Development of an In Vitro Model of Muscle Atrophy for Screening of Small Molecule Therapeutics Targeting the Retinoic Acid Receptors (RARs)

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Background

- Skeletal muscle atrophy is a highly occurring disease caused by aging, undernutrition, cancer, and truly all serious conditions leading to the disuse of muscles.
- Muscle atrophy is also very common in space flights where astronauts experience up to 20% of muscle loss.[1]
- Even though its importance to clinical and space medicine, there are still no pharmaceutical therapies for it.
- Muscle atrophy is caused by a complex series of signaling mechanisms in which the activation of the FOXO transcription factor by the previous mentioned conditions upregulates the transcription of the ubiquitin ligases MAFbx and MuRF-1 causing the increase rate of protein degradation and consequently muscle loss.[2]

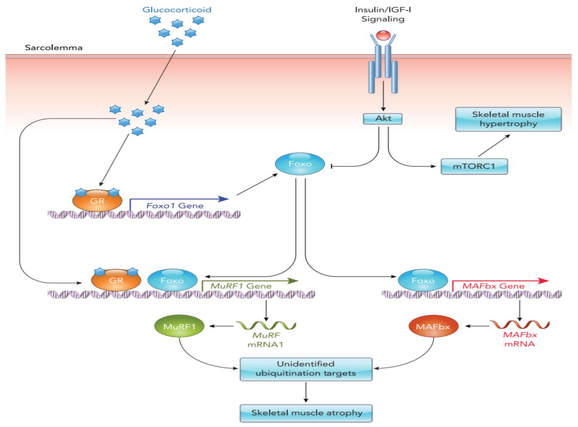
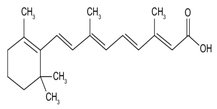


Figure 1. Signaling pathway of muscle atrophy involving MAFbx and MuRF-1. [2]

- Retinoic Acid Receptors (RARα, RARβ, and RARγ) are nuclear transcription factors that regulate differentiation, proliferation and apoptosis in skeletal muscle cells by a variety of different mechanisms.
- Retinoic Acid Receptors are activated by retinoids: a group of small molecules that are hydrophobic, lipid-soluble, and of small size.
- Common retinoids include Vitamin A metabolites and other active synthetic analogs. The most significant retinoid is All -Trans Retinoic Acid (ATRA).



All-Trans Retinoic Acid

- Various studies have shown the importance of the retinoic acid signaling pathway in skeletal muscle cells. RARs promote myogenic differentiation in C2C12 cells [3], maintain satellite cells in immature state [4], promote repair of skeletal muscle cells in mice [5], enhance glucose metabolism [6], and finally induce myotube hypertrophy trough the IGM2 protein. [7]
- RARs ability to allow interaction with biological or synthetic ligands and its significant role in skeletal muscle regeneration makes them excellent therapeutic targets for muscle atrophy.
- Therefore, developing an in vitro model of muscle atrophy to identify small molecule therapeutics targeting the retinoic acid receptors could lead to the discovery of promising treatments.

Objective: Develop an in vitro model of muscle atrophy to screen for small molecules therapeutics targeting the retinoic acid receptors (RARs)

Results

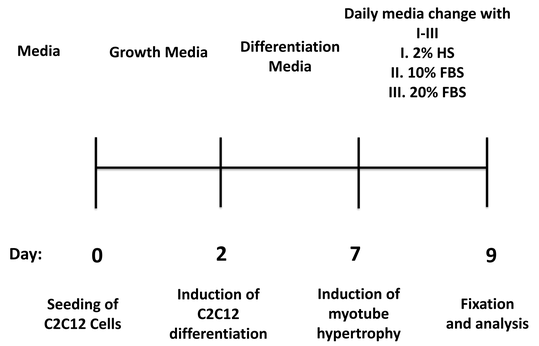


Figure 2. Timeline of C2C12 hypertrophy model. C2C12 myoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% streptomycin (P/S) in 5% CO2 at 37°C for 2 days. Subsequently, the myoblasts were seeded onto 48-well plates at a density of 5.0 x 10^3 cells/cm^2 on day 0 and incubated for 48 hours. After reaching confluency, the media was then replaced by DMEM containing 2% horse serum (HS) and 1% streptomycin and the myoblasts were cultured for a further 5 days with daily media change. Next, myotubes were grown in DMEM with 10% and 20% of FBS for 2 days to cause hypertrophy, respectively.

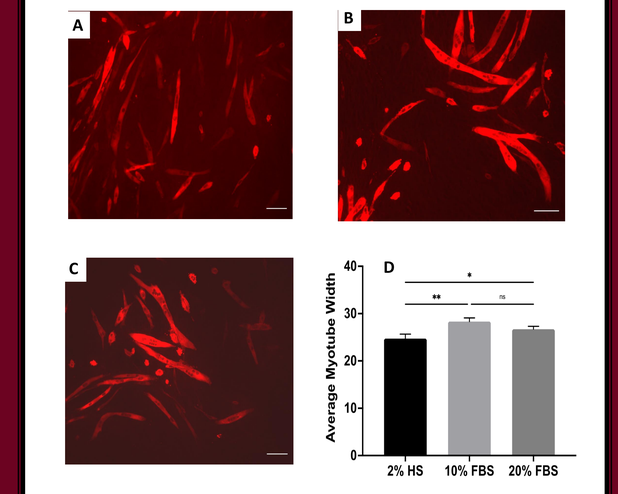


Figure 4. Immunofluorescent images of myotubes during hypertrophy. Immunofluorescence images of (**A**) myotubes receiving only serum starvation media **(B)** myotubes in DMEM + 10% FBS and **(C)** myotubes in DMEM + 20% FBS taken on day 7 and stained with myosin heavy chain (MyHC). This data was quantified by taking the average diameters of the myotubes (15 images per treatment) using ImageJ and is shown in panel **(D)** as mean diameter + standard deviation of the triplicates. One way ANOVA with Tukey Post-Hoc test was conducted, and* represents p<0.05 with ** corresponding to p<0.01. Scale bar, 100 µm.

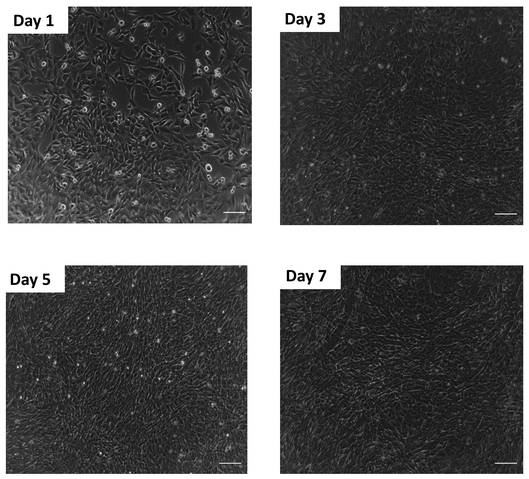


Figure 3. Differentiation of C2C12 myoblasts to myotubes following serum starvation. Differentiation media (DMEM + 2% HS + 1% P/S) was added on day 2 for 5 days with daily media change and phase contrast images were taken of undifferentiated C2C12 cells (**day 1**) and differentiating C2C12 cells at various timepoints (**day 3, 5 and 7**)**.** Scale bar, 100 µm.

Conclusions

- C2C12 cells can differentiate into myotubes using standard protocol.
- Hypertrophy was achieved by both 10% and 20% FBS treated myotubes compared to control.
- There was statistically no difference in mean myotube diameter between treatment with 10% and 20% FBS.

Future studies include:

- Serum starving hypertrophic myotubes with 2% HS for 2 days to induce atrophy as stated in literature
- Optimizing the in vitro atrophy model by treating the hypertrophic myotubes with 1, 0.5 and 0% horse serum.
- Evaluating alternative in vitro atrophy mechanisms such as treatment with Dexamethasone.
- Treating atrophic myotubes with small molecule therapeutics such as ATRA, R667 and other retinoids to inhibit and/or reverse atrophy.

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