**Specific Aims Draft 2**

**Mario Bertogliat**

**Introduction**

Aortic valve disease 1 (AoVD1) is a group of congenital heart malformations that affect the valve through which blood is pumped from the heart to the rest of the body. A prominent phenotype of AoVD1 is stenosis1, in which calcific (Ca2+) mineral deposition causes valve hardening and impairment in function leading to heart murmur, dizziness, and shortness of breath, and chest pain2. The main factor involved in AoVD1 is NOTCH1 which encodes a transmembrane receptor responsible for proper heart valve formation3. Upon ligand binding, NOTCH1 is cleaved and its intracellular domain translocates to the nucleus initiating transcriptional activation of enhancers key to aortic valve smooth muscle cell (SMC) differentiation3. The NOTCH1 gene contains many highly conserved N-terminal Epidermal growth factor (EGF)-like calcium binding motifs that are thought to play a major role in ligand binding in this developmental process4. It has long been established that mutations in NOTCH1 shift the transcriptional profile from proper SMC differentiation to a more osteoblastic program, resulting in the AoVD1 stenotic phenotype5,6; however, it is unknown what role the EGF-like calcium binding motifs play in this shift.

My **objective** is to determine how mutations within the EGF-like calcium binding domain contribute to aortic valve stenosis. Zebrafish (*Danio rerio*) will be used as a model organism in this study as they have an easily manipulated genome7 and possess a similar cardiovascular system in which improper heart valve formation can be easily observed through translucent skin8,9. I **hypothesize** that mutations within conserved EGF-like calcium binding motifs will result in impaired NOTCH1 signaling and resultant aortic valve calcification. My **long-term goal** is to categorize aberrant interactions due to these mutations and how they contribute to the improper osteogenesis seen in aortic valve stenosis.

**Aim 1: Determine and mutate conserved EGF-like calcium binding domains between humans and zebrafish.**

**Rationale:** Mutations within highly conserved regions, like that of the EGF-like calcium binding motifs in NOTCH1, have been observed in clinical settings regarding AoVD1. These mutations may interfere with ligand binding and the initiation of intracellular signaling required for proper valvular SMC differentiation. In addition, interference of intracellular signaling may shift differentiation towards a more calcific phenotype.

**Approach:** Through NCBI, find the most closely related NOTCH1 homolog in zebrafish and then confirm through ENSEMBL. Obtain the FASTA sequence of both humans and zebrafish, align in MEGA, and identify conserved EGF-like calcium binding motifs. Create a CRIPSR-cas9 system to target each conserved EGF-like calcium binding motif in zebrafish and screen for aortic valve malformations.

**Hypothesis:** Mutation of conserved EGF-like calcium binding regions will lead to aortic valve stenosis.

**Aim 2: Determine gene expression changes induced by EGF-like calcium binding domain mutations.**

**Rationale:** NOTCH1 plays an important role in the transcriptional regulation of factors required for SMC differentiation and its impairment shifts this regulation toward an osteoblastic profile. Mutations within the EGF-like calcium binding domain may interfere with ligand binding and resultant intracellular signaling leading to a downregulation of SMC factors, and an upregulation of osteoblastic factors.

**Approach:** Obtain aortic valve tissues from each line produced in Aim 1 that displayed stenotic valve formation. Extract RNA, create cDNA, perform RNA-seq, and determine differentially expressed genes between WT NOTCH1 and mutant NOTCH1 zebrafish.

**Hypothesis:** Mutation of conserved EGF-like calcium binding regions will lead to a shift from SMC differentiation to osteoblast differentiation.

**Aim 3: Determine aberrant interactions caused by EGF-like calcium binding domain mutations.**

**Rationale:** NOTCH1 interacts with several ligands, is cleaved by multiple proteases, and translocates to the nucleus to influence important differentiation factors. Mutations within the EGF-like calcium binding domain is likely to interfere with or impede interactions key to this tightly regulated process and lead to aortic valve calcification.

**Approach:** Obtain aortic valve tissues from each line produced in Aim 1 that displayed stenotic valve formation. Using the BioID platform label cellular extracts with the addition of biotin, lyse and purify proteins in order to separate biotinylated proteins, digest with trypsin, and perform mass spectrometry to determine interacting proteins. Compare differentially expressed interactions between WT NOTCH1 and mutant NOTCH1 zebrafish.

**Hypothesis:** Mutation of conserved EGF-like calcium binding regions will impair proper interactions in NOTCH1 signaling and SMC differentiation leading to interactions seen in osteoblast formation.

**References**

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