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Human and swine DPSCs form vascular-like network after angiogenic differentiation in comparison to endothelial cells — A quantitative analysis

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Abstract

Introduction—The aim was to quantify vascular network formation capacity after angiogenic induction of human and swine dental pulp stem cells (DPSCs) in comparison to endothelial cells.

Methods—Primary human (h) DPSCs or swine (s) DPSCs were induced in endothelial growth medium (EGM) for 7 days. The expression of endothelial marker, von Willebrand Factor (vWF) was determined by immunostaining. Induced (iDPSCs) and non-induced (n-iDPSCs) were seeded at different cell numbers onto Matrigel for vascular network formation assays and analyzed after 4, 8, 12, and 18 h in comparison to human endothelial cells (hMECs). Quantitative analysis of vascular tubule formation was performed using ImageJ. The vascular network formation was also conducted by co-culturing of n-iDPSCs and iDPSCs.

Results—vWF was detected by immunofluorescence in both n-iDPSCs and iDPSCs (human and swine). Time-lapse microscopic observation showed that the vascular network was formed by iDPSCs, but not n-iDPSCs. After 4 h, iDPSCs showed vascular network formation while n-iDPSCs started to aggregate and formed clusters. iDPSCs displayed a similar capacity to form vascular networks in Matrigel compared to hMECs based on quantitative analysis. iDPSCs had a higher capacity compared to ihDPSCs or hMECs ($p < 0.05$) in forming the network structures including segments, nodes and mesh. iDPSCs than ihDPSCs and hMECs ($p < 0.05$). Co-culture experiment showed that n-ihDPSCs co-localized on the angiogenic tubules and vascular networks formed by ihDPSCs.

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Conclusions—Our findings indicate that iDPSCs in combination of their non-induced counterparts may be utilized as a future clinical strategy for enhancing angiogenesis during the process of pulp-dentin regeneration.

Keywords

Angiogenesis; co-culture; dental pulp stem cells; Matrigel; tubule formation

Introduction

Angiogenesis plays a critical role in the success of engineered tissue regeneration. This is particularly important for cell-based therapy as the transplanted cells need blood supply for nutrients to survive in order to initiate tissue regeneration. In the case of cell-based pulp regeneration, angiogenesis in the root canal space faces a greater challenge as the source of any nutrient supply is restricted to the apical foramen. Due to this limitation, cell-based pulp regeneration was only considered possible if dealing with a tooth with wide open apex. Even so, it was proposed that the pulp regeneration process may need to be incremental from apical third toward coronal third (1). Demonstrated from the proof-of-principle experiments, vascularized pulp tissue was regenerated using either a tooth slice model or tooth fragment model in mice when the blood supply can be easily established during pulp regeneration (2, 3).

However, if we expect to utilize cell-based pulp regeneration on humans with most teeth having small apical foramen, other regimens will be needed to achieve the goal. Several studies have reported that dental pulp stem cells (DPSCs) can express several angiogenic growth factors (4, 5), indicating their ability to induce angiogenesis. It has been shown that stem cells from human exfoliated deciduous teeth (SHED) have the potential to become endothelial-like cells (EC-like) evidenced by the expression of endothelial cell (EC) markers in vivo (6). Limited evidence has also indicated that DPSCs have the potential to differentiate into EC-like (7–10). However, no effort has been made to utilize this premise as a strategy in the context of enhancing pulp regeneration. Here we took the first step using an in vitro system to test whether angiogenically induced DPSCs form vascular network in comparison to that of endothelial cells (ECs). Co-culturing of non-induced DPSCs (n-iDPSCs) and induced DPSCs (iDPSCs) was also conducted to assess spatial relationship between the n-iDPSCs and iDPSCs during the vascular network formation.

Materials and Methods

Cell culture

Freshly extracted human and swine teeth were stored in serum-free cell culture medium, α -Minimum Essential Medium (α -MEM; HyClone, Logan, UT, USA) and transported to the laboratory for processing by a previous published protocol (3). Human teeth were from 15–25 year old healthy donors in the Oral Surgery Clinics at Boston University (BU) or University of Tennessee Health Science Center (UTHSC). The patient sample collection in this study conformed to exempt protocols approved by the Institutional Review Board of BU (#H-28882) and UTHSC (12-01937-XM). Swine teeth were collected from Sinclair

miniature swine (12–17 months female, Sinclair Bio-Resources; <http://www.sinclairbioresources.com/>). Animal procedures followed a protocol (#2092) approved by the Institutional Animal Care and Use Committee at UTHSC. Pulp tissue was removed from teeth, minced, and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase (Sigma-Aldrich, St. Louis, MO) for 30–60 min at 37°C. Cells were seeded into six well plates and cultured in a standard medium with α -MEM supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Woodland, CA), 2 mM L-glutamine, 100 mM L-ascorbic acid-2-phosphate, 1% antibiotic-antimycotic (Life Technologies, Grand Island, NY) and maintained in 5% CO₂ at 37°C. Isolated heterogeneous population of DPSCs were passaged at 1:3 ratio when they reached 80% confluence and expanded to passage 3 for experimentation. Human Microvascular Endothelial cells (hMECs) (passage 9, obtained from Dr. Yi Lu, UTHSC, Memphis, TN (11)) were cultured in endothelial growth medium EGM (endothelial growth medium)-2™ (Lonza, Walkersville, MD, USA) until 80% confluency for experimentation.

Endothelial Induction of DPSCs

Human (h) or swine (s) DPSCs at passage 3 and 70% confluency were cultured under standard (non-induced) or angiogenic (induced) conditions. For the angiogenic condition, cells were cultured with EGM-2™ for 7 days. Non-induced DPSCs are denoted as n-iDPSCs (n-ihDPSCs or n-isDPSCs) while induced DPSCs are denoted as iDPSCs (ihDPSCs or isDPSCs).

Immunofluorescence

Cells were cultivated in wells of 24-well plates coated with a thin layer of Matrigel (3 mg/mL, Basement Membrane Matrix, BD Bioscience), fixed with methanol at –20°C for 10 minutes and rinsed with phosphate buffered saline (PBS). Cultures were incubated with a primary antibody against the endothelial cell marker von Willebrand factor (vWF) (rabbit anti-human, 1:100 dilution Sigma-Aldrich, cat# F3520) for overnight, washed with PBS, and incubated with Alexa Fluor conjugated goat anti-rabbit IgG (Abcam, cat#ab150077) for 30 min. Counterstaining of the cells with DAPI was performed and the cells were observed under fluorescence microscopy. The quantification of the fluorescence intensities was performed using the fluorescence images and ImageJ software. Three images taken from 3 randomly selected areas in the stained cell cultures per group were measured. In each image, the outline of each cells were drawn and the area, mean fluorescence along with the background readings were measured (12, 13). The total cellular fluorescence (TCF) was calculated by the following formula: TCF = integrated density – (area of selected cells $\times$ mean fluorescence of background readings).

In Vitro Angiogenesis Tubule Formation Assay

96-well plates were coated with 50 μ L/well of chilled Matrigel solution (10 mg/mL) without air bubbles and incubated for 1 h at 37°C to solidify. n-iDPSCs, iDPSCs or hMECs were trypsinized and seeded onto the solidified Matrigel in the wells at different cell numbers (1×10^4 , 1.5×10^4 , 2×10^4 , or 3×10^4 cells/well or 353, 530, 706, 1060 cells/mm², respectively) in 150 μ L EGM-2 medium and incubated at 37°C. The endothelial tubule-like vascular

network formation was observed at 4, 8, 12, and 18 h of incubation under an inverted microscope.

The images were captured at $\times 4$ in phase contrast mode and the vascular network was analyzed using ImageJ software according to a method reported by Chevalier et al (14). Briefly, the vascular networks were segmented and skeletonized. The trees were then analyzed by detection of nodes (the branching points), segments (the length of single branches), mesh (the closed loops), and total length of tubules of the cellular meshed network organization (15).

Co-culture of n-ihDPSCs and ihDPSCs

n-ihDPSCs at passage 1 were seeded into wells of 12-well plates and transduced with pLentiCMV-GFP vectors (7×10^8 IU/mL, UCLA Vectorcore, Los Angeles, CA) at MOI of ~ 30 with the presence of polybrene (4 $\mu\text{g}/\text{ml}$). n-ihDPSCs at passage 3 were labeled with Vybrant Dil (V2288, Invitrogen, Carlsbad, CA, USA) (denoted as n-ihDPSCs-VD). n-ihDPSCs-GFP expanded to passage 3 were cultured with EGM-2 for 7 days for angiogenic induction, termed ihDPSCs-GFP. A 96-well plate was coated with 50 $\mu\text{L}/\text{well}$ of Matrigel. ihDPSCs-GFP (1.5×10^4) in 100 μL EGM-2 medium were seeded onto Matrigel in each well, immediately followed by seeding n-ihDPSCs-VD in 50 $\mu\text{L}/\text{well}$ of standard medium into the same well. Various cell ratios of ihDPSCs-GFP : n-ihDPSCs-VD were used – 2:1, 5:1, 10:1 and 20:1, with ihDPSCs-GFP stayed at 1.5×10^4 cells/well. The well was then mixed by swirling. Network formation and localization of cells were observed under a fluorescence microscope at 4 h after cell seeding.

Data analysis

One-way ANOVA was used to compare single factor among three groups for the quantitative analysis of cell fluorescence intensity and angiogenesis network formation. When the main effect found to be statistically significant, post-hoc comparisons were examined by Tukey HSD (Honestly Significant Difference) test. Data are reported as mean $\pm$ SD and values are considered significant when $p < 0.05$. All analyses were performed using SPSS.

RESULTS

Endothelial marker expression of induced DPSCs

After 7 days of angiogenic induction, iDPSCs exhibited slight morphological changes. The cell body tended to be less spread out resembling hMECs. ihDPSCs exhibited elongated cell extensions between cells which was also seen in hMECs. n-isDPSCs in general showed less spindle shaped morphology but more triangular or trapezoid compared to human counterparts. After induction, they appeared more ovoid and round (Fig. 1A). Immunofluorescence staining of the EC marker vWF revealed that n-iDPSCs and iDPSCs both expressed the marker (Fig. 1B). The quantification of the fluorescence intensities showed that hMECs expressed significantly higher levels of vWF than those of n-ihDPSCs, but not other cell groups. No difference was detected between non-induced and induced groups (Fig. 1C).

Vascular network formation analysis

The vascular network formation on Matrigel was determined by seeding various number of cells/well. Endothelial tubule formation assay showed that 1.5×10^4 cells/well for ihDPSCs, 1.5×10^4 cells/well for isDPSCs and 2×10^4 cells/well for hMECs were optimal seeding numbers for angiogenic tubule formation (Fig. 2). isDPSCs appeared to begin forming vascular networks at 1×10^4 cells while other two cell types did not. hMECs required a higher cell density to form the networks compared to iDPSCs and appeared to be more sensitive to the right cell density in order to form networks whereas iDPSCs seemed more flexible.

We then determined the optimal timing to form vascular networks after cell seeding. At 4 h, iDPSCs showed vascular network formation while n-iDPSCs did not form any tubules or vascular networks throughout the experimental period up to 18 h. hMECs sustained the networks up to 18 h, however, cells gradually clumped together over time. ihDPSC networks also lost integrity after 8 h and formed large cell clusters increasing in size at 18 h. isDPSCs tended to sustain the network forms although cells lost the tubule formation assembly after 8 h (Fig. 3).

Next we applied a quantitative method (14) to analyze the in vitro angiogenesis assays of these cells. The quantitative analysis was based on the images taken at 4 h presented in Fig. 4A. The imaging program established tubular network skeletons from the cell images and identified segments, mesh and nodes as shown in Fig. 4B. The calculated numbers of these parameters were then compared among the three cell groups (Fig. 4C). With respect to formation of nodes, segments and mesh that constitute the network of vascular-like structures, ihDPSCs displayed a similar capacity to form vascular networks to hMECs ($p > 0.05$) while isDPSCs had a higher capacity compared to ihDPSCs or hMECs ($p < 0.05$).

Co-culture of n-ihDPSCs and ihDPSCs

We next determined the possible dual role of DPSCs because of i) their potential to differentiate into EC-like after induction forming vascular network similar to the capacity of ECs, and ii) potential to exhibit stem cell qualities as non-induced cells. We utilized the in vitro angiogenesis tubule formation 3D model to see if n-ihDPSCs co-cultured with ihDPSCs would establish a similar relationship as observed in vivo between ECs and DPSCs, i.e., perivascular location of DPSCs (16). To be able to observe two population of cells, we labeled the cells with green or red fluorescence. We found that ihDPSCs (green) formed vascular networks as expected, while n-ihDPSCs (red) co-localized with the vascular forming ihDPSCs as shown in Fig. 5. The more n-ihDPSCs were used, the more such cells were seen co-localized with the tubule forming ihDPSCs instead of scattering non-attached.

Discussion

Our quantitative analysis indicated that both human and swine DPSCs can be induced to behave similarly to ECs, forming vascular-like network in the standard in vitro angiogenesis tubule formation assay. Additionally, co-culturing of ihDPSCs and n-ihDPSCs in the angiogenesis tubule formation setting, the n-ihDPSCs co-localized adjacent to the tubule

forming ihDPSCs. This suggests that given an environment simulating the in vivo conditions, n-iDPSCs may behave resembling pericyte-like cells in terms of attaching to the vascular structures.

The formation of tubular network on Matrigel is highly specific to ECs while other cell types form other structures (17, 18). When ECs which typically have cobble stone morphology plated on Matrigel, they differentiate into a network of tubules that mimic a capillary system (19). Our present study showed that when iDPSCs plated on the Matrigel, they adhered onto it, migrated toward each other and formed capillary-like tubules, which matured into tubular networks over 4–18 h. In contrast, n-iDPSCs did not show any vascular development. A previous study reported the similar findings, however, the angiogenic tubules were not organized throughout Matrigel and after 8 h they formed large cell clumps (8). We noticed that n-iDPSCs already expressed similar levels of vWF to iDPSCs in both human and swine systems while n-iDPSCs were not able to form vascular-like network in Matrigel, suggesting that vWF expression is not a determining factor for such functions. Other angiogenic factors may be important in the involvement of this process which require further investigation.

Optimized cell seeding density and identifying the stable phase of the vascular network formation are important for the analysis of angiogenesis in the Matrigel. As evidenced in our data, too high or too low of plating densities resulted in no vascular tubule formation. The reported average cell density of ECs in the literature is 563 cells/mm² (20). The present study showed that the optimal cell density for iDPSCs was 519 cells/mm², while hMECs was 692 cell/mm². At optimal seeding densities, iDPSCs formed similar vascular network including segments, nodes and mesh compared to hMECs. In addition, ihDPSCs started to form cell clumps after 18 h while isDPSCs preserved networks even after 3 days.

As mentioned earlier, re-establishing vascularity during the stem cell-based pulp regeneration is critical for the success of this process. The faster the angiogenesis can occur and establish, the better survival of the transplanted stem cells in the root canal space can be obtained in order to initiate the tissue regeneration. When performing non-cell based pulp regeneration, re-establishing the vascularity relies on the angiogenesis from the blood vessels in the periapical region. It would take time for the newly formed vessels to reach the coronal third. If the root canal space is transplanted with stem cells, the survival of the cells depends on the timely supply of nutrition either from diffusion or from newly formed blood vessels. Mature ECs are terminally differentiated cells with a low proliferative capacity (21) and perivascular network is quite difficult in the root canals with closed root apex (22).

When using tooth slice model to test pulp regeneration, the blood supply is good, therefore the pulp regeneration can occur with good quality (2). Using the tooth fragment model in our previous studies, we have demonstrated that if the apex is large (~2–3 mm), canal is wide and the tooth is short, transplanted cells in the canal space may survive well and produce good quality pulp regeneration (3). However, when longer tooth fragment with small apical openings (<1 mm) were used, the cell-based pulp regeneration could only reach a few millimeter into the canal from apex (data not shown). In a dog animal model performing orthotopic pulp regeneration in the dog teeth, it was demonstrated that the use of a subpopulation of DPSCs, CD105⁺, that secrete considerable amount of angiogenic factors

can allow the formation of vascularized pulp with relatively small foramen opening (~0.6–0.7 mm) (23).

To further enhance the vascularization during pulp regeneration, especially if we were to deal with long canals of small apex (normally no larger than ~0.4 mm of foramen opening), other approaches are likely needed. One approach is to incorporate endothelial cell populations in the transplanted cell pool, which has been tested and studied especially in the field of bone regeneration (24). This concept has also been reported in pulp regeneration (25). However, ECs are difficult to harvest and it is certainly not feasible to obtain them from the same host as an autologous cell source. Alternatively, we can take the advantage of DPSCs endothelial differentiation potential to enhance the angiogenesis during the pulp regeneration. This way only one cell type is needed.

We also showed that ihDPSCs cultivated sequentially with n-ihDPSCs at various ratios (ihDPSC : n-ihDPSC) had the ability to form vascular network in 3D Matrigel. It appeared that the ratio at 2:1 allowed more n-iDPSCs to cover the tubule forming iDPSCs. It has been reported that the ratios between endothelial cells and pericytes range from 1:1 to 10:1 with the central nervous system vasculature having the most pericyte coverage (1:1 to 3:1) (26). It is unknown what the ratio is in the human pulp vasculature. Our observation that n-iDPSCs can bind to vascular network formed by iDPSCs and this pericyte-like behavior may resemble the real pericyte in vivo that provides stabilization and integrity of angiogenic tubules (27). Further in vivo studies are needed to determine whether the combination of n-iDPSCs and iDPSCs can lead to enhanced angiogenesis thereby yielding a better quality of the regenerated pulp.

In conclusion, our data indicate that it may be possible to use DPSCs as the sole cell source, instead of using additional endothelial cell type such as hMECs, to give rise to vasculature; or at least to enhance the angiogenesis during the regeneration process as a pulp regeneration strategy. It is likely that the transplanted n-iDPSCs may also give rise to odontoblast-like cells forming dentin-like structures on the root canal walls as indicated from reported studies (3, 23). This approach of using both induced and n-iDPSCs may be extended to the pulp regeneration studies using swine as a large animal model, as well as possibly used for pulp-dentin regeneration for humans in the future.

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References

1. Huang GT, Sonoyama W, Liu Y, Liu H, Wang S, Shi S. The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. *J Endod.* 2008; 34:645–51. [PubMed: 18498881]
2. Cordeiro MM, Dong Z, Kaneko T, Zhang Z, Miyazawa M, Shi S, et al. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. *J Endod.* 2008; 34:962–9. [PubMed: 18634928]

3. Huang GT, Yamaza T, Shea LD, Djouad F, Kuhn NZ, Tuan RS, et al. Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. *Tissue Eng Part A*. 2010; 16:605–15. [PubMed: 19737072]
4. Tran-Hung L, Laurent P, Camps J, About I. Quantification of angiogenic growth factors released by human dental cells after injury. *Arch Oral Biol*. 2008; 53:9–13. [PubMed: 17764655]
5. Bronckaers A, Hilkens P, Fanton Y, Struys T, Gervois P, Politis C, et al. Angiogenic properties of human dental pulp stem cells. *PloS One*. 2013; 8:e71104. [PubMed: 23951091]
6. Sakai V, Zhang Z, Dong Z, Neiva K, Machado M, Shi S, et al. SHED differentiate into functional odontoblasts and endothelium. *J Dent Res*. 2010; 89:791–6. [PubMed: 20395410]
7. d'Aquino R, Graziano A, Sampaolesi M, Laino G, Pirozzi G, De Rosa A, et al. Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. *Cell Death and Differ*. 2007
8. Marchionni C, Bonsi L, Alviano F, Lanzoni G, Di Tullio A, Costa R, et al. Angiogenic potential of human dental pulp stromal (stem) cells. *Int J Immunopathol Pharmacol*. 2009; 22:699–706. [PubMed: 19822086]
9. Nakashima M, Iohara K, Sugiyama M. Human dental pulp stem cells with highly angiogenic and neurogenic potential for possible use in pulp regeneration. *Cytokine and Growth Factor Rev*. 2009; 20:435–40. [PubMed: 19896887]
10. Liu W, Gong Q, Ling J, Zhang W, Liu Z, Quan J. Role of miR-424 on angiogenic potential in human dental pulp cells. *J Endod*. 2014; 40:76–82. [PubMed: 24331995]
11. Zhang J, Lu A, Beech D, Jiang B, Lu Y. Suppression of breast cancer metastasis through the inhibition of VEGF-mediated tumor angiogenesis. *Cancer Ther*. 2007; 5:273–86. [PubMed: 18548129]
12. McCloy RA, Rogers S, Caldon CE, Lorca T, Castro A, Burgess A. Partial inhibition of Cdk1 in G2 phase overrides the SAC and decouples mitotic events. *Cell Cycle*. 2014; 13:1400–12. [PubMed: 24626186]
13. Gavet O, Pines J. Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. 2010; 18:533–43.
14. Chevalier F, Lavergne M, Negroni E, Ferratge S, Carpentier G, Gilbert-Sirieix M, et al. Glycosaminoglycan mimetic improves enrichment and cell functions of human endothelial progenitor cell colonies. *Stem Cell Res*. 2014; 12:703–15. [PubMed: 24681520]
15. Marquez-Curtis LA, Sultani AB, McGann LE, Elliott JA. Beyond membrane integrity: Assessing the functionality of human umbilical vein endothelial cells after cryopreservation. *Cryobiology*. 2016; 72:183–90. [PubMed: 27182035]
16. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res*. 2003; 18:696–704. [PubMed: 12674330]
17. Kleinman, HK., Martin, GR. *Seminars in Cancer Biology*. Vol. 2005. Elsevier; 2005. Matrigel: basement membrane matrix with biological activity; p. 378–86.
18. Arnaoutova I, George J, Kleinman HK, Benton G. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis*. 2009; 12:267–74. [PubMed: 19399631]
19. Tran-Hung L, Mathieu S, About I. Role of human pulp fibroblasts in angiogenesis. *J Dent Res*. 2006; 85:819–23. [PubMed: 16931864]
20. Smith, EJ., Staton, CA. *Angiogenesis assays: A critical appraisal of current techniques*. John Wiley & Sons, Ltd; 2007. Tubule formation assays; p. 65–87.
21. Hristov M, Erl W, Weber PC. Endothelial progenitor cells mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol*. 2003; 23:1185–9. [PubMed: 12714439]
22. Saghir MA, Asatourian A, Sorenson CM, Sheibani N. Role of Angiogenesis in Endodontics: Contributions of Stem Cells and Proangiogenic and Antiangiogenic Factors to Dental Pulp Regeneration. *J Endod*. 2015; 41:797–803. [PubMed: 25649306]
23. Iohara K, Imabayashi K, Ishizaka R, Watanabe A, Nabekura J, Ito M, et al. Complete pulp regeneration after pulpectomy by transplantation of CD105+ stem cells with stromal cell-derived factor-1. *Tissue Eng Part A*. 2011; 17:1911–20. [PubMed: 21417716]

24. Yu H, VandeVord PJ, Mao L, Matthew HW, Wooley PH, Yang SY. Improved tissue-engineered bone regeneration by endothelial cell mediated vascularization. *Biomaterials*. 2009; 30:508–17. [PubMed: 18973938]
25. Dissanayaka WL, Hargreaves KM, Jin L, Samaranayake LP, Zhang C. 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*. 2015; 21:550–63. [PubMed: 25203774]
26. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell*. 2011; 21:193–215. [PubMed: 21839917]
27. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol*. 2005; 7:452–64. [PubMed: 16212810]

Statement of clinical relevance

This work was to test a potential clinical strategy for cell-based pulp-dentin regeneration by determining if we can induce DPSCs into endothelial-like cells. If yes, combination of non-induced DPSCs and induced DPSCs may enhance the angiogenesis and regeneration of pulp-dentin.

Figure 1.

Morphological characteristics of iDPSCs and immunofluorescence analysis of vWF expression. (A) Cultured hDPSCs, sDPSCs at passage 3 were induced with EGM-2 medium for 7 days. (B) Expression of vWF (green) by DPSCs. hMECs were used as positive controls. DAPI (blue) was for nucleus counter stain. IgG isotype staining (hMECs) was used as a negative control. (C) Quantitative analysis of the cell fluorescence levels. (n= 3; Significant, *p<0.05). (Scale bar: 100 μ m for all images).

Figure 2.

Tubular network formation of DPSCs at different cell numbers on Matrigel. hDPSCs or sDPSCs at passage 3 were induced with EGM-2 for 7 days and seeded at various cell numbers on Matrigel/well in 96-well plates. Representative images taken at 4 h after seeding showing 1.5×10^4 cells/well for ihDPSCs and isDPSCs, and 2×10^4 cells/well for hMECs appeared the optimal cell numbers for angiogenic tubule formation (n=3, from three different donors) (Scale bar: 200 μ m for all images).

Figure 3.

Tubular network formation of DPSCs on Matrigel. Representative images showing tubular network formation at 4, 8, 12 and 18 h after cell seeding (1.5×10^4 cells/well for ihDPSCs and isDPSCs, and 2×10^4 cells/well for hMECs). At 4 h, iDPSCs showed vascular network formation while n-iDPSCs formed clusters of cells which increased in size after 18 h. (Scale bar: 400 μ m for all images).

Figure 4.

Quantitative analysis of angiogenic tubule formation. (A) Representative images of tubular networks on Matrigel formed by ihDPSC, isDPSC and hMECs 4 h after cell seeding in vitro. (Scale bar: 400 μ m for all three images). (B) Corresponding extracted skeletons of tubular networks from images shown in (A), identifying segments (yellow color), mesh (blue color) and nodes (red color) by ImageJ. (C) Comparison of parameters of tube formation among cell groups (n = 3, from three different donors). (Significant, *p<0.05)

Figure 5.

Vascular network formation by ihDPSCs in Matrigel in the presence of n-ihDPSCs. Labeled ihDPSCs-GFP (green) and n-ihDPSCs-VD (red) were co-cultured at different ratios. 1.5×10^4 /well of ihDPSCs were seeded and paired with various ratios of n-ihDPSCs.

(A) Representative bright field of images of co-cultures with different ihDPSCs : n-ihDPSCs ratios (2:1, 5:1, 10:1 and 20:1) showing vascular network formation. (B) Representative merged images of ihDPSCs-GFP and n-ihDPSCs-VD co-cultured at different ratios. (C, D) Higher magnification of ihDPSCs : n-ihDPSCs at 5:1 (C) or 2:1 (D) showing n-ihDPSCs-VD attached onto the tubule forming ihDPSCs-GFP. (E) Higher magnification showing

merged images of ihDPSCs : n-ihDPSCs (2:1) with variable number of n-ihDPSCs-VD co-localized and attaching onto the tubule forming ihDPSCs-GFP. Scale bars: (A, B) 200 μm ; (C, D, E) 50 μm .