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Heparan sulfate regulates vasculogenesis of dental pulp stem cells

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Introduction

Dental pulp stem cells（DPSCs）can differentiate into vascular endothelial cells and display sprouting ability under endothelial induction[1-3]. During this process, DPSC responses to the extracellular microenvironment and cell-matrix interactions are critical for determining their ultimate cell fate. Heparan sulfate（HS）proteoglycans, which are composed of core protein and covalently binding HS glycosaminoglycan chains, are a major component of extracellular matrix（ECM）in all mammalian tissues[4]. HS could enhance the binding force between growth factors and related receptors, modulating a variety of biological activities, including vascular formation and tumor progression[5]. It was reported that proliferation and angiogenesis of vascular endothelial growth factor（VEGF）-stimulated endothelial cells were dependent on HS, which is also highly involved in the blood vessel permeability[6,7]. Furthermore, recent studies revealed that HS together with basic fibroblast growth factor promoted ischemic heart repair after myocardial infarction, and HS-bound $VEGF_{165}$ enhanced functional recovery from cerebral ischemia[8,9]. One of the earliest studies showed that HS proteoglycans were a critical regulator for hematopoietic lineage differentiation, indicating a possible relationship between HS and the vasculogenic development[10]. In addition, HS has been implicated in the differentiation of embryonic stem cells into endothelial cells in mouse embryoid bodies, which was further supported by an *in vivo* zebrafish embryo model[11]. Despite these exciting results obtained based on embryonic models, the role of HS in endothelial differentiation and neovascularization of mesenchymal stem cells remains unknown yet.

To elucidate the role of HS in the vasculogenesis of DPSCs, we explored the vasculogenic behaviors of DPSCs under HS deficiency, by using surfen molecule, which acts as an HS antagonist[12]; and by silencing *exostosin 1*（*EXT1*）, which exerts a key function in regulating the HS initial biosynthesis[13]. In addition, the role of HS in vascular formation was investigated *in vivo* by transplanting *EXT1*-silenced DPSCs into mice subcutaneously[14]. Here, our research findings to demonstrate that HS plays an important role in the vasculogenesis of DPSCs are summarized.

Inhibition of DPSC vasculogenesis by HS antagonist

A capillary-sprouting assay was carried out to evaluate the sprouting ability and reticular structure formation of DPSCs in three-dimensional

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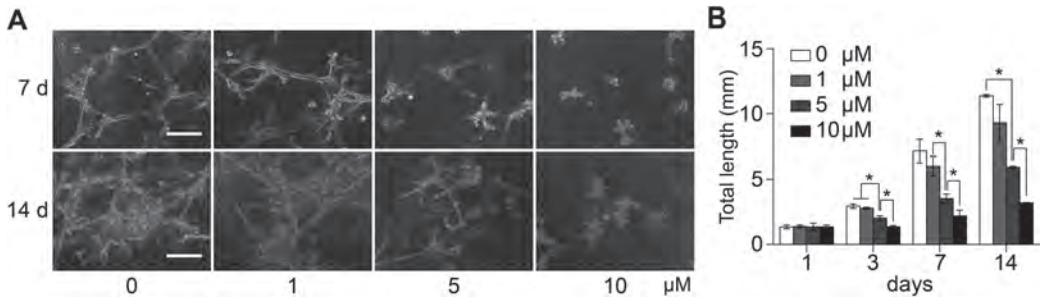

Figure 1. Capillary-sprouting of DPSCs under surfen exposure.（A）Representative bright-field images of DPSCs with sprouting formation induced by endothelial differentiation medium containing 1, 5, or 10 μM surfen for 7 and 14 d. Scale bars: 200 μm.（B）Quantification of total lengths of sprouts at corresponding time points（*P < 0.05; mean ± SD, n=4）. Adapted from Li et al.[14], Copyright 2023, with permission from Sage.

culture. It was revealed that DPSCs possessed great sprouting properties and reticular structure was gradually formed by inducing endothelial differentiation for up to 14 days（Fig. 1A）. However, the sprouting ability of DPSCs was suppressed by the addition of surfen to the media. Total lengths of sprouting branches were significantly decreased in DPSCs cultured with increasing dosages of surfen（Fig. 1B）. These results indicated that reduction of HS production by surfen suppressed endothelial differentiation of DPSCs. It was reported that positively charged surfen could bind to the negatively charged heparin/HS by electronic interactions[12]. Surfen occupied the binding site between heparin/HS and growth factors, subsequently altering various cellular biological activities dependent on heparin/HS, including vasculogenesis[15]（Fig. 2）.

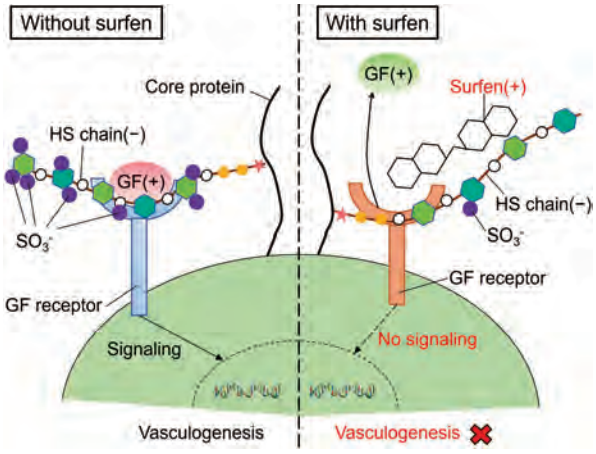

Figure 2. Possible mechanism of the suppressed vasculogenesis of DPSCs by surfen stimulation. Surfen binds to HS by electronic interaction, which interferes the combination between HS and growth factors（GFs）, subsequently inhibiting cellular activities dependent on HS, including vasculogenesis.

Gene expression in *EXT1*-silenced DPSCs

Low-passage DPSCs were transfected with short hairpin RNA specific for *EXT1* or scramble sequence control with green fluorescent protein（GFP）. Following induction with endothelial differentiation medium for 14 days, transfected DPSCs were evaluated for mRNA expression levels by real-time PCR. It was observed that expression of pro-angiogenic markers *vascular endothelial growth factor A*（*VEGFA*）and *C-X-C motif chemokine ligand 1*（*CXCL1*）were significantly lower in *EXT1*-silenced DPSCs（with the fold changes of 0.39 ± 0.07 and 0.07 ± 0.04, respectively）, when compared with scramble sequence-transduced DPSCs（Fig. 3）. Conversely, the expression of stemness-related marker *Nanog* in *EXT1*-silenced DPSCs was significantly higher than that of control cells. These data suggested that HS deficiency inhibited endothelial differentiation potential and, at least in part, maintained the stemness property of DPSCs.

Impact of HS on vascular formation *in vivo*

Dental pulp tissue engineering model using tooth slice/scaffold has been developed as a research

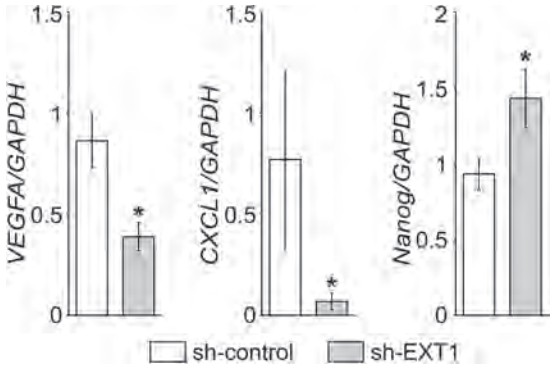

Figure 3. Gene expression of *EXT1*-silenced DPSCs. mRNA expression of *VEGFA*, *CXCL1*, and *Nanog* was evaluated in *EXT1*-silenced (sh-EXT1) and scramble sequence-transduced DPSCs (sh-control) after 14 days of endothelial differentiation (**P* < 0.05; mean ± SD, *n* = 4). Adapted from Li et al.[14], Copyright 2023, with permission from Sage.

method for investigating neovascularization and dentinogenesis of dental stem cells[16-18]. To investigate the role of HS on DPSC vasculogenesis *in vivo*, the biodegradable poly-L-lactic acid scaffolds seeded with *EXT1*-silenced or GFP-transduced control DPSCs were implanted into immunodeficient mice subcutaneously. Five weeks after transplantation, it was shown that the number of blood-containing vessels significantly decreased in scaffolds seeded with *EXT1*-silenced DPSCs compared with specimens loaded with control DPSCs (Fig. 4A). In addition, immunofluorescence staining revealed that control DPSCs formed blood cell-containing vessels positive for human-specific von Willebrand factor (vWF) (Fig. 4B). This result indicated that vascular endothelial cells originating from DPSCs formed nascent blood vessels and anastomosed with the host vasculature. Taken together, these data demonstrate that EXT1 plays an important role in vascular formation of DPSCs, and HS is essential for regulating vasculogenic processes of DPSCs *in vivo*.

Conclusion

These findings demonstrated that HS acts as a key regulator on the vasculogenic process of DPSCs.

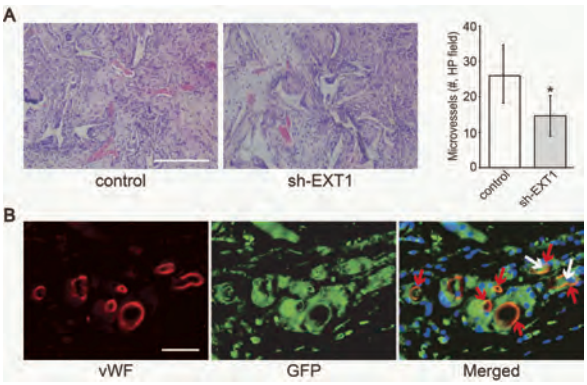

Figure 4. Vasculogenesis of *EXT1*-silenced (sh-EXT1) and GFP-transduced control DPSCs *in vivo*. (A) Histological observation and semiquantitative analysis of blood-containing vessels formed within cell-loaded scaffolds (**P* < 0.05; mean ± SD, *n* = 5). Scale bar: 200 μm. (B) Immunofluorescence staining of microvessels positive for vWF (red) and GFP (green). Cell nuclei are stained in blue. Red arrows indicate vWF-positive vessels originating from GFP-transduced DPSCs. White arrows indicate circulating blood cells inside microvessels formed by DPSCs. Scale bars: 20 μm. Adapted from Li et al.[14], Copyright 2023, with permission from Sage.

Future investigations into the underlying functional signaling mechanisms will provide strong evidence for the potential utility of HS applications in dental pulp regeneration and tissue engineering.

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Conflicts of interest

The authors declare no conflict of interest.

References

1) Sasaki, J.-I., Zhang, Z., Oh, M., Pobocik, A. M., Imazato, S., Shi, S. and Nör, J. E. (2020): VE-cadherin and anastomosis of blood vessels formed by dental stem cells. *J Dent Res*, **99**, 437-445.

2) Katata, C., Sasaki, J.-I., Li, A., Abe, G. L., Nör, J. E., Hayashi, M. and Imazato, S. (2021): Fabrication of vascularized DPSC constructs for efficient pulp regeneration. *J Dent Res*, **100**, 1351–1358.
3) Zhang, Z., Oh, M., Sasaki, J.-I. and Nör, J. E. (2021): Inverse and reciprocal regulation of p53/p21 and Bmi-1 modulates vasculogenic differentiation of dental pulp stem cells. *Cell Death Dis*, **12**, 644.
4) Li, J.-P. and Kusche-Gullberg, M. (2016): Heparan sulfate: biosynthesis, structure, and function. *Int Rev Cell Mol Biol*, **325**, 215–273.
5) Gómez Toledo, A., Sorrentino, J. T., Sandoval, D. R., Malmström, J., Lewis, N. E. and Esko, J. D. (2021): A systems view of the heparan sulfate interactome. *J Histochem Cytochem*, **69**, 105–119.
6) Xu, D., Fuster, M. M., Lawrence, R. and Esko, J. D. (2011): Heparan sulfate regulates VEGF165- and VEGF121-mediated vascular hyperpermeability. *J Biol Chem*, **286**, 737–745.
7) van Wijk, X. M. and van Kuppevelt, T. H. (2014): Heparan sulfate in angiogenesis: a target for therapy. *Angiogenesis*, **17**, 443–462.
8) Shi, J., Fan, C., Zhuang, Y., Sun, J., Hou, X., Chen, B., Xiao, Z., Chen, Y., Zhan, Z., Zhao, Y. and Dai, J. (2019): Heparan sulfate proteoglycan promotes fibroblast growth factor-2 function for ischemic heart repair. *Biomater Sci*, **7**, 5438–5450.
9) Chan, S. J., Esposito, E., Hayakawa, K., Mandaville, E., Smith, R. A. A., Guo, S., Niu, W., Wong, P. T., Cool, S. M., Lo, E. H. and Nurcombe, V. (2020): Vascular endothelial growth factor 165-binding heparan sulfate promotes functional recovery from cerebral ischemia. *Stroke*, **51**, 2844–2853.
10) Gupta, P., Oegema, T. R. Jr., Brazil, J. J., Dudek, A. Z., Slungaard, A. and Verfaillie, C. M. (1998): Structurally specific heparan sulfates support primitive human hematopoiesis by formation of a multimolecular stem cell niche. *Blood*, **92**, 4641–4651.
11) Harfouche, R., Hentschel, D. M., Piecewicz, S., Basu, S., Print, C., Eavarone, D., Kiziltepe, T., Sasisekharan, R. and Sengupta, S. (2009): Glycome and transcriptome regulation of vasculogenesis. *Circulation*, **120**, 1883–1892.
12) Schuksz, M., Fuster, M. M., Brown, J. R., Crawford, B. E., Ditto, D. P., Lawrence, R., Glass, C. A., Wang, L., Tor, Y. and Esko, J. D. (2008): Surfen, a small molecule antagonist of heparan sulfate. *Proc Natl Acad Sci USA*, **105**, 13075–13080.
13) Reijmers, R. M., Groen, R. W., Rozemuller, H., Kuil, A., de Haan-Kramer, A., Csikós, T., Martens, A. C., Spaargaren, M. and Pals, S. T. (2010): Targeting EXT1 reveals a crucial role for heparan sulfate in the growth of multiple myeloma. *Blood*, **115**, 601–604.
14) Li, A., Sasaki, J.-I., Inubushi, T., Abe, G. L., Nör, J. E., Yamashiro, T. and Imazato, S. (2023): Role of heparan sulfate in vasculogenesis of dental pulp stem cells. *J Dent Res*, **102**, 207–216.
15) Weiss, R. J., Gordts, P. L., Le, D., Xu, D., Esko, J. D. and Tor, Y. (2015): Small molecule antagonists of cell-surface heparan sulfate and heparin-protein interactions. *Chem Sci*, **6**, 5984–5993.
16) Gonçalves, S. B., Dong, Z., Bramante, C. M., Holland, G. R., Smith, A. J. and Nör, J. E. (2007): Tooth slice-based models for the study of human dental pulp angiogenesis. *J Endod*, **33**, 811–814.
17) Sakai, V. T., Zhang, Z., Dong, Z., Neiva, K. G., Machado, M. A., Shi, S., Santos, C. F. and Nör, J. E. (2010): SHED differentiate into functional odontoblasts and endothelium. *J Dent Res*, **89**, 791–796.
18) Sakai, V. T., Cordeiro, M. M., Dong, Z., Zhang, Z., Zeitlin, B. D. and Nör, J. E. (2011): Tooth slice/scaffold model of dental pulp tissue engineering. *Adv Dent Res*, **23**, 325–332.