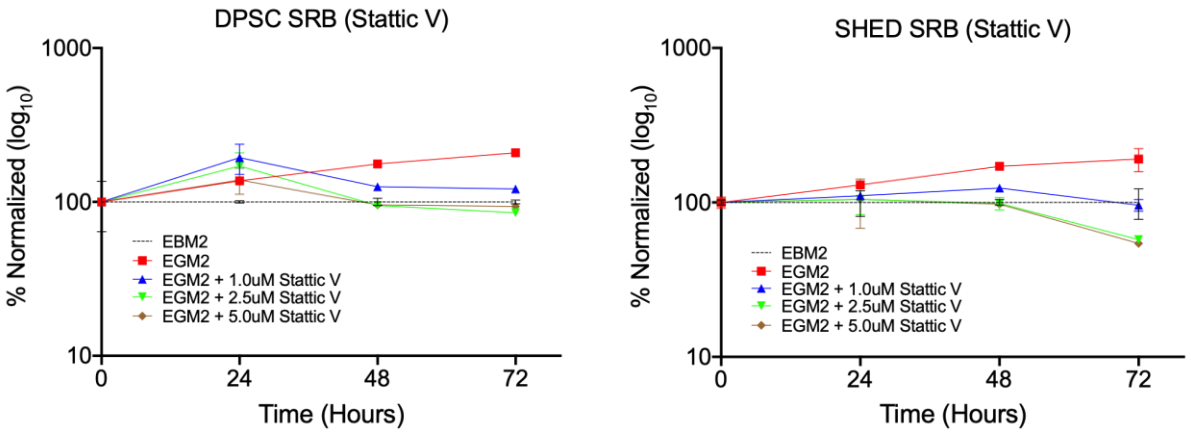

Figure 2.19. Cytotoxicity Assay for Stattic V (STAT3 inhibitor) for DPSC/SHED. Sulforhodamine B colorimetric (SRB) assay for cytotoxicity on DPSC/SHED with 1.0, 2.5, or 5.0μM Stattic V (STAT3 inhibitor) up to 72 hours ($p<0.05$).

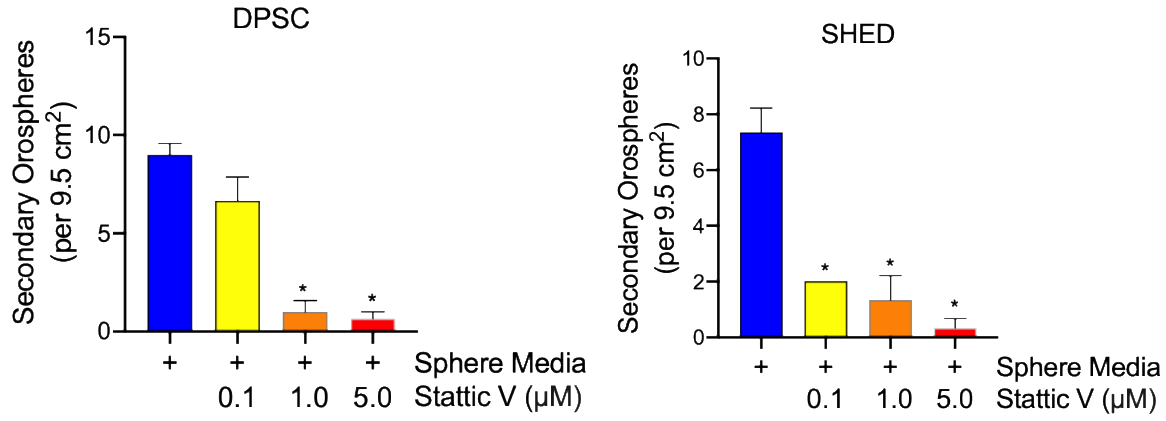

Figure 2.20. STAT3 inhibition decreases secondary orosphere formation. Secondary DPSC/SHED orosphere counts in Sphere Media [SM] containing 1.0, 2.5, or 5.0μM Stattic V (STAT3 inhibitor) at 7 days ($p<0.05$).

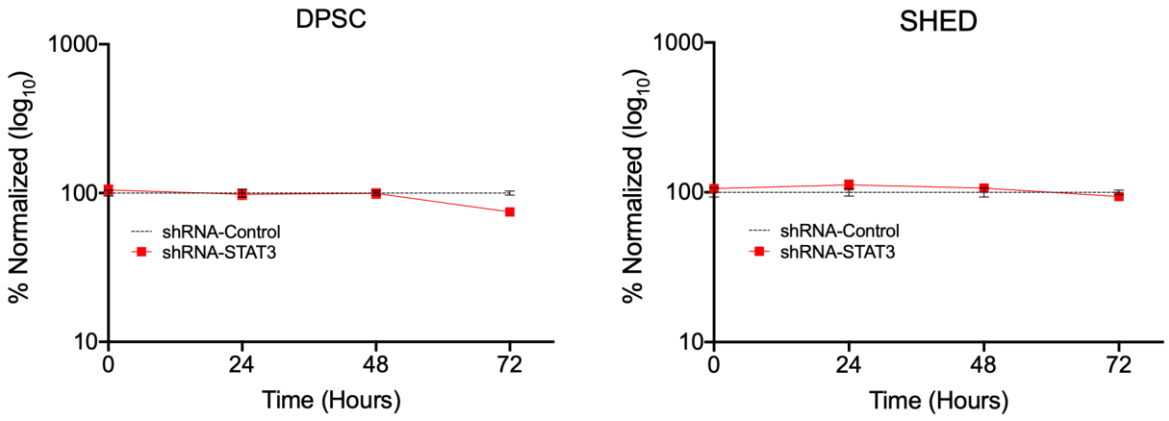

Figure 2.21. Cytotoxicity Assay for STAT3-silenced dental pulp stem cells. Sulforhodamine B colorimetric (SRB) assay for cytotoxicity on DPSC or SHED stably transduced with shRNA-Control (scrambled sequence) or shRNA-STAT3) up to 72 hours ($p<0.05$).

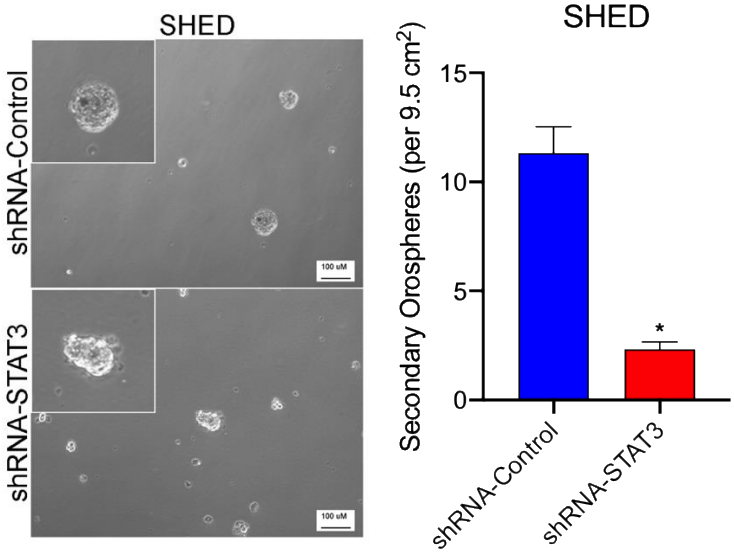

Figure 2.22. STAT3 silencing inhibits secondary orosphere formation. [Left] Phase contrast microscopy of stably transduced SHED-shRNA-Control or SHED-shRNA-STAT3 secondary orospheres cultured in Sphere Media [SM] at 7 days; [Right] Secondary orosphere counts in stably transduced SHED-shRNA-Control or SHED-shRNA-STAT3 cultured in SM at 7 days. Asterisk depicts $p<0.05$.

STAT3 pathway is critical for the establishment of perivascular niches

To determine the role of the STAT3 pathway in the generation and maintenance of perivascular niches *in vivo*, dental pulp stem cells stably transduced with shRNA-STAT3 (or control vector) were seeded in poly-L-lactic acid scaffolds (PLLA) and transplanted in the subcutaneous space of immunodeficient mice (Figure 2.23). After 28 days, scaffolds were retrieved and processed for histology. We observed a decrease ($p<0.05$) in the number and area of blood vessels in the tissues generated with STAT3-silenced cells (n=6) when compared to tissues generated with control cells (n=6) (Figure 2.24, 2.25). Concomitantly, we observed a significant decrease in the number of ALDH1^{high} and Bmi-1^{high} cells in tissues generated by STAT3-silenced cells when compared to tissues generated by control cells (Figure 2.26). These results demonstrate the important role of the STAT3 pathway in the establishment of the perivascular niche *in vivo*.

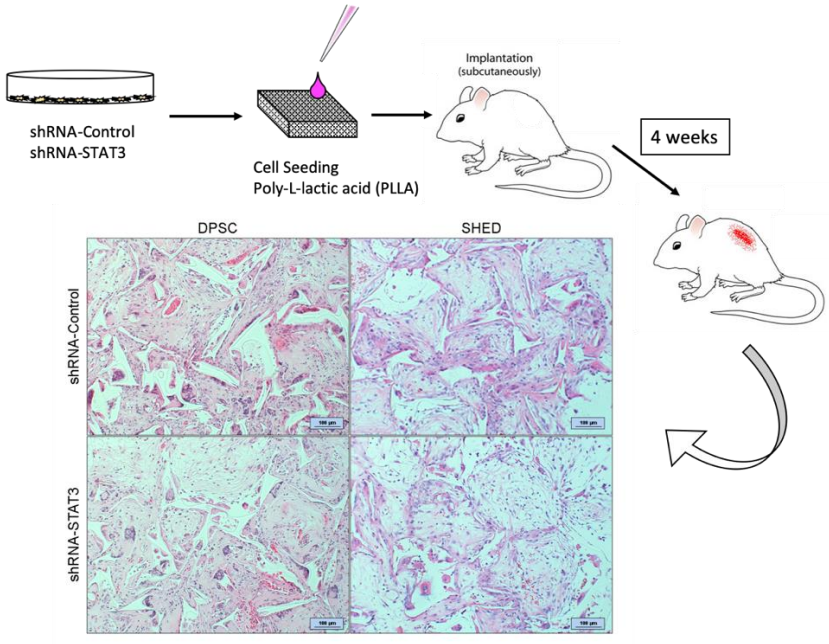

Figure 2.23. Graphical representation of experimental model.

DPSC or SHED stably transduced with either shRNA-Control or shRNA-STAT3 (10^6 cells) were mixed with growth factor reduced Matrigel and seeded into biodegradable scaffolds that were transplanted subcutaneously in immunodeficient mice. Representative hematoxylin and eosin (H&E) images of resulting tissues 28 days after transplantation.

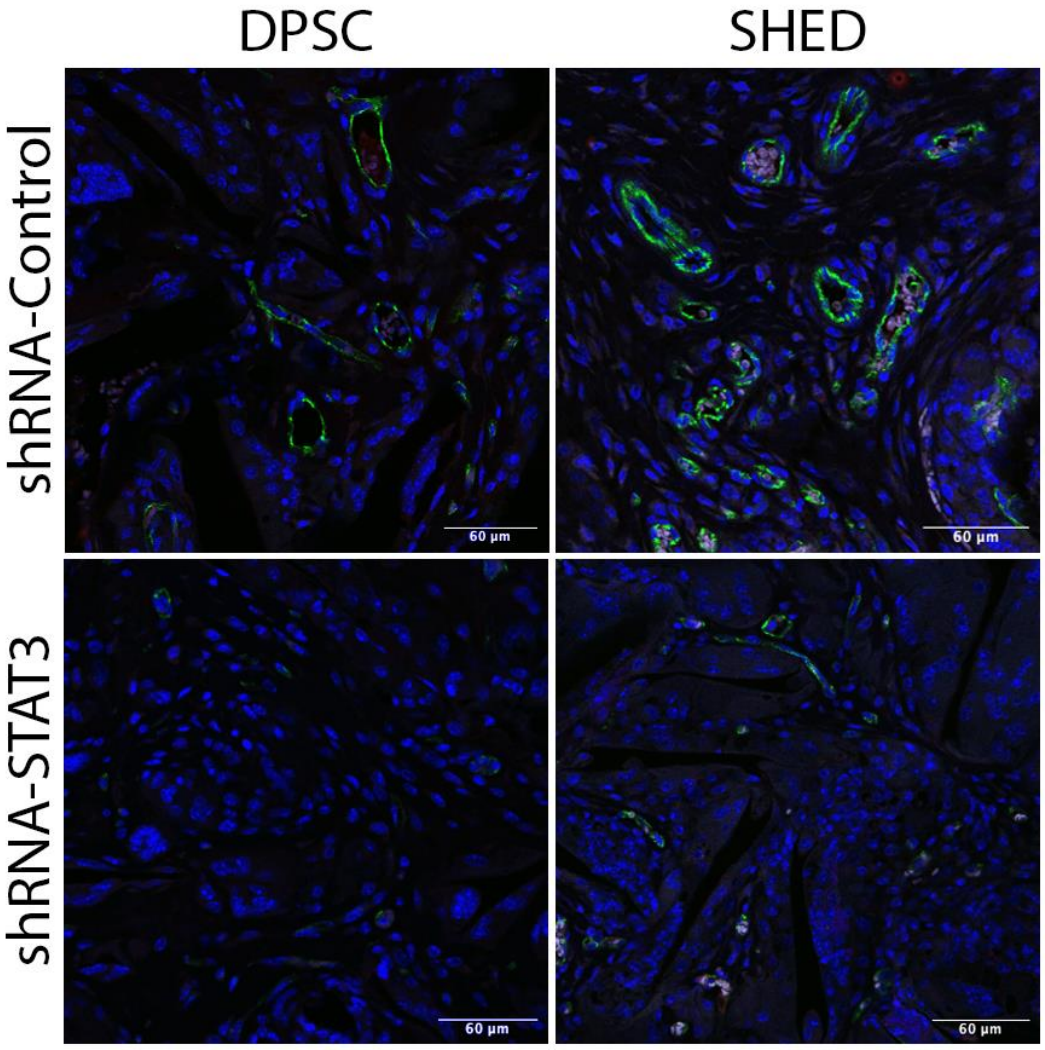

Figure 2.24. STAT3 silencing results in decreased vasculogenesis *in vivo*. Representative confocal microscopy images of scaffolds containing DPSC or SHED stably transduced with either shRNA-Control or shRNA-STAT3. CD31 immunofluorescence depicts blood vessels (green) and DAPI staining depicts nuclei (blue).

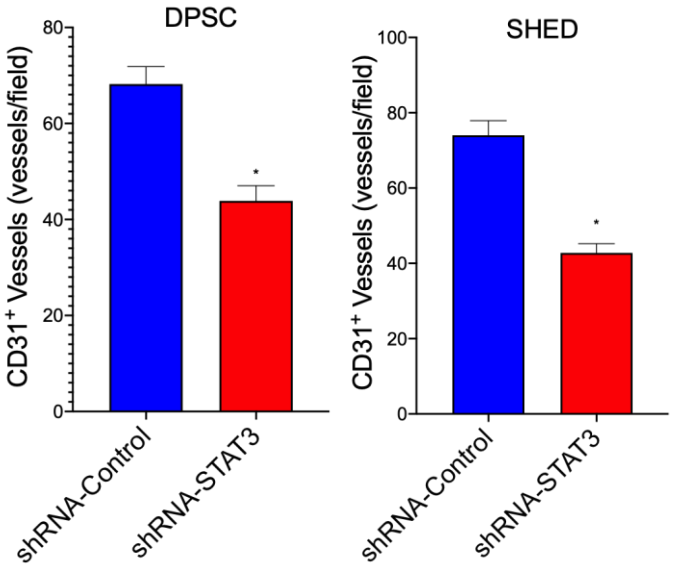

Figure 2.25. STAT3 silencing results in decreased microvessels *in vivo*. Graph depicting the quantification of $CD31^+$ blood vessels generated by DPSC or SHED stably transduced with shRNA-STAT3 (versus shRNA-Control) 28 days after transplantation. Asterisk depicts $p<0.05$.

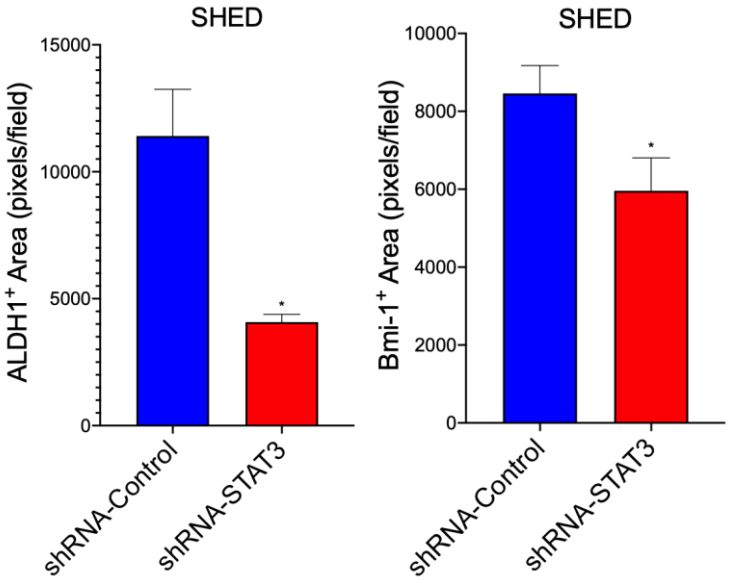

Figure 2.26. STAT3 silencing reduces stem cell populations *in vivo*. Graph depicting the quantification of $ALDH1^{high}$ surface area (pixels/field) in tissues generated by SHED-shRNA-STAT3 (*versus* SHED-shRNA-Control) in normalized confocal microscopy images. (**F**) Graph depicting the quantification of $Bmi\text{-}1^{high}$ surface area (pixels/field) in tissues generated by SHED-shRNA-STAT3 (*versus* SHED-shRNA-Control) in normalized confocal microscopy images. Asterisk depicts $p<0.05$.

STAT3 pathway mediates vasculogenic potential in dental pulp cells

As tissues generated with STAT3-silenced dental pulp stem cells showed a decrease in vascularity, we decided to study the role of STAT3 in vasculogenic potential differentiation of these cells *in vitro* using the capillary sprouting assay. We observed a decrease in the number of capillary sprouts ($p<0.05$) when dental pulp stem cells were treated with sub-lethal concentrations of Tocilizumab or Stattic V (Figure 2.27, 2.28). Similarly, a decrease in number of sprouts was observed when SHED cells stably transduced with shRNA-STAT3 were tested in this assay (Figure 2.29).

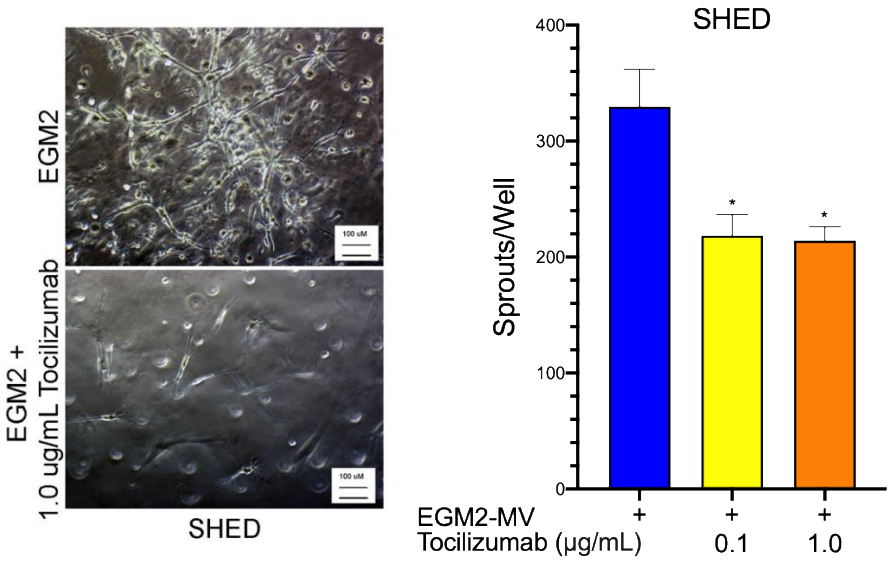

Figure 2.27. IL-6 inhibition decreases vasculogenesis. [Left] Representative phase contrast microscopy images of SHED cultured in growth factor reduced Matrigel with endothelial growth medium-2 (EGM2-MV) supplemented with 0 or 1 µg/mL Tocilizumab for 7 days; [Right] Graph depicting the number of capillary sprouts generated by SHED cultured in growth factor reduced Matrigel with endothelial growth medium-2 (EGM2-MV) supplemented with 0, 0.1 or 1 µg/mL Tocilizumab for 7 days. Asterisk depicts $p<0.05$.

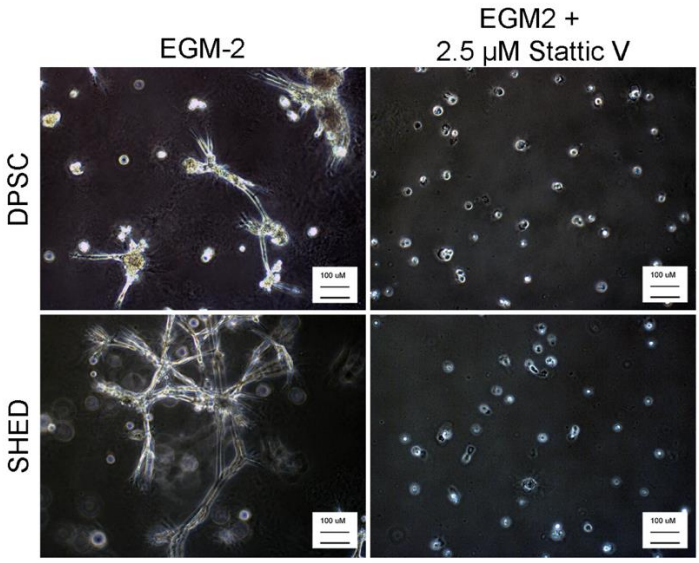

Figure 2.28. STAT3 inhibition decreases vasculogenic potential in dental pulp stem cells. Phase contrast microscopy images of DPSC or SHED cells cultured in growth factor reduced Matrigel with EGM2-MV supplemented with 0 or 2.5 µM Stattic V for 7 days.

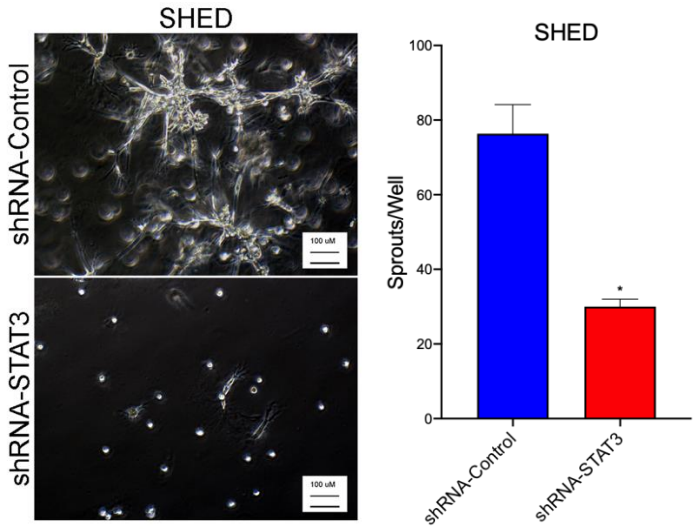

Figure 2.29. STAT3 silencing reduces capillary sprout formation. [Left] Phase contrast microscopy images of SHED stably transduced shRNA-Control or shRNA-STAT3 cultured in growth factor reduced Matrigel with EGM2-MV medium for 7 days; [Right] Graph depicting the number of capillary sprouts generated by SHED-shRNA-Control or SHED-shRNA-STAT3 cultured in growth factor reduced Matrigel with EGM2-MV for 7 days. Asterisk depicts $p<0.05$.

Discussion

Perivascular niches, where stem cells reside near blood vessels, have been initially described in bone (Kollet et al. 2006; Sugiyama et al. 2006; Sacchetti et al. 2007). A seminal study from the Shi laboratory demonstrated co-localization of blood vessels and stem cells in bone marrow and in the dental pulp (Shi et al. 2003). More recently, the role of endothelial cell-stem cell crosstalk in the maintenance of stem cell pools has been unveiled in physiology (Ding et al. 2012) and in cancer (Krishnamurthy et al. 2010). However, the nature of the molecular crosstalk between endothelial cells and stem cells within the perivascular niche in the dental pulp remains unclear.

Aldehyde dehydrogenase (ALDH) has been extensively investigated as a marker of self-renewing stem cells (Corti et al. 2006; Ginestier et al. 2007). Investigation of dental pulp tissues suggested that ALDH1^{high} cells co-localize with cells expressing mesenchymal stem cell surface markers, such as Stro-1 and CD90 (Machado et al. 2016). Further, Bmi-1 was shown to be a major regulator of self-renewal in various stem cell populations, including dental stem cells (Molofsky et al. 2003; Liu et al. 2006; Biehs et al. 2013). Here, we demonstrated that ALDH1^{high} and Bmi-1^{high} cells are preferentially found in close proximity to blood vessels in human dental pulps, suggesting the existence of perivascular niches. We observed that endothelial cell-secreted factors induce self-renewal of dental pulp stem cells, as demonstrated by the increase in the efficiency of secondary orosphere formation using the orosphere assay with cells cultured in low attachment conditions (Reynolds et al. 1992; Dontu et al. 2003; Krishnamurthy et al. 2013). These data provided the first evidence for the functional impact of having stem cells in close proximity to blood vessels in the dental pulp, i.e. endothelial cell factors can

potentially enhance asymmetric stem cell division and self-renewal of stem cells which would enable the maintenance of stem cells pools while other stem cells undergo differentiation.

The function of Bmi-1 is conserved throughout different stem cell populations. In the context of dentistry, Bmi-1 was shown to be involved in tooth development using the continuously growing murine incisor model (Molofsky et al. 2003; Park et al. 2003; Biehs et al. 2013). Here, we demonstrated the ability of dental pulp stem cells to form orospheres in ultralow attachment conditions, and to be serially passaged into secondary orospheres. We showed that Bmi-1 expression was induced when dental pulp stem cells where evaluated in secondary orospheres. These data suggest that Bmi-1 expression is correlated functionally with self-renewal capacity in dental pulp stem cells. Notably, treatment with PTC-209, a Bmi-1 inhibitor, resulted in a decrease in the number of secondary orospheres. These data suggest that Bmi-1 function correlates with increased self-renewal capacity of dental pulp stem cells, and further establish Bmi-1 as a viable indicator of self-renewal of dental pulp stem cells.

The *in vitro* sphere assay presents some limitations. First, orospheres are suspended in culture, and not fixed into a flat or three-dimensional surface, which may limit one's ability to differentiate aggregation from true sphere formation, particularly in early stages of sphere formation. In addition, the formation of primary orospheres may require a higher density of cells, resulting in increased possibility of cell aggregation that is indistinguishable from true orosphere formation. To avoid such limitation, we focused on the ability of dental pulp stem cells to form secondary orospheres. When serially passaging primary orospheres, the resulting cells were able to form secondary

orospheres in low attachment conditions. Under these conditions, a higher proportion of cells derived from primary orospheres were able to form orospheres, consistent with findings in other stem cell populations (Reynolds and Weiss 1996). Notably, chemical inhibitors may have a different cytotoxic impact on cells in suspension (*i.e.,* orospheres) when compared to cells grown in standard culture conditions. As the sulforhodamine B (SRB) assay measures the protein levels on fixed cells attached to cells on standard attachment conditions, the SRB assay is not feasible to perform on cells in suspension. As such, the specific culture conditions should be taken into consideration as one interprets the results of these studies. Despite these limitations, we observed a significant decrease in secondary orosphere formation in the presence of sublethal doses of PTC-209, confirmed with SRB assays. Nevertheless, despite the limitations of the sphere assay, our results suggest that Bmi-1 functions as an important regulator of self-renewal in dental pulp stem cells.

As we observed the localization of $ALDH1^{high}$ or $Bmi\text{-}1^{high}$ cells near $CD31^{+}$ endothelial cells, we hypothesized that endothelial cell-secreted factors induce the self-renewal of dental pulp stem cells. We showed that there is a significant increase in secondary sphere formation and an induction in Bmi-1 expression and number of secondary orospheres in dental pulp stem cells cultured in presence of endothelial cell conditioned medium. These results suggest that endothelial cell-secreted factors promote self-renewal of dental pulp stem cells via Bmi-1. These findings highlight the significance of the endothelial cells in the maintenance of stem cell populations within the perivascular niche.

Knowing that endothelial cell-derived interleukin-6 (IL-6) promotes the self-renewal of oral cancer stem cells (Krishnamurthy et al. 2014), and that the STAT3 pathway mediates self-renewal in several stem cell types (Chen et al. 2015; Kiger et al. 2001; Niwa et al. 1998; Raz et al. 1999; Tulina et al. 2001), we studied here the impact of endothelial cell-IL-6 on STAT3 activation in dental pulp stem cells. We observed here that endothelial cell-derived IL-6 promotes the self-renewal of dental pulp stem cells through STAT3 signaling and induction of Bmi-1 expression. This provided a mechanistic explanation for the functional role of endothelial cell-secreted factors on the maintenance of stem cell pools in the perivascular niche.

When we transplanted STAT3-silenced dental pulp stem cells into immunodeficient mice, we observed similar morphological features to those observed in control specimens, with dense connective tissues interspaced with areas of loose connective tissue. Generally, the denser areas of tissue presented perfused, mature blood vessels. The looser connective tissue area contained smaller blood vessels with fewer red blood cells. Despite similar morphologies, microscopic analyses showed fewer perivascular niches (*i.e.,* blood vessels surrounded by stem cells) when STAT3-silenced dental pulp stem cells were transplanted into mice. We also observed that STAT3 silencing decreases the self-renewal and vasculogenic potential of dental pulp stem cells *in vitro*. These results suggest that STAT3 is a master regulator of the development and maintenance of perivascular niches in the dental pulp.

Through mechanistic experiments using Tocilizumab and Stattic V, we concluded that Bmi-1 is regulated by the IL-6/STAT3 signaling pathway. Frequency distribution analyses on immunofluorescence images in healthy human dental pulps showed that

ALDH1^{high} and Bmi-1^{high} cells reside near perivascular areas. As such, it is plausible that the self-renewing subpopulation of dental pulp stem cells located in these perivascular areas is regulated by the STAT3 pathway by endothelial cell-secreted IL-6. Interpretation of the results from the *in vivo* model for vasculogenic potential suggests that this pathway is essential for the establishment of the perivascular niche. This suggests that the STAT3 pathway is critical for the maintenance of the self-renewing dental pulp stem cells expressing high levels of ALDH1 and Bmi-1 in the perivascular areas within the dental pulp.

In summary, this study showed evidence of a functional crosstalk between endothelial cells and stem cells in the dental pulp tissue. We demonstrated that this crosstalk is initiated by endothelial cell-secreted IL-6 that binds to IL-6R in dental pulp stem cells to activate STAT3 signaling and Bmi-1 expression. We also showed that this pathway is critical for the establishment and maintenance of perivascular niches *in vivo.* As such, we propose that the function of endothelial cells in the dental pulp is not limited to enabling influx of oxygen and nutrients. Rather, these results suggest that the maintenance of pools of stem cells in the dental pulp tissue requires signaling events triggered by nearby vascular endothelial cells.

Chapter III

Conclusions & Future Directions

The interaction of stem cells with other cellular components of their niche is critical for self-renewal and the maintenance of the stem cell pool, and for the determination of their differentiation fate through multipotency. Mesenchymal stem cells play a vital role in the maintenance of various tissues (da Silva Meirelles et al. 2006; Crisan et al. 2008) and we strongly believe that mesenchymal stem cells and their niche are critically important in the context of dental tissue regeneration. The perivascular niche has been shown to provide key molecular cues for the maintenance of diverse stem cells populations, including physiological and pathological (*e.g.,* cancer) stem cells. These findings suggested the existence of conserved mechanisms of stem cell maintenance. While new therapeutic strategies are being developed to target the interaction of the perivascular niche with cancer stem cells as a possible approach for cancer treatment, purposeful efforts to regenerate the stem cell niche might be important for tissue engineering. Likewise, as anti-cancer therapies attempt to inhibit self-renewal of cancer stem cells (*e.g.,* therapeutic use of Bmi-1 inhibitors), it might be beneficial to induce self-renewal pathways to expand physiological stem cell populations for tissue engineering. Thus, studies focused on the understanding of conserved mechanisms regulating the biology of stem cell niches will provide valuable insights on the function and maintenance of stem

cells, and may have a positive impact on the development of strategies that enhance the long-term outcomes of regenerated tissues and organs.

Self-renewal is highly controlled and regulated by various signaling pathways and is initiated through a host of factors and stimuli. As such, there is possibility of redundancy and interplay among other signaling pathways (*e.g*. Notch, Hedgehog) in the regulation of dental pulp stem cell self-renewal. There also may be a complex network of feedback loops among signaling pathways that may precisely regulate the self-renewal of dental pulp stem cells during homeostasis in a dose-sensitive manner. In other words, under physiological conditions, the cells comprising the perivascular niche may not only be secreting certain key factors, but the quantity of these factors may play a critical role in balancing the extent of self-renewal of dental pulp stem cells. If there is a disruption in the activation or repression of these signaling pathways during pathogenesis (*i.e.* bacterial contamination via deep carious lesions or trauma), this could lead to a signaling response from stem cells to respond to external stimuli to asymmetrically divide into differentiated cells for tissue regeneration. As such, understanding the role of alternative pathways, and how they are regulated during homeostasis, may provide additional mechanistic clues to understand the physiology of the dental pulp tissue.

Furthermore, this regulatory feedback loop may be bi-directional, or even multi-directional (*i.e.* involving multiple cell types). As our results show the effect of endothelial cell-derived factors to regulate dental pulp stem cells, these stem cells may inversely provide signaling cues to endothelial cells within the perivascular niche. Further, dental pulp stem cell-derived factors may also contribute to the maintenance of the stem cell niche in an autocrine fashion.

When considering the stem cell niche, there may be other cell types that may be contributing to self-renewal, such as neural cells. The existence of neurovascular stem cell niches has also been suggested, indicating the function of molecular crosstalk among endothelial cells, neural cells, and stem cells (Zhao et al. 2014). This type of histological organization of blood vessels, stem cells, and neural structures might be required to enable tissue maintenance during homeostasis and healing upon tissue wounding. With more detailed mechanistic and functional exploration of other components of the stem cell niches, key signaling pathways can be targeted to regenerate these niches and maintain the long-term viability of the tissue.

While the premise of this project was to study the maintenance of the self-renewing stem cell population within the dental pulp, more specifically during homeostasis, the knowledge gained here may shed insight on the molecular signaling pathways that are activated during other processes, such as wound repair and tissue regeneration. Future studies exploring key signaling pathways that determine the fate of dental pulp stem cells during wound repair would allow us to identify pathways that are potential candidates to target for tissue regeneration.

In summary, these studies showed evidence of a functional crosstalk between endothelial cells and stem cells in the dental pulp tissue. We demonstrated that endothelial cell-derived IL-6 induces dental pulp stem cell self-renewal via STAT3 signaling through Bmi-1 function. We also illustrated that this pathway is critical for the establishment and maintenance of perivascular niches in the dental pulp. As such, we propose that the function of endothelial cells in the dental pulp is not limited to enabling influx of oxygen and nutrients. Rather, these results suggest that the maintenance of

pools of stem cells throughout the life of the dental pulp requires signaling events triggered by nearby vascular endothelial cells. Our findings underscore the significance of understanding cellular interactions within the perivascular stem cell niche and suggest that the IL-6/STAT3 pathway plays a critical role in the self-renewal and maintenance of stem cells in the dental pulp.

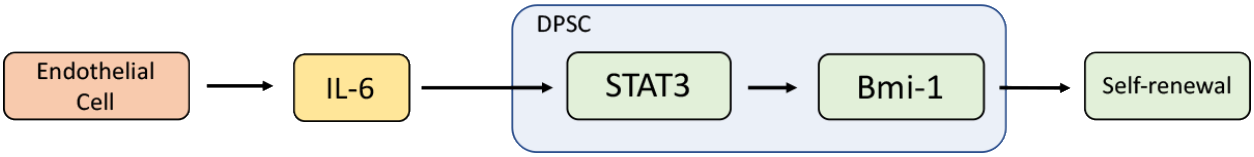

Figure 3.1. Graphical representation of the proposed mechanism for regulation of self-renewal of dental pulp stem cells.

The main **conclusions** of this research project are:

- Endothelial cell-derived IL-6 signals through IL-6R to activate STAT3 and induce Bmi-1 expression in dental pulp stem cells
- The STAT3 pathway is necessary to establish and maintain perivascular niches through the process of self-renewal
- The endothelial cell-dental pulp stem cell crosstalk mediated by IL-6 is required for the maintenance of the stem cell pool

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