Expression of Down Syndrome Critical Region 1 Represses Vascular Branching in *Xenopus laevis* Larva

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A variety of morphological differences exist in blood vessels. Among them for example, large vessels such as aorta and small vessels such as capillaries differ in their sizes and branching frequencies. Although intense investigations have increased our understanding of molecular mechanisms in blood vessel formation, regulative mechanisms of either size or branching frequency of blood vessels still remains to be uncovered. Understanding these unexplained mechanisms may enable us to induce blood vessels of desired size and branching frequency which ultimately lead to new angiogenic therapies targeted to cure morphologically heterogenic vessels.

In order to isolate genes regulating the size and branching of vessels, models for large vessels with low branching frequencies and small vessels with high branching frequencies were developed using fibrin gel assay. Specifically, these two vascular structures were constructed from human umbilical vein endothelial cells (HUVECs) cultured on Cytodex 3 beads. HUVEC/Cytodex 3 beads were then embedded in fibrin gel and treated with different doses of vascular endothelial growth factor (VEGF) for 7 days to produce the two distinct vascular structures. These experiments revealed that at concentrations of 15.0 and 2.5 ng/mL, VEGF could induce large and small vascular structures respectively. The large vascular structure had low branching frequencies **(Fig. 1A)**, and an average diameter of 6.8 ± 2.2 unit lengths. On the other hand, the small vascular structure had high branching frequencies **(Fig. 1B)**, and an average diameter of 1.6 ± 0.7 unit lengths. The average number of sprouts emanating from Cytodex 3 beads was 2.0 ± 0.9 in the low-branching vessels with large diameter and 12.0 ± 2.0 in the high-branching vessels with small diameter.

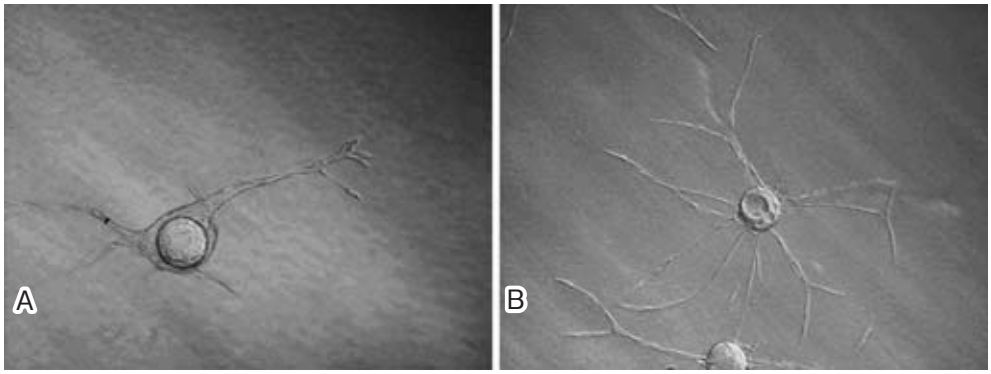

Fig. 1 Different concentrations of VEGF generates different structures of the vasculature. HUVECs cultured on Cytodex3 beads were embedded in fibrin gel. In the presence of 15.0 or 2.5ng/mL VEGF, HUVEC / Cytodex3 beads were cultured for 7 days. Images were captured with an Olympus IX71 microscope with a×4 objective lens.

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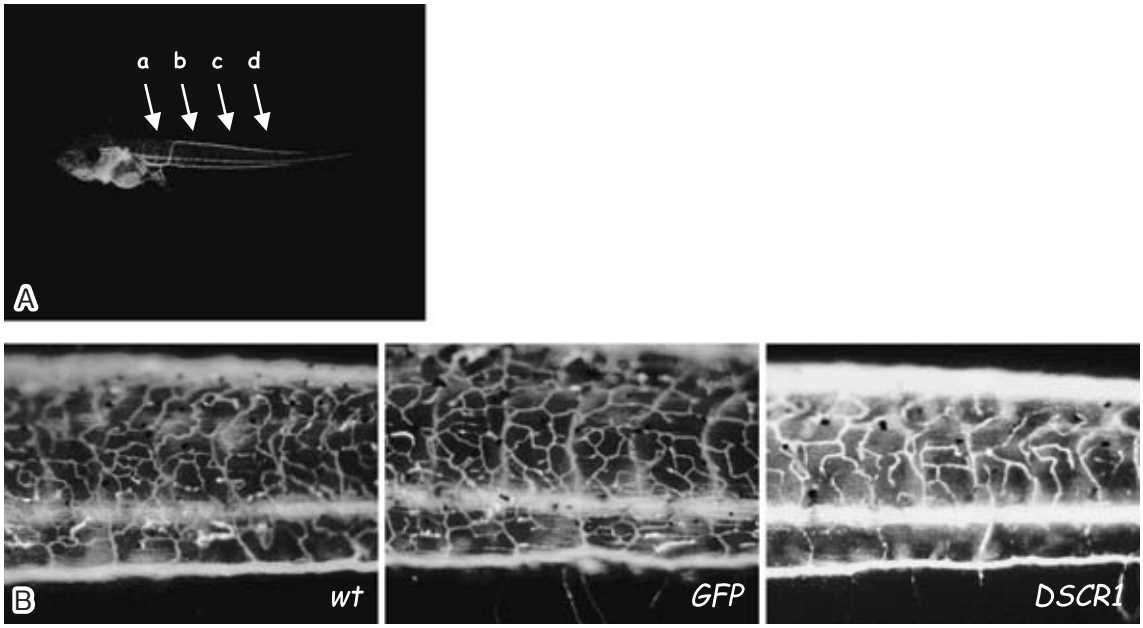

Fig. 2 Microangiography of *X. laevis* larva. **A**: Microangiography of wild type *X. laevis* larva. The morphology of vasculature in area a ～ d was examined. **B**: Microangiography of wild type, GFP cRNA-injected, and DSCR1 cRNA-injected larvae at stage 47. Vessels were stained with Indian dye and photographed with an Olympus SZX 16 stereoscopic microscope.

Using these large and small vascular models, cDNA microarray AceGene Human Oligo Chip (Hitachi Software Engineering Co., Ltd., Yokohama, Japan) was applied to identify the genes, that are responsible for differences in size and branching frequency. Roughly 50 genes were identified as candidate differentially expressed genes. Among them, the Down Syndrome Candidate Region1 (DSCR1) gene was highly expressed in large vessels, and its difference was maintained at a stable rate at 24, 48, and 72 hours following the treatment. As DSCR1 has been recently shown to be up-regulated by VEGF and to function as a growth suppressor in cultured cells, its role in vascular formation was considered to be noteworthy. Furthermore, calcineurin, the only known target for DSCR1, regulates development of heart, cardiac hypertrophy, and axonal outgrowth. These observations all prompted a potential of DSCR1 to regulate the vascular formation process. Therefore, DSCR1 was selected as the first candidate gene whose role in vascular formation was examined.

To clarify the functional role of DSCR1 during vascular formation *in vivo*, *Xenopus laevis* larvae were used because of their transparent body. First, DSCR1 cDNA was isolated from *X. laevis* and inserted into pT7TS plasmids to synthesize DSCR1 cRNA. The synthesized cRNA was then purified, and 1 μg of DSCR1 cRNA was injected into fertilized eggs. At stage 47～48, Indian dye was injected into cardiac ventricle of *X. laevis* larvae with a capillary needle to visualize the entire vasculature. Wild-type and GFP cRNA-injected larva were used as controls. There were no drastic changes in the number and size of aortic arches. However, microvessels sprouting from intersegmental vessels were poorly branched in DSCR1 cRNA-injected larva. Attenuation of bifurcation was observed in a wide area (a～d), as indicated in **Figure 2A**. For the quantification of branching frequency, the number of branching points was counted in a 0.5 mm^2 area. There were 22.3 ± 4.4 branching points in wild type, 19.3 ± 3.9 in GFP-injected larva, and 12.7 ± 2.9 in DSCR1-injected larva. The divergence frequency was markedly reduced by DSCR1 cRNA **(Fig. 2B)**. To confirm that the reduction in branching frequency in *X. laevis* larvae was caused by the over-expression of DSCR1 protein, Western blotting was carried out. DSCR1 was detected at all the time points in DSCR1 cRNA-injected larva (1, 3, and 7 days after the DSCR1 cRNA injection). In contrast, the expression of DSCR1 was not detected in wild type or GFP-injected larvae at any time point. Since microvessels started branching at around day 7 in control larvae, expression of DSCR1 at day 7 was in good accordance with the suppression of vascular bifurcation.

In the present study, a negative functional role of DSCR1 during vascular development was revealed. DSCR1 suppressed branching of microvessels sprouting from intersegmental vessels in *X. laevis* larva. Similarly, large vessel structures, which expressed DSCR1 at high levels, had fewer sprouts in the fibrin gel assay. All of these results indicated DSCR1 as a negative regulator of angiogenic sprouting. However, the precise mechanism by which DSCR1 suppresses vascular sprouting remains to be further clarified. What molecules bind to DSCR1? What molecules execute the suppression of angiogenic sprouting by DSCR1? Furthermore, for clinical application, understanding the mechanisms of vascular sprouting regulated by DSCR1 will provide helpful information in the near future. In addition to the analysis of DSCR1, functional analysis of other candidate genes, identified with by microarray analysis, are currently being evaluated as factors regulating other morphologic features of vasculatures.