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BMP4-Smad8 Signaling Mediates myomiR Suppression in Duchenne Muscular Dystrophy

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Background

- Duchenne muscular dystrophy (DMD) is an X-linked recessive skeletal muscle wasting disease due to loss of dystrophin.

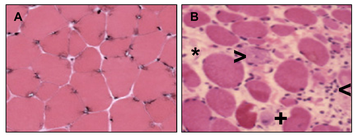


Figure 1 – Histological findings in normal (A) and Duchenne muscular dystrophy (DMD) muscles (B). Hematoxylin and eosin staining demonstrates myofiber degeneration, chronic regeneration: endomysial fibrosis (*) & internally nucleated fibers (>), and recent regeneration: basophilic fibers (+) and inflammation (<).

- Dystrophic signaling is typified by chronically upregulated tumor growth factor beta (TGFβ) superfamily receptor ligands, such as TGFβ1 and BMP4.
- There is also dyregulation of a subset of micro-RNAs that are enriched in muscles, myomiRs, which impairs normal muscle function.
- Receptor Smads, R-Smads, are the intracellular TGFβ receptor mediators.
- R-Smads include two canonical pathways the Smad2 / Smad3 and Smad1 / Smad5 / Smad8 pathways.
- Smad8's role in the potential dysregulation of DMD is undefined.
- Smad8, is highly upregulated in skeletal muscle wasting disease and function is previously implicated as a transcriptional regulator of skeletal muscle mass, differentiation, and microRNA expression.
- We sought to determine the importance of Smad8 as a contributor to DMD using mouse and human skeletal muscles tissues.

Hypothesis: BMP4 / Smad8 functions as a negative regulator of myomiRs and promotes dystrophic disease in skeletal muscles.

Methods

Human muscle samples
- Biopsies from paraspinous muscle were collected from DMD male patients (Age = 15 to 20 years) undergoing scoliosis surgery under an approved Institutional Review Board protocol (IRB- 300002164). Normal muscle samples (Age = 8 - 59 years) were selected from the archive of remnant muscle biopsy tissues at the UAB Division of Neuromuscular Disease.

Mouse strains and muscle samples
- All mouse strains were approved for experimental studies by the UAB Institutional Animal Care and Use Committee. The *mdx*^{5cv} mouse strain and wildtype C57BL/6J strain were obtained from Jackson Laboratories.

qPCR analysis of miRNA and total RNA
- Total and micro-RNA were isolated from muscle tissue using the miRVana miRNA Isolation Kit. Real-time quantitative PCR was performed using the Applied Biosystems ViiA7 instrument. Relative expression values (RQ) were calculated using the ΔΔCT method with normalization to internal reference genes (Gapdh or Actb). Relative quantity (RQ) is shown relative to control muscle after normalization to either RNU48, RNU6, or Gapdh.\

RNA-Seq Analysis
- 3 DMD muscle vs. 3 non-DMD samples and BMP4-treated C2C12 myoblasts vs. vehicle were were analysed by RNA-Seq analysis. Samples were aligned to human or mouse reference genomes. Then differential gene expression was performed using DESeq analyses.

Western blot
- Protein lysates were obtained from homogenized tissues in either T-PER or M-PER lysis buffer with 1x Halt™ Protease Phosphatase Inhibitor Cocktail. Primary antibody utilized were Smad1/5/8, p-Smad1/5/8.

C2C12 Transfection and myotube fusion index
- C2C12 cells were obtained from ATCC. Cultured in growth or differentiation medium in 6 well plates. siGFP or siSmad8 (Horizon Discovery) were transfected at 60% confluency using lipofectamine. Cells were then harvested after 3-5 days post-transfection. BMP4 recombinant protein (R&D System) was used for stimulation of cells at the specified concentration.
- Myotube fusion index (MFI) was measured by immunocyotfluorescence after formalin fixation of transfected C2C12 cells. Cells were stained with MF20 (myosin heavy chain) antibody and imaged with confocal microscopy and MFI was calculated as fraction of myonuclei in MF20+ cells (myotubes) relative to total nuclei.

Statistics
- Statistical analyses were performed in Graphpad Prism 8. T-test with correction for multiple comparisons were used to assess differences between control and disease groups unless noted otherwise.

Results I

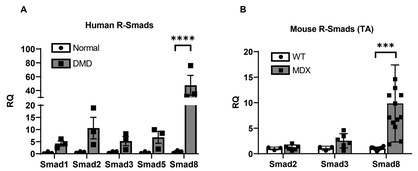


Figure 2. Smad8 mRNA is selectively upregulated in DMD and *mdx*^{5cv} (MDX) skeletal muscles. (A) Smad8 mRNA expression levels, determined by qPCR, show a significant increase in human DMD versus healthy control muscle (Normal). (B) Smad8 mRNA levels were also increased in the tibialis anterior (TA) muscle at 6 months of age. Data points show biological replicates for each group. Bars show mean SEM. P values: ** < 0.01, *** < 0.001, and **** < 0.0001. RQ, relative quantity. As published[1].

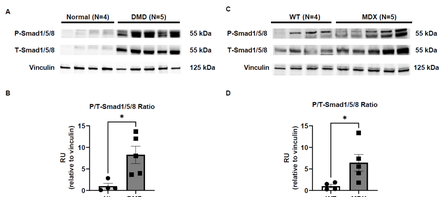


Figure 3. Smad1/5/8 total (T-) and phosphorylated (P-) protein is increased in dystrophic skeletal muscles. (A) Human DMD muscle shows increased T-Smad1/5/8 and P-Smad1/5/8 by western blot. (B) Densitometric analysis showed an increased fraction of P-Smad1/5/8 compared with T-Smad1/5/8 (P/T-Smad1/5/8 ratio). (C,D) 9-month-old *mdx*^{5cv} tibialis anterior muscle showed a similar pattern of increased P/T-Smad1/5/8 ratio. Vinculin is shown for comparison of loading. Data points show biological replicates for each group. Bars show mean SEM. P-value: *<0.05. RU, relative units. As published[1].

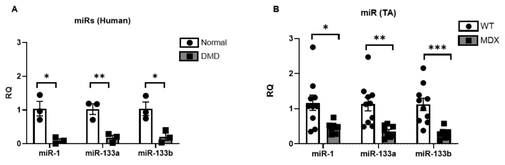


Figure 4. miR-1, miR-133a, and miR-133b are repressed in DMD and *mdx*^{5cv} (MDX) skeletal muscles. (A) DMD human skeletal muscles show reduced miRNA expression levels by qPCR. (B) 6-month-old *mdx*^{5cv} (MDX) mouse tibialis anterior muscles show reduced miR-1, miR-133a, and miR-133b compared with wildtype (WT). Data points show biological replicates for each group. Bars show mean ± SEM. p values: * < 0.05, ** < 0.01, and *** < 0.001. RQ, relative quantity. As published[1].

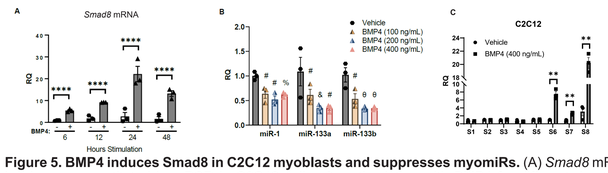


Figure 5. BMP4 induces Smad8 in C2C12 myoblasts and suppresses myomiRs. (A) *Smad8* mRNA levels were quantitated by qPCR in C2C12 cells after stimulation with BMP4 for different durations in growth media (GM). *Smad8* mRNA induction peaked at 24 hours. (B) qPCR results show a dose-dependent suppressive effect on miR-1, miR-133a, and miR-133b at 24 h. (C) BMP4-induced Smad8 (S8) results in increased inhibitory Smad6 (S6) and Smad7 (S7) in C2C12 myoblasts. Smad1 (S1), Smad2 (S2), Smad3 (S3), Smad4 (S4), and Smad5 (S5) were unchanged. Bars show mean ± SEM of 3 biological replicates per group. P values (compared to vehicle): % < 0.05, ** <0.01, # < 0.01, & < 0.001, **** < 0.0001, and θ < 0.0001. ANOVA used for analysis of A and B. RQ, relative quantity. RU, relative units. Adapted[1].

Results II

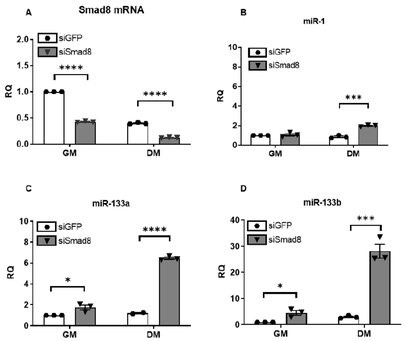


Figure 6. *Smad8* silencing in C2C12 cells increases miR-1, miR-133a, and miR-133b expression by qPCR. (A) Smad8 silencing (siSmad8) achieved >50% reduction of Smad8 mRNA compared with siRNA control (siGFP) in growth (GM) and differentiation media (DM). (B–D) miR-1, miR-133a, and miR-133b increased after Smad8 knockdown. Data points show biological replicates for each group. Bars show SEM. P values: * < 0.05, *** < 0.001, and **** < 0.0001. RQ, relative quantity. As published[1].

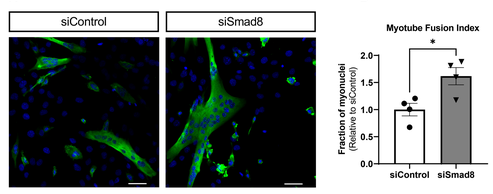


Figure 7. Myotube fusion index is increased after Smad8 silencing (siSmad8). (A) Representative immunocytofluorescence imaging shows C2C12 cells at day 4 of differentiation after transfection of siSmad8 or siControl (siGFP). Cells were stained for MF20 (green) to identify myotubes and 40,6-diamidino-2-phenylindole (blue) for myonuclei. Scale bar = 50 μm. (B) Myotube fusion index was increased significantly relative to siControl (P value: * < 0.05). As published[1].

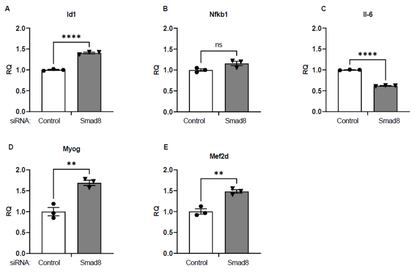
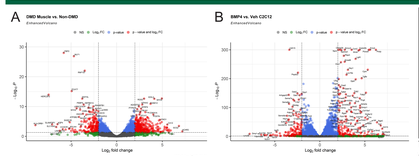


Figure 8. Smad8 silencing promotes DMD-mitigating mRNA patterns in C2C12 cells. After transfection of siSmad8 or siControl (siGFP), qPCR was used to assess mRNA expression levels of (A) Id1, a known transcriptional target of Smad8, (B) Nfkb1, (C) Il-6, a pro-inflammatory cytokine, (D) Myog, muscle regulatory factor, and (E) Mef2d, muscle regulatory factor. Bars show mean ± SEM of 3 biological replicates per group. p values: ** < 0.01, **** < 0.0001. RQ, relative quantity. As published[1].

Results III



Human Gene Symbol	DMD FC	miR Target	Description
HCAR2	145.8		hydroxycarboxylic acid receptor 2
DHRS9	36.9	miR-133a	dehydrogenase/reductase 9
ACVR1C	3.8*	miR-1	activin A receptor type 1C
NIPA1	-3.94	miR-1, miR-133a/b	NIPA magnesium transporter 1
UNC13C	-75.3	miR-133b	unc-13 homolog C

Table 1. Selected DMD Genes and fold change (FC) expression compared with control. *, q-value = 0.09

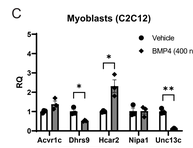


Figure 9. Transcriptomic Changes in DMD Muscle and BMP4 Treated C2C12. (A) DMD muscles were compared to 3 non-DMD muscle samples and (B) C2C12 cells stimulated with BMP4 were compared with vehicle. Principal component analysis demonstrated stratification of the DMD and BMP4 stimulated groups compared with controls. DESeq analysis in DMD muscle revealed 1,257 upregulated and 1,132 downregulated transcripts with fold-change ≥ 2.0 and q-value ≤ 0.05. Similarly in BMP4-treated C2C12, there were 296 upregulated and 172 downregulated transrips. (C) qPCR of BMP4-treated C2C12 myoblasts shows *Dhrs9* and *Hcar2* are increased where as Unc13c is decreased. Red and green lines indicate fold change direction observed in human DMD muscle. P-value: *<0.05, **<0.01.

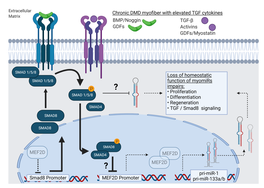


Figure 10. Proposed model of aberrant Smad8 / myomiR signaling in DMD. Wide and dashed lines represent incrveased and reduced signaling, respectively. Chronic upregulation of TGF receptor ligands leads to increased Smad8 in DMD. We hypothesize that Smad8 is involved in a negative transcriptional regulator of myomiRs important for homestatic muscle regulation, potentially through myogenic transcription factor like MEF2D. Created with Biorender.com.[1]

Conclusions

- In DMD and *mdx*^{5cv} skeletal muscles:
  - Smad8 mRNA and protein is upregulated
  - myomiRs, miR-1, miR-133a, and miR-133b are repressed
- BMP4 stimulation of C2C12 leads to:
  - ↑ Smad8 activation and ↑ inhibitory Smad6 > Smad7
  - ↓ myomiRs
- Silencing Smad8 in C2C12 leads to:
  - ↑ myomiRs
  - ↑ myogenic transcription factors, MEF2D and MYOG
  - ↓ pro-inflammatory cytokine, IL-6
  - ↑ myotube fusion index
- Smad8 is a negative regulator of miR-1, miR-133a, and miR-133b in muscle and BMP4-Smad8 appears to be a driver of dystrophic pathology in DMD.

Future Directions:
- Conditional knockout of Smad8 in *mdx*^{5cv} mouse model using muscle specific Cre Lox lines (Smad8 flox, Myf5-Cre, Pax7-Cre)
- Over-express miR-1, miR-133a, miR-133b to assess for attentuation of dystrophic disease in cell culture
- Analysis of transcriptomic changes in DMD including investigation of pathways and gene networks which may be explained by BMP4/Smad overactivation

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References:
1. Lopez MA, Si Y, Hu X, Williams V, Qushair F, Carlyle J, Alesce L, Conklin M, Gilbert S, Bamman MM, Alexander MS, King PH. Smad8 Is Increased in Duchenne Muscular Dystrophy and Suppresses miR-1, miR-133a, and miR-133b. Int J Mol Sci. 2022 Jul 7;23(14). doi: 10.3390/ijms23147515. PubMed PMID: 35886863; PubMed Central PMCID: PMC9323105.