Progress Report Year 2 MPS/ISMRD Grant to Heather Flanagan-Steet – Investigating the Role of Cathepsin Proteases in MLII Cardiac Pathology

In the last year we have made progress (detailed below) on several aspects of the aims outlined in this grant. In addition to progress on the proposed studies, we have made two important advances that will have a major impact on our ongoing pursuit of molecular mediators of MLII cardiac pathology. These include 1) the generation of five unique TALEN-mediated MLII mutant lines and 2) the isolation of a separate MLII mutant line from a sperm TILLING screen. During this year we not only established pure – outcrossed stable lines, but we also confirmed the genetic and biochemical characteristics of these lines. These animals are essential to confirm all of our morpholino-based findings, for analyses of later stage aspects of disease, and for small molecule screens. Importantly, several of the lines exhibit the same craniofacial and cardiac anomalies noted in morpholino-generated animals. When published we will credit MPS/ISMRD for contributing to the establishment of these vital tools.

<u>Aim 1</u>: Assess individual contribution of cathepsin K and L toward cardiac defects in ML zebrafish.

1) *Cathepsin expression and generation of transgenic animals.* Immunohistochemical analyses of cathepsin K and L demonstrate that both proteases are expressed in multiple heart tissues, including the myocardium, endocardium, and epicardium. These analyses have guided the choice of tissue specific promoters for transgenic constructs that will be used to generate zebrafish expressing fluorophor tagged WT and mutant versions of Cts K and L. Toward this goal, we have now successfully shown that following mRNA injection, the CtsK-mCherry fusion protein is both expressed and active in developing embryos. Establishing these parameters was critical before proceeding with transgenesis. These animals, as well as multiple ctsk variants/mutants, particularly those that mimick MLII, are in production.

2) *Inhibition of cathespin K in MLII.* To assess CtsK's contribution toward ML cardiac pathology, its expression or activity was inhibited in the MLII background. CtsK expression was genetically inhibited using one of two gene specific morpholinos and its activity reduced pharmacologically using the FDA-approved cathepsin K inhibitor Odanacatib. All aspects of cardiac morphology and function, including formation of the AV valve, unidirectional blood flow, and heart contraction were substantially improved by these treatments.

<u>Aim 2</u> – Determine whether increased cathepsin activity impacts TGFß signaling in MLII hearts.

1) Are there differences in either TGFß or BMP signaling in WT and MLII hearts? In combination with TGFß and BMP-reporter transgenic animals, immunohistochemical analyses of pSmad levels was used to assess differences in signaling between WT and MLII hearts. These analyses revealed important changes in these pathways that were restored by inhibition of cathepsin K.

Aim 3 – Determine which patient mutations are pathogenic for cardiac dysfunction.

To identify which patient mutations are associated with altered cardiac morphology and function, we have introduced mRNA bearing specific lesions in the morphant background and compared their ability to rescue heart phenotypes with ML animals co-injected with WT mRNA. We have analyzed three different sets of mutations including several within the DMAP domain (including K732N), three mutations within Notch domain 1 (C442Y, C461G, and C468S), and one mutation in Notch domain 2. Thus far analyses have been limited to gross morphology. Unlike WT *gnptab* mRNA, which reduces cardiac edema, restores normal cardiac morphology, and increases blood flow in ≥85% of the animals analyzed, mRNA bearing the K732N mutation did not significantly restore any of the tested parameters. Therefore, as previously noted in the cartilage, the DMAP domain is essential for normal phosphotransferase function – and mutations within it are highly likely to be pathogenic in both the cartilage/bone and the

heart. In contrast, preliminary analyses of the three Notch domain 1 mutations suggest that the C442Y lesion, which has been associated with MLIII, is less pathogenic in the heart than the ML-intermediate lesion C468S. This is supported by the fact that mRNA"rescue" experiments show improved cardiac morphology and reduced edema in 85% of the MLII animals injected with the C442Y-containing mRNA versus only 40% recovery with the mRNA carrying the C468S lesion. mRNAs bearing either the C461G (Notch 1/MLIII) or C505Y (Notch2/MLIII) rescued gross cardiac pathology ~60% of the time, suggesting that in addition to differences in pathogenic severity between MLII and MLIII, different mutations within a single ML class may have different degrees of pathogenicity. More in depth experiments are currently underway. Collectively these data support the idea that genotype-phenotype correlations can be established in this system, and as such may eventually combine with patient data to predict the severity and course of mutation specific tissue pathologies.

Summary of progress to date:

Although complete understanding of the complicated nature of MLII cardiac pathology still demands more work, we are highly encouraged by the findings to date. First, the fact that both cathepsin K knockdown and Ctsk inhibitors improved MLII heart development is very promising. These data not only support a central role for cathepsin(s) in primary pathogenesis but point to FDA-approved drugs for future consideration. Second, the fact that both cathepsin K and TGFß signaling are involved in cardiac pathology suggests common mechanisms underlie ML skeletal and heart dysfunction. If true, this would not only be an important advance for future scientific investigation, but also mean that an individual modality may therapeutically improve both systems. Third, the tools generated thus far and those underway will serve as novel platforms to both further investigate these and other emerging mechanisms and to rapidly screen the efficacy of potential drugs.