**Introduction**

Duchenne Muscular Dystrophy (DMD) • Lethal X-linked recessive disease associated with progressive muscle weakness. • Mutations in the dystrophin gene, which is a component of the dystrophin glycoprotein complex (DGC) present at the plasma membrane of skeletal and cardiac muscle cells. • DGC links the internal cytoskeleton to the extracellular matrix, stabilizing the plasma membrane. • Lack of dystrophin destabilizes the DGC, which leads to calcium (Ca²⁺) influx and Ca²⁺ overload. This hinders the ability of muscle cells to contract and relax normally.

Therapies for Dystrophin-associated Cardiomyopathy • No existing cure for DMD. • ACE inhibitors and β-blockers improve left ventricular function and normalize heart size. • Poloxamer 188 inserts into and blocks Ca²⁺ influx from membrane micro-tears. Improved ventricular geometry in mdx mice and block the development of acute cardiac failure during a dobutamine mediated stress protocol.

Significance Incidence of cardiomyopathy is nearly 100% for DMD patients and it has become a major cause of mortality. Now that an increasing number of DMD patients are reaching the later stages of the disease associated with the onset of late-onset cardiomyopathy in DMD patients.

Goals Long-term goal is to create a calcium buffering system at the plasma membrane of cardiac muscle cells lacking dystrophin. Conceptually, upon calcium influx, low-affinity high-capacity calcium binding proteins at the plasma membrane will have the capacity to transiently trap calcium ions in order to reduce calcium overload in cardiac myocytes. Here we show the results of a study in which three membrane localization sequences, Myr, Gag, and PLCbeta1b, were compared for plasma membrane localization efficiency in HEK 293 cells, neonatal rat ventricular myocytes (NRVM), and adult rat cardiac myocytes.

**Working Model**

- Ca²⁺ binding proteins localized to the inner leaflet of the cell membrane using a membrane targeting sequence.
- When extracellular Ca²⁺ leaks into the cell through microtears in the plasma membrane, the Ca²⁺ binding proteins will transiently trap Ca²⁺ ions at the membrane.
- Potential to reduce Ca²⁺ overload in cardiac myocytes and consequently reduce the symptoms associated with late-onset cardiomyopathy in DMD patients.

**Methods**

Cloning of plasmid vectors (A) Design double-stranded DNA sequence (gBlocks™ Gene Fragments, IDT, Coralville, IA) containing each of the three sequences Myr, Gag, or PLCbeta1b. (B) Cut the pDC316-GFP vector and the gBlocks with the necessary restriction enzymes and ligate to create the recombinant vectors.

HEK 293 cells (A) Lipofectamine transfection (B) Plasma membrane staining with Alexa Fluor® 594 and fixation with 4% paraformaldehyde (C) Image with confocal microscopy

Neonatal Rat Ventricular Myocytes (NRVM) (A) NRVM isolation (B) Lipofectamine transfection (C) Fix, stain, and image with confocal microscopy

Adult Cardiac Myocytes (CM) (A) Obtain adenosoviruses (Ad) containing positive control GFP, Myr, and PLCbeta1b (B) Viral gene transfer (C) Fix, stain, and image with confocal microscopy.

**Results**

Figure 1 Confocal imaging of HEK 293 cells treated with recombinant vectors. GFP is depicted in green and plasma membrane stain WGA Alexa Fluor® 594 is depicted in red. 40x magnification. In (1a), GFP is distributed evenly in the positive control. In (1b) and (1c), Myr and Gag induced GFP localization to the plasma membrane, but not exclusively to the plasma membrane. In (1d), PLCbeta1b did not induce plasma membrane localization.

Figure 2 Percent membrane localization of Gag, Myr, and PLCbeta1b in HEK 293 cells. After vector transfection into HEK 293 cells, the no sequence positive control (n=24 cells) showed 8% membrane localization, Gag (n=51 cells) had 90% localization, Myr (n=44 cells) had 82% localization and PLCbeta1b (n=43 cells) had 4% localization. The asterisk indicates that Gag demonstrated the highest percent localization, which was approximately 10% greater than Myr.

**Conclusion**

Through this study, we found that the Gag sequence induces the best membrane localization of GFP in HEK 293 cells. However, the Gag sequence was ineffective at membrane localization of GFP in neonatal rat cardiac myocytes and adult rat cardiac myocytes.

Next Steps: • Do a literature search and complete optimization experiments to achieve successful membrane localization of Gag. • Test the localization of calcium binding proteins (crt and csq) bound to the gag sequence in HEK 293 cells and neonatal rat cardiac myocytes. • Develop Adenovirus containing plasma membrane-specific Ca²⁺ buffers to test function in adult rat cardiac myocytes.

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**References:**