# **In Vivo Cell Biology Approach to Defining the Natural History of HHT-Associated AVMs** A Final Report

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*HHT Foundation dollars at work…. This research grant was provided by a generous donation from an anonymous donor. Dr. Roman's work will lead to important HHT breakthroughs.*

It is generally believed that by the time of diagnosis, arteriovenous malformations (AMMs) have been developing over relatively long periods of time. But, what if we could follow development of these lesions in a model organism that develops HHT-associated AVMs at a predictable time in a predictable location and allows unfettered access to the cellular behaviors that lead to these malformations? What could we learn from this type of study? If we understand the cell behaviors that lead to these lesions, we can make educated hypotheses regarding the molecular events that underlie these behaviors. This will ultimately allow scientists to develop therapeutic strategies to correct these defects in the absence of sufficient ALK1 or Endoglin function.

The model that we use to study the role of ALK1 signaling in vascular development and AVM prevention is the zebrafish embryo. Zebrafish embryos are very small (about 2-3 mm in length), externally fertilized, optically clear, and develop very fast, so they are accessible for and amenable to continuous observation. And, with the advent of transgenic tools for this model system, we have unparalleled access to endothelial cell behaviors in the live embryo. The endothelial cells that line the blood vessels in our fish sport a variety of fluorescent proteins that allow us to identify sub-cellular structures (for example, the endothelial cell nucleus or membrane) and easily recognize particular behaviors (for example, cell division). These fluorescent proteins can be excited very deep within the zebrafish embryo using an infrared laser, and emitted light can be captured using a specialized microscope, allowing us to visualize events that would otherwise be obscured. ***By imaging endothelial cells in these embryos in three dimensions over time, we can reconstruct the events that give rise to AVMs.***

Over the past year, under the auspices of the HHT Foundation International, we have used zebrafish embryos carrying a variety of fluorescent transgenes and harboring a mutation in *alk1* (HHT2) to track AVM development in real time. These fish consistently develop AVMs around 36-to-40 hours after fertilization in vessels that lie just beneath the developing brain. ***Our results support our previously published work suggesting that AVM development involves two distinct and separable steps***, and provide new insight into the aberrant cell behaviors that underlie each of these steps. **In step one**, loss of Alk1 function leads directly to increased endothelial cell migratory behavior within newly-forming arteries: more cells enter these growing vessels from a pool of endothelial progenitor cells, and some cells zoom past their neighbors and well beyond the "boundaries" that normal endothelial cells respect (Figure 1). Ultimately, this behavior results in increased endothelial cell number and increased caliber of these vessels, which can therefore carry more blood flow. **In step two**, downstream vessels that do not normally express *alk1* increase their caliber, and normally transient connections between arteries and veins are maintained and enlarged, thereby forming high-flow AVMs. Intriguingly, these secondary events only happen in the presence of blood flow. Our preliminary fluid dynamic analysis of downstream vessels involved in Step 2

demonstrates that the biomechanical forces within these vessels are initially very high (Figure 2, left panel): this makes sense, because blood flowing from enlarged arteries into smaller vessels will result in an increased biomechanical load within the smaller vessels. Because endothelial cells are well-equipped to react to these forces, it is reasonable to hypothesize that Step 2 in AVM development is a normal response to changes in hemodynamic environment. In fact, our analysis supports this idea, demonstrating that the final vessel conformation in *alk1* mutants serves to lower hemodynamic forces to normal levels (Figure 2, right panel). Interestingly, our time lapse imaging demonstrates that the mechanism by which these downstream vessels increase their caliber and form AVMs does not involve increased migration but relies primarily on endothelial cell hypertrophy: the cells simply get bigger, thereby increasing vessel caliber and thus decreasing mechanical stress. Therefore, although the end result is vessel enlargement, very different cellular behaviors and thus molecular pathways underlie the two steps of HHT-associated AVM development.

**So where do we go from here?** We plan to use the imaging data collected over the past year as preliminary data to support an application for funding from the National Institutes of Health. The proposed five-year project will focus on identifying the changes in gene expression and cytoskeletal behaviors that cause cessation of migration downstream of Alk1; determining the molecular mechanisms involved in increasing endothelial cell size in response to enhanced hemodynamic forces; and, along with bioengineering colleagues, developing biomechanics-based growth models that predict arterial enlargement and AVM development in zebrafish *alk1* mutants. **If we can mechanistically define these two steps in AVM development, we identify logical targets for HHT therapeutics.**

*The HHT Foundation provided the funding necessary to conduct this initial research study. Dr. Roman will now leverage the results of her work with the National Institutes of Health (NIH) to gain funding for a comprehensive study that will target HHT therapeutics. We are grateful for Dr. Roman's dedication to HHT research and the financial support of our members that make this research possible.*

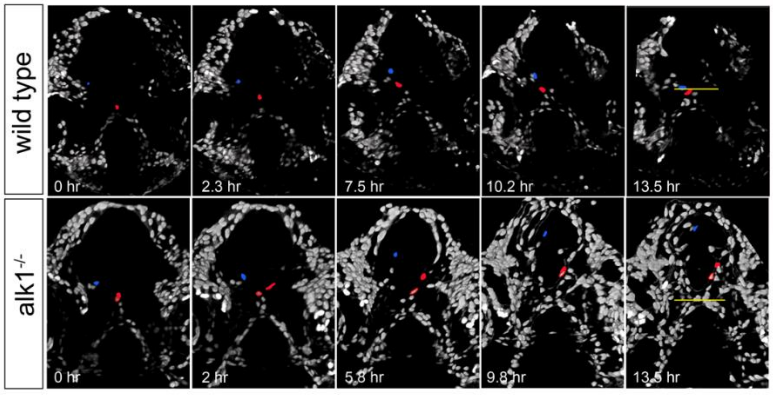


**Figure 1: Enhanced migration of ECs in *alk1* mutant zebrafish embryos.** Stills from time lapse analysis of vessel development in wild type (top) and *alk1* mutant (bottom) embryos, beginning around 27 hours after fertilization. The red-colored cells remain relatively stationary during this period of development in wild type embryos and do not cross into downstream vessels (boundary denoted by yellow line), whereas they migrate much further in *alk1* mutants and fail to respect this boundary.

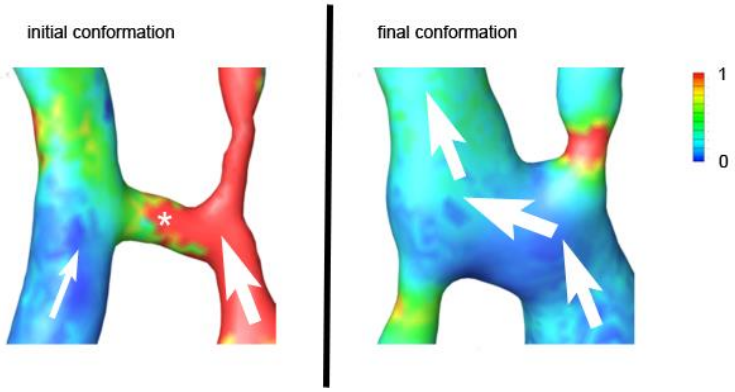


**Figure 2: Arteriovenous shunting reduces biomechanical force in *alk1* mutants.** Initially (left panel), shear stress levels are high (red) in the *alk1* mutants. The final vessel conformation (right panel) decreases shear stress (blue) but results in dramatic vessel enlargement and arteriovenous shunting through what is normally a transient drainage vessel (asterisk in left panel). In each panel, artery is at right, vein is at left. Arrows depict direction and relative magnitude of flow. Analysis performed in collaboration with Dr. Kerem Pekkan and Prahlad Menon, Carnegie Mellon University.