

Progress Report Year 1 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 also recently made two additional advances that will have a major impact on our ongoing pursuit of molecular mediators of MLII cardiac pathology. These include 1) the generation of a TALEN-mediated MLII mutant line and 2) the isolation of a separate MLII mutant line from a sperm TILLING screen. These two unique zebrafish lines are essential to confirm all of our morpholino-based findings, for analyses of later stage aspects of disease, and for small molecule screens. When published we will credit MPS/ISMRD for contributing to the establishment of these vital tools.

Aim 1: 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 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. Therefore the final DNA constructs for transgenesis are currently in production at Gene Art. Initial injections for the generation of transgenic lines should be completed by early next year.

2) *Inhibition of cathepsin 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. Preliminary analyses of multiple aspects of cardiac morphology and function, including formation of the AV valve and unidirectional blood flow, suggest both treatments substantially improve MLII cardiac development. Additional experiments to confirm and extend these findings are currently underway.

Aim 2 – Determine whether increased cathepsin activity impacts TGF β signaling in MLII hearts.

1) *Are there differences in either TGF β or BMP signaling in WT and MLII hearts?* In combination with TGF β and BMP-reporter transgenic animals, immunohistochemical analyses of pSmad levels is being used to assess differences in signaling between WT and MLII hearts. In parallel studies pharmacological inhibition of TGF β /BMP receptor activation is being used to address the pathogenic significance of noted differences in pathway activation. These studies are currently underway. Once these analyses are completed we will address whether genetic or pharmacological inhibition of cathepsin K also affects pSmad levels and reporter transgene activation in MLII hearts.

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 $\geq 85\%$ 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 all studies are still in progress and much work remains, we are highly encouraged by the findings to date. First, the fact that both cathepsin K knockdown and treatment with Odanacatib 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 like Odanacatib for future consideration. Although these findings are preliminary and need to be independently confirmed, they are certainly encouraging. Second, the fact that both cathepsin K and TGF β signaling may be involved in cardiac pathology suggests common mechanisms may 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.

Comment on year 1 budget residual funds:

As clear from the financial report of year 1’s expenditures there was a \$2300 carry-over. I wanted to qualify that this money has essentially been spent, but because the “reagents” are in production they will not post on this year’s financial report. \$800 of the remaining balance will be paid to the Zebrafish TILLING Project Consortium at The Fred Hutchinson Cancer Research Center. This money covers part of the cost for them to screen for the *gnptab* mutant, which is now identified and being raised for shipment to us. The remaining \$1500 is also already allocated to pay Gene Art for synthesizing one of the large DNA constructs necessary to generate the cathepsin transgenic animals.