# Chapter 7

## Summary Findings and Therapy Perspective For DMD

Duchenne muscular dystrophy is a childhood onset, lethal, progressive degenerative disease. The lack of fully functional dystrophin protein (Dp427) from the causal gene *DMD/DYS* makes skeletal and cardiac muscles overtly vulnerable to exercise-activated damage. The ongoing damage drives an inflammatory response which further impairs regeneration. Dp427 is also thought to be required during the proliferation of muscle stem cells, which is believed to affect regeneration directly. Dystrophic muscles have deficient glycolysis, which is necessary during the early part of the exercise when energy stores like phosphocreatine are declining, and the mitochondrial metabolism is yet to reach total capacity. Many ion channels, including calcium reabsorption $Ca^{2+}$/ATPase (SERCA) responsible for calcium persistence, use glycolysis-derived ATP for function. In turn, the higher calcium in dystrophic muscles can increase oxidative stress, mitochondrial function, and dynamic de/re-polymerization of cytoskeletal elements like non-sarcomeric f-actins and microtubules in the sarcolemma region, disrupting membrane integrity. The glucose from glycolysis accumulates as glycerol-3-phosphate, and pyruvate is derived from the pentose phosphate pathway as well as from amino acids. This process keeps the balance in amino acid-protein metabolism tilted more towards catabolism than synthesis. The lower glycolysis flux, especially during exercise, can increase glucose persistence, further interfering with mitochondrial fatty acid oxidation and exacerbating dystrophic muscle energy deficiency. The literature survey led us to hypothesize that glycolytic deficiency is the primary pathology as it precedes myogenic differentiation. It is most likely to generate a clear susceptibility pattern of most affected fast-glycolytic muscles to lastly affected cardiac muscles seen in dystrophy patients. These aspects are discussed in detail in the introductory chapter.

Though ligand Jagged1 and its cognate Notch receptors (Notch1-4) are required for tissue-specific stem cells, including muscle-specific satellite cells and normal myogenesis, they are non-responsive to exercise. This pathway is perturbed in dystrophic and aged muscles; its role is restricted to the stem cell niche. The role of this pathway in fully differentiated and contracting skeletal muscles remains unknown. Given that pathology in DMD is exercise/activity induced, it is difficult to hypothesize how overexpression of Jagged1 might

have rescued the large and severe golden retriever model of DMD (GRMD). To circumvent this problem, we focused on pathological features mitigated by Jagged1 overexpression in zebrafish, another severe model of DMD – as described in Chapter 2.

The CRISPR/Cas9 knock-out strategy targeting exons 6 and 7 of the DMD gene in early zebrafish embryos generated a severe disease in the larvae. The dystrophic larvae exhibited bent tails, small swim bladders and survived for less than 15 days. The confirmation for knockout was done by genomic PCRs and immunohistochemical localization of the dystrophin protein. Behavioral assays revealed a progressive decline in swimming capacity in dystrophic larvae. Human Jag1 CDS containing a plasmid with CMV promoter was co-injected with CRISPR reagents for rescue. Bipolar imaging revealed better muscle structure in the Jagged1 overexpressing group (rescue) than in DMD larvae, which was further confirmed by β-dystroglycan immunostaining. This was also reflected in the significantly improved swimming capacity of rescued dystrophic larvae, as discussed in Chapter 3. Additionally, it was found that Jagged1 overexpression increased the percent of proliferating cell population from larval tails though the identity of this population could not be ascertained.

Similarly, the identity of apoptotic cell populations from larval tails, which was found to be much higher in the dystrophic group than in the control or rescue group, could not be determined. Cytoplasmic oxidative stress was reduced in the rescue group, which was higher in the dystrophic group than in control larvae, as expected. Mitochondrial oxidative stress increased only in the dystrophic group overexpressing Jagged1 compared to DMD or control zebrafish larval muscles. The downstream effectors of the Jag1-Notch pathway involved in the rescue could not be discerned due to various reasons like multiple receptors and the combinatorial and transient transcriptional response of this pathway, as shown in Chapter 4. The significant limitations of zebrafish studies include temporary and non-tissue-specific overexpression by dilution and degradation of the plasmid. The FACS analysis was done without antibodies, limiting the identification of cells undergoing apoptosis or proliferation. The use of genetically modified lines with fluorescent satellite cells could have helped in testing the possibility of inherent higher myogenic cells due to jagged1 overexpression. Nonetheless, the study confirmed the conserved nature of Jagged1-mediated rescue and identified some of the pathological processes mitigated in the rescue.

Immortalized DMD patients derived myoblast cell lines offered two major advantages. The primary benefit of results unconfounded by complications from off-target tissues. Secondly, a

myogenin/MyoG expression window specific effects of jagged1 which could not be done in the zebrafish model. However, one limitation (also confronted during experiments about the cell lines) is that they have lentiviral insertions of hTERT and hCDK4 along with antibiotic resistance, making them less amenable to lentivirus mediated Jagged1 CDS integration and antibiotic selection. Additionally, there is no evidence of Notch receptor signaling involved in the rescue. Hence, the synthetic 17 amino acid peptide of the Jagged1 extracellular domain described in the literature was added to the myoblast cultures after 24 hours in the differentiation medium. These were allowed to grow for 5 days without adding Jagged1-peptide at the first change of media, and hence, peptide treatment is restricted to 24-48 hours of differentiation. Calcimycin was added after 5 days to induce intracellular calcium in myotubes. As expected, DMD and Control cell lines grew and differentiated usually in culture with or without Jagged1 peptide, though myotube width was lower in dystrophic myotubes. Calcimycine did not induce increased detachment/delamination in dystrophic myotubes with or without Jagged1 peptide treatment. The study could not identify differentially expressed notch target genes in response to Jagged1-peptide treatment significantly. Possible notch receptors or downstream effectors involved by Jagged ligand presence could not be identified. Inherent Jagged1 expression and its intron-derived miRNA-6870 remained low and did not show differential expression between DMD and control myoblasts or early differentiating myocytes. Mitochondrial oxidative stress increased during Jagged1-peptide treatment only in the dystrophic lines, similar to the zebrafish finding described in Chapter 5.

To better understand the mechanism of this rescue, original “escaper” GRMD transcriptomics data was reanalyzed. The significant differentially expressed genes (DEGs) were fed into the “Enrichr” tool to identify pathways mitigated in the escaper dogs. Major pathways mitigated belonged to the glyoxal pathway, TCA, and proteasomal degradation. Using only transcriptomics data has limitations, and there is no way to validate these findings. Hence, data from freely available transcriptomics, proteomics, and metabolomics platforms for mouse model (mdx), Golden retriever (GRMD), and human patients were analyzed. Differentially expressed genes, proteins, and metabolites generated the pathways similarly with “*Enrichr*” and “*MetaboAnalyst*” tools. The pathways were compared to find common pathways across species and platforms. Surprisingly, glyoxal and pyruvate metabolism pathways became familiar to all three species and platforms. Though DMD was first described as a metabolic disease with severe metabolic deficiencies described throughout the literature, glyoxal was never considered a major causal factor. However, glyoxal is a highly reactive toxic byproduct

of glycolysis and three other carbon metabolites. This fits the hypothesis that dystrophic muscles cannot coordinate the glycolysis in the membrane compartment, leading to increased glyoxal production, as described in Chapter 6.

From the current study, it can be deduced that energy metabolism is a major defect of dystrophic muscles, and jagged1 expression during the myogenin/MyoG differentiation window rewires the metabolism. Jagged1 possibly functions via multiple and non-canonical pathway/s but needs to be studied further in detail. How Jagged1 rewires metabolism also needs research in the future. There are currently very few therapies directed at metabolic rewiring. Based on the abovementioned findings, metabolism could be a promising therapeutic avenue for this debilitating disease.