Lab Spotlight: Using Zebrafish to Understand the Mechanism of HHT Pathogenesis

By Beth Roman, PhD

It is well established that heterozygous mutations in activin receptor-like kinase 1 (*ACVRL1*, which encodes the protein, ALK1), endoglin (*ENG*), and *SMAD4* result in hereditary hemorrhagic telangiectasia (HHT) (1-3), which is characterized by a predisposition to the development of direct connections between arteries and veins, or arteriovenous malformations (AVMs). However, how these proteins function within endothelial cells to establish and maintain normal arterial-venous separation remains unknown. My laboratory uses a zebrafish model of HHT to uncover the molecular and cellular errors that lead to AVMs.

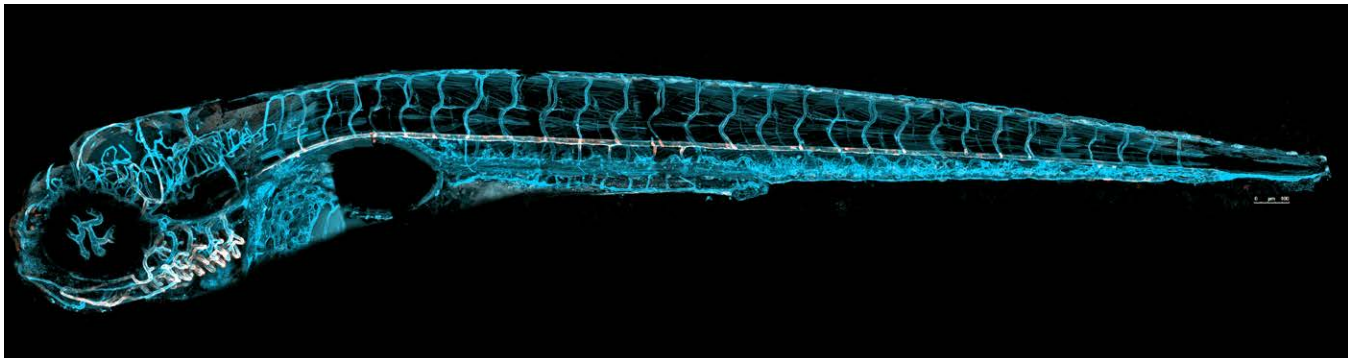
Why Zebrafish?

Zebrafish embryos are an excellent model for studying vertebrate vascular development and HHT. These 2-mm long, optically transparent embryos develop rapidly, initiating heartbeat and circulation through a stereotypically patterned vasculature by ~26 hours post-fertilization. These attributes, combined with the ease of engineering fluorescent transgene expression to mark specific cell types, allow real-time imaging of vessel development at cellular resolution. Importantly, zebrafish vascular development is guided by the same molecular signals as human vascular development, including dependence on ALK1 signaling: zebrafish *acvrl1* homozygous mutants invariably develop embryonic lethal high-flow cranial AVMs at ~40 hours post-fertilization (4). Using this zebrafish model, we have made significant contributions to the understanding of the molecular mechanisms of Alk1 signaling, the natural history of HHT-associated AVMs, and the regulatory mechanisms controlling *acvrl1* gene expression. Our goal is use this knowledge to establish novel access points for development of HHT therapeutics.

New Components of the ALK1 Signaling Pathway

ALK1 is a transforming growth factor beta (TGF-ß) superfamily type I receptor serine/threonine kinase that is predominantly expressed on the plasma membrane of arterial endothelial cells. Upon extracellular ligand binding to a molecular complex containing ALK1, a TGF-ß family type II receptor, and endoglin, the type II receptor phosphorylates ALK1, and ALK1 then phosphorylates intracellular proteins SMAD1, SMAD5, or SMAD8. Phosphorylated SMADS 1/5/8 bind to SMAD4, translocate to the nucleus, bind specific regulatory sequences within genomic DNA, and alter expression of associated genes. Using zebrafish genetics, we demonstrated that the critical Alk1 ligand during embryonic development is bone morphogenetic protein 10 (Bmp10): knockdown of *bmp10* expression generates embryonic lethal AVMs identical to those that develop in *acvrl1* mutants (5). BMP10 is produced exclusively by the vertebrate heart and is detected in serum (5-8), supporting the idea that ALK1 activation requires a circulating endocrine ligand.

The genes directly regulated downstream of BMP10/ALK1/phospho-SMAD are currently unknown. In zebrafish *acvrl1* mutant arterial endothelial cells, we see loss of expression of the mRNA encoding the vasoconstrictor, endothelin-1, and increased expression of the mRNAs encoding the promigratory chemokine receptor, Cxcr4, and the Notch ligand, Dll4 (5, 9, 10). Although normalizing expression of these genes individually does not prevent AVM development (9, 10), it is possible that these changes in gene expression reflect an enhanced migratory and vasodilatory state that may be targeted for therapy.



Two-photon image of transgenic 5-day old zebrafish with all endothelial cells blue and alk1*-positive endothelial cells gray/orange. Lateral view, head is to the left.*

Cellular Mechanisms of AVM Development

Although the genes responsible for 80 to 95 percent of HHT were identified 20 years ago (1, 2), we do not understand how ALK1 signaling influences endothelial cell behavior or why deficits in ALK1 signaling lead to AVMs. Our analysis of AVM development in zebrafish *acvrl1* mutants revealed that AVMs arise via a two-step process (9). In Step one, endothelial cell number and caliber increase in *acvrl1*-positive cranial arteries just upstream of the prospective shunt. This event is the direct result of *acvrl1* loss-of-function and is phenocopied by loss of blood flow. In Step two, normally transient vessel segments are maintained between enlarged cranial arteries and drainage veins. These segments progress to high-flow, embryonic lethal AVMs. This second step in AVM development is not genetically determined: *acvrl1* mutants do not retain these vessel segments in the absence of blood flow.

Our two-step model of AVM development suggests that blood flow plays two distinct and opposing roles in AVM development. In wild type embryos, blood flow prevents AVMs by inducing both *acvrl1* expression (see below) and ALK1 activity (via circulating Bmp10) to limit vessel caliber. In *acvrl1* mutants, blood flow precipitates AVMs downstream of enlarged arteries (5, 9). Current studies are focused on defining how blood flow affects endothelial cell migration within the blood vessel wall and determining whether mechanical force and/or circulating factors mediate effects of blood flow on these two distinct steps of AVM development.

Control of ACVRL1 Gene Expression

Because HHT is an autosomal dominant disease caused by haploinsufficiency, enhancing expression of the wild type copy of the disease gene may have therapeutic benefit. We discovered that in zebrafish, arterial endothelial cell *acvrl1* expression requires blood flow (9), and unpublished work demonstrates exquisite sensitivity to both blood flow and intact Bmp10/Alk1 signaling at the level of transcription. These data suggest that Bmp10/Alk1 activity is required to maintain *acvrl1* expression via positive feedback, but we cannot rule out roles for mechanical force or circulating factors in addition to Bmp10 in control of *acvrl1* expression.

Toward Development of Targeted HHT Therapies

Current drug therapies available to HHT patients inhibit angiogenesis or enhance clotting, but none have proven effective in reducing bleeding over the long term or in reversing HHT pathogenesis (11). As such, there is a pressing need to develop targeted therapeutics for HHT patients. Our research suggests several novel approaches. Based on the recent success of BMP9 administration in rescuing pathology in a haploinsufficient mouse model of pulmonary arterial hypertension (12), we propose that BMP10 ligand therapy may enhance signaling through wild type ALK1/ENG and thereby overcome haploinsufficiency in HHT. Based on the changes in cell behaviors and gene expression associated with AVM development in our zebrafish model, we suggest that dampening arterial endothelial cell migration, limiting vasodilation, or manipulating mechanotransduction pathways may have therapeutic benefits in HHT. Finally, based on our finding that *acvrl1* expression is regulated by blood flow in zebrafish, we postulate that enhancing this as yet undefined molecular regulatory pathway may increase ALK1 signaling beyond a threshold required to maintain normal vascular connections.

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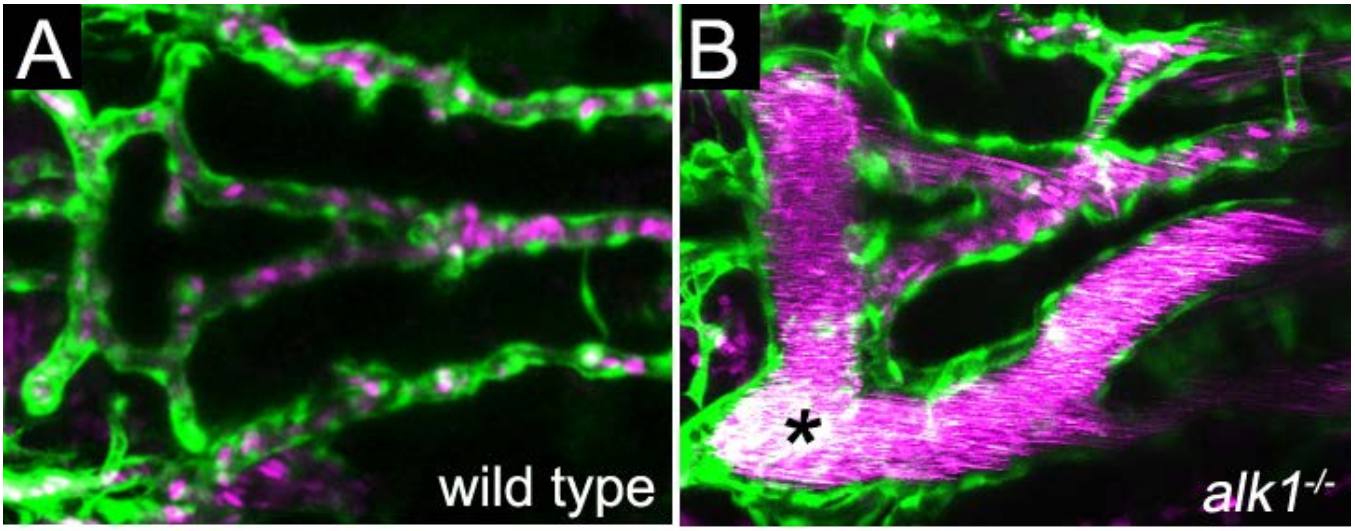

Dorsal cranial vasculature in 2-day old wild type (A) and alk1 *mutant (B) zebrafish. Endothelial cells green, red blood cells magenta. Asterisk marks AVM in* alk1 *mutant.*