Current and Future Views on Pulpal Angiogenesis

3

Petra Hilkens, Ivo Lambrichts, and Annelies Bronckaers

3.1 Introduction

Dental pulp tissue has important functions in the maintenance of tooth vitality, providing nutrients and oxygen, innervation, pain sensation, an immune response and formation of reparative dentine after injury. It can be injured through trauma, excessive wear or invasion by cariogenic oral bacteria, which can ultimately lead to acute irreversible immune/inflammatory events and destruction of the pulp tissue. Preservation of pulp viability is a major challenge in endodontics, as devitalised teeth are more vulnerable and prone to tooth loss later. Treatment of immature teeth, in particular, remains to be a challenge as any factor that interferes with normal pulp physiology may conflict with the completion of root development. Adequate revascularisation is a determining factor in successful dental pulp tissue preservation. To understand the process of pulpal blood vessel formation, the general molecular mechanisms of neovascularisation during embryogenesis and adult life are first discussed.

3.2 The Principles of Blood Vessel Formation

Within the human body, an extensive network of arteries, veins and capillaries can be found which are responsible for the oxygen and nutrient supply, waste removal and transportation of a plethora of different cell types and molecules. Depending on its function and location, the vasculature also displays different tissue-specific and organ-specific features, which are already determined during embryonic development.

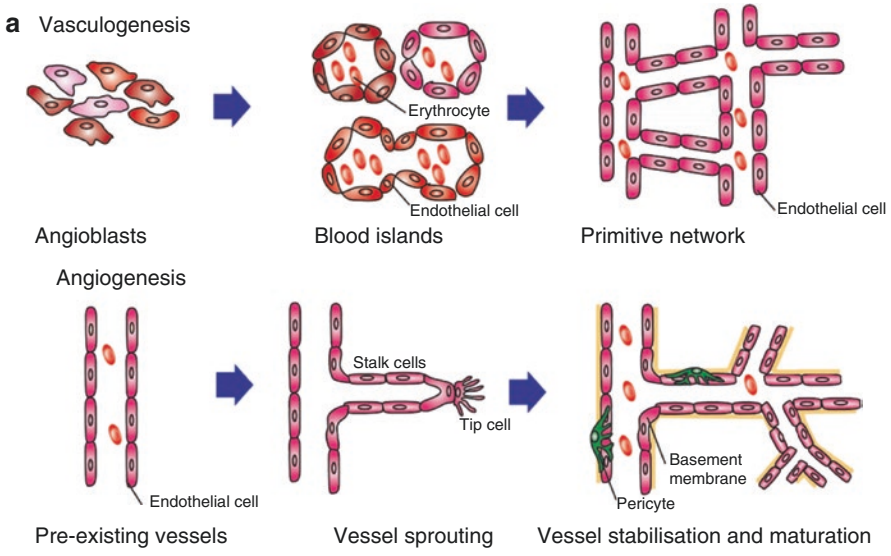
With regard to blood vessel growth and maturation, three different mechanisms can be distinguished, namely, vasculogenesis, angiogenesis and arteriogenesis.

P. Hilkens (✉) · I. Lambrichts · A. Bronckaers
Morphology Research Group, Biomedical Research Institute, Hasselt University, Diepenbeek, Belgium
e-mail: petra.hilkens@uhasselt.be

H. F. Duncan, P. R. Cooper (eds.), *Clinical Approaches in Endodontic Regeneration*,
https://doi.org/10.1007/978-3-319-96848-3_3

3.2.1 Vasculogenesis

During embryonic development, the primitive vascular network is initially formed by vasculogenesis (Fig. 3.1a). This process comprises the recruitment of mesodermally



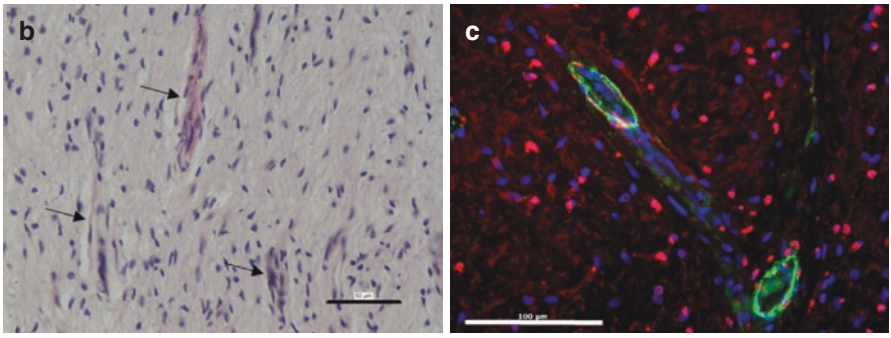


Fig. 3.1 (**a**) Schematic representation of vasculogenesis and angiogenesis. Vasculogenesis is defined as the formation of a primitive vascular network by mesodermally derived endothelial precursor cells, i.e. angioblasts. During embryonic development, angioblasts will first form primitive blood islands and differentiate into endothelial cells and erythrocytes. Subsequently, a primitive vascular plexus is formed which is further extended through angiogenesis. Angiogenesis comprises the formation of blood vessels out of pre-existing vessels during embryogenesis as well as in the adult human body. Specialised endothelial phenotypes will develop due to extensive growth factor signalling and give rise to immature blood vessels. Briefly, tip cells will migrate towards certain chemotactic stimuli, followed by the proliferation of stalk cells. The formation of intracellular vacuoles will eventually lead to the development of a vessel lumen. During the final phase of angiogenesis, i.e. blood vessel maturation, a stable vascular network emerges by anastomosis of adjacent sprouts, the formation of a basement membrane and the recruitment of pericytes. (**b**) Hematoxylin-eosin staining of human pulp tissue, indicating the presence of blood vessels (black arrows). Scale bar = 50 μm. (**c**) Immunofluorescent staining of human pulp tissue against VEGF (red) and CD146 (green). DAPI (blue) was used to stain the nuclei. This staining indicates a pronounced expression of CD146 in the pulp blood vessels, while the surrounding stromal cells express high amounts of VEGF. Scale bar = 100 μm

derived endothelial precursor cells or angioblasts that will coalesce. This begins in the yolk sack and will give rise to a primitive vascular plexus which provides the yolk sac circulation as well as the first primitive network inside the embryo, forming vessels such as the cardinal vein and the dorsal aorta. Subsequently, this network is enlarged by processes such as angiogenesis and arteriogenesis which are the key processes of blood vessel formation in adult life (Jain 2003; Swift and Weinstein 2009).

3.2.2 Angiogenesis

Generally speaking, angiogenesis can be defined as the formation of new capillaries from pre-existing blood vessels (Fig. 3.1a). Although the concept of angiogenesis entails both capillary sprouting and intussusception, i.e. the internal division of previously formed vessels, capillary sprouting is considered to be the most predominant form of vascular development within the adult human body (Swift and Weinstein 2009).

As any multistep biological process, the initiation and continuation of capillary sprouting are strongly coordinated by a myriad of growth factors, chemokines and other proteins. In general, these regulatory mechanisms are characterised by a distinct balance between activation and inhibition of blood vessel formation with the inhibiting factors being the most dominant, thus maintaining a quiescent state. In the presence of hypoxia or any other condition that requires oxygen and nutrients and thus new blood vessel formation, the excess production of stimulatory proteins causes a so-called 'angiogenic switch', tipping the balance towards blood vessel growth (Bronckaers et al. 2014; Distler et al. 2003).

One of the main cell types in the angiogenic process are endothelial cells (ECs). As previously mentioned, these cells normally remain in a quiescent state, enclosed by a vascular basement membrane and mural cells such as pericytes and smooth muscle cells that provide support, protect the cells from the environment and prevent the cells from detaching (Potente et al. 2011). Extensive growth factor signalling, e.g. after a wound or inflammation, leads to endothelial activation and the transient development of specialised endothelial phenotypes (Phng and Gerhardt 2009). Together with vascular endothelial growth factor (VEGF), extensive Notch signalling enables the establishment of a well-coordinated pattern of tip and stalk cells (Potente et al. 2011; Phng and Gerhardt 2009). In response to pro-angiogenic signalling, tip cells will facilitate proteolytic breakdown of the vascular basement membrane by secretion of matrix metalloproteinases (MMPs) and subsequently lead the migrating front of endothelial cells by scanning the environment for angiogenic cues with their filopodia (Potente et al. 2011; Adams and Alitalo 2007). Stalk cells, on the other hand, will proliferate abundantly in order to extend the formed vessel sprout. The formation of intracellular vacuoles, which will fuse with vacuoles of adjoining ECs, will eventually lead to the development of a vessel lumen. Through the production of extracellular matrix (ECM) components, stalk cells also safeguard the integrity of the formed vessel sprout (Potente et al. 2011; Phng and Gerhardt 2009). Finally, a stable vascular network emerges by anastomosis of adjacent sprouts and the recruitment of mural cells. After perfusion, pro-angiogenic signalling will diminish and the ECs return to a resting phenotype (Potente et al. 2011).

3.2.3 Arteriogenesis

Arteriogenesis, i.e. the maturation and stabilisation of the nascent vascular structures, encompasses the active recruitment of pericytes and smooth muscle cells, the deposition of a supportive ECM and the tissue-specific specialisation of the vessel wall in order to ensure vascular function (Jain 2003).

During embryonic development, arteriovenous specification and endothelial differentiation, i.e. vessel specialisation, are not only determined by haemodynamic load but also comprise specific molecular interactions involving VEGF, Notch and members of the Eph receptor kinases and their ephrin ligand, which will eventually lead to a well-organised, tissue-specific network of arteries, veins and capillaries (Jain 2003; Swift and Weinstein 2009).

Similar to vasculogenesis and angiogenesis, vessel maturation is tightly regulated by different molecular signalling pathways. Transforming growth factor-beta (TGF-β), for example, is closely involved in the production of ECM as well as the induction and differentiation of mural cells. The recruitment of pericytes, on the other hand, is mediated by platelet-derived growth factor receptor-beta (PDGFR-β) signalling. Angiopoietin 1 and 2 (ANGPT1 and ANGPT2) also play a critical role in the process of arteriogenesis. Binding to their receptors, Tie1 and Tie2, promotes vessel stabilisation and enables leak resistance by tightening endothelial cell junctions. Interactions between ECs and mural cells are controlled by sphingosine-1-phosphate receptor signalling by causing changes in cytoskeleton organisation and cell adhesion (Jain 2003; Gaengel et al. 2009).

3.3 Blood and Lymph Vessels in the Dental Pulp

3.3.1 Tooth Development

During mammalian tooth development, strongly regulated reciprocal interactions take place between neural crest-derived mesenchymal tissues and ectodermally derived dental epithelium. Tooth morphogenesis and differentiation comprise different stages, with each exhibiting their own specific spatio-temporal events, eventually leading to tooth eruption (Jussila et al. 2013). Given the close relationship between the neurovascular supply of the tooth and tooth morphogenesis, mesenchymal invasion of both nerve fibres and blood vessels already occurs during the late cap stage and/or early bell stage (Jussila et al. 2013; Nait Lechguer et al. 2008). In addition, changes in vascular pressure presumably play a role in the timing and rate of tooth eruption, although definitive conclusions cannot be made at present (Burn-Murdoch 1990; Kjaer 2014; Wise and King 2008).

3.3.2 Vascular Anatomy of the Dental Pulp

The main vascular supply of the dental pulp originates from the maxillary artery, which branches off the external carotid artery. The maxillary artery flows into the

dental artery, which enters the dental pulp through arterioles, forming the pulp microvasculature (Kim 1985). In terms of structural arrangement, the pulpal microcirculation is characterised by a strong hierarchical organisation: the arterioles spread throughout the central part of the dental pulp and eventually form a subodontoblastic capillary network (Kim 1985; Yu and Abbott 2007). This peripheral capillary plexus displays strong regional differences, ranging from a fishnet organisation in the roots to a dense network of hairpin-shaped capillaries in the pulp horn (Kim 1985). The blood vessels are mainly lined by a continuous layer of endothelium, except for the subodontoblastic capillaries which display endothelial fenestrations, reflecting the distinct metabolic demand in this region (Berggreen et al. 2010). The blood eventually drains into venules, which mainly comprise the central part of the pulp tissue and exit the tooth through the apical foramen (Kim 1985).

The pulpal microvasculature also displays certain specialised features, such as the presence of vascular shunts. These anastomoses are either arteriovenous, venous-venous or U-turn loops and presumably play an important role in the regulation of blood flow, given the direct connection between arterioles and venules which bypasses the aforementioned capillary plexus (Kim 1985; Yu and Abbott 2007).

Next to an extensive vascular network (Fig. 3.1b, c), dental pulp tissue also contains lymphatic vessels, which can be identified by their expression of VEGF receptor-3 (VEGFR-3) and lymphatic endothelial hyaluronan receptor-1 (LYVE-1) (Pimenta et al. 2003; Berggreen et al. 2009). These thin-walled vessels originate in the coronal region of the dental pulp and can be clearly distinguished from venules due to the absence of erythrocytes and the presence of wall discontinuities (Nanci 2008). Lymphatic vessels exit the dental pulp, either through large vessels in the apical foramen or through lateral canals in the radicular region (Berggreen et al. 2010; Nanci 2008).

3.3.3 Importance and Regulation of Dental Pulp Vasculature

As any vascular network, the main function of the pulpal microcirculation is to provide sufficient oxygen and nutrients to the tissue's residing cells as well as remove waste products. Studies have also described the relatively high interstitial tissue pressure within the dental pulp tissue, as compared to the vascular blood pressure. However, the constant tissue fluid volume within the pulp tissue indicates an important role for the dental pulp's microvascular network in the management of both the intraluminal vascular pressure and the pressure within the pulp tissue itself (Yu and Abbott 2007; Heyeraas 1989).

Given the low compliance of the dental pulp, the lack of a collateral blood supply and its important role in pressure and blood flow maintenance, strict regulation of the pulpal circulation is of utmost importance in order to safeguard the health of the dental pulp tissue (Yu and Abbott 2007). In physiological circumstances, vascular tone is regulated at different levels, i.e. through local, neurological and humoral mechanisms (Berggreen et al. 2010).

The dental pulp is a strongly innervated tissue, containing afferent sensory fibres, parasympathetic and sympathetic nerve fibres which are closely associated with the

pulpal vascular system (Rodd and Boissonade 2003; Zhang et al. 1998; Caviedes-Bucheli et al. 2008). In addition to sympathetic vascular regulation, a wide array of neuropeptides released by sensory nerve fibres also actively modulates the pulp's vasculature, in particular through vasodilation. More specifically, substance P, calcitonin gene-related peptide (CGRP) and neurokinin A have been shown to cause long-lasting increases in pulpal blood flow upon tooth stimulation (Caviedes-Bucheli et al. 2008).

With regard to the local regulation of pulpal blood flow, different vasoactive agents have been shown to regulate vascular resistance according to the tissue's needs (reviewed in (Berggreen et al. 2010)). The production of NO, for example, has been detected in endothelial cells as well as odontoblasts and plays an important role in the regulation of vasodilation in a number of animal models (Berggreen et al. 2010; Berggreen and Heyeraas 1999; Toda et al. 2012). Endothelin-1 (EDN-1), on the other hand, has been shown to cause a dose-dependent reduction in pulpal blood flow both in vitro and in vivo (Yu et al. 2002). Aside from locally produced regulatory agents, pulpal blood flow can also be controlled at a humoral level, by vasoactive agents which reach the dental pulp through vascular transportation such as adrenaline, dopamine and angiotensin II (Berggreen et al. 2010).

3.4 Inflammation and Angiogenesis

As already mentioned, the dental pulp is a heavily innervated and vascularised tissue which serves many specialised physiological functions. Although its enclosure within the dentinal walls provides both mechanical support and protection, the pulp tissue is very vulnerable to insults such as trauma, caries and infections once the structural integrity of the pulp chamber is compromised. In case of acute inflammation, the resulting vasodilatation will cause an increase in pulp tissue pressure, given the low compliance of the tissue (Yu and Abbott 2007). Due to the resilient, gelatin-like ground substance of the dental pulp tissue, these pressure differences and the resulting cell death remain localised, except in the case of chronic inflammation which can lead to overall tissue necrosis (Yu and Abbott 2007; Heyeraas and Berggreen 1999). The severity of the inflammatory process can thus be considered as a determining factor for the onset of regeneration and repair, as research has shown that low-grade inflammation may induce angiogenesis and stem cell-mediated regeneration, while continuing inflammation leads to tissue destruction and molecular inhibition of regeneration (Cooper et al. 2010). In a rat model of apical periodontitis, for example, a gradual upregulation of VEGF isoforms and their receptors was detected in vascular ECs, inflammatory infiltrate, osteoclasts and stromal cells over a period of 21 days, which suggests extensive vascular and bone remodelling (Bletsa et al. 2012). Artese et al., on the other hand, reported a significant downregulation of both VEGF expression and microvessel density in human dental pulp tissues from patients suffering from irreversible pulpitis (Artese et al. 2002).

In addition to caries and infection, orthodontic tooth movement can also evoke an inflammatory response and thus affect pulp blood flow and angiogenesis

(reviewed in (Javed et al. 2015)). Derringer et al., for example, observed an increased microvascular density in dental pulp tissue of teeth undergoing orthodontic force application for 2 weeks (Derringer et al. 1996). In accordance with these data, the same researchers reported the release of angiogenic growth factors, more specifically epidermal growth factor (EGF), bFGF, VEGF, TGF-β and PDGF, in response to orthodontic force (Derringer and Linden 2003, 2007). A number of studies also mentioned a transient change in pulp blood flow after prolonged exposure to orthodontic forces (reviewed in (Javed et al. 2015)).

As mentioned previously, neuropeptides play an important role in maintaining pulp homoeostasis through their regulation of pulpal blood flow. In addition to normal pulp physiology, neuropeptides also contribute to both neurogenic inflammation and regeneration and repair (Caviedes-Bucheli et al. 2008). In case of occlusal trauma, for example, the subsequent neurogenic inflammation and release of neuropeptides such as substance P promote angiogenesis, either by directly modulating endothelial cell behaviour or by stimulating paracrine mechanisms. The resulting increase in vascularity will promote mineralised tissue formation, both as a defence and repair mechanism (Caviedes-Bucheli et al. 2017). Neuropeptides can also modulate the inflammatory response and regulate angiogenesis through their interaction with dental pulp (stem) cells (Caviedes-Bucheli et al. 2008). An upregulation of CGRP in dental pulp tissue was observed after orthodontic force application, presumably leading to an increased angiogenic response within the dental pulp (Caviedes-Bucheli et al. 2011). Accordingly, El Karim et al. previously demonstrated an altered expression of angiogenic growth factors such as hepatocyte growth factor (HGF), EGF and placental growth factor, after in vitro exposure of dental pulp fibroblasts to different neuropeptides (El Karim et al. 2009).

3.5 The Promotion of Dental Pulp Angiogenesis: What are the Options?

3.5.1 Cell-Free Approaches

Angiogenesis requires a complicated interplay of numerous growth factors, cytokines and ECM components. Consequently, the first revascularisation strategies involved the application of angiogenic factors in biodegradable scaffolds. A wide variety of scaffolds have been tested in preclinical tooth regeneration studies or in vitro studies. Natural biomaterials include proteins such as collagen, fibrin and silk and polysaccharides such as chitosan, hyaluronic acid, alginate and agarose. Examples of synthetic biomaterials are organic polymers like polylactic acid (PLA) and poly(lactic-co-glycolic) acid (PLGA) or inorganic calcium phosphate materials such as hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) (reviewed in (Sharma et al. 2014)). These scaffolds are used as vehicles to deliver angiogenic growth factors, which do not only attract blood vessels but also induce stem cell homing. For instance, angiogenesis and tissue regeneration were augmented in subcutaneously implanted tooth slices treated with VEGF in immunodeficient mice

(Mullane et al. 2008). Collagen scaffolds loaded with FGF-2 successfully induced blood vessel formation and tissue regeneration in human roots implanted into the dorsum of rats (Suzuki et al. 2011). Combined delivery of bFGF, VEGF or PDGF with nerve growth factor (NGF) and bone morphogenetic protein-7 generated cellularised and vascularised tissues in real-size, native human teeth in mouse dorsum after 3 weeks of implantation (Kim et al. 2010).

Besides using single or combinations of recombinant proteins, natural cocktails of such factors can also be applied. Blood platelets, blood clots and consequently blood platelet concentrates such as platelet-rich plasma (PRP), platelet-rich fibrin (PRF) and leucocyte- and platelet-rich fibrin (L-PRF) contain a plethora of angiogenic factors including VEGF, FGF-2, thymidine phosphorylase and PDGF (Masoudi et al. 2016). A good example of the regenerative potential of blood platelets and their derivatives is the current practice in regenerative endodontics, which entails the induction of a blood clot by lacerating the periapical tissue. The blood clot serves as a scaffold for new ingrowing blood vessels but is also considered to attract stem cells from the apical papilla (SCAPs) towards the root canal. Blood platelet concentrates are cost-effective, contain a plethora of growth factors and can be autologously used. In a recent triple blind clinical trial, the use of PRP, PRF and induced bleeding in revascularisation of teeth with necrotic pulp and open apex was compared. PRP performed better than both other approaches with respect to periapical wound healing. All three treatments were comparable on grounds of root lengthening and lateral wall thickening (Shivashankar et al. 2017).

3.5.2 Stem Cell-Based Approaches for Pulp Revascularisation

As vascular access within the human tooth is localised at the apical foramen, the success of revascularisation and revitalisation approaches is largely determined by the size of this apical opening. In comparison to cell homing-based methods, which induce pulp revascularisation and healing in teeth with apical sizes ranging from 1.1 to 1.5 mm, stem cell-based approaches have been proven to successfully regenerate vascularised pulp tissue in pulpectomised canine teeth with apical foramen of 0.7 mm (Hilkens et al. 2015; Iohara et al. 2011). As stem cells do not only replace tissue but also produce of a broad range of (angiogenic) growth factors, these cells are widely studied in pulp revascularisation and regeneration.

During embryonic development as well as in the adult human body, several stem cell populations can be distinguished with each exhibiting their own characteristics. Given the elaborate ethical concerns associated with the isolation and use of embryonic stem cells, induced pluripotent stem cells (iPSCs) have proven to be valuable alternative source of pluripotent stem cells. In 2006, Takahashi et al. reported the creation of iPSCs through genetic reprogramming of somatic cells (Takahashi and Yamanaka 2006). Since then, extensive characterisation of these stem cells pointed out not only their elaborate differentiation potential but also their ability to promote angiogenesis in vitro and in vivo (reviewed in (Clayton et al. 2015)). Theoretically speaking, iPSCs can thus be considered as the stem cell type of choice in dental pulp

revascularisation and regeneration. However, several disadvantages are associated with the use of these stem cells, such as differences in reprogramming efficiency, teratoma formation and activation of oncogenes associated with viral cell transformation (Malhotra 2016). Therefore, multipotent or adult stem cells, in particular mesenchymal stem cells (MSCs) and dental stem cells (DSCs), are assumed to be the most favourable cell type for application in regenerative dentistry.

3.5.2.1 Mesenchymal Stem Cells

In 1970, Friedenstein et al. reported the presence of so-called colony-forming unit fibroblasts, which were later on defined as MSCs (Friedenstein et al. 1970). Elaborate characterisation of these stem cells led to the establishment of minimal criteria, defined by the International Society for Cellular Therapy (ISCT), which these stem cells have to fulfil. More specifically, MSCs have to be adherent to plastic under standard culture conditions; they have to express cell surface markers CD73, CD90 and CD105 and lack the expression of CD14, CD34, CD45, CD79a and HLA-DR; and they display an in vitro trilineage differentiation capacity into adipogenic, chondrogenic and osteogenic cells (Dominici et al. 2006). Within the human body, MSCs can be found in a variety of different tissues, such as bone marrow, tendons, umbilical cord, adipose tissue and teeth, with bone marrow-derived MSCs (BM-MSCs) being one of the most widely studied and applied sources of MSCs (Arana et al. 2013; Bi et al. 2007; Huang et al. 2009; Kim et al. 2013).

In addition to an elaborate differentiation potential, BM-MSCs also display pronounced angiogenic properties. Secretome analysis identified the expression of a vast array of angiogenic growth factors, including but not limited to angiogenin, ANGPT1 and ANGPT2, FGF-2, HGF, insulin-like growth factor-1 (IGF-1), interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), MMPs, TGF-β and VEGF (reviewed in (Bronckaers et al. 2014)). Besides the promotion of endothelial proliferation, migration and tubulogenesis in vitro (Estrada et al. 2009; Gruber et al. 2005; Potapova et al. 2007), BM-MSCs have also been shown to ameliorate angiogenesis in multiple animal models of peripheral artery disease, myocardial infarction and cerebral ischemia (reviewed in (Bronckaers et al. 2014)). With regard to their potential application in regenerative endodontics, BM-MSCs were reported to successfully generate pulp-like tissue in a rat pulpectomy model. However, the authors did not describe any signs of proper tissue vascularisation after transplantation (Ito et al. 2017). Zhang et al., on the other hand, demonstrated the regeneration of pulp-like tissue with a pronounced vascularisation after SDF-1-induced stem cell homing of systemically administered BM-MSCs (Zhang et al. 2015). Similar results were found after in situ transplantation of a $CD31^-$ side population of BM-MSCs in a canine model (Ishizaka et al. 2012). Despite their aforementioned high angiogenic potential, both the invasive and traumatic isolation procedures of BM-MSCs emphasise the need for an alternative source of MSCs (Holdsworth et al. 2003).

Adipose tissue-derived mesenchymal stem cells (AD-MSCs), for example, can be relatively easy isolated through liposuction. Similar to BM-MSCs, these stem cells also secrete several angiogenic growth factors, such as HGF, IGF-1, TGF-β and VEGF (Nakagami et al. 2005; Rehman et al. 2004). AD-MSCs were

successfully applied in animal models of ischemic heart disease, wound healing and peripheral vascular disease (reviewed in (Zhao et al. 2017)). With regard to the use of AD-MSCs in dental pulp revascularisation and regeneration, a $CD105^+$ subpopulation of these stem cells was not able to form a significant amount of vascularised, pulp-like tissue in a canine pulpectomy model (Iohara et al. 2011). In contrast, Hung et al. described the successful regeneration of innervated and vascularised tooth implants by AD-MSCs and dental pulp stem cells (DPSCs). Potential differences in vascularisation rate of the newly formed tissue were not reported (Hung et al. 2011). When comparing the regenerative potential of $CD31^-$ side populations of DPSCs, BM-MSCs and AD-MSCs in a canine model with complete apical closure, Ishizaka et al. detected no significant differences between these stem cell populations with regard to their neovascularisation potential (Ishizaka et al. 2012). However, transplantation of a granulocyte colony-stimulating factor (G-CSF)-mobilised population of AD-MSCs led to the formation of a significantly lower volume of pulp-like tissue with significantly less angiogenesis in comparison to a similar population of DPSCs (Murakami et al. 2015)

3.5.2.2 Dental Stem Cells

Given their inherent capacity to repair and regenerate dental tissues, DSCs are one of the most widely studied stem cell populations for regenerative endodontic procedures. DPSCs, stem cells from human exfoliated deciduous teeth (SHEDs), SCAPs and dental follicle precursor cells (FSCs), in particular, have been successfully administered in different in vivo models of dental pulp regeneration (Hilkens et al. 2015; Ratajczak et al. 2016). According to the previously mentioned minimal criteria of the ISCT, DSCs are considered to be mesenchymal-like stem cells (Dominici et al. 2006; Huang et al. 2009). Over the past decade, an increasing amount of research has been performed regarding the ability of DSCs to promote angiogenesis in vitro and in vivo.

Angiogenic Properties of Dental Stem Cells

With regard to the angiogenic properties of DSCs, a number of studies have indicated their ability to secrete a broad range of angiogenic growth factors and stimulatory proteins such as angiogenin, ANGPT1 and ANGPT2, FGF-2, CSF, dipeptidyl peptidase IV, EDN-1, IGF-1, insulin-like growth factor-binding protein-3, IL-8, HGF, MMPs, MCP-1, PDGF, urokinase-type plasminogen activator and VEGF (reviewed in (Ratajczak et al. 2016)). Furthermore, the DSC secretome also contains a substantial amount of inhibitory proteins, more specifically endostatin, pentraxin-3, pigment epithelium-derived factor, plasminogen activator inhibitor-1, tissue inhibitor of matrix metalloproteinases and thrombospondin-1 (reviewed in (Ratajczak et al. 2016)).

Given their secretion of stimulatory as well as inhibitory proteins, the potential influence of DSCs on the behaviour of ECs has been widely investigated in different in vitro models (Ratajczak et al. 2016). With regard to endothelial proliferation, for example, our research group demonstrated no considerable impact of DPSCs, SCAPs or FSCs on the proliferation of human microvascular endothelial cells

(HMECs) (Hilkens et al. 2014). In contrast, hypoxia-preconditioned DPSCs were reported to cause a time-dependent augmentation of endothelial proliferation (Aranha et al. 2010), which confirmed earlier findings by Iohara et al., describing a pronounced increase in the proliferation and survival of human umbilical vein endothelial cells (HUVECs) caused by a $CD31^{-}/CD146^{-}$ subpopulation of DPSCs (Iohara et al. 2008). Next to endothelial proliferation, DPSCs and SCAPs have been proven to successfully induce endothelial migration towards a chemotactic gradient of proteins (Hilkens et al. 2014). DSCs are also able to promote endothelial tubulogenesis, as was shown in a variety of direct and indirect co-culture systems (Hilkens et al. 2014; Tran-Hung et al. 2006; Yuan et al. 2015; Dissanayaka et al. 2012; Janebodin et al. 2013). Regarding the potential influence of DSCs on the angiogenic process as a whole, our group and others identified a marked increase in the number of blood vessels after application of DPSCs or SCAPs in the chicken chorioallantoic membrane assay (Hilkens et al. 2014; Bronckaers et al. 2013; Woloszyk et al. 2016).

In addition to paracrine regulation of angiogenesis, MSCs are also understood to promote angiogenesis in a direct manner by differentiating into ECs (Sieveking and Ng 2009). With regard to DSCs, DPSCs, SCAPs and SHEDs in particular have been shown to successfully differentiate towards ECs (reviewed in (Ratajczak et al. 2016; About 2014)). DPSCs, for example, were reported to co-differentiate into endotheliocytes, following osteogenic differentiation of a sorted subpopulation (d'Aquino et al. 2007). Differentiated DPSCs were also able to form extensive capillary networks in vitro, as was shown by Marchionni and others (Barachini et al. 2014; Marchionni et al. 2009). Similar results were found for SHEDs, indicating a VEGF-induced upregulation of endothelial markers as well as capillary sprouting in vitro and in vivo (Bento et al. 2013; Cordeiro et al. 2008; Sakai et al. 2010; Zhang et al. 2016). Endothelial differentiation of SCAPs was recently reported by Bakopoulou et al., describing both the upregulation of endothelial markers and the development of capillaries in normoxic culture conditions. Ischemic preconditioning of the cells even led to the establishment of a more pronounced endothelial phenotype (Bakopoulou et al. 2015).

Dental Stem Cells in Dental Pulp Revascularisation and Regeneration

Over the past 15 years, a substantial amount of studies have been published concerning the potential application of DSCs in regenerative endodontic procedures. Both DPSCs and SCAPs have been proven to be a potent cell-based approach for the regeneration of vascularised, pulp-like tissue in a wide variety of in vivo models. Takeuchi et al., for example, reported the successful regeneration of dental pulp tissue after administration of a mobilised subpopulation of DSPCs in an ectopic root transplantation model. While there was no difference in vascularisation rate between the described cell homing-based approaches, the observed capillary density in the newly formed tissue was considerably higher after transplantation of DPSCs supported by a collagen gel (Takeuchi et al. 2015). Kuang et al. showed the formation of vascularised, pulp-like tissue containing a significantly higher number of blood vessels after transplantation of nanofibrous spongy microspheres containing hypoxia-primed DPSCs compared to DPSCs cultured under normoxic conditions

(Kuang et al. 2016). When combining DPSCs with VEGF in an ectopic root transplantation model, a notably higher amount of tissue was formed in comparison to root canals containing solely DPSCs. In terms of vascularisation, however, no significant differences between the experimental conditions were detected (Li et al. 2016). In situ transplantation of constructs containing either canine DPSCs and PRF or DPSCs alone led to a significant promotion of blood vessel formation in comparison to PRF particles alone (Chen et al. 2015). Another combined approach was used by Dissanayaka et al., describing the regeneration of vascularised pulp-like tissue after transplantation of a hydrogel containing DPSCs and HUVECs in an ectopic root transplantation model. This combined method led to more pronounced vascularisation in comparison to the root fragments containing DPSCs alone (Dissanayaka et al. 2015).

As previously described by Rombouts and others, the interaction between DSCs and their micro-environment is a crucial factor, not only in the engraftment of the transplanted cells but also in the regulation of their intrinsic behaviour such as the secretion of paracrine factors (Rombouts et al. 2017; Tran and Damaser 2015). Recent work from our group demonstrated the successful regeneration of vascularised, pulp-like tissue in 3D-printed, hydroxyapatite scaffolds. However, quantification of the vascularisation rate pointed out a significantly lower amount of blood vessels/mm² in the constructs containing DPSCs and/or SCAPs when compared to the negative control condition. These data, together with the observed formation of mineralised tissue within the stem cell constructs suggest a preferential osteogenic/odontogenic differentiation of DSCs rather than the expected promotion of angiogenesis within the applied time frame, which emphasises the determining role of the micro-environment at the time of transplantation partly determined by the experimental conditions such as the choice of scaffold material and duration of construct transplantation (Hilkens et al. 2017).

3.6 Conclusion and Future Perspectives

Over the past two decades, substantial advances have been made in unravelling the angiogenic process and in the application of pro-angiogenic proteins, blood platelet products and stem cells as approaches to induce blood vessel formation and subsequent dental pulp regeneration in animal models. In addition, recent innovations in tissue engineering, such as the use of bioprinted scaffolds, could play a promising role in regenerative dentistry. Athirasala et al., for example, recently developed printable alginate hydrogels with fractions of dentine matrix, which enhanced odontogenic differentiation of SCAPs encapsulated in these hydrogels (Athirasala et al. 2018). Nanotechnology, another promising scientific development, has made it possible to deliver proteins or drugs by means of vehicles such as agarose beads, collagen sponges, alginate gels and hydrogel microspheres, thereby allowing a slow and prolonged release of these substances into the micro-environment.

Despite the high potential of angiogenic approaches such as stem cells, 3D printing and recombinant proteins, more advances are needed before these therapies can

enter the clinic. Cost-effectiveness, for example, as large-scale stem cell propagation, bioprinting or production of bioscaffolds, is currently non-existent or expensive. In addition, application of the aforementioned vascularisation approaches is labour intensive and still requires extensive monitoring. However, clinical translation remains complicated as multiple issues still need to be resolved, such as timing, dose and the presence of a suitable micro-environment/scaffold. The majority of the studies in the literature are based on subcutaneous implantation models in mice with healthy human teeth. These assays lack any signs of inflammation and bacterial infections and are often performed in immunocompromised animals. This is far removed from the clinical situation where pulp regeneration is mostly needed in pathological conditions such as necrosis, inflammation and apical periodontitis. Moreover, endodontic procedures entail the removal of the necrotic tissue and disinfection of the root canal which is potentially harmful for the biological tissues and their regenerative potential. Development of new animal models mimicking the clinical situations is thus needed to ascertain successful dentine-pulp regeneration with these procedures. In conclusion, despite the recent advances made in dental tissue engineering and the promotion of angiogenesis more specifically, there is still a long road ahead with regard to the application of effective revascularisation and regeneration treatment protocols in endodontics.

References

About I (2014) Pulp vascularization and its regulation by the microenvironment. In: Goldberg M (ed) The dental pulp: biology, pathology, and regenerative therapies. Springer, Berlin, pp 61–74

Adams RH, Alitalo K (2007) Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 8(6):464–478

Arana M et al (2013) Adipose tissue-derived mesenchymal stem cells: isolation, expansion, and characterization. Methods Mol Biol 1036:47–61

Aranha AM et al (2010) Hypoxia enhances the angiogenic potential of human dental pulp cells. J Endod 36(10):1633–1637

Artese L et al (2002) Vascular endothelial growth factor (VEGF) expression in healthy and inflamed human dental pulps. J Endod 28(1):20–23

Athirasala A et al (2018) A Dentin-derived hydrogel bioink for 3D printing of cell laden scaffolds for regenerative dentistry. Biofabrication 10:024101

Bakopoulou A et al (2015) Angiogenic potential and secretome of human apical papilla mesenchymal stem cells in various stress microenvironments. Stem Cells Dev 24(21):2496–2512

Barachini S et al (2014) Plasticity of human dental pulp stromal cells with bioengineering platforms: a versatile tool for regenerative medicine. Micron 67:155–168

Bento LW et al (2013) Endothelial differentiation of SHED requires MEK1/ERK signaling. J Dent Res 92(1):51–57

Berggreen E, Heyeraas KJ (1999) The role of sensory neuropeptides and nitric oxide on pulpal blood flow and tissue pressure in the ferret. J Dent Res 78(9):1535–1543

Berggreen E et al (2009) Characterization of the dental lymphatic system and identification of cells immunopositive to specific lymphatic markers. Eur J Oral Sci 117(1):34–42

Berggreen E, Bletsa A, Heyeraas KJ (2010) Circulation in normal and inflamed dental pulp. Endod Topics 17:2–11

Bi Y et al (2007) Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med 13(10):1219–1227

Bletsa A, Virtej A, Berggreen E (2012) Vascular endothelial growth factors and receptors are up-regulated during development of apical periodontitis. J Endod 38(5):628–635
Bronckaers A et al (2013) Angiogenic properties of human dental pulp stem cells. PLoS One 8(8):e71104
Bronckaers A et al (2014) Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. Pharmacol Ther 143(2):181–196
Burn-Murdoch R (1990) The role of the vasculature in tooth eruption. Eur J Orthod 12(1):101–108
Caviedes-Bucheli J et al (2008) Neuropeptides in dental pulp: the silent protagonists. J Endod 34(7):773–788
Caviedes-Bucheli J et al (2011) The effect of orthodontic forces on calcitonin gene-related peptide expression in human dental pulp. J Endod 37(7):934–937
Caviedes-Bucheli J et al (2017) Angiogenic mechanisms of human dental pulp and their relationship with substance P expression in response to occlusal trauma. Int Endod J 50(4):339–351
Chen YJ et al (2015) Potential dental pulp revascularization and odonto-/osteogenic capacity of a novel transplant combined with dental pulp stem cells and platelet-rich fibrin. Cell Tissue Res 361(2):439–455
Clayton ZE, Sadeghipour S, Patel S (2015) Generating induced pluripotent stem cell derived endothelial cells and induced endothelial cells for cardiovascular disease modelling and therapeutic angiogenesis. Int J Cardiol 197:116–122
Cooper PR et al (2010) Inflammation-regeneration interplay in the dentine-pulp complex. J Dent 38(9):687–697
Cordeiro MM et al (2008) Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. J Endod 34(8):962–969
d'Aquino R et al (2007) Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. Cell Death Differ 14(6):1162–1171
Derringer KA, Linden RW (2003) Angiogenic growth factors released in human dental pulp following orthodontic force. Arch Oral Biol 48(4):285–291
Derringer K, Linden R (2007) Epidermal growth factor released in human dental pulp following orthodontic force. Eur J Orthod 29(1):67–71
Derringer KA, Jaggers DC, Linden RW (1996) Angiogenesis in human dental pulp following orthodontic tooth movement. J Dent Res 75(10):1761–1766
Dissanayaka WL et al (2012) Coculture of dental pulp stem cells with endothelial cells enhances osteo-/odontogenic and angiogenic potential in vitro. J Endod 38(4):454–463
Dissanayaka WL et al (2015) The interplay of dental pulp stem cells and endothelial cells in an injectable peptide hydrogel on angiogenesis and pulp regeneration in vivo. Tissue Eng Part A 21(3-4):550–563
Distler JH et al (2003) Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q J Nucl Med 47(3):149–161
Dominici M et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317
El Karim IA et al (2009) Neuropeptides regulate expression of angiogenic growth factors in human dental pulp fibroblasts. J Endod 35(6):829–833
Estrada R et al (2009) Secretome from mesenchymal stem cells induces angiogenesis via Cyr61. J Cell Physiol 219(3):563–571
Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3(4):393–403
Gaengel K et al (2009) Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler Thromb Vasc Biol 29(5):630–638
Gruber R et al (2005) Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. Tissue Eng 11(5-6):896–903
Heyeraas KJ (1989) Pulpal hemodynamics and interstitial fluid pressure: balance of transmicrovascular fluid transport. J Endod 15(10):468–472

Heyeraas KJ, Berggreen E (1999) Interstitial fluid pressure in normal and inflamed pulp. Crit Rev Oral Biol Med 10(3):328–336
Hilkens P et al (2014) Pro-angiogenic impact of dental stem cells in vitro and in vivo. Stem Cell Res 12(3):778–790
Hilkens P et al (2015) Dental stem cells in pulp regeneration: near future or long road ahead? Stem Cells Dev 24(14):1610–1622
Hilkens P et al (2017) The angiogenic potential of DPSCs and SCAPs in an in vivo model of dental pulp regeneration. Stem Cells Int 2017:2582080
Holdsworth MT et al (2003) Pain and distress from bone marrow aspirations and lumbar punctures. Ann Pharmacother 37(1):17–22
Huang GT, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88(9):792–806
Hung CN et al (2011) A comparison between adipose tissue and dental pulp as sources of MSCs for tooth regeneration. Biomaterials 32(29):6995–7005
Iohara K et al (2008) A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp. Stem Cells 26(9):2408–2418
Iohara K et al (2011) Complete pulp regeneration after pulpectomy by transplantation of CD105+ stem cells with stromal cell-derived factor-1. Tissue Eng Part A 17(15-16):1911–1920
Ishizaka R et al (2012) Regeneration of dental pulp following pulpectomy by fractionated stem/progenitor cells from bone marrow and adipose tissue. Biomaterials 33(7):2109–2118
Ito T et al (2017) Dental pulp tissue engineering of pulpotomized rat molars with bone marrow mesenchymal stem cells. Odontology 105:392
Jain RK (2003) Molecular regulation of vessel maturation. Nat Med 9(6):685–693
Janebodin K et al (2013) VEGFR2-dependent angiogenic capacity of pericyte-like dental pulp stem cells. J Dent Res 92(6):524–531
Javed F et al (2015) Influence of orthodontic forces on human dental pulp: a systematic review. Arch Oral Biol 60(2):347–356
Jussila M, Juuri E, Thesleff I (2013) Tooth morphogenesis and renewal. In: Huang GT, Thesleff I (eds) Stem cells in craniofacial development and regeneration. Blackwell-Wiley, Hoboken, NJ, pp 109–134
Kim S (1985) Microcirculation of the dental pulp in health and disease. J Endod 11(11):465–471
Kim JY et al (2010) Regeneration of dental-pulp-like tissue by chemotaxis-induced cell homing. Tissue Eng Part A 16(10):3023–3031
Kim DW et al (2013) Wharton's jelly-derived mesenchymal stem cells: phenotypic characterization and optimizing their therapeutic potential for clinical applications. Int J Mol Sci 14(6):11692–11712
Kjaer I (2014) Mechanism of human tooth eruption: review article including a new theory for future studies on the eruption process. Scientifica (Cairo) 2014:341905
Kuang R et al (2016) Nanofibrous spongy microspheres for the delivery of hypoxia-primed human dental pulp stem cells to regenerate vascularized dental pulp. Acta Biomater 33:225–234
Li X et al (2016) Pulp regeneration in a full-length human tooth root using a hierarchical nanofibrous microsphere system. Acta Biomater 35:57–67
Malhotra N (2016) Induced pluripotent stem (iPS) cells in dentistry: a review. Int J Stem Cells 9(2):176–185
Marchionni C et al (2009) Angiogenic potential of human dental pulp stromal (stem) cells. Int J Immunopathol Pharmacol 22(3):699–706
Masoudi E et al (2016) Platelet-rich blood derivatives for stem cell-based tissue engineering and regeneration. Curr Stem Cell Rep 2(1):33–42
Mullane EM et al (2008) Effects of VEGF and FGF2 on the revascularization of severed human dental pulps. J Dent Res 87(12):1144–1148
Murakami M et al (2015) Trophic effects and regenerative potential of mobilized mesenchymal stem cells from bone marrow and adipose tissue as alternative cell sources for pulp/dentin regeneration. Cell Transplant 24(9):1753–1765
Nait Lechguer A et al (2008) Vascularization of engineered teeth. J Dent Res 87(12):1138–1143

Nakagami H et al (2005) Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. Arterioscler Thromb Vasc Biol 25(12):2542–2547
Nanci A (2008) Dentin-pulp complex. In: Ten Cate's oral histology: development, structure, and function. Mosby Elsevier, St. Louis, MO, pp 191–238
Phng LK, Gerhardt H (2009) Angiogenesis: a team effort coordinated by notch. Dev Cell 16(2):196–208
Pimenta FJ, Sa AR, Gomez RS (2003) Lymphangiogenesis in human dental pulp. Int Endod J 36(12):853–856
Potapova IA et al (2007) Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. Stem Cells 25(7):1761–1768
Potente M, Gerhardt H, Carmeliet P (2011) Basic and therapeutic aspects of angiogenesis. Cell 146(6):873–887
Ratajczak J et al (2016) The neurovascular properties of dental stem cells and their importance in dental tissue engineering. Stem Cells Int 2016:9762871
Rehman J et al (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 109(10):1292–1298
Rodd HD, Boissonade FM (2003) Immunocytochemical investigation of neurovascular relationships in human tooth pulp. J Anat 202(2):195–203
Rombouts C et al (2017) Pulp vascularization during tooth development, regeneration, and therapy. J Dent Res 96(2):137–144
Sakai VT et al (2010) SHED differentiate into functional odontoblasts and endothelium. J Dent Res 89(8):791–796
Sharma S et al (2014) Biomaterials in tooth tissue engineering: a review. J Clin Diagn Res 8(1):309–315
Shivashankar VY et al (2017) Comparison of the effect of PRP, PRF and induced bleeding in the revascularization of teeth with necrotic pulp and open apex: a triple blind randomized clinical trial. J Clin Diagn Res 11(6):ZC34–ZC39
Sieveking DP, Ng MK (2009) Cell therapies for therapeutic angiogenesis: back to the bench. Vasc Med 14(2):153–166
Suzuki T et al (2011) Induced migration of dental pulp stem cells for in vivo pulp regeneration. J Dent Res 90(8):1013–1018
Swift MR, Weinstein BM (2009) Arterial-venous specification during development. Circ Res 104(5):576–588
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
Takeuchi N et al (2015) Similar in vitro effects and pulp regeneration in ectopic tooth transplantation by basic fibroblast growth factor and granulocyte-colony stimulating factor. Oral Dis 21(1):113–122
Toda N, Ayajiki K, Okamura T (2012) Neurogenic and endothelial nitric oxide regulates blood circulation in lingual and other oral tissues. J Cardiovasc Pharmacol 60(1):100–108
Tran C, Damaser MS (2015) Stem cells as drug delivery methods: application of stem cell secretome for regeneration. Adv Drug Deliv Rev 82-83:1–11
Tran-Hung L, Mathieu S, About I (2006) Role of human pulp fibroblasts in angiogenesis. J Dent Res 85(9):819–823
Wise GE, King GJ (2008) Mechanisms of tooth eruption and orthodontic tooth movement. J Dent Res 87(5):414–434
Woloszyk A et al (2016) Human dental pulp stem cells and gingival fibroblasts seeded into silk fibroin scaffolds have the same ability in attracting vessels. Front Physiol 7:140
Yu C, Abbott PV (2007) An overview of the dental pulp: its functions and responses to injury. Aust Dent J 52(1 Suppl):S4–S16
Yu CY et al (2002) An in vivo and in vitro comparison of the effects of vasoactive mediators on pulpal blood vessels in rat incisors. Arch Oral Biol 47(10):723–732

Yuan C et al (2015) Coculture of stem cells from apical papilla and human umbilical vein endothelial cell under hypoxia increases the formation of three-dimensional vessel-like structures in vitro. Tissue Eng Part A 21:1163

Zhang JQ, Nagata K, Iijima T (1998) Scanning electron microscopy and immunohistochemical observations of the vascular nerve plexuses in the dental pulp of rat incisor. Anat Rec 251(2):214–220

Zhang LX et al (2015) Systemic BMSC homing in the regeneration of pulp-like tissue and the enhancing effect of stromal cell-derived factor-1 on BMSC homing. Int J Clin Exp Pathol 8(9):10261–10271

Zhang Z et al (2016) Wnt/beta-catenin signaling determines the vasculogenic fate of post-natal mesenchymal stem cells. Stem Cells 34:576

Zhao L, Johnson T, Liu D (2017) Therapeutic angiogenesis of adipose-derived stem cells for ischemic diseases. Stem Cell Res Ther 8(1):125