Development of Skeletal Muscle Tissues in Collagen Structures

Mina R. Ghobrial[1]; Jonathan M. Grasman, PhD[2]; David L. Kaplan, PhD[1]
[1]Tufts University, Medford, MA; [2] New Jersey Institute of Technology, Newark, NJ

Abstract and Introduction

Volumetric muscle loss (VML) is characterized by the loss of more than 20% of skeletal muscle tissue, and results in a permanent loss of tissue structure and function.[1] The current gold standard of treatment is an autologous tissue transfer, which may result in complications such as donor-site morbidity or infection and is also limited by inadequate vascularization and innervation.[2] There is a clear need to understand how skeletal muscle is innervated to enhance patient specific outcomes. To this end, our approach is to develop a skeletal muscle tissue model to interrogate these components both separately and as a whole.

In this study, we evaluated the efficiency of murine myoblast (C2C12) differentiation in biopolymer hydrogels [collagen type-I (collagen) or fibrin] supplemented with extracellular matrix (ECM) proteins [fibronectin (FN), collagen type-IV (CIV), or laminin-111 (LM)], in different geometric configurations.

We found that myoblast differentiation was maximized with a single needle construct (Fig. 1B) and when hydrogels were supplemented with LM. These findings are essential to generate mature differentiated muscle tissue, and ongoing work is seeking to incorporate endothelial and nerve cells to assess impact on outcomes.

Materials and Methods

A two-dimensional study was carried out to determine which ECM protein concentrations best promoted efficient differentiation. A three-dimensional study was then conducted, using these concentrations and 6.5 mg/mL bulk collagen type-I gels (1.0 x 0.75 x 0.2 cm) cast in a PDMS construct (Fig. 1A).

Two-Dimensional Experiment

- Myoblasts seeded at concentration of one million/mL
- Supplements
  - FN and LM: 1, 10, 100 µg/mL
  - CIV: 2.5, 25, 50 µg/mL
  - Negative Control: 0 µg/mL
- Seeded in 24 well plates

Three-Dimensional Experiment

- Myoblasts seeded at concentration of ten million/mL
- Supplements
  - Collagen: FN100, CIV25, LM100
  - Fibrin: FN100, CIV50, LM1
- Seeded in one or two needle constructs (Fig. 1-B,C)

All Samples

- Collagen (2.5 mg/mL) and Fibrin (3.0 mg/mL)
- Growth Medium (3 days) → Differentiation Medium (4 days) → Fixed in 4% PFA

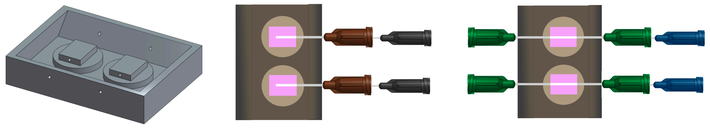

Figure 1. Schematic of 3D constructs. (A) Polycarbonate mold used as a negative for PDMS constructs. (B) One needle and (C) two needle constructs with 1.5 inch 22G (black) and 25G (blue) needles threaded through 0.5 inch 19G (brown) and 21G (green) needles, respectively.

Staining and Data Analysis

- Stained for Myosin (MF-20, 1:50), Actin (phalloidin 1:500), DAPI (1:1000)
- Imaged using 10x (2D) and 4x (3D) objective
- Efficiency of differentiation was quantified as the ratio between the percent myosin positive pixels in each field of view and normalized to actin expression (Fig. 2).
- For 2D images, DAPI was used to count the number of cells. The ratio of myosin positive pixels was then compared to the cell count, as another method to quantify efficiency (Fig. 5).

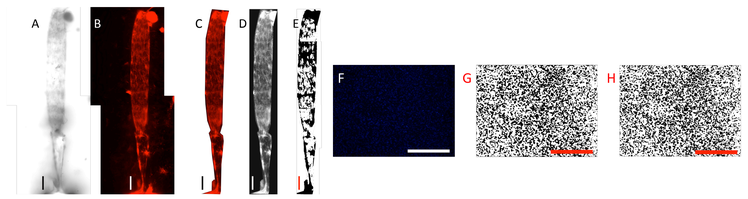

Figure 2. Data gathering protocol. For actin and myosin stains, [in 3D (A) brightfield and (B) stained images are stitched. (C)The stained channel is then isolated based on the outline of the brightfield image. For both 3D and 2D,] (D) in ImageJ, the file is transformed into an RBG stack. (E) The threshold function is then used to isolate pixels that are positive for the stain of interest. ImageJ "measure" feature is then used to quantify the percent coverage. For images of (F) DAPI in 2D, the image is (G) converted to binary, (H) watershed, and particles sized 50-infinity pixels are analyzed. Scale = 500µm

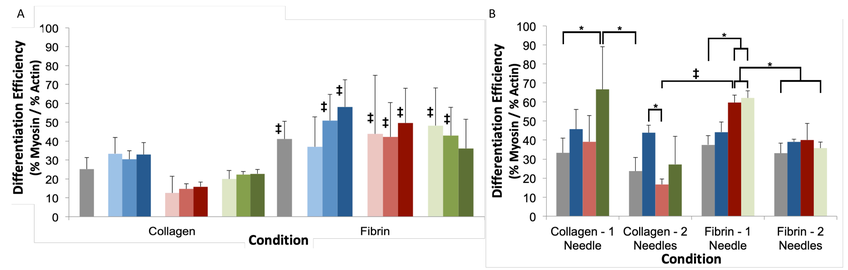

Figure 3. Quantification of differentiation efficiency in myoblasts. Differentiation efficiency across conditions in (A) 2D and (B) 3D. [* indicates $p<0.05$ between supplements of a given hydrogel (collagen or fibrin), as determined by ANOVA and Tukey HSD; ‡ indicates $p<0.05$ between collagen and fibrin of like conditions (2D, 1 needle, or 2 needle), as determined by two-tailed t-test].

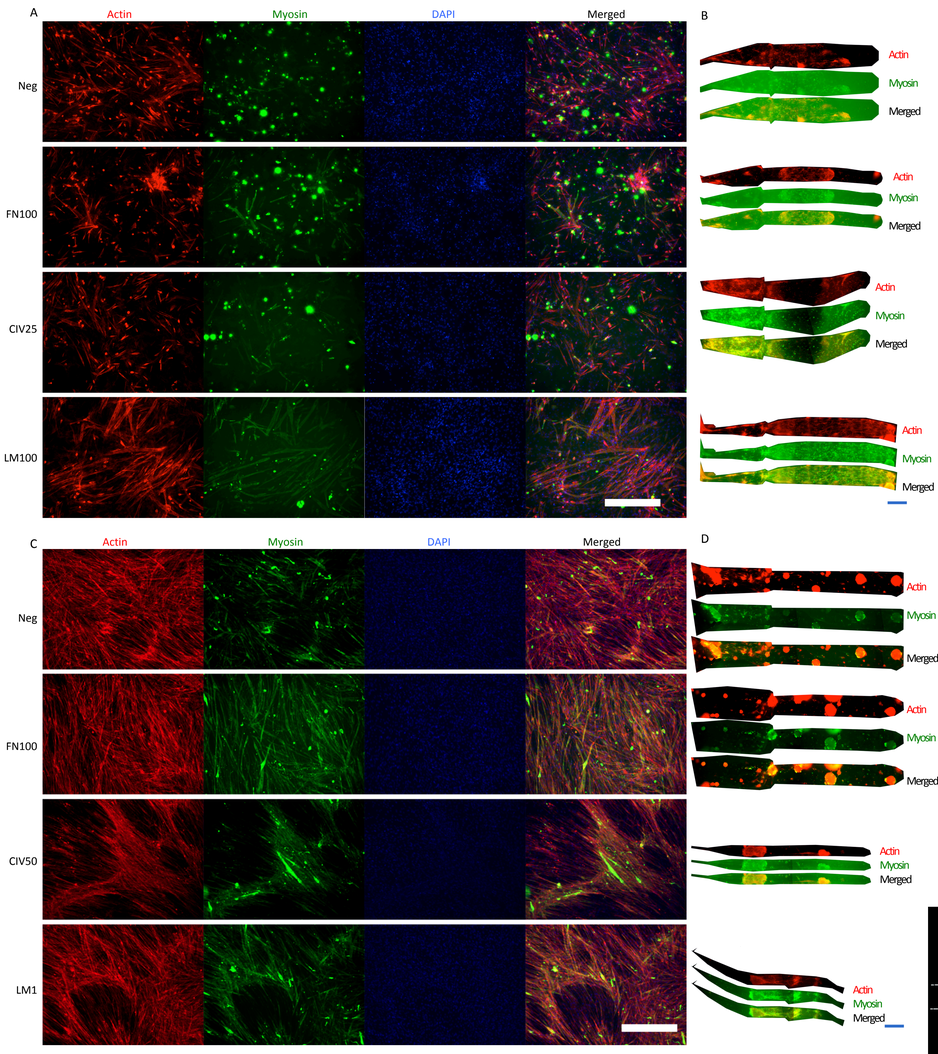

Figure 4. Representative images of 2D collagen (A) and fibrin (C) wells, captured using 10x magnification, and collagen (B) and (D) fibrin channels captured using 4x magnification. Actin (red), myosin (green), DAPI (blue), and overlays of red, green and blue (2D), red and green (3D) are shown. Only the supplement concentrations and channel configurations resulting in the highest differentiation efficiency are shown. Scale = 500 µm

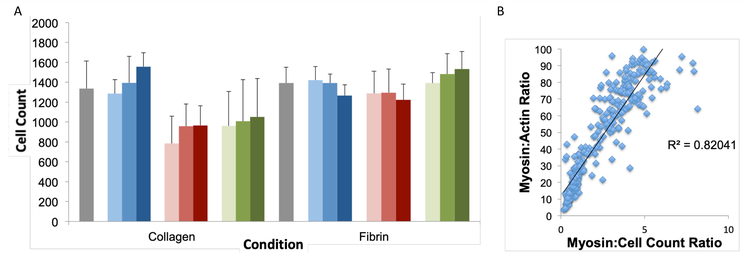

Figure 5. Cell counts as a measure of differentiation efficiency. (A) 2D cell count and (B) comparison of myosin:cell count and myosin:actin ratios as measures of differentiation efficiency. (No significant within-group differences were found.)

Results and Discussion

Differentiation Efficiency

- The ratios of myosin positive pixels per cell were initially calculated. However, in 3D, a cell count could not be determined. Instead, a myosin to actin ratio was used as quantification of differentiation efficiency. A plot of Myosin:Cell vs. Myosin:Actin found a strong correlation between these values (Fig. 5B).

Two-Dimensional Experiment

- **Myoblasts differentiated significantly more efficiently in fibrin than in collagen, across all supplement concentrations except for FN1 and LM100.**
- Differentiation occurred most efficiently in collagen supplemented with FN100, CIV25, and LM100 and fibrin supplemented with FN100, CIV50, and LM1. This decision was made based on:
  - qualitative trends in the data (Fig. 3A).
  - myotube morphology (Fig. 4-A,C). Long, polynucleated myotubes are consistent with differentiation.
- No significant differences in nuclei count were found within each supplement group, implying that seedings were successfully conducted with similar myoblast concentrations across each condition. (Fig. 5A).

Three-Dimensional Experiment

- Myoblasts seeded in the one-needle constructs differentiated more efficiently than in two-needle constructs when supplemented with LM in fibrin.
- Within the 2-needle study, myoblasts seeded in collagen differentiated more efficiently when supplemented with FN compared to LM.
- Within the 1-needle study, fibrin supplemented with CIV and LM supported more efficient differentiation than the negative control.
- **Differentiation was more efficient in collagen supplemented with LM in single needle constructs compared to all other seeding conditions.**

These results indicate that hydrogel composition and configuration, together, affect differentiation efficiency in myoblasts.

Conclusions and Future Work

We demonstrated that muscle tissue seeded within a one-needle fabricated hydrogel significantly enhanced myoblast differentiation compared to two-needle designs. Our data support the hypothesis that supplementation in collagen (LM) and fibrin (LM and CIV) hydrogels significantly enhances myoblast differentiation.

As we eventually create a model of volumetric muscle loss, further studies regarding innervation and vascularization will need to be completed. Previous studies have shown that endothelial cells release neurotropic factors, which direct axonal growth.[4] Preliminary findings interested in identifying a similar effect suggest that the presence of muscle tissue may affect the directionality of neuronal growth (Fig. 6).

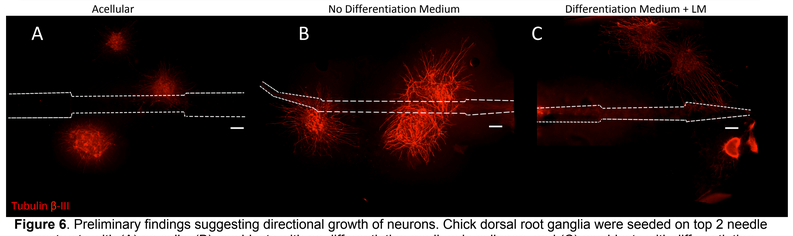

Figure 6. Preliminary findings suggesting directional growth of neurons. Chick dorsal root ganglia were seeded on top 2 needle constructs with (A) no cells, (B) myoblasts with no differentiation medium in collagen, and (C) myoblasts with differentiation medium and LM in collagen. Channels indicated by dashed lines.
Scale = 500 µm

References

1. Corona, B. T., *et al.* J Rehabil Res Dev., 2015; 52(7) 785.
2. Ichihara, S., *et al.* Injury, 2008; 39, 29.
3. Grasman, J. M., *et al.* Adv. Funct. Mater., 2018, 28(48).
4. Grasman, J. M., *et al.* Nature, 2017, 7(1).

Acknowledgements

This work was supported by the NIH (P41EB002520 and F32-DE026058) and the Tufts Summer Scholars program.