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MYOTUBES NEGATIVELY REGULATE FAP-TO-ADIPOCYTE DIFFERENTIATION *IN VITRO*

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Introduction

Skeletal muscle can be a highly regenerative environment, allowing for the repair of injured muscle fibers without scarring or long-term damage, however, it is a complex process involving many different cell types including myogenic and fibro-adipogenic progenitor (FAP) cells in varying differentiation states. The presence or absence of these cells, in their varying states of differentiation, determine the effectiveness of tissue repair. In addition to the presence or absence of cells in the right differentiation state, a complex array of factors play a role in successful muscle regeneration including genetic predispositions of the individual and the current environmental state of the tissue such as the current disease state, and the degree of injury.

The regenerative environment of muscle in healthy individuals is typically stable; however, it can be influenced by the degree of tissue damage and exhaustion or reduction in progenitor cells. In certain disease states, such as Duchenne Muscular Dystrophy (DMD), the regenerative process goes awry and progessive loss of regenerative function occurs.[1] The DMD phenotype arises due to mutations in the dystrophin gene, resulting in no dystrophin production or a lack of functional dystrophin protein. Dystrophin is a critical structural protein found in skeletal muscle and its absence results in severe muscular atrophy and tissue loss. When functional dystrophin is unavailable in the muscle fibers of DMD patients, their muscles tear, break down, and undergo an endless cycle of regeneration resulting in the eventual deposition of fibrotic and fatty tissue with no muscle function. Afflicted patients often become wheelchair-bound before their teens and typically do not survive beyond their late teens or early twenties.[1]

Beyond genetic disorders, a degenerative environment can be produced through Volumetric Muscle Loss (VML) injury.[2] [3] VML is caused by traumatic or surgical loss of muscle tissue resulting in the development of functional issues with the traumatized muscle.[4] This can commonly occur from gunshot wounds, car accidents, or crush injuries. In the case of VML injuries that involve 20% or more of the muscle, the tissue is no longer able to self-repair, and is instead burdened by fibrotic infiltration and associated pathologic secondary conditions. This results in further muscular degeneration, pain, stiffness, and loss of funciton.[5] [6]

In DMD and VML, the dysregulation of cell signaling influences FAP cells, resulting in the deposition of fatty and fibrotic tissue. FAP cells are muscle-resident fibrogenic/adipogenic progenitor cells that may play a role in regulating muscle healing. Interestingly, FAPs do not appear to be important for the development of skeletal muscle; however, a growing body of evidence suggests that these cells may play a critical role in appropriate healing following injury.[7] Thus, the crosstalk between mature muscle, skeletal muscle progenitor cells (skMDC), and FAP cells are critical for proper muscle homeostasis and repair after injury.

Skeletal muscle and FAP cell crosstalk *in-vitro* studies

The purpose of the current study is to begin to elucidate the influence that FAP cells and skMDCs have on one another at various stages of their differentiation processes. To date, significant work has established various cross-talk mechanisms between skeletal muscle and other cell types/organs.[8] In the current study, FAP cells (Cat: FP1111 Lot: T01827-75M.0.2.P2) and skMDCs (Cat: SK-1111 Lot: P01616-21M.1), purified at Cook Myosite, were mono or co-cultured in 0.4µm transwell plates (Corning Cat: 3460) and then analyzed.

Differentiation

skMDC differentiation was performed by culturing the cells for 8 days in differentiation medium (MD-5555) using the Cook MyoSite skMDC Differentiation protocol (see Related Information). Adipocyte differentiation was performed using the Cook MyoSite FAP cell differentiation protocol (see Related Information) for 14 days. For transwell co-culture experiments, skMDC were seeded onto the top of a transwell plate and grown for two days in basal medium (MB-2222) supplemented with growth media and supplement (MS-3333). Then, the media was changed to MD-5555 differentiation media. Differentiation media was then changed again on Day 5 and myotubes were apparent on Day 8 in the top of the transwell. Importantly, no cells were observed to have migrated through the transwell plate into the bottom well. On Day 8 Cook MyoSite FAPs were seeded into the bottom of the transwell plate and grown in SFM+10% FBS for one day to attach, and then cultured in adipocyte differentiation medium

for a total of 14 days, with one media change on Day 7. During this period, the myotubes remained intact in the top of the transwell plate, while the FAP cells were differentiating in the bottom of the plate. Upon completion of the differentiation assays, perilipin staining and targeted gene expression analysis were performed on the cells.

Immunocytochemistry (ICC)

Plates were washed 2x with PBS and then fixed for 15 minutes with 1% PFA diluted in PBS, rinsed in PBS 2x and permeabilized with 0.3% TX-100 diluted in PBS for 15 minutes. Upon completion of fixation and permeabilization, the cells were blocked with 5% donkey serum in 0.1% TX-100 in PBS (blocking buffer) for 1 hour. Rabbit anti-perilipin (Invitrogen Cat: PA5-55046) was diluted 1:200 in blocking buffer and applied for 1 hour. Samples were then washed 2x with blocking buffer at room temperature and anti-rabbit Alexa-Flour Plus 555 (Invitrogen Cat:A32794) diluted to 1:200 in blocking buffer and spiked with 1:500 DAPI (Sigma Cat:D8417-1mg), was applied to the cells and incubated for 1 hour at room temperature in the dark. Upon completion, cells were washed 2x in blocking buffer and imaged. To quantify adipogenic index, FIJI was utilized to determine the area covered in perilipin and DAPI staining.[9]

Gene Expression

For gene expression analysis, two wells of a 12-well plate of differentiated cells were harvested in RLT buffer (Qiagen Cat: 79216) and RNA was extracted using a Qiagen Qiacube with the Cells and Tissues module. For undifferentiated cells, 500,000 cells were pelleted and resuspended in RLT buffer before extraction. RNA was then placed into a 1-step RNA-Ct buffer (Life Technologies Cat:4392938) with targets for IPO8 (as the housekeeping control gene) (Applied Biosystems Cat: 4453320 Gene: Hs0018533), PPARy (Applied Biosystems Cat: 4453320 Gene: Hs01115513), or UCP1 (Applied Biosystems Cat: 4453320 Gene: Hs01084772), and then run using the standard cycling procedure on a QuantStudio 7 Pro. For PDGFRα and myogenic genes, a gene card analysis was run in which RNA was converted to cDNA utilizing a cDNA reverse transcriptase kit (Applied Biosystems Cat: 4369914). cDNA was then loaded into a custom gene card designed to probe a variety of myogenic, senescence, and FAP cell related genes (presented here: PDGFRα (Gene: Hs00998018), MYF5 (Gene: Hs00271574), myogenin (Gene: Hs00231167), and troponin (Gene: Hs00913333)). The gene card was then run on a QuantStudio 7 Pro under standard cycling conditions, and an analysis was performed to correlate gene expression to the IPO8 housekeeping gene.

Differentiated skMDCs Reduce FAP-to-Adipocyte Differentiation and Preferentially Differentiates FAPs into White Adipose Cells *In-Vitro*

To better understand the crosstalk between FAPs and skeletal muscle, transwell experiments were performed. Cook MyoSite skMDC were grown and differentiated into myotubes on the top well of a transwell plate and then Cook MyoSite FAP cells were seeded and differentiated into the bottom well post-myogenic differentiation. This allowed for growth factor transmission and chemical communication between the FAP cells and differentiated myotubes without concerns of confluence inhibition or physical interaction influencing cell fate. Differentiation of skMDCs was noted in the top of the transwell utilizing brightfield imaging, and myotubes were found to remain intact over the course of adipocyte differentiation. In parallel, skMDCs and FAPs were differentiated separately to compare directly to the FAPs differentiated in the presence of myotubes.

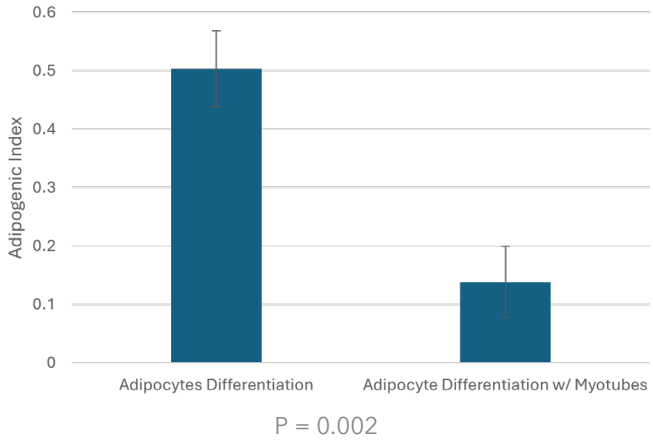
Adipogenic differentiation progressed and, in both instances, with and without the presence of myotubes, adipocytes were observed; however, fewer adipocytes were observed when cultured in the presence of myotubes as seen in the ICC and quantified by adipogenic index (area of perilipin staining/area of nuclei)[10] (Figure 1). Gene expression analysis of undifferentiated FAPs, FAPs differentiated into adipocytes alone, or FAPs differentiated into adipocytes in the presence of myotubes demonstrated drastic gene expression changes with a massive reduction in UCP1 and continued expression of PDGFRα in the samples with myotubes present (Figure 2).

Myotubes that were cultured in adipogenic differentiation medium for 14 days did not demonstrate significant gene expression changes when compared to myotubes harvested immediately after differentiation. Myogenin and troponin expression remained

consistent between the two groups. When compared to undifferentiated skMDCs, MYF5 expression in the myotube group markedly decreased during differentiation as expected, whereas the differentiation genes myogenin and troponin were upregulated despite not being expressed in the undifferentiated cells (Figure 3).

PDGFRα is a characteristic marker of FAP cells and is not typically expressed in mature adipocytes.[11] This current work reinforced this, as during adipogenic differentiation of FAP cells alone, PDGFRα expression was reduced to below detectable levels. Interestingly, expression of PDGFRα was maintained through adipogenic differentiation when in the presence of mature myotubes (Figure 2). UCP1 is a marker characteristic of brown/beige fat,[12] which was markedly expressed during adipogenic differentiation of FAP cells alone, however, in the presence of myotubes, UCP1 expression was diminished.

Figure 1 - Perilipin staining demonstrates a reduction in adipogenic index with myotubes



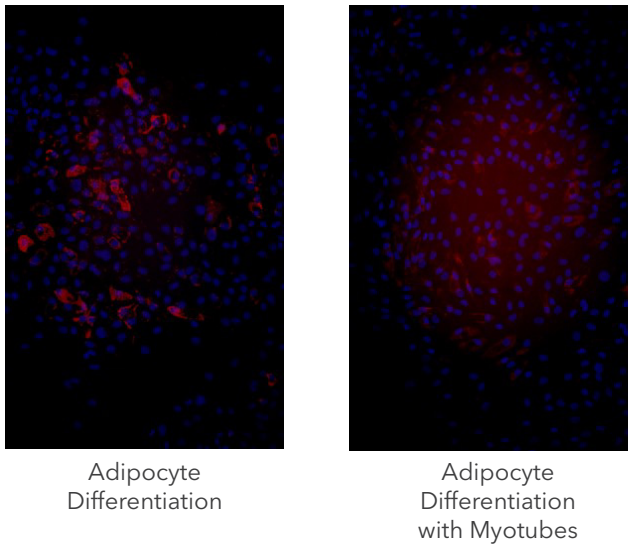


Figure 2 - Genomic analysis suggests more immature FAP/adipocytes in the presence of myotubes

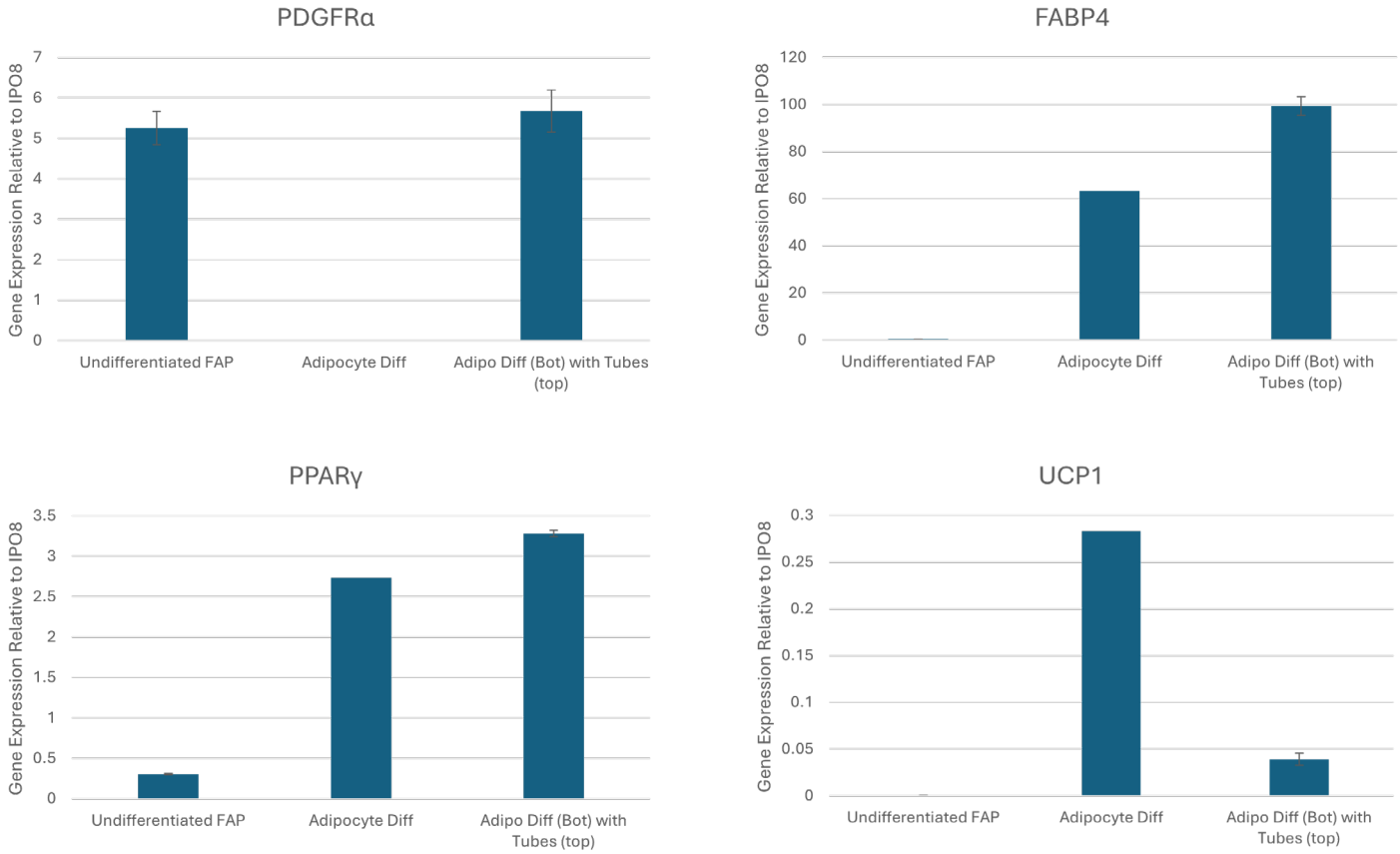


Figure 3 - Myotube formation appears to be unimpacted by incubation in adipocyte differentiation medium

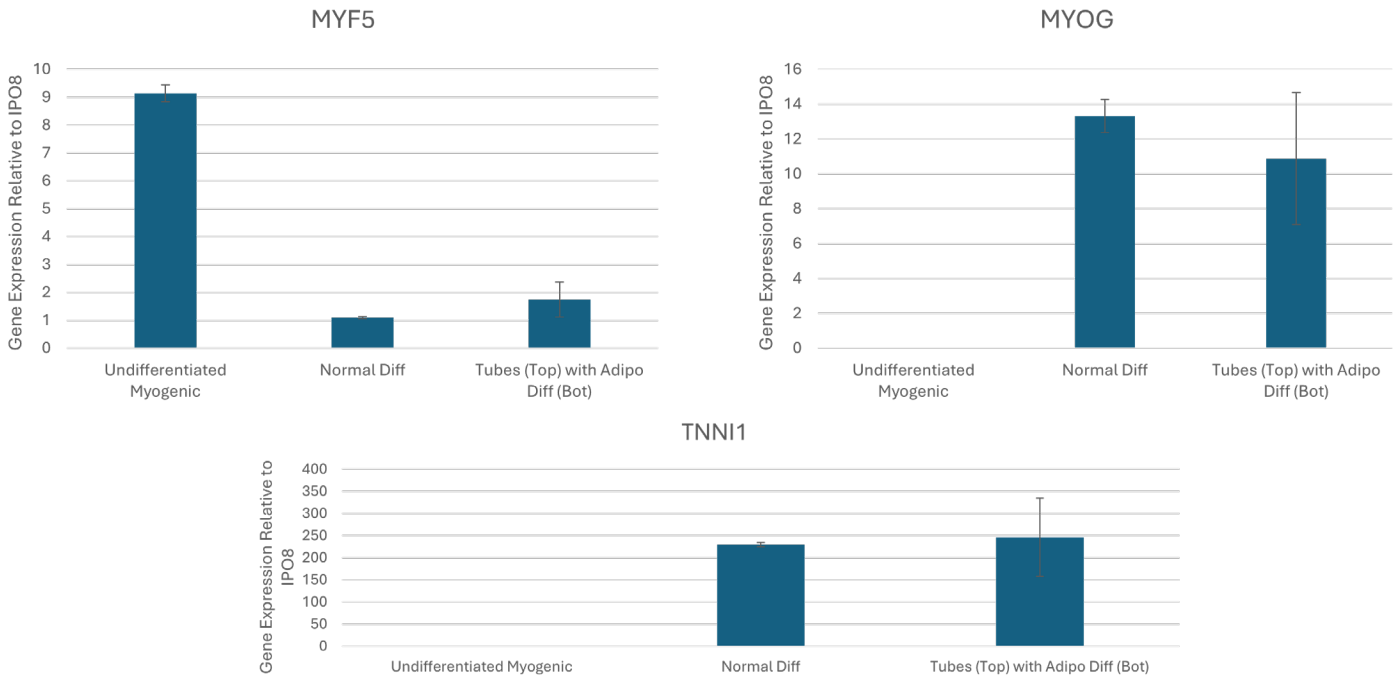
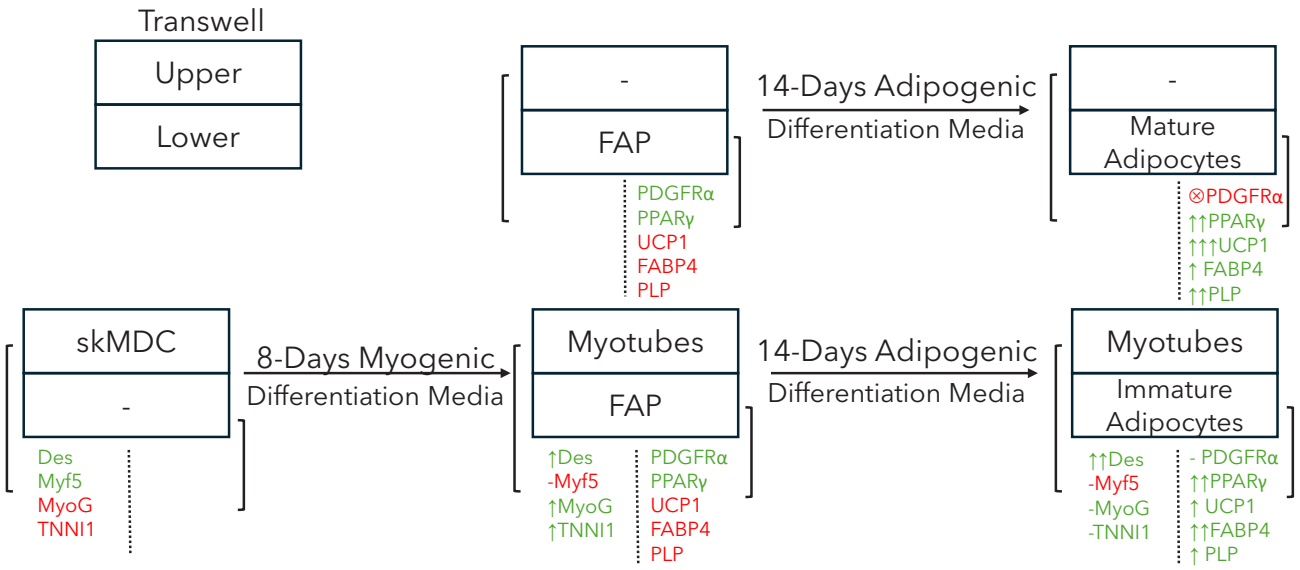


Figure 4 - Results Summary



Conclusions

Disease-induced adipogenesis of muscle tissue can result in a progressive and permanent loss of muscle function. These data demonstrate that *in vitro* cross-talk between FAP cells and differentiated myotubes regulates the differentiation of FAP cells into adipocytes. ICC, using perilipin as a marker of adipocyte differentiation, showed that adipocyte differentiation was attenuated in the presence of myotubes. These types of results are not unprecedented: in a manuscript by Wedell-Neergaard, it was demonstrated that IL-6 signaling can reduce adipose tissue mass with exercise.[13] Analysis of RNA expression in FAP cells differentiated in adipocyte differentiation medium alone suggested they undergo complete differentiation with expression of the brown fat marker UCP1 during this protocol. However, when this differentiation is stimulated in the presence of differentiated skMDC myotubes, UCP1 expression

was massively reduced while PDGFRα expression remained constant (Figure 4), which could indicate that the cells were either too immature and had not yet browned, or that the adipocytes were being driven more towards a white fat phenotype. It has been demonstrated that exercising muscle can result in the browning of adipose tissue.[14] However, without the presence of exercise, it appears that this effect is not produced, at least not *in vitro*. These data suggest that the myotubes may exert an influence on FAP cells by maintaining them in a primordial state, even when being stimulated to become adipocytes. Additional investigation will be required to further elucidate the factors involved in this adipocytic reduction. A better understanding of what factors are being released by mature myotubes that inhibit fat formation and deposition could lead to therapies to prevent this accumulation from occurring in diseased and severely injured skeletal muscle.

Perspective: The Availability Barrier to FAP Cell Research

Compared to the research being performed in myogenic cells, FAP cell research is still in its early stages with new discoveries happening every day in this interesting and impactful cell type. This small study, performed at Cook MyoSite, required the growth of tens of millions of FAP cells to perform, which required months to isolate, culture, and characterize. This underscores one of the main challenges in working with human FAP cells, availability. If the field is to move forward with human FAP cell research, cells need to be made available across a wide range of donor demographics and health conditions. Once made available, this will allow for more work to be translated from rodent models into confirmatory testing in human cells.

In addition to availability, these cells need to be characterized and trusted. Characterization takes considerable time and effort to ensure the cell's purity and potency. FAP cells should reliably differentiate into fibroblasts and adipocytes with known differentiation protocols and be mostly purified away from desmin-positive myogenic cells which could contaminate/influence the results of testing. As shown here, myotubes have the potential to impact FAP cell differentiation and surely myoblasts could do the same. In conclusion, if FAP cell research is to move forward quickly and efficiently, human FAP cells of high purity and known differentiation potential need to be made available to the field to ensure robust translation of rodent work into human cells.

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Cook MyoSite Products Used

Cook MyoSite FAP Fibro-Adipogenic Progenitor Cells

Catalog Number: FP-1111
Lot: T01827-75M.0.2.P2

Cook MyoSite skMDC Skeletal Muscle-Derived Cells

Catalog Number: SK-1111
Lot: P01616-21M.1

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