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The Use of αSMAGFP to Define Progenitor Cells of the Dental Pulp.

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ABSTRACT

The Use of αSMAGFP to Define Progenitor Cells of the Dental Pulp

Reza Fatahi Ph.D.
University of Connecticut, 2013

It has been proposed that perivascular cells of the dental pulp are a source of odontoblast-like cells. However, the differentiation potential of perivascular cells remained undefined. In the present study we utilized alpha-smooth muscle actin promoter driven expression of green fluorescent protein (αSMA-GFP) and αSMACreERT2xAi9 models to define the ability of perivascular cells to give rise to second generation of odontoblasts secreting dentin.

Dental pulp cells were collected from unerupted molars of P5-P7 neonatal pups. The αSMAGFP+ dental pulp cells were a small population residing in close proximity to endothelial cells in vivo. During growth and mineralization of primary pulp cultures, there was a significant increase in the number of αSMAGFP+ cells resulting from both the acquisition of GFP in new cells, and from the mitotic activity of αSMAGFP+ cells.

Analysis of the cultured pulp cells showed that the αSMAGFP+ expressed markers of MSCs. Sorted αSMAGFP+ cells derived from fresh tissue digests had the ability to differentiate into a mineralizing cell type that expressed osteocalcin and collagen type 1α1, and significantly less dentin sialophosphoprotein (Dsp). In contrast, the
sorted αSMAGFP- population represented an enriched odontoblast progenitor cell population that expressed high levels of *Dspp*.

The role of the perivascular cell expressing αSMA during the process of reparative dentinogenesis was examined using a red fluorescent protein (tdTomato) reporter expressed by perivascular cells and their progeny after pulp exposure. The presence of perivascular cells was not initially detected at the site of injury. Seven days following injury, tdTomato+ cells were detected at the injury site. The presence of reparative dentin lined by tdTomato+ cells was found six weeks following injury.

Our findings provide evidence that perivascular cells expressing αSMA constitute one of the progenitor populations in the dental pulp able to differentiate into odontoblast-like cells giving rise to reparative dentin.
The Use of αSMAGFP to Define Progenitor Cells of the Dental Pulp

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B.S. Cleveland College, 2005

A Dissertation
Submitted in Partial Fulfillment of the
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Doctor of Philosophy Dissertation

THE USE OF $\alpha$SMA-GFP TO DEFINE PROGENITOR CELLS OF THE DENTAL PULP

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To my pedar Mostafaa Fataahi Raaviz from Kerman and my maadar Giti Rajaeei from Ardabil and Yazd. My family, all of them everywhere, Sadri, Raaviz, the west, and others.

To Queen Farah for her indescribable personal losses in one generation, unspeakable crimes that were ushered and imposed from the jealousies that came to surface during the Shah's final interviews in 1974 and 1978, moments before his sabotage and assassination in the style of the poisoning of Alexander Letvenenko.

My baby cousin, my hero, for his recognized bravery in the Battle of Fallujah, and Sergeant of the Guard in the United States Marine Corps. His academic excellence, sportsmanship, and knowing how to be a man.

To the good people, who are bona fide figures of intelligence because of their purity of hearts and minds. The families keeping a watchful benevolent eye on me during my eight years.

The support from others I learned, shared ideas, and who helped me out during rough patches, who were kind and inspiring, and taught me the meaning of how to live life: A Lichtler, A Dongari, J Grasso, J Grasso, A Hand, B Kream, B Upholt, C Jacome, D Repic, G Maxwell, J Tanzer, J Xi, J Igwe, K Sagonmonyants, L Davis, L Klobutzer, L Wang, M Sarfarazi, M Frozoni, M Kronenberg, ML Stover, S Eisenberg, S King, S Wadhwa and others. B Rodgers at the very beginning and the very end.

My advisors, I Kalajzic and M Mina, for their unconditional help and good intentions, teaching me life, and supreme patience during my last months. The NIH for their support and kindness throughout during my training (R01-DE016689, U24-DE06495, T32-DE007302 and T90DE021989)
Beauty, enough to drive any simple sane naïve boy off the rails; and nature, enough to drive any simple sane naïve boy back on.

"I still hope, if i can serve energy and absorb the meaning of my experiences, that I shall be able to ascend one of the little hills. a little hill, but still a hill, and not a ridge. Be that as it will be, even from my ridge, I look back on two decades through which good friends stood together. Moved forward a little, dreamed that the world can be better, and tried to make it so. Tasted the joy of small victories, wounded each other, made mistakes, suffered much injury, and stood silent in the chamber of lies. For all of this, I am grateful. That much I have. That much cannot be taken from me. Barcelona fell, but you were not there, and i was not there, and perhaps if we had been the city would have stood, and the world would have been changed and better. But we are here, and here together we remain, and our city won't fall, and if it should, better that we lie buried in its ruins than found absent a second time."

Dalton Trumbo

"I believe that everything that you do bad comes back to you. So everything that I do that's bad, I'm going to suffer from it. But in my mind, I believe what I'm doing is right. So I feel like I'm going to heaven."

Tupac Shakur
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I. INTRODUCTION

Primary and secondary dentinogenesis

The primary odontoblast is a specialized cell type that is found exclusively around the dental pulp of a tooth. The odontoblast is derived from the mesenchymal compartment of a developing tooth and is responsible for the deposition of a primary and secondary dentin [1, 2]. The primary and secondary dentin are tubular matrices. Within the dentin tubules are odontoblast processes that either partially or fully extend the length of dentin [2-4]. The term 'primary dentin' describes the dentin deposited by odontoblasts before tooth eruption whereas the term secondary dentin describes the dentin deposited after tooth eruption [5]. The odontoblasts responsible for the secretion of primary and secondary dentin also secrete reactionary dentin in response to mild stimuli.

The odontoblasts are responsible for the secretion of a dentin extracellular matrix. Initially, the extracellular matrix is produced as an organic matrix primarily composed of collagen and non-collagenous proteins (NCPs) [1, 6, 7]. The predentin that is produced by the odontoblast is analogous to the osteoid produced by osteoblasts, where and when the matrix has not yet fully mineralized [8]. The inorganic content of the predentin continues to increase over time. The organic predentin component contributes to the mineralization of the dentin tissue. Specifically, certain NCPs are known to regulate the process of mineralization [8, 9]. The NCPs are anionic proteins that are categorized into four groups: proteoglycans, glycoproteins, the γ-carboxy glutamic acid (GLA)-containing proteins, and the serum associated proteins. An example of NCPs are the phosphorylated proteins known as the Small Integrin
Binding Ligand N-linked Glycoproteins (SIBLINGs) [7, 9]. These include Dentin Matrix Protein-1 (DMP1) [10, 11], Bone Sialoprotein (BSP), Osteocalcin [12], and the splice variants of the gene Dentin sialophosphoprotein (Dspp): Dentin Sialoprotein (DSP) and Dentin Phosphoprotein (DPP) [6, 9, 13, 14, 15].

The dentin proteins DSP and DPP are found in bone and dentin [16]. The concentrations in these proteins in dentin is much higher than in bone. Half of the remaining organic component in dentin, after accounting for collagen type I, is made up of DPP alone [6, 7, 14]. Therefore, the high expression of DSPP has been previously used to distinguish odontoblasts from osteoblasts and other cell types [13, 17, 18].

Evidence supports that the primary odontoblast is a post-mitotic cell that lasts for the lifetime of the individual [19]. Earlier studies have outlined the different phases or life cycle of an odontoblast during the processes of primary and secondary dentinogenesis [20, 21]. More recently, investigators have discovered that autophagic activity in the odontoblast decreases with age [22, 23]. As a result, there is an increase in the intracellular concentration of lipofuscin, which is a slowly progressive accumulation of waste products. Conversely, in younger odontoblasts, large autophagic vacuoles do not contain as much lipofuscin [22]. Lipofuscin accumulation results from a loss of autophagic activity, where the robustness of autophagic activity and number of autophagic vacuoles were chosen to define cellular aging [23].

Studies conducted with transgenic animals have provided further insight into the different stages of odontoblast differentiation. The differentiation of mesenchymal
progenitor cells derived from the dental pulp occurs along a dentinogenic pathway *in vitro*, and demonstrated that the 3.6Col1a1-GFP transgene was activated in cells at early stages of polarization, while the 2.3Col1a1-GFP transgene was activated at a later stage of polarization just before or at the time of the differentiation of secretory and/or functional odontoblasts [24]. The activity of 3.6Col1a1-GFP was previously characterized to define cells at different stages of maturation along the differentiation to a Col1a1 expressing cell type such as an osteoblast [25]. Further studies showed that the DMP1-GFP transgene was first activated in the secretory/functional odontoblasts engaged in the secretion of predentin and then transiently expressed at high levels in newly differentiated odontoblasts [26]. Expression of DMP1-GFP was downregulated in highly differentiated odontoblasts [24, 26].

Following primary dentinogenesis, the secretory stage of the odontoblast life cycle may be re-activated, as in reactionary dentinogenesis. This is highlighted by an increase in the number of organelles, especially secretory vesicles that are actively participating in the predentin formation [21]. Following the secretory stage, though, there is autophagic activity in the odontoblast and a subsequent reduction in the number of organelles. The reduction includes a decrease in the number or concentration of secretory vesicles. The odontoblast is in a ‘transition period’, at this time. The odontoblast may decrease or increase the number of organelles and return to the ‘secretory stage’ of activity, or the odontoblast may continue to decrease the number of organelles, shrink in size, and become a resting or ‘aged’ odontoblast [22].
Reparative dentinogenesis

An injury may eliminate an odontoblast by apoptosis or cell death [22]. The most common threat to odontoblasts is the infectious process known as caries. Caries is an infectious process of the tooth surface initiated by the pathogen Streptococcus mutans. Caries is a progressive disease, and results in the degradation of the extracellular matrix, and production of acidic waste by-product. A non-lethal exposure of caries to the odontoblast may be the initiating cascade of a dental pulp inflammatory response [27, 28]. An odontoblast potentially has the ability to initiate an inflammatory response following stimulation by the caries [29]. If the caries is not cleared, the acidic by-products spread and irreversibly damage odontoblasts. Furthermore, the infection may worsen and spread into the dental pulp, and will cause necrosis of the dental pulp and tooth itself. The infection may then continue to spread to the adjacent alveolar bone, by way of foramina at the apex of the root. Another major cause of odontoblast death is mechanical trauma, such as in the removal of a carious lesion. Especially in the case of a deep carious lesion or pulp exposure, the chance that the underlying odontoblasts at the site of treatment will have been irreversibly damaged is almost certain.

It remains unknown exactly what initiates the cascade of events that leads to reparative dentinogenesis. However, it is known that the primary dentin matrix is rich in bioactive molecules, such as the SIBLING family of proteins, and transforming growth factor-β1 (TGFβ1) among others [7, 30-32]. Due to the abundance and variety of biomolecules found within the dentin that may be released following injury, it is speculated that the response of the dental pulp to the released biomolecules contributes to the initiation of reparative dentinogenesis [30, 32-34].
Local release and/or distress factors from the odontoblast itself may contribute to the process of reparative dentinogenesis [35]. Besides the odontoblast itself, the environment is rich in nervous and vascular tissue [36], and these may also contribute to cascade of events leading to reparative dentinogenesis. It is generally agreed that there are three broad phases of reparative dentinogenesis: 1) recruitment of macrophages to the site of injury and resolution of tissue debris at the site of injury, 2) angiogenesis and the appearance of fibroblasts and endothelial cells, and 3) the formation of odontoblast-like cells and reparative dentin at the site of injury [37].

Immediately following injury, there is recruitment of macrophages and other cell types to the site of injury. The macrophages remove debris resulting in an environment suitable for healing and repair [30]. The exact role of the macrophage during reparative dentinogenesis remains ill-defined. Specifically what actions, if any, macrophages have on fibroblasts, progenitor cells, or odontoblasts remains unknown [35]. What is known, is that a robust angiogenic response occurs to replace the damaged blood vessel micronetwork [38]. Nonetheless, it is agreed that the release of mitogenic and/or chemotactic factors at the site of injury creates a gradient that may recruit a population of progenitor cells, or induce the differentiation of local mesenchymal cells into odontoblast lineage that leads to the secretion of reparative dentin [19, 32, 34, 39].

Reparative dentin was first identified as a tissue that is distinct from dentin and bone, and was described as a “form of dental bone that presents the most evident similarity to proper bone” [40]. Mummery and others noted that the cells associated with
reparative dentin were few in number and were more broad in morphology than primary odontoblasts [41, 42]. The regenerated odontoblast-like cells were described to have a morphology more similar to osteoblasts than to primary odontoblasts. Others have since confirmed this observation [43].

Primary and secondary dentin are tubular, and unique in that they are associated with primary odontoblasts and their processes [1, 7]. Reparative dentin has a unique morphology and biochemical composition that is distinct from primary and secondary dentin, and is more similar to bone [9, 10, 12, 44, 45]. The distributions of NCPs in primary and secondary dentin are similar to each other and different from reparative dentin [6]. There is higher expression of DSP and DPP in primary and secondary dentin, DMP1 and less expression of BSP, with respect to bone [9, 14]. In contrast, reparative dentin and bone are similar to each other and contain significantly less expression of DSP and DPP, and more expression of BSP and OC [8, 10, 12, 44, 45]. Furthermore, the morphology of bone is known to be atubular and cellular, thus, distinct from primary and secondary dentin. Interestingly, the morphology and distribution of NCPs in the reparative dentin is more similar to bone, than to dentin [43, 45].

Pulse labeling experiments have consistently demonstrated that there is a unique and robust mitogenic activity at blood vessel walls, following irreversible odontoblast injury [46, 47]. The results of numerous studies demonstrate that there may be two distinguishable populations of mitogenic progenitor cells following injury. One population resides at or near the site of injury, and the other population resides in close association with blood vessels [48]. As a result, there have been two competing hypotheses for the origin of odontoblasts giving rise to reparative dentin.
The first is that regenerated odontoblast-like cells are derived from a perivascular source that is away from the site of injury [33, 46, 47]. The competing hypothesis states that the sources of regenerated odontoblast-like cells include pre-existing adjacent odontoblasts and/or the fibroblasts located at or near the site of injury [39, 49].

Transplantation and *in vitro* studies support the presence of mesenchymal progenitor cells within the dental pulp [50]. A transplantation study of the dental pulp utilizing the 2.3Col1a1-GFP transgene provided more evidence that the dental pulp contains undifferentiated progenitor cells [51]. The 2.3Col1a1-GFP transgene was used to show that there were no differentiated cells prior to transplantation, and subsequently following transplantation, there were populations of differentiated cells that expressed the transgene [51]. Furthermore, two populations of terminally differentiated cells were identified: one had an elongated morphology similar to odontoblasts with *Dspp* expression, and the other cells were more round or cuboidal without processes, devoid of *Dspp* expression that closely resembled osteoblasts [4, 51].

Reparative dentinogenesis is generally not a robust process and depends on many variables including the host immune and genetic response, materials, age, and nutrition among others [52]. The more recent identification of progenitor cells that express markers of mesenchymal stem cells within the dental pulp has opened the possibility to better define the cells involved in reparative dentinogenesis [53].
**Mesenchymal stem cells**

The mesenchymal stem cell (MSC) was identified by Friedenstein, as a cell with the ability to form colonies and to differentiate into different tissues. Expecting to study hematopoietic cells *in vitro*, he soon characterized rapidly dividing fibroblasts that were named ‘stromal cells’. These fibroblasts were found to be able to differentiate into osteoblasts, chondrocytes, and adipocytes *in vitro* and *in vivo*, and were then termed ‘mesenchymal stem cells’ [54, 55].

It has been demonstrated that adult stem cells or mesenchymal stem cells are present in many different tissue types [56-58], including dental pulp [59, 60]. The stem cell is defined by two attributes: the ability to self-renew and the ability to differentiate into multiple tissue types [59-61]. The details of the relationship between mesenchymal stem cells and tissue homeostasis remains undefined [34, 39].

Studies have identified that dental pulp derived mesenchymal progenitor cell populations expressed markers normally associated with perivascular cells, such as alpha-smooth muscle actin (αSMA) [62-65]. Positive immunoselection for cells of the blood vessel wall, showed that they expressed markers of MSCs. The STRO-1 and CD146 antigens are characteristic of perivascular cells and antibodies targeting these antigens were used to isolate cells via magnetic or flow-cytometric cell sorting [57, 61, 66, 67]. These isolated cells were clonogenic, multipotential, and expressed other markers of perivascular cells *in vitro*, including αSMA [57, 61].

The expression of αSMA in porcine [64] and human [62, 63] dental pulp cultures showed that αSMA expression was initially detected at low levels, and there was an
increase in αSMA expression over time and number of passages in vitro. The αSMA + cells was detected surrounding mineralized nodules and suggested a significant role of these cells during mineralized nodule formation in vitro [64]. The differentiation of dental pulp stromal cells coincided with the loss of αSMA expression in vitro [62, 64].

Interestingly, these findings support that MSCs are derived from the blood vessel wall [57, 61]. It has been shown that the in vitro phenotype of MSCs derived from the dental pulp and other tissues and organs is indistinguishable from the in vivo phenotype of perivascular cells [57, 61]. However, these studies did not use fresh tissue isolates, and instead, used tissue cultures in which the expression of perivascular cell markers had the potential to be acquired, hence, not to be representative of true perivascular cells. This has raised concern whether or not MSCs are the cell type that participates during reparative dentinogenesis in vivo. Although the answer to this question remains unknown, there is good reason to support a perivascular cell origin for regenerated odontoblast-like cells.

Other methods to define or characterize MSCs of the dental pulp included utilizing the expression of cell surface markers that were characteristic of embryonic stem cells. Multipotential dental pulp progenitor cells that were expanded and passaged in vitro were reported to express markers of embryonic stem cells, such as: Oct 4, SSEA-3 and -4, Tra1-60 and -81, and Nanog [68]. Furthermore, these cells co-expressed a perivascular cell-like phenotype in vitro [68]. Studies utilizing transgenic animal models have provided an alternative means to isolate vascular smooth muscle cells. The Anxa5-LacZ transgenic animal model was used to capture live perivascular cells from the murine embryo and adult brain meninges, where they
were shown to have multilineage potential [69].

Alternative markers that have been used to define MSCs included β1 integrin and the embryonic stem cell marker low-affinity nerve growth factor receptor [70]. The extracellular matrix contains proteins that interact with integrins, and this process mediates cell-to-extracellular matrix interactions. Local fibroblasts have the potential to assume different phenotypes depending on the severity of extracellular matrix disruption and among these phenotypes, it has been shown that fibroblasts have the ability to acquire the expression of perivascular cell markers such as αSMA [71, 72]. The role of integrin signaling is also critical for regulating angiogenesis and tissue healing following injury [73, 74].

The challenge of defining a perivascular cell has not been made easy, due to the lack of specific markers to define perivascular cells [75, 76]; and also that markers such as αSMA may be expressed in non-perivascular cells [71, 72, 77]. There are few dynamically expressed molecular markers that are presently used to identify perivascular cells, and the expression of these markers can vary in a tissue specific manner or due to changes in the angiogenic state of a tissue, such as in development, healing or tumorigenesis [78-81].

**The perivascular cell and αSMA expression in the dental pulp**

The perivascular cells are also referred to as vascular pericytes, smooth muscle cells, and mural wall cells. The perivascular cell is functionally significant to blood vessel wall stability, development, and homeostasis [82, 83]. Perivascular cells reside on the abluminal surface of the blood vessel wall. Blood vessels are
composed of an inner lining that is formed by endothelial cells. The endothelial cell may be surrounded by another layer of vascular smooth muscle cells, to form larger structures such as arteries. Therefore, the distribution and phenotype of perivascular cells vary, according to the size and location of the blood vessel. The dental pulp has a central arteriole, lined with perivascular cells and no smooth muscle cell layer [84, 85]. The remaining significant blood vessel population is found within the odontoblast layer during primary dentinogenesis [86]. This layer of blood vessels migrates away and forms the sub-odontoblastic layer of blood vessels following the completion of primary dentinogenesis [84, 86]. The sub-odontoblastic layer of blood vessels has the ability to relocate to the odontoblastic layer during reactionary dentinogenesis, when a more rapid blood supply to the odontoblasts is necessary [87].

The density of perivascular cells varies according to the characteristics of its host organ [88]. The brain houses the highest density of perivascular cells in the body [89, 90]. This is because the endothelial cells in the brain form a continuous tight junction and they interact with perivascular cells to form the blood brain barrier and to protect the central nervous system from potentially harmful and toxic agents. Within the thymus, perivascular cells derived from cranial neural crest cells have been shown to participate in the egress of mature thymocytes into the bloodstream [91].

The ontogeny of perivascular cells is not completely understood, and it has been shown that the cranial neural crest cells may be a source of perivascular cells of select organs [91], including the dental pulp [92]. Blood vessels are formed first as simple endothelial tubes, and they subsequently recruit mesenchymal cells that form the smooth muscle layer and pericytes. This is accomplished through reciprocal signaling events [93]. The blood vessels play a major role during the process of
inflammation by regulating the egress of monocytes and lymphocytes from the bloodstream to whatever site of injury that may have perfusion [88].

Current investigations, such as determining the activity of serum responsive elements (degenerate CarG sequences) are aimed at defining the genetic and molecular heterogeneity among different cell populations expressing αSMA [94, 95]. The tyrosine kinase receptor PDGFRβ is another widely used marker to define perivascular cells [56, 96]. Mice deficient in PDGFRβ or its ligand PDGF have a severely reduced number of perivascular cells, and subsequently experience embryonic hemorrhagic lethality [82].

More recently, perivascular cells have received attention for being tissue resident progenitor cells [56, 57], and therefore a potential cell source for tissue regenerative therapies. In the human dental pulp and bone marrow, perivascular cells were isolated based on their expression of the melanoma cell adhesion molecule (MCAM, CD146). Transplantation of the sorted CD146+ cells resulted in the formation of dentin-pulp and bone-marrow complexes, respectively. Interestingly, the authors reported that the majority of freshly isolated CD146+ cells co-expressed αSMA [59-61].

The expression of αSMA in the neonatal dental pulp had been previously characterized using αSMA monoclonal antibodies [97, 98]. The expression of αSMA was detected in the dental follicle, and perivascular cells of the dental pulp. The αSMA+ cells of the developing dental pulp are enriched in the apical region and in continuous expression with the dental follicle [18, 97]. Progenitor cells of the dental
A follicle can differentiate into a cell type with osteoblast characteristics and devoid of *Dspp* expression [18].

There is presently no single antigen that specifically defines the perivascular cell, and the markers used *in vivo* are the same as the markers used to define mesenchymal stem cells *in vitro* [57, 99]. The documented plasticity of perivascular cells, known as modulation, and their tight interconnection to the vascular wall further complicate the identification and isolation of individual perivascular cell types. Modulation is the phenotype switching of perivascular cells from a proliferating to contractile state, or vice versa [100]. This phenomenon may indicate that perivascular cells are primed to act as a source of progenitor cells (i.e. during proliferative state) following inductive influences.

Cellular expression of αSMA has been found to increase acutely and transiently following injury [58, 101], and proposed to play a major role during tissue repair [102]. Furthermore, the expression of αSMA has been described to localize to multipotential mesenchymal progenitor cells [57, 58, 61, 99]. In the integument, granulation tissue fibroblasts (myofibroblasts) deposit a new collagen matrix and contract at the site of an open wound [102]. It remains unclear whether cells that express αSMA are derived from the vascular wall, quiescent fibroblasts, or from a common progenitor or stem cell.

Since αSMA expressing cells have been reported to participate in the process of tissue regeneration and in the etiology of various pathologies, the αSMAGFP transgenic mouse line is particularly useful in further studies of the biology of perivascular cells and myofibroblasts. In this reporter system a 1.1 kilobase (kb)
sequence of the αSMA promoter drives the expression of GFP. Characterization of this animal model confirmed that GFP expression correlated to αSMA expression in a site-specific manner in vivo [103]. Our group has demonstrated the utility of the αSMAGFP model to study mesenchymal progenitor cells of the bone marrow- and adipose derived stromal cell populations [58], and osteoprogenitor cells of the periodontium [18]. Although the literature supports a hypothesis that regenerated odontoblast-like cells are derived from a perivascular cell source, details of the association between mesenchymal progenitor cells, and tissue regeneration in vivo remain unknown and subject to additional studies.
II. SPECIFIC AIMS

The goal of this study is to examine if cells expressing αSMA in the dental pulp can give rise to a new generation of odontoblast-like cells during reparative dentinogenesis. We utilized the αSMA driven expression of fluorescent reporters as a marker of perivascular cells. The dentinogenic potential of perivascular cells of the dental pulp were examined in vivo and in vitro using a variety of methods.

Specific aim #1: Analyses of the expression of the αSMAGFP transgene in unerupted molars.

Specific aim #2: Characterization of the expression of the αSMAGFP transgene during dentinogenesis of primary pulp cultures.

Specific aim #3: Defining the dentinogenic potential of αSMAGFP+ cells of the fresh dental pulp.

Specific aim #4: Defining the role of perivascular cells expressing αSMA in reparative dentinogenesis.
III. MATERIALS AND METHODS

Animals: Animals used for this study have been described previously and were in the syngeneic C57/BL6 background [58, 104]. To identify perivascular cells we utilized transgenic mice in which a 1.1 kilobase sequence of the αSMA promoter (termed SMP8, containing the murine vascular smooth muscle alpha-actin promoter and first intron) drives the expression of enhanced Green Fluorescent Protein (GFP; αSMAGFP). The αSMACreERT2 mice in which the expression of Cre was linked to the activity of the αSMA promoter was recently developed [104]. CreERT2 permits temporal induction by delivery of tamoxifen and tissue specificity by directing the expression of Cre under the activity of a tissue-specific promoter [105]. When crossed with Ai9 reporter mice, recombination can be detected by expression of red fluorescence (TdTomato) in transgenic αSMACreERT2xAi9 mice [104, 106].

Tissue preparation for histology: Various tissues including maxillas, mandibles and teeth were obtained from P5-P7 neonatal transgenic and wild type animals and processed for visualization of GFP as described before [18]. Briefly, tissues were fixed in 4% paraformaldehyde, and decalcified in 14% EDTA, 0.5% paraformaldehyde for 1 week at 4°C under gentle agitation. Following decalcification, samples were washed with phosphate buffered saline (PBS), placed in 30% sucrose in PBS solution overnight and embedded in cryomatrix (Thermo Shandon, Kalamazoo, MI, USA). Seven and forty micrometer sections were obtained using the Leica CM3050S cryostat (Leica Microsystems, Bannockburn, IL, USA) and mounted using a CryoJane tape transfer system (Instrumedics, NJ, USA), and processed for various analyses.
**Analysis of GFP expression in vivo:** Sections were visualized using a Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY, USA) equipped with a fluorescein isothiocyanate (FITC)/Texas Red dual filter cube. The microscope was equipped with a motorized stage and controlled with the AxioVision Rel 4.7 graphical user interface program customized to be used with the Zeiss systems (Zeiss systems, Thornwood, NY). Images were obtained by Axiocam digital camera using the dual fluorescent filter, which allowed the GFP signal to be distinguished from the light red autofluorescent background in the marrow space, muscles and decalcified bone.

**Immunocytochemistry:** Sections were washed and hydrated with PBS then treated with 3% H$_2$O$_2$ for 15 min to inactivate endogenous peroxidase activity. Non-specific binding was blocked with 5% normal rat serum for 60 min at room temperature. After washing with PBS, the sections were incubated with rat anti-mouse CD31 primary antibodies (1:100 dilution; BD Biosciences, San Jose, CA, USA) at 4°C overnight, washed and incubated with anti-rat IgG biotinylated secondary antibodies (1:200 dilution) for 1 h at RT, and then SA-HRP (1:200 dilution) was added for 15 minutes at RT. The reaction complexes were visualized using the 3’-diaminobenzidine reaction (Vector Laboratories, Burlingame, CA, USA). The tissue sections were then washed, mounted, and coverslipped using 50% glycerol solution. Images were then recorded in brightfield and fluorescence.

**Monolayer cell culture system:** The coronal portions of the pulps from molars were isolated from P5-7-day-old transgenic mice and non-transgenic littermates and prepared for primary cultures as described previously before [107]. Briefly, a cell suspension was prepared using digestion buffer [1.5 U/mL of collagenase P
(Worthington Biochemical, Lakewood, NJ, USA), 0.05% trypsin (Invitrogen, Grand Island, NY, USA) in PBS] and rotated for 35 min at 37°C. Cells were pelleted by centrifugation at 500 g for 5 min and resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen USA) containing 20% fetal bovine serum (FBS; Hyclone, USA) and filtered through a 70-µm pore size cell strainer. Cells were counted using 0.4% Trypan Blue.

Cell were plated at 5x10⁴ cells/cm² and grown until confluence in “growth medium” consisting of DMEM and 20% FBS supplemented with 40U/mL penicillin and 40µg/mL streptomycin and 2mM glutamine (Invitrogen, USA).

**Induction of mineralization in cultures:** When cells reached confluence, media was switched to ‘mineralization inducing medium’, consisting of: α-minimal essential medium, 10% FBS, 50 µg/ml of ascorbic acid, 4mM β-glycerophosphate, and antibiotics. Media was refreshed every other day until mature mineralized nodules were formed.

**High-density culture system:** Dental pulp cells were resuspended in growth medium at a density of 5.0x10⁶ cells/ml prior to plating in supercharged tissue culture wells as 10µl droplets, as similar to previous work [108]. The plates were incubated for 1 hr at 37ºC prior to the addition of media (10% FBS in DMEM). Once confluence was achieved, “mineralization inducing medium” was added to cultures for 5 days. Media was refreshed every other day until mature mineralized nodules were formed.

**Analysis of αSMAGFP expression in vitro:** The expression of αSMAGFP was visualized using an Olympus IX50 inverted reflected light fluorescence microscope
(Olympus America, Inc., Melville, NY, USA). A specific excitation wavelength was obtained using filters for emerald (exciter, D470/40; dichroic, 495LP; emitter, D525/50). Images were captured using a SPOT Camera (Diagnostic Instruments, Sterling Heights, MI, USA).

**Detection of mineralized nodules:** Mineralized nodules in cultures were examined using two methods: xylenol orange (XO) and von Kossa staining as described before [58]. To examine mineralization via XO staining, 20µM of XO was added to the culture media overnight. Cells were washed with PBS and visualized using fresh media. XO staining was visualized using a Texas Red filter cube (excitation 596nm, emission 615nm) on the Olympus IX50 inverted microscope equipped with an IX-FLA inverted reflected light fluorescence (Olympus America Inc., Melville, NY, USA). After visualization, the same cultures were washed with PBS and fixed with 10% formalin for 10 minutes at RT and processed for von Kossa staining. Light-protected 5% silver nitrate solution was added to the cultures, and tissue culture plates were crosslinked via ultraviolet light using the Stratalinker UV crosslinking system (Stratagene, USA). Cultures were washed with water and air dried. Tissue culture plates stained with von Kossa were digitally imaged with a scanner and quantified using standard image analysis software (Adobe Systems, San Jose, CA, USA).

**Detection of alkaline phosphatase activity in vitro:** Histochemical staining for alkaline phosphatase (AP) activity, characteristic of mesenchymal progenitor cells, was carried out using a commercially available kit (Alkaline phosphatase kit; Sigma Diagnostics, Inc., St Louis, MO, USA) according to the manufacturer's instructions.
**Time-lapse microscopy:** The automated imaging system consisted of a Zeiss Axiovert 200M microscope with automated stage positioning and a stage-mounted incubating chamber, maintained at 37°C, 5% CO\textsubscript{2} in 95% humidified air. Primary dental pulp cultures in monolayer were cultured in 6-well plates with 2 ml of media. The expression of GFP in these cultures was monitored for 24 hours following plating. Images were recorded every 30 minutes at 10x objective in brightfield, and with an emerald filter to detect GFP.

**Flow cytometric analysis and cell sorting:** Fresh digests and cultured cells at various time points (days 3, and 7) were prepared for FACS analysis by mild trypsin/EDTA digestion as described before [17]. After digestion cells were centrifuged, wash and resuspended at a concentration of 500,000 cells per ml. The expression of GFP in 20 to 100 thousand cells was analyzed by the BD LSR-II flow cytometer (Becton-Dickinson, San Jose, CA) equipped with a solid state Coherent Sapphire Blue laser (Coherent Inc., Santa Clara, CA) exciting GFP at 488nm and a 530/30 emission filter. Data were processed using Cell Quest software. Values represent mean±SE determined from at least three independent experiments in which dental pulp cells obtained from non-transgenic littermates were used as controls.

For cell sorting: FACS sorting based on GFP expression was performed on freshly digested dental pulp cells using the FACSAria cell sorter equipped with the same laser. Upon separation, GFP+ and GFP- populations live cells were collected in DMEM containing 20% FBS, and plated as a high-density spot culture as described in previous section.

**Immunophenotype analysis:** The freshly digested dental pulp, subconfluent
cultures at day 3 and confluent cultures at day 7 were examined by flow cytometry for the expression of CD45, CD31, CD90, and Sca-1. For these experiments, cells were resuspended in 2% FBS in PBS and filtered through a 70-µm cell strainer at a density of 1 x 10^6 cells per ml. Standard titration experiments were carried out on representative dental pulp, lymphocyte, and macrophage cell samples.

Approximately 100,000 cells were incubated with pretitrated antibodies (1:800 APC anti-CD45, 1:200 PerCP-Cy5.5 anti-CD31, 1:200 APC anti-CD90.2, and 1:200 APC anti-Sca-1). The immunofluorescent expression was recorded on a LSR-II flow cytometer, and the phenotype analysis was carried-out using the accompanying FACSDiva software (BD Biosciences, San Jose, CA, USA). At least 10,000 cells were analyzed per sample with the flow cytometer. All antibodies were purchased from e-Bioscience (San Diego, CA, USA).

Cell cycle analysis: The BD LSR-II flow cytometer uses a solid state UV laser allowing complex analysis of side population cells in the context of forward and side scatter analysis via Hoechst staining. Cell cycle analysis was utilized to indirectly observe the proliferation of cultured cells. Hoechst 33342 (Molecular Probes, Carlsbad, CA, USA) was added to samples prior to analysis. Primary cultures at day 2 were lifted off tissue culture dishes with 0.25% trypsin and 1mM EDTA. The samples were resuspended in 2% FBS in PBS supplemented with 5 mg/ml Hoechst 33342. The samples were analyzed with a LSR-II flow cytometer. Data analysis was carried out using ModFit Lt software (Verity Software House, Inc., Topsham, ME, USA). The distribution of cells was presented as G_0 + G_1 (DNA= 2n), S (DNA= 2n < x< 4n), and G_2 + M (DNA= 4n) phases on the ModFit software.
**Gene expression analysis:** Total RNA was isolated in TRIzol reagent (Invitrogen) according to the manufacturer's protocol and treated with RNase-free DNase to eliminate genomic DNA. Isolated RNA was reverse transcribed by Superscript II Reverse Transcriptase (Life Technologies) with random primers. Gene expression in the cultures was examined by qPCR analysis using the $2^{-\Delta\Delta CT}$ method as described previously [109]. For TaqMan qPCR reactions, one nanogram of cDNA was combined with 5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ), 2.5 µl H₂O and 0.5 µl TaqMan primers. TaqMan primers for *Bsp*, *Dmp1*, *Dspp*, *Gapdh*, *Osteocalcin* and *Type I α-1 collagen* were purchased from Applied Biosystems (table I).

The samples were run in duplicate for target and endogenous GAPDH. Samples represented end-point analysis of cultures induced to mineralization: i) sorted GFP+ cells, ii) sorted GFP- cells, iii) unsorted dental pulp cells, and iv) unsorted bone marrow stromal cells. The data were processed by iCycler iQv3.1 software and analyzed in Prism v5.1 (GraphPad Software, USA).

**Tooth injury model:** Six-week old mice were processed for pulp exposure as described before [43]. Mice were anesthetized with xylazine and ketamine (100 mg/kg ketamine and 10 mg/kg xylazine) and a cavity was drilled with a carbide burr (diameter 0.04 mm) on the palatal aspect of the maxillary first left molar in the center of the tooth according to the mesio-distal plane on all mice until the pulp was visible through the transparency of the dentine floor of the cavity. A pulp exposure was subsequently created mechanically using an endodontic hand file of 0.15 mm
diameter with a 2% taper. This approach enabled control of pulp exposure size to approximately 150 µm (size of the tip of the file). Pulp capping was performed using mineral trioxide aggregate (Pro-Root MTA®; Dentsply Maillefer) mixed with sterile water following the manufacturer's recommendations. Mineral trioxide aggregate was placed in contact with the pulp using the tip of a probe, and condensed gently with a sterile paper point. The cavity was then sealed with light-cured composite resin associated with a one-step adhesive system. Animals were treated with buprenorphine HCl (Reckitt Benckiser Pharmaceuticals Inc, Richmond, VA) for analgesia at a dose of 0.08 mg/kg for 5 days following surgery. As a control group, pulp exposures were restored with light-cured composite resin without any capping material analyzed immediately after exposure.

**Tissue isolation and analysis following pulp exposure:** Animals were euthanized after zero, one, three, seven, and 44 days following pulp exposure by intracardiac perfusion with 4% paraformaldehyde and processed for histological analysis, as before [43]. Three six-week old animals were analyzed at each time point. The six-week old animals were perfusion fixed with 4% paraformaldehyde prior to being immersion fixed with 4% paraformaldehyde overnight during processing for both paraffin and frozen embedded sections. A perfusion pump was used to infuse 30 ml of 0°C 100-mM sodium phosphate buffer (PBS) (pH 7.4) followed by 30 ml of 0°C 4% freshly dissolved paraformaldehyde in PBS at a rate of approximately 2.5 ml per minute.

After perfusion, maxillary arches were isolated, cleaned from soft tissue, trimmed and fixed in 10% formalin solution for additional 24 hr. Samples were decalcified for 7 days in 15% EDTA, 0.5% of formalin (pH 7.5) at 4º C and then embedded in paraffin
following the standard protocols or processed for frozen section as described above. Paraffin cross-sections of 7 µm were placed onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA) deparaffinized with xylene, rehydrated and processed for epifluorescence analysis. To visualize GFP signal, deparaffinized or frozen sections were mounted with 50% glycerol in PBS. The fluorescence signal in these sections was examined in 10 sections through the region of injury and repair from each mouse using an Axio Observer.Z1 for epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA). Sections were examined with FITC filter and Texas Red filter. The images were captured and analyzed using manufacturer’s graphical user interface program AxioVision Rel 4.7 (Carl Zeiss).
IV- RESULTS

1- Defining αSMAGFP expression in the healthy dental pulp in vivo.

Many studies implicated the role of perivascular cells as a source for progenitor cells of the dental pulp [46, 47, 61, 110]. However, there was no direct evidence to support the function of perivascular cells as the origin of progenitor cells of the dental pulp. Markers to define perivascular cells include: αSMA, PDGFRβ, CD146, and others [18, 57, 58, 61]. αSMA is an intracellular antigen characteristic of perivascular cells [76]. Intracellular markers such as αSMA limit the ability to work with live cells. To observe the expression of an intracellular antigen, a cell must be fixed and permeabilized before the labeling and analysis of the antigen can take place.

The green fluorescent protein (GFP) reporter model functions by the production of the fluorescent reporter, GFP, under the regulation of a defined gene elements, and provides opportunities to work with live cell populations [111]. The GFP model is a non-invasive system that allows the identification and isolation of cell populations, in the absence of the need for cell fixation and permeabilization [111]. Therefore, we utilized a GFP model where the expression of GFP was controlled by regulatory elements in the αSMA gene, known as the αSMAGFP transgenic mouse model [18, 58, 65, 103].

i) αSMAGFP in the dental pulp

First, we examined the pattern of αSMAGFP expression in unerupted P5-P7 molar teeth. The P5-P7 molar teeth were characterized by the presence of a well-shaped
crown with enamel and primary dentin, secreted by ameloblasts and odontoblasts, respectively. Strong expression of αSMAGFP was detected in the dental follicle (Fig. 1A,B) that encircled the developing tooth. αSMAGFP expression was not detected in the ameloblasts (Fig.1A), but was present in the stellate reticulum (‘r’ in Fig.1A) and dental follicle.

At the developing dentin-pulp junction, αSMAGFP expression was not detected in odontoblasts (Fig.1A) that were identified by their columnar morphology and position relative to the primary dentin. The expression of αSMAGFP was present inside the pulp (Fig.1A) and αSMAGFP+ cells formed well-organized structures that were torus in geometry and appeared to outline the shape of large blood vessels in the P7 molar (Fig.1B), entering the pulp from the apex and the dental follicle (Fig.1B).

ii) Correlation of αSMAGFP to CD31 and endothelial cells

The perivascular location of αSMAGFP+ cells was confirmed by examining their relationship to endothelial cells of blood vessels, using CD31 antibody. The CD31 antibody binds to an extracellular cell-adhesion antigen characteristic of endothelial cells.

The expression of CD31 was detected in the endothelial cells of blood vessels in the dental pulp (arrowheads in Fig.2A). Epifluorescence analysis of the same section showed expression of αSMAGFP in cells in close/direct proximity to CD31 (arrows in Fig.2B).
To exclude the possibility of co-expression of αSMAGFP and CD31 in the same cell, their expression in the dental pulp was examined by flow cytometry. Freshly digested cells from the dental pulp of αSMAGFP transgenic animals were labeled with CD31 antibodies conjugated to the fluorophore PerCP-Cy5.5. FACS analysis showed that less than 0.5% of αSMAGFP+ cells stained with CD31 antibodies and vice-versa, indicating that in the dental pulp, αSMAGFP and CD31 defined two distinct populations (Fig.3). This analysis showed that dental pulp contained approximately 5% αSMAGFP+ and CD31+ cells, respectively (Fig.3). Therefore, αSMAGFP is a marker of perivascular cells within the healthy dental pulp in vivo.

iii) Immunophenotype of cells in the dental pulp

We next examined the expression of selected surface markers of the mesenchymal stem cell-like populations in αSMAGFP+ cells in the uninjured and unerupted dental pulp of P5-P7 transgenic animals by fluorescence-activated flow cytometric analysis. The MSC-like population in the dental pulp was examined using CD90 and Sca-1 antibodies [17], known markers of mesenchymal stem cells [112, 113].

Freshly isolated pulp from uninjured and unerupted molars contained a very low percentage (approximately 1%) of cells with hematopoietic lineage phenotypes (CD45), a very high percentage (approximately 90%) of cells expressing CD90, and a low percentage (approximately 10%) of cells expressing Sca-1 (Fig.4). The results are consistent with earlier work that characterized CD45, CD90, and Sca-1 expression in the fresh dental pulp of P5-P7 animals [17]. The rich expression of CD90 coincides with a developing status of the P5-P7 molar [17], and presence of an
enriched population of odontoblast progenitor cells. Furthermore, approximately 1-2% of cells were CD90+/GFP+ and Sca1+/GFP+.

2- Expression of αSMAGFP in primary dental pulp cultures

i) αSMAGFP expression during dentinogenesis of primary dental pulp cultures

Next, we proceeded to characterize the expression of the αSMAGFP transgene in vitro during growth and mineralization of primary pulp cultures. Previous studies in our laboratory [17, 24, 26, 107] have shown that in monolayer systems, primary pulp cultures from P5-P7 molar teeth proliferate and reach confluence at day 7. Induction of mineralization by media containing 50µg/ml of ascorbate and 4mM β-glycerophosphate results in the formation of robust mineralized nodules. Under these conditions, the first sign of mineralization is around day 10, with increases thereafter [17]. At days 18 and 21, almost the entire culture dish is covered by a sheet of von Kossa stained mineralized tissue [24, 107].

Using these culture conditions, we examined the expression of αSMAGFP transgene in primary pulp cultures. Epifluorescence analysis of primary pulp cultures from transgenic animals showed that expression of αSMAGFP initially was limited to a very small population of cells. The expression of the αSMAGFP transgene increased continuously during the proliferation phase of the pulp cells (Fig.5A-H). After addition of mineralization media, and during the formation of multilayered unmineralized and mineralized nodules, there were continuous decreases in the expression of αSMAGFP transgene (Fig.6 A-H), suggestive of a negative correlation between the
expression of αSMAGFP transgene and matrix formation/secretory function of odontoblasts.

This possibility was explored by comparing the expression of αSMAGFP with xylenol orange (XO) supravital staining of the same culture at day 21 (Fig.7). At this time, primary pulp cultures contained extensive mineralization, as previously reported [17]. Our analysis showed lack of detectable expression of the αSMAGFP transgene (Fig. 7A,B) at sites of mature mineralized nodules that were identified by XO stain (Fig. 7B). αSMAGFP+ cells were detected around the mineralized nodules, and not within mineralized nodules. These observations indicated the lack of αSMAGFP transgene expression in differentiated cells of the mineralized nodules in vitro (Fig.7), and are consistent with the lack of αSMAGFP expression in odontoblasts, ameloblasts, cementoblasts, and osteoblasts in vivo (Fig.1).

ii) Changes in the GFP+ population during primary dental pulp cultures.

Changes in the percentage of cells expressing the αSMAGFP transgene at different time points in primary cultures were examined by FACS (Table 2). Analysis of fresh dental pulp digests showed very few cells expressed the αSMAGFP transgene, initially. Three days after plating, the primary pulp cultures displayed a significant increase in the number of αSMAGFP+ cells, compared to day 0. The subconfluent cultures at day 3 contained approximately 75% αSMAGFP+ cells. At day 7, when cultures had reached confluence, as much as 99% of cells in cultures were αSMAGFP+ (Table 2).
We next examined the expression of MSC-like populations in these cultures. The expression of these markers was correlated with the expression of the αSMAGFP transgene by FACS analysis of sub-confluent cultures at day 3, and confluent cultures at day 7. As with our in vivo studies, the presence of MSC-like cells was examined by the expression of CD90 and Sca-1 [17]. Furthermore, the expression of the pan-hematopoietic marker, CD45, was used to identify hematopoietic cells in vitro.

Analysis of primary cultures at day 3 showed that primary pulp cultures contained very few (approximately 1%) CD45+ cells, approximately 40% CD90+ cells, and approximately 20% Sca1+ cells (Fig.8 A-C). Approximately 95% of cells that expressed CD90 or Sca1, also expressed αSMAGFP transgene (Fig.8 B-C).

In confluent cultures at day 7, nearly all cells (approximately 95%) expressed the αSMAGFP transgene. At this time point, there were increases in the number and percentage of cells expressing CD90 (approximately 60%) and Sca-1 (approximately 40%) (Fig.9 B-C). Nearly all cells expressing CD90 and Sca-1 also expressed the αSMAGFP transgene (Fig.9 B-C). These findings show that in primary pulp cultures, αSMAGFP expression is detected in cells expressing markers of mesenchymal progenitor cells.

iii) Mechanism of increase in the number of αSMAGFP+ cells

We focused our attention to better understand the mechanisms of the increase in the number of αSMAGFP+ cells in vitro. The increases in the percentage of the
αSMAGFP+ cells in vitro could be due to activation of transgene in αSMAGFP– cells (that did not initially express the transgene natively) and/or the proliferation of existing αSMAGFP+ cells. To distinguish between these possibilities, αSMAGFP+ and αSMAGFP– populations were analyzed by time-lapse imaging and cell cycle analysis.

Primary cultures in monolayer were analyzed over time by a series of sequential images of specific regions of the culture to capture the earliest events in vitro (Fig. 10). We imaged these cultures during the first twelve hours of tissue culture by phase-contrast and epifluorescence using the FITC filter, to examine whether αSMAGFP transgene expression may be acquired de novo in vitro.

The primary dental pulp cultures initially contained a very limited number of cells that expressed the αSMAGFP transgene. At 3 hours after initial plating, we did not detect αSMAGFP expression in new cells. By 6 hours after initial plating, αSMAGFP expression was detected in cells that previously did not express the transgene. At 9 hours, the expression of the αSMAGFP transgene continued to be detected in new cells that previously did not express the transgene. This pattern continued at 12 hours following initial plating, when the expression of αSMAGFP was amplified and brighter than it was at 9 hours (Fig.10).

The mechanism of the increase in number of cells expressing the αSMAGFP transgene was also studied by examining the DNA content/cell-cycle of cells in cultures derived from αSMAGFP transgenic mice using FACS analysis with the Hoechst33342 nuclear-binding fluorophore. The analysis was performed in day 2 primary cultures that contained approximately equal numbers of αSMAGFP+ and
αSMAGFP– cells (Fig.11). Cell cycle analysis showed significant differences in the rates of proliferation (G2/M + S) between αSMAGFP– (1%) and αSMAGFP+ (82%) populations (Fig.11). Nearly all (approximately 99%) αSMAGFP– cells were diploid, and were most likely in G0 cell cycle arrest (Fig.11B). In contrast, approximately 18% of αSMAGFP+ cells were diploid (Fig.11C), and the majority of αSMAGFP+ cells were active in the cell cycle (Fig.11C).

iv) Endogenous expression of αSMA, SM22α, and PDGFRβ in vitro

To ensure that expression of the αSMAGFP transgene is correlated with the expression of endogenous αSMA, we examined the expression of αSMA in primary pulp cultures by immunocytochemistry in sub-confluent cultures at day 3, and confluent cultures at day 7 (Fig.12). αSMA protein was expressed by the majority of cells at days 3 and 7 (Fig.12 A, A’). The pattern of αSMA expression was indistinguishable from the expression of the αSMAGFP transgene in similar cultures.

We also examined the expression of other markers of perivascular cells in these cultures using antibodies against SM22α and PDGFRβ [99, 114]. Immunohistochemistry showed staining in the majority of cells at day 3, and day 7 (Fig.12). The specificity of the antibodies and staining methods was confirmed in control experiments using the macrophage cell line RAW264.7 cells, plated in parallel and similar conditions. Immunostaining at day 3 and day 7 did not reveal expression for αSMA, SM22α, or PDGFRβ in the macrophage cultures (data not shown).
The patterns of αSMA, SM22α, and PDGFRβ expression in the primary cultures were similar to each other and to the pattern of αSMAGFP expression. This finding supports that the expression of αSMAGFP correlates to αSMA protein, and other markers of perivascular cells such as SM22α and PDGFRβ.

3- Mineralization potential of sorted αSMAGFP populations

The results of the previous section showed the presence of a mixture of αSMAGFP− and αSMAGFP+ populations in primary pulp cultures. Also, a high rate of cell-cycle activity and/or cell proliferation was detected in αSMAGFP+ cells in vitro and image analysis showed activation of this transgene in αSMAGFP− cells. This made it difficult to study the behavior of αSMAGFP+ and αSMAGFP− cells independently. Therefore, as the next step, we studied the behavior of purified αSMAGFP+ and αSMAGFP− populations derived from the fresh dental pulp digest.

The fresh dental pulp digests were processed for FACS analysis and cell sorting to isolate and separate purified populations of αSMAGFP+ and αSMAGFP− cells based on the expression of GFP (Fig.13 A,B). Reanalysis of the sorted populations confirmed the purification of these subpopulations (Fig.13 B,C). Both populations were plated in high-density in small-volume (50,000 cells per 10µl) droplets. The cultures were treated with standard growth medium, until they reached confluence at day 3. Unlike in monolayer cultures, robust mineralization was detected 7 days later, at day 10.

At day 10, cultures were analyzed for the presence of alkaline phosphatase (ALP) activity and mineralization by using ALP and von Kossa staining, respectively. ALP is
expressed by odontoblasts and osteoblasts [59, 62, 115, 116]. The von Kossa stains calcium-rich areas brown or black, depending on the intensity of mineralization and/or time exposed to the stain. Controls in these experiments included unsorted dental pulp cultures seeded and grown in identical conditions as sorted cells and primary bone marrow stromal cell (BMSC) cultures. The BMSCs were initially plated and grown in monolayer and terminated at day 21, when these cultures contained significant matrix. BMSC cultures at day 21 were analyzed for the extent of mineralization, and gene expression analysis.

Analysis of cultures at day 10 showed ALP expression, and the presence of robust mineralization in cultures derived from unsorted cells (Fig.14 A,B) and cultures derived from αSMAGFP− cells (Fig.14 C,D). Cultures derived from αSMAGFP+ cells showed robust ALP activity and little mineralization (Fig.14 E,F).

The percent area stained by von Kossa in various cultures was quantified in these cultures. The extent of mineralization (percent area stained with von Kossa, per 10µm²) was similar between cultures derived from unsorted dental pulp, and cultures derived from αSMAGFP− cells (Fig.14G). The extent of mineralization in cultures derived from αSMAGFP+ cells was significantly less than in cultures derived from unsorted dental pulp cells, and similar to BMSC cultures.

i) Dentinogenic potential of perivascular cells

Analyses of the αSMAGFP+ and αSMAGFP− cultures at day 10 showed distinct differences in the extent of mineralization between these cultures. To gain insight into the dentinogenic potential of these two populations, we examined the expression of
selected markers of differentiation, including: Bone sialoprotein (Bsp), Osteocalcin (Oc), Dentin matrix protein-1 (Dmp1), Dentin sialophosphoprotein (Dspp), and Type I α-1 collagen (Col1a1) by quantitative real-time polymerase chain reaction (qRT-PCR) method. RNA was isolated and processed for qRT-PCR analysis of the genes listed in Table 1. Unsorted dental pulp, and BMSC cultures were utilized as controls. The expression of genes was calculated relative to the levels in unsorted dental pulp cultures, that was arbitrarily set to value equal one (Fig.15 and Table 3).

The gene expression profile of the unsorted BMSCs showed significantly higher levels of Bsp and Oc, and lower levels of Dmp1 and Dspp, as compared to unsorted dental pulp cultures. The relative levels of Col1a1 expression were similar between these cultures. The patterns and levels of Bsp, Oc, Dmp1, Dspp, and Col1a1 in the αSMAGFP– group was similar to the unsorted dental pulp cultures. There was no significant difference in the levels of gene expression between the unsorted dental pulp cultures and the αSMAGFP– group (Fig.15, Table 3).

In αSMAGFP+ cultures, the relative levels of Bsp and Col1a1 were similar to the levels in αSMAGFP– cultures. In αSMAGFP+ cultures, the expression of Oc was significantly higher than in αSMAGFP– cultures, and more similar to BMSC cultures. Dmp1 and Dspp were expressed in lower levels in αSMAGFP+ cultures compared to αSMAGFP– cultures. The relative levels of Dmp1 expression in αSMAGFP+ cultures were similar to levels of BMSC cultures. The level of Dspp expression in αSMAGFP+ cultures was 10-fold higher than in BMSC cultures, and 500-fold lower than in αSMAGFP– and unsorted pulp cultures (Fig.15 and Table 3).
4- Lineage tracing of perivascular cells during the process of reparative dentinogenesis

To gain a better understanding of the roles of perivascular cells expressing αSMAGFP transgene as progenitors giving rise to second generation of odontoblasts during reparative dentinogenesis [34, 45], we used an in vivo tooth injury model that involved pulp exposures [43]. In this tooth-injury model, reparative dentinogenesis was stimulated by mechanical exposure of the pulp. The pinpoint pulp exposure caused death of primary odontoblasts at the site of injury [43]. The mechanical damage initiated a cascade of healing to promote the protection of the dental pulp and tooth vitality, and leading to the formation of reparative dentin [34, 37, 43, 47].

The study by Frozoni et al. utilized the aforementioned 3.6Col1a1-GFP transgenic mouse, and showed a delay in healing and reparative dentinogenesis in mice of the C57Bl6 background compared to CD1 mice [43]. Furthermore, this study, similar to other studies, showed that mineral trioxide aggregate (MTA) enhanced healing [117], and promoted recruitment of progenitor cells to the site of injury and reparative dentin formation [43].

Our studies using αSMAGFP allowed us to characterize the expression of this transgene to perivascular cells in healthy tissues, such as the dental pulp. However, the αSMAGFP model was not sufficient to define the role of perivascular cells during the process of reparative dentinogenesis. αSMAGFP is not expressed by mature odontoblasts and osteoblasts. Also, αSMAGFP may be expressed by other cells during tissue injury, where disruption of the extracellular matrix [73, 74] and escape of serum from the rich microvasculature of the pulp [84, 118, 119] can contribute to the
activation of αSMA in non-perivascular cells [95, 120]. The non-inducible Cre recombinase system also does not provide any way to specify the expression of Cre recombinase to track perivascular cells.

However, if the Cre functioned on a temporal-specific basis, then we would be able to distinguish αSMA expression in perivascular cells from other cell types. Perivascular cells would be the only cells expressing αSMA in the healthy dental pulp, and a temporal specific induction would allow us to pulse label perivascular cells and chase them, following injury of the tooth. This is made possible by the inducible Cre transgenic model. The CreERT system allows us to temporally define a population, and their progeny, following induction with tamoxifen [105]. The αSMACreERT2 model expresses Cre under the regulation of the αSMA gene elements [104]. αSMACreERT2 mice are bred with Cre-dependent Ai9 reporter mice (Rosa26-tdTomato) that constitutively express a reporter in an inactive state until it is recombined by Cre recombinase enzyme. Treatment of dual transgenic mice (αSMACreERT2xAi9) with tamoxifen induces recombination and tdTomato expression in cells (tdTomato+) expressing the αSMACreERT2 transgene [104].

In a set of preliminary experiments, we utilized αSMACreERT2xAi9 dual transgenic mice to perform lineage analyses on a perivascular cell population and their progeny [104], during the process of reparative dentinogenesis. Six-week old animals were injected intraperitoneally with a single dose (37.5 mg/kg) of tamoxifen four days before surgery. Pulp exposures were created on the occlusal surface of the first maxillary molar of αSMACreERT2xAi9 transgenic mice in C57Bl6 background (n = 9), and tamoxifen treated αSMACreERT2 animal in C57Bl6/129 background (n = 1), and a dual-transgenic animal in C57Bl6 genetic background that was not treated with
tamoxifen (n = 1). Direct pulp capping of exposed teeth was performed using MTA followed by restoration with a light-cured adhesive system and composite resin for all teeth. Animals were euthanized at three (n = 3), seven (n = 3) and 44 (n = 3) days after pulp exposure and capping. The maxillary arch was isolated, fixed and processed for histological and epifluorescence analysis to examine reparative dentinogenesis, as described previously [43].

At three days following injury, tdTomato+ cells were detected within the dental pulp at areas in close association with blood vessels, but not at the site of injury (Fig.16A,A'). tdTomato+ cells were also detected in the periodontal ligament and bone marrow (Fig.16). Consistent with previous results [43], dentin bridge formation was not detected seven days following injury. However, at day seven, tdTomato+ cells were present at the site of injury where the MTA was deposited (Fig.16B,B').

At 44 days following injury, there was a well-defined calcified bridge detected along the exposure site underneath the MTA. The reparative dentin was surrounded by tdTomato+ cells (Fig.16C,C', Fig.17). There were decreases in the number of tdTomato+ cells in the pulp, as compared to day 3 and day 7 following injury. The expression of tdTomato was not detected in the αSMACreERT2 single transgenic animal injected with tamoxifen, and the αSMACreERT2xAi9 dual transgenic animal not injected with tamoxifen, supporting the specificity of tdTomato expression.
V- DISCUSSION

Among the earliest literature available in histology, investigators observed the formation of reparative dentin by odontoblast-like cells that differed from primary odontoblasts in morphology and secretion of atubular dentin [40, 41]. Furthermore, it was speculated that reparative dentin formation originated from cells of the blood vessel wall [110]. Later, nucleotide base labeling provided additional support that regenerated odontoblasts are post-mitotic progeny of perivascular cells [47].

The results from previous in vitro and in vivo studies supported the idea that odontoblast progenitor cells are derived from a perivascular cell origin [46, 47, 61, 110]. However, there was no direct evidence showing this; and evidence to show the ability of perivascular cells to differentiate into the odontoblast lineage was needed.

Studies of various organs have developed multiple criteria to assist in the identification of perivascular cells. These include the expression of markers such as alpha-smooth muscle actin (αSMA), cell morphology, and physical association to the endothelium. Perivascular cells may be defined by the expression of a variety of markers, including αSMA [76], desmin and vimentin [121], PDGFRβ [56, 57, 99], CD146 [57, 61], NG2 [122, 123] and others.

The identification of perivascular cells by antibodies against cytoskeletal elements, including αSMA, requires fixation and permeabilization of cells for their visualization. Therefore, it was not possible to track cells expressing intracellular markers such as αSMA in real-time. To address these limitations, our group utilized a transgenic animal model that expresses green fluorescent protein (GFP) under the regulation of
elements in the αSMA gene (αSMAGFP), in the present study, to gain further insight into the roles of perivascular cells during reparative dentinogenesis.

Our studies showed that αSMAGFP was expressed in cells that were in close association with endothelial cells that stained positive for CD31 antibody. The fluorescence activated flow cytometry (FACS analysis) provided further support that αSMAGFP+ population was a separate and distinct group of cells from the CD31 population. Our studies also showed that the αSMAGFP transgene is expressed in cells within the dental pulp, dental follicle, and stellate reticulum. The perivascular location of the αSMAGFP transgene in our study is consistent with the expression of endogenous αSMA in the dental pulp [97, 98], and other tissues [57, 58].

Consistent with previous results [17], the murine P5-P7 dental pulp contained an abundance of CD90+ cells. The expression of CD90 co-localized to approximately half of the total αSMAGFP+ population in situ in the murine P5-P7 dental pulp. This observation is consistent with the work published by Crisan et al. that showed CD90 was characteristic of some, but not all, perivascular cells [57]. In their studies, and others, the endothelium was labeled with CD31 or CD34 antibodies [57, 58]. The endothelium plays a major role alongside progenitor cells, and is correlated to the presence and/or function of progenitor cells during development and tissue healing [124-126]. More studies are necessary to define the role of the endothelial cells in αSMAGFP+ perivascular cell development and maintenance in the dental pulp. The αSMAGFP transgenic animal model may also be utilized to further define the correlation of αSMAGFP to other extracellular antigens such as NG2 and CD44 for their use in the identification and/or isolation of perivascular cells.
The expression of the αSMAGFP transgene was acquired in the majority of cells in pulp cultures

Culture techniques are commonly used to study the behavior of odontoblast progenitor cells of the dental pulp. Utilization of primary cultures of dental pulp was essential for establishing the ability of dental pulp cells to produce mineralized tissue, thus, evidence for a population of progenitor cells within the dental pulp [50, 63, 127]. In studies of human [62, 63] and porcine [64] dental pulp cultures, others detected increasing levels of αSMA expressed over time in monolayer systems. Initially, upon plating, αSMA expression was detected at low levels [62-64]. The expression of αSMA increased significantly over time and passage in vitro [62-64].

Similarly, dental pulp cultures derived from mature and erupted teeth of transgenic αSMAGFP animals were initially found to have low expression of transgene [65]. This was followed by a significant increase in αSMAGFP transgene expression over time in vitro [65]. In our study, although only a small number of cells expressed αSMAGFP in vivo (2-5%), the expression of the αSMAGFP transgene increased rapidly during primary tissue cultures. By the time cells had reached confluence in primary pulp cultures, nearly all the cells were expressing the αSMAGFP transgene.

We were interested in mechanisms that contributed to the rapid and robust increase of αSMAGFP transgene expression early in culture. We utilized cell-cycle analysis as an indirect measure of cell proliferation. Our results indicated that αSMAGFP+ cells are highly proliferative. We then analyzed the earliest events in primary pulp cultures by time-lapse imaging to distinguish between de novo acquisition of αSMAGFP transgene expression, and expression by progeny of dividing cells. Time-lapse
analysis indicated that many cells acquired αSMAGFP transgene expression during their first 12 hours of tissue culture. Therefore, the rapid increase in the number of αSMAGFP+ cells also results from the activation of the transgene. However, this observation has to be taken with reserve as αSMAGFP could be expressed at low level that is below detection. With the time, signal can increase and cells can begin to proliferate.

Numerous mechanisms can modulate the expression of αSMA, including: sphingosine 1-phosphate [128, 129], TFGβ and FGF [130, 131], IFNγamma [72, 132], and notch signaling [133]. However, there may be other mechanisms for the increase of αSMA expression. Two of these are the integrin system [74] and the serum response factor elements [95, 120]. Tissue cultures mimic tissue injury, where the mechanical signals detected by the integrins may play a role in the activation of αSMA. Dental pulp tissue cultures are also serum dependent, and cells will not survive without an appropriate amount of serum or serum replacement.

Nearly all the cells that expressed the MSC markers CD90 and Sca-1 *in vitro* co-expressed the αSMAGFP transgene. The expression of CD90 and Sca1 have previously been utilized *in vitro* to identify mesenchymal progenitor cells [17, 112, 113]. Our studies were consistent with previous results [17], and showed the absence of significant expression of the pan-hematopoietic marker, CD45, *in vitro*. The P5-P7 dental pulp is characterized by the absence of hematopoietic cells *in vivo* [17]. The number of CD45+ cells in the dental pulp increases over time with age and maturation of the tooth [17].
Mesenchymal progenitor cells express markers of vascular smooth muscle cells in vitro

As our data suggests that αSMAGFP expression was acquired in culture by many cells that did not initially express the transgene, the αSMAGFP transgene was no longer a specific marker of perivascular cells in vitro. Therefore, αSMA expression is not specific to perivascular cells under adverse conditions such as tissue culture and injury. It is 40 years since it was first shown that mesenchymal cells in vitro have muscle-like qualities with the ability to contract or deform their matrix [134, 135]. A contractile behavior of dermal fibroblasts, following injury, was also identified [136, 137]. The contractility was associated by the expression of αSMA [71, 79, 137]. It was then known that αSMA expression can be acutely and transiently expressed in myofibroblasts [71].

The contractility defined a behavior and role for αSMA during the process of healing by providing a mechanism to restabilize the extracellular matrix following injury [71, 74], allowing for the formation of new blood vessels, and the migration of progenitor cells to the site of injury [138]. More recent studies of the bone showed that following injury there is an acute and transient expansion of the number of cells expressing αSMA [101] and αSMAGFP [58], following injury. A recent study showing expression of αSMA more than forty days following tooth injury found that expression of αSMA is present underneath the site of injury, and limited to sites of re-vascularization of the injured dental pulp [139].

Other studies have shown that mesenchymal progenitor cells derived from a variety of tissue sources express markers of perivascular cells [18, 56-58, 99, 140]. To
examine if this was true of cells derived from the dental pulp, we examined the expression of endogenous markers of perivascular cells at day 3 and day 7 of primary pulp tissue cultures in monolayer. DAPI was used as a counterstain to visualize the total cell population in vitro. The distribution of antigens relative to DAPI counterstain showed that the majority of cells expressed αSMA, and there was no distinguishable difference in the patterns of expression between the transgene and endogenous αSMA. Therefore, our findings using the αSMAGFP transgene did not represent a difference between transgene expression and αSMA protein expression.

We proceeded to examine the expression of other markers of perivascular cells, namely SM22α and PDGFRβ [99, 114].

Although in our study staining was not done in the same cultures, our analysis showed that the majority of cells in primary tissue cultures also expressed other markers of perivascular cells at those times, under identical conditions. Charbord’s group communicated that mesenchymal progenitor cells derived from the bone marrow expressed the equivalent of a perivascular cell phenotype in vitro [99]. Subsequently, the same pattern held true for mesenchymal progenitor cells from other tissues of origin, including adipose [56-58, 140] and periodontal ligaments [18].

*The αSMAGFP transgene is not expressed by functional odontoblasts and osteoblasts*

In our study, the αSMAGFP transgene was expressed robustly early during tissue cultures, and diminished over time following the formation of a mineralizing matrix. The expression of the αSMAGFP transgene steadily decreased over time, until it was no longer detected. The decrease in expression was directly correlated to an
increase in mineralization. A previous study by Brock et al. showed that αSMA expression was not detected at sites of mineralized nodule formation in vitro [64]. Furthermore, following the differentiation of cells and formation of mineralization in vitro, expression of αSMA was found only at sites surrounding mineralized nodules [64]. This indicates that αSMA+ cells may be heterogeneous in function, where some cells did not differentiate and continued to express αSMA at sites near to progenitor cells, or that the progenitor cells remained in an undifferentiated state of existence.

Another study by Alliot-Licht et al. also demonstrated that odontoblast differentiation in dental pulp cultures is correlated to a loss of αSMA expression in vitro [62]. The administration of dexamethasone led to a more than two-fold decrease in the number of cells expressing αSMA in vitro. Treatment with dexamethasone also caused a major increase in the number of non-viable cells in culture [62]. The cells that survived were found to express greater amounts of alkaline phosphatase and Dspp relative to cells that were not treated. This suggests that the diminished expression of αSMA may have been fully or partially related to the differentiation of cells. Indeed, the authors concluded that the decrease in αSMA expression was related to the commitment of cells into the dentinogenetic lineage in vitro, as previously reported [116]. Interestingly, the authors characterized the effect of dexamethasone on the expression of STRO-1 under a set of different conditions that showed an increase in STRO-1 expression following treatment with dexamethasone [62]. The expression of αSMA is known to correlate to the expression of STRO-1 [141-144]. Dental pulp mesenchymal cells that were magnetically sorted on the basis of STRO-1 expression were found to co-express αSMA [61]. Dexamethasone treatment also caused a decrease in incorporation of thymidine, indicative of a decrease in cell proliferation [62]. The decrease in the number of cells that expressed αSMA, and/or the hastened
differentiation of cells may have contributed to the loss of αSMA expression.

In the study by Marty-Roix et al., mature odontoblasts were derived from the coronal portion of porcine dental pulp, and plated at low density. The odontoblasts were detected to stain intensely for the expression of αSMA [127]. The authors did not observe the process of differentiation, and instead viewed the characteristics of mature cell types in vitro. It is known that odontoblasts express antigens, such as myosin heavy chain at their processes, for their functions [145]. It is possible that odontoblasts have the ability to express αSMA under conditions such as tissue culture following tissue dissociation and immediately following injury (i.e. surviving odontoblasts adjacent to the site of injury). Our studies were limited to undifferentiated mesenchymal cells [4, 17], and we did not isolate mature odontoblasts or observe their behavior immediately following injury with the αSMAGFP transgene.

The Zhao et al. study utilized outgrowth cells from the dental pulp of adult transgenic animals that were then passaged and induced to differentiate, and they concluded that αSMAGFP transgene colocalized to functional odontoblasts or osteoblasts, within robust mineralized nodules [65]. Although αSMAGFP may be detected by the use of antibodies, the fluorescence is no longer active at sites of robust mineralized nodule formation. However, it is not clear from their study if cells had lost the expression of αSMAGFP by the time they had differentiated and formed a mineralized matrix. The half-life of αSMA protein [146, 147] and GFP [148] do not exceed 48 hours, and our study and others show that αSMA [62-64] and αSMAGFP expression [18, 58] is lost once cells in monolayer have differentiated into functional odontoblasts or osteoblasts within mature mineralized nodules in vitro. Such a
conclusion is consistent with our findings in vivo, where mature odontoblasts and osteoblasts are not detected to express the αSMAGFP transgene, and do not express αSMA protein [97].

*Perivascular cells of the dental pulp that express αSMA can differentiate into odontoblast-like cells in vitro*

Based on the visual detectability of GFP, cell populations were purified by FACS to test the dentinogenic potential of GFP+ perivascular cells. Certainly, the primary limitation of this study design and the previous time-lapse analysis was the arbitrary designation of what is GFP negative. GFP expression that is not visually detected may be detectable by the flow cytometer. Visual detectability extends multiple logarithmic units away from zero on the flow cytometric histogram’s fluorescence scale, and does not equal to zero expression of GFP. Therefore, this study did not distinguish between cell populations expressing GFP in the non-visually detectable range. With this caveat in mind, we examined the differentiation potential of perivascular cells of the P5-P7 dental pulp. Since αSMAGFP expression was representative of perivascular cells in vivo, we sorted cells from fresh digests of the dental pulp based on the expression of GFP. The limiting factor to this technique is contamination by the dental follicle. The dental follicle expresses abundant αSMAGFP, and is connected to the dental pulp at the apex. To minimize contamination, the apex of the dental pulp was completely removed to exclude the dental follicle during tissue isolation and surgery.

Cells were sorted and/or purified according to their expression of GFP. Once they reached confluence, nearly all cells of both GFP+ and GFP- populations expressed
the αSMAGFP transgene. Cultures established from the GFP– population formed robust mineralized nodules with relatively high levels of Dspp expression that was almost identical to the unsorted dental pulp. The robust mineralization formed from the GFP– population was similar to the mineralization formed by unsorted dental pulp cells, suggesting that the GFP– population was enriched in committed odontoprogenitor cells that is supported and consistent with previous results [17, 24, 26, 107].

Our findings showed that the αSMAGFP+ population was limited population of cells (2-5%) that displayed mineralization potential and Dspp expression, albeit significantly less relative to the αSMAGFP– or unsorted dental pulp populations. These observations revealed the dentinogenic potential of the limited number of αSMAGFP+ perivascular cells in the healthy P5-P7 dental pulp. The αSMAGFP+ perivascular cells of the P5-P7 dental pulp represent a heterogeneous and small fraction of cells with dentinogenic potential that was not appreciated in previous studies, due to the presence of large numbers of committed odontoprogenitor cells.

The potential of αSMAGFP+ perivascular cells of the P5-P7 pulp to differentiate into multiple lineages remains undefined. However, previous results showed a general absence of multilineage potential in cells derived from the unerupted P5-P7 molar [17]. This may be due to the absence of multilineage progenitor cells, the overwhelming presence of odontoblast progenitors and/or CD90+ cells, or from a change in multilineage potential of αSMA+ perivascular cells and/or other dental pulp cells over time. There is a possibility that paracrine actions from hematopoietic cells of the mature dental pulp may contribute to increased plasticity of the perivascular cell and/or existence of multilineage mesenchymal stem cells. It would be worthwhile
to examine the effects of conditioned media from macrophages and/or lymphocytes on the plasticity of committed progenitor cells, such as the dental follicle, rodent incisor, or dental pulp fibroblasts of P5-P7 animals.

Our findings show that there are at least two populations of progenitor cells found within the dental pulp of P5-P7 mice. The dental pulp of P5-P7 is rich in odontoblasts progenitor cells and CD90 expression. It is an unerupted and developing organ that may resemble an embryonic organ in a post-natal environment. The vast majority of cells in the dental pulp of P5-P7 animals represented the population that is most characteristic of a dental pulp tissue culture: they were dentinogenic in nature and had an extensive ability to form a mineralized matrix \textit{in vitro}. The other cells represented a much more defined population with different characteristics of mineralization potential and gene expression signature. The αSMAGFP+ perivascular cells expressed significantly higher levels of Oc, and significantly lower levels of Dspp; and formed significantly less amount of mineralization compared to the unsorted dental pulp cells.

A recent study demonstrated that odontoblast progenitor cells of the rodent incisor come from multiple sources, including a perivascular cell source [149]. The authors utilized a non-inducible or global Cre lineage trace model that labeled perivascular cells of the healthy dental pulp, and observed the dynamics of reporter activity following injury, and during the process of odontoblast differentiation at the cervical loop of rodent incisors. The rodent incisor is a unique continuously growing organ, and the findings may have limited relevance relative to molars and human incisors. A recent study by Balic and Mina showed that the mesenchymal progenitor cells from dental pulp of the rodent incisor behave different from mesenchymal progenitor cells
derived from molars *in vitro* [150]. Nonetheless, two populations of odontoblast progenitor cells were identified within the dental pulp of rodent incisors: one from a perivascular cell origin and one from a non-perivascular cell origin [149].

*Perivascular cells that express αSMA contribute to the process of reparative dentinogenesis in vivo*

Since the αSMAGFP transgene is not expressed in mature cell types, such as odontoblasts and osteoblasts, it cannot be utilized for defining the dynamics of perivascular cell contribution during the process of reparative dentinogenesis *in vivo*. Furthermore, the αSMAGFP transgene may be expressed following injury, by a variety of cell types other than perivascular cells. As a result the αSMAGFP animal model did not provide a good model for examining the roles of perivascular cells in reparative dentinogenesis.

To address whether perivascular cells directly contribute to the formation of reparative dentin, we utilized the αSMACreERT2xAi9 dual transgenic mouse line that allowed induction of red fluorescent protein (tdTomato) expression, following treatment with tamoxifen. Prior to induction by tamoxifen, the tdTomato reporter includes a ‘stop’ code that is flagged by loxP sites and, as a result remains in the ‘off’ configuration. Upon treatment with tamoxifen, tamoxifen binds to the Cre-ERTxAi9 protein allowing it to translocate to the nucleus where the Cre recombinase causes recombination and deletion of the ‘stop codon’, thus, allowing the tdTomato reporter to be expressed. We injected a single pulse dose of tamoxifen into healthy six-week old dual transgenic animals four days prior to injury of molars. Control of the expression of the Cre-ERT:Ai9 recombinase by the αSMA gene regulatory elements
effectively limited the recombination and the subsequent ‘permanent’ expression of tdTomato to perivascular cells and their progeny \textit{in vivo}. We were interested to examine the dynamic localization of perivascular cells, and their progeny, during the process of reparative dentinogenesis.

A preliminary experiment was conducted to assess whether perivascular cells expressing $\alpha$SMA contribute to the formation of odontoblast-like cells, during the process of reparative dentinogenesis. Our results show at three days following injury, tdTomato+ cells were detected within pulp at areas away from the site of injury, but not at the site of injury. At seven days following tooth injury, tdTomato+ cells were present at the site of injury, and at six weeks following tooth injury, tdTomato+ cells were found lining the newly formed reparative dentin.

There was very good correlation between the patterns of tdTomato and 3.6Col1a1-GFP transgene expression during reparative dentinogenesis [43]. Seven days following injury, cells expressing the 3.6Col1a1-GFP transgene were present at the site of tooth injury. Six weeks following tooth injury, cells expressing the 3.6Col1a1-GFP transgene were present lining the reparative dentin. Therefore, our results provide strong evidence that perivascular cells and/or their progeny are odontoblast-like progenitor cells that directly participate during the process of reparative dentinogenesis.

Our results from the cell sort provides direct evidence that $\alpha$SMAGFP+ perivascular cells have a unique differentiation phenotype, relative to GFP– and unsorted dental pulp cells. Other studies utilizing biochemical techniques support our conclusion that regenerated odontoblasts have a unique phenotype \textit{in vivo}. Early and more recent
studies took note that regenerated odontoblasts resembled osteoblasts and/or reparative dentin resembled bone [40, 110, 151]. These cells were shown to express less DMP1 and DSPP, and more OC and BSP than primary odontoblasts [10, 12, 117]. The reparative dentin matrix formed by these cells is a unique, atubular, cellular or acellular matrix [43, 151] that resembles bone tissue.

**Conclusion**

Our studies showed that the αSMAGFP transgene identifies perivascular cells in the healthy dental pulp. In primary pulp cultures derived from the unerupted molars of αSMAGFP transgenic animals, there was a rapid increase in the percent of αSMAGFP+ cells that was caused by activation of transgene in the αSMAGFP− population, and high mitotic activity of αSMAGFP+ cells. Our studies of the sorted αSMAGFP+ population *in vitro* showed that these limited group of cells have mineralization and dentinogenic potential, albeit much less than the αSMAGFP− population. The preliminary lineage tracing experiments using αSMA-CreERT2xAi9 animals and experimental pulp exposure showed the contribution of cells expressing the transgene to odontoblast-like cells in reparative dentinogenesis.
**VI- TABLES and FIGURES**

Table 1

<table>
<thead>
<tr>
<th>Gene ID</th>
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<tr>
<td>Osteocalcin</td>
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Figure 1
Figure 3
Figure 4

A. CD45

B. CD90

C. Sca1

dSWA/GFP+ vs PE+
Figure 5

Day 0 | Day 3 | Day 5 | Day 7
---|---|---|---
A | C | E | G
B | D | F | H
Figure 7
Table 2

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<tr>
<td>Day 0 (n=5)</td>
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<tr>
<td>Day 3 (n=6)</td>
<td>74.35 ± 2.81%</td>
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<tr>
<td>Day 7 (n=5)</td>
<td>97.3 ± 1.63%</td>
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Figure 8

A. CD45  

B. CD90  

C. Sca1  

αMAGFP+ PE+
Figure 9

A) CD45  

B) CD90  

C) Sca1  

PE+  

cSMAGFP+  

$11.4 \pm 4.8\%$  

$1.2 \pm 0.7\%$  

$0.1 \pm 0.02\%$  

$0.3 \pm 0.01\%$  

$0.5 \pm 0.04\%$  

$0.8 \pm 0.05\%$  

$1.2 \pm 0.03\%$  

$5.5 \pm 2.1\%$  

$6.2 \pm 2.0\%$  

$6.7 \pm 1.9\%$  

$7.4 \pm 2.8\%$  

$9.2 \pm 3.6\%$  

$10.2 \pm 4.9\%$  

$1.6 \pm 0.7\%$  

$1.8 \pm 0.8\%$  

$3.2 \pm 1.3\%$  

$4.8 \pm 2.0\%$  

$6.3 \pm 2.5\%$  

$11.2 \pm 6.4\%$  

$0.2 \pm 0.02\%$
Figure 10
<table>
<thead>
<tr>
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<th>(A) Total population</th>
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<th>(C) GFP positive cells</th>
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<tr>
<td>G0/G1</td>
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<td>99%</td>
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<tr>
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<td>7%</td>
<td>0%</td>
<td>35%</td>
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<td>S</td>
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</table>
Figure 12

Day 3

A

A'

Day 7

B

B'

SM22α

C

C'

αSMA

PDGFRβ
Figure 13

A) Non-transgenic

B) Transgenic

C) GFP- population reanalysis

D) GFP+ population reanalysis

2.3%

>99%

>95%
Figure 14

ALP

von Kossa

unssorted

A

B

C

D

GFP negative

GFP positive

E

F

G

GFP negative

GFP positive

GFP negative

bone marrow

per cent area stained/10 mm²

unsorted
dental pulp

unsorted
dental pulp

unssorted
dental pulp
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<td>Type I collagen</td>
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Figure 17
Table 1: List of primers used for qRT-PCR analysis.

Figure 1: The expression of αSMAGFP transgene in P5-P7 molars in vivo. Images of frontal frozen sections of molars isolated from P5 (A) and P7 (B) transgenic mice. Section A is 7µm thick and B is 40µm thick. Images were captured using a dual-filter cube. αSMAGFP transgene is detected in green and is localized inside and outside the dental pulp. Within the dental pulp is the presence of the αSMAGFP transgene in a limited number of fibroblast-like cells that may be captured as a collection of cells lining a blood vessel. The expression of αSMAGFP is abundant in the surrounding dental follicle. αSMAGFP is also expressed in the stellate reticulum.

The expression of αSMAGFP is not detected in the layer of ameloblasts or odontoblasts. There is rich expression of αSMAGFP transgene at the apex. The apex represents a continuum of αSMAGFP transgene expression from the dental follicle, and into the dental pulp. The areas in yellow (indicated by arrow) and orange (indicated by arrowhead) indicate high intensity GFP expression captured with the dual filter cube. Abbreviation: a = ameloblasts; f = dental follicle; o = odontoblasts; p = pulp; r = stellate reticulum; x = apex. Scale bar, 100 µm.

Figure 2: The correlation of expression of αSMAGFP with endothelial cells in dental pulp in vivo. Images of a frontal section through a molar from a P6 transgenic mouse processed for CD31 immunohistochemistry. In P6 molars, endothelial cells (arrowheads in A) are stained with CD31 antibody. The same
section was then imaged under epifluorescent light. αSMAGFP+ cells (arrows in B) are present lining the areas stained by CD31. Note the close association of cells expressing αSMAGFP (arrows in B) with the walls of blood vessels stained with CD31 antibody (arrowheads in A). Scale bar, 100 µm.

**Figure 3: αSMAGFP and CD31 are expressed in two distinct populations.** FACS analysis of the expression of CD31 (X axis) and αSMAGFP (Y axis) in freshly isolated pulp cells from P5-7 mice. Fresh dental pulp tissues from P5-P7 transgenic pups were incubated with primary PerCP-Cy5.5 conjugated CD31 antibodies and analyzed by flow cytometry. Note that freshly isolated dental pulp cells from unerupted molars contained two independent populations. One population expressed the αSMAGFP transgene (2.9 ± 0.7%) and the other expressed CD31 (4.9 ± 1.7%). Values represent mean value ± S.E. of the three independent experiments.

**Figure 4: Cell surface profile of fresh dental pulp digests.** Flow cytometric analysis of the expression of CD45, CD90, and Sca-1 (X-axes) in freshly isolated dental pulp cells from P5-7 transgenic mice. αSMAGFP expression is shown on the (Y-axes). Note the limited number of cells that expressed CD45 and Sca-1, whereas a high percentage of cells expressed CD90. Approximately half of GFP+ cells co-expressed CD90 in the fresh digests. Values represent mean ± S.E. of three independent experiments.

**Figure 5. The expression of αSMAGFP during the proliferation phase of primary dental pulp cultures.** Cultures were derived from coronal pulp of P5-P7 αSMAGFP transgenic mice. Images of the same areas in culture at different time
points that were captured under phase contrast (A,C,E,G) and epifluorescent light (B,D,F,H). There was a robust increase in the expression of the αSMAGFP transgene during the proliferation phase of cultures. Scale bar, 100 μm.

**Figure 6. The expression of αSMAGFP during the differentiation phase of primary dental pulp cultures induced into the dentinogenic lineage.** Mineralization was induced at day 7 by the addition of mineralization inducing media. Images of the same field were captured at different time points under phase contrast (A,C,E,G) and epifluorescent FITC light (B,D,F,H). Note the steady decrease in αSMAGFP transgene expression over time during the differentiation of cells *in vitro*. Scale bar, 100 μm.

**Figure 7. The correlation of sites of mineralization and αSMAGFP expression.** Primary dental pulp cultures obtained from transgenic αSMAGFP animals were grown for 21 days. Image ‘A’ represents overlay of images of the same field that were captured under phase contrast and FITC epifluorescent light, and processed using Adobe Photoshop. Image ‘B’ represents overlay of the same field used in ‘A’ that were captured under epifluorescent lights using the FITC and TexasRed filters. αSMAGFP+ cells were captured with the FITC filter and xylenol orange (XO) signal with the TexasRed filter. XO staining is observed at sites of mineralized nodule formation (indicated by *). αSMAGFP transgene is present at sites adjacent to, and not within, mineralized nodules (indicated by arrowhead). Scale bar, 400 μm.

**Table 2: The increase of cells expressing αSMAGFP transgene during proliferation phase of primary dental pulp cultures.** Cultures were derived from coronal pulp of P5-P7 αSMAGFP transgenic mice. Cells expressing αSMAGFP
transgene were quantified using FACS analysis. Note the robust increase in the expression of the αSMAGFP transgene during the proliferation phase of cultures. Values represent mean ± S.E. of the three independent experiments.

**Figure 8. Characterization of cell surface markers at day 3 of primary cultures.**
FACS analyses of the expressions of CD45, CD90, and Sca-1 (X-axes) cell surface markers in primary pulp cultures derived from P5-7 transgenic mice, at subconfluence (day 3). αSMAGFP expression is shown on the (Y-axes). Values represent mean ± S.E. of three independent experiments.

**Figure 9. Characterization of cell surface markers at day 7 of primary cultures.**
FACS analyses of the expressions of CD45, CD90, and Sca-1 (X-axes) cell surface markers in primary pulp cultures derived from P5-7 αSMAGFP transgenic mice at day 7. αSMAGFP expression is shown on the (Y-axes). Values represent mean ± S.E. of three independent experiments.

**Figure 10. Time-lapse microphotography of primary dental pulp cultures.** Serial images of the same field under phase contrast (upper row) and epifluorescence (lower row) of cultures during the first 12 hours, following initial plating. The arrowhead denotes a αSMAGFP– cell that was followed over time in culture. Note that this cell acquired the expression of transgene over time. The star denotes a αSMAGFP+ cell that was followed over time in culture. Scale bar, 100 µm.

**Figure 11. Cell-cycle analysis at day 2 in primary dental pulp cultures.** Primary cultures were established from αSMAGFP transgenic animals and analyzed at day 2 for cell-cycle activity in the αSMAGFP– and αSMAGFP+ populations. Hoechst33342
was utilized to label the DNA content of cells in total population of cells at day 2 (A), the αSMAGFP− population (B), and the αSMAGFP+ population (C). The table (D) shows the percentage of cells distributed according to the status of cells in the cell cycle. G0/G1 indicates populations where DNA content equals 2n. S and G2/M indicate populations where DNA content exceeds 2n. Values represent mean ± S.E. of three independent experiments.

**Figure 12. The expression of markers of perivascular cells in primary dental pulp cultures.** Primary cultures from non-transgenic animals were labeled with antibodies against αSMA, SM22α, and PDGFRβ at day 3 (A-C) and day 7 (A’-C’). The αSMA antibody was conjugated with Cy3. The PDGFRβ and SM22α antibodies were conjugated with FITC. Images were captured under epifluorescent light using TexasRed filter for αSMA, and FITC filter for SM22α and PDGFRβ. Cultures were counterstained with DAPI to view cell number *in vitro*. Note that the majority of cells in pulp cultures expressed these markers. Macrophage cell line RAW264 cells did not express any of these markers *in vitro*. Scale bar, 100 µm.

**Figure 13. Fluorescence activated sorting based on the expression of αSMAGFP in fresh dental pulp tissue digests.** Dental pulps were isolated from P5-P7 non-transgenic (A) and transgenic (B) animals and processed for FACS analysis and cell sorting. Reanalysis of the purified αSMAGFP− (C) and αSMAGFP+ populations (D) show purity of isolation and sorting exceeded 99% for the GFP− population. Flow analysis shows αSMAGFP expression along the x-axes in the FITC channel, and the side scatter of cells (SSC) along the y-axes.
**Figure 14. Mineralization potential of perivascular cells of the dental pulp.** The sorted αSMAGFP+ and αSMAGFP– populations were cultured and induced to differentiate as described in *Materials and Methods*. Images of alkaline phosphatase (A,C,E) and von Kossa (B,D,F) stained cultures, at day 10 when the formation of robust mineralization was detected. Alkaline phosphatase and von Kossa staining were used to detect mineralizing and mineralized tissue, respectively. G is a histogram showing percent area stained with von Kossa per representative 10µm² of high-density/small-volume cultures at day 10, and BMSCs in monolayer at day 21. The extent of mineralization in unsorted dental pulp cultures were similar to that in cultures derived from αSMAGFP– population. The unsorted bone marrow and αSMAGFP+ populations formed similar amounts of mineralization. The amount of mineralization formed by αSMAGFP+ perivascular cells was significantly less than the αSMAGFP– population. Note the nodular pattern of mineralization in αSMAGFP+ cultures indicating that not all αSMAGFP+ cells had mineralization potential *in vitro*. Values represent mean ± S.E. of three independent experiments. Significance (*p<0.05) was determined relative to unsorted dental pulp cultures.

**Figure 15. Gene expression profile of mineralized cultures at day 10.** Cultures from unsorted dental pulp cells, αSMAGFP– cells, and αSMAGFP+ cells were established at high density as described in *Materials and Methods*. Cultures of primary bone marrow stromal cells (BMSCs) were plated in standard monolayer as described in *Materials and Methods*. RNA was isolated from the high-density/small-volume cultures at day 10 and from BMSCs was derived from cultures at day 21. The RNA was processed for gene expression analysis using qRT-PCR with the primers listed in Table 1. The dash line represents the levels of expression of each transcript/ GAPDH in unsorted dental pulp cells that was arbitrarily set equal to one.
Note that the expression of Col1a1 is similar in all groups. The expression of genes derived from BMSCs is different from unsorted dental pulp. In brief, Oc and Bsp are expressed at significantly higher levels in BMSCs as compared to unsorted dental pulp. The patterns and levels of expression of various genes in αSMAGFP– cultures are similar to unsorted dental pulp cultures. The profile of Bsp expression in the αSMAGFP+ cultures was similar to unsorted dental pulp. The profile of the Dmp1, Dspp, and Oc expression in αSMAGFP+ cultures is similar to those in BMSCs. Values represent mean ± S.E. of the two independent experiments. Statistical significance (*p<0.05) was calculated relative to expression of genes derived from unsorted primary pulp cultures.

Table 3: Gene expression profile of mineralized cultures derived from dental pulp, bone marrow, and perivascular cells in vitro. qRT-PCR analysis of RNA isolated from various cultures. Data was imported to Microsoft Excel and analyzed using ∆∆CT method to examine the changes in the expression of Type I α 1 collagen, Bsp, Dmp1, Oc, and Dspp relative to Gapdh. Gene expression was calculated relative to expression derived from unsorted primary cultures. Values represent mean ± S.E. of the two independent experiments.

Figure 16. Dynamic localization of perivascular cells, and progeny, during reparative dentinogenesis. Histological sections stained with hematoxylin (A,B,C) and epifluorescence (A’,B’,C’) of the same sections from teeth of double transgenic animals analyzed following pulp exposure. Sections were first analyzed under epifluorescence using dual filter TRITC cube to capture tdTomato expression and then processed for histology and visualized under light microscopy. (A and A’) are
images of a section through a pulp capped with MTA after 3 days and shows the presence of tdTomato+ cells within the dental pulp (indicated by arrows). Note the absence of tdTomato+ cells at the site of injury. (B,B’) are images from a tooth capped with MTA after day 7 and shows that tdTomato+ cells were present at the site of injury (indicated by arrows). Note the close association of tdTomato+ cells with the MTA (indicated by arrows). (C and C’) are images from a tooth capped with MTA after 44 days following tooth injury. The tdTomato+ cells were present in the dental pulp and also at the site of injury (indicated by arrows), where tdTomato+ cells lined reparative dentin. b = bone; c = dentin chip; d = dentin; l = periodontal ligament; p = pulp; (arrows) = tdTomato+ cells; (star) = site of injury. Scale bar, 100µm.

Figure 17. Reparative dentin lined by tdTomato+ cells six weeks following injury. These are higher magnification images of the same section shown in 16C. Histological sections stained with hematoxylin (A and A’) and epifluorescence analyses of the same section (B and B’) of teeth from dual transgenic animals analyzed 44 days (six weeks) following tooth injury. A calcified bridge is formed underneath the site of injury capped with MTA. Note the numerous tdTomato+ cells around the calcified bridge (indicated by arrows). b = bone; d = dentin; p = pulp; rd = reparative dentin; (arrows) = tdTomato+ cells; (star) = site of injury. Scale bar, 100µm.
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