Angiogenesis in the apical papilla of immature permanent teeth

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“It always seems impossible until it’s done.”

Nelson Mandela.
Abstract

Dental trauma, caries or developmental anomalies may result in reversible or irreversible injury to the dental pulp in immature permanent teeth and this presents a clinical conundrum. Immature teeth possess a more favourable healing potential, and this is partly contingent on the vascularity, and angiogenic potential of the resident cells and growth factors. Moreover, the apical papilla is a tissue unique to these teeth and plays an important role in root development and healing. Angiogenesis is a fundamental process in development and disease and is incumbent upon an intricate interplay of angiogenic genes, growth factors and their cognate receptors. The dental pulp is not a “doomed organ” (Rebel 1922) and is capable of healing. Hence, current therapies are aimed at preservation of pulp vitality and facilitating apexogenesis, but treatment outcomes are not always predictable. An increased understanding of angiogenic signalling in the apical region at a molecular level may improve this. This research explored the angiogenic potential of the apical papilla in healthy immature permanent human teeth and serves as a baseline for understanding potential responses to disease. As well as examining protein expression in the apical papilla tissue, the distribution and relative mRNA levels of angiogenic growth factors (VEGF, Ang-1, Ang-2), receptor proteins (VEGFR2 and Tie-2), and cell-surface markers (CD34 and CD45) were investigated.

Immunohistochemistry and gene expression experiments conducted on the apical papilla showed that this tissue was comprised of angiogenic growth factors and receptors and few resident cells and blood vessels, demonstrating evidence that it is capable of partaking in and promoting angiogenic signalling. Endothelial cells were the predominant cells expressing these markers but expression on other cell types including fibroblasts and immature mesenchymal cells suggests they play contributory roles. VEGF, Ang-1, and Tie-2 exhibited greater immunopositivity and higher cell counts than Ang2 in the apical papilla, while CD34 and CD45 were sparsely distributed. Real-time polymerase chain reaction corroborated these results and revealed differences in gene expression between the apical and coronal regions. The differential expression of VEGF and angiopoietins and their receptor mRNA and protein in the apical papilla suggests the potential for these growth factors to contribute to physiological root development and pulp healing following injury. This study has enhanced our understanding of angiogenesis in healthy dental pulps, and contributes to the body of knowledge related to potential use of angiogenic-modulatory factors in vital pulp therapies.
Acknowlegements

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<th>Full Form</th>
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<tbody>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>ANG</td>
<td>Angiopoietins</td>
</tr>
<tr>
<td>Ang-1</td>
<td>Angiopoietin-1</td>
</tr>
<tr>
<td>Ang-2</td>
<td>Angiopoietin-2</td>
</tr>
<tr>
<td>AP</td>
<td>Apical papilla</td>
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<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
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<tr>
<td>bFGF</td>
<td>basic-Fibroblast growth factor</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CEJ</td>
<td>Cemento-enamel junction</td>
</tr>
<tr>
<td>CFZ</td>
<td>Cell-free zone</td>
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<tr>
<td>Cq</td>
<td>Quantification cycle</td>
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<tr>
<td>CRZ</td>
<td>Cell-rich zone</td>
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<tr>
<td>ds-DNA</td>
<td>Double-stranded deoxyribonucleic acid</td>
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<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
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<tr>
<td>DPSC</td>
<td>Dental pulp stem cells</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
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<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factors</td>
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<tr>
<td>GE</td>
<td>Gene expression</td>
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<tr>
<td>GOI</td>
<td>Gene of interest</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
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<tr>
<td>HERS</td>
<td>Hertwig’s epithelial root sheath</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor-1α</td>
</tr>
<tr>
<td>HKG</td>
<td>Housekeeping gene</td>
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<tr>
<td>HMAR</td>
<td>Heat-mediated antigen retrieval</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LCA</td>
<td>Leukocyte common antigen</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic-acid</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain-like receptors</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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Chapter One — Literature Review

Key Words: Angiogenesis, Apical papilla, Immature permanent teeth, Healing, Dental pulp, VEGF, Angiopoietins.

1.1 Introduction

Dental injury to an immature permanent tooth is common and poses a clinical challenge. Such injuries are often associated with damage to the pulp, apical papilla (AP), periodontal ligament (PDL), and Hertwig’s epithelial root sheath (HERS), and lead to a range of clinical sequelae ranging from mild tooth discoloration to pulp infection and cessation of root development (Andreasen et al. 1988).

The dental pulp has one main vascular pathway via the apical foramen. It is rich in blood vessels and possesses the capacity to heal (Glass & Zander 1949), but this is not always predictable. The stage of tooth development (Andreasen et al. 1986), proximity to a blood supply (Magloire et al. 1996), and the presence of inflammation (Schmalz & Galler 2011) are important. Following insults from dental trauma, dental caries, or developmental anomalies that involve the pulp, the main vascular supply may be damaged. Management of immature permanent teeth following pulp injury is challenging. These teeth have incomplete root development with thin parallel or divergent walls, which are weak and prone to fracture. Traditional management involved apexification procedures to create an apical barrier against which to place a root filling (Frank 1966). However, root apexification does not result in continued root development and obturation of a wide canal with an open apex poses difficulty in achieving an adequate seal from the apical tissues. Contemporary treatment recommends a conservative approach with preservation of the apical pulp and/or apical papilla in order to maintain pulp vitality and facilitate apexogenesis and root maturation (Bogen et al. 2008). However, treatment outcomes are often unpredictable (Wigler et al. 2013).

In immature permanent teeth, healing entails an absence of clinical signs and symptoms of infection, maintenance of pulp vitality, continued root development and a normal periapical appearance radiographically (Cohenca et al. 2013). Pulps of immature permanent teeth demonstrate more favourable healing compared with mature teeth (Friedlander et al. 2014). This is mainly due to the presence of a wider/open apical region in proximity to a blood supply and a short distance from the apex to pulp horn. Moreover, immature permanent teeth
are associated with an AP that has known roles in tooth root development, tissue regeneration and healing (Sonoyama et al. 2006, 2008, Huang 2008).

The AP is the soft, gelatinous tissue loosely adherent to the root apex, beneath the mature pulp. It is largely avascular, but is adjacent to the apical cell-rich zone of the radicular pulp, and the dental follicle which gives rise to the periodontal ligament, cementum and alveolar bone (Abe et al. 2007, Friedlander et al. 2014). In event of a pulp injury, cells of the AP may survive and contribute to pulp healing, continued root development and maturation (Sonoyama et al. 2008, Huang 2008, Bogen & Kuttler 2009).

Wound healing in the pulp-dentine complex may occur by repair or regeneration (Goldberg 2011, About 2013, Ricucci et al. 2014, Simon et al. 2014), but irrespective of the type of healing, cell populations, growth factors and a blood supply are required. Re-establishment of a blood supply is necessary and involves the process of angiogenesis (Dvorak 2005). Angiogenesis is a complex multi-step process, involves the growth of new blood vessels from pre-existing vasculature and is tightly regulated by growth factors (Iruela-Arispe & Dvorak 1997, Toyono et al. 1997, Gerwins et al. 2000, Karamysheva et al. 2008, Shimabukuro et al. 2009). Under homeostatic conditions, a careful balance exists between their pro- and anti-angiogenic signals (Jia et al. 2004).

Vascular endothelial growth factors (VEGF) are a family of platelet-derived growth factors. VEGF-A, hereby referred to as VEGF unless otherwise stated, is a key pro-angiogenic growth factor during development, healing and disease (Ferrara & Henzel 1989, Carmeliet 2005, Mattuella et al. 2007a). VEGF is expressed in endothelial cells, odontoblasts, fibroblasts, pulp stem cells, in the dentine matrix and is upregulated in response to inflammation (Artese et al. 2002, Telles et al. 2003, Derringer & Linden 1996, 1998, 2003, 2004, Güven et al. 2007, Bletsa et al. 2012). Vascular endothelial growth factor receptor-2 (VEGFR2) is the primary proangiogenic receptor for VEGF (Shibuya 2006, Mattuella et al. 2007b) and is almost exclusively expressed on endothelial cells (Witmer et al. 2002).

Angiopoietins (Ang) and VEGF play synergistic roles in vascular growth and development (Lobov et al. 2002, Felcht et al. 2012). Ang-1 is an agonist and important for vessel remodelling and maturation of the immature vasculature (Davis et al. 1996, Sundberg et al. 2002), while Ang-2 is associated with blood vessel instability (Maisonpierre et al. 1997, Fiedler & Augustin 2006). Both are essential for the initiation of angiogenesis and bind with relatively high affinities to the receptor, tunica interna endothelial cell kinase-2 (Tie-2) (Fiedler et al. 2003).
Immunosurveillance is recognised in the dental pulp, particularly associated with odontoblasts (Farges et al. 2009, 2013, Gaudin et al. 2015) but the presence of inflammatory markers in the AP remains unclear. Angiogenesis is up-regulated in the presence of inflammation (Imhof & Aurrand-Lions 2006) and may be altered following \textit{in vitro} induced mechanical pulp cell injury (Tran-Hung et al. 2006, 2008). Under pathological conditions, local pulpal angiogenesis is vital in the pulpal healing process (Baume \textit{et al}. 1980, Smith \textit{et al}. 1990). This study aims to investigate the angiogenic activity in the AP of immature permanent teeth associated with healthy pulp tissues. Identification and quantification of growth factors and receptors that regulate angiogenesis may offer novel therapies for the management of dental pulps in immature permanent teeth.

1.2 The pulp-dentine complex

The pulp and dentine are embryologically, histologically and functionally coupled, and both are of neural crest origin. The pulp arises from the dental papilla and peripherally contains specialised odontoblast cells whose primary role is the secretion of dentine that surrounds the pulp and is intimately connected to it via dentinal tubules (Trowbridge 2003).

1.2.1 Dental pulp development

Tooth development is a continuous process, however traditionally, it has been divided into bud, cap and bell stages (Kollar & Lumsden 1979). It is during the cap stage that formation of the pulp-dentine complex begins. The cells of the enamel knot induce the formation of the dental papilla and cell proliferation via the release of growth factors. During the bell stage, the invaginating epithelium partially envelops the dental papilla. This condensation of ectomesenchyme that surrounds the enamel organ and dental papilla forms the dental sac and eventually becomes the periodontal ligament (Nanci 2013).

Late in the bell stage, differentiation of dental papilla cells into odontoblasts occurs (Nanci 2013). This commences at the site of the future cusp tip(s) and migrates towards the cervical loop. The pre-ameloblasts that line the dental papilla induce differentiation of odontoblasts (epithelial-mesenchymal interactions). As differentiation begins, a layer of pre-odontoblasts align themselves along the basement membrane. They cease to divide, instead, they elongate into columnar cells with basal nuclei. At this stage, they are still relatively undifferentiated. This differentiation is not only under the organised influence of the epithelium, but also involves several signalling molecules (Thesleff & Hurmerinta 1981, Thesleff & Sharpe 1997, Puthiyaveetil \textit{et al}. 2016).
Production of the outer layer of dentine, i.e. mantle dentine, results in the formation and maturation of an extracellular matrix, comprising collagen fibrils and proteoglycans. The odontoblasts migrate towards the pulp as predentine is formed and line the outer aspect of the pulp chamber. A projection from each odontoblast forms the odontoblast process. The dentinal tubules form around these odontoblast processes.

The location of the dental papilla becomes progressively more apical as the crown of the tooth forms during the late bell stage. With the beginning of root development, the dental papilla is referred to as the AP (Sonoyama et al. 2006).

1.2.2 Dental pulp cells

1.2.2.1 Odontoblasts

Odontoblasts are terminally differentiated cells lining the periphery of the pulp at the pulp-dentine interface. Odontoblasts have an extension/process that arises from the cell body and reaches into the dentine where it is ensheathed within a dentinal tubule (Arana-Chavez & Massa 2004).

The functional stage of an odontoblast varies through its life and this is accurately reflected in its morphology, which ranges from an active secretory phase to an older, quiescent phase. As these cells are ‘permanent,’ once lost, they are incapable of further mitosis (Ruch et al. 1995).

Odontoblasts have several roles to play. Their most important role is the secretion of the organic dentine matrix. This unmineralised matrix is primarily composed of Type 1 collagen and non-collagenous matrix proteins (Arana-Chavez & Massa 2004). Odontoblasts have also been postulated to be involved in pain transduction (Chung et al. 2013) and have a role in the innate immune response of the dental pulp. These cells exhibit toll-like-receptors (TLRs), and produce cytokines, chemokines and anti-microbial peptides (Levin et al. 1999, Farges et al. 2009, Farges et al. 2013).

1.2.2.2 Fibroblasts

Fibroblasts are the most abundant cells in the dental pulp and their main role is in the synthesis and maintenance of the pulp matrix, composed of collagen and ground substance (Nanci 2013). Fibroblast morphology reflects their functional activity. When young, these cells have a plump cytoplasm, large nucleus and abundant organelles. As they age, their nucleus appears denser while they appear as flattened spindle-shaped cells. Fibroblasts also play a vital role in repair following injury (Artese et al. 2002, Tran-Hung et al. 2006, 2008). They are ‘stable’ cells and have a reduced ability to divide with age (Kumar 2012).
Fibroblasts have an established role in contributing to the inflammatory response and angiogenesis necessary for healing. They produce an array of inflammatory mediators involved in innate immunity including IL-6 and IL-8, and express TLRs and Nod-like receptors (Dongari-Bagtzoglou & Ebersole 1996, Aranha et al. 2010, Nakanishi et al. 2011, Bainbridge 2013). In addition, angiogenic growth factors such as VEGF and angiopoietins are able to be released from fibroblasts which in turn act on endothelial cells to exert pro- and anti-angiogenic effects (El Karim et al. 2009) thus regulating vascular homeostasis and healing (Tziafas et al. 2000, About 2011). Fibroblasts have also been shown to secrete angiogenic growth factors and participate in healing in the pulp-dentine complex, but the effect of these factors on their phenotype and behaviour has not been elucidated yet (Aranha et al. 2010).

1.2.2.3 Dental stem and progenitor cells

Dental stem cells may either exist as progenitor cells, or as ‘true’ stem cells. Progenitor cells are characterized by having a limited capacity for self-renewal but they do maintain their multipotentiality and the ability to follow several cell populations (Gronthos et al. 2000). Stem cells are characterized by three properties: they demonstrate high proliferation potential, are capable of unlimited self-renewal, and upon division, the daughter cells give rise to cells which eventually become differentiated. The ability of these stem cells to differentiate into cells of different lineages is thought to depend on their extent of differentiation and their commitment, and on the local microenvironment within which they reside (About 2013, Chmilewsky et al. 2014).

The peripheral cell rich zone (CRZ) of Höhl and the central pulp contain a pool/reserve of undifferentiated mesenchymal cells that may be progenitor cells and can participate in healing. These cells have been well characterised and are capable of partaking in repair, dentinogenesis and angiogenesis, depending on the stimulus and on the local microenvironment (Gronthos et al. 2000, Gronthos et al. 2002). However, the signalling events that regulate these processes are still not clear (Sonoyama et al. 2008).

Dental mesenchymal stem cells have been isolated from the dental pulp (DPSC) (Gronthos et al. 2000), human exfoliated deciduous teeth (Miura et al. 2003), periodontal ligament (Seo et al. 2004), bone-marrow (Shi & Gronthos 2003) and from mesenchymal stem cells residing in the apical papilla (SCAP) of immature permanent teeth (Sonoyama et al. 2008). Studies have also revealed a population of stem cells in the dental follicle (Morsczeck et al. 2005). These cells demonstrate multilineage potential in vitro, differentiating into odontogenic, osteogenic,
chondrogenic, adipogenic and neurogenic tissues in the presence of established lineage-specific factors.

Several stem cell and progenitor cell niches are present in the coronal and apical dental pulp. One has been identified associated with blood vessels (Feng et al. 2011, Mitsiadis et al. 2011, Lizier et al. 2012, Dimarino et al. 2013, Oh & Nör 2015). DPSC were first isolated associated with blood vessels in the pulp (Gronthos et al. 2000, Shi & Gronthos 2003, Lovschall et al. 2007) and were shown to be able to proliferate and differentiate into multiple cell lineages including odontoblast-like cells able to form dentine/pulp-like complex when implanted into the subcutaneous space of immunocompromised mice (Gronthos et al. 2000, Shi et al. 2005). Téclès et al. (2005) showed that undifferentiated progenitor cells are able to migrate in response to odontoblast injury following pulpal trauma and elaborate a dentine matrix. In addition to their perivascular niche, separate niches in the CRZ of Höhl and the central pulp stroma (Nanci 2013, Mitsiadis et al. 2011) have been reported. It is important to bear in mind that only 1% of the pulp cell population are stem cells (Smith et al. 2005), and better outcomes for regeneration are observed in younger patients as the regenerative cell pool decreases as a person ages (Stocum et al. 2001).

The AP is unique to immature permanent teeth and has been shown to contain a novel population of stem/progenitor cells distinct from DPSCs (Sonoyama et al. 2006, Abe et al. 2008, Huang et al. 2008) and with a greater proliferative and regenerative capacity compared with DPSCs (Sonoyama et al. 2006, 2008). Further, SCAP may be the source of primary odontoblasts for root dentine formation, while DPSCs are likely to be the source of replacement odontoblasts (Sonoyama et al. 2008).

1.2.2.4 Immune cells

Immune cells including macrophages, plasma cells, mast cells, lymphocytes and neutrophils are critical to the defence response of the pulp (Izumi et al. 1995, Jontell et al. 1998). Macrophages are located predominantly in the CRZ. Histologically, they appear as large, oval or even oblong cells with an intensely stained nucleus. Their key role is phagocytosis.

In health, B lymphocytes are scarce but T lymphocytes are recognized as normal residents of the dental pulp (Hahn et al. 1989, Jontell et al. 1998). Polymorphonuclear leukocytes (neutrophils) are not commonly found in health but increase markedly during acute inflammation (Gaudin et al. 2015).
Antigen-presenting cells (APC) such as dendritic cells are located within, under and around the outermost odontoblast layer (Okiji et al. 1997). They are usually located in a perivascular and perineural location. APCs are important for immunosurveillance and act as ‘sentinels,’ whereby they capture and present antigens to the T lymphocytes. Dendritic cells have long, cytoplasmic extensions that can extend into the odontoblast layer, especially in event of microbial insults or other injury.

1.2.2.5 Endothelial cells

Endothelial cells line all blood vessels and are key cells in the process of vascular development and angiogenesis (Sepp et al. 1997, Lamalice et al. 2007, About 2011, Dissanayaka et al. 2012). These cells arise from mesodermal cells that have undergone successive differentiation into hemangioblasts. Hemangioblasts at the periphery of primitive blood islands give rise to angioblasts; these cells are the precursors of endothelial cells. Endothelial cells are surrounded by mural cells called pericytes in small blood vessels (Allt & Lawrenson 2001) and by vascular smooth muscle cells in larger vessels. A finely regulated reciprocity exists between endothelial and mural cells. These mural cells not only guide endothelial cells to induce angiogenesis, but can also secrete angiogenic factors (Gerhardt & Betsholtz 2003, Ribatti et al. 2011).

Histologically, endothelial cells appear flat with a centrally placed nucleus. These cells form flat configurations on the inside of the blood vessels and at the junctions between adjacent cells.

Injury to the dental pulp may be associated with injury to the microvasculature and endothelial cells. Injured endothelial cells release various signalling molecules such as VEGF and FGF and chemotactic factors to stimulate angiogenesis, the inflammatory response and healing (Martin et al. 1997, Mathieu et al. 2005). Adhesion molecules expressed by these cells are important in the recruitment of progenitor and inflammatory cells. In event of endothelial cell damage, other cell populations such as fibroblasts and dental pulp progenitor/stem cells may contribute to healing (About 2011).

1.2.3 Zones of the dental pulp

Histologically, the pulp is divisible into four distinct zones, from the pulp-dentine junction to the centre of the pulp (Baume 1980) (Figure 1.1).
1.2.3.1 Odontoblast layer

This is the outermost layer of the pulp and is located adjacent to the predentine. This layer comprises the cell bodies of odontoblasts, since their processes lie within dentine.

In addition, neurovascular structures and APCs may be located amongst the odontoblasts (Tsuruga et al. 1999). Odontoblasts assume a palisaded appearance and are connected to each other by gap and tight junctions. They vary in morphology from being tall columnar in the coronal pulp to being flattened and cuboidal in shape in the apical radicular pulp (Arana-Chavez & Massa 2004).

1.2.3.2 The cell-free zone of Weil

The cell-free zone (CFZ) of Weil is a narrow zone and is relatively acellular. Capillaries, unmyelinated nerve fibres, and fibroblast cytoplasmic processes traverse through this zone (Nanci 2013).
1.2.3.3 The cell-rich zone of Höhl

This is the zone beneath the CFZ and is composed of a high proportion of fibroblasts, pericytes and small blood vessels. Macrophages, APCs and stem/progenitor cells may also be present, along with a rich capillary plexus. In event of odontoblast death, cells from this zone may migrate to the site of injury and differentiate to replace the odontoblast population (Tziafas & Kodonas 2010, Lin et al. 2014).

1.2.3.4 The central pulp

This is the core of pulp tissue, and composed of loose connective tissue, immature cells and large nerves and blood vessels, in addition to the pulp fibroblasts. In health, inflammatory cells are also present but these are few (Gaudin et al. 2015).

1.2.4 Dentine and dentinogenesis

Dentine is a mineralized tissue and comprises the bulk of the tooth. Functionally, it provides support to the underlying pulp via dentinal tubules, whilst providing elasticity to the overlying enamel (Mjör & Nordahl 1996). Dentine is secreted by odontoblasts, which differentiate from the ectomesenchymal cells of the dental papilla (Goldberg et al. 2011). This process is highly organized and results in the production of an organic matrix, which later becomes mineralized (Bhaskar 2011).

1.2.4.1 Types of dentine

Goldberg (2011) classified different types of dentine based on the varied functions of dentine, and on the differing odontoblastic activity.

1.2.4.1.1 Primary dentine

Primary dentine is the first formed dentine and consists of mantle and circumpulpal dentine. Mantle dentine is the dentine first-formed, and is found at the periphery of the tooth, i.e. next to the enamel. This layer is relatively atubular, with only thin and few tubules present, and is poorly mineralized, which makes this dentine resilient and suitable for dissipation of forces at the dentine-enamel junction. Circumpulpal dentine, as the name suggests, lines the pulp chamber, and is the dominant portion of dentine. It is laid down at a rate of 4-20 μm per day and is not homogenous.

Predentine is the unmineralised matrix secreted by odontoblasts and is composed of collagen fibres and non-collagenous proteins. It stains lighter than the mineralised dentine when stained with hematoxylin and eosin (H & E). During the period of active dentinogenesis, predentine assumes its greatest thickness; this thickness gradually diminishes with ageing.
(Nanci 2013). Primary dentine continues to be laid down until completion of root development.

1.2.4.1.2 **Secondary dentine**

Secondary dentine starts at the completion of root development and persists throughout life, albeit at a much slower deposition rate (0.4 μm). Secondary dentine is largely continuous with primary dentine, with a similar amount of mineral to organic material. A larger proportion of secondary dentine is deposited on the floor and roof of the pulp chamber. Over time, continued secondary dentine deposition in the radicular portion of the tooth contributes to root thickening and a subsequent reduction in the pulp space.

1.2.4.1.3 **Tertiary dentine**

Tertiary dentine is produced in response to an insult, be it biological, chemical or mechanical, with the final aim being protection of the integrity of the pulp (Smith et al. 1995). While primary and secondary dentine are laid down physiologically along the whole pulp-dentine interface, tertiary dentine is secreted by those cells directly affected by the insult. The tubular structure of tertiary dentine may be continuous with that of secondary dentine, but there tend to be fewer and more haphazardly arranged tubules, or in some cases, atubular. Two types of tertiary dentine have been reported in the literature, reactionary and reparative (Goldberg 2011). Reactionary dentine is formed from existing odontoblasts, while reparative dentine entails the recruitment of pulp progenitor cells and their subsequent differentiation into odontoblast-like cells (Sloan et al. 2001, Tziafas 2004, Mattuella et al. 2007, Sloan & Smith 2007, Sloan & Waddington 2009, About 2013).

1.2.5 **Root development**

Root development is a dynamic process and commences after enamel formation (Zeichner-David et al. 2003). Once the stellate reticulum and stratum intermedium cells have disappeared, the inner and outer enamel epithelium in the distal cervical loop form the HERS. HERS plays an important role in root development and root shape, radicular dentinogenesis and cementogenesis, and in development of the periodontium (Andreasen et al. 1988, Sonoyama et al. 2007, Nanci 2013). Similar to crown formation, epithelial-mesenchymal interactions induce the progenitor cells in the dental papilla and radicular pulp to differentiate into odontoblasts that secrete dentine matrix formation with subsequent mineralization. HERS eventually begins to disintegrate, allowing the dental sac mesenchymal cells to establish contact with the newly formed dentine (Huang et al. 2009).
1.2.6 The apical region of immature permanent teeth

Over the last decade, the apical region of immature permanent teeth has been the focus of considerable investigation in the field of pulp-dentine regeneration and hard tissue engineering (Sonoyama et al. 2006, Abe et al. 2007, Abe et al. 2008, Huang et al. 2008, Sonoyama et al. 2008, Friedlander et al. 2009). Cells recovered from the AP of such immature permanent teeth demonstrate a multilineage differentiation potential and high growth activity (Abe et al. 2007, Sonoyama et al. 2008, Chrepa et al. 2017). Huang in his study (Huang et al. 2008) on the AP described this tissue as a “hidden treasure.”

The histology of the apical region (Abe et al. 2007, Sonoyama et al. 2008, Friedlander et al. 2014) of immature permanent teeth includes the apical CRZ, the AP, periodontal ligament and dental follicle. This review will be limited to discussion of the pulp and AP.

1.2.6.1 The apical cell-rich zone

The apical CRZ is a cap-shaped richly cellular band extending across the open root apex, separating the mature radicular pulp from the immature AP tissue (Figure 1.2). It contains numerous small blood vessels, proliferating cells and fibroblasts although its precise role in angiogenesis and healing has not been fully elaborated.

1.2.6.2 The apical papilla

The AP is a soft gelatinous tissue loosely adherent to the apex of the immature tooth root, beneath the apical cell rich zone. When viewed under a light microscope, it contains plump immature cells and fibroblasts in a loose largely avascular connective tissue stroma (Sonoyama et al. 2008, Friedlander et al. 2014).

Recent research has shown that the cells from the AP have a much greater proliferative rate and population doubling capacity when compared to DPSC (Bakopoulou et al. 2011). In addition, in vitro and clinical studies have shown that the cells of the AP have the potential to survive trauma or infection and may contribute to healing (Sonoyama et al. 2008, Xu et al. 2009, Friedlander et al. 2014).
1.3 Vascular supply of the dental pulp and apical papilla

The dental pulp in immature teeth is highly vascular with arterioles and venules running centrally and peripherally, respectively. Immature teeth have wider or open apices and shorter roots and are in closer proximity to the blood supply.

Kramer used extracted human permanent teeth and injected Indian ink into the vessels to reveal the detailed architecture of the pulpal microvasculature. Blood vessels travel via the apical foramen or foramina along with nerve bundles and enter the tooth via arterioles, which travel up into the central portion of the root pulp and branch off and spread towards the odontoblast layer, under which they ramify to form the capillary plexus (Kramer 1968). As these arterioles enter the coronal pulp, they spread out laterally towards the dentine, and give rise to a capillary network in the subodontoblastic plexus (Provenza 1958, Kramer 1960). Drainage occurs via post-capillary venules. While these studies have provided us with knowledge of the vascular framework of the dental pulp, further understanding around the response of the vasculature to injury including angiogenesis is required.
1.4 Angiogenesis

Angiogenesis is an intricate, multistep process (Folkman & Klagsbrun 1987, Folkman & Shing 1992) resulting in the growth of new blood vessels from pre-existing vasculature (Friesel & Maciag 1995, Polverini 1995, Kaufmann et al. 2004). This process is initiated by quiescent endothelial cells which become “activated.” (Risau 1997). Physiological angiogenesis occurs in adult tissues during wound healing (Tonnesen et al. 2000), formation of the corpus luteum and endometrial growth (Seval et al. 2008, Schiessl et al. 2009), while pathological angiogenesis is a hallmark of conditions ranging from uncontrolled inflammation to atherosclerosis to neoplasia (Ahmad et al. 2000, Carmeliet 2005, Fiedler & Augustin 2006, Demasi et al. 2012).

Angiogenesis involves extracellular matrix secretion and reorganisation, proteolytic enzyme secretion, vascular membrane breakdown, endothelial cell mitosis and migration to form a capillary sprout, folding of cells to form a vessel lumen, capillary loop formation and subsequent anastomosis (Folkman & Shing 1992, Dvorak 2005, Carmeliet & Jain 2011). This process is initiated and regulated by a close interplay of numerous cytokines and growth factors (Figure 1.3). These angiogenic growth factors may have stimulatory or inhibitory roles, and often a “switch” or an imbalance in the activity of the pro- and anti-angiogenic factors determine a transition to unchecked angiogenesis and consequent pathology.

Figure 1.3 – The angiogenic process. Reproduced from Griffioen and Molema (2000) Pharmacological Reviews 52, 237-68.
Angiogenic capillary sprouting leads to an increase in the number and density of vessels, which serve to maintain vascular homeostasis, by removing waste metabolites, participating in immune defence, bringing in oxygen, nutrients, growth factors, and improving perfusion in hypoxic tissues. The dental pulp is a richly vascular tissue and has an average capillary density higher than seen in other tissues (Vongsavan & Matthews 1992), but lacks a collateral circulation. This vascularization has been demonstrated to be under local influences through pro- and anti-angiogenic factors and neuropeptides released from the local microenvironment (About 2011, About 2013). Under hypoxic conditions, as may occur with a pulpal injury due to caries or inflammation, these cells can affect the local vascular system directly through neo-angiogenesis, or indirectly through trophic actions on endothelial cells via angiogenic factors (El Karim et al. 2009). The dentine matrix (Roberts-Clark & Smith 2000), and other pulpal cells such as fibroblasts, endothelial cells and nerve cells all play a role in the vascularization process, each through a coordinated release of pro- and anti-angiogenic growth factors (Wakisaka 1990, Aranha et al. 2010, Dissanayaka et al. 2012).

Previous research has provided us with a greater understanding of the spatial arrangement of cells, structures and blood vessels associated with the pulp of healthy, immature permanent teeth (Friedlander et al. 2014), and a pilot study has further identified angiogenic growth factors in the apical papilla that may aid in healing (unpublished data). A small number of angiogenic genes, namely the angiopoietins have been identified in healthy pulp tissue but the angiogenic potential and cell signalling within the apical papilla merits further investigation.

1.4.1 Angiogenic growth factors and receptors

Growth factors are diffusible chemical peptide signalling molecules that stimulate growth, differentiation, survival and repair of cells (Kim et al. 1993, Sloan et al. 1999, Ferrara 1999, Smith 2003, Tran-Hung et al. 2008). They may act either directly to regulate cell function, or indirectly to regulate the expression of other growth factors by different cell types. Several growth factors have been identified play a role in the healing and repair of dental tissues (Robert-Clarks & Smith 2000, Tziafas et al. 2000, Smith & Lesot 2001, Shimabukuro et al. 2005, Mattuella et al. 2007, Nakajima et al. 2014, Angel-Mosqueda et al. 2015). VEGF is the primary regulator of angiogenesis but other growth factors have a recognised role in facilitating this process. The angiopoietin family of growth factors have a recognised role in physiological and pathological angiogenesis particularly in the later stages around vascular stabilisation (Staton et al. 2010).
Pulpal injury may be associated with the release of an array of growth factors from the dentine matrix, along with a more local release from supportive dental pulp cells (About 2013). Pulpal fibroblasts and endothelial cells are able to secrete angiogenic factors in response to pulpal injury, and play an important role in pulpal healing (Tran-Hung et al. 2006, El Karim et al. 2009). However, scant knowledge exists on whether these growth factors are differently distributed in the crown and root portions of immature permanent teeth, or if the angiogenic activity in the AP is related to expression of markers of immunity.

1.4.1.1 Vascular endothelial growth factor
The VEGF family is comprised of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, platelet-derived growth factor (PDGF) and placental growth factor (PlGF). VEGF-A (VEGF) is a homodimeric 34-42 kDa glycoprotein. It is a key pro-angiogenic growth factor during growth and development, healing and disease (Ferrara & Henzel 1989, Leung et al. 1989, Matsushita et al. 2000, Artese et al. 2002, Mattuella et al. 2007a). The following review will be limited to VEGF-A/VEGF only.

VEGF-A
Amongst the pro-angiogenic growth factors, VEGF has been regarded as the most important factor (Senger et al. 1983, Ferrara 2003, Mattuella et al. 2007a, Virtej et al. 2013). It is the key regulator of vascular formation, as it is a prerequisite in the initiation of proliferation, permeability, invasion, migration, activation and survival of immature vessels (Leung et al. 1989, Ferrara et al. 1996, 2003, Dvorak et al. 1999, Byrne et al. 2005, Mattuella 2007a). Alternative mRNA splicing has yielded five different isoforms of VEGF (Tammela et al. 2005), but VEGF165 predominates in dental tissues, and is potent in stimulating angiogenesis (Park et al. 1993, Keyt et al. 1996, Mullane et al. 2008).

VEGF is produced by various cells, including endothelial cells (Eichmann & Simons 2012), vascular smooth muscle cells (Ishida et al. 2001), and pericytes (Ribatti et al. 2011) contributing to its functional roles in development, and normal homeostasis postnatally, during wound healing, and in disease states including inflammation and neoplasia (Ferrara 1999, Artese et al. 2002, Hoeben et al. 2004, Byrne et al. 2005, Carmeliet 2005). The importance of VEGF comes from experiments in gene-deficient mice where lack of a single VEGF gene allele leads to impaired angiogenesis due to lack of endothelial cells (Carmeliet 1996, Ferrara 1996). Additionally, tooth slice/scaffolds seeded with stem cells from deciduous teeth and subsequently implanted into immunodeficient mice have been shown to differentiate into odontoblasts and endothelial cells, with the addition of VEGF (Sakai et al. 2010, 2011).
VEGF is expressed in odontoblasts, dentine matrix and in dental pulp cells (Booth et al. 1998, Artese et al. 2002, Roberts-Clark & Smith 2000, Botero et al. 2006, Scheven et al. 2009). It acts in an autocrine fashion to enhance chemotaxis, cell proliferation, and/or differentiation (Mattuella et al. 2007a). The dentine matrix contains a reservoir of angiogenic growth factors, particularly VEGF, and these are released in response to injury (e.g. a carious lesion), to contribute to the healing response in the pulp-dentine complex (Roberts-Clark & Smith 2000, Zhang et al. 2011). During bacterial invasion the release of lipoteichoic acid (LTA) and lipopolysaccharide (LPS) from gram positive and negative bacteria results in the upregulation of VEGF and inflammatory cytokines in the pulp (Ben-Av et al. 1995, Botero et al. 2003, 2006, 2010, Telles et al. 2003, Chu et al. 2004, Yang et al. 2004, Li et al. 2005, Konishi et al. 2005, Soden et al. 2009). As well as responding to the release of endotoxins, VEGF expression and angiogenesis are induced in the presence of tissue hypoxia as occurs following a traumatic or a carious pulp injury (Shweiki et al. 1992, Detmar et al. 1997, Semenza et al. 2000, Michiels et al. 2004). Activation of genes encoding pro-angiogenic growth factors such as VEGF occurs via a hetero-dimeric protein called hypoxia-inducible transcription factor-1 (Hif-1) (Forsythe et al. 1996, Carmeliet et al. 1998). Aranha et al. (2010) conducted an in vitro immunohistochemistry (IHC) study to investigate the responses of human dental pulp cells and pulpal fibroblasts in a hypoxic environment. The results showed a greater expression of VEGF, and endothelial cell proliferation in human dental pulp cells, demonstrating the importance of these cells in pulp healing.

### 1.4.1.2 Vascular endothelial growth factor receptors

There are several tyrosine kinase receptors for VEGF but VEGFR-1 and VEGFR-2 are most associated with the activities of VEGF. VEGFR-1 is a member of the VEGFR family, and binds VEGF but weakly (de Vries et al. 1992, Park et al. 1994, Maru et al. 1998, Olofsson et al. 1998) while VEGFR-2 is the main receptor for VEGF (Gille et al. 2001, Ferrara et al. 2003) and has a high affinity for VEGF with a kDa of approximately 75–125 pM (Terman et al. 1992).

#### 1.4.1.2.1 Vascular endothelial growth factor receptor 2

VEGFR-2 is encoded by the KDR gene. VEGFR-2/KDR is the primary pro-angiogenic VEGF receptor and appears to mediate the major growth and permeability actions of VEGF. It is now regarded as the major mediator of the angiogenic, mitogenic, and permeability-enhancing effects of VEGF (Waltenberger et al. 1994, Keyt et al. 1996, Ferrara et al. 2003). When bound to VEGF, VEGFR-2 undergoes dimerization and ligand-dependent tyrosine-phosphorylation in intact cells and results in an amplified proangiogenic signal. This cascade
of events induces cell proliferation, chemotaxis and/or endothelial cell differentiation (Gille et al. 2001, Mattuella et al. 2007b). VEGFR-2 is primarily associated with endothelial cell function and vascular homeostasis and is mainly expressed on the surface of endothelial cells and endothelial progenitor cells. VEGFR-2 is also present on human dental pulp cells (Matsushita et al. 2000), DPSC, odontoblasts (Miwa et al. 2008), vascular smooth muscle cells, and tumour cells (Price et al. 2001). Bletsa et al. (2012) demonstrated VEGFR2 expression on inflammatory cells and fibroblasts in pulps with apical periodontitis, an inflammatory disease of the periradicular tissues. Hypoxia is also shown to upregulate the expression of VEGFR2 and VEGFR1 (Brogi et al. 1996, Gerber et al. 1997).

Little information exists on the role of VEGFR-2 in reparative pulpal healing and tooth development. Both primary (Mattuella et al. 2007b) and immature permanent teeth (Guan & Wang 2012) express VEGFR-2. The intensity of staining in primary teeth was noted to be in the vicinity of the subodontoblastic layer, a site where angiogenesis may be more important. In comparison, the staining in the permanent teeth was noted to be more uniform within the pulp. In the immunohistochemical study on deciduous human molars by Miwa et al. (2008), both VEGF and VEGFR-2 were increasingly expressed from the cap (especially intense in the inner enamel epithelium) stage to the late bell stage. Their expression and role in root development is however, unclear.

1.4.1.2.2 Vascular endothelial growth factor receptor 1

VEGFR-1 is encoded by the FLT-1 gene. VEGFR-1/FLT-1 appears to play a negative role in angiogenesis, by serving as a decoy receptor for VEGF, or by suppressing signalling mechanisms through VEGFR2 (Maru et al. 1998, Ferrara et al. 2003).

1.4.1.3 Angiopoietins

Angiopoietins are a family of angiogenic growth factors that play important roles in vascular development (Thurston et al. 2003, Fagiani & Christofori 2013), but their role within the pulp has not been well described. ANG are secreted glycoproteins with a dimeric molecular weight of approximately 75 kDa. There are four members of the ANG family, Ang-1, Ang-2, Ang-3 and Ang-4 (Davis et al. 1996, Maisonpierre et al. 1997, Gale & Yancopoulos 1999, Kim et al. 1999, Valenzuala et al. 1999).

All of the known ANG have a predilection for vascular endothelium and bind primarily to the receptor Tie-2 (Dumont et al. 1992, Partanen et al. 1992, Sato et al. 1993), and it is not wholly clear whether there are independent ligands for the second Tie receptor, Tie-1, or, whether the known ANG can in some way or under some circumstances also engage Tie-1.
ANG and VEGF play synergistic and coordinated roles in vascular growth and development (Holash et al. 1999, Thurston 2002, Lobov et al. 2002, Saharinen et al. 2010, Felcht et al. 2012). Unlike VEGFs, angiopoietins do not play a role in the initial stages of capillary sprouting and endothelial cell proliferation, rather they play an important role in the latter stages of regulation of vascular integrity and remodelling. Besides their role in angiogenesis, they have also been shown to be important modulators in inflammation (Thurston et al. 1999, Lemieux et al. 2005, Roviezzo et al. 2005, Witzenbichler et al. 2005, Fiedler et al. 2006). Tissue hypoxia, for instance secondary to an inflammatory stimulus is known to upregulate the expression of both Ang-1 (Park et al. 2003) and Ang-2 (Oh et al. 1999, Mandriota et al. 2000, Pichiule et al. 2004).

1.4.1.3.1 Angiopoietin-1

Ang-1 is predominantly constitutively expressed in perivascular cells such as endothelial cells, pericytes, smooth muscle cells, fibroblasts and some tumour cells (Stratmann et al. 1998, Nakajima et al. 2014).

Ang-1 is an angiogenic agonist and important for vessel remodelling and maturation of the initially immature vasculature (Suri et al. 1996, Sundberg et al. 2002). Following vessel maturation, Ang1 binds to and activates Tie2 in a paracrine agonist manner and contributes to the vascular stability and homeostasis of the mature vasculature via the PI3K-AKT cell-survival signalling pathway (Asahara et al. 1998, Yancopoulos et al. 2000, Brindle et al. 2006, Fiedler et al. 2006). Thus, Ang-1 plays a protective and anti-inflammatory effect on the endothelium and limits its ability to be activated by exogenous cytokines. Ang-1 regulates VEGF-induced blood vessel formation and vascular permeability with angiogenic inhibitory roles (Thurston et al. 1999, 2000).

Ang-1 has been researched in perivascular cells, but little is known on how it affects other cells. Nakajima et al. (2014) in their pioneer study on mice teeth discovered that Ang-1 and Tie2 were co-expressed by odontoblasts in both immature and mature teeth, and consequently in the dentine matrix and in predentine too, likely suggesting a role in dentinogenesis. Capillary endothelial cells identified the presence of Tie 2 in the pulp horn.

1.4.1.3.2 Angiopoietin-2

Ang-2 is mostly expressed by endothelial cells located at the leading edge of proliferating blood vessels, and stored in Weibel-Palade bodies (Maisonpierre et al. 1997, Fiedler et al. 2004). Ang-2 and Tie-2 have also been described in pericytes (Wakui et al. 2006, Cai et al. 2008).
Ang-2 may be homologous to Ang-1 (Thurston et al. 2003), and has high affinity for Tie-2 in an autocrine fashion, but it has the potential to either activate or antagonise Tie-2 under different circumstances and behave like a partial agonist (Maisonpierre et al. 1997, Korff et al. 2001, Yuan et al. 2009, Thomas et al. 2010, Felcht et al. 2012). Seminal work by Maisonpierre and co-workers (1997) proposed that Ang-2 antagonises the phosphorylation of its cognate receptor, mediated by Ang-1. The molecular basis for its varying actions have not been fully understood yet, but the cell type, degree of endothelial cell confluence, duration of stimulation, Ang-2 concentration, cytokine milieu, and presence of co-receptors such as Tie-1 may all play a role (Kim et al. 2000, Thurston 2003). Ang-2 is an autocrine dynamic regulator of the Ang-Tie2 axis (Scharpfenecker et al. 2005, Fiedler et al. 2006), and may be involved in providing a key signal in commencing angiogenic remodelling (Holash et al. 1999). This then allows the endothelium to revert to a more plastic state typical of developing immature vessels. In contrast to Ang-1, Ang-2 is pro-inflammatory and induces endothelial permeability (Roviezzo et al. 2005, Fiedler et al. 2006). Evidence is also pointing to the possibility the function of Ang-2 may be vascular bed and organ-specific, but the molecular mechanisms behind this have not been unravelled yet.

Ang-2 expression is influenced by various endotheliotropic cytokines, such as VEGF, bFGF, and by environmental factors such as hypoxia, for example, in sites of inflammation (Oh et al. 1999, Mandriota et al. 2000, Pichiule et al. 2004). In the presence of VEGF, Ang-2 induces endothelial cell migration, proliferation and an increase in capillary diameter. In the absence of VEGF, Ang-2 induces endothelial cell apoptosis and vascular regression (Maisonpierre et al. 1997).

Few studies have described the role of Ang-2 in the pulp. Most of the research work on Ang-2 has been carried out in the field of wound healing and placental development (Kampfer et al. 2001, Geva et al. 2002, Bitto et al. 2008). Research has shown that Ang-2 is also constitutively expressed in a wide range of tumour cells (Ahmad et al. 2001, Bach et al. 2007, Demasi et al. 2012), and it is thought that an overexpression of this growth factor by newly formed tumour vessels can lead to hypoxia, induction of VEGF expression and angiogenesis. It is unknown if similar events occur during pulp tissue hypoxia and healing.

1.4.1.4 Angiopoietin receptors

1.4.1.4.1 Tie-2 receptor

Tie-2 (Partanen et al. 1992, Dumont et al. 1992, Iwama et al. 1993) is a phospho-tyrosine kinase receptor that is mainly expressed in the vascular endothelium, and primarily functions
as a receptor for Ang-1 (Davis et al. 1996, Brindle et al. 2006, Fiedler et al. 2006). Larger blood vessels have been shown to express Tie2 in greater abundance compared with smaller-sized vessels (Schnürch & Risau 1993).

Ang-1 and Ang-2 both bind to the same site in the extracellular domain of Tie-2 with relatively high affinities (Fiedler et al. 2003). This binding of Ang-1 mediates receptor autophosphorylation and promotes endothelial cell migration and survival.

Recent evidence is pointing to the role the Ang-1/Tie-2 signalling system plays in the latter stages of blood vessel growth, as they form, remodel, organize and form complex reticular networks. El Karim et al. (2009) in their in vitro study concluded that neuropeptides such as vasoactive intestinal polypeptide (VIP), calcitonin gene related peptide, substance P and neuropeptide Y can result in the expression of certain angiogenic growth factors (including Ang-2) from pulp fibroblasts. It is known that Ang-2, in the presence of VEGF, plays a crucial role in the initiation and stabilisation of angiogenesis through competitive binding with the Tie2 receptor. Thus, pulpal cells secrete pro-and anti-angiogenic factors, and are responsive to them too (El Karim et al. 2009, Aranha et al. 2010, About 2011).

1.5 Inflammation of the dental pulp

The dental pulp is housed within rigid dental tissues i.e. enamel and dentine, that support and protect it. Once this hard tissue barrier is breached, the integrity of the pulp is jeopardized, as noxious insults from the external environment (e.g. caries/trauma) are able to gain access, and elicit inflammatory reactions within the dental pulp (Brännström & Lind 1965). Absence of collateral circulation means that minor increases in tissue pressure may result in compression and local venular collapse, however, there exists poor correlation between clinical and histological diagnoses especially in the diagnosis of immature irreversibly inflamed teeth (Tziafas et al. 2000, Ricucci et al. 2014, Goldberg 2014, Goldberg et al. 2015).

1.5.1 Healthy pulp

The healthy pulp exhibits no signs or symptoms and responds ‘normally’ to pulpal sensibility tests. The symptoms elicited from pulp tests are mild, with no lingering response. Clinically, such teeth exhibit no evidence of caries, or mechanical exposures.

Even in the absence of pulpal irritants, immune cells such as T lymphocytes (CD8+ and CD4+ cells), macrophages, dendritic and natural killer cells are residents of healthy human dental pulp. They play an important role in immunosurveillance (Gaudin et al. 2015). Additionally, regulatory T lymphocytes are also present wherein they interact with APCs such as dendritic
cells and odontoblasts to ‘fine-tune’ the innate and adaptive components of the immune response.

1.5.2 Reversible pulpitis

Irritants to the pulp result in inflammation (Seltzer et al. 1963). The intensity of inflammation depends upon the character and duration of the noxious insult (Levin 2003). Mild insults, such as early carious lesions, attrition or shallow cavity preparations may result in no or mild inflammatory changes in the pulp (Brännström 1961, Brännström & Lind 1965).

The initial pulpal reaction to a mild antigenic challenge is usually reversible. Low amounts of pro-inflammatory cytokines and growth factors and extracellular matrix components are sequestered from dentine as a result of dentine demineralization due to cariogenic bacteria (Roberts-Clark & Smith 2000, Smith & Lesot 2001, Smith 2003, Goldberg & Smith 2004, Smith et al. 2012). This may promote reactionary dentine formation with surviving odontoblasts and other pulp cells up-regulating their secretory activity to form tertiary dentine. This reparative layer serves as a physical barrier and is protective to the pulp.

At a cellular level, the non-specific innate immune system swings into action and is mediated by histamine, bradykinin and arachidonic acid metabolites (Torabinejad 1994). Neutrophils also play an important part in the process, along with protease inhibitors and neuropeptides. In conjunction with this non-specific inflammatory response, an immune response to the antigenic challenge occurs. If injury persists, a more specific adaptive immune system is brought into play, with immuno-competent cells such as macrophages, T and B lymphocytes expressed in inflamed pulps. MHC Class 2 molecules expressing APCs such as dendritic cells have also been noted (Jontell et al. 1998).

In the case of more severe injury, the odontoblasts and pulp cells in the vicinity of the injury die and may be replaced by a new cell population similar to the odontoblasts, and will lay down a reparative tertiary dentine matrix. This new generation of odontoblast-like cells may arise from different potential populations within the dental pulp, and may include the CRZ adjacent to the odontoblasts, fibroblasts and the progenitor/DPSC (Ricucci et al. 2014). While the ability of the pulp-dentine complex to respond to a variety of insults by tertiary dentine deposition has long been recognised, there is an increasing body of evidence to suggest this is reparative in nature (Ricucci et al. 2014, 2017).

Interleukins-1α (IL-1α), IL-6 and IL-37 inflammatory markers have been well characterised in the coronal pulp. IL-1α is a well-researched pro-inflammatory cytokine and has been
implicated in several inflammatory diseases, including pulpitis and apical periodontitis (Matsuo et al. 1994; Tan-Ishii et al. 1995; Bletsa et al. 2004). It is secreted by monocytes, macrophages, vascular cells and fibroblasts (Wisithphrom K et al. 2006). IL-6 is a multifunctional cytokine produced by various cell types and can act on different cells such as endothelial cells, epithelial cells, B cells and T cells (Motro et al. 1990). It mediates the host’s immuno-inflammatory response subsequent to tissue injury or infective processes (Barkhordar et al. 1999). It is present in the un-inflamed pulp in negligible amounts, and increases dramatically in event of pulpitis (Barkhordar et al. 1999; El Salhy et al. 2013; Sabir & Sumidarti 2017). In contrast, IL-37 is a recently discovered anti-inflammatory cytokine and has also been detected in several cell types such as dendritic cells, monocytes and epithelial cells (Tete et al. 2012). It has been shown to inhibit the expression and production of other pro-inflammatory cytokines such as IL-1α and IL-6; suggestive of its role in counteracting excessive inflammation in a negative-feedback mechanism (Nold et al. 2010; Dinarello & Bufler 2013). However, scarce information exists on the presence of these inflammatory markers in the AP, and further research in this field is warranted.

1.5.3 Irreversible pulpitis

In irreversibly inflamed pulps, there is often partial or complete necrosis of the coronal pulp and often chronic inflammatory cells, such as lymphocytes, macrophages, and plasma cells are present (Ricucci et al. 2014). Bacteria may be present in the pulp horn and a severe acute inflammatory reaction with neutrophils persists in the surrounding areas. However, the remainder of the radicular pulp may be less inflamed or healthy and amenable to vital pulp therapy.

From a histological standpoint, the presence of a necrotic focus with bacterial presence appears to symbolize the transition between a reversible and an irreversible state of the pulp (Ricucci & Siqueira 2013). However, the limitations of our current clinical diagnostic tests make accurate determination of the state of the pulp difficult (Seltzer et al. 1963, Mejäre et al. 2012, Ricucci et al. 2014).

Irreversible pulpal injury to an immature permanent tooth, whether due to trauma or caries, represents a clinical quandary, as it may result in an immature tooth with arrested root development and subsequent thin roots with wide open dentinal walls with a greater propensity for root fracture with repeat trauma (Frank 1966, Cvek 1992). Recent evidence suggests vital pulp therapy (VPT) as a feasible treatment modality in the management of teeth with irreversible pulpitis (Aguilar & Linsuwanont 2011), as the possibility exists to retain
some vital pulpal tissue and therefore permit continued root development. Some of the benefits of VPT are it is a conservative, non-invasive, less complex and financially viable method (McDougal et al. 2004, Camp 2008, Witherspoon 2008, Eghbal et al. 2009, Asgari et al. 2013).

The apical papilla and HERS may survive in an immature permanent tooth with irreversible pulptis, as vital tissue may exist beneath the inflamed coronal pulp. These tissues may then participate in the pulpal healing process (Andreasen et al. 1988, Huang 2008, Sonoyama et al. 2007).

1.5.4 Management of pulptis

Over the past few years, treatment for reversible and irreversible pulptis in immature permanent teeth has been increasingly directed at conservative therapies with preservation of remaining vital pulp and/or the apical papilla tissue (Bogen et al. 2008).

Vital pulp therapies including indirect pulp capping, direct pulp capping or partial pulpotomy are recommended to promote pulp healing in cases of reversible pulptis (Mejàre & Cvek 1993, Barthel et al. 2000, Al-Hiyasat et al. 2006, Bjørndal et al. 2010, Bjørndal et al. 2014), while complete pulpotomy and revitalisation procedures have been recommended (Holland et al. 2001, Shimizu et al. 2012) in cases of irreversible pulptis, but all are dependent on a blood supply and angiogenesis (Saghiri et al. 2015).

Traditionally, irreversible pulptis has been managed by apexification, but VPT is now recommended in the first instance (Bogen et al. 2008, Camp 2008, Aguilar & Linsuwanont 2011, Asgary et al. 2013). Complete pulp removal with loss of vitality leaves immature teeth with thin dentinal walls, and a tooth that is susceptible to fracture (Cvek 1992, Hargreaves et al. 2012). Moreover, with loss of odontoblasts, apexification treatment does not permit continued root development (Bose et al. 2009, Shabahang 2013). Recognising that the apical papilla tissue may survive infection and contribute to healing, procedures which stimulate blood clot inside the root canal as a matrix for cell growth have been shown to result in apexogenesis, although these are not always predictable (Iwaya et al. 2001, Banchs & Trope 2004, Thibodeau 2007, Jung et al. 2008).

1.6 Healing following pulp injury

Pulpal healing is evaluated clinically by the absence of signs and symptoms, hard-tissue bridge formation, continued root development, a positive response to sensibility testing, or evidence of pulpal mineralization/obliteration (Tziafa et al. 2014, Tziafa et al. 2015). The
outcome of clinical treatments is affected by the stage of tooth development, presence of inflammation and infection, and on the extent of damage to the pulpal microcirculation (Fong & Davis 2002, Bogen et al. 2008). While the size of the pulp exposure does not affect healing \textit{per se}, poorer outcomes have been observed for VPT treatments in complete pulp exposures greater than 5 mm and is reflective of the level of microbial contamination (Chailertvanitkul et al. 2014). True healing can only be evaluated histologically and the clinical presentation does not necessarily reflect the histology (Seltzer et al. 1963, Mejāre et al. 2012). Healing in the pulp-dentine complex may occur by repair and/or regeneration (Tziafas et al. 2000, Ricucci et al. 2014).

1.6.1 Reparative healing

Pulp healing by repair is most common in the pulp-dentine complex (Smith et al. 1995, Tziafas et al. 2000, Téclès et al. 2004, Ricucci et al. 2014, 2017). If tissue damage is severe or of a chronic nature and is associated with damage to both the parenchymal cells and stromal components, then reconstitution of tissue components by regeneration cannot take place and the original tissue is replaced with a fibrous scar tissue. Ricucci et al. (2014) in their histological study on cellular changes in the pulp associated with caries and restorations, demonstrated that the formation of reparative dentine could not be regarded as “regeneration” since the hard tissue so formed in the pulp lacked the characteristic features of true dentine. Most recently, similar histological observations have been made when assessing the healing of immature teeth with deep caries (Ricucci et al. 2017).

1.6.2 Regenerative healing

Regeneration is the ideal wound healing outcome and is the replacement of damaged tissue with new tissue that restores the architecture and the functionality to its original state (Bressan et al. 2012). In the past decade, an increasing number of researchers have attempted to regenerate the pulp-dentine complex (Bose et al. 2009, Hargreaves et al. 2012, About 2013, Goldberg et al. 2015). Regeneration is incumbent upon the presence of stem cells, growth factors and a matrix/scaffold (Nakashima & Akamine 2005).

The dental pulp is endowed with immense regenerative potential which can be harnessed in event of pulp injury whether by trauma, dental caries or by developmental anomalies. Tissue regeneration strategies encompass both cell-based and cell-free approaches. The most common approach entails a complex and carefully controlled interaction between stable cells
that have survived the injury, labile cells such as mesenchymal stem cells, morphogenic factors and cytokines, and lastly scaffolds to provide an optimal environment for tissue regeneration to occur (Vacanti & Langer 1999, Nakashima et al. 2009, Nakashima et al. 2011). A more detailed knowledge of the science behind tissue regeneration is necessary but further discussion on the topic is beyond the scope of this review.

1.7 Laboratory techniques for evaluating the dental pulp and apical papilla

1.7.1 Tissue samples

Maxillary incisors are the most frequently traumatized teeth but are not readily available for laboratory studies. Instead, third molar teeth provide a suitable research model for examining the pulp and the apical papilla of immature permanent teeth (Kullman et al. 1992, Gronthos et al. 2002, Mullane et al. 2008, Sonoyama et al. 2008, Tran-Hung et al. 2008). Access to these teeth is easy, and they may be removed with minimal surgical trauma (Ikeda et al. 2006). Previous studies conducted in our laboratory have found that an age-range of 17-23 years provides suitable numbers for examination of the AP in third molar teeth (Friedlander et al. 2014). Samples are fixed in 10% neutral buffered formalin for 24-48 hours to preserve the antigenicity and tissue architecture (Pollard et al. 1987, Arber et al. 1996, Prento & Lyon 1997). For teeth, a decalcification process is necessary to enable sections to be cut for histology or IHC (Walsh et al. 1992). Decalcification in 10% ethylene diaminetetraacetic acid (EDTA) is preferred for IHC as it is gentler on the tissue than stronger decalcifying agents such as formic or nitric acid (Brain 1966, Rosen 1981), however use of EDTA requires a lengthy decalcification period and frequent replenishing (Page et al. 1982, Christgau et al. 1998). Heat-mediated antigen retrieval (HMAR) is usually employed to break the protein cross-links of formalin-fixation and to expose antigen sites for binding (Shi et al. 1991). Heat is often preferred to protease-mediated antigen retrieval (Huang et al. 1976) as enzyme treatments are harsher on the tissues and can cleave a larger protein into smaller peptides yielding false positive results (Polak & Van Noorden 2003).

1.7.2 Immunohistochemistry

Immunohistochemistry may be defined as the use of a labelled or marked antibody as a specific agent for isolation or localisation of the amplified target tissue or cell antigens in-situ (Polak & Van Noorden 2003, Ramos-Vara 2005). Manual IHC has been extensively developed since the 1940s (Coons et al. 1941, Coons & Kaplan 1950) to provide valuable information on the localization of proteins in formalin-fixed, paraffin-embedded (FFPE)
tissues and its expression in health and various diseases (Taylor & Burns 1974). It is now regarded as a routine and an important technique for diagnosis and research where it is effective for detecting the expression and spatial arrangement of a protein of interest in the whole tissue. The advent of automated immunostainners has allowed greater flexibility, optimized use of reagents, improved efficiency and standardization (Herman et al. 1999, Bánkfalvi et al. 2004, Zeheb 2006, Prichard 2014, Prichard 2015). Antibodies can be equally and even more efficiently optimised with automated immunostainners because they also provide the option to modify the variables in the process according to the antibody to be investigated. However, the results of an automated immunostainer are still incumbent on the quality of tissue specimens provided manually and its handling, fixation, embedding and tissue preparation.

The antibody-antigen complex can only be visualised with the light microscope if it is labelled to the primary, secondary or tertiary antibodies of a detection system. The first label (transporter molecule) ever used was far too weak to be visually detectable. Subsequently, labels such as fluorescent compounds, enzymes, radioactive labels have been developed, with enzymes being most commonly used. Detection systems may be ‘direct’ or ‘indirect’ (Figure 1.4).

Direct detection methods are one-step processes with primary antibodies conjugated directly to the label (Coons & Kaplan 1950). Whilst direct IHC is efficient, it lacks sensitivity. Current IHC methods are indirect wherein the first antibody is unlabelled, but the second layer is labelled (Polak & Van Noorden 2003). This permits detection of smaller amounts of the

Figure 1.4 – Direct and indirect detection methods.
epitope, with greater sensitivity. The most popular indirect detection methods are avidin-biotin complex (ABC) methods and polymer labelling methods, however background staining can be a problem with ABC and this has largely been superseded by polymer-based systems.

Polymer-based systems have higher sensitivity and specificity, as they utilize a polymer backbone to allow multiple antibodies and antigens to be conjugated. Moreover, background staining is considerably reduced or eliminated due to endogenous avidin or biotin. However, one limitation of this system is a restricted choice of primary antibodies to select from. This has been addressed with EnVision™ (Sabattini et al. 1998, Vyberg & Nielsen 1998). EnVision+ is a relatively new indirect dextran polymer visualization system for IHC. Being a two-step method, the IHC assay time (and labour time) is decreased considerably compared to the three-step ABC method. The procedure is simplified too, thereby minimizing the possibility of technical errors. EnVision™ is however, more expensive. Mild staining of nuclei and other structures has also been reported following use of EnVision™ after heat induced enzyme retrieval in a tris/EDTA buffer.

IHC is widely used but it does have some limitations. Multiple variables may impact upon the sensitivity and specificity of antigen detection and may therefore yield false-positive or false-negative results. Some of these variables include tissue fixation, tissue processing, drying protocol, antigen retrieval methods, antibody concentration, staining and visualization techniques (Engel & Moore 2001). It is also less sensitive and specific when compared to polymerase chain reaction-based techniques.

### 1.7.3 Gene expression studies

Whilst IHC is beneficial in analysing the presence and spatial arrangement of particular markers in a whole tissue, it is unable to provide information on gene expression and the amount of protein present. This information can be obtained using quantitative polymerase chain reaction (qPCR) (Higuchi et al. 1992, Bustin 2000, Gachon et al. 2004, Nolan et al. 2006). qPCR is a rapid and accurate method for detection and quantification of mRNA levels. It is a highly sensitive, rapid, easily reproducible and powerful technique that permits gene expression analysis in several different types of tissue, and for a number of genes (Heid et al. 1996, Schmittgen et al. 2000, Wilhelm & Pingoud 2003, Kubista et al. 2006). Post-amplification manipulation is not required either (Wong & Medrano 2005).

Several approaches have been utilised in qPCR to detect the amplified PCR product. Fluorescent signals may be generated by fluorescent dyes specific for double-stranded DNA
(ds-DNA), or by fluorophore-labelled oligonucleotide probes that are sequence-specific (Wilhelm & Pingoud 2003) (Figure 1.5).

![Figure 1.5 – Real-time PCR detection methods – DNA binding dyes (A) and hydrolysis probes (B).](image)

- Quenched fluorophore; ☆ DNA polymerase; ☀ Light-emitting fluorophore; Q – Quencher fluorophore; R – Reporter fluorophore.

SYBR®-Green I is an asymmetric cyanine dye and is the most often used ds-DNA specific fluorescent dye in qPCR (Zipper et al. 2004). These dyes emit fluorescence when bound to the minor groove of ds-DNA, and the intensity of the signal increases in proportion to the concentration of ds-DNA accumulated during cycling. This detection chemistry is relatively simple, robust and cheaper when compared to the TaqMan® probes (Ponchel et al. 2003). However, it is less sensitive and specific than TaqMan® as amplified non-specific products such as primer-dimers may mask the signal from the specific products (unless the assay is optimised). False-positive results may result (Simpson et al. 2000). Hence TaqMan® detection chemistry was employed in our research project.

The TaqMan® detection assay, also called a 5’ nuclease assay (due to the 5’ nuclease activity associated with Taq DNA polymerase), employs sequence-specific probes which fluoresce upon probe hydrolysis to detect the amplified product (Holland et al. 1991). This probe is labelled with a reporter dye at the 5’ end, and a quencher dye at the 3’ end (Gibson et al. 1996) (Figure 1.6). During the PCR extension step, the bound and quenched probe are degraded by the DNA polymerase when annealed to the target sequence. This permits a separation of the reporter from the quencher dye moiety and an increased fluorescent signal (Heid et al. 1996).
Prior to comparing transcription profiles, it is important to understand that the starting material, reverse transcription efficiency, RNA extraction, presence of inhibitors in various samples may vary. To account for these variables, qPCR relies on normalization to a control gene, also called a housekeeping gene (HKG) (Karge et al. 1998, Dheda et al. 2004). This HKG should not only be constitutively expressed in the target tissue being studied, but should also remain stable in between samples and when used under different research conditions (Thellin et al. 1999). However, many of the HKG demonstrate marked variability in expression in different tissues (Suzuki et al. 2000, Glare et al. 2002) and so accurate analysis of gene expression warrants a very careful selection of the HKG to ensure normalisation. An additional limitation of this technique is that degraded or incorrectly quantified RNA would yield erroneous results. The accuracy of gene expression may also be affected by deoxyribonucleic acid (DNA) contamination, and DNase I is often employed to better mRNA extraction efficiency.
The ABI Prism software plots fluorescence against the cycle number and generates a plot that represents the accumulation of amplified product (amplicon) over the course of the reaction cycles in ‘real time.’ The software is also used to determine a definite baseline value, threshold value and quantification cycle (Cq) numbers. Baseline value is the fluorescence signal during the early PCR cycles, usually in the 3-15 cycle range, wherein there is little, if any change in the fluorescent signal. Threshold value refers to the fluorescence signal that is indicative of a statistically significant increase over the baseline value. The cycle number at which the signal crosses the threshold is referred to as the Cq value.

1.8 Significance of this research

Anterior teeth are the most frequently traumatized teeth (Lauridsen et al. 2012), with an observed prevalence of around 19% reported in a retrospective study on New Zealand children (Dearing 1984, Chan et al. 2011). Trauma to these teeth prior to the completion of root development is common and incurs significant financial costs and affects self-esteem and confidence (Lee & Divaris 2009). The cost of dental treatment and ongoing care is often significant and treatment may be protracted. The presence of the apical papilla offers the potential for healing but requires an immune response and vascular supply. Outcomes are variable so it is important to have a baseline understanding of health in order to predict the response of the dental pulp to caries or trauma.

This in vitro study aims to investigate the angiogenic activity in the AP from immature permanent teeth with healthy dental pulps. The whole apical tissue will be examined, recognizing the role of cells, blood vessels and the tissue stroma in contributing to healing and physiological development.

Angiogenic growth factors and receptors have been identified on several cell types in the pulp, and elsewhere in the body they play important roles in angiogenesis, vascular remodelling and wound healing. Findings from this study will enhance our understanding of the growth factors and receptors involved in angiogenesis in developing permanent teeth and adds to the body of knowledge to assist in development of more predictable therapies for immature permanent teeth.

1.9 Thesis aims

To investigate angiogenic gene expression, at the mRNA and protein level, in the apical papilla tissue of human immature permanent teeth associated with healthy dental pulps.
1.10 Thesis hypothesis

Angiogenic signalling exists within the apical papilla tissue of immature permanent teeth in healthy dental pulps, and that differences between the apical papilla and the mature pulp exist, at least in part, due to differences in their angiogenic potential.

1.11 Ethical approval and Māori consultation

Ethical approval has been granted by the University of Otago Human Ethics Committee (Health) (Ref H15/002), and Māori Consultation has been undertaken with the Ngāi Tahu Research Consultation Committee, as this research is considered important to Māori (Appendix A).
Chapter Two — Materials and Methods

2.1 Ethical approval

Ethical approval for use of tissue for this study was obtained from the University of Otago Human Ethics Committee (Health) – Approval number Ref. H15/002 (Appendix A).

2.2 Experiment One - Immunohistochemical examination of the apical papilla

2.2.1 Experimental Aim

To investigate the distribution of angiogenic factors VEGF, ANG1, ANG2, the receptor Tie2, and the cell-surface markers CD34 and CD45 in the AP of immature permanent teeth using IHC, and to analyse them qualitatively and semi-quantitatively.

2.2.2 Inclusion criteria

- Immature permanent teeth with healthy pulps that were being extracted as part of an overall treatment plan
- Healthy patients, of any ethnic group aged 17-23 years of age
- No underlying systemic disease
- Non-smokers
- Informed consent, both verbal and written

2.2.3 Exclusion criteria

- Smokers
- Underlying systemic disease
- Patients requiring antibiotic cover for dental treatment
- Pregnancy
- Mature tooth
- Necrotic pulp

2.2.4 Sample collection

Permanent third molar teeth with incomplete root development and an AP were selected for the study (Figure 2.1). Teeth and attached soft tissue were collected from twelve healthy participants (10 for use in study and 2 for antibody optimisation) who were being treated at the Faculty of Dentistry at the University of Otago, Dunedin, New Zealand, for routine care.
Third molars were being extracted for clinical or pathological reasons and the researchers were not involved with clinical treatment planning. Patients willing to participate in the study, and those who met the inclusion criteria were explained the nature of the research project, were requested to ask any questions pertaining to the project, following which both verbal and written informed consent were obtained.

Unerupted permanent immature third molars at a similar stage of root development were extracted with an attached AP by experienced oral surgery clinicians at the Department of Oral Surgery, Faculty of Dentistry at the University of Otago (Figure 2.1).

![Diagram of an immature permanent third molar depicting the location of the apical papilla. Reproduced from Volponi et al. 2010.](image)

**Figure 2.1 – Diagram of an immature permanent third molar depicting the location of the apical papilla. Reproduced from Volponi et al. 2010.**

### 2.2.5 Sample preparation and fixation

Immediately following extraction, the specimens were sectioned below the cemento-enamel junction (CEJ) and placed in a tissue fixative of 10% neutral buffered formalin for 48 hours prior to decalcification and tissue processing.

#### 2.2.5.1 Hard tissue decalcification

Specimens were placed in a 10% EDTA solution with agitation at 4°C for 8-11 weeks for complete hard tissue decalcification (Appendix B). This solution was replenished every three days (Sanjai et al. 2012). Following decalcification, specimens were trimmed to ensure the correct plane of section. These specimens were agitated in phosphate-buffered saline (PBS) for approximately 24 hours prior to tissue processing and embedding in paraffin.
2.2.6 Section preparation

Fourteen sections (4 µm thick) from each specimen block were cut using a microtome (Microm HM 340 E, Thermo Fisher Scientific Inc., Waltham, USA). One section from each specimen block was stained with H&E (Shandon Varistain 24-3, Diversified Equipment Company Inc., Lorton, USA) to verify the histological plane of section and the AP. Specimen sections were placed on positively-charged slides (Huang et al. 1983) (Histobond® slides, Paul Marienfeld GmbH & Co. KG, Lauda-Königshafen, Germany), and incubated in an upright position at a temperature of 60°C for 120 minutes followed by an overnight incubation at 50°C (Thurby et al. 2009). Sections were then stored at 4°C until needed for future use.

H&E stained sections were viewed under a light microscope at varying magnifications up to 1000x (Leica DM Digital Microscope, CTR 5000, Leica Microsystems, Wetzlar, Germany). All specimens were histologically examined and diagnosed by two oral pathologists at the Oral Pathology Centre.

2.2.7 Primary antibody optimisation and immunohistochemistry protocol

An IHC working method was optimized manually for the primary antibodies anti-VEGF, anti-ANG1, anti-ANG2, anti-Tie2, anti-CD34, and anti-CD45 using a positive control (Section 2.2.7.3) and a decalcified sample. In order to accomplish this, varying antibody concentrations, antigen-retrieval methods, blocking agents and wash buffers were employed for each primary antibody. The specificity of the selected antibodies is listed in Table 2.1.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Antigen specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-VEGF</td>
<td>Vascular endothelial growth factor, key regulator of angiogenesis</td>
</tr>
<tr>
<td>anti-ANG1</td>
<td>Angiogenic agonist- important for vessel remodelling and maturation</td>
</tr>
<tr>
<td>anti-ANG2</td>
<td>Pro-inflammatory, negative regulator of angiogenesis</td>
</tr>
<tr>
<td>anti-Tie2</td>
<td>Primary receptor for ANG1</td>
</tr>
<tr>
<td>anti-CD34</td>
<td>Hematopoietic/ endothelial cells</td>
</tr>
<tr>
<td>anti-CD45</td>
<td>Leucocyte common antigen (LCA), leucocytes and all hematopoietic cells except mature erythroid cells</td>
</tr>
</tbody>
</table>
2.2.7.1 Antigen retrieval

Two antigen retrieval conditions were compared at each concentration to establish a reproducible working method for the primary antibodies under investigation.

1. No antigen retrieval.

2. Heat-mediated antigen retrieval was performed on the sections by placing them in a pre-heated sodium citrate buffer (pH 6) in a water bath (Shi et al. 1991, Morgan et al. 1994). Initially, a temperature of 80°C was tested for 10 and 20-minutes immersion periods.

2.2.7.2 Primary antibody concentration

Optimal antibody concentrations for the primary antibodies under investigation were sourced from previous studies carried out using the same antibody within the Immunopathology Research group—Sir John Walsh Research Institute (SJWRI), University of Otago, and from the antibody data sheets from the respective manufacturers. A doubling technique was used to test different concentrations in order to identify the concentration that would yield optimal staining with minimal non-specific background staining.

2.2.7.3 Positive and negative control specimens

Positive tissue controls were used to check the specificity of the antigen-antibody binding. Archival pyogenic granuloma, human tonsil and human appendix specimens known to contain the particular antigen for the antibodies were run in parallel with the primary antibodies. Non-specific antibodies replaced the primary antibody and acted as the negative controls (Hewitt et al. 2014). Non-specific mouse IgG1 antibody (X0931, Agilent technologies, Dako Denmark) was used when testing anti-VEGF, anti-CD34, and anti-CD45. Non-specific rabbit immunoglobulin fraction (solid-phase adsorbed, X0936, Agilent technologies, Dako Denmark) was used as a negative control when testing anti-ANG1, anti-ANG2, and anti-Tie2. Their concentrations were matched to the test antibody concentrations. This is tabulated below in Table 2.2.
Table 2.2 - Primary antibody concentrations and positive control tissue employed in antibody optimisation

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Catalogue number</th>
<th>Isotype</th>
<th>Clonality</th>
<th>Antibody concentration (µg/ml)</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-VEGF</td>
<td>sc7269</td>
<td>IgG</td>
<td>Monoclonal mouse C-1</td>
<td>6 1.2</td>
<td>Pyogenic granuloma</td>
</tr>
<tr>
<td>anti-ANG1</td>
<td>ab102015</td>
<td>IgG</td>
<td>Polyclonal rabbit</td>
<td>2 1</td>
<td>Pyogenic granuloma</td>
</tr>
<tr>
<td>anti-ANG2</td>
<td>ab153934</td>
<td>IgG</td>
<td>Polyclonal rabbit</td>
<td>5 2.5</td>
<td>Pyogenic granuloma</td>
</tr>
<tr>
<td>anti-Tie2</td>
<td>sc324</td>
<td>IgG</td>
<td>Polyclonal rabbit</td>
<td>4 2 1.34</td>
<td>Pyogenic granuloma</td>
</tr>
<tr>
<td>anti-CD34&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Ventana-790-2927</td>
<td>IgG</td>
<td>Monoclonal mouse</td>
<td></td>
<td>Ventana Autostainer</td>
</tr>
<tr>
<td>anti-CD45&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Ventana-760-4279</td>
<td>IgG</td>
<td>Monoclonal mouse</td>
<td></td>
<td>Human appendix</td>
</tr>
</tbody>
</table>

Antibody manufacturers – ab- Abcam, sc-Santa Cruz Biotechnology

<sup>1</sup> Automated IHC technique was employed for both CD34 and CD45
2.2.7.4 Manual immunohistochemistry

Immunohistochemistry for Tie2 was conducted manually, as this antibody had not been previously investigated in our laboratory. After labelling all specimen and control slides, tissue sections were deparaffinised in xylene and rehydrated through descending grades of ethanol. These sections were then rinsed in tap water, followed by distilled water. A wash in PBS (pH 7.4) for 2-3 minutes was followed by a rinse in distilled water. Heat-mediated antigen retrieval was employed for each of the primary antibodies under investigation. Sections were immersed in a tri-sodium citrate buffer (pH 6) at 80°C for 10 minutes (Shi et al. 2001) followed by a PBS wash and then allowed to cool down in tap water for 90 seconds. Endogenous peroxidase activity was quenched by an immersion of 3% hydrogen peroxide in methanol for 10 minutes. A 5-minute rinse with PBS followed. These were subsequently incubated with the optimized dilutions of primary antibodies at 4°C overnight in a humidified chamber. Non-specific rabbit immunoglobulin fraction (solid-phase adsorbed, X0936, Agilent technologies, Dako Denmark) was used as a negative control replacing anti-Tie2. Following incubation, all sections were washed with two 5-minute washes of PBS. The first wash contained 1% skim milk (dissolved in PBS) to reduce background staining. The slides were then dried with blotting paper to remove any excess PBS prior to incubation with 10 µl of the secondary reagent EnVision™ HRP System anti-mouse (K4002, Dako, Glostrup, Denmark), or anti-rabbit (K4003, Dako, Glostrup, Denmark) for 30 minutes at room temperature in the humidified chamber (Heras et al. 1995). Following a 2-minute wash with PBS, tissue sections were incubated with the chromogen 3,3’-diamino-benzidine (DAB; Sigma Aldrich, D3939, St. Louis, MO, USA) for 3 minutes. A rinse with PBS followed, and a subsequent wash with distilled water for 2 minutes. Counterstaining was carried out with Gill’s haematoxylin for 30 seconds, followed by running tap water (until clear), Scott’s tap water (10 dips), and dehydrated in ascending grades of alcohol, and lastly xylene for 5 minutes each. DPX (Sigma-Aldrich, 44581, St. Louis, USA) was used as a mountant for the slides.

2.2.7.5 Automated immunohistochemistry

Immunohistochemistry for VEGF, ANG-1, ANG-2, CD-34 and CD-45 was conducted on a Ventana Benchmark XT™ Autostainer (Ventana Medical Systems, Inc., Roche Diagnostics, Indianapolis, USA). The Ventana Autostainer was employed to carry out deparaffinisation, primary antibody incubation, antigen-retrieval, secondary antibody incubation. The DAB incubation was done using an ultraView Universal DAB Detection Kit (Ventana Medical Systems, Inc., Tuscon, USA). Following this, secondary antibody titration, counterstaining, bluing, dehydration and mounting were performed manually. Negative tissue controls were
treated with non-specific mouse IgG1 (X0931, Agilent Technologies Dako Denmark) in place of anti-VEGF, anti-CD34 and anti-CD45. Non-specific rabbit immunoglobulin fraction (solid-phase adsorbed, X0936, Agilent technologies, Dako Denmark) was used as a negative control when replacing anti-ANG1 and anti-ANG2.

2.2.8 Immunohistochemistry qualitative and quantitative analysis

The sections were examined using a light microscope (Leica CTR5000, Leica Microsystems, Wetzlar, Germany) at 10x, 20x and 40x and 100x objective magnification and photomicrographs of the representative fields in the correct orientation were captured using a digital camera (Leica DM5000B; Leica Microsystems, Wetzlar, Germany). The presence and site of staining, antibody specificity for protein along with non-specific background staining were evaluated for all tissue sections. Positive immunostaining was confirmed if a distinct copper-brown coloured end-product was seen at the site of the antigenic target(s). The distributions of each of the proteins’ expression were qualitatively described. An attempt to grade the intensity of staining was not made. Morphological structures with a lumen containing red blood cells, and exhibiting immunopositivity to the anti-CD34 antibody were considered to be blood vessels.

The immunoreactivity of VEGF, ANG-1, ANG-2 and Tie2 in the apical papillae were examined quantitatively. Due to the small size of the specimen, and the sparse nature of the angiogenic protein expression, it was decided to image the whole AP specimen. Cell counting was performed using the image-analysis software “ImageJ” v1.50 (National Institutes of Health, Bethesda, MD, U.S.A.). Images of the whole AP specimen were taken at high-power views (400x magnification) for final cell counting. Immunopositive cells were counted with a digitally-superimposed square graticule grid board using the “Grid” plug-in. Next, the “Cell-counter” plug-in was used. The positively stained cells were counted by pointing and clicking in the image window. Final cell counts were expressed as an absolute number of positively stained cells per image area.
2.3 Experiment Two - Gene expression

2.3.1 Experimental aim
To compare the mRNA expression of (1) the angiogenic growth factors VEGF, ANG1, ANG2, their receptors KDR and Tie2; (2) inflammatory marker genes IL-37, IL1A, IL6; and (3) cell-surface marker genes CD34, PTPRC, CD44 and CD83 in mature coronal pulp tissue compared with the AP using qPCR.

2.3.2 Sample collection
Study specimens were collected from healthy patients who were being treated as part of their routine dental care at the Faculty of Dentistry at the University of Otago, Dunedin, New Zealand.

2.3.2.1 Inclusion criteria
- Immature permanent teeth with healthy pulps which were being extracted as part of an overall treatment plan
- Healthy patients, of any ethnic group aged 17-23 years of age
- No underlying systemic disease
- Non-smokers
- Informed consent, both verbal and written

2.3.2.2 Exclusion criteria
- Smokers
- Underlying systemic disease
- Patients requiring antibiotic cover for dental treatment
- Pregnancy
- Mature tooth
- Necrotic pulp

Fourteen participants with permanent immature third molars at a similar stage of root development were recruited for the study. These participants were different to the IHC participants. Only one tooth from each participant was used. Samples from eight participants were required for the experiments but additional samples were collected to ensure the use of high quality RNA. Following extraction by an experienced oral surgery clinician or an oral and maxillofacial surgeon, teeth were immediately immersed in a RNase-retarding solution of RNeasy® (R0901, Sigma Aldrich, Merck, Germany) in a nuclease-free tube (Lader et al.)
Fourteen teeth were sectioned transversely in a dental operatory with a high-speed diamond bur at a level 2-3mm below the CEJ; the coronal pulp excavated and soaked in a 1.5 ml microcentrifuge tube, and the excavated AP was placed in a separate 1.5 ml microcentrifuge tube, and stored at -20°C until required (Conde et al. 2012). Tubes were RNase-free or treated with RNase AWAY™ (10328011, ThermoFisher Scientific Inc.) to eliminate any RNase or DNase contamination.

### 2.3.3 RNA extraction

The coronal pulp and apical papilla tissues from eight participants stored in RNAlater® were removed from the -20°C freezer and allowed to come to room temperature.

A PureLink® RNA Mini Kit (Ambion®, Life Technologies) which employs a phenol-chloroform extraction combined with a spin-column was used to isolate total RNA (Chomczynski & Sacchi 1987). The protocol used includes a phase separation, binding to the spin-column, a wash and an on–column PureLink® DNase treatment.

The coronal pulp and the apical papillae samples were incubated with 500 µl of TRIzol® reagent (guanidinium isothiocyanate) in a laminar flow-hood at room temperature for approximately five minutes. A pair of sterile suture scissors were used to cut the tissue into smaller pieces to facilitate better penetration of TRIzol®, and a plastic mortar-pestle (soaked in RNase AWAY™) was used to aid complete dissolution of the tissues (Lader et al. 2003, Miyamoto et al. 2009). Chloroform (100 µl) was added and this was followed by vigorous agitation by hand horizontally for 15 seconds, and left to incubate for two minutes. Following centrifugation at 12,000 g for 15 minutes at 4°C, approximately 300 µl of the clear upper-phase containing the RNA was transferred into a fresh RNase-free tube. An equal amount of 70% RNase-free ethanol was added and vortexed before being applied to the spin-column.

Following centrifugation at 12,000 g for 60 seconds at room temperature, a wash buffer I (350 µl) was added and centrifuged again at 12,000 g for 60 seconds at room temperature. The flow-through was discarded and the cartridge inserted into a new collection tube. With the RNA bound to the spin cartridge, the genomic DNA was removed using an on-column PureLink® DNase treatment protocol. A mastermix was prepared with the DNase Reaction Buffer (8 µl), DNase (10 µl) and RNase free water (62 µl). The mastermix (10 µl) was applied to the spin column and left to incubate for 15 minutes at room temperature. Following further washes with wash buffer I and II the spin column was placed into a new collection tube and centrifuged at 12,000 g for 60 seconds to dry the membrane. The spin column was then transferred to a fresh RNase-free recovery tube. The membrane-bound RNA was eluted.
with RNase-Free water (50 µl) following a 1-minute incubation at room temperature and centrifuged at 12,000 g for two minutes at room temperature. The purified total RNA was stored at -20°C. The quantity and quality of the purified total RNA was spectrophotometrically assessed using the NanoVue Plus™ (GE Healthcare, Little Chalfont, UK). An absorbance ratio (A$_{260}$:A$_{280}$) of 1.8-2.0 was considered to represent good quality total RNA for qPCR analysis.

### 2.3.4 cDNA synthesis

cDNA synthesis was achieved using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA), from the purified total RNA.

All the components of the kit, and the samples were allowed to thaw on ice prior to use. A 2X Reverse transcription master mix was prepared containing RT buffer (10x, 2 µl), dNTP Mix (25x 0.8 µl), RT Random Primers (10x, 2 µl), MultiScribe™ Reverse Transcriptase (1.0 µl), and Nuclease-free H$_2$O (4.2 µl). In a 100 µl PCR reaction tube, the 2X mastermix (10 µl) was combined with the purified RNA (300ng/10 µl) and briefly centrifuged. The cDNA synthesis was carried out in a thermal cycler (PTC-100® Programmable Thermal Controller; MJ Research, Waltham, USA) using the following temperatures: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for five minutes, cooled to 4°C and removed to -18°C until required.

### 2.3.5 Quantitative polymerase chain reaction

The coronal pulp and AP mRNA expression level of 12 genes was determined. The genes of interest included five genes which have been identified as playing a role in angiogenesis, four cell-surface markers, three inflammatory markers and three HKGs. Custom 96-well TaqMan® FAST qPCR assay arrays (Life Technologies) were used with amplification and subsequent detection performed using the QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems, Foster City, USA).

Briefly, the TaqMan® Fast mastermix and synthesized cDNA were brought to room temperature and centrifuged briefly. The cDNA was diluted to 160 ng/90 µl using DNase- and RNase-free water to which the TaqMan® Fast mastermix (2x, 90 µl) was added and mixed well. The reaction mix (10 µl) was dispensed into each of the 96 wells on each plate. Six cDNA samples (three control/coronal pulp and three test/apical papilla samples) were run on each plate with a total of three plates run. The plate was sealed with a MicroAmp® Optical adhesive film, and centrifuged at 1000 rpm for 60 seconds.
Amplification was achieved using a QuantStudio 6 system (Life Technologies, Foster City, USA). Thermal cycling parameters used were according to manufacturer’s instructions: polymerase activation (hot start) at 95°C for 20s, 40 cycles at 95°C for 3s and annealing and extension at 60°C for 30s. The threshold value was set at 0.05 and the background cycles were set at 3-10 cycles. Following amplification and real-time data acquisition, the threshold and Cq value were adjusted manually so as to obtain an intersection of the amplification curves in the linear portion of the log plot.

2.3.6 Data analysis using comparative quantitative polymerase chain reaction method

Cq values were determined using the QuantStudio 6 Sequence Detector Software v 2.0.6 (Life Technologies, CA, USA). Data analysis was performed using GraphPad Prism software V7 (GraphPad Software Inc., San Diego, USA).

NormFinder software (Department of Molecular Medicine, Aarhus University Hospital, Denmark) was used to determine the suitability of housekeeping genes, B2M, HPRT1 or RPLP0, for gene normalisation (Andersen et al. 2004, Nicot et al. 2005, Bustin et al. 2010).

Cq and the original gene expression level are inversely proportional to each other. As the product is doubled with each subsequent cycle, the original gene expression level is expressed as:

\[ L = 2^{-Cq} \]

The \( \Delta \Delta Cq \) method of comparative quantification was used to normalize the expression level of a GOI (Livak & Schmittgen 2001).

\[
Cq_{GOI} - Cq_{Hkg} = \Delta Cq_{sample}
\]

\[
Cq_{GOI} - Cq_{Hkg} = \Delta Cq_{calibrator}
\]

\[
\Delta Cq_{sample} - \Delta Cq_{calibrator} = \Delta \Delta Cq
\]

The fold-difference was then calculated between the coronal pulp and apical papilla samples.

\[
\text{Fold-difference} = 2^{\Delta \Delta Cq}
\]

Fold-regulation transcribes/ translates these fold-difference results. Fold-difference values of >1 imply an induction or an up-regulation, and the fold-regulation is equal to the fold-difference. In contrast, fold-difference values < 1 indicate a repression or down-regulation,
and the fold-regulation is the negative inverse of the fold-difference. A threshold value of 38 was set as the limit of detection below which no signal could be detected.

Wilcoxon matched-pairs signed rank test was used to compare the differences in expression between the coronal pulp and AP in the angiogenic growth factor and receptor, inflammatory cell marker and cell-surface marker groups. A $p$ value of $< 0.05$ was deemed statistically significant.
Chapter Three — Results

3.1 Immunohistochemistry—Results

3.1.1 Primary antibody optimisation

3.1.1.1 Section adherence

Decalcified tissue sections frequently lifted from the charged slides when IHC was performed using the autostainer. This was not observed for the soft tissue specimens. The HMAR method contributed to adverse tissue adherence and resulted in mild tissue lifting, complete tissue detachment or folding, rendering some of the specimens unsuitable for further analyses. This was addressed with employing manual IHC for all decalcified hard tissue specimens, and the use of the Ventana Benchmark XT™ autostainer for all soft tissue specimens.

3.1.1.2 Optimal working dilution of primary antibody

Optimisation of each primary antibody on soft and decalcified tissues resulted in a working concentration and retrieval method as shown in Table 3.1.

Table 3.1 - Optimised antigen retrieval and primary antibody concentration for soft and decalcified specimens with manual and automated IHC

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Antigen retrieval method</th>
<th>Antibody concentration µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-VEGF</td>
<td>Heat 80°C 10 mins</td>
<td>6</td>
</tr>
<tr>
<td>anti-ANG1</td>
<td>Heat 80°C 10 mins</td>
<td>4</td>
</tr>
<tr>
<td>anti-ANG2</td>
<td>Heat 80°C 10 mins</td>
<td>2.5</td>
</tr>
<tr>
<td>anti-Tie2</td>
<td>Heat 80°C 10 mins</td>
<td>2</td>
</tr>
<tr>
<td>anti-CD34</td>
<td>No Retrieval</td>
<td>Ventana Autostainer</td>
</tr>
<tr>
<td>anti-CD45</td>
<td>CC1 AR for 28 minutes</td>
<td>Ventana Autostainer</td>
</tr>
</tbody>
</table>

CC1- cell-conditioning 1
AR- Antigen retrieval

Non-specific background staining was detected with anti-Tie2, anti-ANG1 and anti-ANG2 primary antibodies. Extended washes, 1% skim milk power in PBS and agitation were used to minimise this.
### 3.1.2 Anatomy of the apical papilla

The AP is a creamy-white, soft, gelatinous tissue, and is loosely adherent to the apex of the immature tooth root (Figures 3.1 A, C). It may be easily detached from the root apex/apices (Figure 3.1 B).

![Figure 3.1 - Gross anatomy of the apical papilla (AP). (A) Sectioned roots from an immature human permanent third molar depicting AP tissue attached to the root apices (arrows). (B) Detached AP from the root apex (black arrow). Red arrow points to coronal pulp tissue. (C) Decalcified immature permanent tooth specimen shows sectioned roots (marked in indelible ink) with AP attached to the root apices (arrows), prior to being embedded in paraffin.](image)

### 3.1.3 Histological examination of Haematoxylin and Eosin staining

#### 3.1.3.1 The apical region of immature permanent teeth

The histology of the apical region of immature permanent teeth encompasses the mature radicular pulp, the apical CRZ, the AP, the periodontal ligament and the dental follicle (Figure 3.2 A). All the permanent third molar teeth obtained for the study had two-thirds root...
development and an attached AP. The HERS was associated with the periodontal ligament and adjacent to the AP. Remnants of dental follicle were often seen (Figure 3.2 D). The apical CRZ separated the mature radicular pulp from the AP. The radicular pulp consisted of a loose connective tissue, with cuboidal odontoblasts adjacent to the dentine with processes that extended into the dentinal tubules. The CRZ with a distinct ‘cap-like’ layer rich in cells and small blood vessels separated the AP from the radicular pulp (Figure 3.2 C). The AP consisted of a loose connective tissue stroma containing small fibroblasts, and other immature cells and a few vessels (Figure 3.2 B). When blood vessels were present, they were generally seen towards the periphery of the AP in proximity to the apical CRZ and the dental follicle. This is depicted in Figure 3.2.

Figure 3.2 – H and E stain of the apical region of a decalcified immature permanent tooth. (A) Peripheral aspect of the apical region demonstrating the relationship of the immature root apex to the AP and periodontal tissues, at low power (100x magnification). (B) Central region of the AP at high power (400x magnification). (C) Relationship between the CRZ (superiorly) and the AP (inferiorly), at medium power (200x magnification). (D) Relationship between the CRZ, AP (superiorly) and the dental follicle (inferiorly), at medium power (200x magnification). (Scale bar 50 µm)

AP- apical papilla, BV- blood vessel, CRZ- cell-rich zone, D – Dentine, F- dental follicle, HERS- Hertwig’s epithelial root sheath, O-odontoblasts, PDL- periodontal ligament
3.1.4 Immunohistochemistry of the apical papilla

Qualitative and semi-quantitative analyses were performed to assess the protein expression in the AP specimens.

3.1.4.1 Qualitative analyses

The presence and distribution of protein expression and antibody specificity was examined in the specimens.

3.1.4.1.1 CD34

CD34 identified endothelial cells consistently in both small and large blood vessels. CD34 is a cell-surface protein; immunopositivity to this antibody was identified on the surface of endothelial cells bordering lumina (immature vessels), and also multi-layered bundles of endothelial cells bordering an indistinct lumen (mature vessels) (Figure 3.3 B).

Immunopositivity to the anti-CD34 antibody was demonstrated in the vascular apical CRZ (Figure 3.3 A), and in the AP, being less vascular (Figures 3.3 A, B). Immunopositivity was seen towards the periphery of the AP, in proximity to the apical CRZ and the dental follicle.

Human tonsil tissue was used as a positive control and this tissue demonstrated immunopositivity, thus confirming the presence of protein (Figure 3.3 C). Conversely, non-specific mouse IgG antibody replaced the anti-CD34 as a negative control, and demonstrated no immunostaining (Figure 3.3 D).
Figure 3.3 – IHC with the anti-CD34 antibody (endothelial cell-marker) in a decalcified immature permanent tooth. (A) Low power view (100x magnification) demonstrating the relationship between the radicular mature pulp, and the CRZ and AP located inferiorly. Remnants of the dental follicle can be seen below. Arrows show immunopositive cells. (B) High power view (400x magnification) of the central region of the apical papilla (inset taken at 1000x magnification). Arrow points to immunopositive cells. (C) Positive control tissue — human tonsil (400x magnification). (D) Negative control, non-specific mouse IgG (400x magnification). (Scale bar 50 µm)

AP - apical papilla, BV - blood vessel, CRZ - cell-rich zone, F - follicle, P - radicular pulp

3.1.4.1.2 CD45

CD45 or leukocyte common antigen (LCA) is a leucocyte marker, and identified leucocytes on both soft and decalcified hard tissues. Few CD45 positive cells were present in the AP in the apical region, and when seen, these were isolated round/ovoid cells in the apical CRZ and AP and morphologically resembled lymphocytes (Figures 3.4 A, B).

Human appendix served as the positive control, and consistently stained positive, confirming the accuracy of the staining (Figure 3.4 C). Conversely, non-specific mouse IgG antibody replaced the anti-CD45 antibody as a negative control, and demonstrated no immuno-staining (Figure 3.4 D).
Figure 3.4 – IHC with the anti-CD45 antibody in a decalcified immature permanent tooth. (A) Low power view (100x magnification) of the relation of the radicular pulp to the CRZ and AP. (B) Medium power view (200x magnification) of the central region of the AP (inset at 400x magnification) shows isolated immunopositive cell. (C) High power view (400x magnification) of positive control tissue — human appendix. (D) High power view (400x magnification) of negative control, non-specific mouse IgG. (Scale bar 50 µm)

AP - apical papilla, CRZ - cell-rich zone, P - radicular pulp

3.1.4.1.3 Vascular endothelial growth factor

Both soft and decalcified hard tissue specimens stained positively and consistently for the anti-VEGF antibody. Anti-VEGF was often identified perivascularly, and plump cells that morphologically resembled fibroblasts were also seen within the AP stroma in addition to endothelial cells lining blood vessels (Figure 3.5 B). VEGF was also expressed extracellularly in the connective tissue stroma. In the decalcified hard tissue sections, immunopositivity to the anti-VEGF antibody apically was demonstrated in the dentine and predentine matrix, and on odontoblasts in vicinity to the dentine tubules (Figure 3.5 A).

A pyogenic granuloma served as the positive control and immunopositivity confirmed the accuracy of the staining protocol (Figure 3.5 C). Conversely, non-specific mouse IgG
antibody replaced the anti-VEGF as a negative control, and demonstrated no immuno-staining (Figure 3.5D).

Figure 3.5 – IHC with the anti-VEGF antibody in a decalcified immature permanent tooth. (A) Apical region of the immature permanent tooth at low power (100x magnification) (B) Central region of the AP taken at high power (400x magnification) showing immunopositive staining of plump cells and endothelial cells (arrows). (C) Positive control tissue — oral pyogenic granuloma high power view (400x magnification). (D) Negative control — non-specific mouse IgG high-power view (400x magnification). (Scale bar 50 µm)

AP - apical papilla, BV- blood vessel, CRZ – cell rich zone, D - dentine, P - radicular pulp

3.1.4.1.4 Angiopoietin-1

All soft and decalcified hard tissue specimens were positive with the anti-ANG1 antibody which ascertained the distribution of the angiogenic growth factor within the tissues. ANG-1 positive staining was mainly associated with endothelial cells (Figures 3.6 A, B). Very few plump cells morphologically resembling fibroblasts also demonstrated immunopositivity (Figure 3.6 B). A pyogenic granuloma served as the positive control and this tissue and immunopositivity confirmed the accuracy of the staining protocol (Figure 3.6 C). Conversely, non-specific rabbit immunoglobulin fraction (solid-phase adsorbed) replaced the anti-ANG1 as a negative control, and demonstrated no immuno-staining (Figure 3.6 D).
Figure 3.6 – IHC with the anti-ANG1 antibody in a decalcified immature permanent tooth. (A) Apical region of the immature permanent tooth at low power (100x magnification). (B) Central region of the AP showing immunopositive endothelial cells (arrows), high power (400x magnification). Inset picture taken at 1000x magnification. (C) Positive control tissue — oral pyogenic granuloma at high power (400x magnification). (D) Negative control, non-specific rabbit IgG at high power (400x magnification). (Scale bar 50 µm)

AP - apical papilla, BV - blood vessel, CRZ - cell-rich zone, D - dentine, EC - endothelial cell

3.1.4.1.5 Angiopoietin-2

Soft and decalcified tissue specimens were positive with the anti-ANG2 antibody which verified the distribution of the angiogenic growth factor within the tissues. Immunopositivity to the anti-ANG2 antibody was predominantly demonstrated within endothelial cells lining blood vessels (Figures 3.7 A, B).

A pyogenic granuloma served as the positive control and immunopositivity confirmed the accuracy of the staining protocol (Figure 3.7 C). Conversely, non-specific rabbit immunoglobulin fraction (solid-phase adsorbed) replaced the anti-ANG2 as a negative control, and demonstrated no immuno-staining (Figure 3.7 D).
Figure 3.7 – IHC with the anti-ANG2 antibody in a decalcified immature permanent tooth. (A) Apical region of the immature permanent tooth at low power (100x magnification). (B) Central region of the AP showing immunopositive endothelial cells (arrows), high power (400x magnification). Inset picture taken at 1000x magnification. (C) Positive control tissue — oral pyogenic granuloma at high power (400x magnification). (D) Negative control, non-specific rabbit IgG at high power (400x magnification). (Scale bar 50 µm)

AP - apical papilla, CRZ - cell-rich zone, D – Dentine, EC - endothelial cell

3.1.4.1.6 Tunica interna endothelial cell kinase-2

An anti-Tie2 antibody was used to identify the receptor for ANG-1 and ANG-2. This was mainly expressed on endothelial cells (Figures 3.8 A, B). A few cells with a plump morphology resembling fibroblasts also expressed the Tie-2 protein (Figure 3.8 B).

A pyogenic granuloma served as the positive control and this tissue and immunopositivity confirmed the accuracy of the staining protocol (Figure 3.8 C). Conversely, non-specific rabbit immunoglobulin fraction (solid-phase adsorbed) replaced the anti-Tie2 as a negative control, and demonstrated no immuno-staining (Figure 3.8 D).
Figure 3.8 – IHC with the anti-Tie2 antibody in a decalcified immature permanent tooth. (A) Apical region of the immature permanent tooth low power view (100x magnification). (B) Central region of the AP high power view (400x magnification) with immunopositive staining of endothelial cells and plump cells (arrows). Inset picture at 1000x magnification. (C) Positive control tissue — oral pyogenic granuloma at high power (400x magnification). (D) Negative control — non-specific rabbit IgG high power view (400x magnification). (Scale bar 50 µm)

AP – apical papilla, BV- blood vessel, CRZ – cell-rich zone, D – dentine, EC- endothelial cell, P – pulp

3.1.5 Semi-quantitative analyses

The image-analysis software “ImageJ” v1.50 (National Institutes of Health, Bethesda, MD, U.S.A.) was used to count the number of immunopositive cells for the angiogenic growth factors and Tie-2 receptor from the AP specimen at high-power (400x magnification). Anti-VEGF was expressed both intracellularly and extracellularly (within the connective tissue stroma). This is recorded in Table 3.2.
Table 3.2 – Number of VEGF+, Ang-1+, ANG-2+ and Tie2+ cells in the apical papilla in soft and decalcified tissue specimens

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Number of positively stained cells/ specimen (n=10)</th>
<th>Mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-VEGF</td>
<td>52, 48, 50, 36, 44, 39, 48, 43, 49, 49</td>
<td>45.8</td>
<td>5.2</td>
</tr>
<tr>
<td>anti-ANG1</td>
<td>16, 20, 14, 23, 21, 19, 9, 11, 14, 17</td>
<td>16.4</td>
<td>4.5</td>
</tr>
<tr>
<td>anti-ANG2</td>
<td>6, 9, 7, 6, 12, 13, 7, 8, 5, 6</td>
<td>7.9</td>
<td>2.7</td>
</tr>
<tr>
<td>anti-Tie2</td>
<td>46, 41, 28, 23, 32, 41, 38, 40, 35, 42</td>
<td>36.6</td>
<td>7.0</td>
</tr>
</tbody>
</table>
3.2 RNA quality and quantity – UV spectrophotometric analysis

Coronal and apical tissue samples from 14 participants were identified as suitable for RNA extraction. The RNA was extracted and converted to cDNA. Mechanically homogenising the tissue proved difficult due to the resultant firm tissue texture and the formation of crystals on some of the tissue, following storage in the RNaLater®. The RNA quality varied from -0.48 to 2.07 (A$_{260}$:A$_{280}$; a ratio of 1.8 - 2.0 considered high quality), and the total RNA quantity ranged from 0 to 2600 µg/50 µl. A total of 16 samples were found to be suitable for subsequent cDNA synthesis and qPCR analysis (Figure 3.9; Table 3.3).

![Flow diagram]

**Participants assessed for suitability (n = 14)**

- **Inclusion for RNA extraction (n = 28/14 pairs)**
  - **Exclusion (n = 12/6 pairs)**
    - Poor quality/quantity of RNA
  - **Inclusion for cDNA and qPCR (n = 16/8 pairs)**
    - **Exclusion (n = 2/1 pairs)**
      - Outlier Cq values
  - **Final analysis (n = 14/7 pairs of coronal pulp and apical papilla)**

**Figure 3.9 – A flow diagram outlining the process of selection of tissue samples.**
Table 3.3 – Mature coronal pulp and immature apical papilla RNA quality and quantity

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Sample ID.</th>
<th>A260 nm</th>
<th>A260/A280</th>
<th>Conc. (ng/µl)</th>
<th>Total RNA (µg/50 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>0.14</td>
<td>1.47</td>
<td>5.3</td>
<td>265</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>0.29</td>
<td>1.88</td>
<td>11.0</td>
<td>550</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>0.11</td>
<td>1.63</td>
<td>5.8</td>
<td>290</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>1.81</td>
<td>-0.48</td>
<td>-0.6</td>
<td>-30</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>0.01</td>
<td>1.45</td>
<td>2.2</td>
<td>110</td>
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Samples not highlighted were omitted from further analysis due to their low quantities (< 25 ng/µl) of RNA.
3.2.1 Regulatory gene expression analysis

3.2.1.1 mRNA transcription analysis

A customised 16 gene x 6 sample TaqMan® Fast 96-Well Plate array was used for mRNA transcription analysis. Cq values were calculated following baseline correction (Appendix C, Table C). The manufacturers r18S plated assay, resulted in a very high amplification fluorescence signal that skewed the data and for this reason the r18S values were excluded from the analysis.

3.2.1.2 Validation of housekeeping gene selection

The mRNA levels of three mRNA transcripts B2M, HPRT1, RPLP0 were analysed. A good HKG or normalisation gene is one that is expressed at the same level in all samples to be compared. HPRT1, B2M and RPLP0 mRNA transcripts were detected at similar levels in the coronal pulp and apical papilla tissues (Figure 3.10). The relative mRNA expression levels of HPRT1 were lower compared to B2M and RPLP0. However, NormFinder identified HPRT1 as the most suitable reference gene with a stability value of 0.942 across the pairs of samples (Appendix C, Table D). The corresponding stability values for B2M and RPLP0 were 1.150 and 1.017 respectively. The stability value for a gene is a direct measure for the estimated expression variation enabling the user to evaluate the systematic error introduced when using the gene for normalization. A lower stability value indicates a more stable gene.
Figure 3.10 - Housekeeping gene expression. A. HPRT1; B. B2M; C. RPLP0. Apical papilla (red dots) and coronal pulp (blue dots) are shown. The dotted line indicates the upper limit of detection. UD – undetected.
3.2.1.3 mRNA transcript analysis

A general trend observed across all the tissue samples revealed the relative mRNA mean expression level of the inflammatory cell marker gene group (IL37, IL1A and IL6) was considerably lower than the angiogenic growth factors (VEGFA, ANGPT1, ANGPT2), their receptors (KDR, TIE2), and the cell-surface marker genes (CD34, PTPRC, CD44, CD83) (Figure 3.11).

![Figure 3.11](image)

**Figure 3.11 - Relative mRNA expression levels.** Mature coronal pulp (C) and apical papillae (A) mRNA expression following normalisation to the HKG HPRT1 is shown. Data is expressed as a linear transformation of the Cq ($2^{-\Delta Cq}$). The floating-bars plot shows the minimum and maximum range. The dashed line indicates limit of detection. Genes are grouped and coloured according to their known functions: angiogenic regulatory genes (red), their receptors (green), cell-surface marker genes (yellow) and the inflammatory cell marker genes (blue). UD – undetected.

When AP and coronal pulps were compared, although not statistically significant, a lower relative mRNA expression level of the angiogenic growth factor VEGFA gene and its cognate receptor gene KDR was found in the AP, when compared to the coronal pulp. As were the cell-surface marker genes CD34 and PTPRC to a smaller degree (Figure 3.11).

Conversely, the angiogenic growth factor ANGPT1 and ANGPT2 genes and their putative receptor gene TEK, inflammatory cell marker gene IL1A, and the cell-surface marker genes CD44, CD83 had higher relative mRNA expression levels in the AP, compared with the coronal pulp. These were not statistically significant.
3.2.1.3.1 *Apical (developmental) versus coronal (mature) tissue mRNA expression*

3.2.1.3.1.1 Angiogenic growth factors and growth factor receptors

VEGFA mRNA was mostly detected at lower levels in the AP when compared to coronal pulp tissue (Figure 3.12 A). In contrast, ANGPT1 and ANGPT2 mRNA were detected at slightly greater levels in the AP (Figure 3.12 B, C). However, these pairings did not reveal any statistically significant differences.

Figure 3.12 - Angiogenic growth factors mRNA expression. A. VEGFA; B. ANGPT1; C. ANGPT2. Data is expressed as a linear transformation of the Cq ($2^{-\Delta Cq}$). The box-whisker plot shows the minimum and maximum range of mRNA detected. UD – Undetected.
Both KDR and TEK mRNA were detected in the apical and coronal tissue (Figure 3.13).

![Figure 3.13 - Angiogenic growth factor receptors mRNA expression. A. KDR; B. TEK. Data is expressed as a linear transformation of the Cq ($2^{-\Delta Cq}$). The box-whisker plot shows the minimum and maximum range of mRNA detected. UD – Undetected.](image)

3.2.1.3.1.2 Cell-surface and surface adhesion molecule markers

The endothelial and dendritic cells cell-surface markers CD34 and CD83, and surface adhesion molecules CD44 and PTPRC (which encodes CD45) were detected at similar levels in both apical and coronal tissues (Figure 3.14).
Figure 3.14 – Cell-surface markers and surface adhesion molecules mRNA expression. A. CD34; B. CD83; C. CD44; D. PTPRC. Data is expressed as a linear transformation of the Cq ($2^{-\Delta Cq}$). The box-whisker plot shows the minimum and maximum range of mRNA detected. UD – Undetected.

3.2.1.3.1.3 Inflammatory cell markers

The mRNA transcript levels of the inflammatory cell-markers IL37, IL1A and IL6 have not been previously investigated in the AP. IL37 was not detected in the AP and two samples analysed showed low levels of expression in the coronal tissue (Figure 3.15 A). IL1A was detected with greater expression in apical tissue compared with coronal (Figure 3.15 B). IL6 was detected in both the AP and coronal pulp tissue (Figure 3.15 C). These differences were not statistically significant.
Figure 3.15 – Inflammatory cell markers mRNA expression. A. IL37; B. IL1A; C. IL6. Data is expressed as a linear transformation of the Cq ($2^{-\Delta Cq}$). The box-whisker plot shows the minimum and maximum range of mRNA detected. UD – Undetected.
3.2.1.4 Fold-regulation

Fold-regulation was calculated for every individual pairing (Table 3.4). A wide range of values were obtained and no distinct correlation could be made between the separate AP and coronal pulp groups (Appendix C, Table E). Closer examination of the fold-regulation values at the individual level revealed a consistent greater relative mRNA expression in the coronal pulp in some participants (e.g. MSt1), and a greater mRNA expression in the AP in others (e.g. JWi4).
Table 3.4 – Fold-regulation of the genes of interest for each participant

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Chapter Four — Discussion and Conclusion

4.1 Discussion

The current study investigated the localisation of selected angiogenic growth factors and receptor proteins in the AP, and compared the mRNA expression levels of the genes associated with these proteins in the immature AP with the mature coronal pulp. This study examined healthy teeth in order to provide a baseline for understanding changes that may occur in the presence of disease.

Our understanding of the events underpinning angiogenesis in immature permanent teeth is still equivocal (Tziafas et al. 2000). We know that angiogenic events are pivotal to pulp development and healing, but knowledge at a molecular level of these processes including the expression and regulation of angiogenic genes is still lacking.

VEGF, Ang-1, Ang-2, Tie-2, CD34 and CD45 were chosen for the IHC experiments due to their recognised roles in angiogenesis and their expression on endothelial and lymphohaematopoietic cells (Ferrara et al. 1996, Trubiani et al. 2003, Fiedler & Augustin 2006, Nakajima et al. 2014, Gaudin et al. 2015). VEGF is the key regulator of angiogenesis especially in the initial stages. Angiopoietins also have a predilection for vascular endothelium, mostly in the latter stages of angiogenesis and these growth factors are regulated by VEGF (Maisonpierre et al. 1997, Carmeliet et al. 2000, Yancopoulos et al. 2000). VEGF and angiopoietins act by binding to their respective tyrosine kinase receptors (Gale & Yancopoulos 1999, Fiedler et al. 2003). VEGF binds to its receptors VEGFR-2 and VEGFR-1 (Terman et al. 1991, Gille et al. 2001, Shibuya 2001, 2006), while the cognate receptor for Ang-1 and Ang-2 is Tie-2 (Partanen et al. 1992, Witzenbichler et al. 1998). The vast majority of studies in the dental pulp have focussed on VEGF (Artese et al. 2002, Mattuella et al. 2007a, 2007b, Miwa et al. 2008, Mullane et al. 2008, Aranha et al. 2010), but there is a paucity of research evaluating the role of angiopoietins and their known contribution to vessel maturation as part of angiogenesis (El Karim et al. 2009, Nakajima et al. 2014). Understanding of this growth factor family and their receptor(s) in the dental pulp is still in its infancy. To our knowledge, this is the first study profiling the localisation of angiopoietins and their receptor in the AP, and drawing a comparison between the mRNA expression levels in the AP and the coronal pulp. The use of IHC and qPCR techniques enabled corroboration that the mRNA expression of individual genes was also present at the protein level.
Teeth are composed of several tissue types of varying consistency i.e. the soft dental pulp and PDL tissue are juxtaposed with the hard mineralised dentine and cementum. This meant histological processing of some specimens was challenging. Artefacts, tissue lifting, folding or detachment were observed particularly with the use of the autostainer in these specimens. These problems have been identified by others, (Christgau et al. 1998, Jones et al. 2001, Engel & Moore 2011, Silva et al. 2011) and the use of thin sections (3-5 µm) on positively charged Histobond® slides (Paul Marienfeld GmbH & Co. KG, Lauda-Königshafen, Germany), dried upright (to remove any trapped water) at a temperature of 60°C for 120 minutes followed by an overnight incubation at 50°C were used to circumvent this problem (Huang et al. 1983, Thurby et al. 2009, Friedlander et al. 2014). Heat-mediated antigen retrieval was performed at a lower temperature for a duration of ten minutes to alleviate any tissue loss that may have resulted from the use of high heat (Biddolph et al. 1999). Manual processing of tissue specimens was gentler on the tissues and resulted in less lifting but does not provide the technical consistency offered by automated staining machine protocols.

Several attempts were made to optimise the anti-VEGFR1 and anti-VEGFR2 antibodies for their use in IHC. These were polyclonal antibodies which are recognised as being more difficult to optimise (Hayat 2002, Taylor et al. 2006) and may also reflect the scarcity we observed in the expression of genes encoding these proteins. The concentration of these primary antibodies was adjusted, along with antigen retrieval and incubation periods, but these were unsuccessful. Due to time and financial constraints, it was decided to perform IHC experiments on the other selected six primary antibodies and analyse gene expression of VEGF1/FLT1 and VEGFR2/KDR. Non-specific background staining was observed with anti-Tie2, anti-ANG1 and anti-ANG2 primary polyclonal antibodies, when EnVision™ HRP System was used as a secondary reagent. A similar finding was reported by Kämmerer et al. (2001). Extended washes, 1% skim milk powder in PBS (Ludány et al. 1993, Miller et al. 1999) and agitation were used to minimise this, but normal serum prior to incubation with the primary antibody was not used. The use of Tween-20 was not considered due to the greater propensity of tissue-lifting when such non-ionic surfactants are employed.

Data from our gene expression studies revealed that the relative mRNA expression of VEGF was greater in the coronal pulp compared with the AP, while the relative mRNA expression levels of ANG1 and ANG2 genes showed a slightly greater expression in the AP compared with the coronal pulp. These findings were not statistically significant. Expression of these angiogenic growth factors in the AP tissue is important to physiological processes. In health, the AP contained a few isolated CD45+ cells indicating the presence of immune surveillance
to respond to injury and presence of the growth factors is essential for development of the vasculature and for pulp and root maturation. A basal level of expression of VEGF, ANG1 and ANG2 suggests these growth factors play a role in physiological angiogenesis in immature permanent teeth and the potential to contribute to healing following pulp injury.

VEGF is a potent angiogenic regulator and is involved in the promotion of endothelial cell survival, induction of endothelial cell proliferation, migration, invasion and activation (Ferrara et al. 1996, 2003, 2005, Thomas et al. 1996, Dvorak 2005). IHC experiments with anti-VEGF antibody identified positive staining on several cell types in high quantities in the AP including endothelial cells and plump cells with large nuclei that morphologically resembled fibroblasts suggesting that non-endothelial cells also have the potential to contribute to angiogenesis. At the developing root end, immunopositivity was also detected in the dentine matrix and odontoblasts lining the mature radicular pulp. These findings are in accordance with other IHC and gene expression studies which show that VEGF is expressed in moderate to high levels in the pulps of non-carious permanent third molars (Artese et al. 2002, Guan & Wang 2012, Virtej et al. 2013). Wang et al. (2007) showed a pronounced cytoplasmic expression of VEGF in pulpal fibroblasts and odontoblasts in immature permanent teeth, significantly greater than in mature permanent teeth. This correlates well with our IHC and gene expression results. Another study from our research group by Friedlander et al. (2014) revealed VEGF was expressed in the AP fibroblasts. The use of IHC double-staining will be considered in the future to more definitively identify the cell types expressing VEGF on the specimens examined.

VEGFR2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF (Shalaby et al. 2005, Mattuellea et al. 2007b). KDR gene encodes for the VEGFR2 protein and our gene expression data showed expression in both crown and apex. This suggests that the putative receptor may be expressed at a level to facilitate angiogenic signalling mediated by VEGF in the crown and the apical papilla. Unpublished data from our research group reveals concordant findings and a slight preferential expression in the coronal pulp, in addition to being mildly expressed in the apical CRZ and in the AP stroma. The polyclonal nature of the antibody and scarcity of protein expression in the AP tissue created difficulties in antibody optimisation for anti-VEGFR2. Further research with gene expression data was deemed more meaningful. Overall, these results are in line with other experiments on immature permanent teeth which demonstrated a high expression of VEGFR2 on both endothelial cells (predominantly) and non-endothelial cells in the coronal dental pulp.
(Matsushita et al. 2000, Mattuella et al. 2007b, Guan & Wang 2012), and very low expression in the AP (Friedlander et al. 2014).

The IHC experiments with the anti-Ang1 antibody on the AP specimens identified positive staining mainly associated with endothelial cells, and scarcely with plump cells that morphologically resembled immature fibroblasts. A low to moderate positive cell-count of the healthy AP specimens suggests that constitutive expression of this angiogenic protein is reflective of its role in physiological angiogenesis and maintenance of vascular quiescence. Ang-1 is anti-inflammatory and serves as a paracrine “switch” to regulate the modulation from an activated endothelium to a resting endothelium through its inhibitory effects on LPS, expression of tissue factor (TF) and adhesion molecules (Asahara et al. 1998, Gamble et al. 2000, Kim et al. 2001, Jeon et al. 2003, Witzenbichler et al. 2005). These results are in concordance with Stratmann et al. (1998) who reported Ang-1 expression in fibroblasts and endothelial cells, although their samples comprised of tumour specimens and are therefore not directly comparable. Ang-1 expression has also been detected in odontoblasts and in the predentine and dentine matrix in immature permanent teeth, possibly reflective of their roles in dentinogenesis and odontoblast homeostasis (Nakajima et al. 2014). This was not detected in our current study samples. A larger sample size and employing a double-staining technique using fibroblast markers such as vimentin or fibroblast surface antigen, and odontoblast markers such as dentine phosphoprotein or dentine sialoprotein are recommended.

IHC experiments with the anti-Ang2 antibody on the AP specimens identified positive staining almost exclusively with endothelial cells. Ang-2 expression was not detected in odontoblasts or fibroblasts. A low positive cell-count of the whole specimen suggests that this growth factor is probably not produced constitutively in the AP associated with a healthy pulp. Literature on wound healing and tumorigenesis has identified that mRNA expression levels of Ang-2 are near imperceptible in the resting endothelium. This, however, increases rapidly with an activated endothelium phenotype as stored Ang-2 is immobilised from Weibel-Palade bodies in endothelial cells (Stratmann et al. 1998, Holash et al. 1999, Fiedler et al. 2004). This corroborates with its role as an autocrine dynamic regulator of angiogenesis and a destabiliser of the quiescent resting endothelium (Scharpfenecker et al. 2005). Ang-2 is pro-inflammatory and induces and upregulates inflammation by sensitising endothelial cells to pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and to VEGF (Lemieux et al. 2005, Roviezzo et al. 2005, Fiedler et al. 2006). Initial experiments on gene-targeted mice revealed that Ang-2 antagonises the actions of Ang-1 and works in tandem with angiogenic growth factors such as VEGF to induce angiogenesis, or vessel regression in the

The aforementioned data on the localisation and mRNA expression levels of Ang-1 and Ang-2 lend credence to the proposed model that physiological vascular homeostasis is maintained by Ang-1/Tie-2 signalling, with the resting endothelium Ang1/Ang2 ratio in support of Ang-1 (Figure 4.1). Nature intended it to be this way, so as to thwart any unregulated angiogenesis due to upregulated Ang-2 signalling. However, in the event of local inflammation, as may be encountered in the low-compliance environment of the dental pulp (Kim et al. 1990), the resting endothelium is primed to promptly respond to angiogenic cues, namely VEGF, and upregulate Ang-2 signalling (El Karim et al. 2009).

![Figure 4.1](image_url)

Figure 4.1 – A proposed model of vascular homeostasis maintained by Ang1/Tie2 signaling, displaying vessel quiescence, vessel activation, vessel regression and angiogenesis. Reproduced from Fiedler and Augustin (2006) Angiopoietins: a link between angiogenesis and inflammation. Trends in Immunology 27, 552-8.

Scant information is available on Tie-2 expression in the dental pulp. Tie-2 is an endothelial cell-specific receptor, its activation promotes vessel assembly and maturation by mediating survival signals for endothelial cells and regulating the recruitment of mural cells. TEK gene encodes for the Tie-2 protein and our gene expression data revealed similar levels of expression in both the crown and apex. IHC experiments with the anti-Tie2 antibody on the AP specimens demonstrated immunopositivity mainly associated with endothelial cells, and
plump immature cells resembling fibroblasts. Together, these results suggest that while the ligands Ang-1 and Ang-2 may be upregulated to promote angiogenesis, the cognate receptor itself may maintain a steady level of expression (Wong et al. 1997). Similar to Ang-1, Tie-2 is also anti-inflammatory and effects its actions by binding to the human A20 binding inhibitor of NF-kappaB activation-2 (ABIN-2), which in turn inhibits inflammatory gene expression and signaling (Hughes et al. 2003). Several cell types (endothelial cells, hematopoietic cells, fibroblasts) express Tie-2, accounting for the increased staining observed on cells that did not resemble endothelial cells (Dumont et al. 1992, Maisonpierre et al. 1993, Sato et al. 1993, Staton et al. 2010). Nakajima et al. (2014) reported Tie-2 expression by odontoblasts, and in the predentine and dentine matrix of immature permanent teeth, but this was not observed in our study.

Cell counting of the entire specimen was carried out due to the small size of the AP as a quantitative measure of the immunopositivity of VEGF, Ang-1, Ang-2 and Tie-2. Although trends were observed between the different specimens, this should be validated with mRNA expression levels due to the inherent possibility of biological tissue variability.

CD34 is an endothelial cell marker and is expressed on hematopoietic cells, endothelial cells, and mural cells such as pericytes which ensheath the vasculature (Trubiani et al. 2003, Digka et al. 2006, Carnevale et al. 2017). The mRNA expression levels of the CD34 gene was expressed at a higher level in the coronal pulp when compared with the AP (not statistically significant), likely reflecting the greater vascularity of the coronal pulp, or a response to a carious stimulus (Güven et al. 2007). Classic literature by Provenza (1958), Kramer (1960, 1968) and more recently by Vongsavan and Matthews (1992) demonstrated the vascularity of the coronal pulp with small vessels often seen in the subodontoblastic region in proximity to the CRZ, especially in the region of the pulp horn. The IHC experiments on the anti-CD34 antibody similarly identified marked immunopositivity in the mature radicular pulp, and sparsely in the central AP. Increased vascularity and CD34 staining was observed adjacent to the AP, i.e. in the apical CRZ and in proximity to the dental follicle. These results are consistent with other published literature (Sonoyama et al. 2006, Abe et al. 2007, Friedlander et al. 2014).

A dearth of literature exists on the expression of CD44 in the pulp and/or AP (Abe et al. 2007, Pisterna & Siragusa 2007, Chen et al. 2013). CD44 is a cell adhesion molecule expressed by several cells including endothelial cells, odontoblasts and leucocytes, and mainly binds hyaluronic acid (Chen et al. 2013). Gene expression studies revealed high mRNA expression levels of this cell surface glycoprotein in the developmental tissue, the AP. This finding is
consistent with the literature that describes this molecules’ expression in early embryonic development (Campbell et al. 1995, Corbel et al. 2000). Chen and co-workers (2013) similarly identified a greater expression of the CD44 protein in the stromal cells of the AP, and in the apical pulp odontoblasts of immature teeth. This expression pattern likely implies its role in the mineralisation of dental pulp cells and odontoblasts, and tooth development (Pisterna & Siragusa 2007, Chen et al. 2013, 2016).

Although not statistically significant, a surprising finding was a greater mRNA expression of the mature dendritic cell marker CD83 (Zhou & Tedder 1995) in the AP. To our knowledge, this is the first study to report on the expression of this marker in the AP. Dendritic cells are potent APC, and their presence in the AP could signify important immunomodulatory interactions at the apical papilla/pulp or the apical papilla/periodontal interface, but the origin of these dendritic cells in a likely un-inflamed AP warrants further clarification. They may originate from either circulating immature dendritic cells or other hematopoietic progenitor cells that may be present in the AP. Laxmanan et al. (2005) demonstrated that VEGF could alter the phenotype of mature dendritic cells (CD83 positive) to immature to participate in tissue repair and healing.

Inflammation is a micro-environmental cue (Chmilewsky et al. 2014). Angiogenesis occurs when numerous such cues, both stimulatory and inhibitory, act on the resting endothelium (Polverini 1995). Endothelium activation is not only a requisite for initiating angiogenesis, but also for inducing inflammation through the surface presentation of adhesion molecules and leucocyte recruitment. This implies that inflammation and angiogenesis may share a common signaling pathway.

An innate level of inflammation, i.e. immune-tolerance, pervades the dental pulp and even clinically ‘normal’ pulps are likely to contain occasional immune cells (Hahn et al. 1989, Jontell et al. 1998, Gaudin et al. 2015). As previously discussed, we observed similar results and this was reflected in a higher mRNA expression level of the leucocyte marker CD45 gene in the coronal pulp compared with the uninflamed AP. This was not statistically significant. IHC experiments on the anti-CD45 antibody revealed sparse immunopositivity on isolated ovoid/round cells in the AP, morphologically resembling lymphocytes. These findings are in concordance with other studies (Sonoyama et al. 2006, Abe et al. 2007, Friedlander et al. 2014, Gaudin et al. 2015). The use of lymphocyte markers and a double-labelling technique could be employed to verify the specific cell-subsets of these CD45 positive cells (Shipkova & Weiland 2012).
The mRNA expression levels of the inflammatory cytokines IL-1α, IL-6 and IL-37 markers in the AP were determined. IL-1α was expressed higher in the AP, when compared with the coronal pulp, but this was not statistically significant. The mRNA expression level of IL-1α in the coronal pulps of the seven samples were imperceptible making it not possible to determine any quantitation for the inflammatory marker gene. Caution must be exercised when extrapolating these results. IL-1α is a pro-inflammatory cytokine synthesized by endothelial cells, fibroblasts, macrophages, neutrophils and has been extensively studied in the dental pulp (Matsuo et al. 1994, Bletsa et al. 2004, Hahn et al. 2007). IL-1α is pro-angiogenic and its production is upregulated in a hypoxic environment and (BenEzra et al. 1990, Shreeniwas et al. 1992). The greater expression of this cytokine in the AP is however, a discordant finding. It is likely that expression levels of IL-1α correlate with its synthesis by apical papilla fibroblasts and endothelial cells, where it induces angiogenesis through its effects on VEGF (Chu et al. 2004).

Contrarily, a greater mRNA expression level of the pro-inflammatory cytokine IL-6 was demonstrated in the coronal pulp compared with the AP. These were not statistically significant. These findings are in concordance with Barkhordar et al. (1999) who reported negligible amounts of the cytokine in clinically ‘normal’ pulp tissue but elevated numbers in inflamed pulps. IL-6 is synthesised by several cell types, including T-lymphocytes, B-lymphocytes, macrophages, vascular endothelial cells (Motro et al. 1990) and pulp fibroblasts (Yamaguchi et al. 2004). IL-6 is also shown to be an indirect inducer of angiogenesis through its induction of VEGF mRNA (Cohen et al. 1996), and its expression levels in the coronal pulp may reflect its roles as a key player in inflammation and angiogenesis.

IL-37 (previously called IL-7) mRNA expression levels were negligibly higher in the coronal pulp than in the AP. IL-37 is an anti-inflammatory cytokine (Tetè et al. 2012) expressed by macrophage, monocytes, dendritic cells, and endothelial cells; it inhibits the production, activation and function of other pro-inflammatory cytokines such as IL-1α, IL-8, IL-6 (Dinarello & Bufler 2013). Its presence in the mature coronal pulp and not in the un-inflamed AP signifies its role in curbing inflammation that follows tissue injury, and restoring tissue homeostasis. Like VEGF, IL-37 is also strongly induced by pro-angiogenic hypoxic signalling, and is shown to enhance the proliferation and migration of endothelial cells in vitro (Yang et al. 2015).

This study adds to the knowledge related to immune surveillance of the pulp and AP in health and that it may have an important role in angiogenic activities required for root development and healing. The selected study specimens demonstrated negligible gene expression of the
inflammatory markers in the AP, and the differing levels of expression of these markers amongst the subjects may be attributed to the individual host response and the varying ages of the participants. This is indicative of the uninflamed nature of the AP (Chrepa et al. 2017, Ricucci et al. 2017). Stressors such as hypoxia and inflammation have been shown to have a proangiogenic effect on the secretome of the apical papilla stem cells, but these conditions were induced in culture (Vanacker et al. 2014, Bakopoulou et al. 2015, Liu et al. 2016) and not the focus of our study. Further research exploring the impact of inflammation on the angiogenic response of the AP would be beneficial.

It is important to recognise that prior histological examination was not performed on the specimens used in gene expression studies as they were immersed in RNAlater® immediately after extraction. Thus, it is possible that some teeth which were clinically diagnosed as ‘normal’ may in fact have included the presence of neutrophils in the pulp. It is also possible that the excavated AP tissues used in the study may have inadvertently included adjacent cell populations. Perhaps, newer techniques such as laser micro-dissection could be employed to isolate previously characterised cells thereby permitting gene analysis of carefully chosen cells in a heterogeneous cell population (Watahiki et al. 2004, Ladanyi et al. 2006).

Whilst this study has provided a closer insight into the mRNA expression levels and spatial localisation of VEGF, angiopoietins and their receptors in the whole AP tissue consistent with their roles in angiogenesis; we are still constrained in our understanding of healing and angiogenesis in immature teeth. The future directions of this research include a greater comprehension of the biochemical and molecular events involved in angiogenesis. This is paramount in helping clinicians offer improved therapeutic treatment options to patients with immature permanent teeth afflicted by dental trauma or other dental insults. Novel approaches harnessing endogenous and exogenous bioactive angiogenic growth factors including angiopoietins for use in vital pulp therapy either topically or in controlled drug-delivery systems to either the coronal pulp tissue or to the immature root apex, could be developed in the future. Proangiogenic effects may augment the outcome of teeth undergoing revascularisation, or avulsion injuries. Antiangiogenic or angiogenic-modulatory therapies could be focussed on manipulating the expression levels of Ang-2, given the constitutive expression of Ang-1.

In summary, results from our IHC experiments displayed the presence of immunopositive blood vessels, immature cells, and angiogenic growth factors and their receptors in the AP from healthy teeth. Gene expression data revealed differences exist in the mRNA expression between the coronal and apical regions of immature permanent teeth, which likely reflects
their different functional roles. Together, these findings suggest that angiogenic signalling exists within the AP and angiopoietins have an important role. This developmental mesenchymal tissue may likely offer a plausible source of cells, vessels and growth factors that could participate in and promote healing and root development (Sonoyama et al. 2006, Abe et al. 2007, Bakopoulou et al. 2011, Lovelace et al. 2011). However, biological variability exists within specimens, and responses at a molecular level are influenced by the presence of inflammation. Further studies with immature teeth with inflamed pulps and larger sample sizes are justified.

4.2 Conclusion

This thesis makes a contribution to the field of pulp biology and angiogenesis and highlights the angiogenic potential of the AP via its resident cells, growth factors and vasculature. The all-encompassing aim of this study was to investigate the angiogenic gene expression and identify protein localisation in the AP of immature permanent teeth; and test the hypotheses that angiogenic signalling exists within the AP and that differences exist between the AP and the coronal pulp, at least in part, due to differences in their angiogenic potential.

Within the limitations of the study, the pertinent aims of the research study were addressed. The findings from our IHC experiments confirmed that resident cells, angiogenic growth factors and their receptors are present in the AP; and upheld the hypothesis that angiogenic signalling exists within the AP. Endothelial cells were the key cells expressing angiogenic growth factors in the AP. Other supportive non-endothelial cells such as fibroblasts, and immature mesenchymal cells are also thought to play an important contributory role. VEGF was abundantly expressed in the AP, while Ang-1 and Tie2 were moderately expressed. Gene expression studies revealed differences in the level of mRNA expression of angiogenic growth factors and their receptors between the immature AP and the coronal pulp which served as a control, but these differences were statistically insignificant. There was greater expression of Ang-1 and Ang-2 genes in the apical papilla, while VEGF was more highly expressed in the coronal pulp, confirming the different roles of these growth factors in physiological and pathological angiogenesis. The relative mRNA expression levels of Ang-2 and VEGF in the AP further suggests synergy between the different angiogenic growth factors. The gene encoding the Tie-2 receptor showed a relatively steady level of expression across both coronal and apical specimens, highlighting the role of Ang-1/Tie-2 as “gatekeepers” and the importance of Ang-1/Tie-2 signalling as the ‘default pathway’ in the maintenance of vascular quiescence. Another key finding was the detection of high levels of
the gene encoding the mature dendritic cell marker CD83 in the AP, reflective of its role in antigen presentation or VEGF-mediated tissue repair. Genes encoding the inflammatory markers were poorly expressed in the AP, implying that resident inflammatory cells are not often present in this developmental tissue.

Further research in immature teeth with a larger sample size comparing healthy with inflamed pulps may help elaborate the effect of inflammation and disease on the phenotype of the AP, with functional implications in development and healing.
Bibliography


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Rebel HH (1922) Über die ausheilung der freigelegten pulpa. Deutsch Zahnheilkunde 55, 3-83.


Shi SR, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for Immunohistochemical staining based on


Appendix A – Ethics Approval, Maori Consultation and Informed Consent

Dr L Friedlander
Sir John Walsh Research Institute
Department of Oral Diagnostic and Surgical Sciences
Faculty of Dentistry

Dear Dr Friedlander,

I am writing to let you know that, at its recent meeting, the Ethics Committee considered your proposal entitled "Angiogenesis in healthy and inflamed dental pulps".

As a result of that consideration, the current status of your proposal is: Approved

For your future reference, the Ethics Committee’s reference code for this project is: H15/002.

The comments and views expressed by the Ethics Committee concerning your proposal are as follows:

While approving the application, the Committee would be grateful if you would respond to the following:

The Committee noted that the Information Sheet could be seen as somewhat complex and suggests revising.

The Committee also suggests that you might want to consider increasing the duration of the storage of the cells given the advances in technology and the potential for future research with the cells.

Please provide the Committee with copies of the updated documents, if changes have been necessary.

The standard conditions of approval for all human research projects reviewed and approved by the Committee are the following:

Conduct the research project strictly in accordance with the research proposal submitted and granted ethics approval, including any amendments required to be made to the proposal by the Human Research Ethics Committee.
Inform the Human Research Ethics Committee immediately of anything which may warrant review of ethics approval of the research project, including: serious or unexpected adverse effects on participants; unforeseen events that might affect continued ethical acceptability of the project; and a written report about these matters must be submitted to the Academic Committees Office by no later than the next working day after recognition of an adverse occurrence/event. Please note that in cases of adverse events an incident report should also be made to the Health and Safety Office:

http://www.otago.ac.nz/healthandsafety/index.html

Advise the Committee in writing as soon as practicable if the research project is discontinued.

Make no change to the project as approved in its entirety by the Committee, including any wording in any document approved as part of the project, without prior written approval of the Committee for any change. If you are applying for an amendment to your approved research, please email your request to the Academic Committees Office:

gary.witte@otago.ac.nz

jo.farrondediaz@otago.ac.nz

Approval is for up to three years from the date of this letter. If this project has not been completed within three years from the date of this letter, re-approval or an extension of approval must be requested. If the nature, consent, location, procedures or personnel of your approved application change, please advise me in writing.

Yours sincerely,

[Signature]

Mr Gary Witte
Manager, Academic Committees
Tel: 479 8256
Email: gary.witte@otago.ac.nz

c.c. Professor R D Cannon Director Sir John Walsh Research Institute
Wednesday, 17 December 2014.

Mrs Lara Friedlander,
Faculty of Dentistry - Sir John Walsh Research Institute,
DUNEDIN.

Tēna Koe Mrs Lara Friedlander,

Angiogenesis in healthy and inflamed dental pulps

The Ngāi Tahu Research Consultation Committee (the committee) met on Tuesday, 16 December 2014 to discuss your research proposition.

By way of introduction, this response from The Committee is provided as part of the Memorandum of Understanding between Te Rūnanga o Ngāi Tahu and the University. In the statement of principles of the memorandum it states "Ngāi Tahu acknowledges that the consultation process outlined in this policy provides no power of veto by Ngāi Tahu to research undertaken at the University of Otago". As such, this response is not "approval" or "mandate" for the research, rather it is a mandated response from a Ngāi Tahu appointed committee. This process is part of a number of requirements for researchers to undertake and does not cover other issues relating to ethics, including methodology they are separate requirements with other committees, for example the Human Ethics Committee, etc.

Within the context of the Policy for Research Consultation with Māori, the Committee base consultation on that defined by Justice McGechan:

"Consultation does not mean negotiation or agreement. It means: setting out a proposal not fully decided upon; adequately informing a party about relevant information upon which the proposal is based; listening to what the others have to say with an open mind (in that there is room to be persuaded against the proposal); undertaking that task in a genuine and not cosmetic manner. Reaching a decision that may or may not alter the original proposal."

The Committee considers the research to be of importance to Māori health.

As this study involves human participants, the Committee strongly encourage that ethnicity data be collected as part of the research project. That is the questions on self-identified ethnicity and descent, these questions are contained in the latest census.

The Committee suggests dissemination of the findings to relevant Māori health organisations, for example the National Māori Organisation for Dental Health, Oranga Nīho and to Professor John Broughton, who is involved in Māori Dental Health, University of Otago.

We wish you every success in your research and the committee also requests a copy of the research findings.
This letter of suggestion, recommendation and advice is current for an 18 month period from
Tuesday, 16 December 2014 to 16 June 2016.

Nāhaku noa, nā

Mark Brunton
Kaiwhakahaere Rangahau Māori
Research Manager Māori
Research Division
Te Whare Wānanga o Otago
Ph: +64 3 479 8738
Email: mark.brunton@otago.ac.nz
Web: www.otago.ac.nz
Participant Information Sheet

<table>
<thead>
<tr>
<th>Study title:</th>
<th>Angiogenesis in healthy and inflamed dental pulps</th>
</tr>
</thead>
</table>
| Principal investigator: | Dr Lara Friedlander  
Sir John Walsh Research Institute  
Faculty of Dentistry  
Position: Senior Lecturer/Endodontist |
| Contact phone number: | 03 4797126 |

Introduction

Thank you for showing an interest in this project that will look at the pulp tissue inside your tooth to identify factors which may improve healing after tooth injury. Please read this information sheet carefully. Take time to consider the information and, if you wish, talk with relatives or friends, before deciding whether or not to participate.

If you decide to participate we thank you. If you decide not to take part there will be no disadvantage to you and we thank you for considering our request.

What is the aim of this research project?

Severe injury to a tooth from decay or trauma (accident) has detrimental effects on those affected and often means the injured tooth cannot be saved. The purpose of this study is to look at the dental pulp and its cells in healthy and diseased teeth at different stages of root development and to establish whether a blood supply and healing can be improved. This will be done in the laboratory by examining your tissue under a microscope and studying your pulp tissue and its cells. It is hoped that
this study will help us understand and develop treatments to improve tooth healing and self-esteem for those who have suffered injury. This study is being carried out at the Faculty of Dentistry, University of Otago.

Who is funding this project?

This study is being funded by the Oral Molecular and Immunopathology Research Group within the Sir John Walsh Institute, Faculty of Dentistry, University of Otago.

Who are we seeking to participate in the project?

We need sixty participants who are having permanent teeth extracted. They must be medically healthy, non-smokers and people who are not diabetic or pregnant. We will be looking at tooth roots that either have, or have not, finished forming so selected participants will be between 17-50 years of age and of any ethnic group.

If you participate, what will you be asked to do?

You will be asked to donate your tooth/teeth once it has been removed as part of your planned dental treatment. To ensure your anonymity, no personal data will be collected and following collection your tooth will be given a laboratory number code. No extra time or dental appointments will be required for this study. An extra 10 minutes will be required at your routine appointment prior to extraction to answer any questions you may have about the study and obtain written consent for participation.

There will be no extra cost incurred by you for participation in this study and there will be no remuneration. Normal fees for extraction of teeth will apply.

Is there any risk of discomfort or harm from participation?

There is no additional harm or discomfort from participating in this study.

What specimens, data or information will be collected, and how will they be used?
Your tooth tissue will be examined under a microscope to study factors in your pulp tissue. Cells may be grown from your tooth in the laboratory to observe how genes responsible for healing are affected.

At the end of the study we would like to store some of your tooth tissue or pulp cells in our secure laboratory for use in future studies looking at pulp healing. Any future studies will be carried out with appropriate ethical approval. Please notify the researchers if you do not want this to occur. The cultural issues associated with storing your sample should be discussed with your family/whānau.

Whole teeth will be securely stored in the tissue bank within the MedLab Dental Oral Diagnostic Laboratory within the Faculty of Dentistry. Cells will be securely stored for up to 5 years. Any tissue remaining at the end of this time will be disposed of as normal clinical waste.

**What about anonymity and confidentiality?**

No material which could personally identify you will be used in any reports in this study. The deidentified data collected will be stored securely so that only those directly involved in the research will have access to the records. Any raw data on which the results of the study depend, will be retained in secure storage for 10 years after the results are published, as required by the University's research policy, after which it will be destroyed.

**If you agree to participate, can you withdraw later?**

Your whole tooth will be required for this study and collected without personal identifying details so will be unable to be returned. You may withdraw consent prior to or immediately after tooth extraction but not following donation.

**Any questions?**

If you have any questions now or in the future, please feel free to contact either:
<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Contact phone number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Lara Friedlander</td>
<td>Senior Lecturer / Endodontist</td>
<td>03 479 7126</td>
</tr>
<tr>
<td></td>
<td>Department Faculty of Dentistry</td>
<td></td>
</tr>
<tr>
<td>Professor Alison Rich</td>
<td>Acting Dean/ Oral Pathologist / Director</td>
<td>03 479 5686</td>
</tr>
<tr>
<td></td>
<td>Medlab Dental Oral Diagnostic Laboratory</td>
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<tr>
<td>Dr Praveen Parachuru</td>
<td>Senior Lecturer / Periodontist</td>
<td>03 479 5189</td>
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<tr>
<td>Mr Harsha De Silva</td>
<td>Senior Lecturer / Maxillofacial surgeon</td>
<td>03 470 3556</td>
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<tr>
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<tr>
<td>Associate Professor Rohana De Silva</td>
<td>Maxillofacial surgeon</td>
<td>03 479 7031</td>
</tr>
<tr>
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<td>Faculty of Dentistry</td>
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This study has been approved by the University of Otago Human Ethics Committee (Health). If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (phone +64 3 479 8256 or email gary.witte@otago.ac.nz). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.
Angiogenesis in healthy and inflamed dental pulps

Principal Investigator:
Dr Lara Friedlander

Email: lara.friedlander@otago.ac.nz
Phone: 03 479 7126

CONSENT FORM FOR PARTICIPANTS
Following signature and return to the research team this form will be stored in a secure place for ten years.

Name of participant: ..........................................................

1. I have read the Information Sheet concerning this study and understand the aims of this research project.
2. I have had sufficient time to talk with other people of my choice about participating in the study.
3. I confirm that I meet the criteria for participation which are explained in the Information Sheet.
4. All my questions about the project have been answered to my satisfaction, and I understand that I am free to request further information at any stage.
5. I know that my participation in the project is entirely voluntary, and that I am free to withdraw from the project at any time prior to tooth donation without disadvantage.
6. I know that as a participant I will donate my whole tooth to this study and this tissue will be de-identified.
7. I understand that there is no additional harm or discomfort in my participation other than that associated with tooth extraction.
8. I know that when the project is completed all personal identifying information will be removed from the paper records and electronic files which represent the data from the project, and that these will be placed in secure storage and kept for at least ten years.
9. I understand that the results of the project may be published and be available in the University of Otago Library, but that either (i) I agree that any personal identifying
information will remain confidential between myself and the researchers during the study, 
and will not appear in any spoken or written report of the study □ I know that there is 
no remuneration offered for this study, and that no commercial use will be made of the 
data.

10. At the end of the study I consent to any remaining samples being disposed of using 
□ Standard disposal methods. 
□ Disposed with appropriate karakia

Signature of participant: ___________________________ Date: ___________________________

I understand that the tooth tissue samples and cells will be securely stored for use in future 
studies, any such study being subject to further ethical approval.

Signature of participant: ___________________________ Date: ___________________________
Appendix B – IHC sample preparation

Decalcification protocol

Post tissue fixation in 10 % neutral buffered formalin for 24 hours at room temperature, the teeth specimens were rinsed with PBS. This was followed by immersion of the specimens in 50-60 mL of 10 % EDTA and agitated at 4°C for 6-8 weeks. This solution was replenished with a fresh solution every 3-4 days. Once the tissue was suitably decalcified, it was trimmed with a sterile surgical scalpel blade, to ensure adequate orientation of the tissue samples. The samples were then placed again in a solution of EDTA for an additional 7-10 days. Prior to tissue processing and paraffin-embedding, the tissue samples were rinsed with two changes of PBS.

10% EDTA solution

- 50 g EDTA (Merck, Germany 1084181000)
- 250 mL distilled water

The pH of the freshly-mixed solution was adjusted to 7.4 by adding sodium hydroxide pellets (Merck, Germany 1064980500) and a 500 mL solution was prepared using distilled water.
## Appendix C – qPCR Tables

### Table A - Gene array in the customized TaqMan® Fast 96-Well Plates

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Table B - PCR TaqMan® Fast 96-Well Plates array individual plate format showing the gene location

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Table C - Cq values - Genes of interest referenced against the housekeeping genes (6 samples per plate). Samples 21 and 22 (shaded in grey) were eventually discarded from further analyses. Rpt – repeat, ND – Not detected.

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Table E – Fold-regulation values of individual Genes of interest

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<th>Fold-regulation (2^{\Delta\Delta C_q})</th>
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### PTPRC

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