Selective Inhibition of G9a/GLP Histone Methyltransferases Unlocks Myogenic Differentiation Potential in Human Fibro-Adipogenic Progenitors

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INTRODUCTION: Fibro-adipogenic progenitors (FAPs) are skeletal muscle resident mesenchymal stromal cells capable of differentiating into fibroblasts and adipocytes, contributing to intramuscular fibrotic and fatty degeneration after injury. Under physiological conditions, FAPs support muscle homeostasis and regeneration by releasing paracrine signaling factors that stimulate the proliferation and fusion of myogenic cells into multinucleated myotubes, but they do not possess the ability for myogenic differentiation on their own. Recent findings have demonstrated the ability of murine FAPs to undergo myogenic differentiation by inhibiting the histone methyltransferases G9a/GLP, which typically silence myogenic genes in FAPs through H3K9 methylation [1]. However, this process has not yet been studied in human FAPs. Thus, this study sought to investigate the potential for human FAP myogenic differentiation following the treatment with a selective G9a/GLP histone methyltransferase inhibitor, A366.

METHODS: Healthy human skeletal muscle was obtained from patients undergoing rotator cuff repair surgery (deltoid). Muscle was digested, and CD45-/CD31-/CD56-/CD34+/PDGFRα+ human FAPs were sorted with flow cytometry. Human FAPs were cultured in either standard FAP, myogenic, fibrogenic, or adipogenic media for 10 days with administration of 50nM A366 every 48 hours or DMSO as a control. Cells were then fixed and used for immunofluorescence (IF) staining to assess for myogenic differentiation, as well as fibrogenic and adipogenic differentiation depending on their media condition (N = 2 biological replicates per treatment and media condition group). Myotube fusion index (MFI) was calculated as the percentage of nuclei in MF20-positive myotubes with at least three nuclei. A subset of FAPs was also cultured in standard FAP media for 21 days, with 50nM A366 administered every 48 hours, and then used for single-cell RNA sequencing (scRNAseq) (N = 3 biological replicates). The datasets were processed using CellRanger and analyzed and clustered using the R package Seurat. For pseudotime analysis, UMAP dimensions from Seurat were imported into Monocle3 and performed according to the Monocle3 tutorial. All data are presented in the form of mean ± SD. Two-way ANOVAs with Šidak's post hoc test were used to compare MFI across treatment and media condition groups. This study was approved by our IRB and ethics committee.

RESULTS SECTION: Human FAPs treated with A366 underwent myogenic differentiation, forming myotubes under both standard FAP and fibrogenic media conditions, with an MFI of 11.0% ± 4.1% and 5.8% ± 0.3%, respectively (**Figure 1A-E**). A366 treatment did not result in any FAP myogenic differentiation under the low-serum myogenic and adipogenic conditions. There was no myogenic differentiation of FAPs with DMSO control under any condition. scRNAseq analyses of human FAPs treated with A366 for 21 days demonstrated differentiated populations of myoblasts, myocytes, and undifferentiated FAPs (**Figure 2A**). Feature plots of key lineage genes showed continued expression of PDGFRα in the myoblast population, co-expressed with MYOD1, before transitioning to myocytes with the expression of MYOG (**Figure 2B**). Pseudotime analysis further validated the differentiation pathway from human FAPs to myoblasts to eventual myocytes with A366 treatment.

DISCUSSION: Inhibition of the histone methyltransferases G9a and GLP, which catalyze H3K9 methylation, has been shown to unlock the myogenic pathway in murine FAPs. In this study, we demonstrate that the pharmacological treatment of human FAPs with a selective G9a/GLP histone methyltransferase inhibitor, A366, reprograms FAPs to facilitate myogenic differentiation. Combined IF staining and scRNAseq analyses revealed that A366-treated human FAPs undergo transdifferentiation into myoblasts, which subsequently mature into myocytes that fuse to form myotubes, consistent with canonical myogenic differentiation pathways. Notably, this reprogramming is only observed in high-serum conditions, such as standard FAP and fibrogenic media, where robust cell proliferation occurs, potentially facilitating increased cell differentiation reprogramming. These findings suggest that human FAPs can be pharmacologically reprogrammed with A366 for myogenic differentiation, presenting a potential avenue to enhance muscle regeneration after injury in translational models.

SIGNIFICANCE/CLINICAL RELEVANCE: Demonstrating the potential for human FAP myogenic differentiation with a drug treatment unlocks the potential to investigate the utilization of this mechanism for improved muscle regeneration after injury in clinical contexts.

REFERENCES: [1] Biferali et al., Sci Adv. 2021.

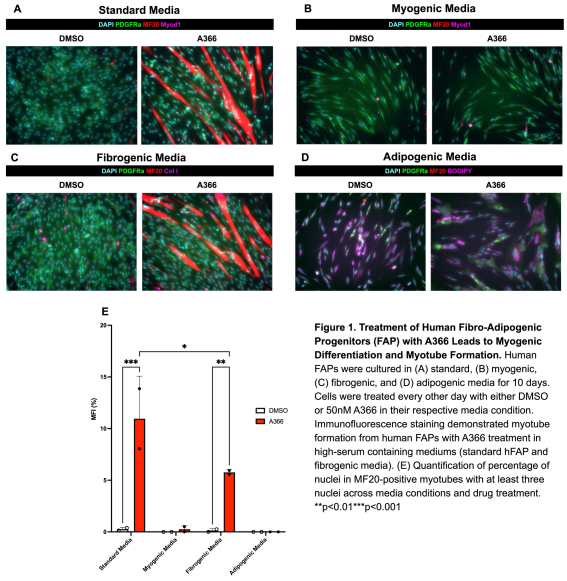

Figure 1. Treatment of Human Fibro-Adipogenic Progenitors (FAP) with A366 Leads to Myogenic Differentiation and Myotube Formation. Human FAPs were cultured in (A) standard, (B) myogenic, (C) fibrogenic, and (D) adipogenic media for 10 days. Cells were treated every other day with either DMSO or 50nM A366 in their respective media condition. Immunofluorescence staining demonstrated myotube formation from human FAPs with A366 treatment in high-serum containing mediums (standard hFAP and fibrogenic media). (E) Quantification of percentage of nuclei in MF20-positive myotubes with at least three nuclei across media conditions and drug treatment. **p<0.01***p<0.001

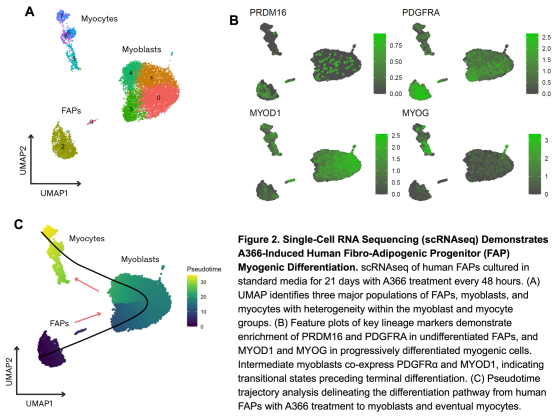

Figure 2. Single-Cell RNA Sequencing (scRNAseq) Demonstrates A366-Induced Human Fibro-Adipogenic Progenitor (FAP) Myogenic Differentiation. scRNAseq of human FAPs cultured in standard media for 21 days with A366 treatment every 48 hours. (A) UMAP identifies three major populations of FAPs, myoblasts, and myocytes with heterogeneity within the myoblast and myocyte groups. (B) Feature plots of key lineage markers demonstrate enrichment of PRDM16 and PDGFRA in undifferentiated FAPs, and MYOD1 and MYOG in progressively differentiated myogenic cells. Intermediate myoblasts co-express PDGFRα and MYOD1, indicating transitional states preceding terminal differentiation. (C) Pseudotime trajectory analysis delineating the differentiation pathway from human FAPs with A366 treatment to myoblasts and eventual myocytes.