Poster #1623

Multiple *In Vitro* Differentiated Skeletal Muscle Models for Screening of Synthetic Muscle Promoters for Gene Therapy

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ABSTRACT

Gene therapy delivery to skeletal muscle for inherited musculoskeletal disorders has been challenging, in part due to the target tissue's large size, mass, and anatomical distribution, and the requirement for high systemic vector doses of adeno-associated virus (AAV) for therapeutic efficacy. High AAV doses have caused dose-limiting toxicity and immunogenic responses, as observed in ongoing clinical trials for inherited muscular dystrophies. Muscle tissue is also an effective target for the therapeutic production of secretory proteins, like enzymes, hormones, antibodies, and clotting factors, following local delivery of the viral vector containing the therapeutic transgene to the muscle. The potential of the muscle as a target for gene therapy highlights a demand for more potent, tissue-specific, and compact regulators of therapeutic gene expression, such as promoters. Stronger promoters can provide therapeutic potency at lower viral vector doses, while a reduction in size allows for more efficient packaging of larger transgenes or expression cassettes. To our knowledge, there is a lack of accessible data comparing transcriptomic profiles across multiple *in vitro* and *in vivo* skeletal muscle models pre- and post-differentiation, which can be used for designing and testing novel muscle-specific AAV therapies. Here, we present multiple *in vitro* differentiated myotube models of skeletal muscle for more efficient evaluation of engineered muscle promoters for gene therapy and their characterization by transcriptomics and immunofluorescence analyses. The differentiation-induced myotube models include C2C12 immortalized mouse myoblast cell lines, primary human myoblasts from the quadriceps, and human induced pluripotent stem cell-derived myoblasts. Transcriptomic changes were assessed before and after differentiation for each model and condition. After validating the presence of key myotube differentiation markers, a set of in-house proprietary muscle promoters were tested in these models and compared against known reference muscle promoters currently used in clinical trials of AAV-delivered treatment for muscular dystrophies. Top performing candidates from our rationally designed compact muscle-based promoters were evaluated in these *in vitro* skeletal muscle models, in addition to *in vivo* assessment in C57BL6 mice, with our results showing higher transgene expression compared to reference muscle promoters. In summary, we show promising reliable *in vitro* differentiated skeletal muscle models characterized via RNAseq and immunofluorescence as well as demonstrate the potential of our promoter engineering platform designed to target specific tissues of interest, like skeletal muscle.

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Development of *in vitro* skeletal muscle models for promoter engineering

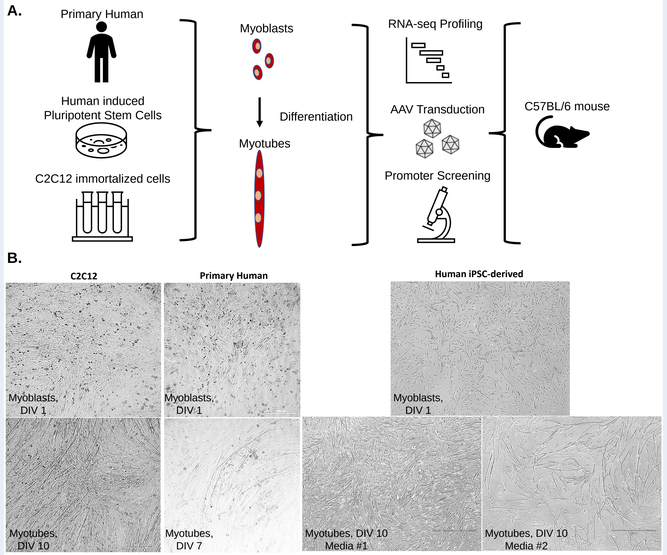

Figure 1. Pipeline for the characterization and promoter evaluation in cellular skeletal muscle models. **(A)** A step-wise approach to the differentiation of skeletal muscle myoblasts, then subsequent RNA-seq and adeno-associated virus (AAV) transduction for promoter screening; **(B)** Brightfield images of skeletal muscle cellular models. Scale bar sizes are indicated.

Transcriptomic profiling of skeletal muscle models

- PCA analyses show myotube models cluster closer to *ex vivo* skeletal muscle samples than their myoblast counterparts (Fig. 2A, D).
- We predicted the cell type composition of our bulk models (Fig. 2B,E). The differentiation of iPSC-myoblasts towards myotubes results in a shift in profile with the skeletal myocyte component increasing between 20% and 35%. Similar changes were not observed in human primary models or C2C12 lines.
- Expression levels of key myocyte markers confirmed iPSC-myotubes' resemblance to reference human skeletal muscles with increased expression of *DES*, myosin heavy chain and troponin genes when compared to iPSC myoblasts (Fig. 2C).
- Primary human myotubes also show changes in key markers but do not match the reference skeletal muscle expression profile as closely, suggesting incomplete differentiation at DIV 7.
- While not perfectly matching the profile of wild-type muscle (B6WT/Muscle), several skeletal muscle-specific markers are overexpressed following the differentiation of C2C12 myoblasts into myotubes.(Fig. 2F).

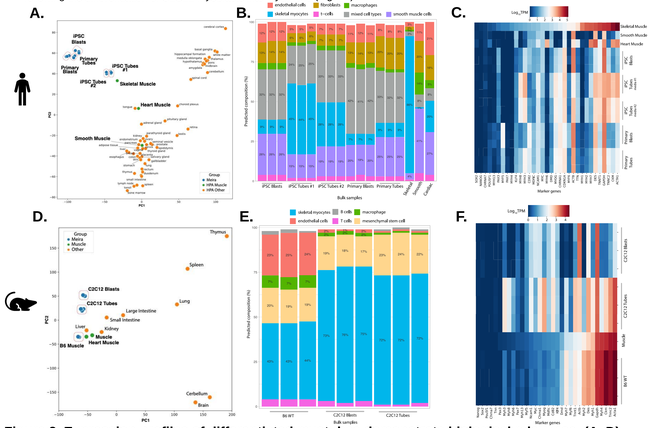

Figure 2. Expression profiles of differentiated myotubes demonstrate biological relevance. **(A, D)** Principal component analysis of transcriptomic profiles of MeiraGTx's skeletal muscle models and reference tissues from public databases; **(B, E)** Bulk signal deconvolution into cell-type proportions; **(C, F)** Heatmaps of select muscle gene markers.

Differentiation induces skeletal muscle gene expression profiles in myotubes

- Differential expression analysis between iPSC myoblasts and myotubes showed 6,498 differentially expressed genes (FDR<0.01).
- Enrichment analysis shows a strong activation of genes involved in muscle contraction and skeletal muscle structure. Proliferation pathways are suppressed following differentiation.
- Fewer genes (n=1,148) were differentially expressed (FDR<0.01) when comparing primary myoblasts to primary myotubes, with only few enriched terms being involved in muscle functions.

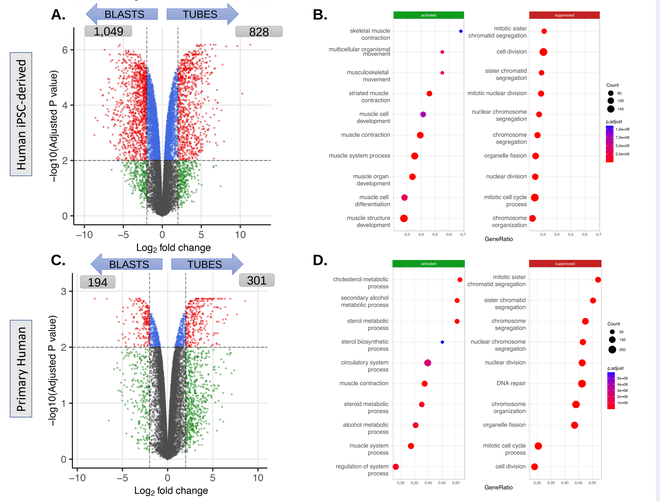

Figure 3. Differentially expressed genes between human myoblasts vs. myotubes. (A, C) Volcano plot showing the distribution of differentially expressed genes between myoblast and myotubes for iPSC and Primary models. Grey boxes indicate genes with adjusted p-value <0.01 and log2 fold change >2; **(B, D)** GeneSet Enrichment Analysis (GSEA) results for Human iPSC-derived and Primary Human models.

Identification of a potent, durable muscle promoter using our skeletal muscle models

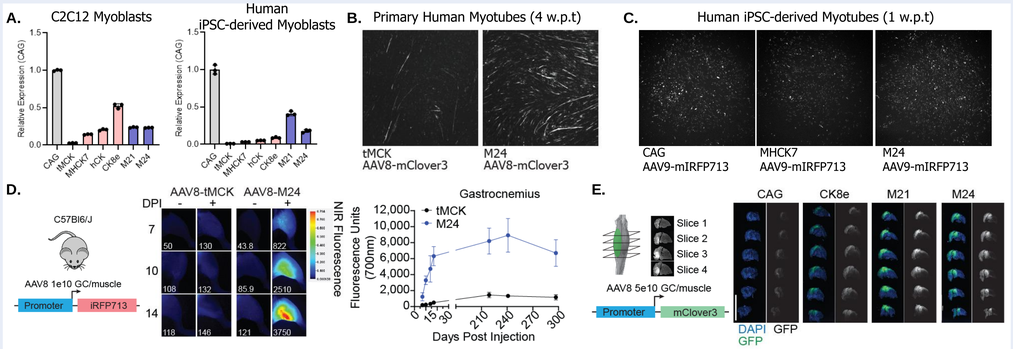

Figure 4. Skeletal muscle models identify a potent muscle promoter: MGTx-M24. (A) Promoter activity was measured using a FACS-based assay in transfected C2C12 and iPSC myoblasts; **(B-C)** Transduction of primary human myotubes after 4 weeks (B) or iPSC-derived myotubes after 1 week (C); **(D)** Bioimaging of adult male mice injected (i.m.) with AAV8-mIRFP713; **(E)** Native fluorescence in the mouse gastrocnemius transduced with AAV8-mClover3. Scale bar: 10 mm.

Conclusions

- We established multiple *in vitro* differentiated skeletal muscle models originating from both mouse and human.
- Transcriptomic analyses identified expected changes in myoblast gene expression profiles following differentiation.
- The expression profile of human iPSC-derived myotubes showed the highest similarity to human skeletal muscle from public databases.
- Using a limited set of markers to confirm cell type differentiation may not be sufficient to fully characterize muscle models, despite being commonly done in the field.
- Our *in vitro* skeletal muscle models allow for efficient promoter screening, which identified MGTx-M24 as a top candidate.
- MGTx-M24 demonstrates higher potency *in vivo* than promoters used in clinical trials, which validates the relevance of our models.
- Collectively, these results show we have established a robust platform to screen engineered promoters for applications to skeletal muscle gene therapies.

References

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