Aortic valve disease 1 (AoVD1) is a congenital heart malformation that affects how blood is pumped from the heart to the rest of the body. A main phenotype of this disease is stenosis, in which calcium deposition leads to valve hardening and impairment1. This calcification causes blood flow deterrence resulting in heart murmur, dizziness, shortness of breath, and chest pain2. The main gene associated with AoVD1 is NOTCH1 which encodes a transmembrane receptor necessary for heart valve formation3. The NOTCH1 receptor is important for transcriptional activation of genetic enhancers critical to aortic valve smooth muscle cell (SMC) differentiation3. Mutations in NOTCH1 alter the transcriptional profile of SMC precursors during differentiation leading to an osteoblastic phenotype (calcium-utilizing bone cells), thereby causing heart valve hardening4,5. NOTCH1 has highly conserved Epidermal Growth Factor (EGF)-like calcium binding domains in the N-terminus that play a major role in extracellular ligand binding during SMC differentiation6. It is known that loss of overall NOTCH1 signaling leads to the transcriptional shift toward osteoblast formation; however, it is unknown how and which EGF-like calcium binding domains contribute.

My **objective** is to determine how and which NOTCH1 EGF-like calcium binding domains contribute to aortic valve calcification. Zebrafish (*Danio rerio*) will be used as a model organism in this study as they have an easily manipulated genome7 and a cardiovascular system in which improper heart valve formation can be easily observed through translucent skin8,9. I **hypothesize** that mutations within EGF-like calcium binding domains conserved between species with complex cardiovascular systems (hearts) will result in impaired NOTCH1 signaling and resultant aortic valve calcification. My **long-term goal** is to determine the mechanism by which mutations in these specific NOTCH1 EGF-like calcium binding domains lead to aortic valve calcification.

**Aim 1: Determine which EGF-like calcium binding domains in NOTCH1 are necessary for aortic valve calcification.**

**Rationale:** EGF-like calcium binding domains are necessary for proper SMC differentiation in the aortic valve, yet it is unclear which are critical for this function. Mutations in domains conserved between organisms with complex cardiovascular systems are likely to lead to the osteoblastic transcriptional shift outlined above and subsequent aortic valve calcification.

**Approach:** Through NCBI, find the most closely related NOTCH1 homolog in model organisms with and without hearts. Confirm through ENSEMBL. Obtain the FASTA sequence of each organism, align in MEGA, and identify three EGF-like calcium binding domains conserved in heart-bearing animals but not simpler organisms. Create a CRIPSR-cas9 system to mutate a conserved amino acid within each of the three selected EGF-like calcium binding domains in zebrafish and screen for calcification (visually and through osteoblast staining). A line possessing the clinically observed R1350L NOTCH1 mutation will be used as a positive control and comparison.

**Hypothesis:** Mutation of EGF-like calcium binding domains conserved between model organism with a heart will lead to aortic valve calcification.

**Aim 2: Identify which EGF-like calcium binding domains are important for maintaining gene expression related to smooth muscle differentiation.**

**Rationale:** When mutated, EGF-like calcium binding domains necessary for proper SMC differentiation in the aortic valve will produce zebrafish with aortic calcification. RNA-seq will illustrate differential gene expression changes in regard to smooth muscle formation and osteogenesis.

**Approach:** Obtain aortic valve tissues from each line produced in Aim 1, the R1350L line, and WT NOTCH1 zebrafish. Extract RNA, create cDNA, perform RNA-seq, and determine differential gene expression between WT NOTCH1 and mutant NOTCH1 zebrafish.

**Hypothesis:** Mutation of conserved EGF-like calcium binding regions will lead to a shift from gene expression highly enriched in smooth muscle formation to gene expression highly enriched in osteogenesis.

**Aim 3: Identify which EGF-like calcium binding domain is important for maintaining protein expression responsible for smooth muscle differentiation.**

**Rationale:** When mutated, EGF-like calcium binding domains necessary for proper SMC differentiation in the aortic valve will produce zebrafish with aortic calcification. iTRAQ will illustrate differential protein expression changes in regard to smooth muscle formation and osteogenesis.

**Approach:** Obtain aortic valve tissues from each line produced in Aim 1, the R1350 line, and WT NOTCH1 zebrafish. Trypsanize cellular extracts, label each line with isobaric tags specific to a smooth muscle protein and an osteoblast protein, combine, and perform liquid chromatography/mass spectrometry to determine line-specific differential protein expression.

**Hypothesis:** Mutation of conserved EGF-like calcium binding regions will lead to a shift from protein expression highly enriched in smooth muscle formation to protein expression highly enriched in osteogenesis.

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