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Fox(y) Regulators of VEGF Receptors

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Vascular development is a multi-step process that initially involves vasculogenesis, the process of *de novo* formation of the primitive vasculature from mesodermal precursors, and angiogenesis, that involves sprouting and remodeling of the primitive vasculature ¹. This is followed by vascular fate specification steps that define formation of arterial, venous and lymphatic vasculatures ². This complex series of steps is regulated by a number of growth factors and their receptors. Among them are vascular endothelial growth factor (VEGFs), angiopoietins, and Notch receptors and their ligands, delta-like ligand 4 (Dll4) and jagged-1. Any abnormalities in this sequence of events lead to either an outright failure of vascular development or formation of abnormally patterned vasculature. The later include arterio venous malformations (AVMs), cranial cerebral malformations (CCMs) and aneurysms among others ³.

Among the numerous growth factors that are involved in these phenomena VEGF-A plays a particularly critical role and is involved both in formation of the initial primitive vascular plexus as well as in subsequent sprouting, remodeling and fate specification steps ⁴. VEGF-A signaling input is tightly controlled so much so that deletion of even a single *Vegfa* allele results in embryonic lethality ^{5, 6}. VEGF-A signals via its two tyrosine kinase receptors (VEGFRs), Flt1 (VEGFR1) and Flk1 (VEGFR2), as well as a non-receptor tyrosine kinase neuropilin-1 (Nrp1). Deletion of VEGFR2 is embryonically lethal due to almost complete failure of vasculogenesis ⁷ while endothelial deletion of Nrp1 leads to multiple vascular abnormalities at a later stage of development ⁸. VEGFR1 in this context is seen mainly as a negative regulator of VEGF-A signaling. Its knockout leads to embryonic lethality due to excessive vasculogenesis and angiogenesis ⁹.

Given the critical role that VEGF-A plays in vascular development and the tight control of its effective concentration range, much effort has been expended to understand the mechanisms that regulate its expression. Several transcription factors have been implicated in control of endothelial-specific gene expressions including homeobox protein B5

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(HoxB5)¹⁰, zinc finger transcription factor GATA2¹¹, basic helix-loop-helix transcription factor Tal1¹² as well as the E26 transformation-specific (ETS) family and forkhead (Fox) family.

ETS genes appear particularly critical, as all known endothelial enhancers and promoters contain multiple ETS binding sites. Furthermore, analysis of the human genome shows a strong association between ETS motifs and endothelial genes¹³. Among the 19 different ETS expressed by human endothelial cells, two have a particular importance as a regulator of endothelial cell development: Etv2 and Fli-1. *Etv2* is expressed at the very early stages of murine vascular development, and is extinguished by E10.5. Mice with homozygous disruption of *Etv2* expression die at mid-gestation with a complete lack of endothelial progenitors, blood islands and vessels as shown by the absence of key vascular markers such as Flk1, Pecam, Tie-2¹⁴. Fli-1 is also expressed very early in hematopoietic and endothelial cells and appears to be upstream of most transcriptional factors¹⁵. But unlike *Etv2*, deletion of *Fli-1* in mice leads to embryonic lethality consecutive to hemorrhages and loss of vascular integrity and defects in hematopoietic while endothelial specification is not affected¹⁶.

Another family of transcription factors critical to endothelial gene expression is Fox with FoxF1 and FoxH1 have been established as key regulators of endothelial genes' expression. A global *FoxF1* knockout in mice is lethal by mid-gestation, with the embryos demonstrating a number of defects including, notably, a lack of vasculogenesis¹⁷. In contrast, FoxH1 has an inhibitory function in vascular specification, as its overexpression in zebrafish impairs vascular development and downregulates *flk1* expression¹⁸.

As important as individual transcription factors are, interactions among them are probably critical to fine specification of vascular development. One such well characterized interaction is the role played by the FOX:ETS motif, which consists of an ETS site and non-canonical FOX site¹³. This motif is highly associated with endothelial genes, *Tal1*, *Tie2*, *Flk1*, and *VE-cadherin* among them¹³. Although Etv2 or FoxC2 alone weakly activate genes with this motif in their enhancers or promoters, the activation is much stronger they bind simultaneously.^{13, 19}

Despite these advances, our knowledge of transcriptional regulators of key endothelial genes is incomplete. In this issue of *Circulation research*, Ren and collaborators describe the role of endothelial FoxF1 in the development of embryonic vasculature²⁰. Although the involvement of FoxF1 in vasculogenesis was already suggested by studies in the global *Foxf1* knockout mice¹⁷, the mechanism behind that observation was not yet elucidated.

FoxF1 is expressed in endothelial cells precursors between E8.5 and E12.5, and in pulmonary, yolk sac and placental endothelial cells at E13.5. Endothelial-specific deletion of the transcription factor results in a reduction in vascular branching in the yolk sac and placenta as well as in the lung of the embryo proper and retinas of the newborn mice. Other prominent abnormalities include growth retardation, cardiac ventricular hypoplasia and endocardia cushions defect. Overall, the deletion leads to a progressive lethality between E13.5 and E16.5. While it is hard to evaluate growth retardation and organ hypoplasia in the

presence of yolk sac and placenta vascular defects, it is tempting to speculate that they are associated with reduced vascular tissue density and are consistent with the notion that vascular density determines organ size ²¹.

On the molecular level, the loss of endothelial FoxF1 induced a profound reduction in expression of a number of endothelial-specific genes, including VEGFR1 and R2, PECAM, CD34, ephrin B2, Sox 17 and Tie2 among others. At the same time, there was a marked increase in Dll4 and Ang2 expression. The reduction in VEGFR2 expression and the consequent decrease in VEGF-driven ERK activation as well as a profound decline in ephrin B2 and Sox17 levels are particularly interesting as these have been linked to the arterial fate specification program ^{22–24}. At the same time, a marked increase in Dll4 would be expected to result in increased Notch signaling leading to decreased vascular branching ^{25, 26} while elevated Ang2 levels may result in vascular destabilization ^{27, 28}. Some or all of these mechanisms may account for alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV) observed in patients with *FOXF1* mutations.

ACD/MPV is characterized by a defect in the development of pulmonary vasculature leading to severe respiratory distress and pulmonary hypertension in few hours after birth and in most cases death within the first month of the patient life ²⁹. Mutations or deletion in *FOXF1* gene occur in ~40% of ACD/MPV cases ³⁰. Mice with endothelial FOXF1 deletion had decreased pulmonary capillary density, an ACD/MVP feature, albeit not a full-blown syndrome. It is tempting to speculate that this is likely due to early mortality and that a less severe defect in FoxF1-driven pathway would lead to these findings.

This interesting work raises multiple questions, notably regarding the regulation of FoxF1 expression in endothelial cells. Indeed, FoxF1 is not expressed in all endothelial cells, and its expression level varies in different developmental stages and tissues ³¹. Although sonic hedgehog (Shh) has been described as an inducer of FoxF1 expression ³², its role in this pathway has not been conclusively established. It would be interesting to compare endothelial knockout of Shh receptor *Patched-1* with the phenotype observed in this study. On a molecular level, it is unclear what ETS family member FoxF1 interacts with or whether it truly acts alone. Lastly, as already discussed, *Foxf1* deficient mice exhibit some but not all features of ACD/MPV. A study in somewhat older animals with the knockout induced by a Cre driver with a better activity in the lung vasculature than *Pdgfb-CreER*^{T2} used in the present study, would go a long way clarifying that.

The latter considerations bring us to the vexing issue of endothelial Cre driver choices. In the case of the paper in question the authors carried out most of the work using Tie2-Cre. This was clearly noticed by an eagle-eyed reviewer who asked the authors to repeat the study with another endothelial Cre driver since Tie2-Cre is expressed in a certain population of hematopoietic and mononuclear cells and thus some of the observed effects may be due to the effect of the target gene knockout in these cell populations rather than in the endothelium proper. Indeed, such has likely been the fate of a number of recently published studies including (in the interest of full disclosure) one from our laboratory ³³. Remarkably, as far as we are aware, there is not a single published study that demonstrates a difference in a vascular development phenotype with the use of Tie2 vs. any other Cre. It is entirely

possible, of course, that when such a difference was detected, Tie2-Cre data were simply dropped from the manuscript. Nevertheless, given this state of knowledge, it seems unreasonable to demand new, expensive and time-consuming studies when the original work was done with Tie2 unless there is a sufficiently justified and strong concern that non-endothelial Tie2⁺ cell populations do really matter. It would seem reasonable to simply acknowledge the limitations of Tie2-Cre system. On the other hand, there are now much better endothelial-specific Cre lines, Pdgfb-CreER^{T2} and Cdh5-CreER^{T2} among them³⁴. On yet another hand, Pdgfb-Cre used in the current study is very effective in the retina but much less so in other vascular beds. So choices do matter but so does the common sense.

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