REVIEW

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Phenotype heterogeneity in dental pulp stem niches

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Abstract

Dental stem niches (DSNs) reside in different dental tissues, being of particular importance in tissue engineering and dental regeneration procedures. The present paper aims to review the DSNs from the view of niche inhabitants, either extrinsic, such as cells of the myeloid lineage, or intrinsic, such as endothelial cells, perivascular cells and spindle-shaped stromal cells, *e.g.*, telocytes. DSNs harbor different dental stem/progenitor cell morphologies, in different stages of differentiation and with various potentialities, the angiogenic potential with respect to regenerative endodontic procedures being emphasized here. It seems therefore important to consider the DSNs as being heterogeneous, for a better understanding of an accurate identification of niche team players in regenerative medicine.

Keywords: dental pulp, apical papilla, Stro-1, CD117, endothelial progenitor cells, endothelial-mesenchymal transformation.

Introduction

The dental stem niche is continuously in focus of various research groups, actually being characterized six human dental stem/progenitor cell types, which reside different dental tissues: (1) dental pulp stem cells (DPSCs); (2) stem cells from exfoliated deciduous teeth (SHED) [1]; (3) periodontal ligament stem cells (PDLSCs); (4) dental follicle progenitor cells (DFPCs); (5) stem cells of the apical papilla (SCAP) and (6) periodontal ligament of deciduous teeth (DePDL) [2, 3]. Only three of these subsets actually belong to the dental pulp: DPSCs, SHED and SCAP [4, 5].

Dental pulp stem cells were initially isolated and characterized by Gronthos *et al.* (2000), who collected normal human impacted third molars, separated the pulp tissue and further processed it to obtain single-cell suspensions [6]. Then, single-cell suspensions of dental pulp and bone marrow were seeded to form colonies. The resulted DPSCs and bone marrow stromal cells (BMSCs), as precursors of osteoblasts, were evaluated by histochemistry, immunohistochemistry and were transplanted on mice to be further evaluated by reverse transcription-polymerase chain reaction (RT-PCR) [6]. Although a similar *in vitro* phenotype was found for those cell populations, the BMSCs formed in transplantation models lamellar bone, while the DPSCs formed dentin-pulp-like complexes [6]. It should be noted here that *in vitro* cells within each colony were characterized by a typical fibroblastoid morphology and displayed thin and moniliform prolongations [6], being quite similar to cultured stromal cells assumed being telocytes (TCs) [7–9], except perhaps the length, but not the morphology, of the cell processes. This may not be speculative, as TCs were found expressing stem cell markers, such as c-kit, nanog and sca-1 [9].

Different original studies and reviews regarding the dental stem niche indicate [2, 10, 11], or not [3, 12], the endothelial potential of differentiation of the respective niche resident stem/progenitor cells. There are studies indicating the angiogenic potential of stem/progenitor cells residing in the dental pulp, which were performed under *in vitro* conditions [13–15]. Such a subset of stem/progenitor cells of the dental pulp have been isolated by their ability to efflux Hoechst 33342 dye and were referred to as side population cells, which possess a mesenchymal stem (stromal) cell (MSC) phenotype and have, among other potentialities, angiogenic potential [16]. However, the standards of identification of MSCs are designed for an *in vitro* diagnosis of such cells, as they include, among different markers and multilineage potential of differentiation, the plastic-adherence of the cells subjected to identification [17]. Nevertheless, not much information

is provided about the angiogenic abilities and mechanisms of the human DPSCs, which were demonstrated having no influence on the proliferation of endothelial cells (ECs), but being able to significantly induce ECs migration *in vitro* [18].

Stro-1, the DPSCs marker

Simmons & Torok-Storb (1991) developed a murine IgM monoclonal antibody, namely STRO-1, binding to "*human bone marrow fibroblast-like cells and to various nonhematopoietic cellular components of the adherent cell layer in long-term bone marrow cultures*" and that can be also used for isolation of stromal precursors in fresh bone marrow aspirates [19]. Since then, Stro-1 was used for isolation of various types of MSCs, particularly those in dental stem niches [20]. Indeed, the positive expression of Stro-1 was assigned in various studies to DPSCs, SHED, SCAP, PDLSCs and DePDL, but not to DFPCs [21].

Although being continuously used for identification of MSCs, the identity of Stro-1's cognate antigen was consistently unexplored [20]. Endothelial expression of Stro-1 was found either positive, or negative, in a tissue-dependent fashion [22]. Endothelia of capillaries and arterioles were found expressing Stro-1 [22, 23], which, in turn, is not expressed in arterial endothelia [23] and is scarcely expressed in the endothelium of veins [22]. In various tissues, Stro-1 and the von Willebrand factor (vWF) were co-expressed in endothelia, but not exclusively [20]. Western blot analysis with Stro-1 antibody detected a single protein band of 75 kDa in endothelial cells, supporting the fact that Stro-1 is intrinsically an endothelial antigen, its expression in MSCs being then speculated as an induced effect [20]. It is so expected that endothelial progenitor cells (EPCs), which express vWF, also express Stro-1. This is supported by various studies, one of these demonstrating that adipose-derived EPCs, as well as stem cells, exhibit the *in vitro* positive expression of Stro-1 and CD34 [24]. Tissue-specific expression of markers should be however taken into account, because although most of the subsets of dental stem/progenitor subpopulations express Stro-1, all these subpopulations were found negative for the expression of CD34 [21].

Stro-1, as well as CD146, are regarded as reliable MSCs markers [25]. It was demonstrated that 5-bromo-2'-deoxyuridine (BrdU) label-retaining dental pulp stem/progenitor cells harbor in the perivascular niche which surrounds endothelia and co-express Stro-1 and CD146 [26]. It was then discussed that these two markers are not absolutely reliable, as they were previously [27] found expressed in other cell types, such as fibroblasts and endothelial cells [26]. In an attempt of clarifying the isolation techniques and specific markers for different adult stem cell types, there were distinguished specific panels of markers for EPCs and MSCs, some of these markers being equally expressed in both cell lineages: CD146, MCAM, MUC18, S-endo-1, and STRO-1 [28]. It appears thus conceivable that a subset of dental stem/progenitor cells should be viewed as EPCs, which can exhibit a TC-like morphology, as it was recently indicated in human, but not dental, tissues [29, 30].

EPCs markers were not fully tested in dental stem/progenitor niches

A study of Okiji *et al.* (1997) found HLA-DR+/CD68+ cells with dendritic morphologies, which formed a reticular network in the human dental pulp [31]. The authors observed that the processes of those cells were contacting endothelial cells and were building a three-dimensional structure around pulp microvessels [31]. According to the specificities of antibodies the authors related their finding to the immune subset of dendritic cells which populate stromal compartments and could not discuss their findings in light of concepts which emerged later, such as that of the universal perivascular/periendothelial stem niche of adult, inhabited by cells which can equally differentiate toward a hematopoietic, or mesenchymal, stem phenotype [32–35]. On other hand, HLA-DR and CD68 are also expressed in monocytes [36], which, in turn, are well known actors during vasculogenesis in adult tissues, being thus able to differentiate in endothelial cells [37]. The CD14+ monocyte-derived multipotential cells, or monocyte-derived mesenchymal progenitors, are fibroblastoid cells, which exhibit mixed features of monocytes, mesenchymal cells and endothelial cells [38] and also express the embryonic stem markers nanog and Oct-4 [39]. However, EPCs were discovered by Asahara *et al.*, in 1997 [40], the same year when Okiji *et al.* published their results on the dental pulp stromal networks built-up by cells they tested for a phagocytic phenotype, although not tested for an endothelial and/or progenitor one. Okiji *et al.* (1997) worked on pulp tissues resulted from intact molars and premolars, which were extracted for orthodontic reasons and seemingly found perivascular progenitors, prior to the EPCs discovery. However, presence of HLA-DR+ and CD68+ dendritic cells was adequately documented in a study performed later on radicular granulomas, when such cells with long cytoplasmic processes were found concentrated in lymphocyte-rich areas, and were evaluated as stronger antigen-presenting cells, as compared with macrophages [41].

Endothelial cells derived from cultured monocytes simultaneously express endothelial (CD31, vWF, Tie-2) and macrophage/monocyte (CD68, CD80, CD45, CD36) antigens, distinctive of endothelial cells derived from culture of more primitive hematopoietic cells which express CD34 or CD133 [42]. In this regard, the negative expression of CD34 in stem/progenitors cells of dental niches [21] should be judged with caution. Moreover, human DPSCs were found co-expressing during their *in vitro* osteoblastic differentiation osteocalcin, flk-1 and, in 30%, endothelial antigens such as vWF, CD31, and CD54 [43]. There were then found stem cells synergically differentiating into endotheliocytes and osteoblasts and it was observed that flk-1 or vascular endothelial growth factor receptor-2 has a pivotal role in coupling the osteogenic and endothelial differentiation, which are thus interdependent processes [43].

Maintaining dental pulp vitality in youth and childhood is crucial for tooth preservation and functionality. Pulpal necrosis and infection may conduct to incomplete root formation, affecting children's growth and well-being [44]. With respect to root development outcome, two distinct clinical situations have been described [45]. In case of

immature teeth with vital pulp, conservative procedures inducing apexogenesis are approached, aiming to preserve pulp vitality, allow root maturation and apex formation and closure [46]. The other situation refers to immature teeth with non-vital pulp. In this situation, apexification is performed, by forming an apical hard tissue bridge, root development being usually arrested [47, 48].

Within this framework, the angiogenic properties of dental stem cells are urged to be addressed to regenerative endodontic procedures. It has been suggested that induction of a blood clot in a disinfected root canal may conduct to growth factors' release and residing stromal cells' attraction [45]. With this regard, the apical size is an important factor for dental pulp regeneration and vascularization, since the apical foramen is the main pathway of communication between dental pulp and periapical tissues. Another important issue is related to angiogenic properties of different dental stem cell populations. The paracrine angiogenic properties of dental stem cells were tested and it resulted they have a predominant pro-angiogenic influence on endothelial migration and tube formation, *in vitro* as well as *in vivo*; seemingly, DPSCs and SCAP have a stronger angiogenic profile and function as compared with DFPCs, as well as with human gingival fibroblasts (HGFs) [49]. A monocytes-deriving lineage populating the dental pulp stem niche should be so distinctively addressed when attempts are made to characterize the dental pulp niche, being also known that DPSCs express, similar to HGFs, angiogenic factors such as vascular endothelial growth factor (VEGF) and monocyte chemotactic protein-1 [18, 49]. The positive expression of vWF or the evidence of Weibel–Palade bodies, in light and, respectively, transmission electron microscopy could associate the origin of dental pulp stem or progenitor subpopulations with the endothelial lineage, being well-known that these are absolutely specific molecular and ultrastructural markers of that lineage. However, stem niches include transitory amplifying cells (TACs), which are progressively differentiating progenitors, thus firm molecular phenotypes could not be postulated. It is difficult, if not impossible, to differentiate between stem and progenitors cells within the niches [50], which are dynamic populations with reciprocal interactions within niches [51].

Telocytes, veil cells of Majno, endothelial progenitors, and the perivascular niche of the dental pulp

The gold standard for TCs identification was initially postulated being an ultrastructural one [52]. As TCs were renamed from interstitial Cajal-like cells (ICLCs), the initial standards for the body of TCs were derived from those postulated for ICLCs, and included at that stage a small amount of cytoplasm surrounding the nucleus which, in turn, was presenting clustered marginal heterochromatin, thin and intermediate filaments, abundant mitochondria, endoplasmic reticulum, a small Golgi complex, and abundant subplasmalemmal caveolae [52]. This description could correspond at that time to a differentiated cell type although criteria of exclusion for a partly differentiated stage (*i.e.*, of TACs) were not provided. Further, the TCs definition as "cells with telopodes" [53, 54] gave allowance to overlooking the ultrastructural details of these cells. Nevertheless, it seems that before TCs were indicated as a peculiar cell type, Majno described such cells as "veil cells" and included them in the perivascular niche [29, 55, 56], which is actually consistently indicated as a stem niche [34, 57, 58]. Perivascular TCs were found expressing c-kit, CD34, vimentin, caveolin-1, VEGF and inducible nitric oxide synthase, and the c-kit-expressing TCs were found inconsistently co-expressing CD34, CD44, α-smooth muscle actin (α-SMA), S100 protein, neuron-specific enolase, and nestin [59]. This feeds the concept of perivascular stem/progenitor cells. On other hand, some of these markers, such as CD34, CD44 and nestin, could as well identify EPCs. In this regard, it should be noted that CD44-expressing DPSCs were demonstrated an angiogenic potential after VEGF induction, and gained expression of vWF [13].

A key marker for identifying ICLCs before they were renamed to TC was the expression of CD117/c-kit [60–63], which was also found expressed in dental pulp stromal networks and was discussed as a possible identifier of ICLCs [5]. Although differences were noted between ICLCs and the classical interstitial Cajal cells (ICCs) belonging to the gastrointestinal tract [62], c-kit was not discussed as a stem/progenitor marker of cells located outside the gastrointestinal tract. Neither were the ICLCs (that will further be renamed to TCs) differentiated by use of specific panels of markers from endothelial progenitors, being known that such circulating progenitor cells, as well as the HSCs, also express c-kit [64]. Recent papers point to a progenitor phenotype of TCs [30, 65–70], and it appears that at least a subset of TCs qualify as EPCs [29]; this should be investigated also within the dental stem niches by mandatory use of electron microscopy. Noteworthy, the concepts regarding TCs were updated and was clearly stated that they are not ICLCs, being different from ICCs in ultrastructure, immunophenotype, electrophysiology, gene profile, and proteomics [71].

An *in vitro* study took into account that Stro-1 is expressed in mural vascular and perivascular cells, being seemingly an early marker of different mesenchymal stem cell populations and inferring possible perivascular niche for these stem cell populations *in situ* [72]. DPSCs were tested then tested for expression of endothelial cells antigens (vWF, CD146), smooth muscle cells, and pericytes (α-SMA, CD146), and a pericyte-associated antigen (3G5) [72]. DPSCs expressed α-SMA and CD146, as well as 3G5 in their majority [72]. This indicates that at least a subset of DPSCs could belong to the perivascular niche.

Telocytes, fibrocytes, fibroblasts … a single actor but multiple characters

It should be discussed further why the expression of CD34 in dental pulp stromal cells should be regarded with caution and how would it relate with the possible presence of TCs within the dental pulp stem niche. One should take into account that CD34 is expressed in various cell types, including MSCs, although a misconception persists that expression of CD34 distinctively indicates cells of hematopoietic origin [73].

As Barth & Westhoff (2007) documented, the earliest reports on CD34+ stromal cells appeared in the mid 90ies

and cells actually indicated as CD34+ "fibrocytes" were termed "stromal cells", or "spindle-shaped" cells, or "dendritic cells" [74]. Their observation that "*probably, the authors were unaware of the fact that the cell populations they investigated and described were identical*" [74] was further supported by Díaz-Flores *et al.* (2014) who reviewed that no matter how CD34+ stromal cells were termed, fibroblasts, fibrocytes, telocytes, dendritic, adventitial, perivascular, etc., they are an unique cell type able of losing the CD34+ phenotype and of gaining expression of mesenchymal stromal cells and their derivate cells (osteoblasts, myofibroblasts, chondroblasts, adipocytes) [75, 76]. In these regards, spindle-shaped stem/progenitor cells within dental niches, which lack, as discussed, the expression of CD34, could be as well regarded as TCs which gained a progenitor potential.

The phenotypic switch of CD34 expression, from positive to negative, could use to differentiate subsets of DPSCs thus being supportive for the growing concept of the dental stem niches heterogeneity [73, 77].

Heterogeneity of DPSCs

A study using different stem markers demonstrated that the dental pulp consists of multiple stem niches rich of nestin-expressing cells [77], which were patterned for MSCs and embryonic stem (ES) markers as presented in Table 1.

Table 1 – ***Histological mapping of stem cells markers expression in SHED (according to [77])***

	Cell-rich zone of DP	Cell-free zone of DP	Odontoblastic layer of DP
Nestin	▪ abundant expression of nestin in undifferentiated MSCs with two different morphologies, fibroblastoid (spindle-shaped) and round epithelial-like (ES-like).	▪ endothelial and neural expression of nestin; ▪ perivascular nestin+ cells.	▪ nestin+ round ES-like cells; ▪ nestin+ large columnar cells (odontoblasts).
Stro-1	negative expression	▪ endothelial and periendothelial expression of Stro-1; ▪ scarce expression of Stro-1 in pulp nerves.	negative expression
Vimentin	▪ endothelial cells; ▪ stromal cells.	▪ endothelial cells; ▪ stromal cells.	N/A
Oct3/4	▪ endothelial cells; ▪ Oct3/4+ round ES-like cells.	▪ endothelial cells; ▪ rather fibroblastoid Oct3/4+ cells.	N/A

SHED: Stem cells from exfoliated deciduous teeth; MSCs: Mesenchymal stem cells; DP: Dental pulp; ES: Embryonic stem; N/A: Not available.

Two distinctive stem/progenitor cells morphologies were indicated, spindle-shaped fibroblastoid cells and round epithelial-like ES-like cells [77]. Previously, human SHED were tested *in vitro* and were found expressing ES markers (Oct-4, SSEA-3, SSEA-4, TRA-1-60, Nanog), MSC markers, and markers (CD13, CD31) indicating cells of the myeloid lineage, these cells being considered as immature DPSCs [78]. In this regard, DPSCs were tested in an *in vitro* study [73] and two distinctive molecular phenotypes resulted after cells formerly selected for c-kit and Stro-1 were separated for the positive or negative expression of CD34 [73]. Both subsets of DPSCs were similar in what concerns their ability to differentiate towards mesodermal lineages, only one of these two demonstrating a specific neurogenic potential [73]. There were discussed then [73] the results of a different *in vitro* study [79] which indicated that the subset of DPSCs expressing Stro-1, c-kit, as well as CD34, represent a subpopulation of MSCs with neural crest origin (Table 2).

Table 2 – ***Molecular distinctive subsets of DPSCs inhabit the dental pulp stem niche (according to [73])***

Markers	DPSCs subsets	
c-kit	+	+
Stro-1	+	+
CD34	–	+
CD271	–	+
Nestin	–	+
	▪ high proliferation capacity; ▪ lower doubling time; ▪ low rate of cell senescence; ▪ at passage 6: active proliferation and maintenance of stemness markers; ▪ osteogenic, myogenic and adipogenic potential; ▪ low efficiency of neurogenic commitment.	▪ slow proliferation kinetics; ▪ reduction of cell density over the culture time; ▪ early induced towards cell senescence; ▪ at passage 6: cell apoptosis, loss of CD34 expression, maintenance of Stro-1 and c-kit expressions; ▪ osteogenic, myogenic and adipogenic potential; ▪ high efficiency of neurogenic commitment, towards neuronal lineage and glial-like cells.

DPSCs: Dental pulp stem cells.

Identification of the positive endothelial and peri-endothelial expression of Oct3/4 in dental pulp cells [77] raises an attractive hypothesis on the sources of DPSCs and should relate that expression to processes of endothelial-mesenchymal transformation (EndMT) able of generating stromal cells with MSCs potentialities. Resident ECs as well as EPCs can undergo EndMT [80], being demonstrated that smooth muscle-like progeny can result from endothelial colony-forming cells, which mirrors the acquisition of a smooth muscle-like phenotype in ECs [81]. Dental pulp endothelia were not previously specifically tested for being able of EndMT, neither was this discussed when expression of Oct3/4 was found positive in dental pulps [77]. A periendothelial Oct3/4-expressing cell could result after the EndMT of a resident EC, which also expresses Oct3/4, by a stereotyped process involving not a cell division, but a strong Ω bending of the EC which brings in contact its endothelial neighbors then it leaves the endothelial wall and passes in the subendothelial tissue, as it was demonstrated experimentally [82].

Noteworthy, a transmission electron microscopy (TEM) study on healthy dental pulps of impacted third molars brought evidence to define three types of periendothelial cells, pericytes, transitional cells and fibroblasts, as reflected by their basement membrane embedding and by the their cytoplasmic dense bodies similar to the endothelial Weibel–Palade bodies; these findings were suggestive for an endothelial origin of pericytes and for a stromal recruitment of these [83].

Conclusive remarks

Further investigations on EPCs in dental stem niches could bring more information needed for outcome improvement following regenerative endodontic procedures. Attempts should be done to pattern in transmission electron microscopy the different phenotypes of the heterogeneous stem/progenitor cells populating the dental stem niches. The endothelial-mesenchymal transformation should be tested for its potential of replenishing the dental pulp stem niches.

Conflict of interests

The authors declare that they have no conflict of interests.

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Author contribution

Authors #1 (PP) and #2 (MCR) have equal contributions to this paper.

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