Semaphorin Signalling in Neuronal and Vascular Development in the Tooth

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ABBREVIATIONS

+/+	Wild type
+/-	Heterozygous
-/-	Homozygous
2H3	Antibody against meduim chain neurofilament
AEC	3-amino-9-ethyl carbazol
ALK5	Activin receptor-like kinase
ALPL	Alkaline phosphatase
ANG	Angiopoietin
BARX1	BarH-like homeobox
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CGRP	Calcitonin gene-related peptide
CNS	Central nervous systm
COUP-TF	Chicken ovalbumin upstream promoter-transcription factor
Dll	Delta like ligand
DLX	Homeodomain transcription factor related to *Drosophila* distal-less
DRG	Dorsal root ganglion
E	Embryonic
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
Eda	Ectodysplasin
EphB	Ephrin B
ERK	Extracellular signal-regulated kinase
EU	Europian union
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Flk1	Fetal liver kinase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GPI	Glycosylphosphatidylinositol
HH	Hedgehog
HIF	Hypoxia inducible factor
HMG	High mobility group box domain
IGF	Insulin-like growth factor
IL	Interlukin

KDR	Kinase insert domain receptor
Lef1	Lymphoid enhancer factor
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
MSX	Vertebrate homologue of *Drosophila* muscle segment homeobox gene
MT-MMPs	Matrix metalloproteinases
NF200	Neurofilament 200
NG2	Chondroitin sulphate proteoglycan
Ngf	Nerve growth factor
NPN	Neuropilin
Nrf2	NF-E2-related factor 2
OCT	Optimal cutting temperature
PAX	Paired box-containing transcription factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PECAM	Platelet endothelial cell adhesion molecule 1
PFA	Paraformaldehyde
PGP9.5	Protein gene product 9.5
PITX1	Paired-like homeodomain
PLC	Phospholipase C
PN	Postnatal
PTEN	Phosphate and tensin homologue deleted on chromosome TEN
RT-PCR	Reverse transcription/polymerase chain reaction
Sema	Semaphorin
SHH	Sonic hedgehog
SMA	Smooth muscle actin
SP	Substance P
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WNT	Vertebrate homologue of *Drosophila* Wingless

SCIENTIFIC ENVIROMENT

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SUMMARY

Background: The tooth is a well-established model in which to study molecular signaling that regulates organ morphogenesis. Previously, organ-specific innervation of the tooth and its molecular regulation has, to some extent, been investigated. The findings show that semaphorin (Sema) signaling is required for innervation of the tooth and that semaphorin signalling is under local control of signaling molecules produced by the tooth. On the other hand, development of dental vasculature, and its regulation and relationship to dental innervation, is currently still obscure. **Objectives:** To investigate in detail development of tooth vascularization during embryogenesis and in the postnatal crown and early root development. Moreover, to compare the early development of tooth vasculature and innervation. To examine, in addition, expression of VEGF mRNAs during tooth organogenesis, as well as *in vivo* functions of SEMA3A and SEMA6A signaling in the development of tooth vasculature, innervation and morphogenesis. **Materials and methods:** RT-PCR on embryonic tooth germs and *in situ* hybridization on histological sections were applied in order to analyse and localize mRNAs. Immunohistochemistry was used to localize proteins in tissue sections. *Sema3A* and *-6A*-deficient mouse mandibular first molar tooth germs and wild-type teeth were used as model organ systems. **Results:** Growth and branching of dental blood vessels and expression VEGF mRNAs during early odontogenesis are regulated in the course of development. *Sema6A*-deficient mice show defects in timing of tooth innervation, but SEM3A and -6A are dispensable for the development of tooth blood vessels. **Conclusions:** Both the maturation of tooth nerve supply and blood vessels are developmentally but differentially regulated, and VEGF is suggested to serve key functions in the development of tooth vasculature. SEM3A and -6A signaling regulates tooth innervation but does not appear to play any essential role in tooth histomorphogenesis or development of its supporting tissues such as blood vessels *in vivo*. **Consequences:** Further experimental and molecular investigations are warranted with regard to gaining a better understanding of regulation of dental vascular development, and maturation, as well as the putative

functions of VEGF signaling on growth and patterning of blood vessels and neurites in the developing tooth.

LIST OF ARTICLES

Article I

Omnia Shadad*, Rajib Chaulagain*, Keijo Luukko and Paivi Kettunen.

Establishment of tooth blood supply and innervation is developmentally regulated and takes place through differential patterning process. (manuscript to be submitted)

Article II

Omnia Shadad, Paivi Kettunen, Masahiko Taniguchi and Keijo Luukko.

Dental vasculature develops independently of SEMA3A signaling and sensory innervation. (manuscript to be submitted)

Article III

Omnia Shadad, Kevin Mitchell, Keijo Luukko and Paivi Kettunen.

SEMA6A regulates sensory innervation, but is dispensable for vascular development and tooth morphogenesis. (manuscript to be submitted)

*Equal contribution

1. INTRODUCTION

1.1 Dentition in vertebrates

The majority of existing vertebrates develop teeth during their lifetime (Sire et al., 2008). Exceptions are for example birds, anteaters and baleen whales, which are edentulous (Naples, 1999; Mitsiadis et al., 2003; Friedman, 2012). Some toothed vertebrates, like fish and reptiles, have the characteristic of preserving/compensating their teeth by adding or replacing them on multiple occasions during their lifetime. This occurs in a highly controlled manner (polyphyodonty) (Whitlock and Richman, 2013). Most mammals, including humans, have the capacity of replacing their teeth only once during life, and have therefore only deciduous and permanent sets of teeth (diphyodonty) (Jernvall and Thesleff, 2012). On the other hand mice retain the same set of teeth for life (monophyodonty) (Jernvall and Thesleff, 2012). Teeth are rooted in the alveolar processes of the maxilla and mandible, which arise from the first branchial arch. Like other vertebrates, mammals also show an adaptation to their diet by having different types of teeth (Rodrigues et al., 2013). A human being has four different types of teeth: incisors to cut food into pieces, canines for tearing food, premolars and molars for crushing and breaking down food. Mouse dentition consists of two incisors and six molars in the jaw, no canines or premolars being available, but there is a long toothless diastema between the incisor and the first molar. Seven vestigial tooth germs have been identified in the maxillary diastema of the mouse. Their development, however, ceases already during embryogenesis and they are removed by apoptosis (Peterkova et al., 1998).

1.2 Development of mouse dentition

1.2.1 Mechanisms involved in mouse mandibular first molar tooth development

Tooth development starts during intrauterine life as a result of sequential and reciprocal interactions between odontogenic epithelium originating from the oral ectoderm, and mesenchymal tissues originating from the neural crest (Fig. 1) (Miletich and Sharpe, 2003; Thesleff, 2003). Like other organs, the development of the tooth is divided into three overlapping stages, which include initiation and morphogenesis as well as cell differentiation that are partly overlapping processes (Kollar and Lumsden, 1979; Peters and Balling, 1999; Thesleff, 2006). The first step of odontogenesis in the mouse is local epithelial thickening (dental lamina) occurring on embryonic day 11 (E11). Thereafter, the dental epithelium increases in size and invaginates into the mesenchyme, which then later starts to accumulate around the dental epithelial cells and form the condensed dental mesenchyme (Peters and Balling, 1999; Nanci, 2003). During this stage, non-dividing epithelial cells are located at the tip of the epithelial bud, and are defined as the primary enamel knot (Luukko et al., 2003). At the cap stage the lingual and buccal sides of the dental epithelium (enamel organ) extend and form the cervical loops, which later at the bell stage enclose the mesenchymal dental papilla. Later the papilla becomes dental pulp, whereas the mesenchymal cells around the dental epithelium and papilla form the dental follicle (Peters and Balling, 1999; Nanci, 2003).

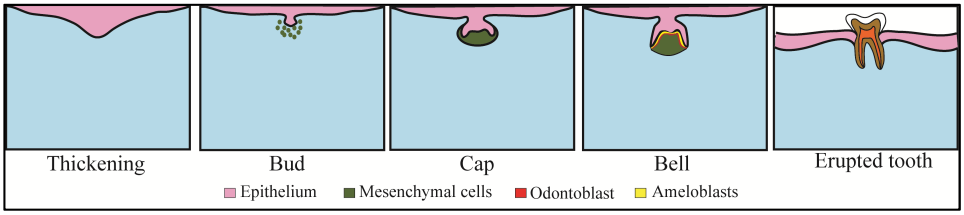

Figure 1. Schematic frontal view of the tooth development stages. Modified from (Tucker and Sharpe, 2004).

During the bell stage the enamel organ cells differentiate into four different cell types, which include inner and outer enamel epithelium cells, stellate reticulum and stratum

intermedium cells. Enamel knots are non-dividing cells, which share the same histological features and regulate tooth development (Butler, 1956; Jernvall et al., 1994; Vaahtokari et al., 1996a; Keranen et al., 1998; Thesleff et al., 2001) before they are eliminated by apoptosis (Vaahtokari et al., 1996b; Coin et al., 2000).

1.2.1.1 Mandibular molar tooth anatomy

A developed mandibular molar tooth (Fig. 2) consists of two parts: a multi-cusped crown, which is the visible part in the oral cavity, and the roots, which are the invisible part of the tooth that are embedded in the gingiva and alveolar bone. The crown of the tooth consists of two hard tissues: enamel that is the outer protective layer and dentin that is the inner hard tissue supporting enamel. The central part of the crown is known as the dental pulp, which is enclosed by the dentin and is intensely vascularized and innervated. The first molar is a two-rooted tooth, the root of which consists of dentin covered by the cementum layer (Nanci, 2003).

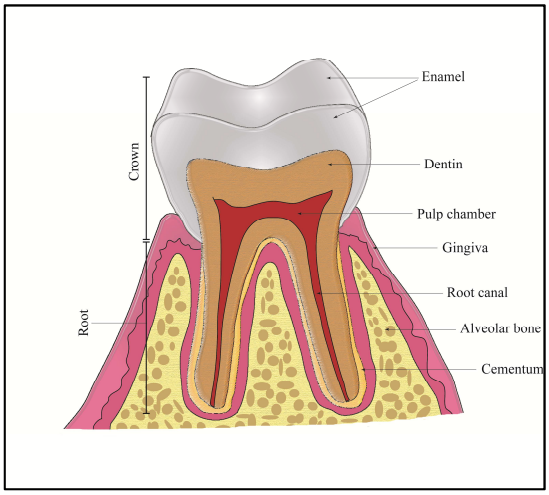

Figure 2. Molar tooth anatomy.

1.2.2 Enamel and dentin formation in the tooth crown

Dentin is a hard tissue, which forms the bulk of the tooth, and is softer than enamel but harder than bone. Odontoblasts, which differentiate from the ectomesenchymal cells of the dental papilla, are responsible for dentin secretion. Dentin formation starts with deposition of predentin, which is mineralised to form mature dentin. Secondary dentin deposition continues as long as the tooth is alive (Lesot et al., 2001; Nanci, 2003).

Enamel, which covers the outer surface of the crown, is highly mineralised and extremely hard. These properties allow the enamel to withstand the mechanical forces that are applied to the tooth. Enamel is secreted by ameloblasts derived from the inner enamel epithelia of the enamel organ. Unlike dentin, enamel formation takes place only before tooth eruption (Nanci, 2003). In the mouse mandibular first molar tooth, the tips of the cusps and the ridges between the cusps were found to be devoid of enamel (Hay, 1961; Luukko et al., 2003).

The process of hard tissue formation (dentin and enamel), which starts during the bell stage of tooth development is induced by molecular signals (Ruch et al., 1995; Lesot et al., 2001). These biological signals include among others, fibroblast growth factors (FGF), transforming growth factor (TGF) beta super family signals, insulin-like growth factor (IGF), bone morphogenetic proteins (BMP) and sonic hedgehog (SHH) (Begue-Kirn et al., 1992; Begue-Kirn et al., 1994; Ruch et al., 1995; Kettunen et al., 1998; Coin et al., 1999; Unda et al., 2000; Gritli-Linde et al., 2002; Takamori et al., 2008).

1.2.3 Root development

After completion of crown formation, root development starts (Cohn, 1957). The stellate reticulum and stratum intermedium cells become diminished in the cervical

loop and as a result a structure defined as an epithelial root sheath consisting of inner and outer enamel epithelia is formed (Berkovitz et al., 2009). It grows apically until the total length of the root is attained. The cementoblasts, cells in the dental follicle, lay down the third hard tissue of the tooth, namely, cementum, on the surface of the root dentin. Many signals and their intracellular downstream signaling pathways, which are active during crown formation are also utilized during root development such as SHH (Nakatomi et al., 2006), BMPs, and MSX2 (Yamashiro et al., 2003). FGF signaling is also involved in regulation of root formation by controlling epithelial root sheath development and thus shifts from crown to root formation (Yokohama-Tamaki et al., 2006). In addition to being necessary for dentin and enamel mineralisation DLX3 and Alkaline phosphatase (ALPL) are crucial for root development and formation of cementum, respectively, as well as for dentin and enamel mineralisation (Hu et al., 2007; Duverger et al., 2012; Millan and Whyte, 2015).

1.2.4 Molecular control of odontogenesis

Teeth develop as a result of reciprocal and sequential interactions between the dental epithelium and mesenchyme. These interactions are regulated by essential signaling molecules, which stimulate transcription factor expression and this regulates expression of other genes (Thesleff, 2014). In the mouse, patterning of the teeth in the jaw has been shown to be controlled by local tissue interactions involving non-*Hox* homeobox and other transcription factors (Miletich and Sharpe, 2003). Location of incisors in the distal region and molars in the proximal region of the jaw are regulated by MSX1 and MSX2, as well as DLX1, DLX2, BARX1 and PITX1, respectively (Tucker et al., 1998; Jussila and Thesleff, 2012). Recently FGF and SHH have been proposed to control dental epithelial stratification at the lamina stage, and further invagination to form a dental bud, respectively (Li et al., 2016).

Five signaling molecule families are considered as key regulators of tooth development: BMP, FGF, WNT, Hedgehog and Ectodysplasin (Eda) families (Satokata and Maas, 1994; Kettunen et al., 2000; Miletich and Sharpe, 2003; Tucker and Sharpe, 2004; Thesleff, 2006; Jussila and Thesleff, 2012). They are repeatedly used during tooth initiation, morphogenesis and renewal (Jernvall and Thesleff, 2012). Interestingly, an integrated network of inhibitors and activators of BMP, FGF, SHH and WNT regulate the number and patterning of teeth along the row of teeth. In addition, the ectodysplasin (EDA) signaling pathway is involved in regulation of tooth size, shape and number (Lan et al., 2014). Many of the signaling molecules are expressed in three transient signaling centers, termed enamel knots. The first center is present in the dental placode stage when tooth development starts, the second center is the primary enamel knot at the bud and cap stage, and the third signaling center is the secondary enamel knot that is seen in the bell stage molar tooth germ. The enamel knots determine the location of the tooth cusps (Jernvall et al., 1998; Thesleff et al., 2001; Thesleff, 2014).

Several transcription factors, such as members of the homeobox, paired-box and zinc-finger transcription factor families, have been demonstrated to be necessary for odontogenesis. One homeobox gene, *Msx1,* has been reported to be regulated by BMP4 and FGF (Vainio et al., 1993; Chen et al., 1996; Bei and Maas, 1998; Kettunen and Thesleff, 1998). In *Msx1* deficient mice arrest of tooth development occurs at the bud stage (Satokata and Maas, 1994). In mice lacking both *Msx1* and *Msx2,* tooth development terminates even earlier at the lamina or early bud stages (Bei and Maas, 1998). In man missense mutation in *MSX1* give rise to tooth agenesis (Vastardis et al., 1996). Other homeobox genes that are induced by FGF8 and BMP4 are *Dlx1* and *Dlx2* (Bei and Maas, 1998). Mice lacking both *Dlx1* and *Dlx2* genes show arrest in the lamina stage in maxillary molar development (Qiu et al., 1997; Thomas et al., 1997). mRNA expression of a paired-box gene PAX9 is controlled by FGF8 and BMPs (Neubuser et al., 1997). In *Pax9* deficient mice all tooth germs stop their development at the bud stage (Peters et al., 1998). In man mutation in *PAX9* give rise

to oligodontia (Stockton et al., 2000). Supernumerary maxillary incisors have been reported in mice that have a *Pax6* mutation (Kaufman et al., 1995). *Lef1*, which is expressed in the enamel knot, is one of the HMG-box genes, which is regulated by BMP4 (Kratochwil et al., 1996). *Fgf4* expressed in the enamel knot, is a transcriptional target of *Lef1* in the dental epithelium. On the other hand FGF4 is fully able to rescue the Lef1$^{-/-}$ tooth germs showing the developmental arrest (van Genderen et al., 1994; Kratochwil et al., 1996; Kratochwil et al., 2002). *Shh* is a dental epithelium expressed gene, the protein product of which regulates dental cell proliferation and tooth morphogenesis (Dassule et al., 2000). *Gli1-3,* a member of the zinc-finger transcription factor family, is expressed in the tooth germ and belongs to the SHH signaling pathway (Hardcastle et al., 1998). *Gli2* deficient mice show tooth defects mainly in the maxillary incisors, and *Gli2/Gli3* double knockout mice lack all teeth. In addition, *Gli2*$^{-/-}$*;Gli3*$^{+/-}$ mice have small molars and mandibular incisors but no maxillary incisors (Mo et al., 1997; Hardcastle et al., 1998). Gli1 is also a part of the BMP-Smad4-SHH-Gli1 signaling network, which is suggested to provide a niche supporting transient Sox2+ dental epithelial stem cells in mouse molars (Li et al., 2015). *Activin βA* is a member of TGFβ superfamily and regulated by FGF8 in dental mesenchyme. On the other hand, Activin *βA* itself stimulates follistatin expression in oral epithelium. In *Activin βA* deficient mice all teeth, except maxillary molars, showed arrested development at the bud stage (Ferguson et al., 1998). Like *Activin βA, follistatin* was found to be dispensable for maxillary molar tooth development (Matzuk et al., 1995; Ferguson et al., 1998). Follistatin however inhibits asymmetrically BMP signaling and ameloblast differentiation in mouse incisors and consequently regulates enamel patterning in the tooth (Wang et al., 2004). The correct amount of BMP in tooth germ is critical for tooth cusp patterning and number of teeth, as displayed in BMP inhibitor ectodin-deficient mice (Kassai et al., 2005). Gene modified mice have shown functions for canonical Wnt/beta catenin signaling in tooth renewal (Jarvinen et al., 2006), molar tooth morphogenesis (Chen et al., 2009) and determination of incisor tooth number (Fujimori et al., 2010). In an ever-growing mouse incisor, cervical loop located stem cells secure cell proliferation and differentiation of tooth specific cells. A stem cell marker, Sox2, which is also

expressed in epithelial dental stem cells, was recently demonstrated to contribute to all epithelial lineages of the incisor via Sfrp5 positive progenitor cells (Juuri et al., 2012).

1.2.5 Blood supply in adult mandibular molar tooth

Like other organs, teeth require a healthy vascular network to maintain their metabolic system: they receive oxygen and nutrients, as well as they eliminate waste material through blood vessels. Starting from the arch of the aorta, the first mandibular molar tooth on the right side gets its arterial supply through the brachiocephalic artery, which gives off a vessel called the right common carotid artery from which branches the right external carotid artery. From the latter arises the right maxillary artery, and this gives a branch – the right inferior alveolar artery, from which arterioles provide the blood supply to the mandibular first molar on the right side. The arterial supply to the molar on the left side is similar, except that the left common carotid artery arises directly from the arch of the aorta (Berkovitz et al., 2009).

The dental pulp is composed of soft connective tissue, which is richly vascularized by blood vessels that enter and exit the pulp through the apical and accessory foramina and lateral canals in the root (Seltzer and Bender, 2012). Smooth muscle cells, enwrapped with arterioles, are associated with nerve bundles, which envelop partially or totally arterioles, in the root and to the crown pulp (Tuisku and Hildebrand, 1995; Steiniger et al., 2013). In addition, smooth muscle cells of arterioles are innervated by sympathetic nerves (Rodd and Boissonade, 2003). The largest arterioles in the human tooth pulp are about 150µm in diameter. Arterioles are principally centrally located in the pulp and give off many thin branches to form an extensive vascular capillary network in the periphery of the pulp adjacent to the odontoblast layer. This is defined as the subodontoblastic capillary plexus (Takahashi et al., 1982). Capillaries are 6-8

μm in diameter. They are connected to postcapillary venules, which form the drainage system in the dental pulp. They have an incomplete coverage of pericytes. The venules have larger lumina than arterioles and their endothelial wall contains a discontinuous arrangement of either pericytes or smooth muscle cells. Capillaries and venules are not associated with nerve bundles (Steiniger et al., 2013). Furthermore, arterio-venous anastomoses have also been identified in the dental pulp (Provenza, 1958; Kramer, 1960). Results pertaining to the existence of lymphatic vessels in dental pulp have not been in full agreement. Recent results, however, strongly suggest that the dental pulp in humans, and some animals like the dog, is devoid of lymphatic vessels (Gerli et al., 2010; Martin et al., 2010). The periodontal space around the dental root is highly vascularized (Chintakanon and Sims, 1994). The blood supply to the periodontal space is derived from inferior and superior alveolar arteries, and in addition, the lingual and palatine arteries have also been suggested to be involved. Furthermore, blood vessels from alveolar bone also contribute to the blood supply of the periodontal ligament (Berkovitz et al., 2009).

1.2.5.1 Development of blood vessels into the dental pulp

In vertebrates the cardiovascular system is the first functional organ system that is developed during embryogenesis (Flamme et al., 1997). At E8.5-9 from the aortic sac of the heart grow the paired ventral aortae, which continue as a primitive aortic arch on the right and left side of the pre-oral gut to be connected with the paired dorsal aorta in the dorsal side of the embryo. Regarding the blood supply of the first branchial (pharyngeal) arch, the primitive maxillary artery branches from the primitive aortic arch at E9-9.5 and the first pharyngeal artery arises from the ventral aorta at E9.5-10. Subsequently, formation of an adult-type vascular system in the face requires complex reorganization of the pharyngeal arch arteries (Hiruma et al., 2002). A primitive blood vessel network is present in the jaw mesenchyme, under the oral epithelium, prior to the histological sign of tooth development. No blood vessels are, however, detected in the dental mesenchyme but rather in the peridental mesenchyme

after initiation of tooth development in the bud stage (Rothova et al., 2011; Yuan et al., 2014). At the cap stage blood vessels have been reported to be present in the dental follicle (Nait Lechguer et al., 2008; Rothova et al., 2011; Yuan et al., 2014). It has also been earlier documented that the first blood vessels from the vascular plexus surrounding the dental papilla and the enamel organ, start to enter the dental papilla at the late cap stage (E15) (Rothova et al., 2011).

Before odontoblast differentiation in the dental pulp, blood vessels are observed in the central pulp relatively distant from the inner dental epithelium both in mouse and rat. When the odontoblasts start to differentiate, blood vessels head towards the odontoblast layer. With the launch of predentin secretion, capillaries, which become fenestrated, start to mingle between the odontoblasts and reach their most peripheral location close to the predentin (Yoshida and Ohshima, 1996). Subsequently, once the formation of dentin slows down, fenestrations in the capillaries are no longer detected and capillaries become continuous. Moreover, capillaries withdraw from the odontoblast layer and become relocated to the subodontoblastic area (Yoshida et al., 1988; Yoshida and Ohshima, 1996). The first blood vessels have been reported to enter the epithelial enamel organ and grow to the stratum intermedium layer next to the ameloblasts at PN2 (Decker, 1967; Nait Lechguer et al., 2008).

Blood vessel formation in the dental follicle has been suggested to occur via vasculogenesis (Yuan et al., 2014), and dental papilla vascularization has been proposed to take place via angiogenesis (Rothova et al., 2011; Yuan et al., 2014). It is likely that formation of new blood vessels into the enamel organ of the molar tooth occurs through vasculogenesis and angiogenesis (Manzke et al., 2005).

1.2.6 Innervation of the tooth

The dental pulp of the tooth is richly innervated by both nociceptive sensory and sympathetic nerves (Mohamed and Atkinson, 1983; Hildebrand et al., 1995; Luukko, 1997; Fried et al., 2000; Kettunen et al., 2005; Haug and Heyeraas, 2006). The sensory nerves are derived from the trigeminal ganglion (Tsuzuki and Kitamura, 1991; Hildebrand et al., 1995; Fried et al., 2000), and the sympathetic nerve fibers are from the superior cervical ganglion (Tsuzuki and Kitamura, 1991; Hildebrand et al., 1995). Currently the evidence for the presence of parasympathetic nerves in the dental pulp is weak (Sasano et al., 1995). Three different types of nerve fibers have been located in the dental pulp, so-called A-beta, A-delta and C-fibers. Myelinated A-beta fibers are responsible for pre-pain sensation (Byers and Narhi, 1999). Myelinated A-delta fibers and unmyelinated C-fibers mediate sharp-acute and dull-thermal pain sensations, respectively (Byers et al., 2003). Generally, besides meditating pain sensation, nerve fibers may regulate vascularization and inflammation as well as immune and healing systems (Hildebrand et al., 1995; Byers and Narhi, 1999; Byers et al., 2003).

The mandibular first molar tooth is supplied by the mandibular nerve, which arises from the inferior alveolar nerve that is a branch of the trigeminal nerve. The nerve axons reach the mandible before there is any histological sign of tooth development. At the early bud stage of tooth development, the molar nerve is directed towards the mandibular first molar tooth (Luukko, 1997; Loes et al., 2002; Kettunen et al., 2005). Later at the bud stage axons give rise to first buccal and then lingual branches next to the tooth germ (Loes et al., 2002). Even though a heavy concentration of nerve fibers is present in the dental follicle already at the cap stage and continues to be present there at the bell stage, branches of nerve fibers invade into the dental pulp for the first time at PN3-4, once a thin layer of dentine and enamel had already been laid down (Mohamed and Atkinson, 1983; Luukko, 1997; Moe et al., 2008). Interestingly, in the mandibular first molar, which contains two roots, sensory nerve fibers invade the

dental pulp from the locations where the future roots will develop prior to any physical sign of root formation (Luukko et al., 2008). It is noteworthy that the pioneer axons of sympathetic innervation follow the sensory nerves and enter the dental pulp at PN9, after the onset of root formation (Moe et al., 2008).

1.3 Mechanisms of vascularization

Vascularization is a multistep process, starting after formation of mesodermal tissue in which precursors of blood vessels and cells differentiate, and finally establish the cardiovascular system. This provides all cells in the organism with gas exchange, fluid, nutrients and removal of waste products. Vascularization, establishing development of some large vessels, is followed by angiogenesis, which modifies the already existing vascular network and causes formation of novel vessel branches from old ones. A vascular network is created before the heart becomes functional. Various parts of the vasculature will acquire different physiological and anatomical phenotypes in order to respond to different requirements of the cells and tissues, in an organ-specific manner. Smooth muscle cells and pericytes, which surround the endothelial cells, are involved in maturation and stabilization of the vessels (Cleaver and Krieg, 2010). Blood vessel networks are distributed in complex tissues and organs in such a way that ensures location of each cell within 100-200um of a blood vessel, which is the diffusion limit for oxygen (Folkman, 1971).

1.3.1 Vasculogenesis

Vasculogenesis is defined as the *de novo* adhesion and accumulation of the individual free angioblasts in the lateral plate mesoderm to form primary vascular plexus and vascular tube primordia of the major blood vessels such as the dorsal aortae, posterior cardinal veins, vitelline veins and the endocardial tube (Risau and Flamme, 1995; Cleaver and Krieg, 1998). Vasculogenesis occurs in two different ways. Angioblasts stay in the place where they originally differentiated in the mesoderm, and

subsequently they further differentiate and mature into endothelial cells without significant migration. Alternatively, angioblasts show a significant migration from their first original position within the mesoderm to associate with other angioblasts in another distant position where the primary vascular plexus is formed (Noden, 1989; Poole and Coffin, 1989; Christ et al., 1990; Noden, 1990; Coffin and Poole, 1991; Cleaver and Krieg, 1998; Schmidt et al., 2007). In addition to intraembryonic mesoderm, vasculogenesis also occurs in extraembryonic mesoderm (Pardanaud et al., 1987; Peault et al., 1988; Ferkowicz and Yoder, 2005) (Fig. 3).

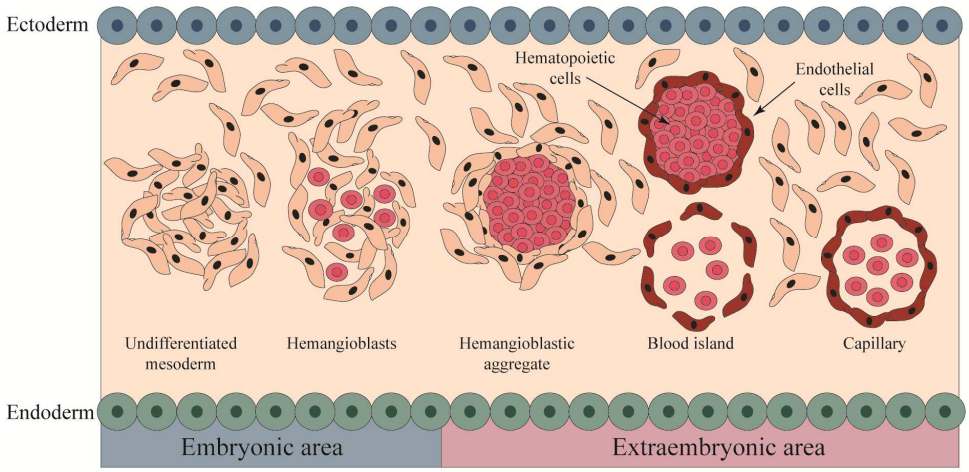

Figure 3. Hemangioblasts are the precursors of both hematopoietic stem cells and angioblasts. Hemangioblasts aggregate and differentiate into hematopoietic stem cells at the center and angioblasts at the peripheral part of the aggregate, forming the blood islands. Adapted from Heart Development and Regeneration.Rosenthal, Nadia. Harvey, Richard P. (Cleaver and Krieg, 2010).

1.3.2 Angiogenesis

After formation of the primary vascular plexus via vasculogenesis, and in order to meet the demands of the more developed and complex organism, an additional advanced blood vessel network is needed and this is generated through a more complex process, termed angiogenesis. Angiogenesis is a process whereby new blood vessels sprout and form from a pre-existing vascular plexus. Angiogenesis is established through two different ways, sprouting of the pre-existing blood vessels to form novel vessel branches to supply avascular tissues, or by splitting of the primary blood vessels to engender new ones via a process called angiogenic remodeling or non-sprouting angiogenesis (Risau, 1997). Sprouting and non-sprouting angiogenesis takes place in parallel during blood vessel development (Caduff et al., 1986; Risau, 1997; Adams and Alitalo, 2007). In addition to being necessary in embryonic development, normal angiogenesis is required in wound healing, in the female reproductive cycle and in formation and growth of bone. Aberrant angiogenesis engenders several pathological conditions (Robinson and Stringer, 2001). Moreover, there is evidence that blood vessel-expressed signals are needed for organogenesis and cell differentiation, like in early development of both the liver and pancreas (Lammert et al., 2001; Matsumoto et al., 2001).

1.3.2.1 Sprouting angiogenesis

The first step in sprouting angiogenesis is removal of pericytes and degradation of the extracellular matrix by enzymes like matrix metalloproteinases (MT-MMPs) that are released from pre-existing blood vessels (Davis and Senger, 2005; Cleaver and Krieg, 2010). However, MT-MMPs appear not to be absolutely necessary for sprouting angiogenesis during embryogenesis because the basement membrane is thin, or even absent during development (De Smet et al., 2009). Subsequently an endothelial cell termed a tip cell becomes different from the other surrounding endothelial cells and starts to sense and respond to guidance cues in their microenvironment and subsequently migrate and navigate forward (Dorrell et al., 2002) (Fig. 4). The

function of the tip cell is to guide the new sprout utilizing numerous filopodia in the leading edge.

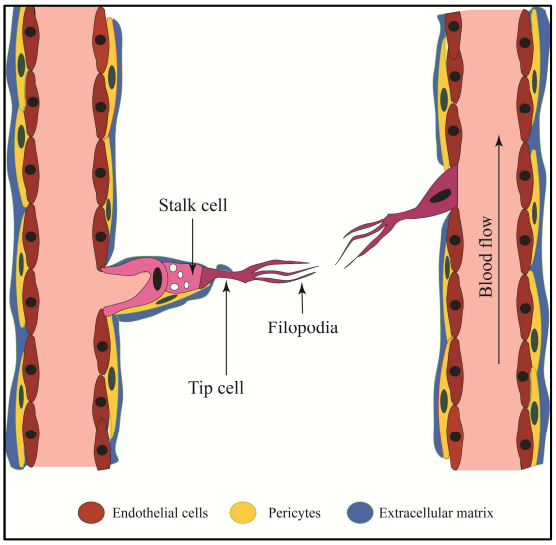

Figure 4. Schematic illustration of the angiogenic sprouting. Adapted from (Adams and Alitalo, 2007).

Tip cells express several growth factors and their receptors such as VEGFR2, VEGFR3, platelet derived growth factor-b (PDGF-B), Unc5B, Delta-like ligand-4 (Dll4), neuropilin-1 (NPN1) and MT1-MMP (De Smet et al., 2009). Endothelial cells abutting the tip cells differentiate into cells, defined as stalk cells, that have the ability to proliferate and subsequently elongate as a new vessel sprout, create a lumen, lay down basement membrane and then connect to the circulation (Marin-Padilla, 1985; Gerhardt et al., 2003; Gerhardt and Betsholtz, 2005). Selection of tip or stalk cell identity is under the control of VEGF/notch-dependent regulatory mechanisms (Geudens and Gerhardt, 2011). Tip cells and stalk cells show functional plasticity and are capable of changing their phenotypes depending on the balance between pro-angiogenic factors and suppressors of endothelial cell proliferation. An important pro-angiogenic factor is VEGF, expression and regulation of which creates a specific extracellular gradient for proper vascular patterning. Migration of tip cells depends on a VEGF gradient, and proliferation of stalk cells is concomitantly regulated by VEGF (Gerhardt, 2008; Ribatti and Crivellato, 2012). Once a vessel branch and blood flow

inside it is established, endothelial cells become non-proliferating and quiescent as well as maintain lumen and create an orderly shaped endothelial cell layer of the blood vessels with a cobblestone appearance. These cells are the third recently identified endothelial cell type called phalanx cells (Mazzone et al., 2009). They express VEGFR1 and VE-cadherin, deposit basement membrane and establish cell junctions (Mazzone et al., 2009).

1.3.2.2 Non-sprouting angiogenesis

Non-sprouting angiogenesis, also known as intussusception, refers to the division and splitting of the main blood vessel lumen by a tiny hole into two parts through insertion of endothelial columns which make a perforation named as a tissue pillar or post. The pillar enlarges gradually without any change in its own structure and results in increased complexity of the vascular network and to formation of new blood vessels (Fig. 5) (Caduff et al., 1986; Burri and Tarek, 1990; Patan et al., 1996; Burri and Djonov, 2002).

A comparison of sprouting and intussusceptive angiogenesis shows that the latter displays a low level of proliferation whereas in sprouting angiogenesis a high level of endothelial cell proliferation is needed. Therefore, intussusception angiogenesis needs less time and energy as well as lower metabolic activity compared to that of sprouting angiogenesis (Burri and Djonov, 2002). Intussusception angiogenesis has been reported to be detected in various tissues and organs such as the lung, kidney, liver, brain, intestinal tract, ovary and uterus (Burri and Djonov, 2002). However, the physiological role and molecular regulation of intussusception are still obscure (Adams and Alitalo, 2007).

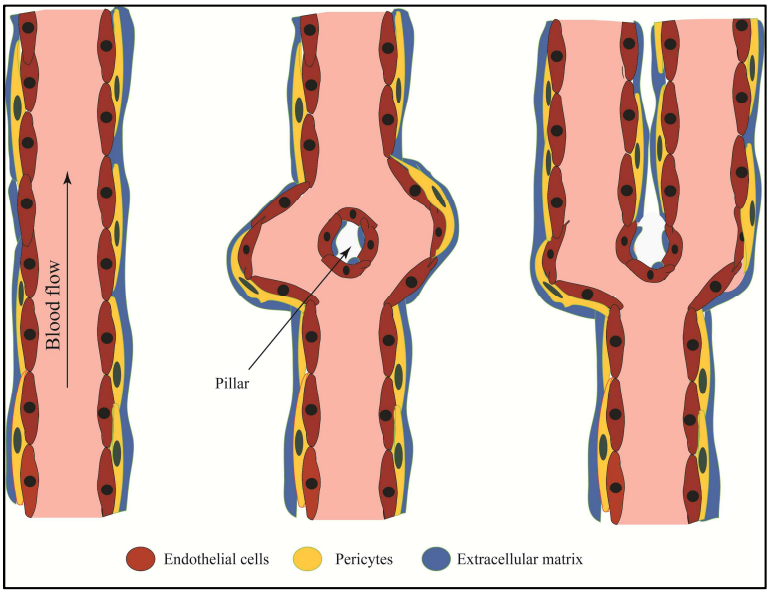

Figure 5. Schematic illustration of non-sprouting angiogenesis. Degradation of the extracellular matrix (ECM) accompanied by perforation of the normal blood vessel lumen by a hole that is known as a pillar, which will gradually increase in size and end up with the splitting of the main blood vessel lumen and formation of two new blood vessels with the deposition of new ECM. Adapted from (Adams and Alitalo, 2007).

1.3.3 Molecular regulation of vascularization

Like any other biological process, blood vessel development is under tight molecular control. Various steps in vascularization such as differentiation precursor and endothelial cells, vasculogenesis, creation of an appropriate microenvironment, tubulogenesis, angiogenesis, arterio-venous patterning and recruitment of mural cells are processes that are regulated by coordinated activity of proteins including angiogenetic molecules, classic axon guidance molecules and morphogens and their nascent intracellular signaling pathways (Carmeliet and Tessier-Lavigne, 2005; Carmeliet and Jain, 2011). In addition, extracellelar matrix molecules and microRNAs are involved in regulation of vascularization (Wang et al., 2008; Cleaver

and Krieg, 2010). Subsequently, selected signaling molecules and their receptors, which are involved in regulation of vascular development, are presented below.

FGFs

Fibroblast growth factor (FGF) was the first purified molecule reported to induce mesoderm in *Xenopus*, and subsequent findings showed additionally that the induced tissues included blood islands (Slack et al., 1987). Later Flamme and Risau demonstrated in dissociated quail epiblasts that FGFs can induce differentiation of endothelial and hematopoietic cells, and in long-term culture vasculogenesis (Flamme and Risau, 1992). During primitive hematopoiesis in the chick, high FGF4 and -8 activity mediated by FGFR2, promotes endothelial cell fate and hinders primitive blood cell differentiation (Nakazawa et al., 2006). Functions for FGF signaling in mouse angiogenesis have also been reported. FGFs are involved in maintenance of vascular integrity (Lee et al., 2000). In the eye FGF signaling has been suggested to be involved in choroidal angiogenesis (Rousseau et al., 2000). In addition, Sprouty-4, which is an FGF inhibitor, hinders angiogenesis by downregulating branching and sprouting of small vessels (Lee et al., 2001a). FGF9 is needed *in vivo* for development of mouse coronary blood vessels (Lavine et al., 2006).

VEGF

In mammals, the vascular endothelial growth factor (VEGF) cysteine knot family includes five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (Ellis and Hicklin, 2008). VEGF-A is also termed VEGF and vascular permeability factor. cDNA of VEGF was characterized in 1989 (Ferrara, 2004). The mouse gene for VEGF resides on chromosome 17 (De Gregorio et al., 1997). The coding region spans about 14 kb and contains eight exons (Shima et al., 1996). Alternative splicing of mRNA generates at least four distinct isoforms in mice, namely VEGF120, VEGF144, VEGF164 and VEGF188 (Ruhrberg, 2003; Ferrara,

2004). A shorter form, VEGF115, has been demonstrated in immortal mouse embryonic fibroblasts (Sugihara et al., 1998). Exon six is responsible for binding of VEGF188 and VEGF144 to heparin-sulphate proteoglycans in extracellular matrix. Exon seven in VEGF164 is responsible for moderate diffusibility of this isoform. VEGF120 lacks exons six and seven and is highly soluble (Ferrara, 2004).

mRNAs coding for VEGF120, VEGF144, VEGF164 and VEGF188 have been reported during mouse embryogenesis. The expression levels of individual isoforms appear to be organ specific, but VEGF164 seems to be the isoform present in the majority of organs during embryogenesis (Ng et al., 2001; Mukouyama et al., 2002; Ruhrberg et al., 2002). Expression of VEGF mRNA is dynamic and prominent in the tissues adjacent to the developing blood vessels (Breier et al., 1992; Dumont et al., 1995; Flamme et al., 1995; Cleaver et al., 1997). Several growth factors and cytokines such as PDGF, TNF-alpha, TGF-alpha, TGF-beta, FGF4, FGF7 EGF, IL-1alpha, IL-1beta, IL6 and IGF upregulate VEGF mRNA expression or stimulate VEGF release from cells (Robinson and Stringer, 2001). *Vegf* expression is additionally upregulated via hypoxia (Lee et al., 2001b; Nanka et al., 2006), that occurs as a result of activation of the transcription factor hypoxia inducible factor-1 (HIF-1) (Campochiaro, 2000). VEGF acts as chemoattractant to the nearby blood vessels to supply the avascular tissues. Once the tissues become vascularized and the requirement for oxygen is met, then the VEGF level diminishes gradually (Stone et al., 1995).

VEGF is secreted as a homodimer and has a molecular weight of about 45kDa (Ferrara, 2004). It is able to trigger proliferation, migration, specialization and survival of endothelial cells (Ferrara, 2004). In addition to having roles in blood vessel formation, VEGF has also neuronal function. It has been reported to trigger Schwann cell proliferation *in vitro* (Sondell et al., 1999b; Sondell et al., 1999a). In addition, it increases number, length and size of neurites in primary CNS neuron cultures (Rosenstein et al., 2003; Khaibullina et al., 2004). VEGF is able to guide migrating brachiomotor neurons in the hindbrain, and stimulate the contralateral growth of retinal ganglion cell axons across the optic chiasm (Tillo et al., 2015). In

the CNS it acts as a chemoattractant to commissural axons through (Erskine et al., 2011; Ruiz de Almodovar et al., 2011). Using VEGF null mice it was shown that *in vivo* VEGF is necessary for early hematopoiesis, embryonic vasculogenesis and angiogenesis. Inactivation of a single VEGF allele resulted in lethality at E11-E12 (Carmeliet et al., 1996; Ferrara et al., 1996). Findings from conditional knockout mice displayed that VEGF-gene dosage is critical during embryogenesis (Ferrara, 2004). One VEGF isoform can support blood vessel development in the mouse, but features of the vascular network depend on which isoform is present. VEGF120 triggers vessels with few and less branches whereas VEGF188 induces formation of abnormally thin and numerous vessels. Other findings have suggested that normal vessel formation is best supported by the VEGF164 isoform (Ruhrberg, 2003; Adams and Alitalo, 2007).

VEGF exerts its functions via tyrosine kinase receptor vascular endothelial growth factor receptor 2 (VEGFR2/KDR (kinase insert domain receptor), in the human; Flk1 (fetal liver kinase), in mice). The expression of VEGFR2 transcripts is initiated at E7 in the embryonic and extraembryonic mesoderm in areas that give rise to the vasculature. Endothelial cells exhibit nearly exclusively VEGFR2 expression (Dumont et al., 1995). VEGFR2 knockout mice die at E8.5-9.5 because they lack vasculogenesis, blood islands and organized blood vessels (Shalaby et al., 1995; Habeck et al., 2002). Like *VEGF*, *VEGFR2* expression is diminished after the tissue blood supply requirement is met (Dumont et al., 1995). VEGFR1 has high affinity for VEGF (de Vries et al., 1992). VEGFR1 is considered to act as a negative regulator of angiogenesis (Hiratsuka et al., 1998; Fong et al., 1999).

Neuropilin-1 (NPN1) is a transmembrane receptor, identified as a third VEGF receptor that is strongly expressed in endothelial cells. Two neuropilins exist, namely NPN1 and NPN2 (Kolodkin et al., 1997; Soker et al., 1998). *Npn1* is expressed in the arteries and *Npn1* null mice show different types of vascular defects (Kawasaki et al.,

1999; Herzog et al., 2001). In contrast, *Npn2* expression is prominent in veins and deletion of *Npn2* unexpectedly gives rise to lymphatic defects rather than vascular defects (Herzog et al., 2001; Yuan et al., 2002). NPN1 binds only to VEGF164 and VEGF189 (VEGF188 in mouse) isoforms (Soker et al., 1998; Tillo et al., 2015). NPN1 plays a critical role in angiogenesis by acting as a co-receptor and enhancing the ability of VEGFR2 to bind VEGF (Soker et al., 1998; Soker et al., 2002; Kofler and Simons, 2015) (Fig. 6). NPN1 can however also exert its angiogenetic function independently of VEGFR2 (Aspalter et al., 2015). NPN1 mRNA is also expressed in sensory and sympathetic neurons (Kolodkin et al., 1997). NPN1 mediates the activity of semaphorin in nervous tissue development and VEGF in development of the heart and vasculature (Gu et al., 2003).

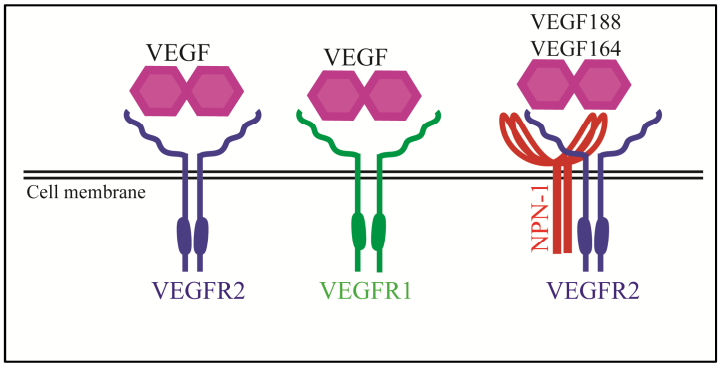

Figure 6. All VEGF isoforms bind to VEGFR2 and VEGFR1 receptors. VEGF164 or VEGF188 isoforms can bind to Nrp-1with the association of VEGFR2. Modified from (Ruhrberg, 2003; Tillo et al., 2015).

PDGF

The PDGF family consists of five dimeric isoforms generated by four gene products, these are PDGF-AA, -AB, BB, -CC and -DD. Platelet derived growth factor-B (PDGF-B) is a ligand for PDGFR-alpha and PDGFR-beta receptors. PDGF-B mRNA is expressed in endothelial cells, especially in tip cells whereas its receptor *PDGFR-beta* is expressed in mural cells (Gerhardt et al., 2003). Studies in transgenic mice

have demonstrated that PDGF-B has a function related to recruitment of pericytes onto capillaries. Data from PDGF-B and PDGFR-beta null mice has shown that the absence of pericytes gives rise to endothelial hyperplasia and defective vascular morphogenesis, subsequently resulting in late embryonic lethality because of significant hemorrhage from capillary vessels (Leveen et al., 1994; Soriano, 1994; Hellstrom et al., 2001). Other molecules involved in recruitment of pericytes, and thus vessel stabilization, are angiopoietin-1 (ANG1)-TIE2, TGF-beta1, activin receptor-like kinase 5 (ALK5) and notch signaling pathway (Geudens and Gerhardt, 2011). In the head, where the majority of smooth muscle cells have a neural crest origin, MSX1 and MSX2 genes define a subset of mural cell precursors necessary for vessel maturation (Lopes et al., 2011).

Notch

The Notch gene family encodes Notch transmembrane receptors 1-4 and plays an essential role in regulating vascular development and remodeling. *Notch1* and *Notch4* genes are expressed in endothelial cells and deletion of *Notch1* gives rise to defects in vascular remodeling (Reaume et al., 1992; Krebs et al., 2000). Notch ligands, which are transmembrane proteins, are encoded by five different genes *Jagged-1* and *-2*, *Dll1*, *-3* and *-4*. Arteries, arterioles and capillaries, but not venules and veins show *Dll4* expression in vasculature (Shutter et al., 2000; Villa et al., 2001). Consequently, *Dll4* heterozygote mice exhibit early embryonic lethality because of arteriovenous malformations (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). Notch signaling is of importance for determination of tip and stalk cell phenotype. The tip cell is associated with high expression of Dll4, which induces Notch expression in adjacent stalk cells and hinders them from acquiring the tip cell phenotype. Subsequently, once notch is activated in stalk cells their proliferation ceases, which is mediated by PTEN (Serra et al., 2015).

Experimental data indicated some time ago that the Notch signaling pathway, which acts downstream of VEGF, has an important role in arterial/venous specification and that it is upstream of EphrinB2/EphB4 signaling (Gridley, 2007). *EphrinB2,* which is expressed in arteries and its receptor *EphB4* that is expressed in veins are also involved in differentiation of arteries and veins. *EphrinB2* null mice indicated for the first time that differences between arteries and veins are at least partly genetically determined (Wang et al., 1998). Differentiation of major arteries in zebrafish was reported to involve notochord expressed SHH, which upregulates *Vegf* in somites and the latter consequently activates Notch signaling (Lawson et al., 2002). Recently, new upstream regulators of Notch signaling during differentiation of arteries and veins have been characterized. Wnt/beta-catenin upregulates expression of Sox17 transcription factor, which in turn, induces Notch signaling in endothelial cells (Corada et al., 2013). Another pathway in artery differentiation downstream of VEGF is the PLCgamma/MAPK pathway (Lamont and Childs, 2006). On the other hand, in vein differentiation COUP-TFII (Nr2f2) downregulates NPN1 expression, which is VEGF receptor. Moreover, activated PI3K/Akt hinders ERK activation in endothelial cells (Gridley, 2007). Findings pertaining to the functions of the Notch pathway in vascular development indicate that the pathway is iteratively used in blood vessel development.

Extracellular matrix proteins and cell adhesion molecules

In the beginning of vasculogenesis the fibronectin-rich extracellular matrix (ECM) forms a microenvironment in which the first blood vessels develop. Angioblasts actively lay down and organize ECM, which shows dynamic changes during vascular development. Observations from fibronectin deficient mice show that fibronectin is required for early vascular development (George et al., 1993; George et al., 1997). Subsequently, a decrease in the amount of fibronectin is followed by deposition of basement membrane proteins laminin and type IV collagen, which endothelial cells deposit themselves. Knockout mouse studies have demonstrated that collagens, for

example type I collagen, is necessary for maintenance of the vascular wall late in development (Lohler et al., 1984). Integrins are cell adhesion molecules, which serve as receptors for fibronectin, collagens, and laminin. Vascular defects have been reported in specific integrin knockout mice (Yang et al., 1993). Vascular endothelial cadherin (VE-cadherin) is a protein located in endothelial cells in adherens junctions. In mice deficient in VE-cadherin, the normally developed primitive blood vessel network falls apart suggesting that VE-cadherin is indispensable for maintenance of immature vessels (Crosby et al., 2005).

1.4 Mechanisms of innervation

The nervous system of the body is composed of the central and peripheral nervous systems. These consist of nerve cells (neurons) and glial cells, which support the neurons. For proper navigation, nerve fibers growing from the neuron, need a motile structure located at their tip, known as the growth cone. Thin filopodia in the growth cone facilitate movement and migration of the nerve fiber by sensing guiding cues in the microenvironment surrounding the growth cone (Thoumine, 2008; Kolodkin and Tessier-Lavigne, 2011). Nerve fiber guidance and navigation is under strict molecular control. Classic guidance molecules are classified into four major groups: Netrins, Slits, Ephrins and Semaphorins (Tessier-Lavigne and Goodman, 1996; Kolodkin and Tessier-Lavigne, 2011). In addition to these, there are other proteins such as morphogens that also have axon guidance functions (Tessier-Lavigne and Goodman, 1996; Kolodkin and Tessier-Lavigne, 2011).

1.4.1 Semaphorins

The semaphorins are a family of secreted, transmembrane, or glycosylphosphatidylinositol (GPI-linked) proteins (Committee, 1999), that were initially identified as axon guidance molecules. They have been found in vertebrates, invertebrates and viruses (Yazdani and Terman, 2006). The term semaphorin is

derived from the word ‘Semaphore’, meaning a communication system used to convey signaling information (Kolodkin et al., 1993; Committee, 1999). Generally, semaphorins are characterized by the N-terminal semaphorin (sema) domain, which contains 500 amino acids and has been shown to be critical for semaphorin function (Koppel et al., 1997; Oster et al., 2003; Gherardi et al., 2004). Plexins, which act as semaphorin receptors, also possess a sema domain (Committee, 1999).

Grasshopper Fasciclin IV (semaphorin 1) was the first semaphorin that was discovered in the semaphorin family. The protein was named as Fasciclin IV because it is expressed on a subset of axon fascicles in the CNS (Kolodkin et al., 1992). The next semaphorin to be identified was in chicken and was originally named Collapsin (semaphorin 3a) because it collapsed dorsal root ganglion (DRG) growth cones (Luo et al., 1993). Later a large number of proteins possessing the same structural domains have been characterized. The unified nomenclature for the semaphorin proteins was generated by the semaphorin committee in (Committee, 1999).

The semaphorin family includes at least 19 different members in vertebrates and 3 different members in invertebrates. The family has been classified into eight different classes based on their general structure and characteristics (Tran et al., 2007) (Fig. 7). The secreted proteins include Classes 2 and 3 and V (the viral semaphorin) semaphorins, whereas all other classes are transmembrane proteins. Classes 1 and 2, 5c represent invertebrate semaphorins and the other classes are vertebrate semaphorins (Committee, 1999; Tran et al., 2007).

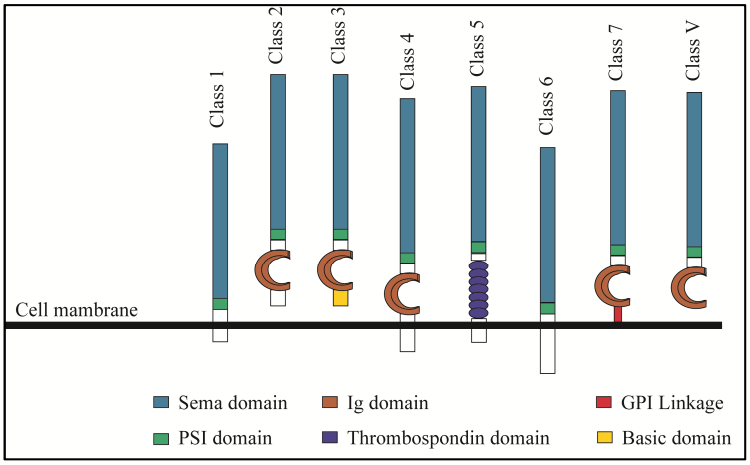

Figure 7. Classification of semaphorins. Class 1, 2 are found in invertebrates. Class 3, 4, 6 and 7 are identified in vertebrates. Class V is a viral semaphorin. Class 5 is divided into (A, B, C) subclasses, A and B are present in vertebrates while C is invertebrate semaphorin. Adapted from (Yazdani and Terman, 2006).

In addition to functioning as chemo-repellent molecules to navigating axons, semaphorins also play an essential role in organogenesis, cancer, the immune system and vasculogenesis, as well as angiogenesis (Kruger et al., 2005; Yazdani and Terman, 2006; Tran et al., 2007). Surprisingly, certain transmembrane semaphorins act as receptors for other members of the semaphorin family (Jongbloets and Pasterkamp, 2014).

1.4.1.1 Semaphorin 3A and its receptors

Class 3 Semaphorins have seven members: SEMA3A-3G (Zhou et al., 2008). The SEMA3A is a secreted chemo-repellent (Fan and Raper, 1995; Messersmith et al., 1995; Committee, 1999). with a molecular weight of 95 kDa (Klostermann et al., 1998). Cleavage of SEMA3A by proteolytic enzymes produces two shorter peptides of 33 and 65 kDa. To be functional SEMA3A must be a homodimer (Adams et al.,

1997; Klostermann et al., 1998; Koppel and Raper, 1998). Using *Sema3A* deficient mice, SEMA3A has been shown to be required for growth and patterning of peripheral cranial nerves such as the trigeminal, facial, glossopharyngeal, accessory and vagus nerves (Taniguchi et al., 1997; Ulupinar et al., 1999; Rochlin et al., 2000). Furthermore, sympathetic neurons and neurites are fasciculated and spinal nerves are abnormally projected (Taniguchi et al., 1997). Data from transgenic mouse studies confirmed results from many earlier *in vitro* studies, which were performed using chicken and mouse tissues (Luo et al., 1993; Fan and Raper, 1995; Messersmith et al., 1995; Puschel et al., 1995; Chedotal et al., 1998). SEMA3A mRNAs are broadly expressed in various compartments of the developing nervous system, and outside of it. Regarding developing peripheral nervous system *Sema3A* is widely expressed in exclusion areas for growing axons. Expression is reduced in general perinatally and is seen only in specific neuronal tissues in adulthood (Wright et al., 1995; Giger et al., 1996; Shepherd et al., 1996; Taniguchi et al., 1997). In addition, *Sema3A* is expressed in endothelial cells and has been suggested to regulate blood vessel development. SEMA3A may have an inhibitory role in angiogenesis (Miao et al., 1999; Serini et al., 2003; Shoji et al., 2003). On the other hand, SEMA3A signal transduced through NPN1 has been reported not to be necessary for blood vessel development using SEM3A deficient mice (C57BL/6 background) (Vieira et al., 2007a).

SEMA3A exerts its functions on nerve fibers through PlexinA receptors and the NPN1 co-receptor (He and Tessier-Lavigne, 1997; Takahashi et al., 1999; Raper, 2000; Rohm et al., 2000; Tamagnone and Comoglio, 2000; Yazdani and Terman, 2006; Sharma et al., 2012) (Fig. 8). Plexins are transmembrane receptors, which are categorized into four groups: A, B, C and D (Tamagnone et al., 1999; Gherardi et al., 2004; Tran et al., 2007).

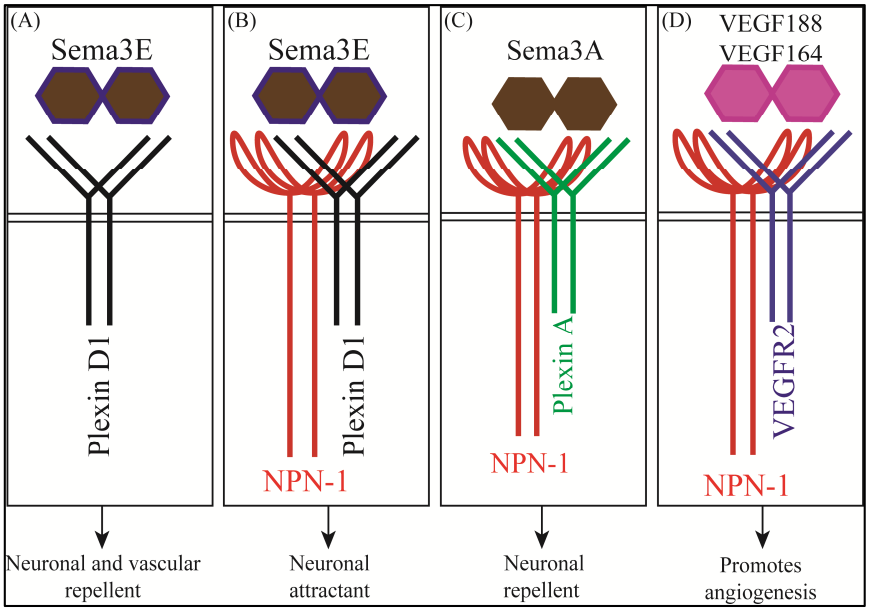

Figure 8. Neuropilin-1 is unique. It acts as a co-receptor in different pathways within different tissues. (A) Sema3E/PlexinD1 in the absence of NPN-1 repels both neuronal and vascular growth (Gu et al., 2005; Chauvet et al., 2007). (B) Sema3E/PlexinD1/NPN-1 stimulates neuronal growth (Chauvet et al., 2007). (C) Sema3A/PlexinA/NPN-1 repels axons (Raper, 2000; Sharma et al., 2012).(D) VEGF164/VEGFR2/NPN-1 or VEGF188/VEGFR2/NPN-1 promotes angiogenesis (Soker et al., 1998; Soker et al., 2002; Tillo et al., 2015).

1.4.1.2 Semaphorin 6A and their receptors

Class 6 semaphorins (SEMA6A-6D) are transmembrane proteins found in vertebrates (Kruger et al., 2005; Yazdani and Terman, 2006). Class 6 semaphorins show a strong structural similarity to class 1 Semaphorin (Yazdani and Terman, 2006). Among the transmembrane semaphorins, class 6 semaphorins own the largest proline-rich intracellular domain (400 amino acids) (Kruger et al., 2005). PlexinA2 and plexinA4 act as principal receptors for SEMA6A (Suto et al., 2005; Haklai-Topper et al., 2010; Nogi et al., 2010). SEMA6A is different from SEMA3A and does not use NPN1 as a co-receptor (Suto et al., 2005; Worzfeld and Offermanns, 2014). The mouse embryonic nervous system, as well as peripheral tissues, display dynamic expression

of SEMA6A mRNAs. Compared to *Sema3A* the expression of *Sema6A* in the nervous system is very different, but in general *Sema6A* and *Sema3A* have frequently been shown to be present in adjacent or complementary expression domains (Zhou et al., 1997; Xu et al., 2000). In developing tooth germ, SEMA6A mRNA has been reported by employing RT-PCR assay (Lillesaar and Fried, 2004). A prominent expression of SEMA6A mRNA has been observed in the thalamus, where it is required for proper axon projection (Leighton et al., 2001). Although SEMA6A and SEMA6C act as chemo-repulsive molecules for the sympathetic and dorsal root ganglion axons, the repulsive activity for SEMA6A is 200-fold less as compared to that of SEMA3A (Kikuchi et al., 1999; Xu et al., 2000). PlexinA4 deficient mice display defects in projection and trajectory of peripheral sensory axons and sympathetic axons (Suto et al., 2005). The SEMA6A-1 recombinant ectodomain was reported to hinder endothelial cell growth and tumor angiogenesis (Kigel et al., 2011). SEMA6A has been demonstrated to regulate endothelial cell survival and proliferation, as well as adult angiogenesis by modulating VEGF/VEGFR2 signaling (Segarra et al., 2012). Recently, SEMA6A was reported to be involved in retinal vascular regeneration by a mechanism in which Nrf2 (NF-E2-related factor 2) in ischemic neurons regulates SEMA6A (Wei et al., 2015). Hence, SEMA6A signaling is suggested to regulate neuronal and vascular development as well as angiogenesis in the adult (Segarra et al., 2012).

2. OBJECTIVES

The hypothesis forming the basis of the study was that the formation of dental blood vessels is developmentally regulated, being dependent on innervation, and that development of tooth morphogenesis, vasculature and innervation is to some extent regulated by the semaphoring signalling.

Main goal

The main goal was to investigate tooth morphogenesis, development of blood vessels and neurites, as well as to investigate molecular regulatory mechanisms controlling these processes.

Secondary goals

1. To investigate in detail, and compare the normal early development of the blood supply and innervation of the mouse mandibular first molar, and to study the expression of VEGF mRNA during odontogenesis using wild type mice.

2. To examine the influence of innervation and SEMA3A on the development of dental blood vessels in the early developing mouse mandibular first molar, using a *Sema3A* deficient mouse strain.

3. To study *in vivo* functions of SEMA6A on histomorphogenesis, innervation and vasculature of the early developing mouse mandibular first molar using a *Sema6A* deficient mouse strain.

3. MATERIALS AND METHODS

The methods used in the project is represented in (Fig. 9)

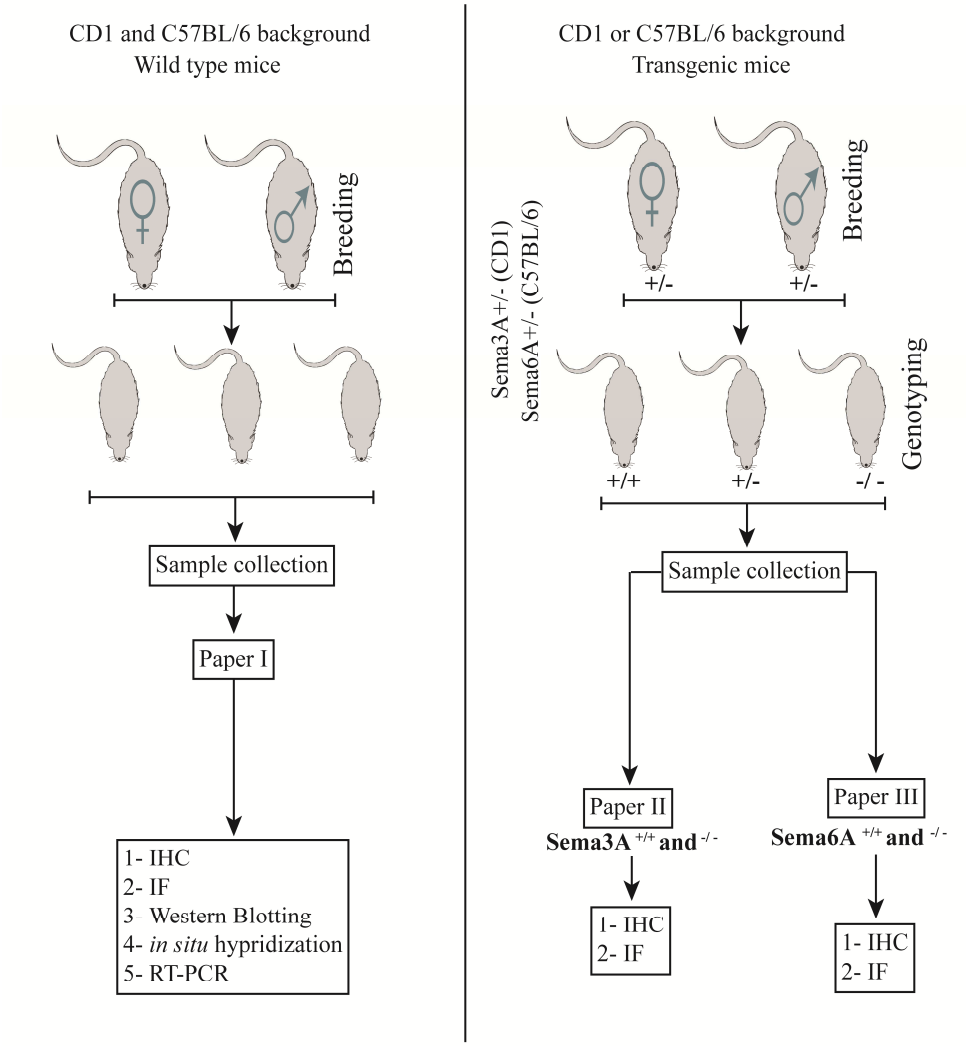

Figure 9. Flow chart of the methods used in the study.

3.1 Animal use and sample preparation

3.1.1 Husbandry of laboratory mice

In this project, the approval to use the laboratory animals was obtained from the Department of Biomedicine, Faculty of Medicine and Dentistry, University of Bergen under the surveillance of the Norwegian Animal Research Authority. All procedures were performed on animals according to the guidelines under the Norwegian Committee for Experiments on Animals, and EU directive 2010/63/EU on the protection of animals used for scientific purposes. Mice in the animal facility were kept in a room with controlled ventilation, temperature and humidity. The room was also kept under a controlled constant light and dark cycle. In addition to standard aspen bedding material, each cage contained at least one transparent dark red mouse house/igloo that reduces stress by providing mice a hiding place from direct light and dominating neighbours, as well as a place for nesting. To reduce boredom mice had access to aspen bricks and paper rolls, which are safe as shredding material. In each cage adequate amounts of food and water was available *ad libitum*. Males and females were generally kept in separate cages, except for breeding where three females were kept together with a male over three nights. To get time-pregnant females each female was monitored for the presence of a vaginal plug every morning during a three-day breeding period.

3.1.2 Tissue collection and Sample preparation (Papers I-III)

To identify each of the offspring in a litter, every single Sema3A and Sema6A genetically modified mouse was ear-punched by cutting off a small piece of the external ear at the age of four weeks. Ear biopsies were collected for genomic DNA isolation, which was used to genotype each mouse using a polymerase chain reaction (PCR) assay. The embryonic and postnatal stages that were analysed in the respective papers are presented in Table 1.

Table 1. Developmental stages in the mouse that were investigated in the study.

	Embryonic stages										Postnatal stages										
	10	11	12	12.5	13	14	15	16	17	18	0	1	2	3	4	5	6	7	8	9	10	11
Paper I	■	■	■		■	■	■	■	■	■	■	■	■		■			■				■
Paper II	■		■	■	■	■		■	■		■					■						
Paper III		■			■	■	■		■		■	■			■			■				

For immunohistochemistry and immunofluorescence studies (papers I-III), heads of mouse embryos and mandibles of postnatal pups were immediately embedded in Tissue-Tek OCT (Sakura Finetek, USA), frozen in carbon dioxide ice and subsequently stored at -80^{o}C. Fresh frozen frontal, horizontal and sagittal sections of the mandibular first molar tooth were cut serially (papers I, II) and identically (paper III), using a Leica CM 3050S cryostat (Leica Microsystems, Wetzlar, Germany), into 16 µm thick sections for the early embryonic stages and 30 µm thick sections for the other stages. Tissues were placed on slides and dried for 30 min at room temperature. 30-50 sections were prepared from a mandibular first molar tooth germ, depending on the developmental stage of the mouse and thickness of the sections.

For *in situ* hybridization (paper I) the heads of embryos and the mandibles of postnatal pups were fixed in 4% paraformaldehyde (PFA) at 4^{o}C overnight. Thereafter the tissues were dehydrated in a graded ethanol series and embedded in paraffin, and serial or identical sections with a thickness of 7 µm were cut using a Leica RM 2235 microtome (Leica Microsystems, Wetzlar, Germany), placed on slides and stored at 4^{o}C until used.

The collected and analysed mice are displayed in (Fig. 10).

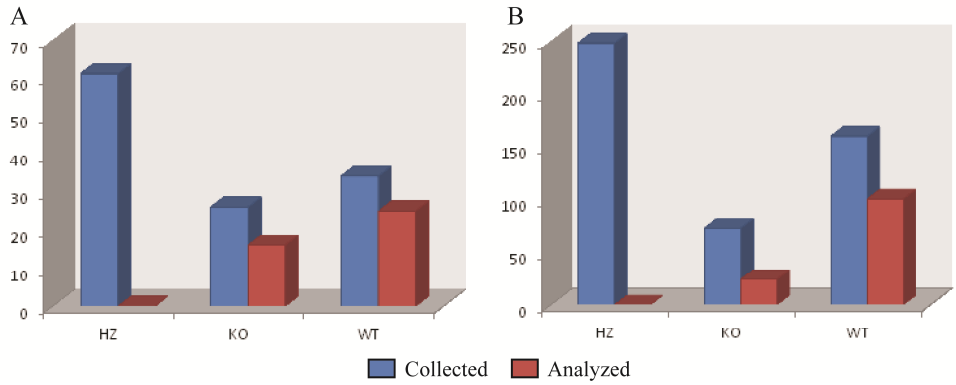

Figure 10. (A) SEMA6A mice. (B) SEMA3A mice.

3.2 Genotyping of transgenic mice (papers II, III)

Genomic DNA was isolated from ear biopsies using the Promega Wizard genomic DNA purification kit (Promega Corporation, Madison, USA; A1620). PCR was carried out using GoTag DNA polymerase (Thermo Scientific, USA, EP0402). Sema3A deficient mice were genotyped with a primer pair 5'-GTTCTGCTCCCGGCTCTAAATCTC-3' and 5'-ATGGTTCTGATAGGTGAGGCATGG-3') (Taniguchi et al., 1997). PCR was performed at 96^{o}C for 5min, 75^{o}C for 5 min, 55^{o}C for 3 min and thereafter for 35 cycles: 96^{o}C for 30 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 2 min. The PCR product for the wild type mouse was 1.2 kb and for the knockout mouse 500bp (Taniguchi et al., 1997) For Sema6A mice genotyping was performed with the following three primers: KST69-2F 5´-GAGATGCACAGCTAACTTCTGGTG-3, KST69-2R 5-TTGAAGCCTGCTCTTAGTGGCTCC-3´ and GTR4 5´-GCTACCGGCTAAAACTTGAGACCT-3´. A pair of primers KST69-2F and KST69-2R amplifies a 1.43-kb wild type product for the wild-type allele that spans the site of the insertion. A primer pair KST69-2F and GTR4 amplifies a 990-kb product for the mutant allele from the intron sequence into the vector (Kerjan et al.,

2005) (personal communication). PCR was carried out at 95°C for 3 minutes, 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 90 seconds. The size of PCR products was determined by agarose gel electrophoresis.

3.3 Antibodies (Papers I-III)

The antibodies used in the project are displayed in Table 2

Table 2. Primary and secondary antibodies.

Primary antibody	**Company**	**Dilution**	**Secondary antibody**	**Dilution**
Goat polyclonal anti-VEGFR2	R&D systems, UK AF644	1:20	Biotin donkey anti-goat Jackson ImmunoResearch, USA 705-066-147	1:250
			FITC conjugated donkey anti-goat Jackson ImmunoResearch, USA 705-096-147	1:50
Rabbit polyclonal anti-Peripherin	Chemicon international, CA, USA AB1530	1:100	Cy3 conjugated goat anti-rabbit Jackson ImmunoResearch, USA 111-167-033	1:100
Rabbit polyclonal anti-Alpha smooth muscle actin	Abcam, England AB5694	1:100	Cy3 conjugated donkey anti-rabbit Jackson ImmunoResearch, USA 711-166-152	1:50
Mouse monoclonal anti-neurofilament 200	Sigma-Aldrich, USA N0142	1:100	FITC conjugated donkey anti-mouse Jackson ImmunoResearch, USA 715-095-150	1:50
Goat polyclonal anti-VEGF164	R&D systems, UK AF493	1µg/ml	Horseradish peroxidase conjugated rabbit anti-goat Dako cytomation, Glostrup, Denmark, P0160	1:2000

3.4 Immunohistochemistry (Papers I-III)

Immunohistochemistry was carried out on fresh frozen samples. Frozen sections were first fixed in 4% PFA for 15 min, then they were washed with phosphate buffered saline (PBS), and afterwards kept in 100% cold methanol for 30 min and washed with PBS. Thereafter, unspecific binding sites in the tissues were blocked with 10% donkey serum (D9663, Sigma Aldrich, Inc, USA) for 30 min at room temperature, and subsequently sections were incubated at 4^{o}C overnight with the primary antibody polyclonal goat anti-VEGFR2 (AF644, R&D systems, UK) (1:20 dilution), which was diluted in 0.2% PBS/(bovine serum albumin) BSA (Nait Lechguer et al., 2008; Walchli et al., 2015). The sections were washed with PBS and incubated at 37^{o}C for one hour with biotin-conjugated donkey anti-goat antibody (705-066-147, Jackson ImmunoResearch, USA) (1:250 dilution). The sections were kept in avidin biotin peroxidase complex (PK 4000, VECTASTAIN Elite ABS kit; Vector Laboratories, USA) at 37^{o}C for 30 min according to the instructions of the manufacturer. 3-amino-9-ethylcarbazole (AEC) (A6926, Sigma-Aldrich, Inc, USA) was used as a chromogenic substrate. No specific staining was seen in control sections under conditions where the primary antibody was omitted. Sections were viewed in a Zeiss Axioskop 2 Plus microscope and the images were captured using a spot Insight digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and the electronic image plates were compiled using Adobe Photoshop CS5 Extended software (Adobe systems Incorporated, USA).

3.5 Immunofluorescence (Papers I-III)

Slides were first fixed in 4% PFA for 15 min, and then rinsed in PBS. Afterwards, sections were placed in 100% cold methanol for 30 min, then rinsed in PBS and blocked at room temperature with 10% donkey serum (D9663, Sigma Aldrich, Inc, USA) for 30 min.

Three different double immunofluorescence-staining experiments were carried out in this thesis:

1. The first primary antibody pair was polyclonal goat anti-VEGFR2 and polyclonal rabbit anti-Peripherin. The slides were incubated at 4^{o}C overnight with the primary antibodies, which were diluted in 0.2% PBS/BSA. Subsequently the sections were incubated in FITC donkey anti-goat (for VEGFR2) antibody for one hour at 37^{o}C, then washed with 1XPBS three times, followed by blocking with 10% normal goat serum for 30 min at room temperature. Thereafter the sections were incubated in Cy3 goat anti-rabbit (for Peripherin) antibody for one hour at 37^{o}C.

2. The second primary antibody pair was polyclonal rabbit anti-Alpha smooth muscle actin and mouse monoclonal antibody against neurofilament 200 (NF200). Cy3 donkey anti-rabbit (for Alpha smooth muscle actin) and FITC donkey anti-mouse (for NF200) antibodies were used as secondary antibodies.

3. The third primary antibody pair was polyclonal goat anti-VEGFR2 and polyclonal rabbit anti-Alpha smooth muscle actin. Cy3 donkey anti-rabbit (for Alpha smooth muscle actin) and FITC donkey anti-goat (for VEGFR2) were used as secondary antibodies.

Afterwards all sections were mounted using mounting medium with DAPI (Vector Vectashield, Burlingame, CA94010). Slides were viewed sequentially with DAPI, Cy3 and FITC filter sets using a Zeiss Axioplan microscope (Carl Zeiss Microscopy, Germany) and three channel images were captured in a Zeiss Axiocam camera (Carl Zeiss 0445-553) and the image plates were compiled using Adobe Photoshop CS5 Extended software (Adobe systems Incorporated, USA).

3.6 In situ hybridization (Paper I)

In situ hybridization was employed in this study in order to analyse mRNA expression patterns of VEGF and VEGFR2 in embryonic and postnatal stages of mouse molar tooth development. The assay was performed as described earlier (Luukko et al., 1996; Kettunen and Thesleff, 1998). Plasmids with the subcloned *Vegf* and *Vegfr2* DNA fragments were linearized by using the appropriate restriction enzymes and *in vitro* transcription of ^{35}S-UTP-labeled antisense and sense RNA probes was executed using RNA polymerase. After a 20-hour hybridization period the slides were covered with NTB-2 emulsion for autoradiography (Eastman Kodak, NY, USA). Subsequently, after a 3-4 -week exposure time, the sections were developed in Kodak D-19 developer and fixed with Unifix (Eastman Kodak) fixative. Counterstaining of the sections was carried out using hematoxylin and the last step was mounting of the slides with Depex (Electron Microscopy Sciences, PA, USA). Sections were observed in a Zeiss Axioskop 2 Plus microscope with high and low magnification objectives, and representative digital bright- and dark-field images were captured with 5 0.15 NA and 10 0.3 NA objectives using a Spot Insight camera. Image plates were compiled using Adobe Photoshop CS4 software.

3.7 RT-PCR (Paper I)

Total RNA was isolated from E14 mouse molar tooth germs using GenElute Mammalian total RNA Miniprep Kit (Sigma cat nr RTN70-1KT) and reverse-transcribed with RevertAid M-MuLV reverse transcriptase for RT-PCR (Fermentas cat nr EP0441). VEGF and GAPDH (Glyceraldehyde 3-Phosphate Dehydrogenase) genes were amplified using Taq DNA polymerase (VWR cat nr 5101600-0100) for 40 cycles using various concentrations of $MgCl_2$ (1.5-2.5 mM). The PCR products were fractionated by electrophoresis in 2% agarose gel. The following primer pairs were used for PCR amplification: VEGF-F (5´-GACCCTGGTGGACATCTTCCAGGA-3´/VEGF-R (5-GGT GAG AGG TCT GGT

TCC CGA-3) and GAPDH-F (5′-GCTGAGTATGTCGTGGAGTC-3′/GAPDH-R (5′-TTGGTGGTGCAGGATGCATT-3′). The primers have been used earlier (Mukouyama et al., 2002; Ruhrberg et al., 2002).

3.8 Western blot (Paper I)

Mandibular first molar tooth germs at E16 were microdissected for western blot analysis and stored at -80^{o}C. The sample was mechanically homogenized in ice in 200 µl loading buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl, pH 6.8 and 0.1% Bromophenol blue). The tissue was subsequently vortexed and centrifuged. A sample of 50 µl was then loaded on a 12% acrylamide gel and subjected to electrophoresis at 200 V for 50 min. Western blot was performed under non-reducing conditions. Proteins were transferred to a nitrocellulosa membrane, 30 V overnight. The membrane was blocked using 5% dry milk in PBS-0.1% Tween 20 (PBST) for 1 hour and then the membrane was incubated with polyclonal goat anti-VEGF164 (R&D systems, UK, AF493) (1µg/ml) at 4^{o}C overnight. Secondary antibody was horseradish peroxidase-conjugated rabbit anti-goat antibody (Dako cytomation, Glostrup, Denmark, P0160) (dilution 1:2000). Incubation time in secondary antibody was for 90 min at room temperature. Visualization of the reaction was performed by ECL (Enhanced chemiluminescence) (Thermo Scientific, USA, 34095) and protein bands were detected with an Image reader (Las-3000 version 2.0 W) scanner. Bio-Rad Kaleidoscope ladder (cat no 161-0375) was used as a molecular weight standard.

3.9 Image processing (Paper I-III)

For *in situ* hybridization images were captured using dark- and bright-field microscopy (Kettunen et al., 2005) using a spot insight digital camera (Diagnostic instruments Inc, Sterling Hight, MI) mounted on a Zeiss Axioskop 2 microscope (Carl Zeiss GmBH, Jena, Germany). Images were taken under bright-field

microscopy by using the same camera and microscope as for immunohistochemistry. For double immunofluorescence Zeiss Axioplan microscope (Carl Zeiss Microscopy, Germany) was used with Zeiss Axiocam camera (Carl Zeiss 0445-553) and the image plates were created using Adobe Photoshop CS5 Extended software (Adobe systems Incorporated, USA).

3.10 Methodological considerations

3.10.1 The mouse as a study model

The laboratory mouse (Mus musculus) is a powerful tool to investigate mammalian development and genetics (Nguyen and Xu, 2008; Segarra et al., 2012; Klein et al., 2013; Oh and Gu, 2013; Yuan et al., 2014). Mice have been exploited in genetic and biomedical studies for more than 100 years (Nguyen and Xu, 2008). Many different advantageous traits make the mouse an excellent study model: mice are easy to handle, small in size, the reproductive cycle is relatively short, and the embryonic period from fertilization to birth is 18-20 days. In addition, mouse anatomy and physiology shows a strong similarity to that of the human. A very significant feature is that the human being and mouse share the majority of genes. After publication of the complete draft sequence of C57BL/6J mouse and comparison of it with the previously published human genome, it was recognized that the difference between the mouse and human genome is less than 5% (Mouse Genome Sequencing Consortium, 2002). Because of well-known genome and sophisticated molecular biology and genetic methods, the mouse genome is easy to manipulate, and transgenic mouse strains can be generated and used to investigate molecular mechanisms in *in vivo* embryogenesis, as well as human diseases. There are only a few disadvantages associated with the use of the mouse as a model animal and research tool. In theory it would be interesting to generate double or triple knockout mice, and inducible transgenic mouse strains as well various Cre-lox combinations.

However, maintenance of mouse colonies in the animal care unit is expensive and husbandry is time-consuming.

Generally, mouse dentition has been used broadly to investigate developmental and adult anatomy and physiology as well as molecular and genetic mechanisms of mammalian tooth organogenesis and innervation (Klein et al., 2013; Luukko and Kettunen, 2014). Especially, the first mandibular molars of the mouse resemble those in the human, both during development and adulthood (Jernvall and Thesleff, 2012). Both human and mouse mandibular first molar teeth are multi-cusped and contain two roots. The mandibular first molar tooth of the mouse was therefore used as a model system in this study. The embryonic and postnatal developmental stages that were used in the project were confirmed by checking the histo-anatomical features in the histological sections viewed in the light microscope (Hay, 1961). In this project *in vivo* studies were performed and these have a major advantage: functions of proteins can be investigated in a three-dimensional manner in tissues/organs.

There exist other vertebrates that have teeth, but they were deemed as not being suitable for the planned studies. Zebrafish, for example, have teeth, but they are very small in size, simpler in form than human teeth, and they are not located in the oral cavity but in the pharynx (Bruneel and Witten, 2015). Toothed mammals such as the vole, rat, cat and ferret have been exploited to some extent in dental studies (Kvinnsland and Heyeraas, 1990; Luukko, 1997; Keranen et al., 1999; Jussila et al., 2014). There are, however, more disadvantages than advantages related to their use in studies on tooth organogenesis.

3.10.2 Detection of mRNAs and protein products in histological sections

Expression domains of VEGF mRNAs, but not the protein product were reported in histological sections in this study. The reason was that by using the *in situ* hybridization technique it is usually possible to visualize location of mRNAs specifically in embryonic mouse histologic sections. Specificity depends on the cDNA fragment, which has been subcloned in the plasmid. Immunohistochemistry using antibody against mouse VEGF164 (R&D systems) was performed using both frozen and paraffin sections, but the antibody did not work in our hands. Earlier successful immunohistochemical staining with antibody against VEGF has been demonstrated, for example in embryonic mouse and human lung (Acarregui et al., 1999; Healy et al., 2000). The positive staining was faint in epithelium and subepithelial matrix in mouse embryonic lung (Healy et al., 2000) but relatively strong in human fetal lung epithelium (Acarregui et al., 1999). Distinct, positive results may be due to the use of different antibodies (Anti-human VEGF antibody from Santa Cruz Biotechnology). However, in (Healy et al., 2000) paper, it is likely that the same antibody, which we used, was utilized (goat anti-mouse VEGF antibody from R&D systems)**.** One possibility is that an antibody works well in some tissues but not so well in others. We used the same VEGF antibody in Western blot and obtained a faint band, showing that it did work, but was not very effective. It must be pointed out, however, that the sample was E16 tooth germ (15 pieces), which is only a small amount of tissue.

3.10.3 Visualization of blood vessels

Antibody against VEGFR2 was used in this study to visualize developing blood vessels in embryonic and postnatal tooth germs by using immunohistochemical and immunofluorescent techniques. The VEGFR2 gene was cloned from a human endothelial cell cDNA library (Terman et al., 1991) and its protein product was shown to be located on the surface of the endothelial progenitor cells and endothelial

cells (Vaisman et al., 1990; Hristov et al., 2003). It has frequently been used to visualize blood vessels during embryogenesis and postnatal tissues (Mukouyama et al., 2002; Gerhardt et al., 2003; Saint-Geniez et al., 2006; Nait Lechguer et al., 2008). VEGFR2 proved to be a good marker for blood vessels in the tooth germ, because it was totally endothelial cell specific.

Other conceivable markers, which alternatively could have been used, are discussed here. CD31, which is also known as Platelet endothelial cell adhesion molecule 1 (PECAM-1) is a specific marker for endothelial progenitor cells and endothelial cells (Hristov et al., 2003; Ilan and Madri, 2003). It has often been used to visualize blood vessels (Mukouyama et al., 2002; Mukouyama et al., 2005; Nait Lechguer et al., 2008; Fantin et al., 2013; Frahm et al., 2013; Oh and Gu, 2013). We employed anti-CD31 antibody for immunohistochemical and immunofluorescent staining, but the results were not as good as we obtained with antibody against VEGFR2.

Anti-CD34 (Hematopoietic progenitor cell antigen CD34) antibody is a marker for endothelial cells (Siemerink et al., 2012; Friedlander et al., 2015). CD34 is a transmembrane glycoprotein protein expressed in the hematopoietic progenitor and stem cells, and in blood vessels forming by vasculogenesis. Its expression is low in vessels forming through coalescence, such as in the cardinal veins, but is otherwise detected in many other blood vessels forming by angiogenesis, and plays an important role in early hematopoiesis (Wood et al., 1997; Walchli et al., 2015).

Antibodies against type IV collagen and laminin (Walchli et al., 2015), which are present in the basement membrane of blood vessels, are also applicable in order to display vessels (Fristad et al., 1994; Vandevska-Radunovic et al., 1997; Nait Lechguer et al., 2008). Of note, type IV collagen and laminin are not specific to blood vessels, but localized in basement membranes in general. We did some test immunofluorescence staining with antibody against type IV collagen, but we were more satisfied with the results we obtained with anti-VEGFR2 antibody.

In this project antibody against Alpha smooth muscle actin was employed to detect smooth muscle cells, which are recruited to cover and stabilize maturing blood vessels, such as arteries, arterioles and veins (Potente et al., 2011; Liu et al., 2015; Meyerholz et al., 2015; Otani et al., 2015; Zhang et al., 2015). Capillaries and postcapillary venules show incomplete envelopment of pericytes, which can be stained to display a capillary network. Pericytes express, for example, NG2 (chondroitin sulphate proteoglycan), PDGFR-beta and desmin (Diaz-Flores et al., 1991; Lindahl et al., 1997; Ozerdem et al., 2001). These markers are not completely specific because mural cells have been reported that express both Alpha smooth muscle actin and NG2 (Ozerdem et al., 2001). Alternatively, there may be some functional plasticity in these mural cells.

Fluorescently and biotin-conjugated lectins have been utilized to demonstrate the distribution of blood vessels (Gerhardt et al., 2003; Hamid et al., 2006).

In addition to the antibodies and lectins, reporter mice can be used to display localization of blood vessels. For example (Larina et al., 2009) reported generation and use of the Tg (Flk1::myr-mCherry) transgenic mouse strain, in which fluorescent protein (mCherry) is targeted to the endothelial cell membrane, which facilitates detection of growth, modification and topography of blood vessels, even in *in vitro* tissue and organ cultures using confocal, fluorescence or stereofluorescence microscopy and time-lapse imaging (Larina et al., 2009). When the strain is crossed with the Tg(Flk1::H2B-EYFP) strain, in which fluorescent protein is located in the nucleus, it is possible to follow individual endothelial cells simultaneously.

Injection of dyes into blood vessels is also used to visualize blood vessels (Kramer, 1951). An especially useful method is use of the vascular carbocyanine dye DiI by injection because DiI´s fluorescence is bright and robust, and for example chicken embryos survive up to 24 hours after injection. The technique is therefore compatible with live imaging (Delalande et al., 2015).

In case arteries and veins are displayed separately arteries can be stained by *in situ* hybridization using the *EphrinB2* probe, and veins with the *EphA4* probe. Furthermore, *EphrinB2* and *EphA4* transgenic mice with LacZ and GFP-reporter may be used, and usually display more obviously vessels (Wang et al., 1998).

3.10.4 Visualization of nerve fibres

Antibodies against peripherin and neurofilament 200 (NF200) were used in this study to visualize embryonic and postnatal dental neurites by using immunofluorescence technique. Peripherin is a major intermediate filament (IF) protein (Portier et al., 1983) with a molecular weight of 57 kDa, which is found in the peripheral and central nervous system (Parysek and Goldman, 1988; Escurat et al., 1990). Peripherin has been used to localize embryonic and postnatal trigeminal neurites as well as sympathetic nerves (Gorham et al., 1990). The protein was named as Peripherin because the expression was strong and specific in peripheral neurons (Escurat et al., 1990). Anti-NF200 antibody has been used widely previously to detect neurites as well (Moe et al., 2012; Oh and Gu, 2013). NF200 is a cytoskeletal intermediate filament protein (Herrmann and Aebi, 2000). When compared to anti-Peripherin antibody the antibody against NF200 appears to give less specific and weaker staining in the dental axons (Moe et al., 2012). Other antibodies that have been used to detect nerve fibres are monoclonal anti-medium chain neurofilament (2H3) antibody (Dodd et al., 1988; Taniguchi et al., 1997). Anti-2H3 antibody has been used in postnatal mice to localize sensory nerve fibres in tooth germ (Moe et al., 2008). PGP9.5 is a cytoplasmic intermediate filament protein and a member of the ubiquitin carboxyl-terminal hydrolases family (Thompson et al., 1983; Wilkinson et al., 1989). Antibody against it (Protein gene product 9.5) has also been used to identify dental nerve fibres (Christensen et al., 1993; Fristad et al., 1994; Luukko, 1997). Calcitonin gene-related peptide (CGRP) and substance P (SP) have been used to localize sensory nerves especially, in erupted and adult teeth (Veerayutthwilai et al., 2006). Neuron-specific beta tubulin, Tuj1, is a structural protein that is involved in axonal transport. It is expressed both in central and peripheral nervous systems (Memberg and Hall,

1995). An antibody against it has been successfully used to show nerve fiber growth and distribution during development of organs such as the eye and limb skin (Mukouyama et al., 2002; McKenna and Lwigale, 2011). The oldest method utilized to visualize neurons is the silver impregnation technique (Davenport, 1930; Bodian, 1936).

4. RESULTS

4.1 Comparison of the time-course of blood vessel and neurite growth and patterning during the development of the mouse first molar tooth germ (Article I)

Prior to visible histological onset of the lower jaw first molar tooth anlage at embryonic day 10 (E10), numerous scattered blood vessels as observed by VEGFR2-immunohistochemistry were seen in the lower jaw mesenchyme. Blood vessels were also present in the mesenchyme adjacent to the developing oral and presumptive dental epithelium. Some blood vessels appeared to be in contact with the basement membrane and epithelium. On the other hand, branches of the developing peripheral mandibular nerve, as earlier shown by peripherin-immunoreaction (Lumsden, 1982; Kettunen et al., 2005; Kettunen et al., 2007), are localized in the mesenchyme in the central part of the developing lower jaw. However, they were not observed in the presumptive area of the molar tooth germ (Kettunen et al., 2005).

At E11 the molar tooth germ was apparent as a local thickening of the oral epithelium. The lower jaw mesenchyme was abundant in blood vessels, but they were no longer found adjacent to dental or oral epithelia. In contrast to blood vessels, nerve fibers were not seen in the developing tooth area at E11. One day later (E12), blood vessels were detected further away from the dental placode and were present in the peridental mesenchyme. It has been reported earlier that the molar nerve (Kettunen et al., 2005) arises from the mandibular nerve and seen to grow towards the molar tooth anlage.

The molar tooth germ is at the bud stage at E13. Dental mesenchyme is condensed around the epithelial bud, but blood vessels and the first neurites are not present in the

dental mesenchymal cell condensate, but are in the peridental mesenchyme around the tooth anlage. Blood vessels had established a plexus pattern whereas the pioneer molar nerve branch was located in the buccal mesenchyme of the tooth germ next to the forming dental follicle.

One day later (E14), the enamel organ is at the early cap stage. Blood vessels were found located in the outer boundary of the dental follicle around the enamel organ. This is the first stage when blood vessels surrounding the tooth germ are present in the dental mesenchyme, which consists of dental follicle and dental papilla. The blood vessel plexus is located in the dental follicle and from there vessels start to grow and invade the dental papilla. The dental papilla later becomes the dental pulp, which in the adult tooth contains blood vessels and nerves. A large number of nerve fibers were now seen in the dental follicle (defined as the mesenchymal target field area) in the lingual and buccal sides of the tooth germ. Using high magnification, in the sections on which immunohistochemistry was applied, blood vessels were seen to show variable staining, such that some small cells appeared to be darker than others, especially in the early stages.

At the early bell stage (E16 and E17) blood vessels were increasingly numerous in the dental follicle as well as in the dental papilla, but they were still absent from the enamel organ. Blood vessels of a larger diameter appeared in the core of the papilla mesenchyme running towards the area of the inner dental epithelium in the crown cusps where the formation of a blood vessel plexus adjacent to the preodontoblast layer was evident. A nerve fiber plexus persisted in the dental follicle.

Between E18 bell stage and PN2 distribution of blood vessels in the dental pulp as well as patterning of the vessels and neurites in the dental follicle were similar as in the early bell stage tooth germ. The first blood vessels ingressed into the epithelial

enamel organ at E18 and were seen within the stellate reticulum located in the middle of the enamel organ.

Later, during PN4-PN11, dentin and enamel secretion takes place. Rich blood vessels and a neurite plexus were evident in the dental follicle area surrounding the tooth germ. In the pulp, large caliber vessels were located in the center, and the capillary plexus was formed at the periphery of the pulp, adjacent to the odontoblast layer. Few vessels were seen within the odontoblast layer. In the center of the pulp some of the large diameter vessels started to anastomose. Within the enamel organ, vessels were increasingly plentiful being located within the stellate reticulum and stratum intermedium layer next to the enamel secreting ameloblasts. At PN4, the innervation of the pulp was found to have commenced by the growth of pioneer nerve fibers from the nerve plexus into the dental follicle and invading the dental pulp.

At PN7, the shape of the crown is mostly determined and root formation is initiated and forms mesial and distal secondary apical foramina. Accordingly, the growing mesial and distal roots are seen in PN11 tooth germ. The patterning of blood vessels was similar as in the previous stages in the pulp, but a vast increase in the number of neurites was obvious in the dental pulp and thicker nerve bundles, in particular, seemed to be associated with blood vessels. Double immunohistochemical staining of horizontal sections (through the pulp close to the future enamel-cementum junction) of PN4, PN7 and PN11 tooth germs using antibody pairs against VEGFR2 and peripherin, as well as NF200 and alpha smooth muscle actin, showed that thick nerve fibers were associated with blood vessels. Moreover, the majority of the blood vessels associated with neurites were covered with smooth muscle cells. Double immunohistochemical staining on sagittal sections of E14 and E16 tooth germs showed that smooth muscle cells were partially enwrapping one blood vessel entering into the dental papilla at E16, but not at E14 when the first blood vessels grow into

the dental papilla. However, the mandibular artery was apparently shielded by smooth cells at E14.

4.2 Expression of VEGF mRNAs in developing tooth germ (Article I)

The cellular expression of VEGF mRNAs was investigated in sections by radioactive *in situ* hybridization. At E11 VEGF mRNAs showed only a low level of expression in the lower jaw mesenchyme, including the presumptive dental one, lying under the thickened dental epithelium. The heart, which was used as a positive internal control shows a prominent expression of *Vegf* (Ferrara et al., 1996; Lagercrantz et al., 1998; Lymboussaki et al., 1999). At the placode stage (E12), the presumptive dental mesenchyme exhibited very weak *Vegf* hybridization, but expression was more obvious in the buccal peridental mesenchyme. At the cap stage (E14), the epithelial signaling center of the tooth, the primary enamel knot, showed a notable level of *Vegf* expression. Moreover, VEGF transcripts were observed in the dental papilla mesenchyme adjacent to the enamel knot.

During the subsequent bell stage, the secondary enamel knots signaling centers appear at the tips of the future cusps and the developing tooth undergoes specific crown morphogenesis. At E16 (early bell stage), *Vegf* expression persisted in the primary enamel knot, stellate reticulum as well as in the dental follicle on the lingual side of the tooth germ. This was also the case in the enamel organ, namely in the secondary enamel knot, inner dental epithelium, stratum intermedium and stellate reticulum and outer dental epithelium. Prior to birth (E18), expression of VEGF transcripts continued in the enamel organ, namely in the secondary enamel knots, inner dental epithelium, stratum intermedium and stellate reticulum cells. Some transcripts were also detected in the outer dental epithelium and cervical loop.

Postnatally, at PN1, the stratum intermedium layer next to the preameloblasts, in particular in the distal slopes of the developing cusps, very intense VEGF staining was observed. Transcripts were also still present in the stellate reticulum. In the dental pulp very faint *Vegf* expression was seen in some areas of the preodonto- and odontoblast cell layers.

The VEGFA gene has eight exons, and as a result of alternative splicing, at least four isoforms have been reported in the mouse, namely VEGF120, VEGF144, VEGF164 and VEGF188, all of which are expressed in embryos (Ng et al., 2001; Mukouyama et al., 2002; Ruhrberg et al., 2002). RT-PCR analysis showed that at E14, cap stage molar tooth germ expresses VEGF120, VEGF144 and VEGF164 isoforms and perhaps also VEGF188, and an isoform shorter than VEGF120. Moreover, Western blot analysis confirmed the presence VEGF protein in E16 molar tooth germs.

4.3 Expression of Vegfr2 in developing tooth germ (Article I)

After investigating distribution of developing blood vessels in the mouse mandibular molar tooth germ, using an antibody against VEGFR2, which is the principal receptor for VEGF, we aimed to analyse the level of VEGFR2 mRNA in the growing blood vessels. We performed radioactive *in situ* hybridization on sections using a *Vegfr2* probe, and the results showed that the expression level of *Vegfr2* was extremely similar in all blood vessels, except in those that were located in the stratum intermedium layer next to the amaloblasts. In the stratum intermedium and adjacent stellate reticulum the intensity of expression was higher than in other tissues Vegfr2 mRNAs and VEGFR2 protein showed apparent, similar expression domains exclusively in blood vessels during the tooth development stages studied.

4.4 Comparison of vascular development during crown development of *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ mouse mandibular first molar (Article II)

At E10, plentiful blood vessels were observed by VEGFR2-immunofluorescence analysis throughout the mandibular process mesenchyme, including the presumptive dental mesenchyme in *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ mice. Few blood vessels were in contact with the basement membrane of the oral epithelium, and some appeared to have grown inside the epithelium. A similar distribution of vessels was seen in both genotypes. Two days later, at the placode stage (E12), many VEGFR2-immunoreactive blood vessels persisted similarly in the mandibular mesenchyme. Whereas blood vessels were observed outside of the presumptive dental mesenchyme, they were devoid from the dental and oral epithelium in wild type and *Sema3A*$^{-/-}$ mice. At E13 VEGFR2-positive blood vessels were present in peridental mesenchyme adjacent to the dental follicle in *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$.

In the E14 *Sema3A*$^{+/+}$ molar tooth germ, small branches of blood vessels had entered into the dental papilla, whereas in the *Sema3A*$^{-/-}$ molar, which was slightly behind in histomorphogenesis, they were about to develop in the same manner. In molars at the early bell stage (E16), several blood vessel branches had ingressed into the dental papilla and were ramified in the core mesenchyme of the lingual and buccal cusps in both genotypes. Some of the vessels in the dental follicle were in contact with the outer dental epithelium already at E16. Pulpal vessels had grown next to the inner dental epithelium at E17, and some of the vessels appeared to be in contact with the inner dental epithelium layer.

At PN0 large caliber vessels were observed in the middle of the pulp and the subodontoblastic blood vessel plexus was formed in *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ molars. Futhermore, blood vessels were present in the stellate reticulum.

At PN5, blood vessels were found to be located in the dental pulp in a similar pattern in both *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ molars. There appeared to be fewer blood vessels in the subodontoblastic region compared to the newborn stage. In the dental epithelium, in addition to the stellate reticulum, vessels had grown to the stratum intermedium layer next the ameloblast cell layer in both genotypes.

4.5 Comparison of the time-course and patterning of neurites and blood vessels in early developing *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ molars (Article II)

To investigate the potential developmental interrelationship between the dental neurites and developing blood vessels, double immunofluorescence staining using peripherin and VEGFR2 antibodies was performed on *Sema3A*$^{+/+}$ and *Sema3A*$^{-/-}$ mandibular first molar sections at E12.5 and E14, when critical stages of dental axon growth and navigation take place. As reported earlier, timing of tooth innervation takes place prematurely and nerve fibers show abnormal patterning and fasciculation in E11-E14 *Sema3A*$^{-/-}$ molars. Accordingly, neurites were ectopically located in the mesenchymal exclusion areas of the developing *Sema3A*$^{-/-}$ (Kettunen et al., 2005; Kettunen et al., 2007). In line with results from earlier studies, ectopic neurites were seen in the presumptive, condensed dental mesenchyme of *Sema3A*$^{-/-}$ but not in *Sema3A*$^{+/+}$ molars at E12.5. At the cap stage premature neurites were present in the dental follicle. Importantly, in spite of these neuronal defects, double immunofluorescence staining revealed no apparent changes or disturbance in the localization and patterning of the blood vessels in the *Sema3A*$^{-/-}$ molars as compared to that in *Sema3A*$^{+/+}$ molars. For instance, blood vessels were not seen in the dental mesenchyme or dental papilla mesenchyme in the *Sema3A*$^{-/-}$ molars, indicating that they apparently had not followed the misrouted and abnormally patterned trigeminal neurites within the tooth target.

4.6 Tooth phenotype in *Sema6A*$^{-/-}$ mice: comparison of vascular development and innervation as well as tooth morphogenesis in *Sema6A*$^{+/+}$ and *Sema6A*$^{-/-}$ molars (Article III)

At the onset of development of the mandibular first molar (E11) in both *Sema6A*$^{+/+}$ and *Sema6A*$^{-/-}$ embryos, blood vessels were broadly and similarly distributed in the mandible mesenchyme, including the area of the presumptive dental mesenchyme. The trigeminal mandibular nerve and its branches were present in the deep mandibular mesenchyme, similarly in both *Sema6A*$^{+/+}$ and *Sema6A*$^{-/-}$ embryos, as shown using double immunofluorescence staining with antibodies against VEGFR2 and peripherin. In both *Sema6A*$^{+/+}$ and *Sema6A*$^{-/-}$ embryos at E13, a large number of blood vessels were located in the outer borderline of the condensed dental mesenchyme surrounding the dental epithelium. At this stage, the trigeminal molar nerve (Luukko et al., 2005) had reached the dental follicle target field area and had given rise to the buccal branch in both genotypes. Immunofluorescence staining showed that there are two parallel blood vessels surrounding the tooth bud, and the navigation route of the buccal neurite branch is outside the outer blood vessel. At E14 blood vessels and their branches were abundant in the dental follicle area in both genotypes. Minor first branches of blood vessels were found to initiate their growth from the plexus towards the dental papilla in *Sema6A*$^{+/+}$ mice, but not in *Sema6A*$^{-/-}$, in which tooth morphogenesis was slightly delayed. Moreover, at this stage, the lingual branch of the molar nerve had emerged and nerve fibers were now similarly found both in the buccal and lingual sides of the dental follicle target field area in the two genotypes. Subsequently, at the late cap-stage, the first blood vessels had extended and were located in the middle of the dental papilla in *Sema6A*$^{+/+}$ and *Sema6A*$^{-/-}$ mice. At the early bell stage (E17) blood vessels were abundant in the dental papilla in both *Sema6A*$^{+/+}$ *and Sema6A*$^{-/-}$ molars, and formation of a blood vessel plexus adjacent to the preodontoblasts became apparent. An increasing number of nerve fibers was evident in the dental follicle target area, where they showed similar development and patterning in both Sema6A genotypes.

In PN0, blood vessels in the tooth germ had grown into the enamel organ in *Sema6A*$^{+/+}$ mice, but not in *Sema6A*$^{-/-}$ mice. Both the patterning and density of nerve fibers were found to be similar around the tooth germ in both genotypes. At PN1, nerve fibers were still present outside the tooth germ in *Sema6A*$^{+/+}$, but had entered the dental papilla in *Sema6A*$^{-/-}$ mice. Later at PN4, blood vessels were observed in the subodontoblastic area and some also in the odontoblast layer in both genotypes. An increasing number of blood vessels was detected in the enamel organ and many had reached the stratum intermedium. At this stage, nerve fibers had entered the dental pulp also in *Sema6A*$^{+/+}$ mice, and both in *Sema6A*$^{+/+}$ and *Sema6A*$^{-/-}$ mice nerve fibers in the pulp had reached the central part of the pulp. At PN7 root formation had initiated, and the density of nerve fibers had increased inside the pulp in both *Sema6A*$^{+/+}$ and *Sema6A*$^{-/-}$ molars. Large caliber nerve bundles were seen in the center of the pulp, and thinner arborizations were present at the pulp–dentin border area. In the middle of the pulp nerve a subset of the fibers was closely associated with blood vessels. No apparent differences were observed in nerve fiber patterning, fasciculation or branching, and blood vessels, between the two genotypes. Morphogenesis of the first molar tooth germ appeared to be similar in *Sema6A*$^{+/+}$ and *Sema6A*$^{-/-}$ mice in the stages studied. There was, however, a slight delay in shape development at E14, but in the stages that followed no differences were any longer detectable.

5. DISCUSSION

The major aim of the study was to investigate the development of tooth vasculature and innervation, their putative interrelationship and molecular signaling mechanisms involved in these processes. To address this main aim, analysis of wild-type mice, as well as two transgenic mouse strains, deficient in *Sema3A* and *Sema6A,* was performed. Initially, blood supply, innervation and normal development of the mouse mandibular first molar tooth germ were described and compared, and the expression of *Vegf* during early tooth development was investigated (Article I). Thereafter, the putative influence of innervation and SEMA3A signaling on the development of dental blood vessels was addressed using the *Sema3A* deficient mouse strain (Article II). Finally, the developmental functions of SEMA6A on tooth histomorphogenesis, innervation and vasculature were investigated employing the *Sema6A* deficient mouse strain (Article III).

5.1 Formation of dental vasculature takes place in a developmentally regulated manner and is spatio-temporally coupled with tooth morphogenesis

Results obtained in this study from the mouse mandibular first molar tooth germ revealed that the development of the tooth vascular supply takes place in a distinct, spatio-temporally regulated manner during odontogenesis. A rich unorganized spread of vasculature was seen in the jaw and presumptive dental mesenchymal area before histological appearance of the first molar tooth germ, indicating that vasculature precedes tooth development, and was present in the presumptive dental mesenchyme. In the thickened dental epithelium stage and at the bud stage, blood vessels were no longer present in the dental mesenchyme, suggesting that local tooth-specific signals had repelled the blood vessels outside of the dental mesenchyme. At the cap stage the first blood vessels grew from the blood vessel plexus in the dental follicle into the dental pulp, proposing yet again that local repelling signals prevented the first

ingrowth of the blood vessels or they were attracted to enter the dental papilla. Similarly, the vascularization of the epithelial enamel organ was developmentally regulated and commenced after a waiting period at E18, before onset of dentin and enamel production. Previously it was reported that blood vessels enter the dental papilla at the late cap stage (E15) or at the early bell stage (Nait Lechguer et al., 2008; Rothova et al., 2011; Yuan et al., 2014), but our results show that ingrowth occurs already at E14. Vascularization of the enamel organ was demonstrated to happen after birth (PN2) (Nait Lechguer et al., 2008), but we saw that blood vessels entered the enamel organ before birth at E18. A set of similar observations has been published for teeth in the cat (Gaunt, 1959).

Maturation of blood vessels is of importance for their function. When differentiation and maturation of various subsets of vasculatures occurs, arteries and arterioles become enwrapped by smooth muscle cells and capillaries become enveloped by discontinuously organized pericytes, which in the face originate from the cephalic neural crest (Hirschi and D'Amore, 1996; Etchevers et al., 2001). Even though pericytes are scarcely located, their actual location is functionally determined and they may prefer to shield epithelial cell junctions (Bergers and Song, 2005). Our findings demonstrated that there was only one arteriole in the dental papilla that started to mature at the early bell stage (E16), as shown by immununohistochemistry performed on all sections through the tooth germ, using an antibody against alpha smooth muscle actin (alpha SMA), Recruitment of mural cells on the dental arteriole occurred two days after the ingrowth of the first blood vessels into the papilla. Recruitment of mural cells appears to be an organ-specific process. At E14 the mandibular artery already displayed an obvious coverage of smooth muscle, but the tooth specific blood vessels had no mural cells. In limb skin, vascular alpha SMA positive cells were seen at E14.5 but only in larger diameter vessels. One day later, at E15.5, the cells had extended to cover smaller vessel branches as well (Mukouyama et al., 2002). On the other hand, periendothelial cells were first seen in the retina after birth at PN2, but they were alpha SMA negative. At PN5 large arterioles are alpha

SMA positive and at PN9 mural cell coverage had additionally extended into thinner arterioles in the retina (Stalmans et al., 2002).

Collectively, our present data shows that the development and patterning of early mouse mandibular first molar tooth germ, as demonstrated earlier for trigeminal innervation of the mouse molar (Luukko et al., 2005; Luukko et al., 2008), is a distinct, step-wise process that takes place in a developmentally regulated, well-defined manner and is intimately coupled with advancing histo-morphogenesis of the tooth germ proper. The developing multicuspid, two-rooted mandibular first molar, has been established as a valuable system for investigation of the molecular mechanisms of organ formation, peripheral innervation and evolution (Thesleff et al., 2001; Luukko et al., 2005; Fried et al., 2007; Luukko et al., 2008). The present findings suggest that the tooth germ provides a useful model for studying the cellular and molecular mechanisms involved in blood vessel development in peripheral organs. Earlier other model systems have been used to investigate the development of vasculature: retina, hindbrain and limb skin (Mukouyama et al., 2002; Tata et al., 2015).

5.2 Development of the dental vasculature is suggested to involve angiogenic sprouting

Vasculogenesis has been used to define the formation of blood vessels by *in situ* development of endothelial cells from angioblasts. In contrast, angiogenesis has been defined as development of novel blood vessels from pre-existing ones by a process called angiogenic sprouting (Risau and Lemmon, 1988; Pardanaud et al., 1989; Yancopoulos et al., 2000). In general, vasculogenesis is thought to control formation of the early vascular tree in the early embryo, whereas an angiogenic process appears to predominate during organogenesis (Pardanaud et al., 1989). Based on the results presented here and earlier data, it appears that during the early stages of odontogenesis, a developing tooth blood supply emerges through an angiogenic

process: blood vessels surrounding the tooth epithelium sprout from the blood vessel network in the jaw mesenchyme. At the cap stage blood vessels grow into the dental pulp from the pre-existing vascular plexus in the dental follicle. Likewise, during later tooth histomorphogenesis, the development of the tooth blood supply takes place by the angiogenic process as supported by the histological observations that new dental blood vessels emerge from the pre-existing blood vessel in the tooth (Gaunt, 1959; Yuan et al., 2014; Ribatti et al., 2015). This receives support from a transplantation experiment in which blood vessels into both mesenchymal dental papilla and the epithelial enamel organ of cultured tooth germs, originated from the host thus ruling out a vasculogenetic process in this context (Nait Lechguer et al., 2008). However, in a recent electron microscopy study, it is of note that an involvement of vasculogenesis has been proposed for the development of blood vessels into the dental follicle (Yuan et al., 2014). Moreover, vasculogenesis has been suggested to occur also in the enamel organ in addition to angiogenesis (Manzke et al., 2005).

5.3 Development of blood vessels in the tooth precedes that of tooth innervation

Comparison of the localization of developing blood vessels and neurites in the mouse mandibular first molar tooth germ, demonstrated that the development of blood vessels in the tooth preceded that of innervation. Before and during the initial stages of tooth histomorphogenesis at E10 and E11, a plentiful number of blood vessels was seen throughout the mandibular process mesenchyme including the presumptive dental mesenchyme. In contrast, the single trigeminal molar nerve, which is the first one to reach and innervate the molar tooth germ, appeared at around E12. This is in agreement with earlier reports (Loes et al., 2002; Kettunen et al., 2005; Kettunen et al., 2007). Later at the placode stage, the distribution of blood vessels was reorganized and dental mesenchyme was devoid of blood vessels. One day later, the first branches of the molar nerve had just reached the tooth target (Luukko, 1997; Kettunen et al., 2005; Kettunen et al., 2007). During later development, while the first

blood vessels entered the dental papilla at the cap stage, the first neurites were allowed to ingress the dental pulp only after several days postnatally, after the onset of enamel formation at around PN4. Based on this data, it can be concluded that development of the blood vessels in the developing tooth precedes that of innervation.

In early stages before enamel formation, no co-localization of nerves and blood vessels was observed by double immunofluorescence analysis in the dental follicle. After ingrowth of nerve fibers into the pulp some thicker nerve fibers appeared to follow, to some extent, pre-existing large caliber blood vessels surrounded by smooth muscle cells in the pulp. When the nerve bundles were defasciculated and were heading to the cusp regions, it was noted that neurites were not associated with capillaries in the periphery of the pulp. Accordingly, in the enamel organ of the tooth, blood vessels were observed at E18, whereas no nerves were observed there during the stages studied up to PN7. Collectively, the present data shows that development and patterning of tooth blood vessels and innervation appear not to be directly spatio-temporally interrelated until postnatal stages when the innervation of the dental pulp commences and some neurites follow the larger blood vessels in the dental pulp.

5.4 Development of tooth vasculature and innervation is suggested to take place in an independent and non-independent manner

The definition of neurovascular congruency (Bates et al., 2002) has been used to describe the phenomenon where blood vessels and neurons run together (Martin and Lewis, 1989; Feig and Guillery, 2000; Bates et al., 2002; Bates et al., 2003; Oh and Gu, 2013). In the adult tooth, peripheral nerves and blood vessels are commonly located in the same areas and next to each other, namely inside the tooth in the dental pulp and in the periodontal space surrounding the tooth root, in which collagen fibers connect the root to the alveolar bone (Hildebrand et al., 1995; Steiniger et al., 2013).

In many organs such as limb skin and the hair follicle, neurovascular congruency has been reported to be established during embryogenesis (Bates et al., 2003; Oh and Gu, 2013). There are earlier findings which have demonstrated that the vasculature does not act as a model for nerve distribution (Martin and Lewis, 1989; Gu et al., 1995; Bates et al., 2002) and where nerve fibers have been shown to regulate arterial differentiation and branching (Mukouyama et al., 2002). The hair follicle, like the tooth, develops as a skin appendage (Thesleff et al., 1995). It has been confirmed that in the hair follicle the patterning of blood vessels and neurites, and the establishment of neurovascular congruency, occur independently of each other but via shared molecular patterning mechanisms (Martin and Lewis, 1989; Bates et al., 2003; Oh and Gu, 2013), i.e. by utilizing the same molecules and receptors, which control separately the development of both systems (Adams et al., 1999; Carmeliet and Tessier-Lavigne, 2005; Gelfand et al., 2009; Adams and Eichmann, 2010).

The present results revealed that formation of dental vasculature preceded ingrowth of nerve fibers, and that although developing dental nerve fibers and blood vessels were observed in the same mesenchymal areas, they showed distinct developmental time-courses and patterning, indicating that nerve fibers did not follow blood vessels before ingrowth of neurites into the pulp occurred. In addition, even though dental neurites showed disturbed patterning, especially in the developing *Sema3A*$^{-/-}$ molars, and in *Sema6A*$^{-/-}$ tooth germs, though to a lesser extent, no apparent changes or disturbances in the development or patterning of the dental blood vessels were detected. Moreover, in *Sema3A*-deficient molars, blood vessels were found not to follow nerve fibers, which were ectopically present in the condensed dental mesenchyme and dental papilla before ingrowth of blood vessels. Earlier, in limb skin, blood vessels were demonstrated to follow misrouted neurites in *Sema3A*$^{-/-}$ mice (Mukouyama et al., 2002). Taken together, it is proposed that the development and patterning of dental blood vessels is not regulated or dependent on peripheral sensory nerve fibers or SEMA3A or SEMA6A signaling during early odontogenesis. It is hypothesized that tooth innervation and vascularization take place via separate

mechanisms involving differential molecular regulation. Postnatally, however, it is possible that dental blood vessels with smooth muscle coverage (arterioles), influence some ingrowing sensory neurites, that may be guided to follow pre-existing dental blood vessels in the dental pulp, in a similar fashion to that proposed for blood vessels and sympathetic nerves (Honma et al., 2002; Damon et al., 2007; Brunet et al., 2014) where sympathetic nerves have been shown to follow arterial smooth muscle cells that produced the axon guidance cue netrin-1 (Brunet et al., 2014). On the other hand, because VEGF has been reported to be expressed in nerve fibers and Schwann cells (Mukouyama et al., 2002) it may be involved in the process in which dental sensory nerve fibers invading the pulp are aligned with blood vessels, which express VEGF receptors, VEGFR2 and NPN1 (Sijaona et al., 2012).

5.5 VEGF is suggested to mediate local tooth organ-blood vessel interactions and regulate development of dental vasculature

VEGF is a secreted protein, and is an important regulator of embryonic blood vessel formation and regulates vasculogenesis and angiogenic sprouting by affecting cell migration and proliferation as shown using *in vitro* studies and various *Vegf* transgenic mouse strains (Carmeliet et al., 1996; Ferrara et al., 1996; Gelfand et al., 2009). Based on the fact that there is limited information regarding mRNA and protein expression, as well as function of VEGF in vascularization during tooth development (Aida et al., 2005; Miwa et al., 2008; Nait Lechguer et al., 2008; Ide et al., 2011; Yuan et al., 2014), and that in general development of blood vessels and nerves involves shared molecular mechanisms (Martin and Lewis, 1989; Adams et al., 1999; Bates et al., 2002; Carmeliet and Tessier-Lavigne, 2005; Gelfand et al., 2009; Adams and Eichmann, 2010; Oh and Gu, 2013), prompted us to investigate *Vegf* expression in the developing mouse mandibular first molar tooth germ. Mouse VEGF has three principal isoforms VEGF120, VEGF164, and VEGF188, which are produced by the majority of embryonic tissues (Mackenzie and Ruhrberg, 2012). Each isoform has a distinct affinity for heparin sulphate, and consequently they form

a VEGF gradient in the tissue (Carmeliet and Tessier-Lavigne, 2005). Our RT-PCR analysis showed that at the cap stage (E14) the tooth germ expresses several VEGF isoforms, such as freely soluble VEGF120 as well as secreted VEGF144 and VEGF164, which both bind to the cell surface and extracellular matrix. The relative ratio of the different VEGF isoforms at the mRNA level differs in various organs, and in different developmental stages in embryogenesis, as well as in adulthood (Ng et al., 2001; Mukouyama et al., 2002; Tillo et al., 2015). It is likely that this reflects the different functions of the various isoforms in the vasculature and organ-specific functions of mature blood vessels (Mackenzie and Ruhrberg, 2012). In the tooth germ VEGF120, VEGF144 and VEGF164 were co-expressed. In addition, we observed two very faint bands, which suggests that also VEGF188 and an isoform that is shorter than VEGF120 are expressed in the tooth germ. Previously immortal mouse embryonic fibroblasts have been reported to produce VEGF115 isoform (Poltorak et al., 1997; Sugihara et al., 1998; Ruhrberg et al., 2002; Ferrara, 2004).

Sectional *in situ* hybridization revealed that *Vegf* exhibits spatio-temporally regulated cellular expression domains in both dental epithelial and mesenchymal tissue components, including enamel knots, dental papilla and follicle region and thus showed apparent correlation with the development and localization of dental blood vessels. At the early stages of molar tooth development at E11 and E12, *Vegf* expression in the developing tooth germ did not show any distinct pattern of expression. This may explain why the early blood vessel network was randomly organized in the jaw mesenchyme. However, later at E14, when tooth specific morphogenesis takes place, development and patterning of dental blood vessels may be attributed to the up-regulated and specific *Vegf* expression domains. Dental papilla mesenchyme and epithelial primary enamel knot signaling center exhibited intense *Vegf* expression during blood vessel ingrowth in the papilla. Later *Vegf* was also seen in the secondary enamel knots. Previously, the enamel knots have been shown to express a variety of signaling molecules, which are critical for tooth development (Jernvall et al., 1994; Thesleff and Jernvall, 1997; Thesleff et al., 2001; Luukko et al.,

2003). Our *in situ* hybridization and RT-PCR results suggest that enamel knot expressed VEGF isoforms generate a VEGF gradient in the dental papilla at the cap stage that attracts blood vessels to grow first into the papilla and later into the cusp mesenchyme and then form there a subodontoblastic blood vessel plexus. Similarly, the expression of VEGF mRNA in stellate reticulum and stratum intermedium that preceded ingrowth of blood vessels into the enamel organ, suggests that VEGF is involved in the vascularization of the enamel organ, and especially provides vascular support for ameloblasts in their enamel production. These proposed functions receive support from the findings in the retina, where an intense VEGF mRNA expression was seen in front of the growing vascular plexus and very little expression was present behind the leading edge of expression (Gerhardt et al., 2003). Moreover, a gradient of VEGF affects migration of the blood vessel tip cell and the concentration of VEGF has an effect on proliferation of stalk cells in the retina (Gerhardt et al., 2003). Our Western blot results indicated that in addition to VEGF mRNA also VEGF protein is synthesized in the developing tooth germ even though the protein level appeared to be low. Earlier it has been reported that there is a good correlation between VEGF mRNA and protein level when VEGF is induced by hypoxia (Shima et al., 1995). This suggests that VEGF is produced during odontogenesis and it is reflected by spatio-temporally changing VEGF mRNA domains. It will be interesting to investigate further the putative mode of actions of VEGF in odontogenesis. The major VEGF signaling receptor VEGFR2 and its mRNA was found to be specifically expressed in the developing blood vessels. Based on these findings, we suggest a model where the tooth target expressed VEGF through binding to VEGFR2 in the tip cell acts as a critical regulator of the development and patterning of tooth vasculature, and by so acting mediates local signaling interactions between tooth target and blood vessels. In addition to VEGFR2, VEGF is able to bind co-receptor NPN1. NPN1 has been shown to modulate angiogenic functions of VEGF (Gu et al., 2003; Vieira et al., 2007b) and its mRNAs are expressed in the dental blood vessels during molar tooth formation (Loes et al., 2001). Besides enhancing the response of VEGFR2 to VEGF164 stimulation during angiogenesis, there is some evidence suggesting that NPN1 may also regulate angiogenesis independently of VEGFR2 (Aspalter et al.,

2015; Kofler and Simons, 2015). Recently, VEGF189 was displayed to bind NPN1 and regulate neuronal patterning in the brain (Tillo et al., 2015).

5.6 VEGF signaling may serve neuronal and non-neuronal functions during odontogenesis

Besides being a critical regulator of the cardio-vascular system, VEGF has been reported to have various *in vitro* and *in vivo* neuronal functions such as in neurogenesis, neuronal survival and migration as well as axon guidance (Mackenzie and Ruhrberg, 2012). VEGF, by binding directly on NPN1, has been shown to promote axon growth and act as a chemoattractive signal independently of semaphorin3A (SEM3A) (Erskine et al., 2011). SEMA3A, which is a diffusible chemorepellant, binds co-receptor NPN1 in addition to class A plexins on axons (He and Tessier-Lavigne, 1997) and is essential for the timing of tooth innervation as well as dental axon pathfinding and patterning (Kettunen et al., 2005; Kettunen et al., 2007). *Npn1* is expressed in dental nerve fibers during mouse molar development, and *Npn1*-deficient mouse embryos show a defect in tooth innervation (Loes et al., 2001; Kettunen et al., 2005). Thus, it is possible that VEGF signaling may be involved in regulation of tooth innervation by directly acting upon developing axons. It is also possible that a balance between SEMA3A and VEGF signaling may also regulate development of dental sympathetic innervation, which in the mouse molar starts at around PN9 (Moe et al., 2008; Long et al., 2009). VEGF has been previously reported to be capable of promoting growth of sympathetic neuritis, together with SEMA3A (Long et al., 2009). On the other hand, the observation that VEGF and NPN1 mRNAs show apparent co-expression in sites such as the dental papilla mesenchyme that does not correlate with localization of neurites or blood vessels (Loes et al., 2001) suggests that VEGF may serve other, organogenetic functions during tooth formation, perhaps acting through NPN1. In support of this, VEGF has been shown to be involved in bone formation and regulates differentiation of chondrocytes and osteoblasts (Carlevaro et al., 2000; Duan et al., 2015).

5.7 Local tissue interactions are proposed to integrate tooth histomorphogenesis, angiogenesis and innervation

Various signaling molecules of different families such as conserved FGF, HH, TGF-ß and WNT are important mediators of odontogenic tissue interactions and regulate formation of the tooth organ proper as well as tooth innervation signaling (Luukko et al., 2008; Cobourne and Sharpe, 2013; Luukko and Kettunen, 2014; Thesleff, 2014). They also integrate their development as shown for FGF, TGFß and WNT signaling (Luukko et al., 2008; Luukko and Kettunen, 2014). Importantly, these key signaling families are also implicated in vasculo–angiogenesis as shown by targeted inactivation of numerous genes in mice (Coultas et al., 2005), and additionally regulate VEGF mRNA expression (Ferrara, 2004; Clifford et al., 2008). For instance, in developing lung, which develops as a result of epithelial-mesenchymal interactions, FGF and SHH signaling, which are essential for proper tooth formation, also regulate *Vegf* expression (White et al., 2007; Scott et al., 2010). Moreover, *Vegf* expression in osteoblasts can be induced by BMP, suggesting that BMP-regulated VEGF signaling may couple angiogenesis to osteogenesis (Deckers et al., 2002). VEGF may also, in turn, regulate BMP2 mRNA and protein expression in endothelial cells, demonstrating an osteogenic role for Vegf by stimulation of Bmp (Bouletreau et al., 2002). Collectively, based on the available data, it is tempting to propose that local tissue interactions, mediated by members of different signaling molecule families, regulate development of dental vasculature. Furthermore, these local interactions are proposed to spatio-temporally integrate formation of the tooth organ proper, its innervation and vascular development. Future investigations are warranted to elucidate further the regulatory mechanisms governing the integration of the tooth supporting tissues with the development of the tooth organ proper.

5.8 SEMA6A signaling regulates timing of dental pulp innervation but not the initial neurite encounter with the tooth germ

Semaphorin signaling serves critical neuronal functions during tooth formation as demonstrated for SEMA3A, which regulates the timing and patterning of tooth innervation and fasciculation of neurites (Kettunen et al., 2005; Moe et al., 2012; Shrestha et al., 2014). In the present study *in vivo* functions of transmembrane SEMA6A were addressed using *Sema6A*-deficient mice. SEMA6A mRNAs are spatio-temporally expressed in the mouse embryo (Zhou et al., 1997) and they have also been reported to be present in the developing mouse tooth using RT-PCR (Lillesaar and Fried, 2004). Localization of nerve fibers in embryonic *Sema6A*$^{-/-}$ molars showed that SEMA6A does not affect the initial nerve encounter with the tooth target or its innervation. It is important to note that neurites were found to penetrate the dental pulp prematurely, already at PN1 in *Sema6A* deficient molars, whereas in the wild-type molar the first nerve fibers were seen in the pulp later after enamel formation at PN4, and this is in line with previous reports (Mohamed and Atkinson, 1983; Moe et al., 2008; Moe et al., 2012). In PN4 and PN7 molars, however, no differences in localization, patterning or arborization of nerves were observed between the wild type and *Sema6A*$^{-/-}$ mice. This indicates that the effect of SEMA6A on pulp innervation i.e. regulation of timing, is time-limited. Indeed, neuronal defects in *Sema6A* and *Sema3A* deficient mice have been reported to undergo correction (White and Behar, 2000; Little et al., 2009; Moe et al., 2012). Taken together the present results indicate that SEMA6A signaling regulates the timing of innervation of the dental pulp but appears not to serve any other essential neuronal function in tooth innervation. Like in *Sema3A*$^{-/-}$ teeth, no disturbances in the development of tooth shape or dental blood vessels were seen in *Sema6A* deficient molars. An exception was the tooth germ in E14 where ingrowth of blood vessels had not occurred. This, however, can be attributed to the delay in tooth morphogenesis, which was later corrected.

5.9 SEMA6A signaling may regulate sympathetic innervation of the tooth

Sympathetic nerves enter the mouse molar pulp after the sensory trigeminal ones at around PN9 (Moe et al., 2008). SEMA6A acts as a repulsive molecule for the sensory and sympathetic axons *in vitro* (Xu et al., 2000; Suto et al., 2005). Because SEMA6A mRNA has been reported to be present in the postnatal mouse molar dental pulp up to PN9 (Lillesaar and Fried, 2004), it is proposed that SEMA6A signaling may regulate sympathetic innervation in the tooth germ as well. In support of this, SEMA6A is a potent regulator of sympathetic axons because *in vitro* a higher concentration of SEMA6A-Fc is required to collapse sensory dorsal root ganglion axons than sympathetic ones (Xu et al., 2000). In addition, the SEMA6A receptor PlexinA4 is expressed in the superior cervical ganglion (Haklai-Topper et al., 2010).

5.10 It is suggested that SEMA6A and SEMA3A exert synergistic and redundant repellent functions during tooth innervation

Although neurites penetrated prematurely into the *Sema6A*$^{-/-}$ pulp, no other apparent distinguishable changes in tooth innervation were found. There is ample evidence suggesting that tooth innervation is controlled by the coordinated action of locally expressed tooth target regulatory molecules of different families such as neurotrophins, in particular nerve growth factor (Ngf) and other semaphorins expressed in the tooth, such as SEMA3A (Fried et al., 2000; Luukko et al., 2005; Luukko et al., 2008; Luukko and Kettunen, 2014). In particular in the developing tooth, tooth-target expressed *Sema3A* has been shown to regulate tooth innervation by controlling dental axon navigation, patterning and fasciculation, as well as determining the timing of pioneer nerve encounter in the tooth germ and dental pulp innervation in both molar and incisor tooth germ fields (Loes et al., 2001; Kettunen et al., 2005). Unlike in the *Sema6A*-deficient molars where nerve fibers are prematurely seen in the dental pulp at PN1, in *Sema3A*$^{-/-}$ teeth, nerve fibers were ectopically present in the condensed dental pulp already at E13, where the nerves persisted until

normal innervation of the dental pulp commenced (Kettunen et al., 2005; Moe et al., 2012). It is suggested that both tooth target expressed cell-membrane-bound (SEMA6A) and secreted semaphorins (SEMA3A) that act as repulsive axon guidance cues, exert synergistic functions during tooth sensory and sympathetic innervation. Moreover, it is possible that the observed defects in tooth sensory trigeminal innervation in *Sema6A*$^{-/-}$ molars may have been compensated, to some extent, by SEMA3A signaling. Similarly, based on redundancy, the effects on tooth innervation in *Sema3A*$^{-/-}$ mice may have been compensated by SEMA6A signaling. In addition, it is possible that neuroregulatory molecules such as NGF and GDNF (Byers et al., 1992; Luukko et al., 1997a; Luukko et al., 1997b; Nosrat et al., 1997; Kvinnsland et al., 2004; Nosrat et al., 2004) and other tooth expressed molecules implicated in tooth neuronal development may, in addition to SEMA3A, act to compensate for the lack of SEMA6A in *Sema6A*$^{-/-}$ teeth. In summary, the present *in vivo* data demonstrates that SEMA6A is an essential, tooth target produced signal, which regulates the timing of early tooth vascularization and dental pulp innervation, but not tooth morphogenesis. The present data show that both cell-membrane-bound semaphorins act to regulate the development of tooth-supporting tissues. Furthermore, they may exert synergistic and redundant functions with other tooth expressed semaphorins.

6. CONCLUSIONS

In this study the development of tooth shape, vasculature and innervation as well as molecular regulation of these processes was addressed in the early developing mouse mandibular first molar. Specifically, it was found that:

-The time-course, maturation and patterning of dental vasculature takes place in a dynamic, spatio-temporally regulated manner that differs from that of tooth innervation and is suggested to be regulated locally, by tooth-target expressed VEGF, which shows a dynamically changing expression pattern in the developing tooth germ.

-Development of the dental blood vessels and their patterning is not dependent on peripheral nerves and SEMA3A-signaling.

-SEMA6A signaling regulates the timing of the innervation of the dental pulp but is dispensable for tooth histomorphogenesis and vascularization.

To conclude, the present data provides further evidence in support of the model that integration of tooth morphogenesis and development of its supporting tissues are developmentally controlled processes and involves developmentally coordinated, local signaling networks.

7. FUTURE PERSPECTIVES

Teeth, which are present in the oral cavity, serve important masticatory functions. The development of the tooth, which provides a central model system to unravel cellular and molecular mechanisms of organ formation, is regulated by local reciprocal cell-cell and tissue interactions. These interactions are mediated by a number of locally produced signals, which together form large integrated signaling networks. Recent research has provided evidence that local interactions are also involved in the development of tooth-supporting tissues, such as tooth innervation. The data presented here provide new knowledge pertaining to the molecular regulation of tooth-supporting blood supply, maturation of dental blood vessels and association of blood vessels and nerve fibers. Proper function of both the blood supply and innervation is essential for the existence and normal function of all teeth in the oral cavity.

In particular, intact vasculature and innervation are indispensable for the success and preservation of the putative biological replacement of teeth in the tooth row, generated by bioengineering. A biological approach, such as the use of stem cells, would seem to be a promising tool in order to manage dental problems in the future. It is apparent, however, that there still exists a great need for further research to gain a better understanding, and to unravel the molecular mechanisms that control both normal tooth development and dental stem cells, as well as how their development and function is coupled with the concomitant development and biology of the tooth-supporting blood vessels and nerve fibers.

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