

# Role of Dental Pulp Stem Cells to Promote Angiogenesis in Cell-based Regenerative Endodontics

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*Maintaining the viability and avoiding necrosis of dental pulp are crucial to preserving the structural integrity and functioning of teeth. In recent years, cell-based regenerative endodontics has emerged as a promising approach to achieve this goal and has gained increasing attention in scientific research; however, in the confined space of the root canal system, hypoxic conditions can be both beneficial and detrimental, as they may promote angiogenesis in the graft to some extent but also lead to tissue necrosis if prolonged. Dental pulp stem cells (DPSCs) have been verified as multipotent cells that can promote angiogenesis and are therefore ideal candidates for realising real dental pulp regeneration within root canals. Thus, we focus on the underlying mechanisms of DPSCs to promote angiogenesis and summarise some preclinical studies and clinical trials involving transplanting of DPSCs to achieve real dental pulp regeneration, in the hope that this intractable source of perplexity in regenerative endodontics may be resolved sooner.*

**Key words:** angiogenesis, dental pulp stem cell, pulp regeneration, pulp repair, regenerative endodontics

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Regenerative endodontics refers to a collection of “biologically based procedures designed to replace damaged tooth structures, including dentine and root structures, as well as cells of the pulp–dentine complex”<sup>1</sup>. Its only officially approved protocol in the clinic as yet, revascularisation, also known as revitalisation, serves to restore the vitality of pulp and promote root maturation of necrotic immature permanent teeth<sup>2–4</sup>. Based on the principles of tissue engineering, this procedure requires high levels of irrigation rather than mechanical debridement to disinfect root canals and induce blood clot formation with sterile files, providing a favourable environment

that contains various types of cells and growth factors for pulp regeneration<sup>5,6</sup>.

The ultimate goal of regenerative endodontics is to achieve dental pulp regeneration, which aims to reconstruct complete vital pulp with both structural and functional tissue *in situ*<sup>5,7</sup>; however, a series of histological assessments of revascularisation in animal and human studies revealed that the new tissues regenerated in the root canals included cementum-, bone- and periodontal ligament–like tissues rather than the ideal formation of pulp–dentine complex<sup>8–10</sup>. This can be explained by the unpredictable composition or concentration of those trapped cells and growth factors in blood clots, leading to unforeseen tissue deposition<sup>11</sup> and even root canal obturation in long-term follow-ups<sup>12</sup>. Since the cells induced in the blood clots are unpredictable, revascularisation may result in uncontrollable outcomes in the process of pulp regeneration. Therefore, real pulp regeneration can be achieved if more controllable pulp stem cells are present in the root canals.

As the first dental-derived mesenchymal stem cells (MSCs) to be isolated and identified in 2000<sup>13</sup>, dental pulp stem cells (DPSCs) possess the ability for self-renewal and multilineage differentiation as well as neurovascular properties, and can be seen as an excel-

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lent stem cell source for transplantation in cell-based regenerative endodontics<sup>14</sup>. Although only a limited number of animal studies and clinical trials have been conducted on this, this promising protocol shows great potential in real pulp regeneration and function restoration<sup>15,16</sup>.

Angiogenesis refers to the formation of new blood vessels from the pre-existing ones and serves as the major mechanism whereby most tissues become vascularised during later embryonic development and adult life<sup>17</sup>. Timely angiogenesis for nutrient delivery and waste removal is believed to be crucial for cell survival in tissue engineering; otherwise, most tissue will undergo malnutrition under hypoxia<sup>18</sup>. Although hypoxia itself can promote angiogenesis of the graft, its speed is too limited to support the metabolism of the whole tissue<sup>19</sup>. Consequently, the inability to construct a sufficient blood supply promptly after cell transplantation can make it highly likely that pulp regeneration will fail.

Considering the neovascular property of DPSCs, we focus on the underlying mechanisms of DPSCs to promote angiogenesis and summarise some preclinical studies and clinical trials on transplanting DPSCs to achieve real dental pulp regeneration in the hope that this intractable source of perplexity in regenerative endodontics can be resolved sooner.

### Angiogenesis: the essence of regenerative endodontics

Dental pulp is a highly vascularised tissue that supports all the other components within it and maintains the normal function of teeth<sup>20</sup>. If it is not promptly treated, contaminated pulp tissue can easily develop from reversible to irreversible pulpitis and even end in necrosis<sup>21</sup>. Consequently, pulpectomy must be performed as a last resort to preserve natural teeth but will lead to the loss of vital pulp and susceptibility to traumatic fracture and bacterial infection of teeth<sup>22</sup>.

Cell-based regenerative endodontics can be an ideal alternative to pulpectomy that has great potential to overcome the aforementioned disadvantages<sup>15</sup>; however, due to being encased by rigid dentine and the lack of a collateral blood supply, transplanted stem cells are desperate for spontaneous angiogenesis to avoid unexpected cell distribution and differentiation in a nutrient and oxygen gradient environment<sup>23</sup>. It is universally accepted that low ambient oxygen concentration cannot maintain the normal metabolism of tissues as the oxygen diffusion distance is limited to 200  $\mu\text{m}$  between the capillary wall and cell membrane<sup>18</sup>; however, hypoxia itself can be the environmental factor

that conduces angiogenesis to some extent.

Hypoxia-inducible factor 1 (HIF-1), a transcription factor expressed under hypoxic conditions by almost all kinds of cells in mammals, consists of two subunits including HIF-1 $\alpha$  and HIF-1 $\beta$ <sup>24</sup> and acts as a regulatory factor to oxygen homeostasis. The hypoxia response elements binding with HIF-1 can help identify target genes for HIF-1 to activate downstream<sup>25</sup>. Subsequently, HIF-1 enhances the transcription of multiple angiogenic growth factors such as vascular endothelial growth factor (VEGF), angiopoietin1 (Ang1), placental growth factor (PlGF), platelet-derived growth factor (PDGF) and angiogenic chemokines such as stromal cell-derived factor 1 (SDF-1), sphingosine-1-phosphate (S1P) and their receptors<sup>26-28</sup>. Those factors can either facilitate the differentiation of stem cells into endothelial cells (ECs) or recruit endothelial progenitor cells to promote angiogenesis<sup>27</sup>. Moreover, the migration speed of ECs subjected to hypoxia will be increased by reducing the expression of vascular endothelial-cadherin (VE-cadherin)<sup>29</sup>. As for DPSCs, hypoxia can enhance the expression of HIF-1<sup>30,31</sup> and activate the secretion of VEGF which is the primary transcriptional target for HIF-1 downstream<sup>31</sup>. Besides, increased expression of SDF-1 by overexpressing HIF-1 $\alpha$  showed great proangiogenic characteristics of DPSCs in hypoxic ambience<sup>32</sup>. Han et al<sup>33</sup> demonstrated that preconditioned stem cells from human exfoliated primary teeth (SHED) by HIF-1 stabilisation showed positive results of a higher level of angiogenesis in the newly formed pulp-like tissue.

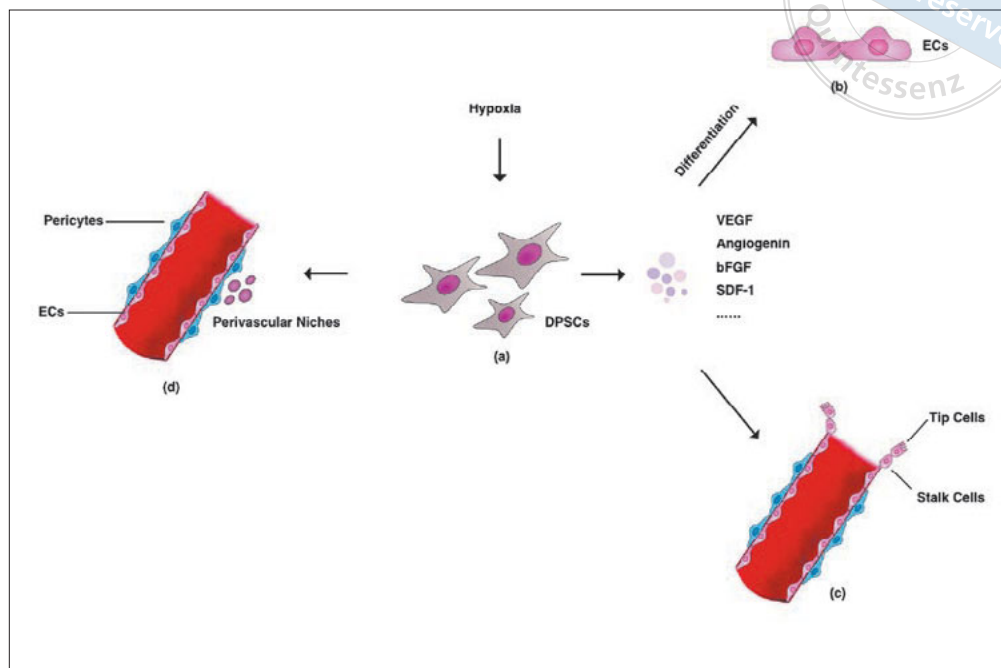
Despite the desirable effect of hypoxia on angiogenesis in the early stages, it cannot always exert a positive effect on it. An extremely low oxygen concentration below 1% and excessive accumulation of HIF-1 will both curb the collective migration velocity of ECs<sup>34</sup>. Thus, relying on hypoxia alone to promote angiogenesis may not be reliable, and the contribution of DPSCs to expedite this process needs to be illustrated in detail to offer a new perspective for desirable outcomes.

### Proangiogenic property of DPSCs: residing in perivascular niches

DPSCs are derived from the cranial neural crest that forms the dental mesenchyme during early embryonic head development. This origin confers DPSCs with typical MSC properties, such as self-renewal, clonogenicity and multi-lineage differentiation<sup>35,36</sup>, implying its proangiogenic potential that is worthy of investigation.

In addition, the proangiogenic property of DPSCs can be attributed to their local microenvironment in

**Fig 1** Summary of the underlying mechanisms for DPSCs to promote angiogenesis. **(a)** DPSCs secrete multiple proangiogenic factors in paracrine under physiological or hypoxic conditions. **(b)** These secreted proangiogenic factors contribute in turn to the differentiation of DPSCs into ECs. **(c)** The secreted proangiogenic factors can modulate the proliferation and migration of specialised ECs, such as tip and stalk cells, to promote angiogenesis in vivo. **(d)** DPSCs adapt to reside in perivascular niches, which can stabilise the neovasculature by acting like pericytes. Figure produced using Affinity Designer (Serif, West Bridgford, UK).



the pulp. Niches refer to the specific microenvironment in which stem cells are located. The concept of the perivascular niche was proposed initially for hematopoietic stem cells in bone marrow, where they reside near the vasculature<sup>37</sup>. This structure has been verified to exist in multiple human organs<sup>38</sup>. In a study by Shi et al<sup>39</sup>, STRO-1, as an early marker present on different MSCs, was used to retrieve DPSCs from pulp tissue. This antigen was also found on dental pulp microvasculature, inferring the existence of perivascular niches for DPSCs in situ. Aldehyde dehydrogenase 1 (ALDH-1) is another marker associated with the stemness of cells. Both Machado et al<sup>40</sup> and Oh et al<sup>41</sup> demonstrated that DPSCs with high ALDH-1 were preferentially located proximally to vasculatures, indicating the presence of perivascular niches. Oh et al<sup>41</sup> also demonstrated that EC-derived interleukin-6 (IL-6) could induce the asymmetric division of DPSCs into either endothelial cell differentiation or an undifferentiated state and thus maintain both the perivascular niches and the multipotency of DPSCs. Consequently, the origin and locality of DPSCs reveal the close relationship between DPSCs and vasculatures in pulp and imply their proangiogenic property. Indeed, a study provided evidence to elucidate the mechanisms of DPSC-mediated angiogenesis, which mainly involve secreting proangiogenic factors or differentiating into ECs to form vasculature<sup>7</sup>. These mechanisms will be elaborated on in detail later in this article.

In addition, intriguingly, the neural origin of DPSCs from peripheral nerve-associated glia has been proven<sup>42</sup>, which shows that DPSCs have a dual origin. The neurogenic potential<sup>43</sup> and the odontogenic and osteogenic ability<sup>44</sup> of DPSCs are generally accepted. Thus, given the easy availability of DPSCs from non-functioning third molars, all the factors above suggest this kind of cell is an optimal candidate for cell-based regenerative endodontics for the benefit of both angiogenesis and dental pulp regeneration.

### Underlying mechanisms through which DPSCs promote angiogenesis

Angiogenesis is a complex process that can be divided into several phases: secretion of angiogenic factors as a response to physiological or pathological stimuli; degradation of the basement membrane and extracellular matrix with the purpose of allowing EC migration; migration and proliferation of ECs to form tubular structures; and maturation or stabilisation of vasculatures<sup>45</sup>. Correspondingly, there are four mechanisms through which DPSCs promote angiogenesis, as mentioned below (Fig 1).

#### *Secretion of proangiogenic factors in paracrine*

As previously aforementioned, DPSCs reside in perivascular niches and interact with ECs to regulate new blood

vessel formation. Hence, multiple signalling molecules secreted by DPSCs can be involved, among which the most detected factor is VEGF. Secretion of VEGF by DPSCs can be observed in almost every condition in both the cell lysates and the conditioned medium (CM)<sup>46</sup>, and is even increased under hypoxia with the induction of HIF-1<sup>47</sup>. Using an angiogenic inhibitor to competitively bind with VEGF-A or knocking down the expression of VEGFR2 on DPSCs both lead to decreased blood vessel formation in vitro and in vivo, respectively, showing the impairment of the angiogenic capacity of DPSCs<sup>48</sup>. Conversely, transfecting DPSCs with high-level overexpression of VEGF showed a great ability for vascular tube formation<sup>49</sup>. In addition to VEGF, other proangiogenic factors secreted by DPSCs can be detected, including angiogenin, Ang1, monocyte chemoattractant protein-1 (MCP-1), hepatocyte growth factor (HGF), IL-8, basic-fibroblast growth factors (bFGF), SDF and PDGF<sup>46,50-52</sup>. Once the vessels are formed, it is imperative that they become stabilised and avoid regression. Compared with cultures of EC alone, the administration of DPSCs in a previous study did stabilise the pre-existing vessel-like structures formed by ECs<sup>53</sup>. The recruitment of mural cells, including pericytes and smooth muscle cells (SMC), plays a pivotal role in stabilising neovasculature and maturation of vessels<sup>54</sup>. In the process of angiogenesis, PDGF-BB can be secreted by DPSCs and as a chemoattractant for SMCs to be recruited and expanded around neovasculature to promote its maturation<sup>55</sup>. Meanwhile, Ang1 secreted by DPSCs activated Ang1/Tie2 signalling to maintain the quiescent of ECs and strengthen the interaction between mural cells and ECs, leading to the stabilisation and maturation of newly formed blood vessels<sup>56</sup>.

Moreover, with the exception of those functioning growth factors, an enzyme named urokinase plasminogen activator was found to be expressed by DPSCs, which can stimulate the degradation of extracellular matrix and may facilitate the proliferation, migration and invasion of ECs<sup>46</sup>. All the proangiogenic factors mentioned above synergistically endow DPSCs with proangiogenic properties to a certain extent.

Intriguingly, however, some other antiangiogenic factors or enzymes have been detected. Insulin-like growth factor binding protein-3 (IGFBP-3) was found to be expressed by DPSCs<sup>46,50</sup> and acted to inactivate the ERK1/2 signalling pathway and Elk-1, leading to the reduced transcription of their downstream proangiogenic target genes, such as PDGF and bFGF<sup>57</sup>. Enzymes like tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1 expressed by DPSCs can inhibit the degradation of extracellular matrix<sup>46</sup>. Thus,

the balance between these proangiogenic and antiangiogenic factors may influence the interaction between DPSCs and ECs and eventually the formation of new blood vessels.

### *Promoting proliferation and migration of ECs*

One of the most important processes for angiogenesis is the proliferation and migration of ECs<sup>45</sup>. Endothelial tip cells, as specialized ECs, serve as the navigators to guide endothelial sprouts with their long filopodial protrusions. Endothelial stalk cells, another specialised type of EC, follow behind the tip cells and elongate the sprouts to form vascular lumens<sup>58,59</sup>. This process has been proven to be modulated by miscellaneous proangiogenic factors<sup>60</sup>. VEGF is known as the primary regulator for angiogenesis in either a physiological or pathological state<sup>61</sup>. Binding with its main signalling receptor VEGFR2, which is predominantly expressed on ECs, will sequentially activate several kinases and guide EC proliferation and migration<sup>62</sup>.

Recent studies have shown that human umbilical vein endothelial cells (HUVECs) incubated in DPSC-CM can proliferate significantly faster and enhance the proliferation rate of HUVECs compared with controls<sup>52,63</sup>; however, Bronckaers et al<sup>46</sup> demonstrated that DPSC-CM did not facilitate the growth and proliferation of ECs compared with the control groups containing 10% foetal bovine serum. It is assumed that this consequence may be attributed to the existence of antiangiogenic factors and insufficient proangiogenic factors to induce the proliferation under malnutrition conditions. The addition of fetal bovine serum which can increase the amount of angiogenic factors can solve the problem<sup>64</sup>. This may suggest that angiogenesis in cell-based regenerative endodontics can be promoted by adding extra proangiogenic factors.

With regard to EC migration, Dissanayaka et al<sup>65</sup> indirectly co-cultured DPSCs and HUVECs using a transwell migration assay to identify the impact of DPSCs on HUVEC migration. It emerged that HUVECs incubated over a DPSC monolayer had formed round spindle-shaped morphologies and were organised in vascular lumen networks<sup>65</sup>. This result can be attributed to the secretion of VEGF by DPSCs which was detected in the DPSC monocultures. In addition, the application of anti-VEGF antibodies inhibited the migration of ECs significantly, which verified the chemotaxis of VEGF further<sup>46</sup>; however, insufficient inhibition of EC migration implied that there may be other factors involved in this process. SDF-1 $\alpha$  and CXCR4 axis has been known to regulate hematopoiesis<sup>66</sup>. In the study by Nam et al<sup>51</sup>,

incubating HUVECs in DPSC-CM in vitro or encapsulating DPSCs and HUVECs in vivo while both were treated with CXCR4 antagonist would inhibit the migration of HUVECs and decrease the formation of microvessel structures. DPSCs express high levels of SDF-1 $\alpha$  and low levels of CXCR4, but the opposite is true for ECs<sup>51,67</sup>. Thus, SDF-1 $\alpha$  serves as another significant mediator in angiogenesis that strongly demonstrates the angiogenic capacity of DPSCs.

### *Differentiated into endothelial-like cells*

DPSC has been verified to possess the ability to differentiate into diverse tissues, such as osteoblasts, adipocytes, neurons and endothelial cells, which makes it an optimal choice for cell-based regenerative endodontics<sup>14</sup>. It is well established that VEGF can induce stem cells into ECs. Through VEGF induction, DPSCs can display some features of ECs by upregulating the expression of von Willebrand Factor, CD31, CD34, CD54, CD105, CD106, VEGFR-1 and VEGFR-2 and VE-cadherin, and by forming capillary-like structures in vitro<sup>68-70</sup>. Use of an angiogenic inhibitor that can compete with VEGF revealed that the endothelial differentiation of DPSCs is dependent on VEGFR-2 activation and its downstream ERK signalling pathway<sup>48</sup>. Although VEGFR-1 shows a higher affinity for VEGF, VEGFR-2 is the main receptor for VEGF to activate downstream signalling pathways. VEGFR-1 displays weak autophosphorylation and is often regarded as a decoy receptor to prevent VEGF from binding to VEGFR-2<sup>62</sup>; however, VEGFR-1 also participates in endothelial differentiation of DPSCs. Zhang et al<sup>71</sup> demonstrated that it was VEGFR-1 that activated the downstream canonical Wnt/ $\beta$ -catenin signalling pathway and mediated the process of DPSC endothelial differentiation in response to VEGF. A more recent study even showed that only 10% to 15% DPSCs can express constitutive VEGFR-1 and that this unique sub-population of DPSCs was primed for angiogenic differentiation<sup>72</sup>. Since DPSCs have various subpopulations with significant heterogeneities<sup>73</sup>, this may imply that sifting and purifying DPSC subpopulations with high angiogenic properties may enhance these properties<sup>74,75</sup>.

After differentiation into ECs and formation of vascular-like structures, these newly formed blood vessels still need to anastomose with host vasculatures to provide nutrients and oxygen to the graft. VE-cadherin is a calcium-dependent protein that mediates endothelial cell-to-cell adhesion and is essential for vascular integrity<sup>76</sup>. Sasaki et al<sup>77</sup> recently revealed the underpinning mechanisms of how DPSC-derived neovasculature anastomoses with host vasculature to form a functional

vascular system. They demonstrated that the induction of VEGF can sequentially activate the ERK signalling pathway and its downstream ERG transcription, which bound to the VE-cadherin promoter and enhanced its expression on DPSCs. Silencing VE-cadherin in DPSCs resulted in either disorganised formation of blood vessels in vitro or a decreased number of functional microvessels anastomosed with host vasculature in vivo, which further verified the role of VE-cadherin in sprouting and anastomosis of DPSC-derived vasculature.

### *Stabilising neovasculatures by acting like pericytes*

Besides ECs, which shape the profile of vascular lumens, mural cells are also essential for vascular formation in angiogenesis<sup>54</sup>. Mural cells surround the ECs and display great abilities to stabilise the neovasculature by inhibiting EC proliferation and migration, regulating endothelium permeability, forming the basement membrane and preventing its regression<sup>54,78</sup>.

It has been recognised that a subpopulation of pericytes could express MSC markers, which indicated another derivation of MSCs<sup>79,80</sup>. Similarly, DPSCs have been shown to possess pericyte-like topography and express different levels of perivascular markers, such as alpha-smooth muscle actin, 3G5, NG2 and CD146<sup>51,81-83</sup>. Considering the close relationship between pericytes and DPSCs, Caplan<sup>79</sup> proposed that pericytes were sources of precursors of MSCs, but not all pericytes were MSCs. Dentine sialophosphoprotein (Dspp), along with other odontogenic genes, were found to be in a transcriptionally permissive state that gave pericytes their multipotent differentiation ability in the dental pulp<sup>80,84</sup>. Feng et al<sup>85</sup> demonstrated that pericytes were able to possess MSC-like properties and could be differentiated into specialised mesenchymal-origin cells such as odontoblasts; however, pericyte-derived odontoblasts accounted for part of the total newly differentiated cells, indicating that not all MSCs were pericyte-derived and that MSCs in dental pulp may have distinct origins.

On the other hand, DPSCs can be induced to differentiate into pericyte-like cells with high positive expression of pericyte markers<sup>86</sup>, acting like pericytes to stabilise vascular-like structures formed by ECs and prevent their degeneration<sup>87</sup>. Moreover, DPSCs treated by transforming growth factor- $\beta$  (TGF- $\beta$ ) can be induced into SMCs with high expression of specific markers and improvement of contractile function<sup>56,88</sup>. Zhang et al<sup>56</sup> demonstrated that DPSCs treated by TGF- $\beta$  can function as pericyte-like cells and express high levels of angi-

opoietin 1, a pro-maturation factor for blood vessels, to significantly inhibit EC migration and endothelial sprouting. Besides, angiopoietin 1/Tie2 signalling can act as a key regulator to maintain the stability of blood vessels by increasing vascular endothelial-cadherin expression and enhancing endothelial cell-cell adhesion<sup>89,90</sup>.

### Applying DPSCs in cell-based regenerative endodontics

Since the first case report of regenerative endodontics was published in 2001<sup>91</sup>, there has been a growing interest in this field among scientists worldwide. With the advancement of tissue engineering concepts<sup>92</sup>, including the use of stem cells, scaffolds and signalling molecules, novel approaches have emerged with the aim of achieving true pulp regeneration, such as cell-based and cell-homing approaches<sup>93</sup>. DPSCs can be obtained inviolately from extra third molars or orthodontic teeth and are easy to isolate and cultivate from pulp tissue. They can also differentiate into multilineage tissues in regenerative endodontics<sup>13,94</sup>. Moreover, DPSCs have been shown to promote angiogenesis, which can overcome the challenge of insufficient blood supply after transplantation<sup>95</sup>.

The application of DPSCs in cell-based regenerative endodontics can be divided into mainly four different approaches to be mentioned below, namely applying DPSCs alone or with scaffolds; applying DPSCs with growth factors; co-transplanting DPSCs with ECs; and prevascularisation *in vitro* beforehand. The application of the above methods in preclinical studies using animal models will be discussed below (Table 1), and the clinical trials will be outlined in detail later.

#### *Applying DPSCs alone or with scaffolds*

Currently, regenerative endodontics used clinically is revascularisation [do you mean 'use of regenerative endodontics in clinical settings involves revascularisation?'], where an induced blood clot functions as a scaffold to trap cell and growth factors, initiating pulp regeneration<sup>96</sup>. Likewise, cell-based regenerative endodontics also entails the involvement of natural or synthetic scaffolds<sup>92</sup>. Scaffolds are porous biomaterials with good biocompatibility that allow seeded cells to adhere, aggregate into 3D structures and be positioned correctly<sup>97</sup>. Moreover, the porosity of scaffolds facilitates the permeation of growth factors and nutrients, supporting cell proliferation, migration and differentiation<sup>98</sup>. Most importantly, scaffolds should be mimetic to

the physical and biochemical microenvironment of the root canal for optimal cell survival and viability<sup>98</sup>.

Collagen, as the most abundant component in the extracellular matrix, plays a pivotal role in angiogenesis. The remodelling of the extracellular matrix, particularly the degradation and assembly of collagen, is attuned with the migration of ECs to modulate the formation of vasculatures<sup>99</sup>. This has made collagen the most widely investigated natural scaffold applied with DPSCs. Combining DPSCs or their CM with collagen scaffolds and subcutaneously transplanting them into immunodeficient mice can increasingly generate pulp-like tissue with well-organised vasculature<sup>100</sup>. Compared with bone marrow and adipose tissue-derived MSCs, DPSCs demonstrated a greater ability to stimulate EC differentiation and promoted endogenous cell migration, both essential steps in angiogenesis<sup>100</sup>; however, in a study by Ravindran et al<sup>101</sup>, DPSCs co-transplanted with collagen scaffolds can be differentiated into ECs only at the periphery. The addition of decellularised extracellular matrix, which contains a rich source of growth factors and phosphorylated proteins, has been shown to significantly improve the deposition of collagen fibres and hydroxyapatite, as well as the formation of pulp-like tissue and organised neovasculature<sup>101</sup>. This may indicate that the presence of additional bioactive molecules can enhance pulp regeneration<sup>102</sup>. As well as natural scaffolds, synthetic ones with excellent properties have also been studied. PuraMatrix (3-D Matrix, Tokyo, Japan) is a liquid synthetic peptide hydrogel that self-assembles and polymerises when exposed to physiological conditions<sup>103</sup>. This makes it a promising candidate for regenerative endodontics, as it can adapt to the variable and complicated shape of the root canal system<sup>103</sup>. Most importantly, compared with other traditional scaffolds such as collagen and fibrin, PuraMatrix can initiate a rapid onset of vascularisation which is essential for transplanted cells<sup>104</sup>. Encapsulating PuraMatrix with DPSCs into full-length human root canals and implanting them subcutaneously into immunodeficient mice can generate functional pulp-like tissue with a microvessel density similar to that of normal human dental pulp<sup>65,105</sup>. This further strengthens the significance of providing an appropriate microenvironment for dental pulp regeneration. With the development of materials and technology, a growing number of novel scaffolds such as self-assembled peptide RAD/Dentonin hydrogel and coagulated platelet poor plasma have been investigated and shown great potential in promoting angiogenesis and tissue regeneration<sup>106,107</sup>.

**Table 1** Summary of the different strategies for applying DPSCs in cell-based regenerative endodontics

Strategies	Examples	Method	Conclusion
DPSC alone	3D DPSC constructs <sup>16</sup>	In vivo (animal)	Transplanting DPSCs can regenerate blood vessel-rich pulp-like tissue without any scaffolds and exogenous growth factors
DPSC with scaffolds	DPSCs with collagen <sup>100</sup>	In vivo (animal)	DPSCs demonstrated greater abilities of promoting EC differentiation and cell migration compared with MSCs from bone marrow and adipose tissue
	DPSCs with extracellular matrix embedded collagen/chitosan scaffold <sup>101</sup>	In vitro and in vivo (animal)	Presence of additional bioactive molecules can enhance the regenerative potential of DPSCs
	DPSCs with PuraMatrix <sup>65,105</sup>	In vivo (animal)	Strengthening the significance of providing an appropriate microenvironment for dental pulp regeneration
DPSCs with growth factors	DPSCs with VEGF loaded microspheres <sup>110</sup>	In vivo (animal)	Regenerated pulp-like tissue extending from apex to middle third of the root
	DPSCs with hierarchical VEGF-loaded nanofibrous microsphere scaffolding system <sup>111</sup>	In vivo (animal)	Sustained and prolonged releasing of VEGF can help to reach successful pulp-like tissue regeneration to full-length of human root canals
	DPSCs with CGF <sup>116</sup>	In vitro	Concentrated growth factor enhanced the proliferation, migration, and odonto-/osteogenic differentiation of DPSCs under inflammatory conditions
DPSCs with ECs	DPSCs with HUVECs in GelMA hydrogel <sup>116</sup>	In vivo (animal)	Formation of highly cellularised and vascularised pulp-like tissue
	DPSCs with HUVECs in PuraMatrix <sup>65</sup>	In vitro and in vivo (animal)	HUVECs were induced to form endothelial vascular tubes by the VEGF secretion from DPSCs
	DPSCs with adipose tissue-derived microvascular fragments <sup>123</sup>	In vivo (animal)	Adipose tissue-derived microvascular fragments contained ECs and pericytes, facilitating the formation of vascularised pulp-like tissue
	DPSCs with induced DPSCs <sup>125</sup>	In vivo	Formation of vascular-like networks and host blood vessels invading from apical foramen
Prevascularisation	Prevascularised construction of DPSCs and HUVECs <sup>128</sup>	In vivo (animal)	Prevascularised microtissues enhanced the neovascularisation in regenerated pulp-like tissue, resulting in anastomose with host vasculatures
	Prevascularised construction of DPSCs and induced DPSCs <sup>129</sup>	In vivo (animal)	Prevascularised constructs possessed greater ability to promote neovascularised pulp-like tissue regeneration compared with DPSC constructs without prevascularisation beforehand

Despite those positive aspects, there are still concerns about the application of scaffolds. Scaffolds should biodegrade synchronously at a controllable rate with pulp regeneration, providing enough room for pulp regeneration and averting the surgical removal of scaffolds<sup>97</sup>; however, this process can be hard to control, especially in the complex and variable internal environment that varies from person to person<sup>108</sup>. Thus, the solution of using scaffold-free tissue engineering technology emerges. In a study by Itoh et al<sup>16</sup>, a rod-shaped 3D DPSC construct was fabricated with a thermoresponsive hydrogel mould. After inserting the DPSC constructs into human tooth root segments and subcutaneously transplanting them into immunodeficient mice for weeks of incubation, regenerated pulp-like tissue was observed<sup>16</sup>. These tissues contained multiple neovasculature perfused with host blood cells,

indicating that transplanted DPSC can be induced to form blood vessels and anastomose with host blood vessels to establish vascular access and ensure blood supply to regenerated tissues<sup>16</sup>. Moreover, DPSCs adjacent to dentine were induced into odontoblast-like cells by the rich source of growth factors in dentine, resulting in mineral deposition<sup>16</sup>. Since instantaneous angiogenesis is essential to the survival of graft material, this experiment demonstrated the great potential of using only DPSCs to regenerate blood vessel-rich pulp-like tissue without the need for scaffolds and exogenous growth factors.

#### *Applying DPSCs with growth factors*

Due to the narrow apical foremen and the single blood supply of the pulp, barely relying on DPSCs may not be

sufficient to achieve desirable angiogenesis. The growth factors secreted by DPSCs activate multiple signalling pathways for promoting cell differentiation, proliferation, migration and neovasculature stabilization, which emphasises the significance of the involvement of growth factors.

It is known that VEGF plays a pivotal role in both angiogenesis and dentine regeneration<sup>61,109</sup> and is the most widely investigated growth factor in regenerative endodontics. Many studies have been conducted to explore the effectiveness of VEGF on pulp regeneration and achieved desirable results. Injecting DPSCs with VEGF-loaded microspheres into human tooth root lumens and transplanting them subcutaneously into nude mice led to significant formation of pulp-like tissue extending from the apex to the middle third of the root, with abundant neovasculature and odontoblast-like cells lining the tubular dentine<sup>110</sup>. To better simulate physiological conditions for full-length human root pulp regeneration, a hierarchical nanofibrous microsphere scaffolding system was fabricated to function as both a cell carrier and a controllable VEGF delivery vehicle<sup>111</sup>. With sustained and prolonged release of VEGF, vascularised pulp-like tissue was regenerated, reaching the coronal third of the canals. Compared to groups that transplanted DPSCs alone, the application of controllable release of VEGF yielded promising results not only in pulp regeneration but also in promoting angiogenesis. Strong immunohistochemical staining of von Willebrand factor and CD31 was observed, with neovasculature distributed throughout the full length of the root canal. Intriguingly, the coronal third of the root canal, which is farthest from the apical foramen, generated the highest number of vasculatures, further proving the angiogenic effect of hypoxia. Apart from VEGF, other growth factors such as SDF-1 and bFGF combined with different scaffolds can also yield satisfactory outcomes in pulp regeneration<sup>112-114</sup>. These cytokines can be used respectively or matched with each other to further promote pulp regeneration and angiogenesis. Derived from platelet concentrate, concentrated growth factor is a reservoir rich in multiple growth factors, such as TGF- $\beta$ 1, PDGF-BB, insulin-like growth factor-1, bFGF and VEGF115. This makes it an ideal bioscaffold for supporting cells and provides ample cytokines at the same time. In a study by Xu et al<sup>116</sup>, concentrated growth factor significantly enhanced the proliferation, migration and odonto-/osteogenic differentiation of DPSCs under inflammatory conditions stimulated by lipopolysaccharides. Merely transplanting concentrated growth factor into beagle dogs' immature teeth revealed the continued de-

velopment of teeth and normal pulp-like tissue regeneration with neovasculature and palisading-arranged odontoblasts. Beyond concentrated growth factor, co-transplanting DPSCs with decellularised extracellular matrix<sup>117</sup>, platelet-rich fibrin<sup>118</sup> and platelet-poor plasma<sup>107</sup>, all serving as cytokine-rich bioscaffolds, provided a variety of promising strategies to promote angiogenesis and dental pulp regeneration.

### *Co-transplanting DPSCs with ECs*

It is well acknowledged that both DPSCs and ECs are significant in the angiogenesis process, in which ECs constitute the wall of vascular lumens whereas DPSCs act as pericytes to stabilise the neovasculature<sup>59,87</sup>. Reports have revealed that HUVECs cultured with CM of DPSCs could markedly promote proliferation, migration and tube formation of HUVECs in vitro<sup>119</sup>. Thus, a novel method, namely co-transplanting DPSCs with ECs into root canals, was used to achieve vascularised dental pulp regeneration. In an in vivo study, it was reported that DPSCs can be encapsulated in GelMA hydrogel scaffolds with HUVECs and subcutaneously transplanted them with human teeth segments into nude mice. This resulted in highly cellularised and vascularised pulp-like tissue with patent blood vessels and identifiable host red blood cells<sup>120</sup>. Compared to transplanting DPSCs alone, co-transplanting DPSCs and HUVECs can enhance HUVECs migration significantly to form vascular tubes through the interaction between the two kinds of cells, which was clearly orchestrated by VEGF secretion from DPSCs<sup>65</sup>.

Apart from HUVECs, adipose tissue-derived microvascular fragments are another source of functional vessel fragments that can be easily harvested from fat tissue<sup>121</sup>. This kind of material contains several significant cell types, such as ECs, pericytes and MSCs. In vivo transplantation has shown that these fragments can rapidly interconnect with each other and even with host blood vessels to form perfused microvascular networks at an early stage<sup>122</sup>. Recently, a study conducted by Xu et al<sup>123</sup> subcutaneously implanted human tooth root segments containing DPSC aggregates and adipose tissue-derived microvascular fragments into immunodeficient mice. This resulted in more positive pulp-like tissue with more obvious and abundant neovasculature, both in quantity and diameter, compared to application of DPSCs only. Adipose tissue-derived microvascular fragments not only supply ECs but also provide ample proangiogenic factors that promote both angiogenesis and odontogenesis, providing perivascular niches for DPSCs and preventing their senescence<sup>121,123</sup>. Apart



from harvesting ECs from adipose tissue-derived microvascular fragments, the multi-lineage differentiation property of DPSCs allows them to be angiogenically induced into endothelial-like cells, providing another rich source of ECs<sup>68</sup>. Induced DPSCs even displayed better angiogenic properties than microvascular ECs in vitro, as the latter were more sensitive to the right cell density seeded on scaffolds. Co-culturing induced DPSCs with non-induced DPSCs demonstrated an analogous capacity to form vascular networks in vitro just as observed between microvascular ECs and DPSCs in vivo<sup>124</sup>. Moreover, in vivo transplantation using ectopic neovascularisation mouse models showed the formation of vascular-like networks and host blood vessels invading from the apical foramen<sup>125</sup>, which was an essential step in promoting pulp regeneration within the root canals.

#### *Prevascularisation in vitro beforehand*

Establishing a sufficient blood supply as soon as possible by promoting angiogenesis and anastomosis with host vasculatures is unquestionably critical for graft survival; however, due to the limitation of host vasculature invasion<sup>19</sup> and the distance of oxygen diffusion<sup>18</sup>, this process may be time-consuming and lead to necrosis of the graft. Constructing prevascularised tissue in vitro beforehand can be a promising solution to the hindrance posed by rapid angiogenesis in tissue engineering and can be perfused apace after transplantation in vivo<sup>126</sup>. Applying this concept in regenerative endodontics with DPSCs also shows giant potential in promoting vascularised pulp regeneration.

When ECs were co-cultured with DPSCs in vitro, PDGF-BB secreted by ECs was revealed to be responsible for the recruitment of DPSCs as perivascular cells, promoting basement membrane deposition and neovascularisation formed by self-assembled ECs. After transplantation in vivo, blood perfusion was enhanced significantly, demonstrating the perspectives of prevascularised tissue constructs in regenerative endodontics<sup>127</sup>. For pulp regeneration, in a study by Dissanayaka et al<sup>128</sup>, self-assembly of DPSCs and HUVECs were constructed into microtissue spheroids, and organised vascular networks were induced by these cells apparently in vitro culture beforehand. Tooth root fragments loaded with those prevascularised microtissues were transplanted subcutaneously into the immunodeficient mice. Compared with the neovascularisation achieved through applying DPSCs alone, prevascularized microtissues enhanced neovascularisation in regenerated pulp-like tissue with higher

numbers of perfused vessels, resulting in anastomosis with the host vasculature. Considering the restricted and impractical selection of HUVECs in the clinic, inducing endothelial differentiation of DPSCs and fabricating prevascularised DPSC constructs in vitro can be another option. Partially due to the limited blood supply from the apical foramen, applying DPSCs alone may result in incomplete pulp regeneration with cavities formed within the root canals<sup>16</sup>. Inducing DPSCs into ECs and prevascularising the cell construct may facilitate full-length root canal pulp regeneration. Thus, the same group of scientists conducted this study and demonstrated that prevascularised DPSC constructs possessed a greater ability to promote higher density neovascularised pulp-like tissue regeneration compared with DPSC constructs without prior prevascularisation<sup>129</sup>. Besides, differentiated DPSCs expressing VE-cadherin can contribute to neovascularisation derived from DPSCs anastomosing with the host vasculature<sup>77</sup>.

#### **Clinical trials for pulp regeneration using a cell-based approach**

Clinical trials can only be conducted based on miscellaneous preclinical studies involving multiple animal models. Compared to a large number of preclinical studies conducted over the past decades, the number of clinical trials is limited and have not made significant progress until recently.

The safety and efficacy of transplanting DPSCs with granulocyte-colony stimulating factor have been certified using immunodeficient mice and pulpectomised dog teeth, respectively. No toxicity or adverse events were observed, and desirable vascularised and innervated pulp regeneration was achieved<sup>130</sup>. Subsequently, the first clinical trial applying DPSCs for pulp regeneration was conducted by the same group of researchers<sup>74</sup>. Five patients aged 22-40 years with irreversible pulpitis were included in the study. Autogenous mobilised DPSCs by granulocyte-colony stimulating factor were transplanted into pulpectomised teeth and followed up for 24 weeks. Four of them showed a positive response to electric pulp testing after 4 weeks, indicating reinnervated pulp regeneration within the root canals. Complete pulp regeneration was detected by MRI and three patients demonstrated lateral dentine formation by CBCT; however, a histological analysis to verify the regenerated tissues in detail could not be performed in this study<sup>74</sup>. Another clinical trial transplanted DPSCs from primary teeth into pulpectomised immature permanent teeth in patients suffering from pulp necrosis and periapical periodontitis

after trauma<sup>15</sup>. Compared with those teeth accepting apexification as controls, experimental groups showed significant root development with a closed apical foramen and thickened dentine walls after 12 months of follow-up. Furthermore, histological analysis was conducted in one case due to accidental retraumatisation of the implanted tooth<sup>15</sup>. This revealed the regeneration of vascularised and innervated 3D dental pulp tissue with odontoblastic differentiation and neuronal marker expression. Moreover, a clinical case used autogenous DPSCs and leukocyte platelet-rich fibrin to design a personalised cell-based trial and yielded desirable outcomes in both radiographic and clinical evaluation for pulp regeneration<sup>131</sup>.

Although current clinical trials have shown positive achievements to acquire dental pulp regeneration by transplanting DPSCs without toxicity and adverse events, there amount of published experimental data remains limited. Thus, more high-quality clinical trials are needed to support the safety and efficacy of this protocol. Apart from this, there are existing challenges in cell-based regenerative endodontics that needed to be resolved, such as the isolation, culture, expansion, and storage of stem cells, as well as facilities and technical problems<sup>93</sup>. In clinical trials, contamination should be tightly controlled; this can be more difficult in the oral cavity compared to animal studies conducted in sterile environments. Otherwise, the presence of micro-leakage can lead to persistent apical periodontitis, which eventually affects pulp regeneration<sup>74</sup>.

## Conclusions and perspectives

Maintaining the viability and avoiding the necrosis of dental pulp are crucial for preserving the structural integrity and function of teeth. Cell-based regenerative endodontics provides a promising way to achieving real dental pulp regeneration, compared with the unforeseen and undesirable results that revascularisation achieves in the clinic currently<sup>132</sup>. DPSCs promote angiogenesis through multiple mechanisms and are easily available, making them an ideal candidate for transplantation; however, they can do much more than this. They reside in the niches of neurovascular bundle and deriving from peripheral nerve-associated glia endow DPSCs with the ability to reinnervate regenerated dental pulp. Moreover, odontoblast-like cell differentiation and dentine-like structure deposition have been observed, resembling the process of dentinogenesis<sup>133</sup>. These properties make it possible to achieve real pulp-dentine complex regeneration with cell-based regenerative endodontics.

Nevertheless, technical, ethical and economic issues must be addressed before this protocol can be adopted officially in clinical practice. Additional bigger animal models applying orthotopic rather than ectopic sites to mimic the physical and clinical conditions in humans are needed to provide more accurate data. More high-quality and standardised clinical trials are necessary for practical transfer. The ultimate goal of regenerating both functional and structural natural pulp can only be achieved through generations of endeavors.

## Conflicts of interest

The authors declare no conflicts of interest related to this study.

## Author contribution

Dr Meng Qi TANG contributed to the literature collection and draft of the manuscript; Drs Ling YE and Bo GAO supervised the study design and revised the manuscript.

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