

# Smad9 is Upregulated in mdx<sup>5cv</sup> Skeletal Muscles & Suppresses myomiR Expression

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## BACKGROUND

- Smad9 (formerly Smad8) is a TGF superfamily related transcriptional factor
- Smad9 is phosphorylated by TGF receptors and translocates to nucleus
- No specific antibodies for Smad9 due to homology with Smad1 and 5
- miRNAs are non-coding RNAs involved in fine-tuning cell signaling
- Restoration of repressed miRNA levels in muscular dystrophy is associated with attenuated histopathology

## HYPOTHESIS

Smad9 is a key negative transcriptional regulator of skeletal muscle-enriched miRNAs (myomiRs) which in turn leads to exacerbation of muscular dystrophy

## METHODS

We used two approaches to study the role of Smad9 as a skeletal muscle biomarker of Duchenne muscular dystrophy:

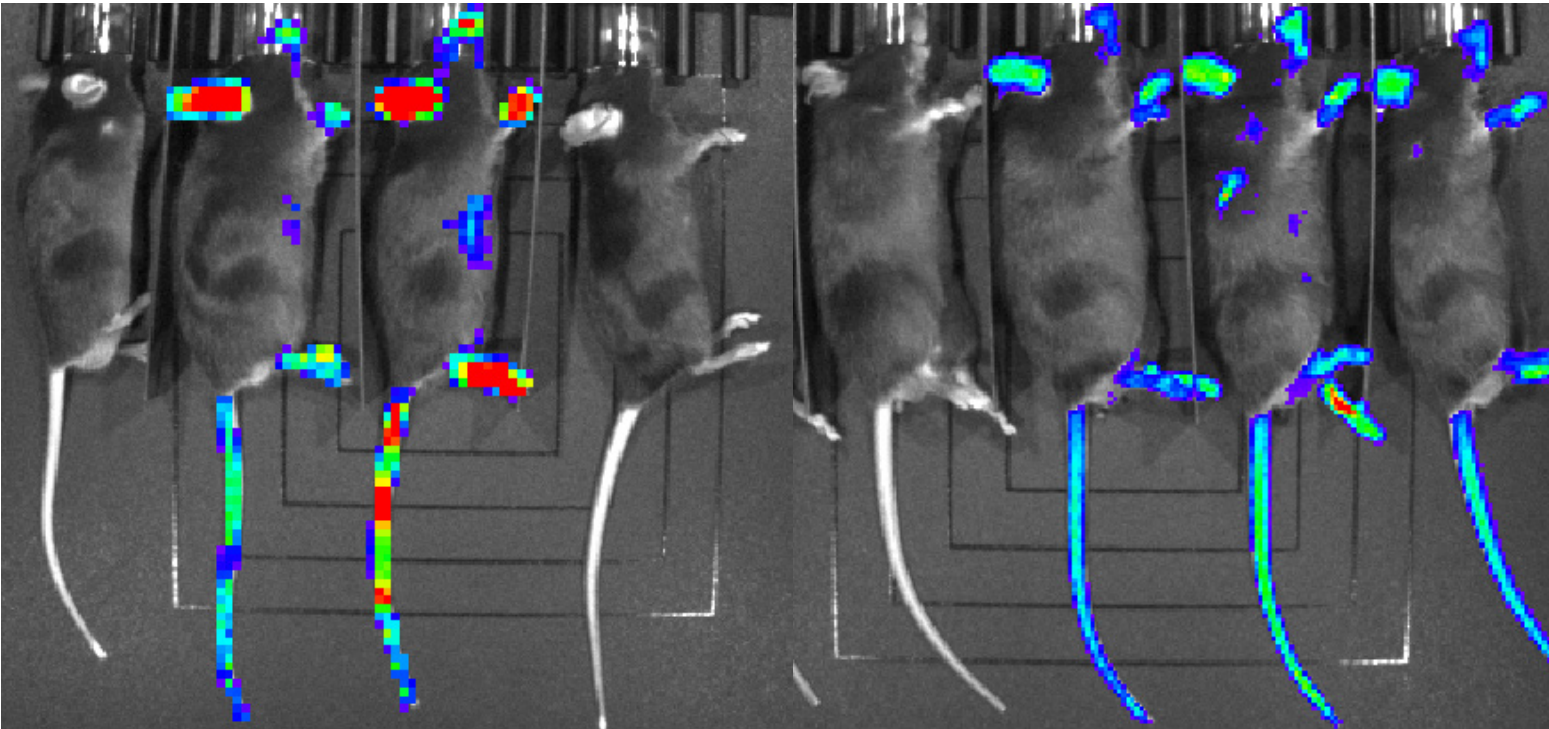
- CRISPR-generated Smad9 knockin mouse with luciferase and eGFP
  - Smad9 reporter mouse was then crossbred with mdx<sup>5cv</sup> mouse
  - Skeletal muscle analyzed by immunofluorescence
- Cell culture myoblasts
  - C2C12 myoblasts transfected with siRNA and over-expression constructs to modulate Smad9 expression

## RESULTS

Smad9 Luciferase and eGFP  
Reporter Mouse Model (KI)

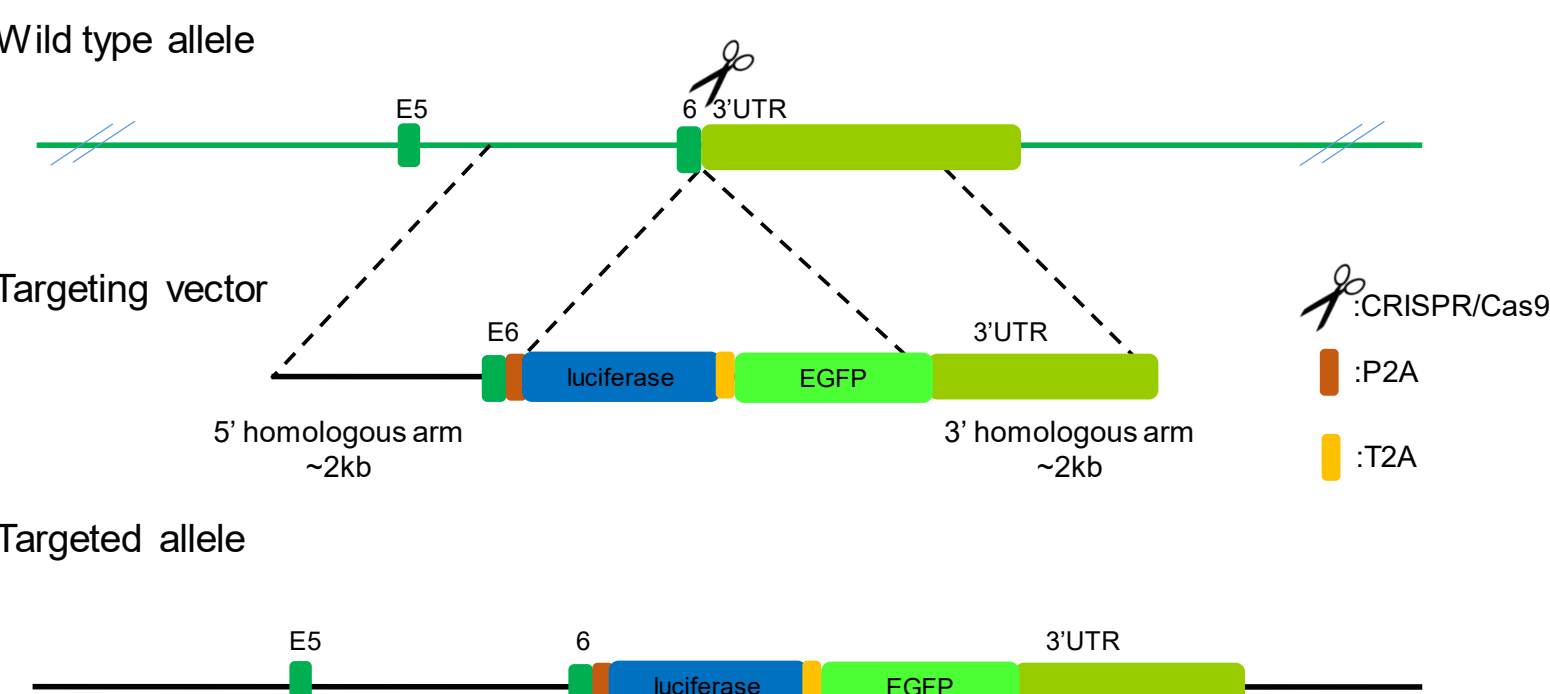
Smad9 KI x mdx<sup>5cv</sup>

Smad9 KI +/+ +/+ +/+ +/+ +/+ +/+ +/+ +/+  
mdx<sup>5cv</sup> +/+ +/+ +/+ +/+ +/+ - - -



Whole animal imaging using the IVIS Lumina Imaging System was performed after injection with D-luciferin. 7-week-old mice shown. Genotypes shown above + = wildtype, - = KI or disease alleles.

### II. Targeting strategy (1) - EGE(CRISPR/Cas9) system



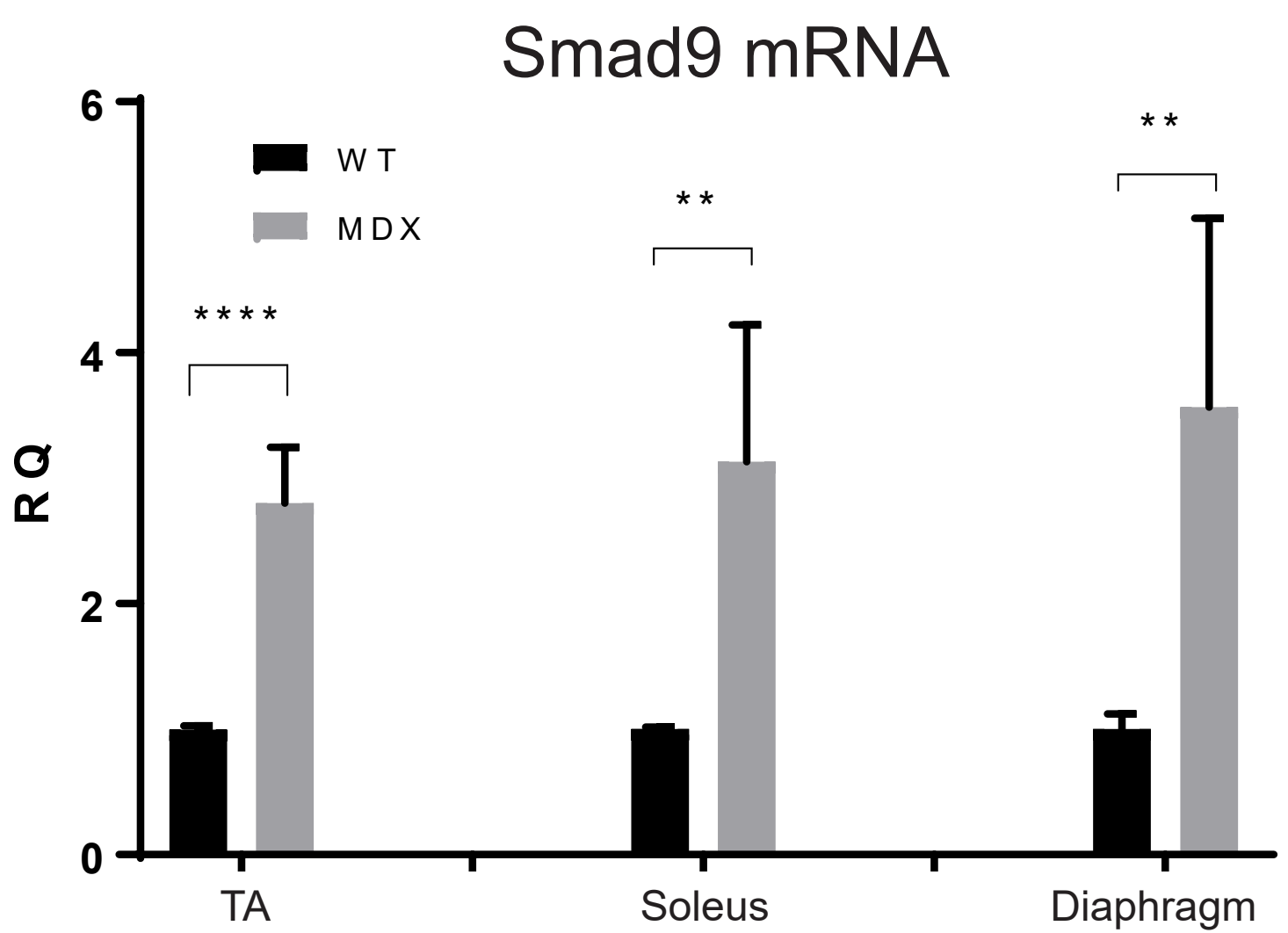
Note: This design is based on transcript-201(NM\_019483)

BIOCYTOGEN

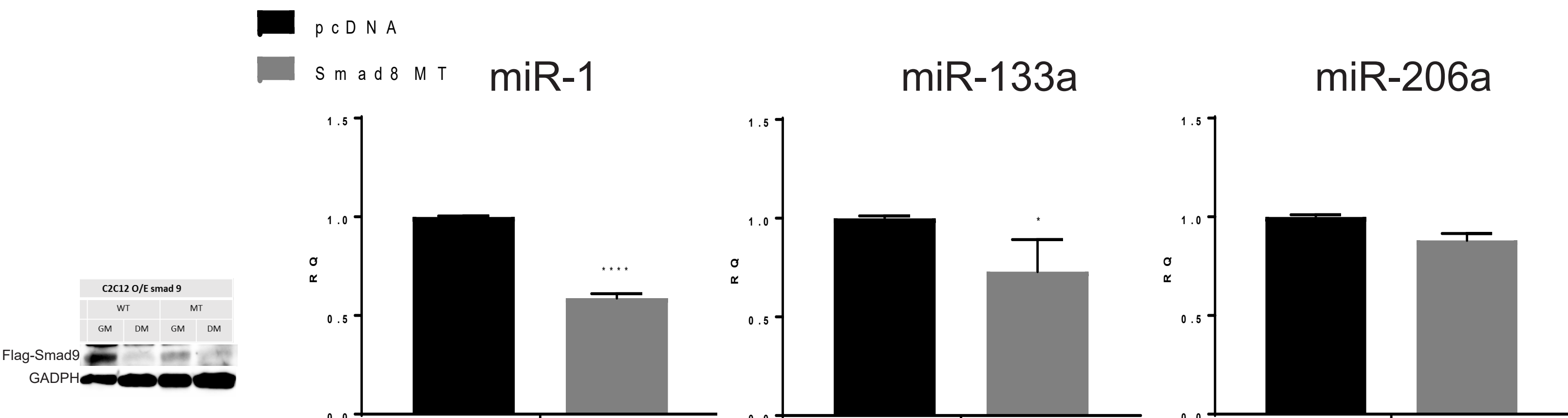
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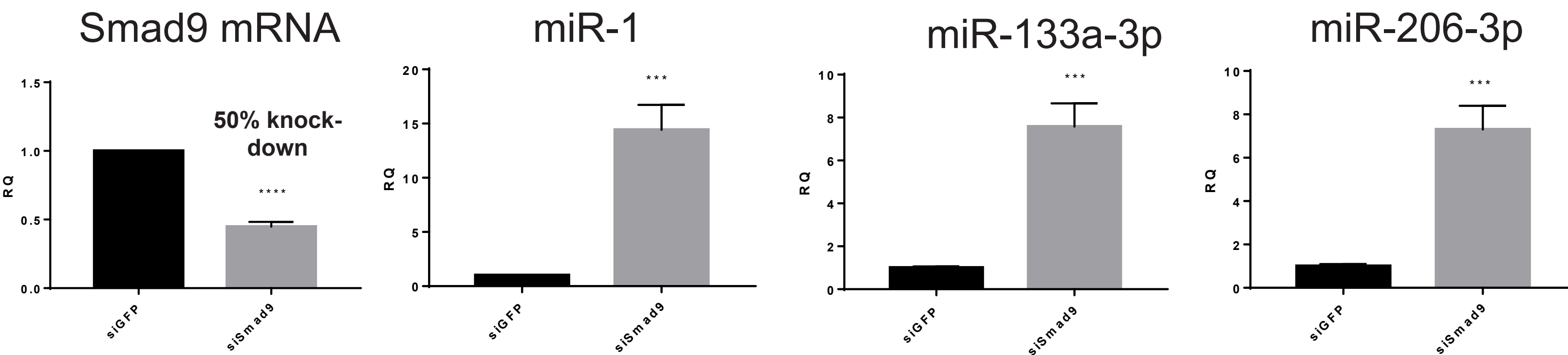
## RESULTS



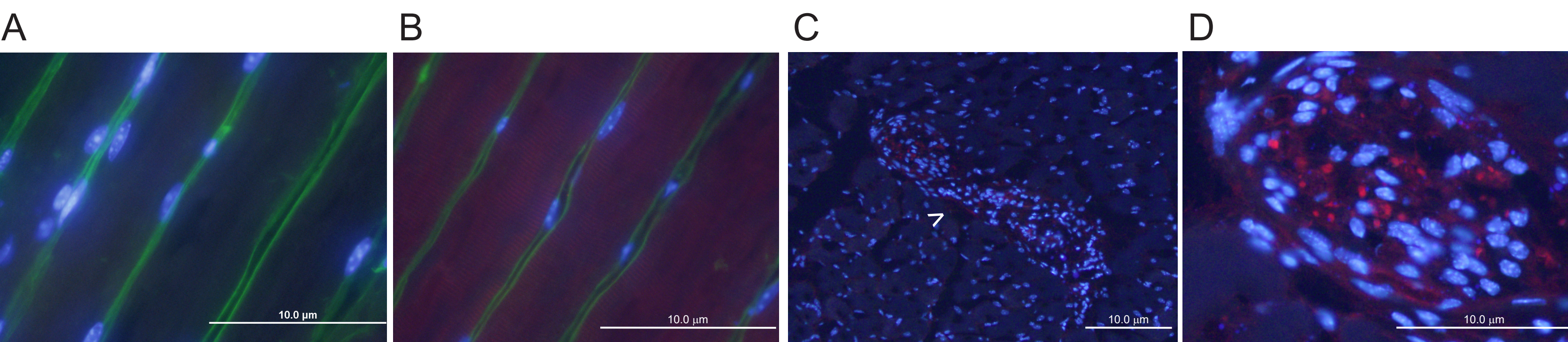
**Figure 1** – Smad9 mRNA by qRT-PCR from mdx<sup>5cv</sup> skeletal muscles at 6 months-old. Smad9 mRNA levels are 3-fold higher in the dystrophic (MDX) muscles compared with wildtype (WT). RQ= relative quantification, TA = tibialis anterior. N=3 for each group. \*\*P<0.0005, \*\*\*\*P < 0.0001.



**Figure 2** - qRT-PCR of myomiR levels in C2C12 cells transfected with constitutively activated over-expressed flag-Smad9. miR-1 and miR-133a show significant reductions in GM. RQ= relative quantification, pcDNA = control plasmid, MT = constitutively activated DVD domain of Smad9. N=6 for each group. GM = growth media, DM = differentiation media. \* P < 0.05, \*\*\*\*P < 0.0001.



**Figure 3** - qRT-PCR of mature miRNA (miR) levels in C2C12 cells transfected with small interfering Smad8 RNA. RQ= relative quantification, siGFP = small interfering control GFP plasmid, siSmad8 = small interfering Smad8 RNA. N=6 for each group. \* P < 0.05, \*\*P<0.0005, \*\*\*\*P < 0.0001.



**Figure 4** - Immunofluorescence of Smad9 KI reporter skeletal muscle shown with anti-luciferase (Red), WGA (Green), and nuclear (DAPI) stainings. Smad9 KI muscles were harvested at 9 weeks age and snap frozen. Shown are primary antibody negative control (A) and anti-luciferase (B) longitudinal sections. Anti-luciferase signal was also seen in the neurovascular bundle (arrow) at 20x (C) and exploded region at 40x (D).

## CONCLUSIONS

- Smad9 mRNA expression is increased in mdx<sup>5cv</sup> skeletal muscles
- Reporter activity suggests Smad9 is located in myofibers and peripheral nerve within muscle
- Smad9 expression is negatively correlated with myomiR expression