Insights into Osteoporosis in Duchenne Muscular Dystrophy: Exploring Intrinsic Differences Between Normal and Dystrophic Osteoclasts

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INTRODUCTION: Duchenne Muscular Dystrophy (DMD) is a progressive muscle wasting disease caused by mutations in the *DMD* gene on the X chromosome. Beyond the characteristic muscle wasting, boys with DMD often suffer from severe osteoporosis and fragility fractures. While some of the causes of osteoporosis in DMD are known, such as loss of ambulation, high corticosteroid use, and release of bone-regulating myokines from dystrophic muscle [1], the pathogenesis is not completely understood. We and others have previously shown higher bone resorption in both DMD patients and dystrophic animal models [1]. However, whether the loss of full-length dystrophin may directly play a role in bone loss remains unknown. Using *in vitro* osteoclastogenesis assays, we observed that OC precursors isolated from dystrophic ($Dmd^{-/Y}$ (mdx)) mice were more osteoclastogenic and hyperactive compared to wild-type (WT) controls. Due to the loss of full-length dystrophin in mdx cells, we hypothesized that dystrophin plays a role in OC differentiation and activity.

METHODS: *1.Osteoclastogenesis.* Standard osteoclastogenesis assays were performed by differentiating bone marrow-derived macrophages (BMMs) isolated from WT and mdx mice to OCs *in vitro.* Only male mice were used because DMD only affects males. At least 3 animals were used for every experiment. To evaluate osteoclastogenesis, mature OCs were TRAP stained, and the size and number of multi-nucleated TRAP+ cells were quantified. Expression of OC marker genes *Nfatc1, Fos, Dcstamp,* and *Ctsk* was evaluated in OCs. *3. OC activity*. To evaluate differences in activity between WT and mdx OCs, mature OCs were seeded onto cortical bone slices, and resorbed area was quantified after 3 days. Actin ring formation was evaluated by staining mature OCs with phalloidin. *4. Evaluation of dystrophin isoform expression.* Expression of the 5 dystrophin isoforms (Dp427, Dp260, Dp140, Dp116, and Dp71) was evaluated by RT-qPCR. Dystrophin expression was characterized in WT and mdx cultures throughout differentiation by immunoprecipitation using a pan-dystrophin antibody, which is able to recognize all isoforms, followed by Western Blot. *5. RNA-sequencing.* To gain unbiased insight into the transcriptional changes between mdx and WT, we performed RNAseq on mature OCs. *6. Agilent Seahorse Assay.* To determine how metabolism may affect for differences in differentiation and activity in mdx OCs, we performed an Agilent Seahorse ATP production assay in BMMs, pre-OCs, and OCs.

RESULTS: *1. Osteoclastogenesis.* TRAP staining revealed that mdx BMMs were more osteoclastogenic than WT. At the end of differentiation, mdx OCs were significantly larger and there were more per well compared to WT cultures (**Figure 1A).** Mdx OCs also had higher expression of *Nfatc1, Dcstamp,* and *Ctsk. 2. Osteoclast activity.* Mdx OCs resorbed a larger area of the bone slices than WT controls (**Figure 1B)** and a greater percentage had actin rings, demonstrating increased activity.*3. Dystrophin expression.* Expression of full-length dystrophin increased during osteoclastogenesis in WT cultures (**Figure 2**), while dystrophin was not expressed in mdx OCs. Expression of Dp260 increased during differentiation of WT OCs, but decreased during differentiation of mdx OCs. Dp140 was not detected at any point during differentiation. Dp116 was expressed in WT and mdx OCs and did not differ between genotypes or change during differentiation. Expression of Dp71 was detected in pre-OCs and OCs in WT and mdx cells but did not differ between genotypes. *4. RNA-sequencing.* There were 882 differentially expressed genes in mdx versus WT OCs, with 404 up-regulated and 478 down-regulated genes (**Figure 3**). Pathway enrichment analysis of revealed GO terms related to inflammation, such as positive regulation of immune response, regulation of leukocyte proliferation, and leukocyte activation. Terms related to lipid metabolism were also enriched. More importantly, data from RNA-seq also detected transcripts for *Dmd* and *Utrn,* as well as members of the dystrophin-associated protein complex (DAPC), including sytrophins, dystroglycans, and dystrobrevins. *5. Agilent Seahorse assay.* By investigating metabolism in mdx and WT OCs throughout differentiation, we observed that total ATP production was higher in mdx BMMs and OCs than WT. This difference was driven by a 30% increase in oxidative phosphorylation. No differences in glycolytic ATP production were observed.

DISCUSSION: These studies suggest that intrinsic differences in OC differentiation potential and activity in DMD may contribute to osteoporosis and high fracture risk. While the complete scope of these differences is not currently understood, our data suggest that some of these differences may be genetic, indicating a not-yet-reported expression of full-length dystrophin in OCs and a possible role of DAPC dysregulation. Furthermore, OCs from dystrophic mice have higher ATP production by oxidative phosphorylation and increased lipid metabolism, which may promote OC hyperactivity. Future work will investigate the DAPC in OCs and establish its role in OC differentiation and activity. We will further explore the metabolic differences in mdx OCs and how those may contribute to increased differentiation and activity.

SIGNIFICANCE/CLINICAL RELEVANCE: Osteoporosis is a severe, yet often overlooked, comorbidity in DMD patients. It worsens disease progression and has been linked to decreased life expectancy. Further understanding the pathogenesis of osteoporosis may lead to novel treatment strategies, including gene therapy to target dystrophin loss, modulation of inflammation, and modulation of lipid metabolism.

REFERENCES: [1] Hurley-Novatny *et al.* Poor bone health in Duchenne Muscular Dystrophy: A multifactorial problem beyond corticosteroid use and loss of ambulation. *Front. Endocrinol.* Nov 2024.

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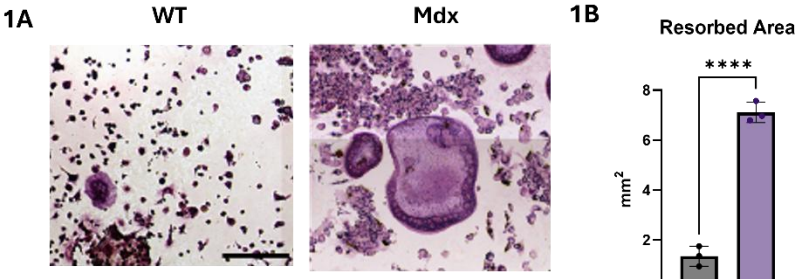
IMAGES:



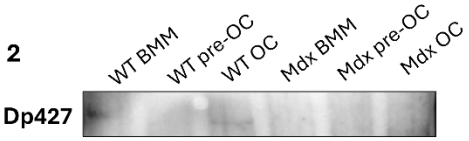


Figure 1. OC differentiation (A) and activity (B)
Figure 2. Dystrophin isoform Dp427
Figure 3. Volcano plot of mdx versus WT gene expression.