

Vascular Endothelial Growth Factor Expression in the Murine Dental Pulp
During Aging and Dentin Regeneration

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Dedication

This master's thesis is dedicated to Malala Yousafzai, an activist for the right to education for all women around the world.

“All I want is an education, and I am afraid of no one.”

When I was frustrated, discouraged or tired you reminded me of the struggle of young women all over the world and of my own educational privilege.

Thank you.

Abstract

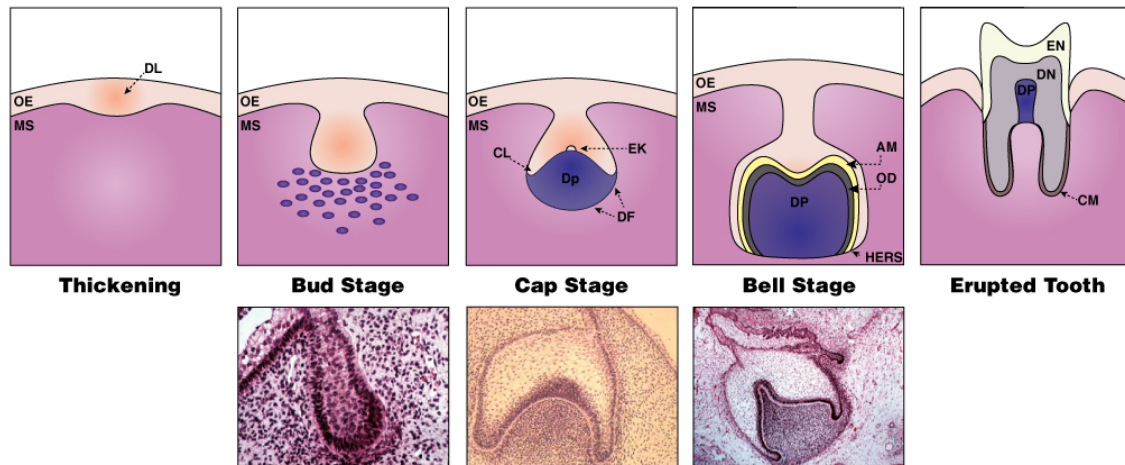
Vascular endothelial growth factor (VEGF) is an important pro-angiogenic growth factor that is essential for maintenance of vascular networks throughout the body, including those in the dental pulp. In addition, it has been suggested that VEGF may play a role in dentin regeneration and mineralization. As well studied as VEGF is, currently there are no studies looking at the effects of aging on VEGF expression in the dental pulp. We proposed a study of the expression levels of VEGF in the dental pulp during aging using a mouse model to test whether VEGF expression decreases with age and whether this decrease causes a decrease in pulpal vascular density and tertiary dentin formation. Tertiary dentin is formed as an internal reparative response to injury to the tooth, which can involve stem cell recruitment from the dental pulp. However, better understanding of this process is needed to develop stem cell and tissue regeneration applications to improve the organization and formation of tertiary dentin following injury. Mandibles from mice in six separate age groups were collected and histological analysis was performed. Mandibular molar sections were stained for antibodies for VEGFR-2, VEGFR-3, VEGF-A, VEGF-C, LYVE-1 (a lymphatic marker) and BS-1 (a vascular marker). Mandibular molars were also isolated from three separate age groups and collected for gene expression analysis using Q-RT-PCR. The immunofluorescence results from the vascularity study showed a significant decrease in blood vessel density with the highest levels found in the neonate and the lowest found in the most aged samples. There was also a distinct qualitative difference in the expression of VEGF-A in the dental pulp and odontoblast layers of samples from each age group that followed the same trend as the vasculature. Lymphatics were not detected in any of the age groups. Q-

RT-PCR results showed that there was a decrease in gene expression in vWF (a blood vessel marker), VE-cadherin (a blood vessel marker), VEGFR-3, VEGF-A and VEGF-C from the neonatal to senescent age groups. VEGFR-1 and -2 were only detected at low levels in the neonatal age group. Surprisingly VEGF-C and VEGFR-3 were highly expressed in the dental pulp and although their expressions also decrease with aging, they were the only VEGF ligand and receptor detectable in the uninjured adult mouse molar. For the second part of the study, to determine if VEGF played a role in dentin regeneration, the first maxillary molars of nine mice were drilled to induce a wound healing response in the tooth. They were allowed time to heal and then the maxillae were collected and stained with 3,3'-diaminobenzidine for VEGF-A and VEGFR-2 expression at three time points post-injury. The histological sections from injured molars showed a dramatic increase in VEGF-A expression fifteen minutes after injury that dissipated with time and returned to near normal levels after four weeks post-injury. VEGFR-2 expression was also elevated soon after injury and then gradually returned to pre-injury levels. Current findings of this study suggest that although the expression of VEGF-A and its receptor VEGFR-2, which has been traditionally viewed as the most potent pro-angiogenic signaling pathway, does decrease with age, this ligand and receptor may play a more important role in regulating the response to tooth injury and dental regeneration. In contrast, VEGFR-3 and its ligand VEGF-C, which have traditionally been associated with only lymphangiogenesis, may be contributing to the regulation of angiogenesis in the dental pulp.

Introduction

Dental anatomy can be broken down into four main components: the enamel, the dentin, the cementum and the dental pulp. The crown of the tooth, which includes the occlusal surface, is covered in a thin layer of highly mineralized enamel. Under the enamel layer is the softer dentin layer, which encapsulates the dental pulp. The dental pulp consists of several cell types including immune cells, fibroblasts and undifferentiated progenitor cells, as well as nerve and blood vessel networks. The root of the tooth is made up of dentin covered by a layer of cementum.

The embryonic development of human and murine teeth is well studied and understood. Both involve the processes of reciprocal signaling of two tissue types found in the embryonic mouth; the oral epithelium and the neural crest derived mesenchyme. At approximately six weeks in the human embryo and days 9-11 in the mouse, the oral epithelium (or oral ectoderm) initiates the first stage in tooth development by way of signaling molecules such as BMP-2, BMP-4 and FGF (D'Souza & Qin, 2012). The ectodermal cell layer begins to thicken and proliferate into the mesenchyme at the sites of future teeth, forming the dental lamina. After the dental lamina is formed, the formation of the tooth in mice and in humans can be classified into 3 separate stages: the bud stage, cap stage and bell stage (Fig. 1) (Tucker and Sharpe, 2004).



AM = Ameloblasts, CL = Cervical Loop, CM = Cementum, DF = Dental Follicle, DL= Dental Lamina, DN = Dentin, Dp = Dental-Papilla, DP = Dental Pulp, EK = Enamel Knot, EN = Enamel, HERS = Hertwig's Epithelial Root Sheath, MS = Mesenchyme, OE = Oral Epithelium

Figure 1. Stages of tooth development. Modified from *Nature Reviews Genetics* 5, 499-508 (July 2004), figure 2(Tucker and Sharpe, 2004).

In the bud stage, as the oral epithelium continues to invaginate into the underlying neural crest derived ecto-mesenchyme, mesenchymal cells begin to condense around the newly formed dental lamina to form a tooth bud. As the cells of the dental lamina continue to proliferate, they form the enamel organ. The cells within the center of the enamel organ are called the stellate reticulum. The transition from the thickening of the dental lamina to the bud stage occurs at approximately embryonic day 13.5 in mice. In humans this process occurs from approximately the 17th embryonic week to age 5 (D'Souza & Qin, 2012).

Tooth crown formation is initiated next in the cap stage. During the cap stage, the dental lamina continues to proliferate into the mesenchyme and folds into a cap-like structure, partially enclosing a cluster of condensed mesenchymal cells now referred to as the dental papilla. The leading edges of the newly folded dental lamina are referred to as the cervical loops. The tissue on the outer edges of the dental papilla, also composed of

ecto-mesenchyme is now referred to as the dental follicle. The epithelium of the cap will eventually give rise to the ameloblast layer, which is responsible for the formation of enamel and the dental papilla will eventually give rise to the odontoblast layer, which is responsible for the formation of dentin, as well as the cementoblasts responsible for cementum and also the tissue of the dental pulp itself. It is during the cap stage that the first enamel knot is formed. The enamel knot will be the future position of the first cusp of the developing tooth. It appears at the tip of the tooth bud at the site of the first epithelial folding. In molar teeth, both murine and human, a second set of enamel knots that determine the multicusped pattern of the teeth will also develop (Nanci, 2008.).

As the dental lamina continues to fold inward, the cap stage gives rise to the bell stage. During the bell stage, morphodifferentiation occurs. The odontoblast layer and the ameloblast layers align along the dentinoenamel junction and begin secreting enamel and primary dentin. As the bell stage progresses, the crown of the tooth is formed. In non-continuously growing teeth (which includes all human teeth and all murine molars) the root is formed when the internal structure of the cervical loop condenses to only a double layer of epithelium, known as Hertwig's epithelial root sheath (HERS), which grows downward and directs root formation (Nanci, 2008.).

As stated earlier, dentin formation begins in the bell stage of tooth development by the aligning of the odontoblast layer in the dental papilla. Dentin, by weight, is comprised of 70% hydroxyapatite, 20% proteins (approximately 90% of which is type I collagen) and 10% water, making it the most bone-like structure of the tooth (Nanci, 2008.). It is less mineralized and brittle than enamel and is continuously laid down by odontoblasts throughout the life of the tooth (Nanci, 2008.). Mature dentin normally

consists of dentin matrix and many microscopic channels called dentinal tubules, which are formed by and made for accommodating the odontoblast cell processes, which often extend the entire width of the dentin layer. The dentinal tubules contain the cell processes of the odontoblasts, but can also provide passage for some nerve fibers. The odontoblasts that form the dentin layer are aligned along the inner surface of the dentin, at the pre-dentin layer, which is the name given to the newest formed dentin of the tooth that has yet to completely mineralize (Nanci, 2008.).

The first type of dentin to be secreted by odontoblasts, and that which abuts the enamel layer, is a kind of primary dentin known as mantle dentin (Smith, 2012). It is thin (approximately 150 μm wide) and lacks the organization of primary and secondary dentin. The rest of the primary dentin layer can be considered all dentin that is laid down before the tooth erupts, which is the majority of the total dentin layer of a mature tooth.

Secondary dentin is that dentin which is laid down after the tooth has erupted. Its formation is slower than primary dentin, but continual over the life of the individual tooth. It is secondary dentin that is responsible for the stenosing of the pulp chamber (Smith, 2012). Histologically, it is very difficult to distinguish secondary from primary dentin as both types have a similar molecular structure, follow similar growth patterns and tend to mineralize to the same degree.

The third type of dentin can be considered pathological and is called tertiary dentin (Smith, 2012). Tertiary dentin formation is the name given to the tooth's ability to secrete dentin in response to an external destructive stimulus on the tooth such as attrition, caries or dental trauma. The reaction of the tooth to these stimuli is complex and categorized based on the level of insult to the tooth and the resulting type of tertiary

dentin formed. Reactionary dentin is formed when an intact and vital postmitotic odontoblast layer of cells begins to secrete a new layer of dentin in response to a mild or moderate external stress. An example could be the accumulation of dentin at the top of a pulp horn in response to mastication forces on the overlying cusp.

In contrast, the process of forming reparative dentin is much more complicated. It is the result of an insult to the tooth great enough to penetrate the enamel and dentin layers and kill the underlying original odontoblast layer (Nanci, 2008.). An example of this could be a pulp exposure as a result of a broken tooth or a deep carious lesion that penetrates to the pulp. When such an injury occurs, progenitor cells from the dental pulp are recruited and begin to differentiate into odontoblast-like cells. They migrate from the pulp core and aggregate in the area of injury where they begin to secrete dentin matrix. The morphology of reparative tertiary dentin is often different from that of reactionary dentin, which can be very similar to primary and secondary physiologic dentin. Usually, reparative tertiary dentin is poorly organized, unevenly calcified, does not contain dentinal tubules and may contain cellular inclusions (Smith *et al.*, 2012).

In order to recruit stem cell progenitors from the pulp and induce their differentiation to odontoblast-like cells capable of secreting tertiary dentin, specific growth factor signaling must take place. Although the exact mechanism for this process is not known, several growth factors have been shown to promote odontoblast-like cell differentiation, including TGF- β 1 (Tziafas *et al.*, 1998), TGF- β 3 (Sloan and Smith, 1999), and bone morphogenic proteins (Nakashima, 1994; Rutherford *et al.*, 1993; Rutherford *et al.*, 1994; Sloan *et al.*, 2000). During injury, these and other growth factors that have been sequestered in the dentin matrix are released into the pulp and are allowed

to interact with the cells of the dental pulp as well as invading immune cells that have localized at the injury site (Roberts-Clark and Smith, 2000; Tran-Hung *et al.*, 2008). It is the complex interaction between these growth factors and the cytokines released by immune cells that leads to the recruitment of dental pulp stem cell progenitors and their differentiation into odontoblast-like cells (Smith *et al.*, 2012).

Another growth factor that has been shown to be sequestered in the primary and secondary physiological dentin layers at high levels is vascular endothelial growth factor A (VEGF-A), a ligand of the vascular endothelial growth factor family (Roberts-Clark and Smith, 2000). Vascular endothelial growth factor (VEGF) is a 45- kDa heparin-binding glycoprotein involved in several essential cell processes. It is a powerful endothelial cell mitogen and is involved in mediating cell differentiation, proliferation and permeability (Artese *et al.*, 2002; Matsushita *et al.*, 2000). In addition, VEGF is also considered the most important growth factor controlling angiogenesis (Grando Mattuella *et al.*, 2007) and lymphangiogenesis (Yancopoulos *et al.*, 2000).

The VEGF family of ligands currently includes 5 known members: VEGF-A, -B, -C, -D and placental growth factor (PLGF), which bind with varying affinity to 3 different tyrosine kinase receptors (Virtej *et al.*, 2013). Ligands VEGF-A, -B and PLGF bind to VEGFR-1. This receptor acts as a negative mediator of VEGF-A, both in soluble and membrane bound forms, by binding excess VEGF-A. It has been shown that homozygous VEGFR-1 mutant mice have excess formation of endothelial cells, demonstrating the regulatory capacity of VEGFR-1 during angiogenesis (Fong *et al.*, 1995). Although it plays a definite role in early embryological angiogenesis, VEGFR-3 is known to bind VEGF-C and VEGF-D and is most commonly associated with

lymphangiogenesis (Taipale *et al.*, 1999). However, recently it has been shown that VEGFR-3 is tumor angiogenesis and growth (Laakkonen *et al.*, 2007) and that knocking down VEGFR-3 will also suppress angiogenesis in some non-pathological adult tissues (Tammela *et al.*, 2008). Although VEGFR-2 is known to bind VEGF ligands A, C and D, it is the signaling between VEGFR-2 and VEGF-A that is known to be the most important factor in angiogenesis. Blocking the signaling pathway between VEGFR-2 and VEGF-A has been the focus of several studies hoping to reduce angiogenesis during tumor production and several drugs have been developed for clinical use in cancer patients for this purpose (Olsson *et al.*, 2006).

In addition to the aforementioned VEGF ligands and receptors, there are two transmembrane glycoproteins that act as co-receptors for VEGFRs, neuropilin-1 and neuropilin-2 (Nrp1, Nrp2). First only thought to bind to semaphorins, responsible for axon guidance during nervous system development, these transmembrane receptors are now known to bind VEGF-A and VEGF-C to promote angiogenesis and lymphangiogenesis respectively by modulating endothelial tip cell extension and preventing tip cell stalling and regression (Xu *et al.*, 2010). Although the exact mechanism for how these co-receptors play a role in the pro-angiogenic and pro-lymphangiogenic VEGF pathways is still not fully understood, the most widely accepted idea of neuropilin/VEGF activity postulates that Nrp1 forms a complex with VEGF-A and VEGFR2 and that Nrp2 forms a complex with VEGF-C and VEGFR3 to enhance intracellular signal transduction pathways (Soker *et al.*, 2000; Mamluk *et al.*, 2002; Favier *et al.*, 2006; Xu *et al.*, 2010; Plein *et al.*, 2014).

The Ligands VEGF-A, -B, -C and -D as well as all three VEGF receptors have been shown to be expressed at some level in the dental pulp. The most pro-angiogenic, VEGF-A is highly expressed in the pulp and is sequestered at high levels in the dentin layer (Zhang *et al.*, 2010). Sequestered dentinal VEGF released during dental injury is a beneficial potential source of this signaling molecule for pulpal angiogenesis. However, VEGF has also been shown to promote differentiation of osteoblast precursor cells to osteoblasts in bone injuries, and to directly affect the osteoprogenitor cells by aiding in mineralization and increasing bone density (Keramaris *et al.*, 2008). Although the physical structure and mineral composition of bone is very similar to the tooth, and much of the molecular signaling for growth and repair involves the same growth factors, to date no studies have been published looking at the possible role that VEGF could play in odontoblast differentiation and dentin mineralization.

It has been demonstrated, however, that angiogenic capacity and VEGF expression decreases with age using the femoral arteries of rabbits as a model (Rivard *et al.*, 1999). There was a significantly higher serum level of VEGF in young Wistar rats versus old after molar extraction (Lin *et al.*, 2008). Therefore, if VEGF plays the complex role of promoting angiogenesis to maintain healthy blood flow at the site of injury and allow for better infiltration of immune cells as well as having a direct effect on the recruitment and differentiation of new odontoblast-like cells and mineralization of tertiary dentin, then can a decrease in the expression levels of VEGF as a result of aging cause an impairment in the tooth's natural ability to recover from dental injury? The aim of this study is to examine the effects of age on the expression levels of VEGF and blood vessel density in the dental pulp of mice in an attempt to better elucidate the role of

VEGF on post-injury angiogenesis, stem cell recruitment, odontoblast differentiation and tertiary dentin mineralization.

Aim 1: A Study of Vascular Endothelial Growth Factor Expression, Blood Vessel Density and Angiogenesis During Aging Using a Mouse Model.

Introduction

Blood vessel formation takes place via two different methods: vasculogenesis and angiogenesis. During vasculogenesis, new blood vessels form from the migration and differentiation of endothelial precursor cells called angioblasts. Vasculogenesis takes place during early development and is responsible for the formation of the primary vascular plexus (Risau and Flamme, 1995). In contrast, angiogenesis is the formation of new vasculature from preexisting vessels. In other words, after the formation of the primary vasculature, new capillaries are formed from the division and expansion of existing vessels via angiogenesis. There are many growth factors that have been shown to induce and regulate angiogenesis, but vascular endothelial growth factor (VEGF), and more specifically VEGF signaling via binding to its receptor VEGFR-2, that have been shown to be a master regulator of this process (Ferrara, 2002).

Lymphangiogenesis is the formation of lymph vessels from preexisting lymph vessels, and like the vascular system, the lymphatic system plays an important role in tissue homeostasis and metabolism as well as immunity. Interestingly, the presence of lymphatics in human dental pulp has been and remains somewhat controversial. Ikeda *et al.* (2012) cites several examples of studies using different light microscopic levels, which have provided supporting evidence of the presence of pulpal lymphatics. However, these studies all predate a study in 2010 by Gerli *et al.* which found that lymphatic vessels could not be detected in any human dental pulp samples using several very

common lymphatic markers (Gerli *et al.*, 2010). The debate on the presence of lymphatic vessels in the dental pulp has yet to be resolved, however, the regulation of lymphatic vessels and the factors involved in the induction of lymphangiogenesis are better understood. Several growth factors and cytokines have been shown to indirectly or pathologically direct lymphatic growth. However, VEGF-C, and to a lesser extent VEGF-D and their receptor VEGFR-3 play the most crucial role in the formation of lymphatic vessels from early embryological development until the completion of the lymphatic system postnatally (Adams and Alitalo, 2007).

Like in many other tissues, the vascular system of the dental pulp is essential for maintaining tissue homeostasis by the transport of nutrients and removal of cellular waste products. Unlike in many other tissues of the body, however, the dental pulp is a completely microcirculatory system with no true arteries and veins. The largest vessels of the dental pulp instead are arterioles (approximately 50 μm in diameter) and smaller venules, which transition into capillary loops. The capillaries of the dental pulp measure approximately 8 μm in diameter (Ikeda and Suda, 2012). Clumps of smooth muscle cells at the transitions between terminal arterioles and capillaries serve as sphincters that tightly regulate the local blood flow and capillary filtration to the adjacent capillary beds, resulting in the possibility of having substantially different circulatory conditions throughout the pulpal tissue (Ikeda and Suda, 2012). This characteristic of dental pulp vasculature is of great benefit to a tissue that is so susceptible to localized injury and inflammation. During aging there is a general decrease in pulpal metabolism. Dental pulp capillary organization is simplified with age and often devolves to a single layer of capillaries terminating directly into venules (Kishi *et al.*, 1990).

Scientists have been classifying the age-related changes in mature human teeth for almost 65 years (Gustafson, 1950). As a result of either long term functional usage or general senescence, or both, several changes in the dental pulp have been observed, including decreasing cellular density, reduction of pulpal space brought on by ever increasing dentinal thickness attributed to dentin secreting odontoblast and odontoblast-like cells, a decrease in the number of cells in the odontoblast layer and a relative increase in fibrous connective tissue and fibrosis.

In relation to the vasculature of the dental pulp, it has been shown that the number of vessels entering the tooth at the apical foramen can decrease with age, the innermost layer of the vessel, the intima, can swell narrowing the lumen and the vessel walls may begin to calcify. In addition, it is important to note that the process of secondary and tertiary dentin formation resulting in the stenosing of the pulp chamber with age can also cause potential issues including the narrowing or even destruction of larger arterioles within the pulp (Ikeda and Suda, 2012). This, in turn, causes a decrease in the surface area of the capillary plexus feeding the odontoblast layer and therefore could potentially affect the viability and dentinogenic capacity of the odontoblasts.

There have been several studies on age-related changes in human dental pulp vasculature (Bennett *et al.*, 1965; Bernick, 1967; Espina *et al.*, 2003), which show that with increasing age there is a decrease in the number of vessels in the dental pulp and in the density of the capillary plexus underlying the odontoblast layer and that the vessels of the dental pulp undergo morphological changes associated with advancing age. However, the role of VEGF in the diminution of dental pulp vasculature during senescence has not been as thoroughly studied. Matsuzaka *et al.* (2008) examined two separate groups of

young (5 week old) and adult (15 week old) Wistar rats for expression levels of VEGF-A using Q-RT-PCR and they found that there was an increase in expression from the young to adult age group (Matsuzaka *et al.*, 2008). The limitation of this study, however, is that the authors did not include a senescent age group in their analysis. To date there are no studies investigating age-related changes in the expression of any VEGF ligand or receptor in mice. However, understanding how advancing age affects the expression of VEGF receptors and ligands in the mouse model is an important component of advancing our understanding by allowing for the usage of transgenic mouse models in the analysis of VEGF signaling and expression for the later translation of findings to human clinical applications. The aim of this study is to perform a more comprehensive age-based analysis of the expression of the VEGF ligands and receptors responsible for angiogenesis (and lymphangiogenesis) and to determine if these expression levels correlate to a decrease in the vascular density of the dental pulp.

Methods

Mandible Isolation

All mouse experiments were performed in accordance with approved Institutional Animal Care and Use Committee (IACUC) guidelines, University of Washington. Left and right hemi-mandibles were dissected from C57Bl/6 (Jackson Laboratory, Bar Harbor, ME). Age groups were designated as follows: 7 days old (n = 3), 1-2 months old (n = 4), 3 months old (n = 3), 4 months old (n = 4), 12 months old (n = 3) and 23 months old (n = 10).

Histology and Immunofluorescence

Hemi-mandibles were fixed in Bouin's solution overnight. A series of 70% ethanol washes were completed to remove excess picric acid. The samples were then left overnight in 70% ethanol. The samples were then submerged in acetic acid-formaldehyde-saline solution (AFS) and left on an agitation plate. The solution was discarded and replaced once per week for 4 weeks. A pin test was performed at the end of 4 weeks to confirm complete decalcification. The samples were processed and paraffin embedded. 10 - 20µm thick sagittal and transverse sections were cut and mounted on slides.

Slides were deparaffinized and stained for haematoxylin and eosin. For immunostaining, slides were deparaffinized and washed with 1% bovine serum albumin (BSA) diluted in 0.1% Triton X-100 and then blocked for non-specific binding with 10% normal goat serum for 1 hour at room temperature. Slides were incubated with primary antibodies (Supplementary Table 1) overnight at 4° C. Negative controls were incubated with corresponding rat, rabbit and mouse γ -immunoglobulin. Stained mandibular tissue sections were then incubated with goat-derived anti-rat, rabbit and mouse Alexa -488, -594 or -647 conjugated antibodies (1:1000) for 1 hour at room temperature. The samples were then washed 3 times with 1% BSA and stained with 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen, Carlsbad, CA, USA) to visualize nuclei.

The tissue sections were analyzed and photographed using a Zeiss Axiovert 200 fluorescence microscope (Thornwood, NY). All immunofluorescence photographs taken using the onboard monochrome AxioCam camera and imaged using the AxioVision 4.8

(Carl Zeiss) imaging system. Background signal was reduced with color settings using Adobe Photoshop CS2 (San Jose, CA).

Blood Vessel Quantification

All background reduction changes were applied to all images before blood vessel quantification. Blood vessel quantification was performed using ImageJ software (NIH, Bethesda, MD). After background reduction was performed, 3 representative images taken from tissue sections of all 6 mouse age groups were reviewed. To be chosen for counting, an image must include the pulp horn, odontoblast layer, subodontoblastic zone and pulp core sections of the tooth. Only images from the first mandibular molars were counted. Counting was carried out using the cell counting function in ImageJ. Blood vessel density was determined as the average number of BS1-lectin positive cells or cell clusters per $50 \mu\text{m}^2$. Small capillaries measuring 25 micrometers in diameter or less were counted, but the larger, main pulpal arterioles were excluded. There were not enough images of the 23-month-old age group samples showing capillaries in the pulp due to extreme tooth wear. Therefore, the 23-month-old age group was excluded from the blood vessel density analysis.

Mandibular Molar Extraction

The mandibles of C56Bl/6 mice of 4 separate age groups were roughly dissected and sectioned into halves. The age groups are as follows: 11 days old ($n = 4$), 6 weeks old ($n = 4$), 6 months old ($n = 1$) and 18 months old ($n = 3$). The protocol for molar extraction for the adult samples began by complete tissue removal of the attached musculature and gingiva using a scalpel and a 31-gauge $\frac{1}{2}$ ml/cc syringe. Under a dissecting microscope,

fine forceps were gently used to break the alveolar bone around the first mandibular molar until it became dislodged from the mandible. The first mandibular molar was collected by this method for all adult samples. Periodontal ligament was thoroughly removed using a syringe, with careful attention to avoid breaking the tooth root.

The neonatal (11 days old) mouse teeth were dissected using the 31 gauge ¹/₂ mL/cc syringe. For all 11-day-old samples, the first, second and third mandibular molars were extracted. The dental sac and gingival tissue was carefully removed under a dissecting microscope. Both left and right side molars were collected for all samples.

Q-RT-PCR Analysis

Molar samples from all 4 age groups were flash frozen in liquid nitrogen and crushed. Total RNA extraction was performed using TRIzol (Life Technologies) according to the manufacturer's protocol. The quantity and purity of the RNA was determined by 260/280 absorbance. First strand cDNA was synthesized from 1 µg of RNA using the High Capacity cDNA Synthesis Kit, using a randomized primer (Applied Biosystems) following the manufacturer's protocol. Primer sequences are shown in Supplementary Table 2.

cDNA (20 ng) was diluted in a final volume of 20 µl per reaction using the Immomix PCR Mastermix from Bioline. PCR was performed using the following thermal cycling conditions; 95°C 7 min for initial activation followed by 95°C/30s; 57°C/30s; 72°C/45s, for 35 cycles, with a final 5-min extension at 72°C. 18S ribosomal RNA was used as a control reference gene. Negative controls lacked cDNA.

cDNA (20 ng) for Q-RT-PCR were prepared using the Maxima SYBR Green/Rox qPCR master mix from Fermentas Life Sciences. Reactions were processed by the ABI 7900HT PCR system with the following parameters: 50°C/2 min and 95°C/10 min, followed by 40 cycles of 95°C/15 s, 60°C/15 s, and 72°C/15 s. Results were analyzed using SDS 2.2 software (Applied Biosystems). The threshold cycle (C_t) value for each gene was normalized to the C_t value of 18S. The relative mRNA expression presented as Relative Quantification; RQ value) was calculated by using the comparative C_t method by the formula:

$$2^{-\Delta\Delta C_t}, \text{ where } \Delta C_t = C_t \text{ target} - C_t \text{ 18S} \text{ and } \Delta\Delta C_t = \Delta C_t \text{ target} - \Delta C_t \text{ calibrator.}$$

Each sample was run in triplicate reactions for each gene. Error bars represent the standard error of mean calculated from three independent experiments.

Statistical Analysis

Statistical analysis of the results of Q-RT-PCR triplicate experiments was performed using the Student's *t*-test. Error bars represent \pm SEM. *P* values ≤ 0.05 were considered to be statistically significant.

Results

Immunohistological staining using fluorescently labeled antibodies for VEGF-A and BS1-biotin, a lectin that binds vessels, across the six designated age groups (7 days to 12 months) showed an obvious qualitative decrease in both VEGF-A and BS1- biotin expression from the neonatal age group to the senescent age group with the largest

apparent decrease in VEGF-A protein level expression between approximately the 3 and 4 months old age groups (Fig. 2).

There is a high level of VEGF-A expression in the odontoblast layer of the neonatal samples as well as visible expression throughout the dental pulp. A lessened but still visible expression of VEGF-A in the odontoblast layer can be seen until approximately the 4th month of age. At 4 months, the expression of VEGF seems to retreat to the dental pulp and is diffuse in nature. VEGF-A expression in the 6-month age group (data not shown) resembles that of the 12-month age group. In these groups, VEGF-A expression is considerably lower than the neonate and 1-3 months old age groups. The pulp chambers of the 23-month-old age group were so stenosed that it was difficult to visualize the dental pulp. Also, in these specimens attrition of the molar crowns was so complete that often there was visible pulpal necrosis and tertiary dentin formation that had either partially or completely occluded the pulp chamber.

BS1-biotin staining revealed a qualitative pattern of blood vessel density that was similar to that of VEGF-A. In the neonatal dental pulp, there were numerous BS1-biotin labeled cells throughout the pulp core as well as located densely just below the odontoblast layer and near the pulp horns. This staining supports the presence of a sub-odontoblastic capillary plexus in the neonate mouse. The location of dense BS1-biotin staining in the sub-odontoblastic cell layer of the dental pulp persists in the 1 months old age group but seems to disappear in the 3-month age group, suggesting that the capillary plexus noted in younger samples has retreated somewhat into the dental pulp core. There is less BS1-biotin expression in the 4-month-old age group than in younger age groups.

The six-month age group (data not shown) resembles the 12-month age group. At 12 months, BS1-biotin staining is weaker but still visible in the pulp core.

Interestingly, there are large, autofluorescent vessels (shown in yellow) in the dental pulp of the 12-month age group. These were noted on several samples and may be a consequence of the method of histological preparation since the poor quality of the 12-month-old murine molars required that the histological sections be cut in the transverse plane versus the sagittal plane.

VEGF-A / BS1

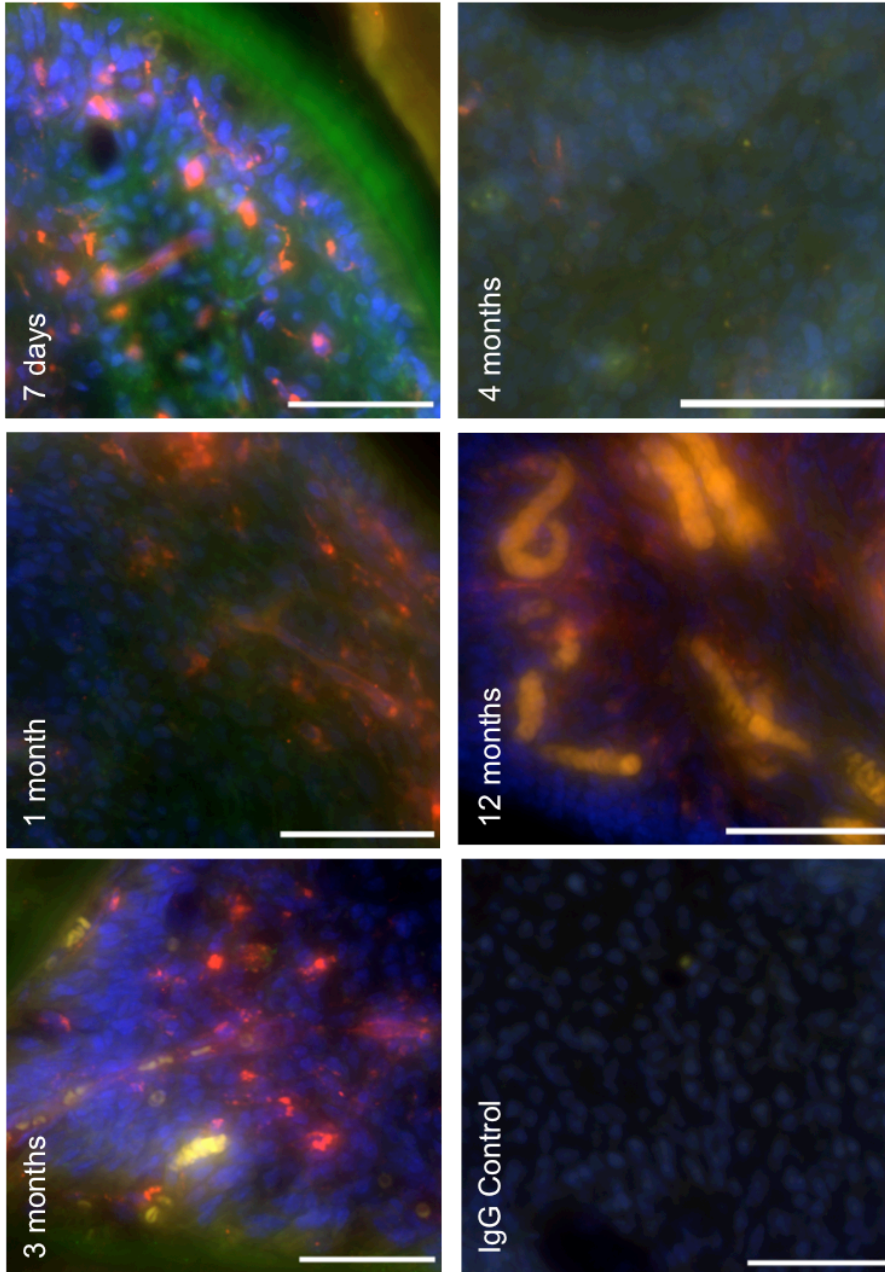


Figure 2. Dental pulp tissue taken from mice of 5 different age groups, immunofluorescently stained with antibodies specific for vascular endothelial growth factor A (Alexa 488 green) and BS1-biotin (Streptavidin 594 red). Staining shows VEGF-A expression is upregulated in the 7-day, 1-month and 3-month age groups, then declines after 3 months and is undetectable in the 12-month age group. There is an appreciable decrease in VEGF-A expression from 7 days to 3 months. BS1 specific staining shows several small capillaries are present in the dental pulp, especially in the sub-odontoblastic layer and the areas near the pulp horns in the 7-day, 1-month and 3-month age groups. Small BS1 positive capillaries are present but less numerous in the 4 month and 12 month age groups. In the 12 month age group, large autofluorescent blood vessels are present in the pulp core. Scale bars = 50 μ m.

To better quantify the difference in blood vessel density between age groups, blood vessel quantification was performed using ImageJ software. Measurements were taken from sagittally sliced tooth sections representing the entire pulp. For purposes of standardization, only images taken from the first molars were used. The blood vessel density (measured as average number of blood vessels per $50 \mu\text{m}^2$) begins highest in the neonate and then drops off gradually in a linear pattern to the oldest age group (Fig. 3). Again, the 23-month-old age group was excluded from this analysis because the pulp chambers were too stenosed to accurately stain and count BS1-biotin positive cells. The blood vessel density (BVD) of the 1- and 3-month age groups are very similar and then there is a significant drop in BVD that seems to plateau in the 4 month through the 12-month age groups. This suggests there is increased blood flow and vascularity in the developing neonatal tooth that decreases as the tooth matures and the animal reaches adulthood and then finally declines even further but plateaus into later adulthood and senescence.

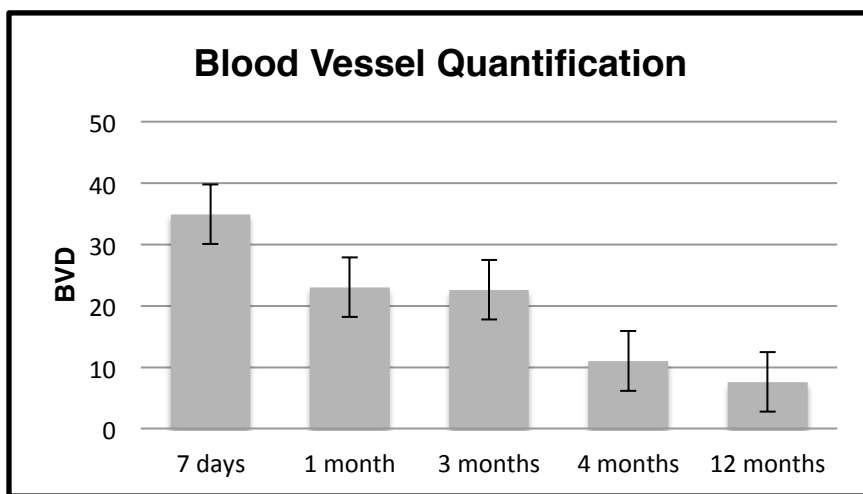


Figure 3. Blood Vessel Quantification. Blood vessel density was measured as the number of BS1-biotin positive cells or cell clusters per $50 \mu\text{m}^2$. Large arterioles were excluded from the counts. 23-month old murine dental pulp sections were excluded from the analysis due to the fact that the pulp cores were too occluded and the crowns too worn to attain accurate cell counts.

Q-RT-PCR analyses were carried out on three murine age groups ranging from neonatal (11 days old) to senescent (18 months old). Q-RT-PCR results for blood vessel markers vWF, (a multimeric glycoprotein expressed on endothelial cells) and VE-cadherin, (or vascular endothelium cadherin, an endothelium-specific cadherin localized at intercellular junctions) showed a slight but non-significant initial upregulation of these gene markers from the neonate to the 6 weeks old age groups followed by a decrease in the 18 months old age group, which was non-significant for vWF but significant for VE-cadherin (Fig. 4a).

Gene expression of VEGF ligands A, C and D and all VEGF receptors were also analyzed via Q-RT-PCR. The results showed that both VEGF-A and VEGF-C were upregulated from the neonatal age group to the 6-week age group and then both fell significantly in the 18-month age group. Interestingly, VEGF-C expression was significantly higher than VEGF-A expression in the 6-week age group and also slightly, but non-significantly higher in the 18-month age group. VEGF-D was only present at very low levels in the neonatal age group and then was undetectable in all other age group samples (Fig. 4c).

Q-RT-PCR results for VEGFR-1 and VEGFR-2 expression revealed that both receptors were present in the neonate age group samples, both at very low levels, but were undetectable in samples older than 11 days. Interestingly, only VEGFR-3 was present and was expressed at higher levels than any other VEGF receptor in all three age groups (Fig. 4d). VEGFR-3 expression followed a pattern similar to the blood vessel markers vWF and VE-cadherin.

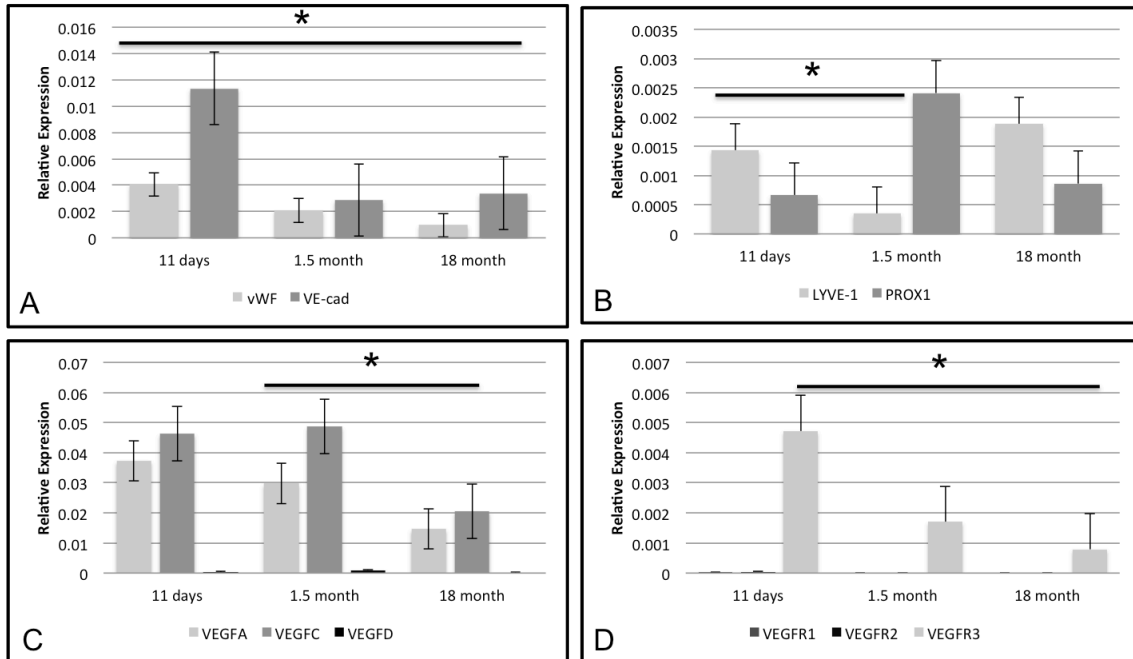


Figure 4. Graphical results of Q-RT-PCR analysis of blood vessel markers, lymph vessel markers, VEGF ligands, and receptors. (A) Q-RT-PCR results for blood vessel markers von Willebrand Factor (vWF) and VE-Cadherin. vWF results show a significant difference between the 11-day and 18-month age groups ($p = 0.04$) and 1.5-month and 18-month age groups ($p = 0.03$). Results for VE-cadherin show a significant difference between the 11-day and 1.5-month age groups ($p = 0.01$) and the 11-day and 18-month age groups ($p = 0.03$). (B) Q-RT-PCR results for lymph vessel markers LYVE-1 and PROX1. LYVE-1 results show a significant difference between the 11-day and 1.5-month age groups ($p = 0.008$). The differences in relative expression of PROX1 between age groups were not significant. (C) Q-RT-PCR results for VEGF ligands A, C and D. Results show a significant difference in relative expression of both VEGF-A and VEGF-C between the 1.5-month and 18-month age groups ($p = 0.02$ and 0.01 respectively). VEGF-D expression levels were almost negligible and therefore were excluded from analysis. (D) Q-RT-PCR results for VEGF receptors. Results show there was a significant difference in VEGFR-3 expression levels between all age groups. ($p = 0.009$, 0.05 and 0.006 respectively). VEGFR-1 and R-2 were expressed at such low levels that they were only detectable at 11 days and Student's *t*-test analysis could not be carried out on those markers.

The expression of the lymphatic markers LYVE-1 (or lymphatic vessel endothelial hyaluronan receptor 1, a cell surface receptor expressed on lymphatic endothelial cells), PROX1 (or Prospero homeobox 1, a lymphatic endothelial nuclear transcription factor) and PDPN (Podoplanin protein, a lymphatic endothelial cell marker) were also analyzed via Q-RT-PCR (Fig. 4b). The results for the expression of LYVE-1 showed that although there was a detectable amount of LYVE-1 in every age group, there was no significant difference in the levels of expression of LYVE-1 between age groups.

The expression of PROX1 and PDPN followed the same pattern but PDPN was expressed at higher levels than PROX1. Both markers were expressed at low levels in the neonate and then rose significantly in the 6-week age group and then dropped significantly in the 18-month age group. There was no significant difference in PROX1 and PDPD expression between the neonate and 18-month-old age groups.

Discussion

From the qualitative immunofluorescent staining results and the quantitative blood vessel density counts derived from histological sections of murine molars from 6 separate age groups, we can conclude that blood vessel density does decrease with age in the mouse dental pulp. The Q-RT-PCR data on the expression levels of the vascular markers von Willebrand factor (vWF) and vascular endothelial cadherin (Ve-Cad) demonstrating a significant decrease in gene level expression during aging further supports this conclusion.

The results of this study also show qualitatively, based on immunofluorescent staining, and quantitatively, based on Q-RT-PCR, that levels of VEGF-A also decrease with advancing age in the mouse dental pulp. Although at what age VEGF-A is expressed the highest and at what age VEGF-A expression begins to decrease is still somewhat elusive, evidence in this study points to the 3rd to 4th month of age as a potentially important time point for changing in the levels of expression of VEGF.

When we examined the other VEGF ligands and all three VEGF receptors, a few interesting observations came to light. First, although VEGF-A is traditionally accepted as the most pro-angiogenic VEGF ligand, and its expression was observed in all age

groups, the expression of VEGF-C was actually much higher than VEGF-A after the neonatal stage of development. Second, VEGFR-2 gene expression is not detectable in this study after the neonatal stage, and is only detectable at very low levels in relation to VEGFR-3 even in the neonate mouse. Third, VEGFR-3 expression is the highest of all three receptors and VEGFR-3 is the only receptor which can be detected by Q-RT-PCR after the neonatal stage in mice. And lastly, the lymph vessel markers, especially LYVE-1, which is the most specific lymph marker used in this study, do not follow the same pattern of expression across age groups as blood vessel markers and VEGF ligands A and C. Most interestingly, they do not follow the same expression pattern across age groups as VEGFR-3 either. In fact, the results of Q-RT-PCR analysis show that the expression pattern of VEGFR-3 across age groups actually most closely resembles the pattern of expression of the blood vessel markers.

These results suggest the possibility that VEGFR-3 may be playing a pro-angiogenic role in the dental pulp in addition to actively promoting lymphangiogenesis. This may explain why the level of expression of VEGF-C, which binds VEGFR-3, is so high. VEGF-C has been shown to be a predominant angiogenic growth factor in tumor cells (Valtola *et al.*, 1999), and VEGFR-3 has been shown to promote angiogenesis in tumors (Laakkonen *et al.*, 2007). However, the angiogenic capacity of VEGFR-3 is not limited to pathogenic tissues. Knocking down VEGFR-3 has been shown to suppress angiogenesis in healthy murine skin and retinal tissues as well (Tammela *et al.*, 2008). Tammela *et al.* (2008) demonstrated that in addition to cases of tumor growth, the angiogenic VEGFR-3 signal is active during settings of angiogenic invasion of tissues, such as early embryonic development and in the retina (Tammela *et al.*, 2008). Xu *et al.*

(2010) also suggest that the presence of certain co-receptors, such as Nrp1 or Nrp2, could promote differential binding of VEGF ligands to endothelial cells of microvasculature, facilitating the function of both ligands VEGF-A and VEGF-C as well as VEGFR3 in angiogenesis. While this study did not test for the presence of these co-receptors in the dental pulp microvasculature, further work could identify if Nrp1 or Nrp2 co-signaling is present in the dental pulp and if their role in this tissue could be contributing to a potential pro-angiogenic VEGFR3 pathway.

Since the dental pulp is a specialized regenerative tissue with active remodeling of the microvasculature that occurs throughout the life of the tooth, it is conceivable that VEGFR-3 could be playing a pro-angiogenic role in the dental pulp. The results of this study are a beginning step in looking at the possible angiogenic role of VEGFR-3 and its ligand VEGF-C in the dental pulp. Since the dental pulp tissue is so specialized, further examination of the vasculature of the dental pulp and the factors that regulate angiogenesis and lymphangiogenesis during embryological development, secondary dentinogenesis and tertiary dentin regeneration are still needed to better elucidate this possibility.

Aim 2: Investigating the Role of Vascular Endothelial Growth Factor in Dentin Mineralization and Regeneration After Injury

Introduction

Once vascular endothelial growth factor was fully characterized and its ligands and receptors were identified almost 25 years ago, the general understanding that VEGF is a pro-angiogenic protein and the key factor in regulating new blood and lymph vessel formation quickly took hold. Since that time, the classical interpretation of VEGF and its role in angiogenesis has led to many lines of research, from the reduction of tumor vasculature via the inhibition of VEGF to therapies for macular degeneration via angiogenic suppression, and many others.

There is now no doubt that the defining role of vascular endothelial growth factor is to promote and maintain angiogenesis (and lymphangiogenesis) in tissues throughout the body. It is important, however, to bear in mind that VEGF plays several other roles at the cellular level that are, at least in part, independent of angiogenesis. Leung *et al.* (1989) first demonstrated the mitogenic effects of VEGF when human 293 cells were transfected with an expression vector containing a bovine or human VEGF cDNA insert and were shown to secrete an endothelial cell mitogen that behaved similarly to native VEGF (Leung *et al.*, 1989).

Alon *et al.* (1995) were able to show VEGF was also capable of promoting cell survival by injecting VEGF at the onset of experimental hyperoxia induced in the eyes of mice. The VEGF injection prevented apoptosis of the endothelial cells and rescued retinal vasculature, acting as a capsular survival factor *in vivo* (Alon *et al.*, 1995). In addition to these findings, it has also been shown that VEGF plays a protective role for endothelial

cells. This was first demonstrated by Yang et al. (1997) who showed that VEGF increased cellular resistance to oxidative stress caused by hydrogen peroxide exposure (Yang and de Bono, 1997). Later, Castilla et al. (2000) completed a study in which bovine aorta endothelial cells were challenged with cytochalasin D (CyD), hydrogen peroxide, and cyclosporine A (CsA) – all cytotoxic substances. Results showed an upregulation of VEGF after exposure to these cells and cell damage when these cells were exposed in conjunction with an anti-VEGF antibody, revealing a mechanism involving autocrine VEGF production for the protection of endothelial cells (Castilla *et al.*, 2000).

It has also been shown that VEGF is important for cell motility and can stimulate the directed, non-random migration of endothelial cells (Stoker, 1991). Interestingly, VEGF has also been implicated in promoting neurogenesis in addition to its pro-angiogenic and pro-lymphangiogenic functions. VEGF receptors have been found on neurons and VEGF has been shown to stimulate neurogenesis through the VEGFR2 signaling pathway *in vitro* and in the rat brain *in vivo* (Jin *et al.*, 2002). It has been shown that VEGF driven neurogenesis takes place in close proximity to blood vessels and may be mechanically linked to angiogenesis (Palmer *et al.*, 2000).

More recently, there have been several interesting studies relating VEGF to bone mineralization and regeneration, even suggesting a possible direct effect of VEGF on osteoprogenitor cell differentiation (Keramaris *et al.*, 2008). It has been shown that VEGF works synergistically with BMP-4 (Peng *et al.*, 2002) and BMP-2 by expressing MMP-9 to promote bone regeneration (Peng *et al.*, 2005) and that VEGF enhances ossification, speeds up bone resorption (Geiger *et al.*, 2007) and promotes bony bridges

(Street *et al.*, 2002). Several studies have also shown that VEGF promotes increased bone mineral density (Kleinheinz *et al.*, 2005; Leach *et al.*, 2006; Tarkka *et al.*, 2003). Murphy *et al.* (2004) showed that the addition of VEGF to a mineralized substrate significantly increased the generation of mineralized tissue compared with mineralized scaffold alone (Murphy *et al.*, 2004). This implies that VEGF indirectly speeds the differentiation and/or maturation of invading osteoblasts and osteoblast precursor cells during new bone development, perhaps by providing a conduit for delivery of osteoinductive soluble signals by increasing angiogenesis, although a more direct function of VEGF was not ruled out.

The function of VEGF in relation to the dental pulp is complex and more recently has become a popular subject of investigation with the discovery and characterization of dental pulp stem cells. It has been shown that VEGF has the ability to induce endothelial cell differentiation from stem cells isolated from human exfoliated deciduous teeth (SHED) (Sakai *et al.*, 2010). Another study (Zhang, 2010) showed that isolated dentin matrix components, including VEGF-A, when given at low dosages have stimulatory effects on endothelial cell proliferation. These molecules are naturally sequestered within the mineralized dentin matrix during dentinogenesis and are released into the dental pulp when an injury to the tooth results in the destruction of dentin matrix, providing an endogenous supply of reparative cytokines and growth factors for the dentin regeneration process.

Tooth development is generally well understood. Dentin formation begins early on in the bell stage of development before birth and prior to eruption and then continues, albeit at a much slower rate, as long as the tooth is viable. In addition to the normal and

slow physiologic production of dentin as the tooth ages, the formation of tertiary dentin takes place when the tooth is injured via caries, attrition, or other trauma. In cases of mild or moderate trauma where a surviving odontoblast layer remains, bioactive molecules, including VEGF, released from the dentin matrix cause an increase in the secretory response of odontoblasts that are in direct tubal contact (via dentinal tubules) with the newly exposed matrix (Smith *et al.*, 1995). VEGF has also been shown to be expressed by odontoblasts and pulpal fibroblasts (Tran-Hung *et al.*, 2006).

In cases of more severe dental trauma where the odontoblast layer has been killed off, a complex series of events takes place in order to induce reparative tertiary dentin formation. First, progenitor cells from the dental pulp must be recruited for odontoblast-like cell differentiation. The mechanism for this process is still unclear (Sloan and Smith, 2007), although peri-vascular cells have been implicated as one potential progenitor source (Fitzgerald *et al.*, 1990). Next, signaling takes place that induces the progenitor cells to differentiate into odontoblast-like cells. It has been shown that dissociated dentin matrix can be autoinductive and induce this odontoblast-like cell differentiation (Smith *et al.*, 1990; Tziafas and Kolokuris, 1990). Finally, as in the previous case, the secretory behavior of the newly formed odontoblast-like cells is upregulated and tertiary dentin formation begins.

Although it is well understood that VEGF is present in the dental pulp during this process and is released from dentin matrix after injury, the exact role of VEGF in tertiary dentin formation remains unclear. Dentin and bone are very similar in structure; they rely on similar signaling pathways, are composed of approximately the same amount of collagen and are mineralized to approximately the same extent. It is possible that the

proven effects of VEGF on bone mineralization, osteoprogenitor differentiation and osteoregeneration could apply equally as well to dentin mineralization and regeneration. The aim of this study is to gain a better understanding of the function of VEGF during tertiary dentin formation by observing *in vivo*, through immunohistochemical analysis, the location and expression levels of VEGF in the dental pulp and dentin layer of murine molars after dental trauma.

Materials and Methods

Dental Microsurgery

Nine C57Bl/6 (Jackson Laboratory, Bar Harbor, ME) mice, all 11 months of age, were anesthetized intraperitoneally using Ketamine / Xylazine / Acepromazine (KXA) in a 50% dilution, 2 µl per 1 gram of body weight (55 mg/kg Ketamine, 5.5 mg/kg Xylazine, 1.1mg/kg Acepromazine). After a 5-10 minute period to allow the subject to reach a deep plane of anesthesia, a ¼ round 0.5 mm carbide burr was used to drill into the first cusp of the first maxillary and mandibular molars. All of the procedures were done with the assistance of an endodontic microscope. In each mouse, the left side of both the mandible and maxilla was drilled. The right side was preserved as a control. Standardization of the technique was difficult to achieve and therefore drilling depths fluctuated from removal of the cuspal dentin but no pulp exposure to complete pulp exposure accompanied by bleeding from the pulp chamber. All mice were given buprenorphine (0.3 mg/ml) upon waking and for the following 24 hours per the manufacturer's protocol if pulp exposure had occurred.

During the procedure, 2 experimental subjects died as a result of anesthetic overdose approximately 15 minutes after drilling had occurred. Of the remaining 7 mice, 1 was sacrificed 2 weeks post-injury and 6 were sacrificed 4 weeks post-injury. The mandibles and maxillae of each mouse were dissected, fixed, decalcified, paraffin embedded, cut and mounted to slides according to the protocol described above.

Immunohistochemistry

Slides were deparaffinized and antigen retrieval was performed by adding 0.5% trypsin-EDTA 10x (Life Technologies) and incubating the slides in a 37° C water bath for 15 minutes. The slides were then washed with 1% BSA for 10 minutes at room temperature. Non-specific binding was blocked by incubating in 10% normal goat serum for 1 hour at room temperature. The slides were then incubated with primary antibodies overnight at 4° C. Negative controls were incubated with corresponding rat, rabbit and mouse γ -immunoglobulin.

Biotin blocking was performed by using the Avidin / Biotin Blocking Kit (Vector Laboratories) according to the manufacturer's protocol. Stained tissue sections were then incubated with anti-rat and rabbit biotinylated secondary antibodies for 1 hour at room temperature. The slides were then incubated with VECTASTAIN RTU peroxidase enzyme reagent (Vector Laboratories) per the manufacturer's protocol.

3, 3'-diaminobenzidine (DAB) substrate (Vector Laboratories) was prepared and added to the slides according to the manufacturer's protocol. Nickel solution was added to the substrate to yield a black colored reaction product. The slides were allowed 7.5 minutes to develop in the case of VEGF-A primary antibody staining and 11.5 minutes to

develop in the case of VEGFR-2 staining. Excess DAB was then washed from the slides. The slides were coverslipped then analyzed and photographed using a Nikon Eclipse e400 Biological microscope with an onboard Nikon DS-1 digital camera, using the Nikon NIS elements F software v.2.30 (Tokyo, Japan).

Results

VEGF-A Expression in Uninjured Versus Injured Dentin

3, 3'-diaminobenzidine staining for VEGF-A reveals high levels of expression in the central dentin layers of the uninjured tooth but not in the periphery of the dentin layer or in the pre-dentin (Fig. 5). Injured cusps show reactionary tertiary dentin does not contain VEGF-A (Fig. 5b, black arrow).

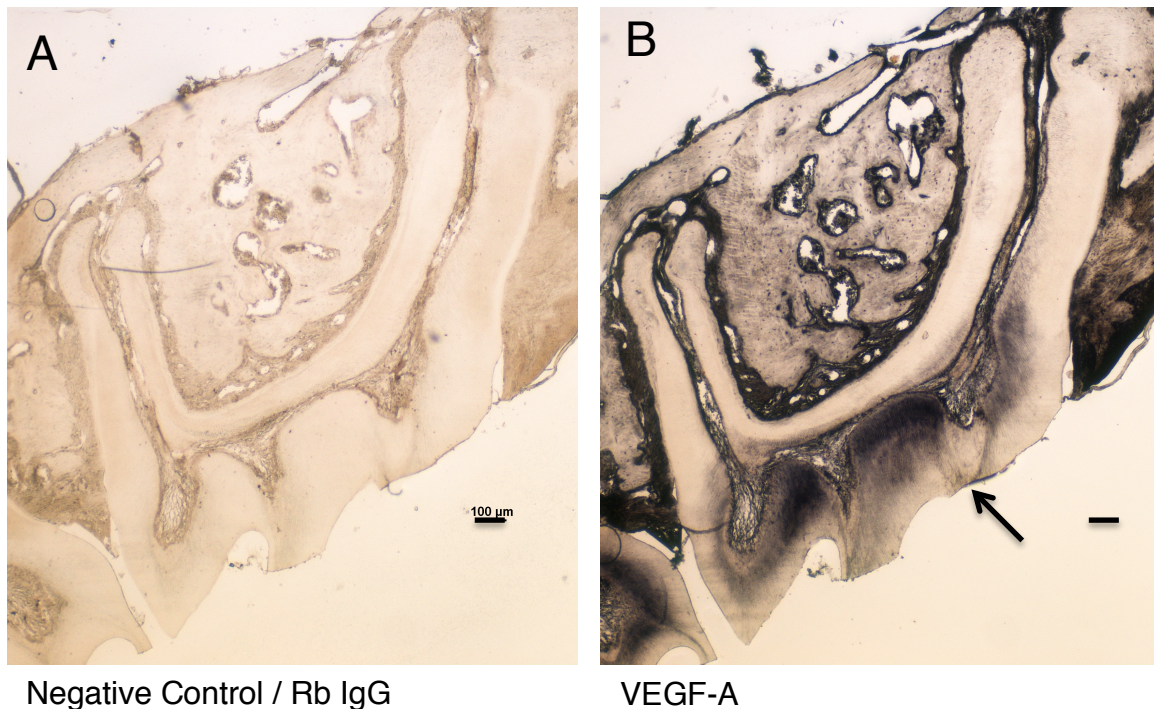


Figure 5. 3, 3'-diaminobenzidine staining for VEGF-A in an injured tooth in sagittal section showing high levels of VEGF-A sequestered in the dentin matrix, but not contained in the newly formed reactionary dentin underlying an injured cusp (black arrow). Note there was no pulp exposure with this injury. Scale bars = 100 μm.

VEGF-A Expression After Injury

Histological sections of injured murine molars DAB stained for VEGF-A showed a marked pattern of increased expression of VEGF-A 15 minutes post-injury that decreased gradually after 2 weeks and approaches the uninjured control sample after 4 weeks (Figure 6).

The sections taken 15 minutes post-injury show the most dramatic VEGF-A positive staining. The upregulation of VEGF-A as evidenced by DAB staining was so intense that in some samples the cellular structure of the dental pulp was obscured. However, the 15-minute post-injury samples showed intense positive staining in almost all cells of the dental pulp, with the exception of red blood cells. There was also intense positive staining of the odontoblast processes within the dentinal tubules that indicate an upregulation of VEGF-A secretion by odontoblasts after injury *within* the dentin matrix where no blood vessels were located (Fig. 6d, blue arrows). In fact, all stained sections, including the control, showed VEGF-A positive staining of the odontoblast processes, although this staining was much more intense 15 minutes post dental injury.

Sections collected 2 weeks post-injury showed a more diffuse pattern of VEGF-A expression in the dental pulp. The cells of the dental pulp continued to express VEGF-A at higher levels than the control but the expression greatly decreased from the 15-minute post injury time point. The odontoblast layer continued to express VEGF-A at higher levels than other cells of the pulp and small arterioles were seen in the odontoblast layer (Fig. 6c, red arrows). It is unclear if these are newly formed vessels resulting from the healing response. However, numerous vessels within the odontoblast and

subodontoblastic cell layers are not noted on the control or 4 week post-injury samples. Four weeks post injury, stained tooth sections showed a continued upregulation in VEGF-A expression, however, it appeared that the levels are approaching an un-injured state. The most remarkable difference between control sections and the sections taken 4 weeks post injury were that the injured sections still showed a marked increase in VEGF-A expression in the odontoblast layer that was absent in the control (Fig. 6b, green arrows).

The control dental pulp sections showed diffuse VEGF-A positive staining throughout the dentin layer in both a globular pattern within the dentin matrix and a linear pattern that followed the paths of dentinal tubules. There were also several VEGF-A positive cells within the dental pulp even in the uninjured control, confirming previous Q-RT-PCR results showing a relatively large expression of VEGF-A under conditions where the tooth was not challenged.

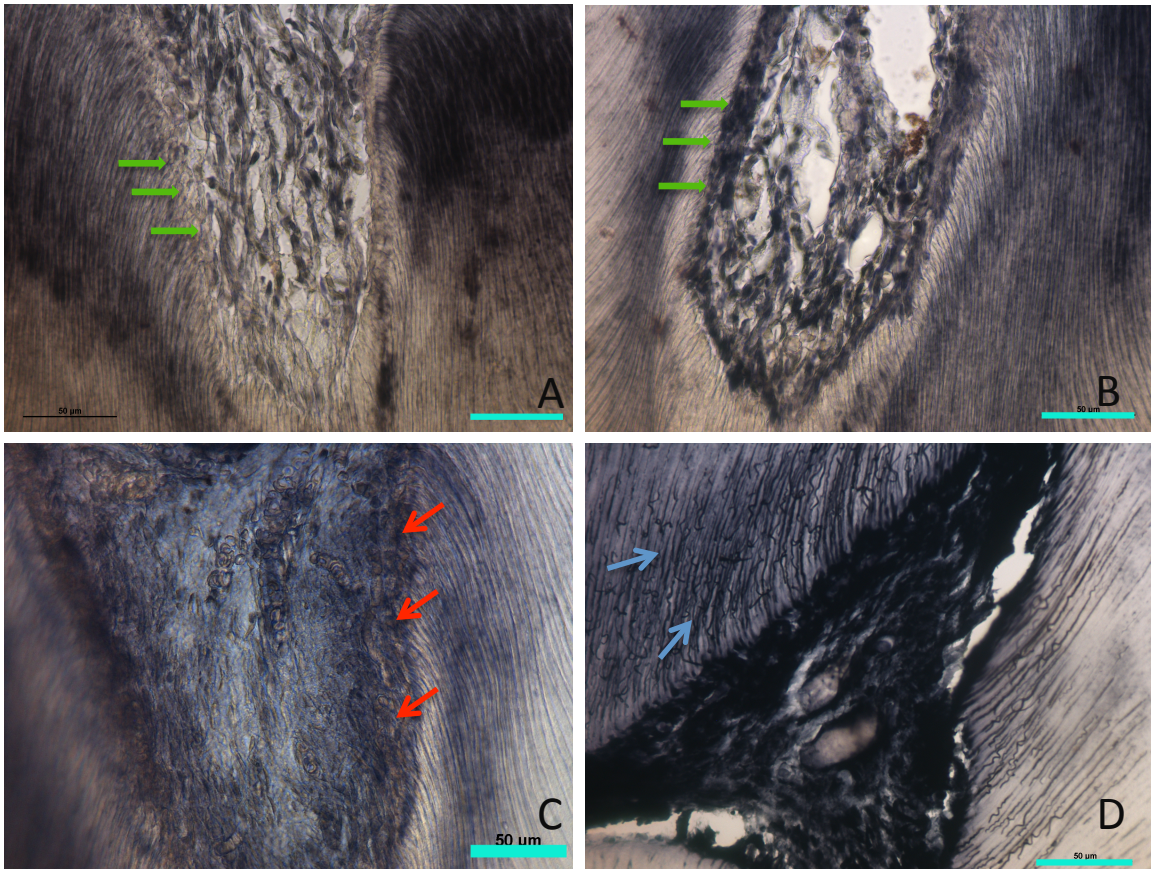


Figure 6. Vascular endothelial growth factor A expression in mouse dental pulp post-injury shown with 3, 3'-diaminobenzidine (DAB) staining. **(A)** Control. Dental pulp from the first cusp of the first maxillary molar of an un-injured control. There is diffuse VEGF-A staining within the dental pulp chamber and heavy staining within the dentin layer that represents sequestered VEGF-A within the dentin matrix. The odontoblast layer is not expressing VEGF-A in the uninjured tooth (green arrows). **(B)** 4 weeks post-injury. Dental pulp from the injured first cusp of the first maxillary molar. The pulp chamber was not exposed during drilling. VEGF-A expression in the pulp has increased and the odontoblast layer is now expressing VEGF-A (green arrows). **(C)** 2 weeks post-injury. Dental pulp of the injured first cusp of the first maxillary molar. VEGF-A expression in the dental pulp space is observably higher. Small capillaries have formed within the odontoblast and sub-odontoblast layer (red arrows). **(D)** 15 minutes post-injury. VEGF-A expression is extremely high in most cells, including the odontoblast layer. The cell processes of the odontoblast layer are also expressing very high levels of VEGF-A in relation to other time points shown (blue arrows). Scale bars = 50 µm

VEGF-A Expression During Dentin Mineralization

VEGF-A staining of sections taken from molars that had had a deep pulpal exposure also shows high levels of VEGF-A expression in the areas of tertiary dentin formation undergoing the process of mineralization (Fig. 7). One injured tooth that had a deep pulp exposure was allowed to heal for 4 weeks. Haematoxylin and eosin staining

revealed the formation of a dentin bridge over an area of the pulp that had begun mineralizing due to severe trauma (Fig. 7a, black arrow). When the sections from the same tooth were stained for VEGF-A, the results showed high levels of VEGF-A in the dental pulp of the adjacent uninjured cusp, in the primary and secondary dentin layers, in the mineralizing portion of the newly formed dentin bridge and in the underlying traumatized dental pulp.

A comparison of an uninjured tooth, an injured tooth with no pulp exposure and a tooth with traumatic pulp exposure revealed VEGF-A was expressed at high levels in the dentin matrix of the uninjured tooth between pulp horns and to a milder extent in the natural reactionary dentin formation resulting from attrition at the top of the pulp horns (Fig. 8a). The injured tooth without pulp exposure showed a large area of newly formed reactionary dentin underlying the site of injury (Fig. 8b). Since the injury was mild and did not penetrate the dentin, it could be assumed that the odontoblast layer underlying the injured area was intact and viable and signals specific to wound healing have caused an upregulation in the secretion of dentin matrix by those odontoblasts. In this mild case of dental injury, the reactionary dentin produced was negative for VEGF-A. In the case of pulp exposure and post-mitotic odontoblast cell death, the entire area of the underlying pulp begins to mineralize. The mineralizing areas of the pulp underlying the dental injury were expressing VEGF-A (Fig. 8c).

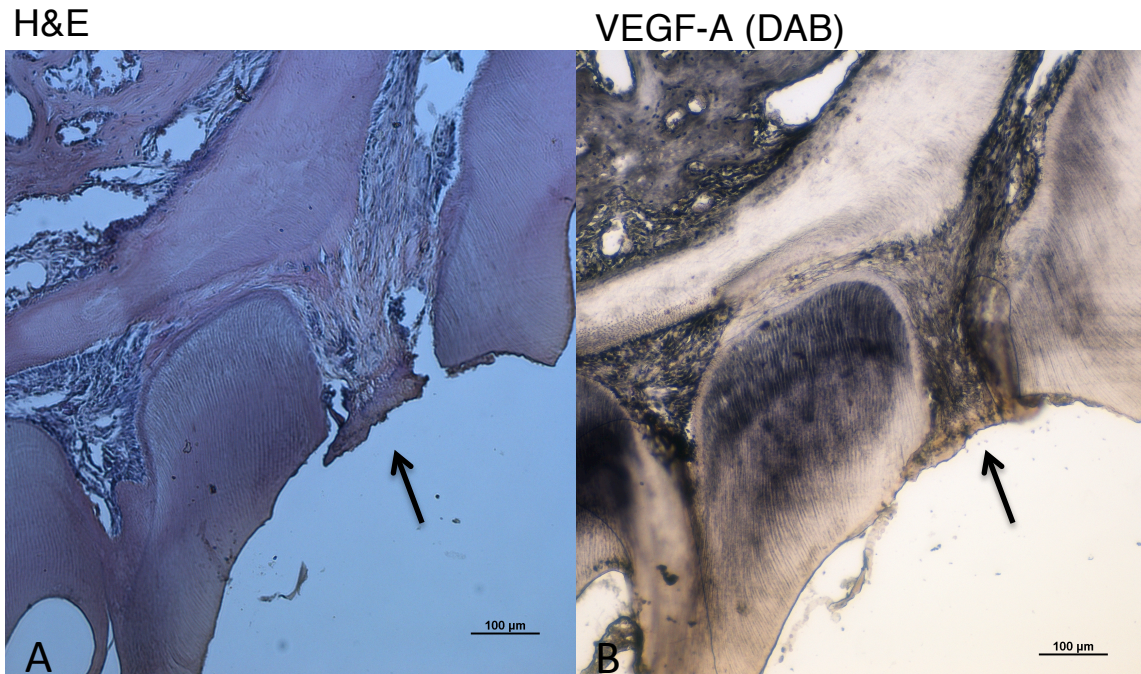


Figure 7. Serial stained dental pulp sections taken 4 weeks after injury. **(A)** Haematoxylin & eosin stained section of murine molar taken 4 weeks post-deep pulpal exposure. Mineralization of the dental pulp underlying the area of trauma has begun to fully mineralize and a dentin bridge has been formed (black arrow). **(B)** A section of the same tooth stained for VEGF-A showing large amounts of VEGF-A sequestered in the primary and secondary dentin layers and high levels of VEGF-A expression in the mineralizing dental pulp tissue. Scale bars = 100 µm.

VEGFR-2 Expression After Injury

VEGFR-2 expression follows a pattern similar to VEGF-A in the injury model, although the expression levels for VEGFR-2 are much lower overall than the expression levels of VEGF-A based on staining intensity. In the uninjured control there is almost no VEGFR-2 positive staining, with the possible exception of a few cells found near or abutting a central arteriole (Fig. 9a). The 4-week post injury section stained for VEGFR-2 shows several positively stained cells throughout the dental pulp, including some in the pulp core and some positive cells in the odontoblast layer (Fig. 9b). The 2-week post injury section shows heavier staining in the pulp core and surrounding central arterioles than in the control and 4-week post injury sections (Fig. 9c). And finally, the 15-minute

post injury sections reveal the heaviest positive staining for VEGFR-2 in the odontoblast layer as well as in cells throughout the dental pulp core (Fig. 9d).

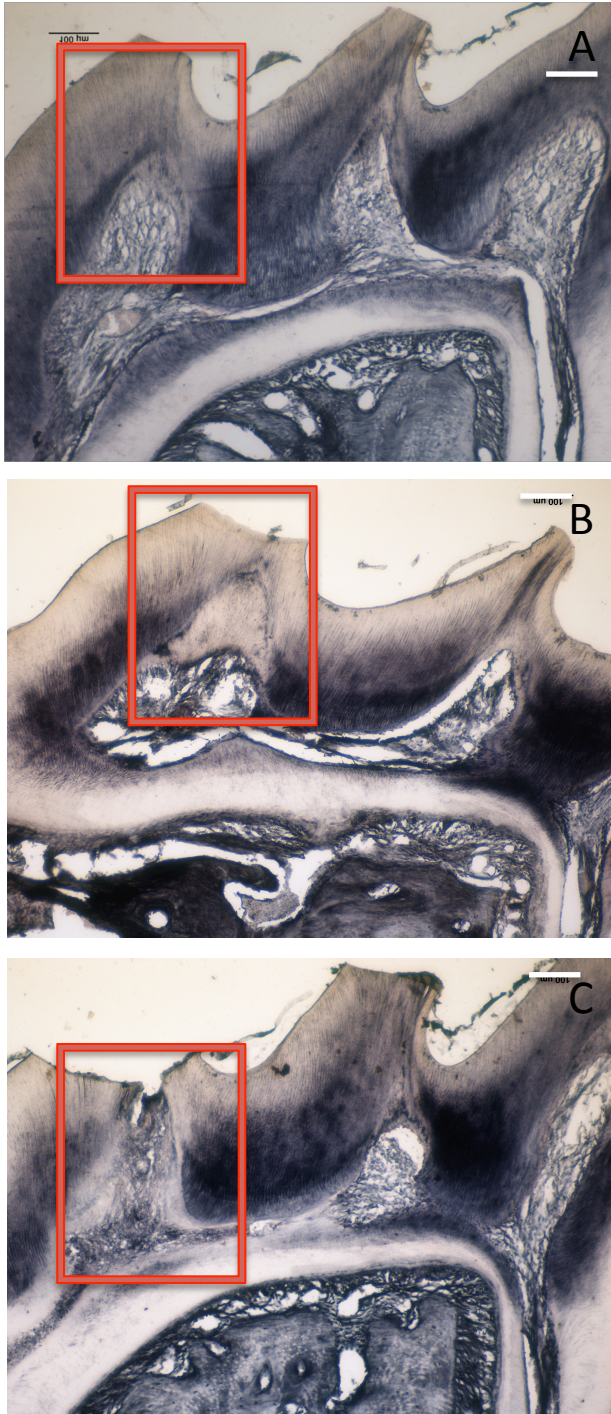


Figure 8. 3,3'-diaminobenzidine (DAB) staining for VEGF-A on three murine molar sections showing various degrees of dental injury. **(A)** Uninjured molar showing diffuse VEGF-A positive staining in the dental pulp and dark VEGF-A positive staining within the dentin matrix. Naturally occurring reactionary dentin formation can be seen at the top of the pulp horns (red box). **(B)** Histological section of an injured murine molar in sagittal section without pulp exposure. DAB staining reveals the dentin of uninjured areas contains large amounts of VEGF-A. The newly formed reactionary dentin secreted in response to drilling of the overlying cusp shows no VEGF-A positive staining although the dental pulp and odontoblast layers are expressing VEGF-A (red box). **(C)** DAB stained histological section of a murine molar with dental injury resulting in pulpal exposure, again showing heavy expression of VEGF-A in the uninjured areas of the dentin. In the area of pulp exposure (red box) the dental pulp has begun to mineralize and reparative dentin has begun to occlude the entire pulpal area underlying the injury. A small dentin bridge has formed at the occlusal surface of the cusp. VEGF-A staining is positive in the areas of mineralizing dentin in the injured pulp horn. Scale bars = 100 μ m.

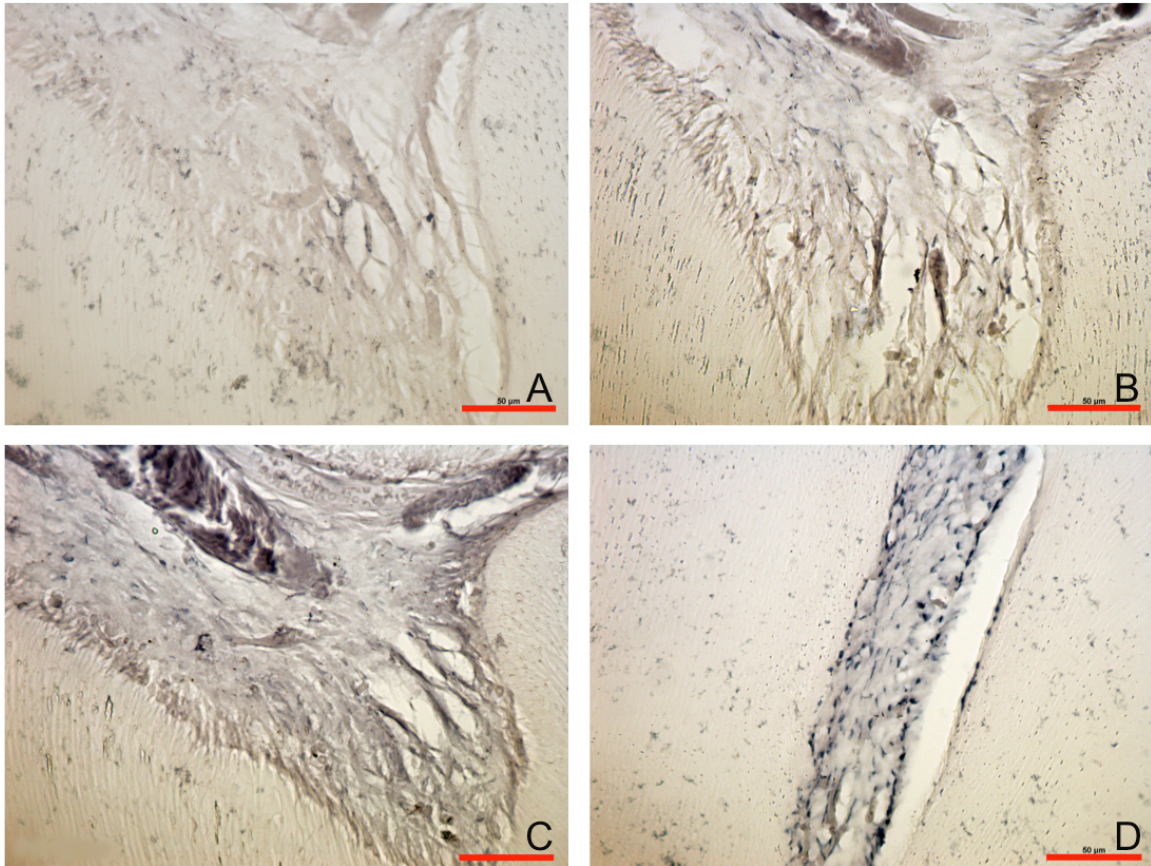


Figure 9. Vascular endothelial growth factor receptor 2 expression in mouse dental pulp post-injury shown with 3, 3'-diaminobenzidine (DAB) staining. **(A)** Control. Dental pulp from the first cusp of the first maxillary molar of an un-injured control. Note that staining residue was unable to be removed from the specimen. In spite of this, very little expression of VEGFR-2 is present in the un-injured dental pulp. **(B)** 4 weeks post-injury. Dental pulp from the injured first cusp of the first maxillary molar. The pulp chamber was not exposed during drilling. There are diffuse and sparse VEGFR-2 positive cells in the dental pulp core with some extension into the odontoblast cell layer. **(C)** 2 weeks post-injury. Dental pulp of the injured first cusp of the first maxillary molar. The pulp core contains more VEGFR-2 positive cells than what was seen at 4 weeks post-injury, including increased positive staining around vessels and in the pulp core. **(D)** 15 minutes post-injury. VEGFR-2 expression revealed by the intensity of the DAB staining is much higher in this sample than in any of the others. The odontoblast layer contains many VEGFR-2 positive cells in addition to those positive in the pulp core. Scale bars = 50 µm.

Discussion

The results of this study provide evidence for an angiogenic independent role of VEGF in the dental pulp during tertiary dentin formation. The observations made through immunohistochemical staining raise several interesting questions and possible explanations.

First, it is easily shown by comparing an injured molar with pulp exposure (Fig. 7) versus an injured molar with no pulp exposure (Fig. 5) that there is very little to no VEGF secreted in reactionary dentin produced by living post-mitotic odontoblasts whereas there is at least some VEGF secreted and sequestered within a newly formed reparative dentin layer that has been formed by odontoblast-like stem cell progenitors recruited from the dental pulp. Although this sequestration of VEGF-A within the reparative dentin layer could be attributed to cellular occlusions that can occur during the reparative dentin formation process, it is unlikely that there would be enough cellular inclusions to account for the intensity of VEGF-A positive staining seen in the reparative dentin samples collected in this study.

Another question brought up by the present findings is: where is all of the VEGF-A observed in the dental pulp at the 15 minute-post injury time point coming from? If it is accepted that 15 minutes is not enough time for new protein synthesis of VEGF-A from dental pulp cells to occur, then only a few other explanations exist. It may be that VEGF is pumped in to the site of injury through the blood stream, delivered by VEGF producing cells such as macrophages and other immune cells, or it is released from the destruction of dentin matrix during injury. The exact answer to this question is still unclear, although all of the mentioned possibilities could be at play simultaneously.

One result of this study which supports the already recognized pro-angiogenic role of VEGF was noted in the 2 week post injury molar samples. In these samples, several small capillaries can be seen invading the sub-odontoblast and odontoblast cell layer (Fig. 6c). As previously mentioned, it is not proven that these vessels were formed as a result of the experimental dental injury, however blood vessels of that size and

number were not observed in any of the control samples or other post-injury time points. This could mean that there is a post-injury vascular invasion response facilitated by VEGF-A and VEGFR-2, possibly as an effort to increase vasculature to that area to provide pro-dentinogenic growth factors.

Results of immunohistochemical staining for VEGFR-2 revealed that there was an observable upregulation of VEGFR-2 in the odontoblast cell layer only 15 minutes post dental injury. This supports a mechanism by which VEGFR-2 is upregulated by odontoblasts in response to injury, which in turn signals the upregulation of VEGF-A in the surviving odontoblast layer, or the reverse response could be taking place where increased VEGF-A from immune cells or crushed dentin matrix is signaling for the increased expression of VEGFR-2 by odontoblasts and other cells of the dental pulp.

Regarding a possible role for VEGF in dentin regeneration, it was noted by viewing the staining results for VEGF-A that there was never any VEGF-A expressed in the unmineralized pre-dentin layer, but that dentin which had mineralized almost always contained large amounts of sequestered VEGF-A, with the exception of reactionary tertiary dentin deposits previously discussed. Additionally, the high level of expression of VEGF-A in the odontoblast layer of the dental pulp, and more specifically in the odontoblast processes which extend far into the dentin layer where there are no blood vessels, supports the idea that VEGF may be playing a role in the process of dentin regeneration either by aiding in progenitor cell recruitment from the dental pulp after severe injury or by contributing by an unknown mechanism to dentin mineralization.

This expression of VEGF-A by odontoblast processes is seen at all time points after injury and arguably in the uninjured molar as well, but to a lesser degree. Although

the pathway and exact mechanism for this idea remains unclear, this study serves as a jumping off point for further investigation into the role of VEGF in dentin regeneration after dental injury.

Although this study is a purely observational analysis of the expression of VEGF-A and VEGFR-2 after dental injury, it provides support for yet another role of VEGF independent of angiogenesis. Further studies are needed to bolster this idea, although it is now apparent that vascular endothelial growth factor is an incredibly important molecule, responsible for a growing list of crucial cellular processes. With additional work, dentin mineralization and regeneration may soon be acknowledged as one of those processes.

Supplemental Materials

Marker	Antibody	Species	Dilution	Company
BS1-lectin-			1:500	Sigma
VEGF-A	Polyclonal	Rabbit	1:100	Abcam

Table 1. List of antibodies

Gene	Forward Primer (5'→3')	Reverse Primer (3'→5')	GenBank Accession Number
<i>VEGF-A</i>	GAGCAGAAGTCCCATGAAGTGAT	CAATCGGACGGCAGTAGCTT	NM_001025250
<i>VEGF-C</i>	GA TTCTCTGCCCCGCTTTG	GGAGGATGCTGTGTTGCTACAA	NM_009506
<i>VEGF-D</i>	TGCGGCAACTTTCTATGACACT	ACTGGCGACTTCTACGCATGT	NM_010216
<i>VEGFR-1 (Flt1)</i>	CGGAGCCATTCCCACAAC	TTGTCTCGGCTCTCCACATTG	NM_010228
<i>VEGFR-2 (Flk1)</i>	ACTGCAGTGATTGCCATGTTCT	CCTTCATTGGCCCCGCTTAA	NM_010612
<i>VEGFR-3</i>	ATCGGCAACCATCTCAACGT	GCTTTGGCGCCTTCTACCA	NM_008029
<i>vWF</i>	GATGTCCAGCTCCCCTTCT	AGGCGTTTCCGAAGTCTACCA	NM_011708
<i>VE-Cadherin</i>	AGCGCAGCATCGGGTACT	TCGGAAGAATTGGCCTCTGT	NM_009868
<i>LYVE-1</i>	CTCGTGCAAGACCTTCCATT	AAGTGTCTTCGGTTGCTCCG	AZ_238936
<i>PROX1</i>	CCCAGAGTGTCTTCCCCTTT	ATACACTACTCGTGGACTCTCG	BC_024201
<i>PDPN</i>	AGAGAACACGAGAGTACAACCA	CGTTTCATCCCCTGCATTATCT	BC_022812
<i>18s rRNA</i>	TTGACGGAAGGGCACCACCAG	GCACCACCACCCACGGAATCG	NM_003278

Table 2. Q-RT-PCR primers used in Aims 1 and 2.

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