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Human Skeletal Muscle Study Through 2D and 3D Model Design

Master's Thesis

submitted in partial satisfaction of the requirements
for the degree of

Master of Science

in Biomedical Engineering

by

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DEDICATION

To

my mother, father, and aunt

in recognition of the value of family and sacrifice

they shaped me into who I am and made every path available to me

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ABSTRACT OF THE THESIS

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Professor Anna Grosberg, Chair

Through the creation of a 2D and 3D model of human skeletal muscle, structural and functional data can be collected. The initiation mechanisms of Facioscapulohumeral muscular dystrophy's (FSHD), a genetic dystrophy that causes increasing muscle degeneration, can be identified through the creation of a reproducible model. Here, we share how the details of various facets and properties of the 2D and 3D design affect a human skeletal muscle *in vitro*. The specifics of how various extra cellular matrix play a role in 2D patterning of skeletal muscle is discussed alongside the intricacies of creating a hydrogel based 3D skeletal muscle bundle. While the models have not yet reached optimal design compared to other techniques, this design is close to reaching a state to produce collectible data.

Chapter 1: Introduction

Skeletal muscle is key to the movement of the human body, and when skeletal muscle is damaged, mobility becomes strictly limited [1,2]. A way skeletal muscle is damaged is through muscular diseases like Myopathies and Dystrophies that cause defects in muscle development, function, and structure [3]. One such dystrophy, Facioscapulobumeral Muscular Dystrophy (FSHD), is caused by abnormal expression of DUX4 gene. The DUX4 overexpression causes increasing damage to muscle structure and function, and as FSHD symptoms progress, patients suffer through increasing muscle degeneration. FSHD can be managed through the use of steroids and physical therapies; however, FSHD is currently incurable and results in total loss of muscle function over time [4]. To investigate a potential cure for FSHD, a model of the muscle function and the initiation mechanisms is crucial. The treatment of FSHD and other muscular diseases will significantly benefit from the creation of a reproducible experimental model.

Because of FSHD cell degeneration, *in vi*vo studies would not be feasible, and primary myocytes collected from the patients directly through needlepoint biopsy or other harvesting methods will have had already experienced degeneration and progressed past disease initiation mechanisms [5]. While human induced pluripotent stem cells (iPSCs) offer the capacity to identify FSHD progression, the differentiation process required to bring iPSCs to myoblasts lengthens the cell culture protocol, and iPSCs do not offer advantages that cannot also be utilized by an immortalized line [6]. Using the Yokomori group's protocol for myocyte differentiation, their two immortalized lines with and without FSHD allow for consistent cells with predictable features that allow for the understanding of the initiation mechanisms surrounding FSHD [7,8,9]. To use these cell lines for the development of treatments, measurements of both physiologically relevant structure and function are essential.

The challenge in collecting structural and functional data is the creation of an accurate *in vitro* 2D or 3D model skeletal muscle that can be used with these cell lines. There are various *in vitro* platforms for measuring structure and function that can be potentially adapted for this project [10]. For example, the printing protocol and design for contractility measurement of the Parker group's heart on a chip offer insights into starting points for controlling myotube alignment and possible controls for a human skeletal 2D model [11]. To further the initial 2D design, a previous study of mouse and human skeletal muscle by the Fienberg group gives an understanding of the optimal conditions needed for microcontact printing for human skeletal muscle, including apt pattern width, and advantages of collagen and laminin ECM [12]. Alternatively, for 3D modeling, the Brusac group has engineered an *in vitro* 3D myotube bundle through the use of 3D PDMS molds that provides a method for contractile force measurements for our 3D model of FSHD afflicted human skeletal muscle [13].

The goal of this project is the creation of a reproducible 2D and 3D *in vitro* model of human skeletal muscle. In the following chapters, we discuss our approaches to methods for the 2D and 3D, and how different facets and properties of the design's components affect the overall structure and function of human skeletal muscle.

Chapter 2: Common Methods

Included in this chapter are the methods shared across both the 2D and 3D design process.

Substrate preparation

Coverslips were prepared as previously described by Drew et al [14]. Briefly, glass coverslips (Fisher Scientific Company, Hanover Park, IL) were sonicated in 95% ethanol and cured overnight

at 65° C. Polydimethylsiloxane (PDMS, Ellsworth Adhesives, Germantown, WI) was prepared in a 1:10 ratio crosslinker to base and spun coated on to glass coverslips. Coverslips were allowed to cure overnight at 65° C. Finally, the large coverslips were cut to size using a diamond scriber (VWR, Radnor, PA)

Silicon Wafer Preparation

Wafers were prepared as previously described by Drew et al [14]. Briefly, stamp designs were drawn using ADOBE ILLUSTRATOR software (Adobe Systems, Inc., San Jose, CA), and were etched into 5 in X 5 in. chrome with sodalime glass masks by a third-party vendor (FrontRange Photo Mask Co., Palmer Lake, CO). Silicon wafers were made through SU-8 deposition using the glass masks in the Bio-Organic Nanofabrication Facility (University of California, Irvine, Irvine, CA). PDMS was poured over the wafer until the desired thickness was reached (60g-80g). It was then cured overnight and released from the wafers. After that, the 25 μm lines separated by 15 μm gaps and similar 20 μm lines by 5 μm gaps patterned regions were cut out and stored for use as stamps.

Seeding and Culture

Cardiomyocytes

As described by Knight et al [15]., neonatal rat ventricular cardiomyocytes were obtained from harvest. Cardiomyocytes were cultured in MEM, 2% FBS, and 1% AB. Following harvest, cardiomyocytes were seeded directly on to patterned and isotropic coverslips. Seeded cardiomyocytes were cultured in 2% FBS media.

Fibroblasts

Fibroblasts were obtained as a byproduct from neonatal rat heart harvests and were collected from preplates as described by Knight et al [15]. Fibroblasts were cultured in media consisting of Minimum Essential Media (MEM, ThermoFisher, Grand Island, NY), 10% Fetal Bovine Serum (FBS, ThermoFisher, Grand Island, NY), and 1% Hyclone Antibiotics Antimycotic solution (AB, GE Life Sciences, Utah). Once the cells reached 80% to 100% confluency, they were passaged using 0.05% Trypsin and seeded. For 2D culture, $4.5x10^5$ Fibroblasts were seeded on patterned and isotropic coverslips. Seeded fibroblasts were cultured in 10% FBS media.

Myocytes

Human primary cultured healthy control myoblasts were immortalized using hTERT with $p16^{INK4a}$-resistant R24C mutant CDK4 (mtCDK4) and cyclin D1 as previously described [16]. After immortalization, CD56-positive cells were selected by magnetic-activated cell sorting conjugated with anti-CD56 antibody (130-050-401, Miltenyi Biotec). 70 single cell-clones were screened, and 30 clones exhibited standard doubling time (~28hr) as primary cells, and efficient myotube differentiation (>70%). Two of them showed spontaneous twitching after 1-2 week differentiation.

Myoblast differentiation was induced as previously described [17]. KYB0-41 cells were plated onto coverslips at a seeding density of ~2.5×10^5 cells/ml in 2 ml of growth medium (high glucose DMEM (11965, Gibco) supplemented with 20% FBS (FB-02, Omega Scientific, Inc.), 1% Pen-Strep (15140122, Gibco) and 2% Ultrasor G (67042, Crescent Chemical Co.)) in each well of a 12-well dish. Approximately 12-16 hours later, differentiation was induced using high glucose DMEM medium supplemented with 2% FBS and ITS supplement (insulin 0.1%, 0.000067% sodium selenite, 0.055% transferrin, 51300044 Invitrogen). New differentiation medium was changed every day.

Imaging and image analysis

The 2D Culture samples were imaged with an IX-83 inverted motorized microscope (Olympus America, Center Valley, PA). Images were taken using an UPLFLN 40x oil immersion objective (Olympus America, Center Valley, PA) and a digital CCD camera ORCA-R2 C10600-10B (Hamamatsu Photonics, Shizuoka Prefecture, Japan). Ten fields of views were selected at random for each sample and imaged at 40x magnification (6.22 μm/pixel).

Matlab Analysis

A custom written Matlab code was used to classify the confluency and maturity of cells. The classification was done through the combination of finding the following parameters: Actin area, Actin Orientation Order Parameter (OOP), z line length, and z line frequency.

Statics

Statistical analysis was done using ANOVA one-way testing with Tukey’s method. Significance was defined as having a p-value of less than 0.05. Sample sizes for Actin Area, OOP, and z-lines were the number of individual wells for each group.

Chapter 3: 2D Model

Described in this chapter are the methods particular to 2D design. The following results are the 2D design process and the future work to further expand this design.

Extracellular matrix patterning

Geltrex (ThermoFisher, Grand Island, NY) was allowed to defrost in the 4° C overnight. PDMS stamps were sonicated in 95% ethanol for 15 minutes while fibronectin aliquots (FN, 0.1 mg/mL (Fisher Scientific, Hanover Park, IL)) were defrosted on ice during preparations or Geltrex was

prepared. In a biosafety cabinet under sterile conditions, Geltrex was gently pipetted into the desired ratio of ice-cold Dulbecco's Modified Eagle's Medium (DMEM, Vancouver, BC) and gently agitated by pipetting the mixture up and down. After sonication finished, the PDMS stamps, using forceps for handling, were dried using compressed nitrogen. To microcontact print, 250 µl of the Geltrex or Fibronectin mixture was pipetted on to the surface of the patterned side of the stamp. For isotropic prints, PDMS coated glass coverslips were ultraviolet-ozone-treated (UVO) for eight minutes to create a hydrophilic surface, and the treated surface was placed in contact with 250 µl of the ECM mixture. Fibronectin printed stamps and coverslips were incubated in the cabinet for one hour while Geltrex printed stamps and coverslips were wrapped in parafilm and allowed to incubate inside the 4°C refrigerator for one and a half hours. After incubation, coverslips were ultraviolet-ozone-treated and returned to the cabinet for printing. Again, using forceps for handling, the excess mixture was tapped off, and the stamps were dried with compressed nitrogen. The coated, patterned side of the stamps was pressed on to the treated coverslips. Fibronectin or Geltrex transfer was immediate. The stamps were carefully removed not to disturb the printed pattern, and printed coverslips were placed in wells and submerged in 1% pluronic acid solution to block adhesion between regions (5g Pluronic F-127, Sigma Aldrich, Inc., Saint Louis, MO, dissolved in 500mL sterile water). Coverslips were allowed to incubate for 5 minutes in Pluronics before being washed three times in Phosphate Buffered Saline to remove excess (PBS, ThermoFisher, Grand Island, NY). Following the last wash, coverslips were left submerged in PBS, and the plate was wrapped in parafilm for storage. Finally, Geltrex printed coverslips were incubated at 37° C for 30 minutes to solidify Geltrex pattern. Printed coverslips were seeded with fibroblasts, cardiomyocytes or myoblasts within two days of printing.

Fixing and immunostaining

Fixing occurred after the desired day of culture. For 2D cultures, media was aspirated from the wells, and the wells were gently washed three times with warmed PBS. To fix the cells, a solution warmed to 37°C of 4% paraformaldehyde (PFA, VWR, Radnow, PA), and 0.0005% Triton X-100 (Sigma-Aldrich, Saint Louis, MO) was added to each well and incubated for 10 minutes at room temperature. Following incubation, the solution was aspirated, and the wells were washed three times with PBS while waiting 10 minutes between each wash. Samples were incubated at 27° C in primary antibodies for nuclei (4,6-Diamidino-2-Phenylindole Dihydrochloride, DAPI, ThermoFisher, Grand Island, NY), actin (Alexa Fluor 488 Phalloidin, ThermoFisher, Grand Island, NY), and sarcomeric α-actinin (mouse monoclonal anti-α-actinin, Sigma Aldrich, Inc., Saint Louis, MO) for 1 hour, washed three times in PBS to prevent background staining, and incubated at 27° C in goat anti-mouse IgG secondary antibodies (Alexa Fluor 633, ThermoFisher, Grand Island, NY) for 1 hour. Once again, the wells were washed three times with PBS and aspirated dry. Coverslips were mounted on glass microscope slides with ProLong Gold Antifade Mountant (ThermoFisher, Grand Island, NY). Clear nail polish was applied to seal coverslips on to microscope slides.

Results

Using the same conditions as the previous printing protocol, here, myoblasts were differentiated into myotubes on top of 20x5 μm lines of microcontact printed Fibronectin (FN). Initial experiments showed actin Orientation Order Parameter (OOP), a function of the uniform probability interval that ranges from 0 for a completely isotropic arrangement to 1 for perfectly

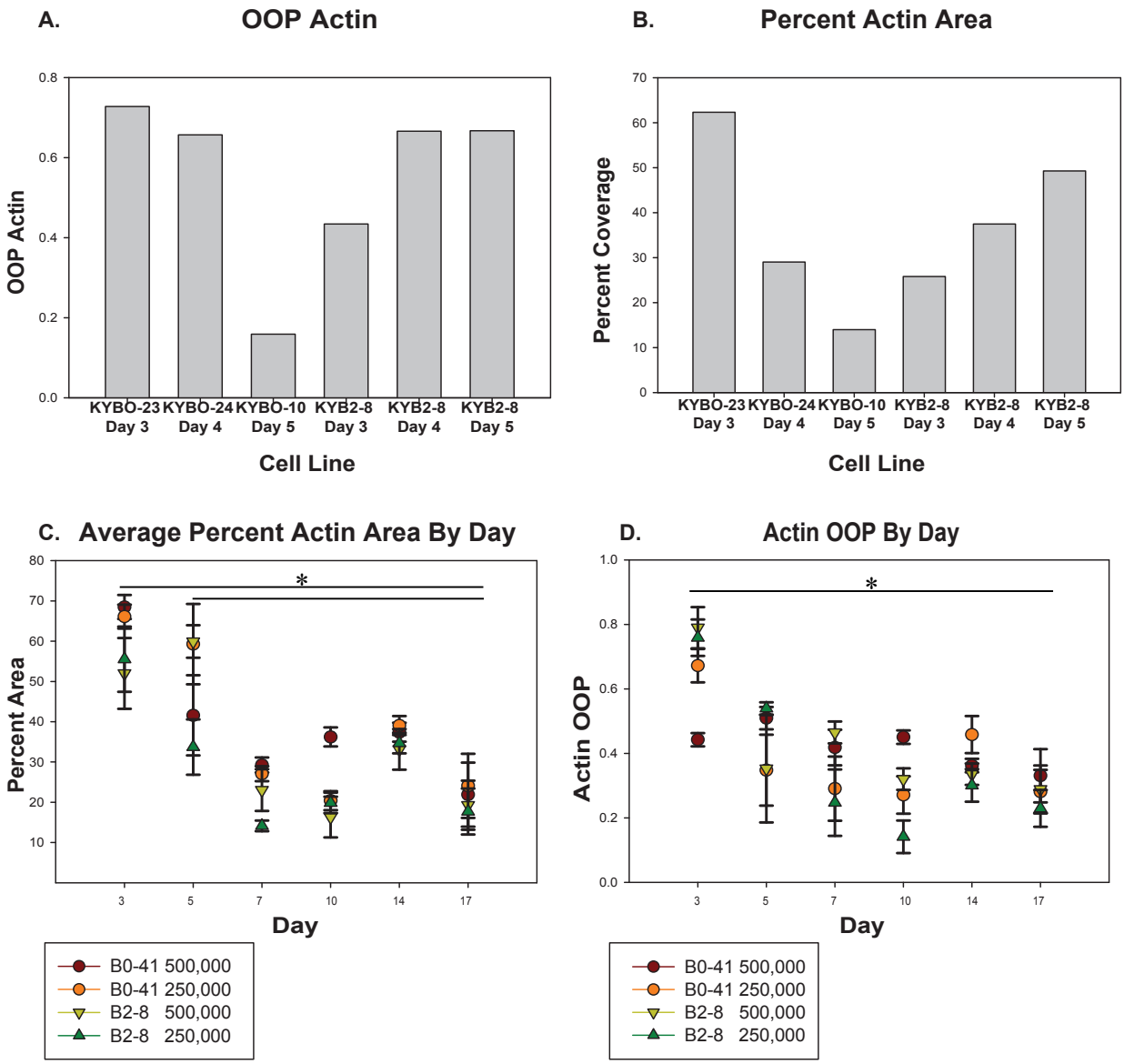

Figure 1. **Initial FN Design Data** (A & B) Fibroblast culture on FN through day 5 of four cell lines. Fibroblast delamination varied by individual coverslips (N=1). (C & D) Fibroblast culture on FN through day 17. Fibroblast confluency was significantly different from day 3 for each cell line and density. Actin area was also significantly less on day 5 from the following days for B0-41 and B2-8 at 250000 and 500000 cells per well in a 12 well plate, respectively. Fibroblast alignment was significantly different for culture days from day 3 for each condition expect b0-41 at 500,000 cells per well (D).

aligned organization, was lower than desired (Fig. 1A & 1B) [18]. As culture time increased, actin area and OOP only decreased as cells were delaminating from the Fibronectin. In order to investigate cell delamination point, the experiment was repeated, culture time was increased, and B0-41 and B2-8 myocytes were seeded at densities of $2.5x10^5$ and $5x10^5$ cells per well of a 12

well plate. After 17 days in culture, cell confluency was inconsistent with only small patches of cells remaining (Fig. 1C & 1D).

Kakulas et al. have shown unstimulated spontaneous twitching of human skeletal muscle recorded between 6-12 days in culture, and this has been confirmed in the selected cell lines by our collaborators [19]. In order to achieve this, a few ECMs that are able to maintain confluent monolayers longer into culture were considered as the extended fibronectin tests proved unfeasible [20].

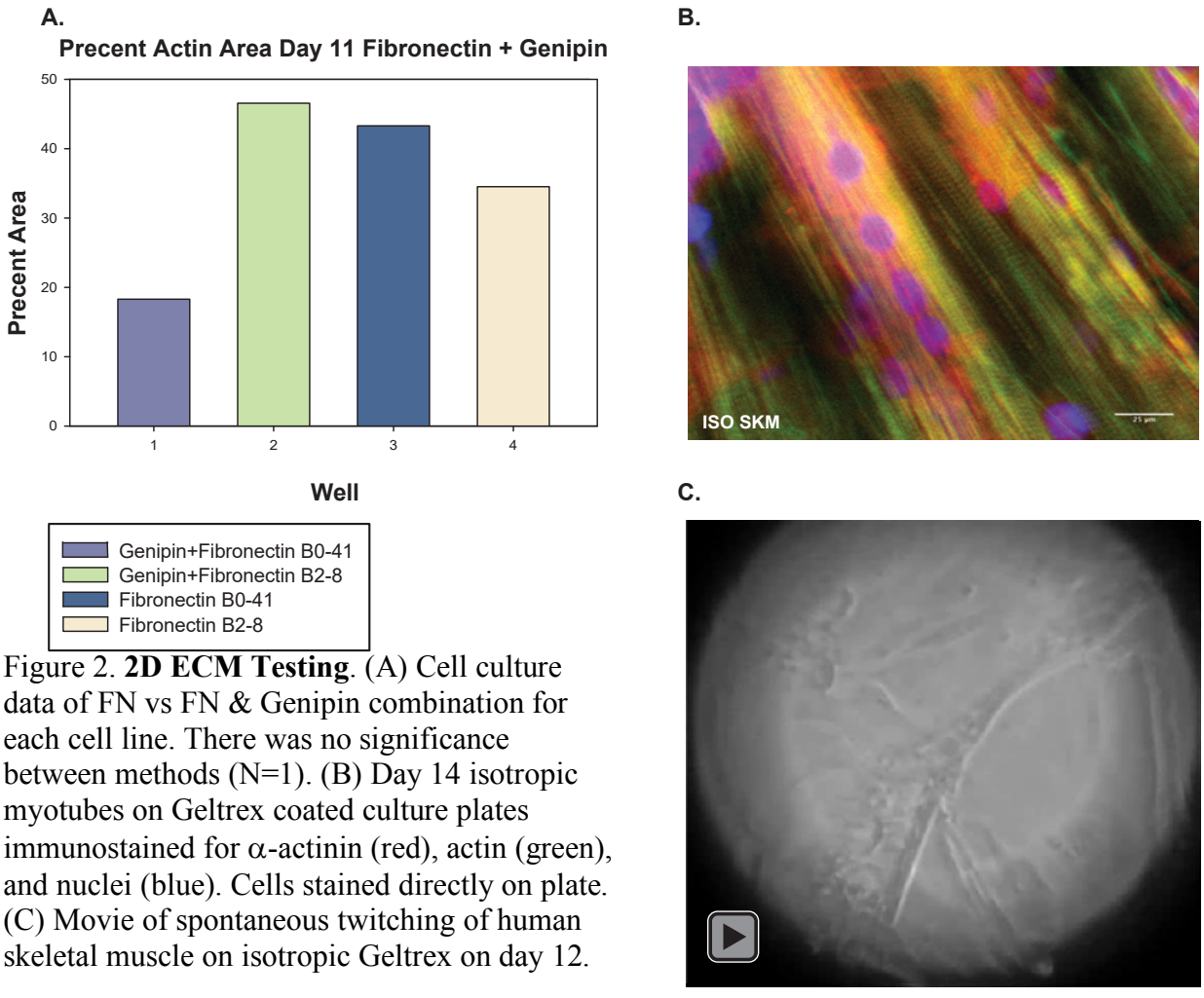

Figure 2. **2D ECM Testing**. (A) Cell culture data of FN vs FN & Genipin combination for each cell line. There was no significance between methods (N=1). (B) Day 14 isotropic myotubes on Geltrex coated culture plates immunostained for α-actinin (red), actin (green), and nuclei (blue). Cells stained directly on plate. (C) Movie of spontaneous twitching of human skeletal muscle on isotropic Geltrex on day 12.

Genipin

Genipin serves as a crosslinker between ECM and the PDMS layer, and its addition has shown to increase the viability of cells longer into culture [21]. Here, Genipin combined with Fibronectin

showed no effect compared to Fibronectin alone; cell confluency was the same or lower with Genipin introduction (Fig. 2A).

STEM CELL Kits

Other projects done by the Grosberg group saw success with the use of cell culture differentiation kits for cardiomyocytes prepared by STEM CELL technologies. We tested the STEM CELL kit for human skeletal muscle that includes both growth and differentiation media and ECM for culture plate preparation. The kit prepared in a 24 well plate maintain highly confluent monolayers across a 14 day time course; however, no twitching was observed.

Geltrex

Geltrex, a basement membrane formulated by ThremoFisher, is often used as a substitute for Matrigel and other gelling hydrogels as it allows for precise control of the gelling properties [22]. The Geltrex preparation in a 24 well plate yielded the highest confluency across a 14 day time course, including spontaneous twitching on day 12 (Fig. 2B & 2C). Since Geltrex provided the highest confluency throughout 14 days in culture and spontaneous twitching was observed, it was chosen as the ECM for the following tests.

In order to achieve a measurable twitch force, a robust and confluent monolayer is essential. Previous experiments have shown that Geltrex would be the best choice for achieving that objective, and it has been shown that Geltrex microcontact printing is possible [23]. So, to optimize, several experiments were performed with various concentrations of Geltrex, and since there is no fluorescent stain for Geltrex, each test was seeded with fibroblasts and kept in culture until confluent (Fig. 3A). These experiments showed Geltrex' binding affinity to each part of the substrate process, and we also confirmed that Geltrex could be laid down through our microcontact printing methods. By moving the Geltrex to the 4° C during the incubation period,

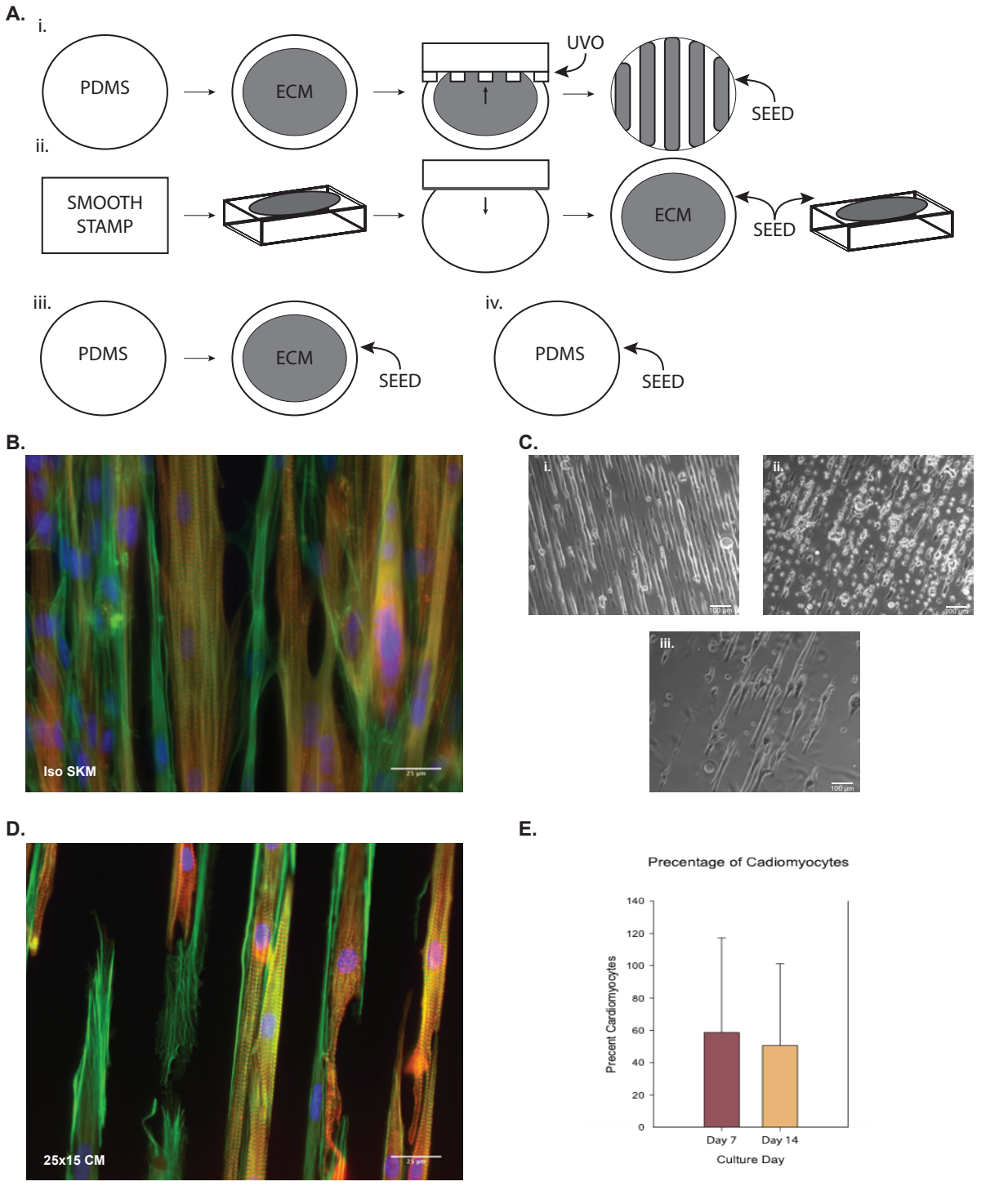

Figure 3. **Geltrex Design**. (A) Schematic of the various methods for Geltrex microcontact printing on PDMS coated coverslips. (B) Isotropic mytotube on Geltrex coverslips plates immunostained for α-actinin (red), actin (green), and nuclei (blue). (C) Patterned 25x15 μm myoctyes on day 1 (i), day 2 (ii), and day 4 (iii). (D) 25x15μm patterned cardiomyocytes immunostained for α-actinin (red), actin (green), and nuclei (blue). (E) Percentage of cardiomyocytes for the set of coverslips after on 7 or 14 days on culture on 25x15μm Geltrex lines (n=6)

this alteration to the previous printing protocol allowed for any Geltrex concentration to be utilized. Now that initial fibroblasts tests aligned and demonstrated that Geltrex microcontact printing was possible, myocytes were seeded at a preliminary concentration of 25 μg/ml or 1:400 ratio of Geltrex to DMEM; however, at 25 μg/ml, myotubes were delaminating as culture time increased. Following several tests of isotropic printing of increasing Geltrex concentration, 200 μg/ml or 1:129 ratio was founded to be the minimum required for more prolonged isotropic culture (Fig. 3B). Despite increasing to a more optimal concentration of 650 μg/ml or 1:40 ratio, patterned myotubes still delaminated with increasing culture time (Fig. 3C). In order to confirm printed Geltrex rather than just isotropic Geltrex would support long term culture, harvested neonatal rat cardiomyocytes were seeded on to 25x15 μm lines. Confluency was maintained throughout the culture with a slight decrease as culture time increased, and spontaneous twitching was observed from culture day 2 through day 11. The percentage of cardiomyocytes in the monolayer was less on day 14 than day 7 although the layer was not significantly different between days (Fig. 3D & 3E).

Despite producing a substrate that maintains confluency for isotropic coverslips, patterned myotubes were still delaminating on later days in culture. Here, we continued experimenting on various cell culture methods to achieve both a confluent monolayer and spontaneously twitching on patterned substrates. Previous experiments have shown myoblasts differentiate better *in vitro* on the edges of culture, so to a localized monolayer on top of the patterned ECM (Fig. 4A), we introduced PDMS reducer, created by pouring PDMS around a 1x1 cm plastic block and cutting the PDMS to size [24]. The reducer creates edges that induce differentiation on top of the area

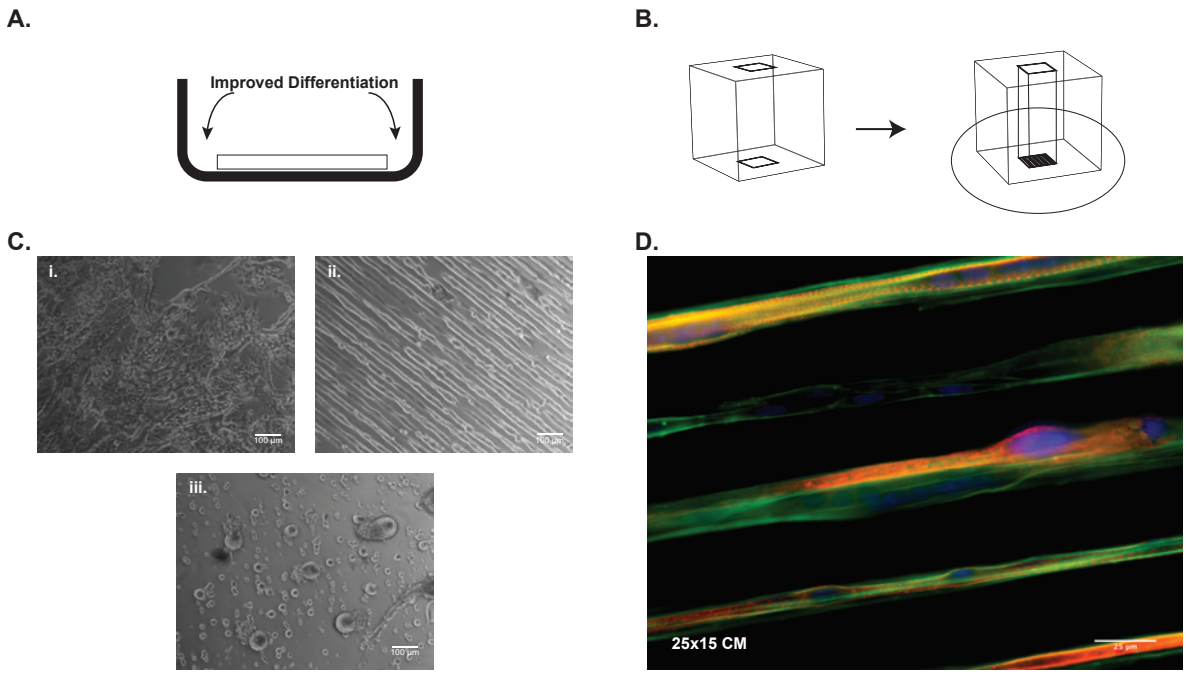

Figure 4. **Geltrex Cell Culture Optimization.** (A) Schematic of myoblast differentiation efficiency (B) Well reducer design. (C) Isotropic myocytes on 1:40 Geltrex on Day 3 (i) Patterned myocytes on 1:40 Geltrex on day 1 (ii) Patterned myocytes on 1:40 Geltrex on day 3 (iii) (D) Patterned myocytes on 1:40 Geltrex seed after differentiation on day 14 immunostained for α-actinin (red), actin (green), and nuclei (blue).

previously microcontact printed rather than the edges of the culture dish (Fig. 4B). Yokomori cell line appeared to be following the same pattern. The introduction of the reducer on top of Geltrex microcontact printed lines did increase cell viability but did not stop cell delamination. Here, cell differentiation is high for isotropic Geltrex; however, the monolayers continue to be inconsistent from delamination of patterned Geltrex (Fig. 4C). Further testing to improve monolayer confluency was to differentiate before patterning. Here, after fusion, myotubes were disturbed and lifted using drops of media then seeded on to isotropic and patterned coverslips. Following seeding, this method saw an improvement in patterned myotube confluence, though this increase was not maintained throughout the coverslips. (Fig. 4D).

Discussion and Future Work

While a confluent monolayer with spontaneous twitching has not yet been seen with this process, the design process exhibited various features of the different parts of the 2D model. The fibronectin culture tests were near to the desired thresholds required for contractility studies, but for the longer culture time needed, a different ECM would be required. Possibly, Fibronectin could be revisited with a different combination of the culture methods which could result in a longer culture time. In the same sense, Genipin may work in combination with the new culture techniques as it has been shown to increase culture time in other applications [25]. Nevertheless, Geltrex proved to be the best ECM to move forward with more combinations of culture methods. We do not yet understand the optimal differentiation day after differentiation to lift the myotubes for patterning and whether or not the reducer will affect the patterning of the myotubes; however, pattern after differentiation emerges as the ideal protocol. As well, patterning wider lines may allow myoblasts more room for differentiation or prevent delamination. Fienberg group showed that wider lines do not increase differentiation efficiency for lines between 50-200 μm in width; however, this may not be applicable as our current design uses lines of either 20 or 25 μm [26]. Line width may play an important role in our differentiation process as our collaborators noted that myocytes delaminating play a function in the myotube fusion.

With the optimal protocol, an aligned, confluent monolayer can have its twitch forces measured. If our cells can reach the threshold of confluence required, we can make further progress with contractility studies. Using a muscular thin film approach, we can identify the twitch force our model can produce. This force can then be compared to the equivalent FSHD model. Alongside force data, our model allows for both structural and functional investigation of FSHD and its mechanisms in 2D.

Chapter 4: 3D Model

In the following chapter, we describe the manufacturing of the PDMS support structure then the hydrogel creation and seeding protocol. Experiments are ongoing for 3D bundle creation, and the results have included from the current design process.

Mold and Channel Fabrication

PDMS masters were 3D printed from plusCLEAR resin using an Asiga pico plus27 3D printer (Asiga, NSW, AU). Following printing, masters were washed twice for 15 minutes and then sonicated for 30 minutes in 95% ethanol to remove excess resin. Masters were post cured for 45 minutes under ultraviolet light. For 12 hours, masters were salinized (UCT Specialties, Bristol, PA) under a vacuum to prevent PDMS adhesion. Following preparation, PDMS was prepared in a 1:10 ratio crosslinker to base and pipetted into masters. Next, for PDMS frames, PDMS was poured directly into dishes until the desired thickness of 5mm was reached. PDMS was degassed until visually all bubbles were removed. PDMS was cured overnight in the oven at 65° C. Carefully, PDMS channels were cut, freed from masters, and returned to the oven. While still hot from the oven, PDMS channels and frames were resized using a 7 mm by 7 mm square punch for the inside of the frame and 11 mm by 11 mm punch for the outer frame in order to prevent buckling when later heated for cell culture. After that, a Velcro tab was cemented in place with 2:1 base to crosslinker PDMS glue using a spacer to press tabs into place during curing.

Formation of Hydrogels

Matrigel (Corning, Tewksbury, MA) and Thrombin (Sigma-Aldrich, Saint Louis, MO) in 1% Bovine serum albumin (BSA, Gibco, Grand Island, NY) in PBS were defrosted overnight on ice in the 4° C refrigerator. PDMS channels and frames were ultraviolet-ozone-treated for sterility, and channels were washed in Pluronics for 45 minutes to prevent hydrogel adhesion and washed

three times in PBS. Flasks at 80% to 100% confluency were passaged using 0.05% Trypsin (Fisher Scientific, Hanover Park, IL), spun, and counted. To create 100 μl bundles, cells pellet was suspended at 1.33 x10^6 myoblasts per 34 μl of growth media, and Fibrinogen mixture was prepared at 20mg per 1 ml of cold DMEM. Following frame and channel preparation, A 96 μl per bundle master mixture (20 μl Matrigel, 20 μl fibrinogen mixture, 22 μl growth media, and 34 μl cell mixture) was quickly and gently mixed not to induce foaming over ice. Master mixture was then mixed with 4 μl of thrombin per bundle, and carefully to not warm the mixture, iced pipette tips were used to transfer 100 μl of hydrogel mixture into Pluronics treated channels already seated inside frames. Hydrogels were allowed to incubate at 37° C for 45 minutes before warmed media was added until just submerging the frames. After day 4 of culture, channels were carefully removed from the bottom of frames and media was replaced with differentiation media. Fibroblast bundles were cultured for 9 days, and skeletal muscles bundles were cultured for 15 days.

Fixing and immunostaining

For 3D culture, bundles were fixed overnight at 4° C in 2% PFA. Fixed samples were washed in PBS and placed in blocking solution (5% chicken serum, 0.2% Triton-X) overnight at 4° C. Samples were incubated in primary antibodies for nuclei (4,6-Diamidino-2-Phenylindole Dihydrochloride, DAPI, ThermoFisher, Grand Island, NY), actin (Alexa Fluor 488 Phalloidin, ThermoFisher, Grand Island, NY), and sarcomeric α-actinin (mouse monoclonal anti-α-actinin, Sigma Aldrich, Inc., Saint Louis, MO) for 24 h at 4° C, washed three times in PBS to prevent background staining, and incubated in goat anti-mouse IgG secondary antibodies (Alexa Fluor 633, ThermoFisher, Grand Island, NY) for 2 hours at 37° C. Once again, the bundles were washed three times with PBS and aspirated dry. Bundles were placed on a cut, glass coverslip, and

coverslips were mounted on glass microscope slides with ProLong Gold Antifade Mountant (ThermoFisher, Grand Island, NY). Clear nail polish was applied to seal coverslips on to microscope slides.

Results

Equally as important as collecting functional data from the 2D model is the creation of the 3D model. Several aspects of the 3D design are still under experimentation, including surface properties, hydrogel concentrations, and cell culture procedures. Since fibrinogen's absorption kinetics with PDMS is not greatly influenced by PDMS's surface hydrophilicity or hydrophobicity, and the addition of BSA to block hydrogel binding with the PDMS support channel, an additional surface is necessary to prompt hydrogel adhesion [27]. Here, a Velcro tab was adhered with 2:1 base to crosslinker PDMS to the interior of frames to facilitate fibrinogen bonding (Fig. 5A). The first experimental tests have been finding the optimal concentrations and volumes for the hydrogel bundles with the correct protocol for preparation. By aliquoting the thrombin separately from the master mixture, the fast gelling property can be precisely controlled. To tests these conditions, frames and channels, as described in the methods, were seeded with our fibroblasts cell solution and hydrogel mixture. By culture day 2, fibroblasts formed a 3D network, and this was maintained until culture day 5 (Fig. 5B). With the confirmation of a 3D fibroblasts network, 3D myoblasts were tested to ensure bundles will localize to the center of channels before channel removal and differentiation (Fig. 5C).

Discussion and Future Work

3D model design creation is still ongoing. Since PDMS channels would need to be removed to allow for differentiation and myotube formation and BSA's ability to block other protein absorption, the UVO process, used in the 2D model to promote patterned ECM adhesion, would

not be sufficient for bonding to frames. Many other 3D models utilize fibrinogen's affinity to bind with plastic micropillars, posts, or frames; however, the ingenuity of the addition Velcro tab

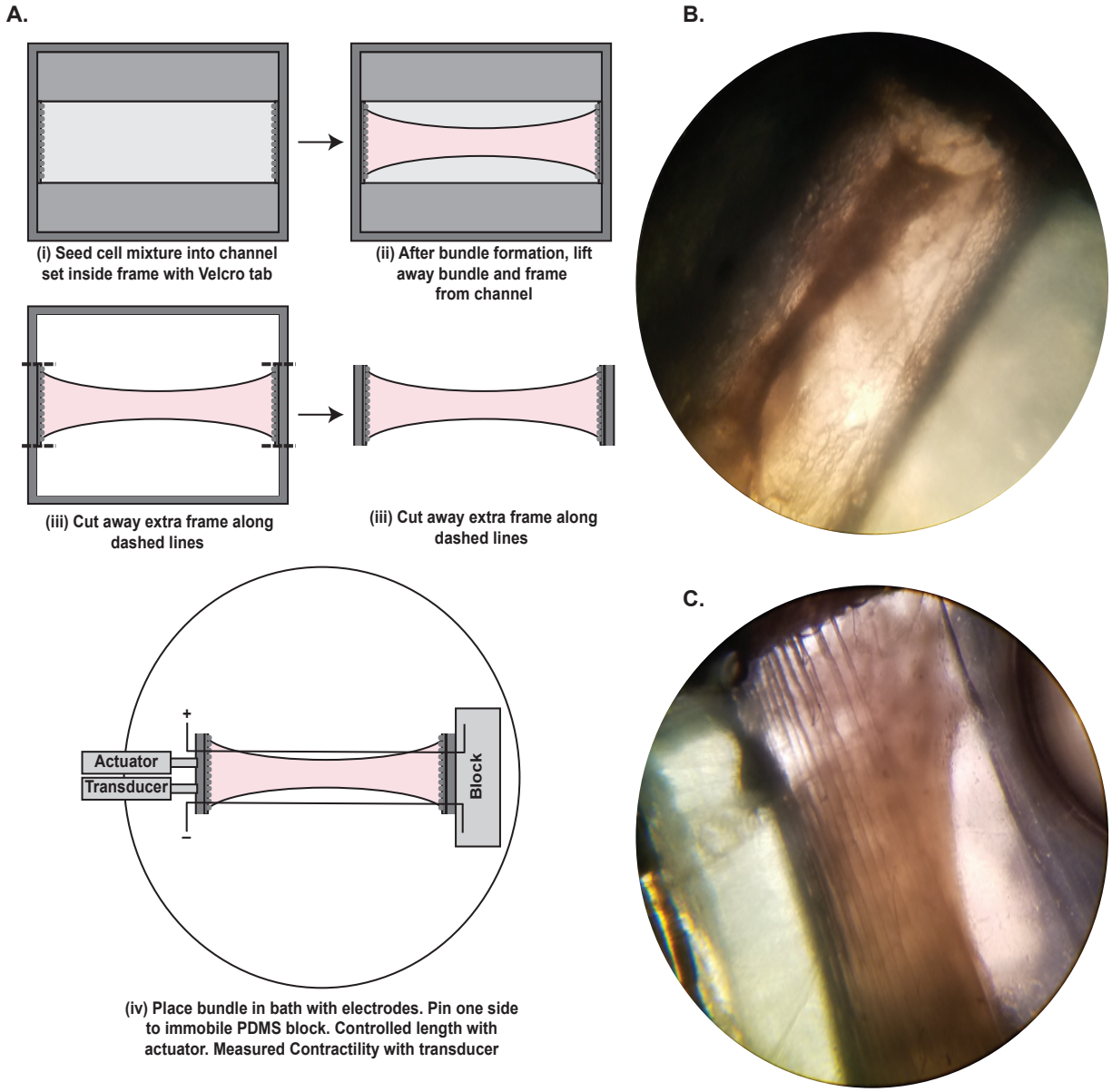

Figure 5. **3D Model Design** (A) Schematic of 3D bundle assembly. (B) 4x image of fibroblast structure in PDMS channel on culture day 1 (C) 4x image of myoblast structure in PDMS channel on culture day 4

allows for the removal of almost the entire of the surrounding frame for force measurements [28]. The fibroblasts' ability to proliferate and their resilience is ideal for primary tests of 3D design. Despite this, the fibroblast network formed in the treated channel has trumped efforts to prevent cell adhesion as fibroblasts possess ability to lay down their own ECM [29]. This weblike network highlights the design's ability to support cells in suspension but prevents the investigation channel removal protocols. Once bundle differentiation is optimized, both force and structural data will be available to be analyzed against the comparable FSHD bundles. The 3D model will be able to produce larger twitch forces than 2D equivalents and will also see changes in structure through cross-sectional analysis. Together with the 2D model's data, our 3D model allows for a holistic structural and functional investigation of FSHD and its mechanisms in 2D and 3D.

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