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Myogenic Differentiation of Embryonic Derived Mesoderm Progenitor Cells

A Thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Bioengineering

by

Manasa Gadde

Committee in charge:

Professor Shyni Varghese, Chair
Professor Gaurav Arya
Professor Ratneshwar Lal

2011

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Chair

University of California, San Diego

2011

DEDICATION

I would like to dedicate this thesis to my family for always believing in me and supporting me. Without their guidance and love, I would not have come this far.

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LIST OF ABBREVIATIONS

ESC	Embryonic Stem Cell
Col I	Collagen I
Col IV	Collagen IV
FBS	Fetal Bovine Serum
hESC	Human Embryonic Stem Cell
HS	Horse Serum
MEF2	Myocyte Enhancer Factor 2
MRF	Myogenic Regulatory Factor
RT	Room temperature

ABSTRACT OF THE THESIS

Myogenic Differentiation of Embryonic Derived Mesoderm Progenitor Cells

by

Manasa Gadde

Master of Science in Bioengineering

University of California, San Diego, 2011

Professor Shyni Varghese, Chair

The ability of embryonic stem cells to differentiate into any cell type provides a potential therapy for skeletal muscle repair. A limitation to such a therapy is the susceptibility of these cells to environmental and chemical signals, making in vitro differentiation into a specific lineage difficult to control and reproduce. Soluble factors, mechanical stress, and cell-cell/matrix interactions influence the cell's commitment to certain lineages. In this study, we aim to determine the media and ECM components that best promote and support differentiation of embryonic-derived

mesoderm progenitor (Hues9D) cells into the myogenic lineage. Hues9D cells cultured in media supplemented with 5% FBS, N2, and hydrocortisone stained positive for Myf5 when analyzed using immunofluorescent staining. Also, immunofluorescent staining revealed positive Myf5 staining of cells grown on collagen I, collagen IV, and laminin. MyoD was expressed in cells grown on laminin and collagen I coated wells. Collagen I and laminin were determined to best support myogenic differentiation of Hues9D cells, while fibronectin best supported proliferation.

I. Introduction

Stem cell engineering is a common research area for treating skeletal muscle defects. Benefits of stem cell engineering are that stem cells are multipotent and can be acquired in large numbers. Drawbacks are their inability to self-renew indefinitely and they cannot be expanded for more than seven to eight passages in-vitro without mutations occurring.[1, 2] These limitations can be overcome by using embryonic stem cells. Embryonic stem cells are pluripotent stem cells that can differentiate into all three germ layers. They have the ability to self-renew indefinitely and can be passaged multiple times in-vitro making but in-vitro behavior of ESCs is very difficult to control.[3-5] Hues9D cells are a cell source derived from embryonic stem cells that display mesenchymal markers. They have the advantage of ESCs in that they do not require a feeder layer to grow and are easier to control in-vitro making them a promising cell source for skeletal muscle treatments.

Hues9D offer multiple advantages over other cell sources but a protocol needs to be established to successfully differentiate these cells into muscle cells. There are many factors that influence what pathway a cell follows such as soluble factors, cell-cell interactions, matrix interactions, dynamic stress, cell shape and polarity, mechanical stimuli, etc.[5] Due to all these factors, it is very difficult to control and replicate ESC differentiation in-vitro. The purpose of this thesis is to determine a protocol for myogenic differentiation of Hues9D cells. My thesis will focus on the use of soluble factors and extra cellular matrix (ECM) interactions for myogenic differentiation of Hues9D cells.

1.1. Aims

1. Determine soluble factors that promote myogenic differentiation of Hues9D cells
2. Identify ECM components that supports myogenic differentiation of Hues9D cells

1.2. Experimental Setup

ESCs were plated on tissue culture plates coated with either fibronectin, laminin, collagen I or collagen IV. Serum concentration in differentiation was tested as follows:

- Differentiation media with no serum
- Differentiation media with following serum supplements:
  - 2% FBS
  - 5% FBS
  - 2% HS
  - 5% FBS

To test the affects of hydrocortisone on ESC differentiation cells were differentiated in media with N2 only, hydrocortisone only or with both as shown below:

- Group 1: Differentiation media with N2
- Group 2: Differentiation media with hydrocortisone
- Group 3: Differentiation media with hydrocortisone and N2

To analyze the coating concentration of ECM proteins, cells were plated on dishes coated with either 25μg/mL or 50μg/mL of protein and then stained. To determine the

matrix that best supports myogenic differentiation, cells were plated on dishes coated with fibronectin, laminin, collagen I or collagen IV and stained for myogenic markers.

1.3. Summary of results

1. Cells that were differentiated in media without serum died overtime.
2. Cells preferred media supplemented with FBS and cells that were in the media supplemented with HS died off
3. Cells favored differentiation media with 5% FBS compared to 2% FBS
4. ESC cells in the differentiation media with both N2 and hydrocortisone stained positive for Myf5. Those in media with N2 or hydrocortisone stained negative for Myf5.
5. Staining revealed better images for a protein concentration of 50 µg/mL
6. Positive Myf5 staining was witnessed on Collagen I, Collagen IV, and Laminin
7. Cells differentiated on Laminin and Collagen I exhibited positive MyoD staining.
8. Fibronectin supported cell proliferations rather than differentiation

II. Literature Review

2.1. Skeletal Muscle

Skeletal muscle is composed of connective tissue and cylindrical elongated cells called muscle fibers (Fig.1). Muscle fibers are a fusion of multiple myoblasts and have many nuclei. Muscle cells are composed of an array of myofibrils, mitochondria, cytosol, t-tubules, terminal cisternae, sarcoplasmic reticulum, and sarcolemma.[6] Myofibrils are made up of sarcomeres which consist of two contractile proteins, myosin and actin. The contractile proteins are responsible for the force generation for muscle contraction. They also contain satellite cells, which allow for regeneration of muscle cells. Muscle fibers also contain two other regulatory proteins called troponin and tropomyosin that are necessary for muscle contraction to occur.[7] Individual muscle cells are surrounded by a layer of connective tissue known as endomysium, which serves to insulate cells from each other. Bundles of muscle fibers are surrounded by the perimysium and epimysium covers the entire muscle to bind the cells together and provide strength and support to the muscle.[6-8]

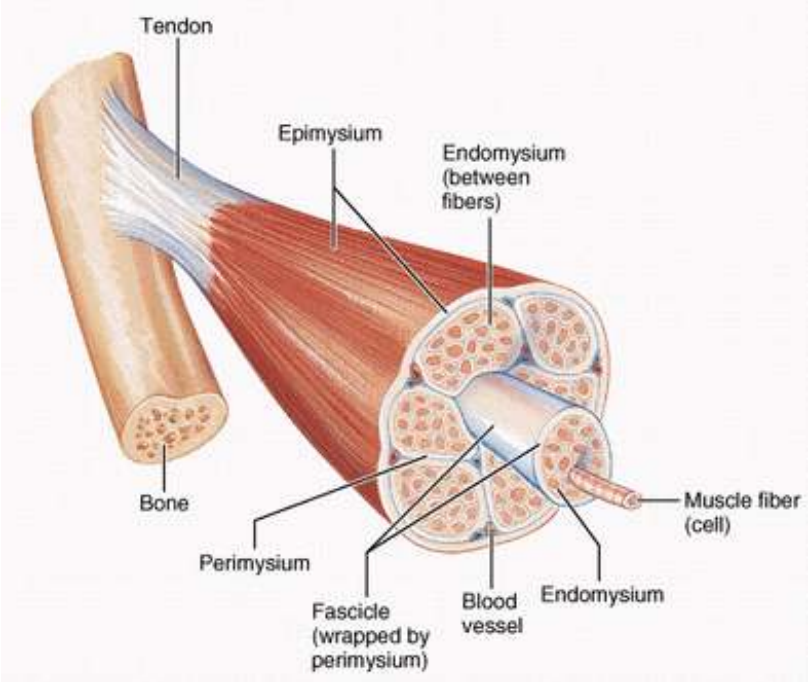


Figure 1: Composition of skeletal muscle[9]

2.2. Skeletal Muscle Engineering

Research focusing on engineering healthy skeletal muscle holds the promise of curing skeletal muscle diseases and repairing muscle damage.[3,10-14] Skeletal muscle injuries are very common and account for 35-55% of sport injuries.[12] Loss of skeletal muscle in patients who are injured or afflicted with muscle wasting diseases such as muscular dystrophy leads to the formation of fibrous tissue. Formation of fibrous tissue prevents the muscle from functioning correctly and can also lead to further damage. Current treatments for skeletal muscle defects are treatments with steroids, surgeries, and physical therapy.[12-14] While these treatments alleviate pain and partly restore muscle function, they do not provide for complete regeneration and repair of the muscle.

2.3. Cell Therapy

As of now, there is no clinically approved cell treatment for skeletal muscle damage but much research is underway in finding a cure. A commonly researched area is cell therapy. Goals of cell therapy are to implant healthy muscle precursor cells into the damaged muscle and have the cells fuse with existing muscle fibers or form new muscle fibers so that healthy muscle can be regenerated. [8,14-16] Transplantation of cells can be allogenic or autologous but there are limitations to each. Using autologous cells decreases the risk of an immune response but acquiring an adequate cell source that can sufficiently provide a large number of cells is hard. Also, acquisition of cells can result necrosis in the tissue surrounding the harvest site.[5,14,16] Another limitation is that if the cells require genetic modification, it is difficult to ensure that the cells will remain altered over time.[5,16-19] Allogenic cells have the advantage that they can be acquired in large numbers and do not require any genetic manipulation but they have a higher risk of eliciting an immune response or graft rejection when implanted in the body.[5,18,20] Due to these limitations, various cells are being studied for their finding a treatment for skeletal muscle loss.

2.4. Cell Sources

Myoblasts

A potential treatment for skeletal muscle damage is the implantation of engineered myoblasts. Rationale for this treatment is that implanting large number of myoblasts will lead to them fusing with the damaged tissue and repairing it.[22] Initial studies have shown both success and failure of this treatment. In some instances

implanted myoblasts were able to fuse with existing muscle fibers [18,23] and in others, the treatments elicited an immune response.[24-28] It was found that myoblasts were damaged by natural killer cells and cytotoxic T cells after implantation.[24] Another reason for failure of myoblast transplant is that the effect is localized and many cells died during the injection of myoblasts to the affected tissue.[24-30] In both cases, the main limitations of using myoblasts are that large number of cells and multiple injection sites are required for the treatment to be successful.

Satellite cells

Satellite cells are another cell source being studied as a possible cell source for the treatment of skeletal muscle damage or loss. Advantages of using satellite cells are their commitment to the myogenic lineage[31,32] and their ability to be passaged multiple times in-vitro.[33] Drawbacks found are massive cell death, immune rejection and limited diffusion of the cells from the injection site.[34,35] Another limitation is that satellite cells have been shown to dedifferentiate in-vitro after a few cycles making it hard to get sufficient number of cells.[36]

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into skeletal muscle among other tissue types.[37] They are considered "immune-privelaged"[38] and have anti-inflammatory properties[39] which makes allografts and xenografts possible. A challenge of using MSCs is their incorporation rate. Brazelton et al. reported that only 5% of transplanted MSCs were able to be fused with existing myofibers. Other disadvantages of using MSCs are that they cannot self-renew

indefinitely, cannot be expanded in-vitro for many passages, and can trans-differentiate into other tissues.[10, 15]

2.5. Embryonic Stem Cells as a Cell Source

Embryonic stem cells are derived from the inner cell mass of the blastocyst and are differentiated in-vitro to form the various cell lines (Fig. 2). They are pluripotent and can transform into any cell type in the body. Concerns with using ESCs are that they are hard to control in-vitro and the formation of teratomas because of their ability to differentiate into all three germ layers. The advantages to using ESCs are that they can self-renew indefinitely, be expanded in-vitro without any mutations for many passages.[2-3]

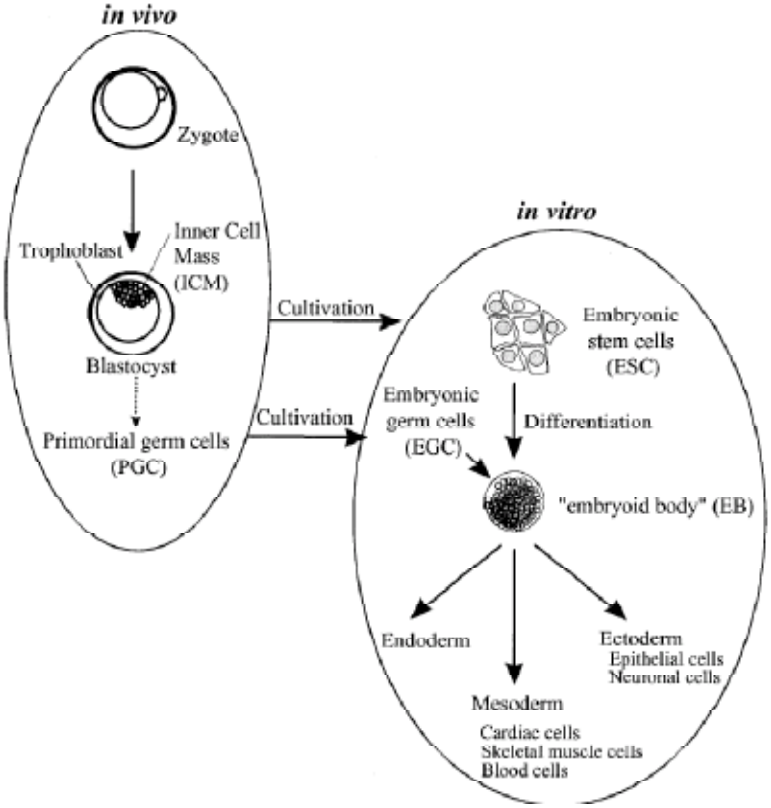


Figure 2: Cultivation and Differentiatin of ESCs[40]

2.6 Myogenesis

In-vivo myogenesis originates from embryonic somites. Various cues from surrounding tissues, mainly dorsal ectoderm, neural tube, and notochord, induce the formation of muscle precursor cells, with cells committed to the myogenic fate then migrating to target muscle groups. The medial portion of the somites gives rise to deep back muscles, while muscle precursor cells migrating from the lateral somites form body wall and limb muscles.[41] Skeletal muscle myogenesis (Fig. 3) is governed by a variety of transcriptional factors known as myogenic regulatory factors (MRFs) and myocyte enhancer factor 2 (MEF2). MyoD and Myf5 are myogenic determinant factors and expressed in the initial stages of myogenic cascade committing the cells into become muscle cells.[3,42] Next, Myog and MEF2 are expressed, promoting the conversion of myoblasts to myotubes. MyoD is still present when myotubes form, and acts with Myog and Myf6 to regulate the gene expression for terminal differentiation and myoblast fusion.[42,45] Rohwedel et al. efficiently differentiated ESCs into myosin heavy chain, desmin, and myogenin positive skeletal muscle cells, They showed the presence of myogenic transcriptional factors in a temporal pattern that reflects in-vivo myogenic pattern.[44]

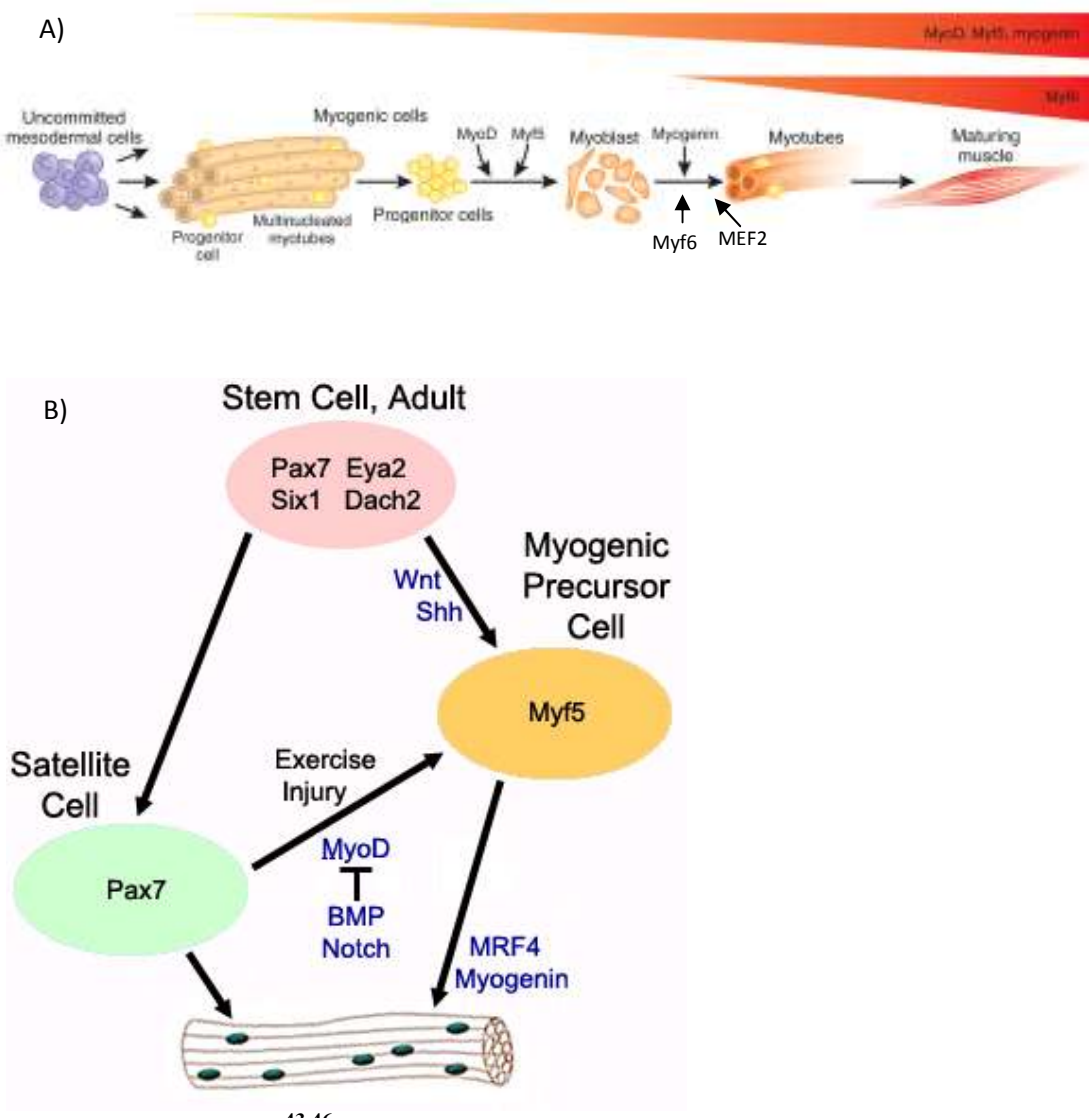


Figure 3: Myogenesis[43,46]
A.Image adapted from [43]; myogenic pathway; B. Transcriptional factors involved in myogenesis[46]

2.7. Transcriptional Factors

Myf5 and MyoD

Myf5 and MyoD are dominant regulators of myogenic progenitor specification and are localized to mononucleated ESCs undergoing in-vitro myogenic differentiation.[44,47-49] Their expression is associated with the initial proliferation and

determination of myoblasts.[47] Both Myf5 and MyoD have overlapping function but Myf5 is connected to myoblast proliferation while MyoD brings about cell cycle arrest and differentiation.[49] Kaul et al showed that mutant mice that were either MyoD (-/-) or Myf5 (-/-) were viable and fertile[50] and Rudnicki et al. revealed that mice born with MyoD (-/-) and Myf5 (-/-) lacked any skeletal muscle and died soon after birth.[51] MyoD (-/-) embryos develop normally and express Myf5 in progenitors, but Myf5 is significantly up-regulated and development of limbs is significantly delayed. [49,51,52] Also, MyoD(-/-) or Myf5(-/-) adults are affected by an increase in satellite cell proliferation and defective regeneration.[49,53-55] Myf5(-/-) embryos exhibit normal limbs, delayed development of myotome and delayed expression of MyoD expression.[52,56]

Myogenin and Myf6

Myogenin and MRF4 are expressed later in the differentiation process when cell fusion starts to occur.[47] Myogenin marks the end of myoblast proliferation and along with Myf6 promotes terminal differentiation and fusion of precursor cells to new or existing fibers.[46,47] Studies have shown that Myog(-/-) or Myf6 (-/-) mouse embryos form Myf5 and MyoD expressing progenitor cells but terminal differentiation of the muscle progenitors is absent[58-60] with the defects being more severe in Myog(-/-) mice. Myog(-/-) mice experience a loss of skeletal muscle and a severe loss of differentiated muscle fibers.[47,61] MyoD (-/-) and Myf6 (-/-) embryos were shown to have more muscle differentiation defects than Myf6 (-/-) embryos since MyoD is crucial for commitment of cells to the myogenic pathway.[62] MyoD(-/-) and Myog (-/-)

and Myf6 (-/-) mutant embryos possess normal numbers of muscle progenitors but fail to form any differentiated muscle fibers.

2.8. Factors Affecting Myogenic Differentiation

Stem cells are surrounded by a complex dynamic microenvironment with which they constantly interact. The microenvironment consists of soluble and insoluble factors, cell-cell interactions, cell-ECM interactions, mechanical and physiochemical signal (Fig.4). These interactions are composed of multiple signals and have been shown to regulate cell behavior such as proliferation and differentiation.[64,65]

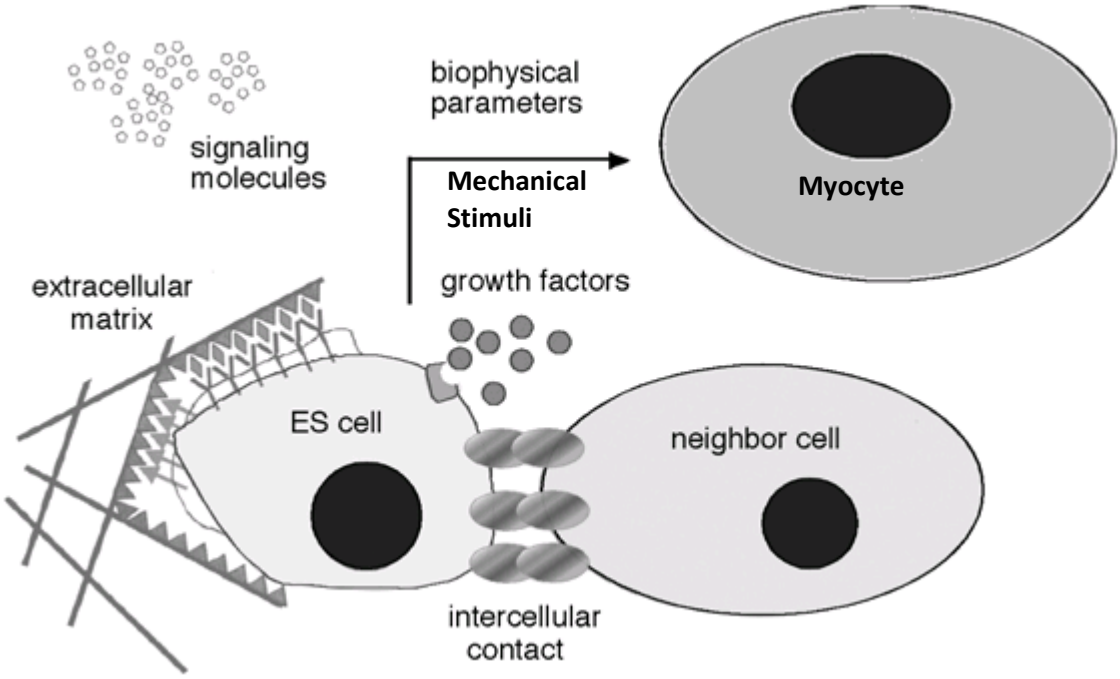


Figure 4: Various Cues Influencing Differentiaion[66]

Extracellular Matrix

Cell-ECM interactions are very complex and have been shown to control cell fate.[67] Rowlands et al. showed interplay of matrix rigidity and ECM protein coating

determined which lineage the cell differentiated to. Myogenic differentiation occurred on all gel-protein combinations that had stiffness > 9 kPa but to varying extents. Highest expression of MyoD1 occurred on gels with a modulus of 25kPa coated in fibronectin and similar levels of expression was observed on 80 kPa collagen I coated gels.[68] Brafman et al. studied the relationship between ECM proteins (collagen I, collagen III, collagen IV, collagen V, laminin, and fibronectin) and hESCs proliferation and pluripotency. They found that the highest proliferation was found on cells seeded on fibronectin and laminin coatings and fibronectin, collagen I, and laminin were able to maintain the pluripotency of hESCs. Also, they determined a mixture of collagen I, collagen IV, fibronectin, and laminin resulted in high proliferation and maintained hESCs pluripotency in long term cultures.[69] Another study showed matrigel and laminin as the preferred ECM coating to sustain stem cell proliferation and myogenic differentiation capacity of the primary porcine muscle stem cells.[70]

Soluble Factors

Cells interactions with factors in media are another factor that influences their behavior. Various components have been shown to promote myogenic differentiation but common ones are dexamethasone, N2, and hydrocortisone. Dexamethasone is used because it has shown to have a positive effect on protein synthesis and a protective effect on muscle cells.[73] It has shown to increase the mRNA levels of myogenic factors such as Myf5, MyoD and MRF4 (Myf6).[74,75] Also, dexamethasone treatment resulted in an increase in creatine kinase levels expressed by muscle cells and myoblast proliferation rate.[76] Another common component of myogenic media is

hydrocortisone which has similar effects as dexemethasone. Hydrocortisone treatment of fetal human myoblasts shortens their doubling time and increases myoblast proliferation rate prior to differentiation.[77] Another effect of hydrocortisone is an increase in the expression of acetylcholine receptors.[78] N2 supplement, a less commonly used component in myogenic media, has been shown to promote myogenesis under serum free conditions. Human embryonic stem cell derived skeletal myocytes terminally differentiated in N2 serum free media expressed MyoD, MyoG and MF20.[79] All these components have previously been reported to promote myogenic differentiation of various cell lines and combining these components could promote stable and efficient myogenic differentiation of Hues9D cells.

III. Materials and Methods

3.1 Media Composition

3.1.1 Growth Media

Composition of the differentiation media is as follows:

- 500mL of DMEM
- 0.5% Pen strep
- 1.0% L-glutamine
- 20% premium select fetal bovine serum (FBS)

3.1.2 Differentiation Media

Composition of the differentiation media is as follows:

- 500mL of DMEM
- 0.5% Pen strep
- 1% L-glutamine
- 5% premium select FBS
- 10nM Dexamethasone
- 50 μM Ascorbic Acid
- Add hydrocortisone solution to a final concentration of 50 μM
    - Dissolve hydrocortisone in 80% ethanol to get ~.05M
    - Dilute it into the media to a final concentration of 50 μM
- Add N2 right before using the differentiation media
    - N2 stock is 100x- needs to be diluted to 1x in the stock media

3.2. Expansion of HUES9-Derived cells

Hues9D cells were obtained from a previous protocol.[72] Cells were cultured in T-175 flasks at 37^{o}C and 5% CO_2 in growth media (500 ml high glucose DMEM supplemented with 20% premium select fetal bovine serum (FBS), .5% Penstrep and 1% L-Glutamine). Media was changed every 3 days until the cells became 70% confluent and they were passaged at this point. Passaging cells consisted of aspirating the media, washing the cells with PBS, adding 8mL of 0.25% trypsin–EDTA for 3 min at 37°C and then neutralizing the trypsin with the addition of growth media. The cells were then collected and counted using the hemocytometer. Cells were then plated in new T-175 flasks at a density of 5000 cells/cm^2 and passaged until they reached their fourteenth passage.

3.3. Preparation of ECM solutions

3.3.1 Human Fibronectin

Fibronectin stock was obtained from Gibco (cat#33016-015). Stock was diluted to a concentration of 50 μg/mL in autoclaved DI H_2O.

3.3.2 Human Laminin

Laminin was obtained from Sigma-Aldrich (cat# L6274). It was diluted in autoclaved PBS to a concentration of 50 μg/mL.

3.3.3 Human Collagen I

Collagen I was obtained from Sigma-Aldrich (cat# C7774) and diluted in sterile 0.01M HCl to a final concentration of 50 μg/mL.

3.3.4 Human Collagen IV

Collagen IV was obtained from Sigma-Aldrich (cat# C5533) and diluted in sterile 0.01M HCl to a final concentration of 50 μg/mL.

3.4. ECM Coating of Coverslips

Coverslips were coated with 250 μl of protein solution and incubated at 37^{o}C for 3 hours. Col I and Col IV coated coverslips were exposed to uv light in culture hoods for 2 hours after incubation for sterilization.

3.5. Cell Seeding

Hues9D cells were seeded onto the coated coverslips at a density of 5000 cells/cm^2. Cells were grown in growth media with media changed every three days until they reached 70% confluency. They then were transferred to differentiation media; this will be considered as day 0. Differentiation media was changed every three days.

3.6. Immunofluorescent Staining

Cells were stained for MHC (Hybridoma Bank, cat#A4.1025-C), Myf5 (Santa Cruz Biotechnology, cat# sc-302), MyoD (Santa Cruz Biotechnology, cat# sc-302), and MF20 (Hybridoma Bank, cat# MF20-c), on days 0, 7, 14, 21, 28 and imaged. Staining procedure is as follows:

- Aspirate medium and wash with PBS once to clean debris
- Wash the samples in paraformaldehyde (about 1mL) for 10 mins at RT
- Aspirate and wash the samples with PBS three times at RT for 10 mins/wash
- Place the samples in secondary buffer for 30 mins at RT

- Add the primary antibodies and place in a humid chamber for 1hr at RT or overnight @ 4^{o}C
    - Use a ratio of 1:100 (Antibody : secondary buffer)
- Wash the samples with PBS, three times at RT and 10mins/wash
- Add the secondary antibodies and place in a humid chamber for 1hr at RT or overnight @ 4^{o}C
    - Use a ratio of 1:250 (Antibody : secondary buffer)
- Wash the samples with PBS, three times at RT and 10 mins/wash
- Mount the samples using DAPI

4% Paraformaldehyde

- Add 30mL of DIH_2O to a 50mL falcon tube and microwave for ~20-25seconds
- Add 2g of paraformaldehyde and 100uL of 6M NaOH to the water and vortex
- Add 5mL of 10x PBS and bring up the volume to 50mL with DI H_2O

Secondary Buffer

- Add 30mL of PBS to a 50mL falcon tube
- Measure and add 1.5g BSA and 50uL of Triton-X-100 to the PBS
- Vortex it until everything is dissolved.
- Bring the volume to 50mL using PBS and vortex

IV. Results

4.1. Optimization of Differentiation Media

4.1.1 Differentiation Media without FBS

Cells placed in growth media supplemented with 20% FBS proliferated on all the ECM. Once they were switched into myogenic media lacking FBS, the cells initially elongated, displaying a myogenic morphology. By day 7, they cells started to die off due to a lack of serum in the media (Fig.5).

A)

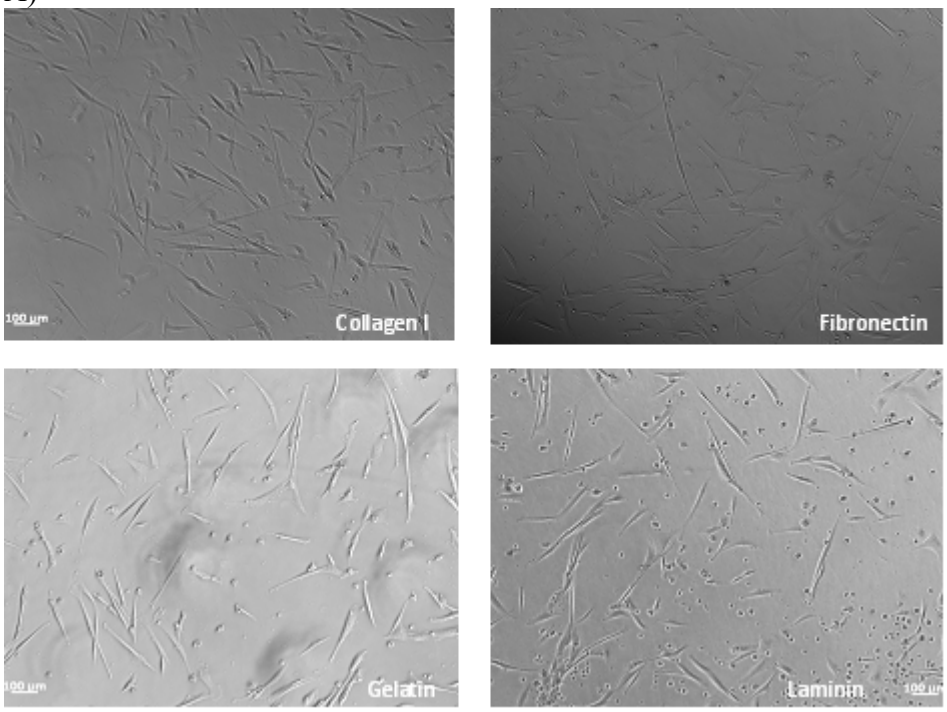


Figure 5: Cells in Myogenic Media without Serum
A) 4 days into myogenic media; B) 7 days into myogenic media.

B)

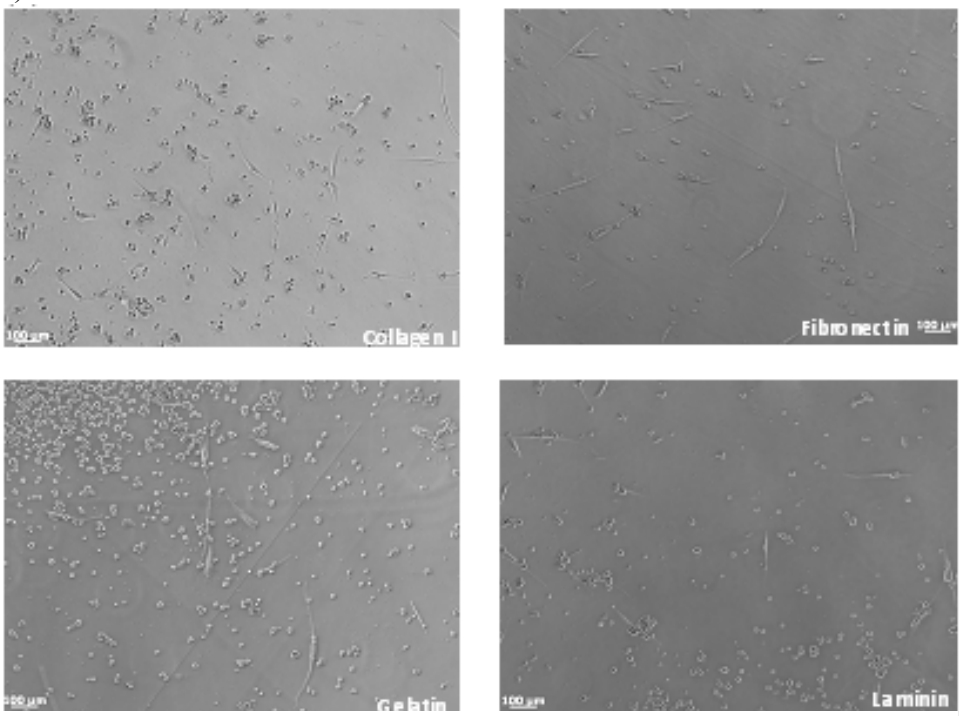


Figure 5: Cells in Myogenic Media without Serum, Continued
A) 4 days into myogenic media; B) 7 days into myogenic media.

4.1.2. Differentiation Media Supplemented with HS or FBS

Cells were once again grown in growth media supplemented with 20% FBS for 3 days. Then they were switched into media supplemented with either 2% HS, 5% HS, 2% FBS, or 5% FBS. Cells are shown below at 2 days into myogenic media (Fig. 6) and 7 days into myogenic media (Fig. 7). Unlike the previous study, cell death was much lower in the groups supplemented with FBS showing that the cells require a low level for survival during differentiation. No live cells were found by day 7 in the HS treatment revealing that cells prefer FBS to HS and that HS cannot support cell viability during differentiation.

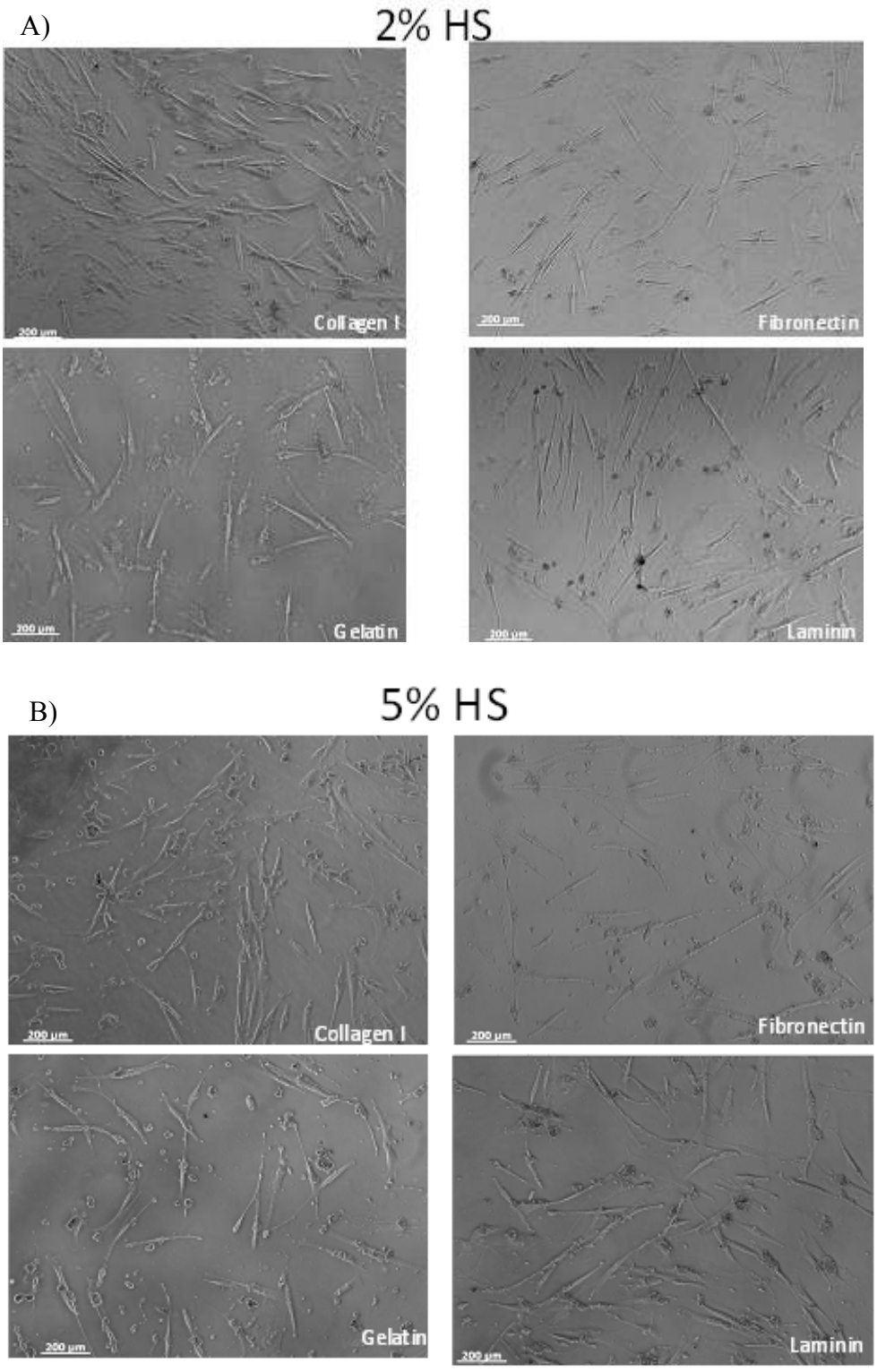


Figure 6: Day 2 of Cells in Myogenic Media Supplemented with Serum
A) 2% HS; B) 5% HS; C) 2% FBS; D) 5% FBS

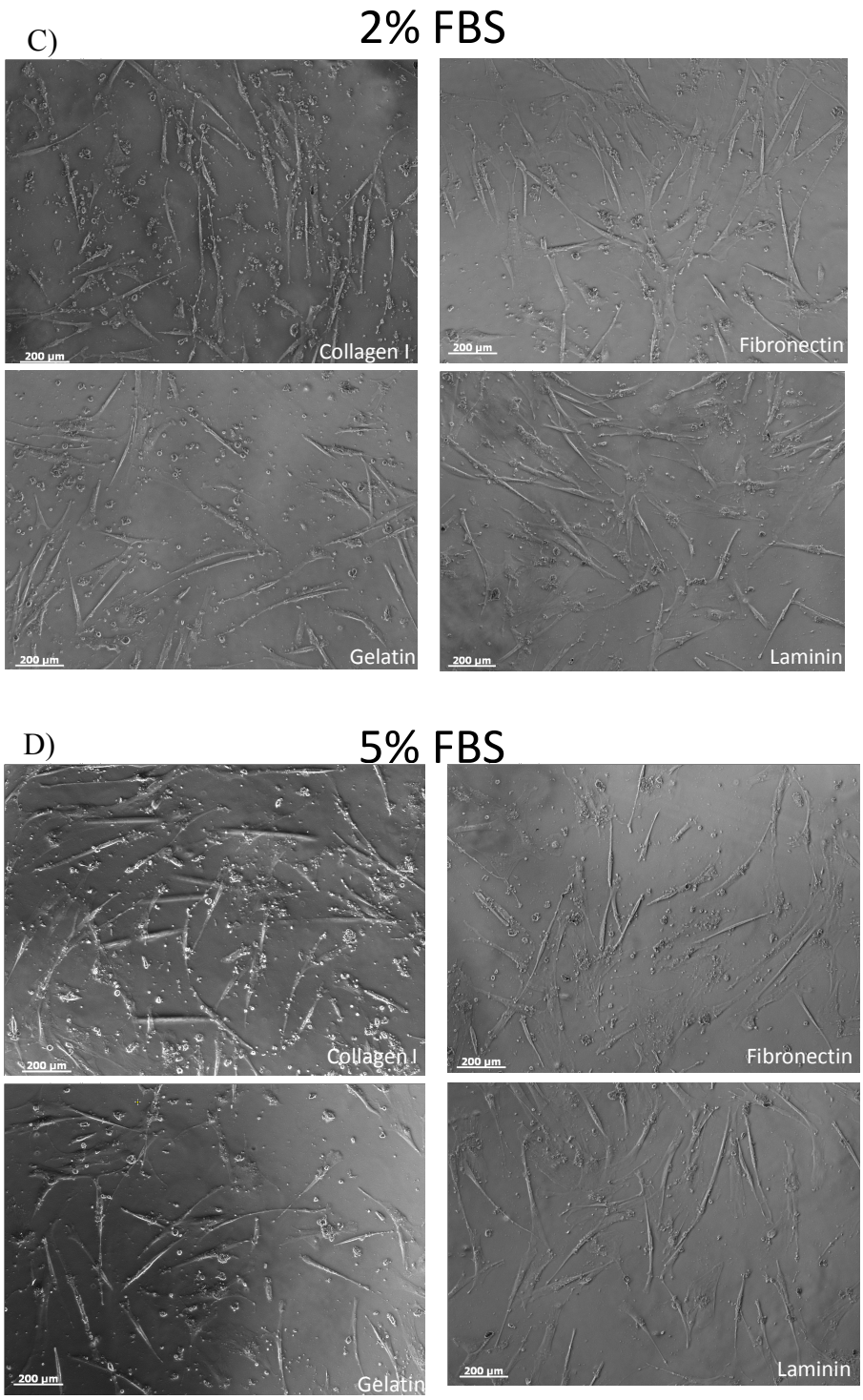


Figure 6: Day 2 of Cells in Myogenic Media Supplemented with Serum, Continued
A) 2% HS; B) 5% HS; C) 2% FBS; D) 5% FBS

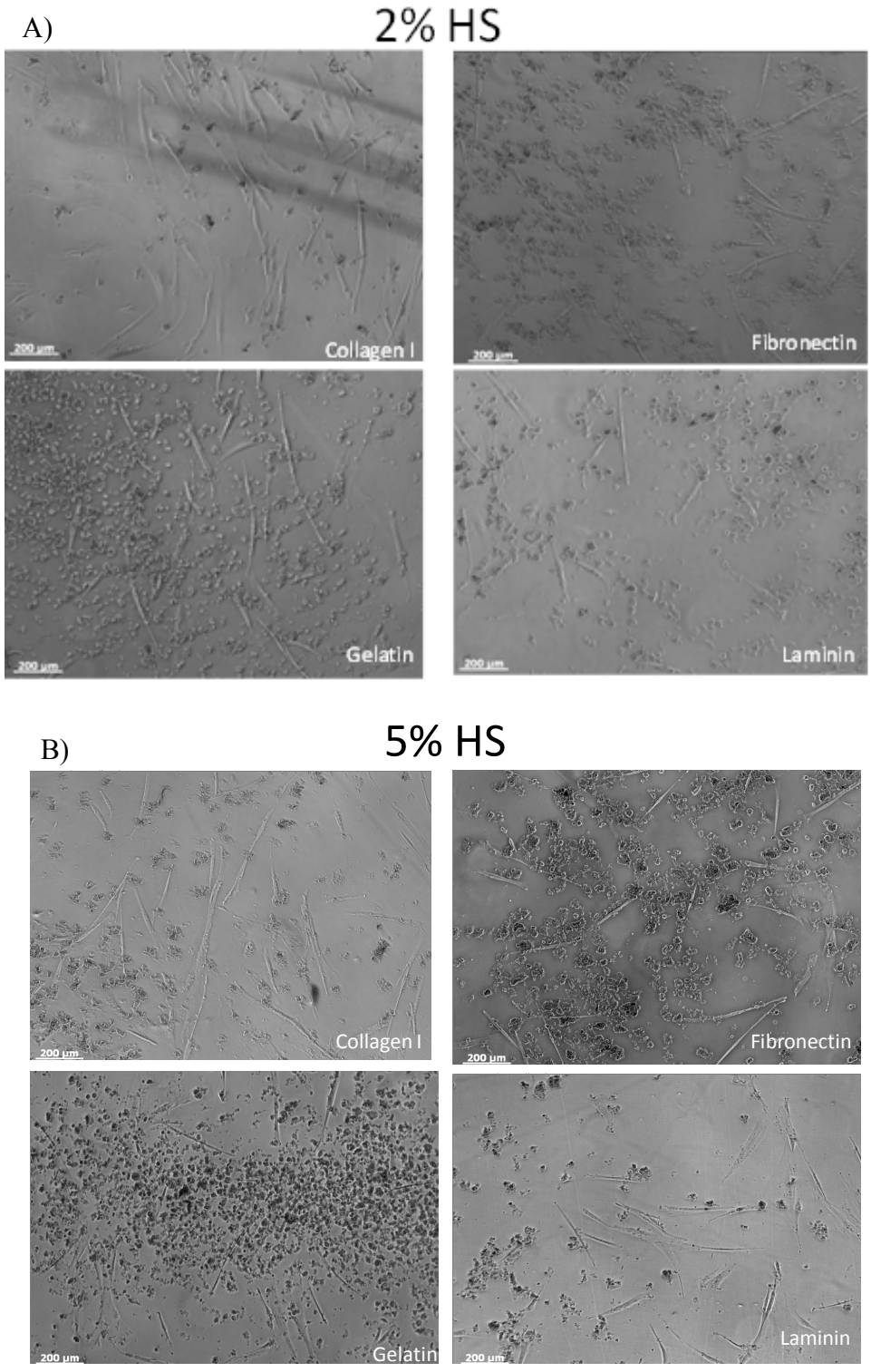


Figure 7: Day 7 of Cells in Myogenic Media Supplemented with Serum
A) 2% HS; B) 5% HS; C) 2% FBS; D) 5% FBS

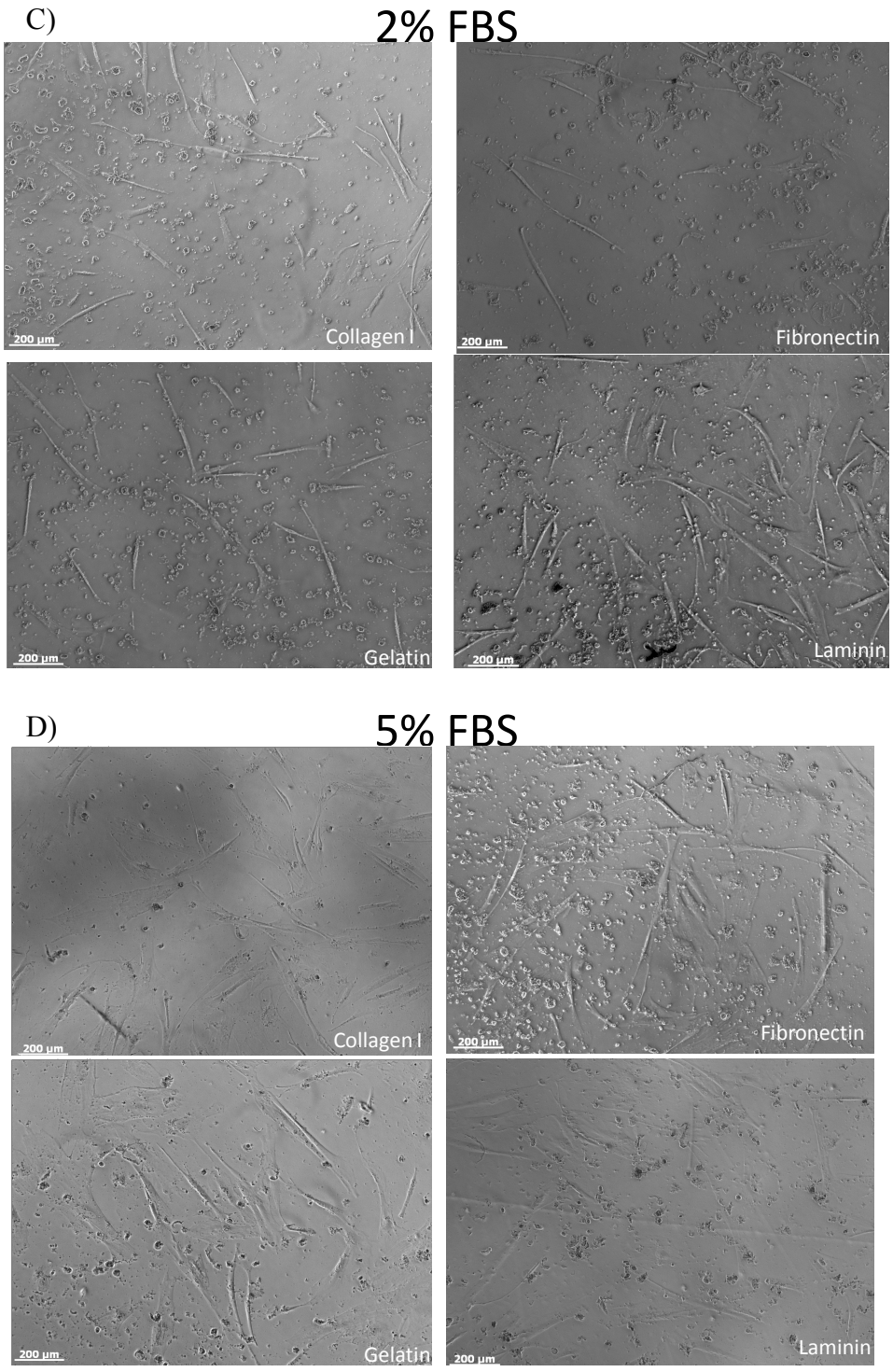


Figure 7: Day 7 of Cells in Myogenic Media Supplemented with Serum, Continued

A) 2% HS; B) 5% HS; C) 2% FBS; D) 5% FBS

4.1.3. Differentiation Media Supplemented with Butyrate and Hydrocortisone

It has been shown in previous studies that treatment with butyrate or hydrocortisone promotes myogenic differentiation. Treatment with butyrate in this study resulted in cell death and it was discovered later that the concentration used was too high (data not shown). Groups treated with hydrocortisone and N2 supplement stained positive for Myf5 whereas the other groups were negative (Fig. 8). Positive Myf5 staining reveals that the cells are in the beginning stages of myogenesis.

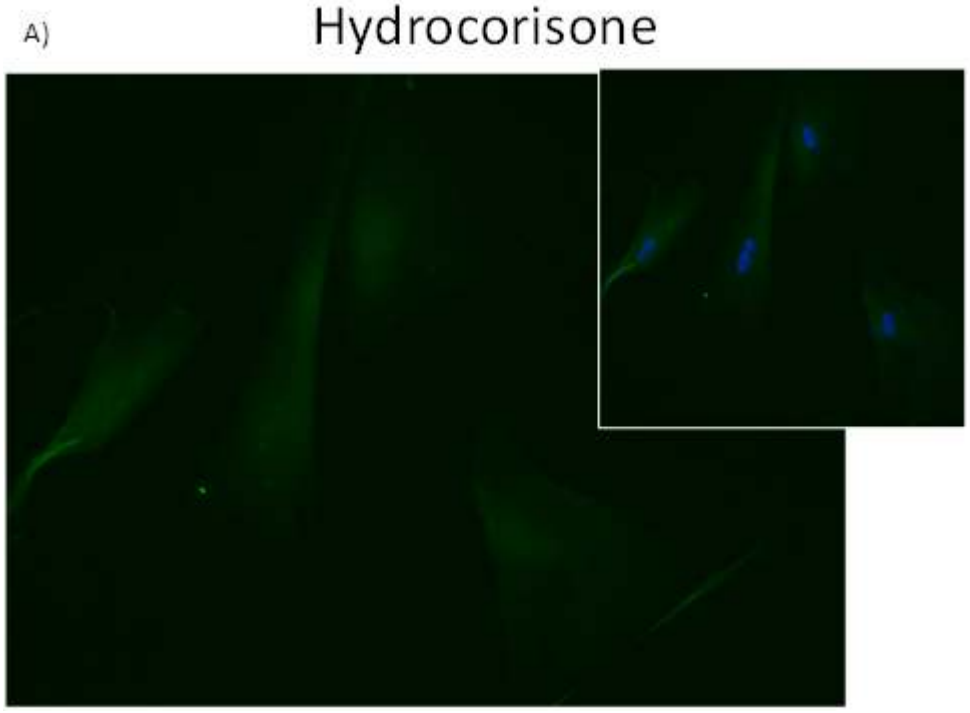


Figure 8: Myogenic media with N2 and/or Hydrocortisone
A) Hydrocortisone treatment; B)N2 treatment; C) Hydrocortisone and N2

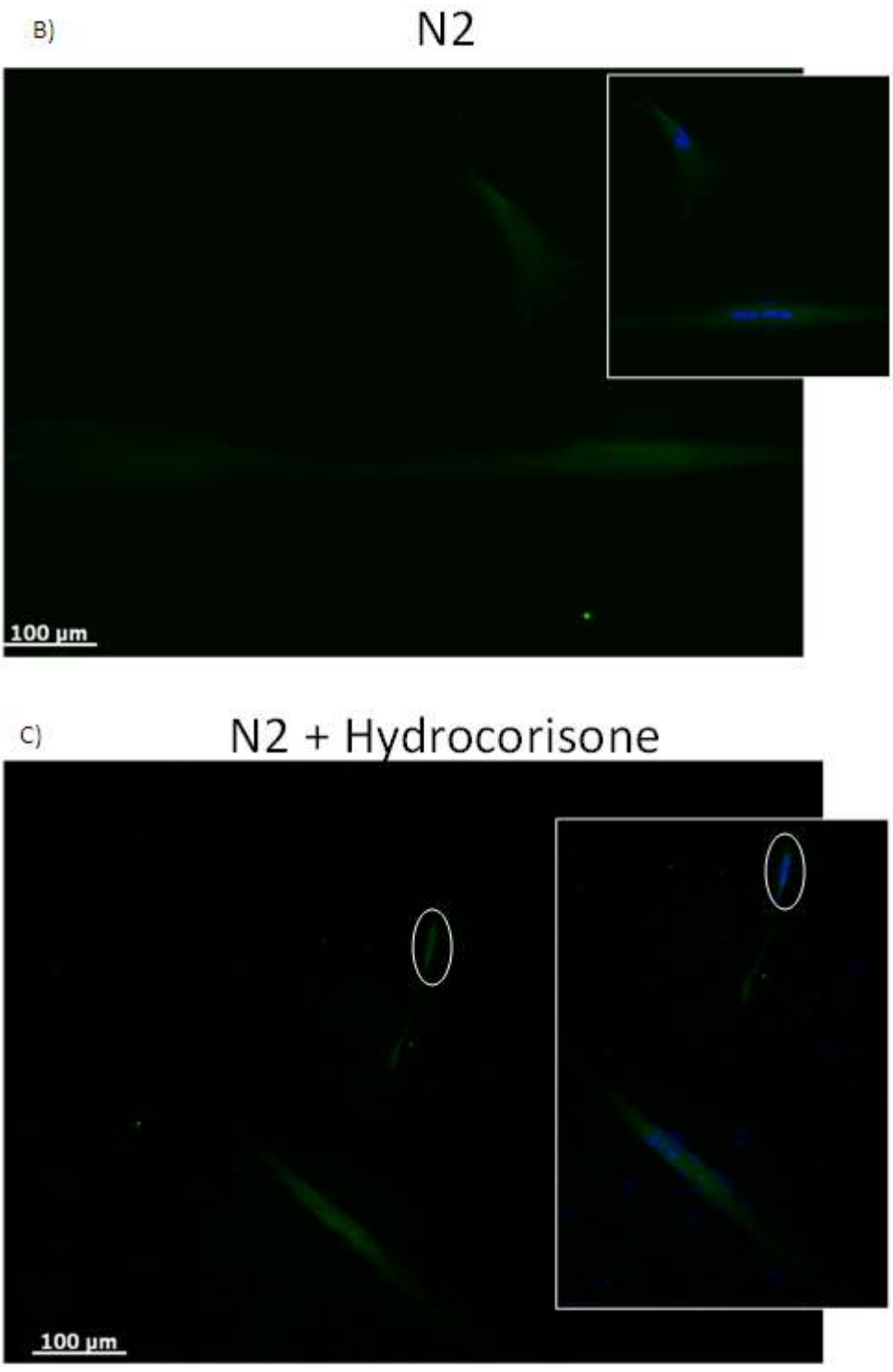


Figure 8: Myogenic media with N2 and/or Hydrocortisone, Continued
A) Hydrocortisone treatment; B)N2 treatment; C) Hydrocortisone and N2

4.2. Optimizing ECM Coating Concentration

Literature has shown that concentration used for ECM coating ranges from 25µg/mL to 50µg/mL. Cells were seeded onto ECMs coated with either 25µg/mL or 50µg/mL of protein. Cell count and staining were performed before the cells were switched into myogenic media as shown below in Fig.9 and Fig.10 respectively**.** Cell count reveals that there is no significant difference in cell count between the two concentrations but proliferation on fibronectin is higher than the other coatings. From the images, better cell stains were acquired from the higher concentrations so 50µg/mL was chosen as the coating concentration. Also, the cells look healthier and less damaged on the higher concentration.

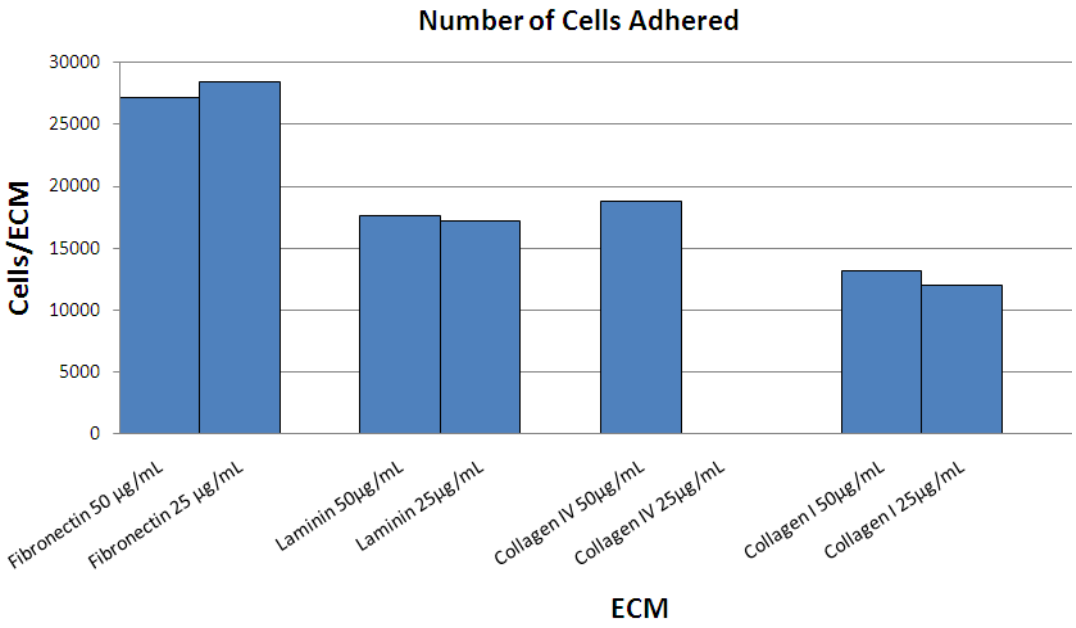
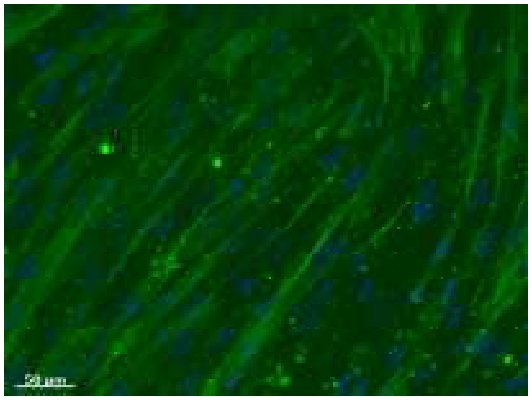
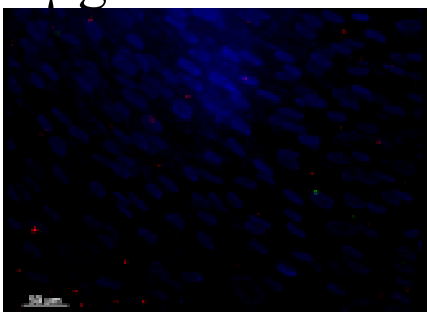
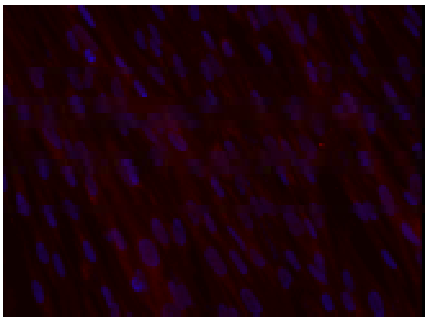


Figure 9: Cell Count

Fibronectin 50 µg/mL

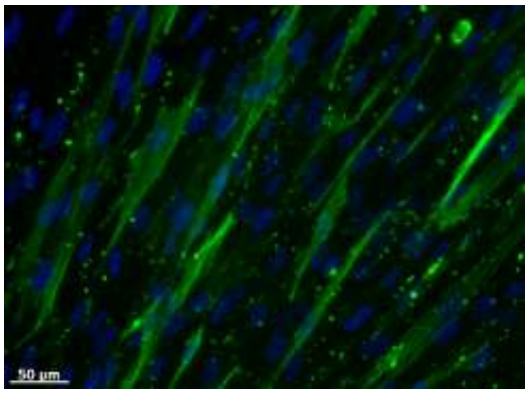


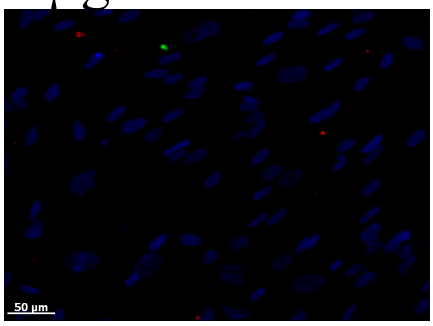


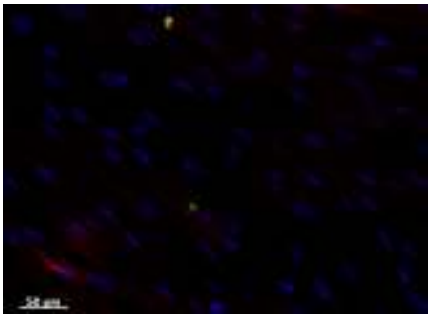


Actin	MHC and MyoD
	MF20 and Myf5

Fibronectin 25 µg/mL



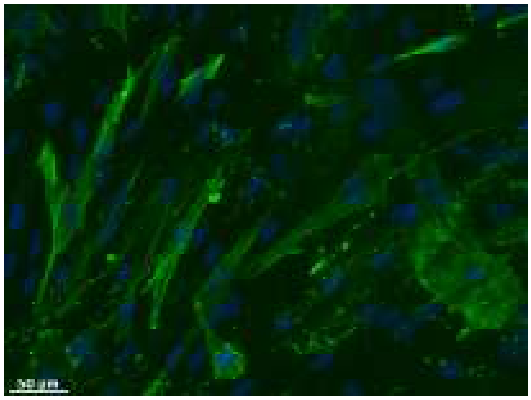


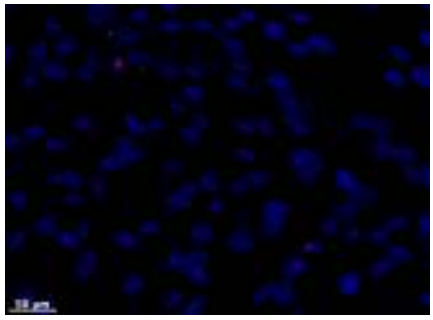


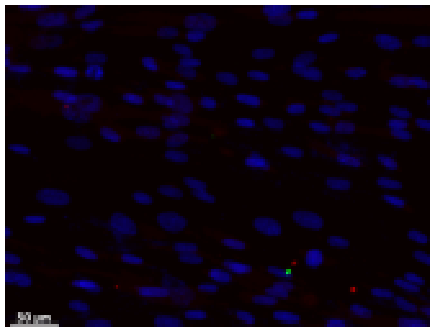
Actin	MHC and MyoD
	MF20 and Myf5

Figure 10: ECM Concentration Cell Staining

Laminin 50 µg/mL

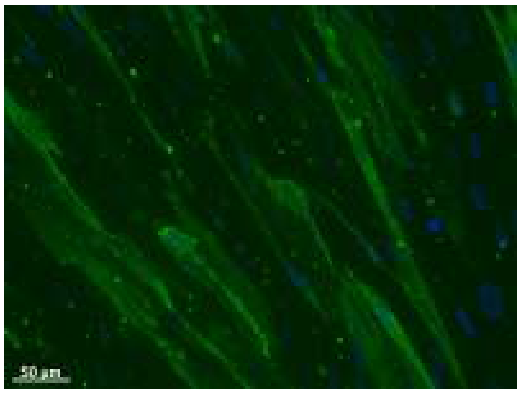


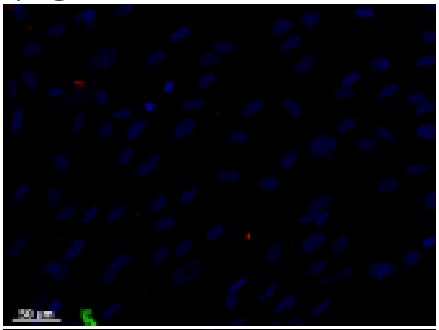


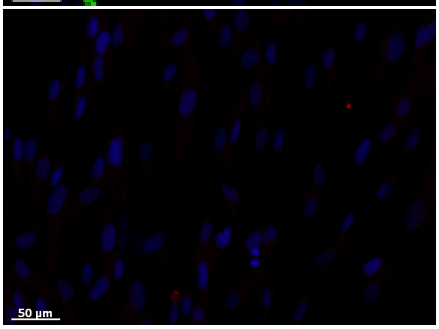


Actin	MHC and MyoD
	MF20 and Myf5

Laminin 25 µg/mL



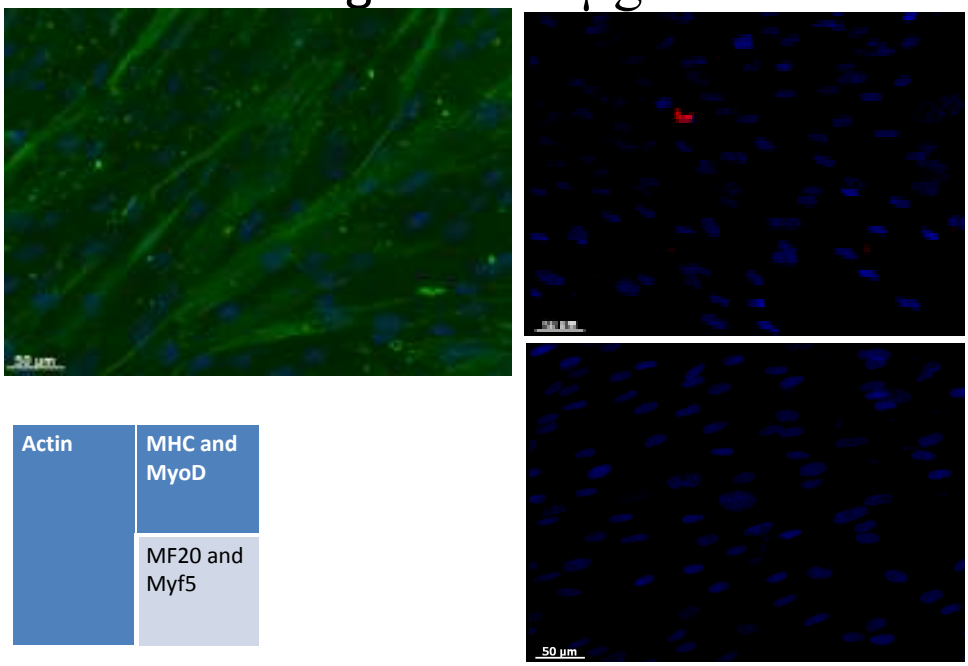




Actin	MHC and MyoD
	MF20 and Myf5

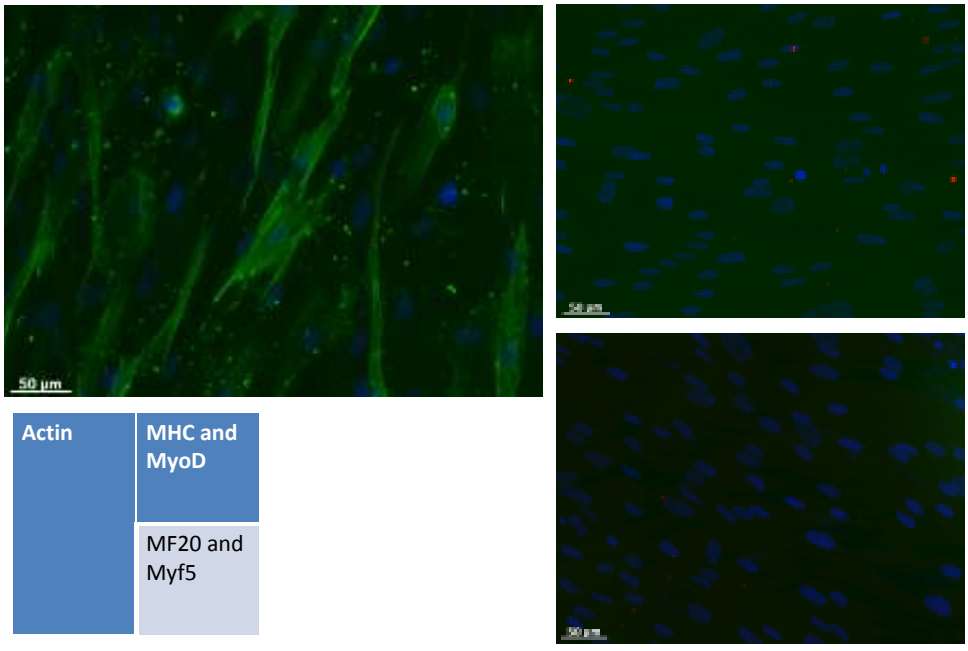
Figure 10: ECM Concentration Cell Staining, Continued

Collagen IV 50 µg/mL



Actin	MHC and MyoD
	MF20 and Myf5

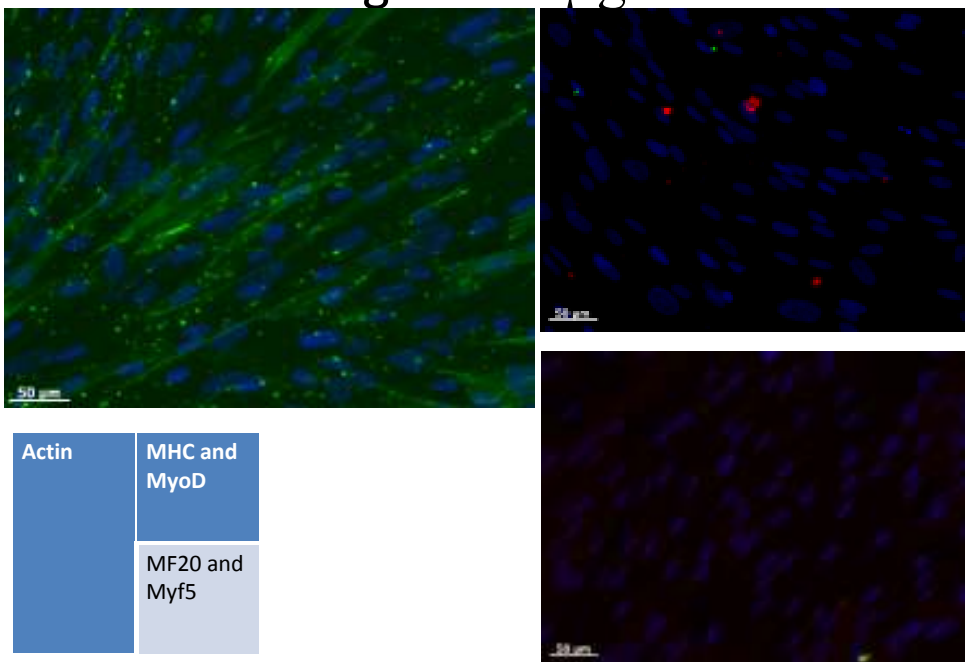
Collagen IV 25 µg/mL



Actin	MHC and MyoD
	MF20 and Myf5

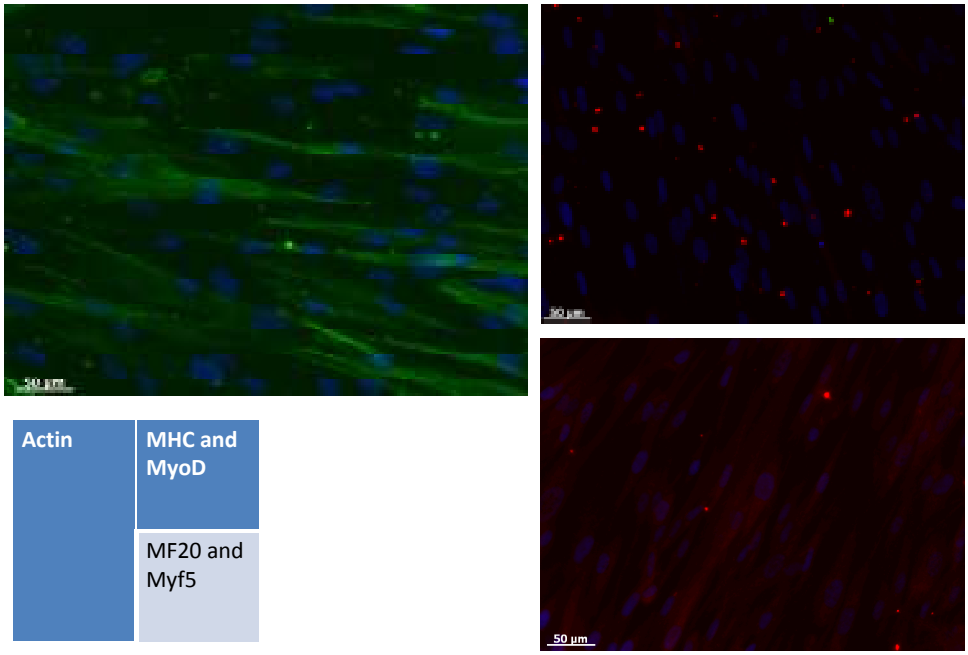
Figure 10: ECM Concentration Cell Staining, Continued

Collagen I 50 µg/mL



Actin	MHC and MyoD
	MF20 and Myf5

Collagen I 25 µg/mL



Actin	MHC and MyoD
	MF20 and Myf5

Figure 10: ECM Concentration Cell Staining, Continued

4.3. Determining ECM that Supports Myogenic Differentiation

Cells were differentiated on collagen I, collagen IV, fibronectin or laminin coatings and differentiation was analyzed through immunofluorescent staining for myogenic markers. Brightfield images of day 0 and day 14 reveal that fibronectin supports cell proliferation since that is the only coating that showed an increase in cell count while all the other coatings showed a decrease in the number of cells adhered (Fig. 11). Cell on all laminin, collagen I, collagen IV coatings were positive for Myf5 on day 14 as seen by the green staining in the nucleus. Positive staining illustrates that cells are in the initial phase of skeletal myogenesis (Fig. 12). Collagen I and laminin coatings stained positive for MyoD staining on day 21 as illustrated by the green staining in the nucleus of the cells in Fig. 13. Appearance of MyoD shows that cells on collagen I and laminin are committed and undergoing myogenic differentiation. Lack of MyoD in fibronectin explains the proliferation of the Hues9D cells because cell cycle hasn't been arrested by MyoD.

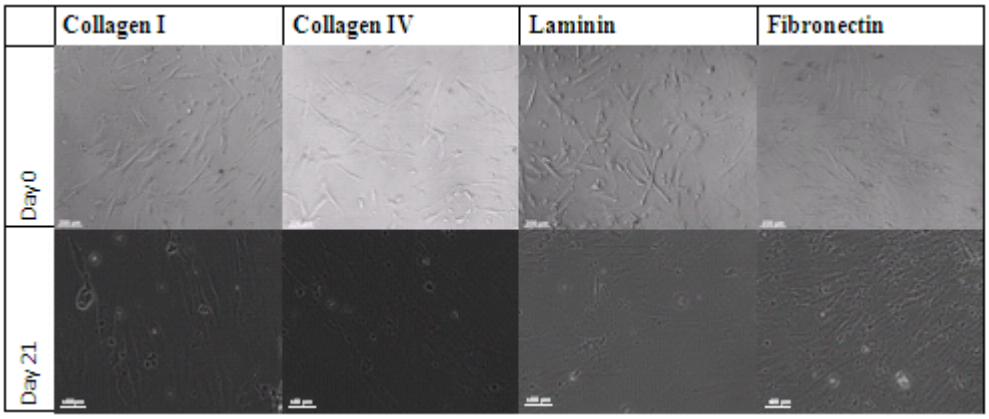


Figure 11: Brightfield Staining
Birghtfield staining of cell on Day 0 and Day 21. Cell count decreased on all the ECM coatings except for fibronectin.

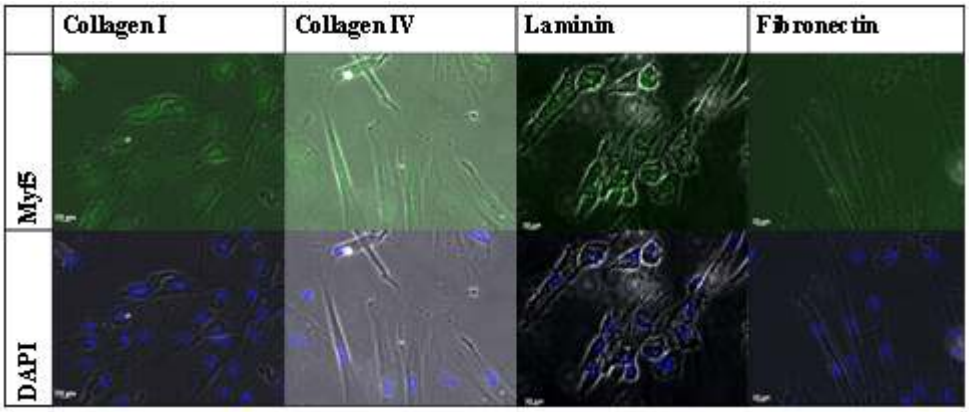


Figure 12: Myf5 Staining
Myf5 staining performed on day 14

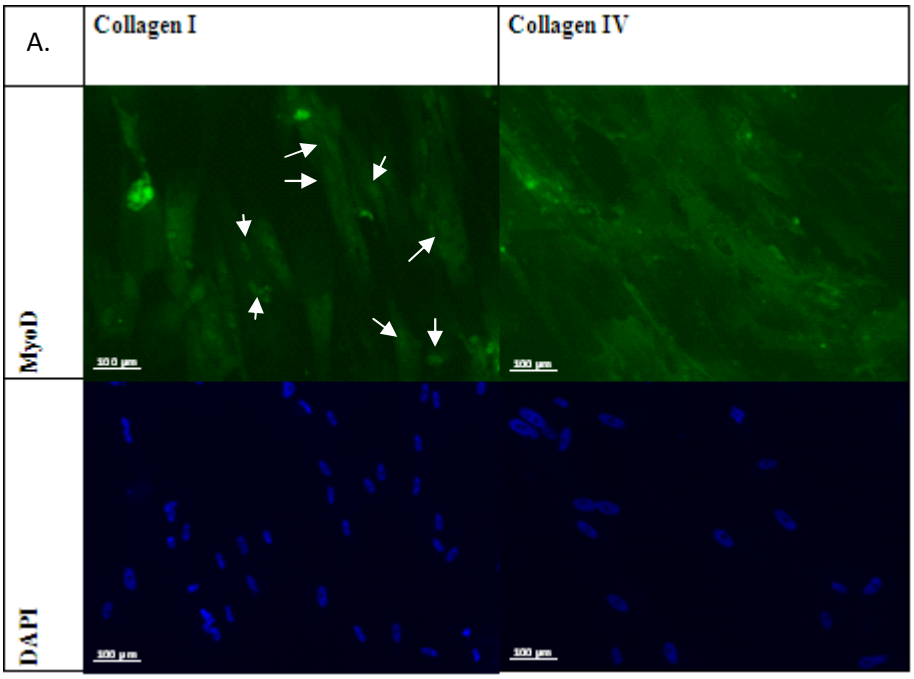


Figure 13: MyoD Staining
A. MyoD staining for Collagen I and Collagen IV; B. MyoD staining for Laminin and Fibronectin. White arrows point to positive staining of MyoD

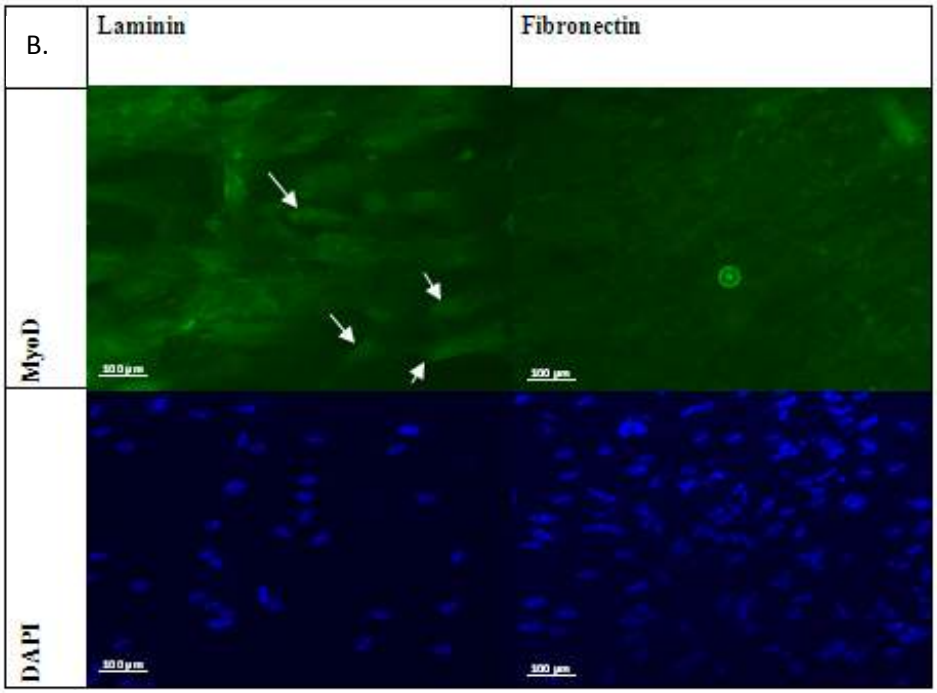


Figure 13: MyoD Staining, Continued
A. MyoD staining for Collagen I and Collagen IV; B. MyoD staining for Laminin and Fibronectin. White arrows point to positive staining of MyoD

V. Discussion

Stem cell differentiation and proliferation is influenced by interplay between soluble and insoluble factors, cell-cell, and cell-ECM interactions, mechanical and biophysical cues. It is a complex dynamic relationship making it hard to efficiently differentiate Hues9D cells in-vitro with repeatable results. While research has been performed on myogenic differentiation, the results do not translate among different cell lines. Cell source used in this study were Hues9D, cells derived from hESCs. Advantages of using HUES9D cells are pluripotency, in-vitro expansion for multiple passages without the need for a feeder layer. This thesis focuses on determining media components and ECM proteins that best support and promote myogenesis of Hues9D cell.

Myogenic media results reveal Hues9D cells require a low concentration of serum in differentiation media for survival. Cells seem to prefer FBS over HS and cell death was lower in cells grown in media supplemented with FBS. Optimal concentration of FBS was found to be 5%, as 2% was too low to support cell viability. Immunofluorescent staining results showed positive Myf5 expressions for cell grown in media supplemented with N2 and hydrocortisone illustrating that the interaction of these factors promotes myogenic differentiation and N2 or hydrocortisone alone could not promote myogenesis.

Stains of cells grown on 50µg/mL had better images when compared to images of cells grown on an ECM coating of 25µg/mL. Cells looked healthier and less damaged on the higher concentration and less cells washed away during the staining process. Cell count performed revealed that fibronectin supported proliferation since

there was s significant difference between the number of cells on fibronectin compared to the other matrices agreeing with earlier studies.[74]

Brightfield images showed elongated and lean cells by 14 days into myogenic media. There morphological changes are consistent with changes that occur in cells undergoing myogenesis.[77] Immunofluorescent staining revealed Myf5 expression on collagen I, collagen IV, and laminin coatings confirming that cells are in the early stage of myogenesis. Positive MyoD expression on laminin and collagen I coatings shows that the cells are committed to myogenesis and proves that laminin and collagen I are the ECM proteins that best support differentiation and lack of MyoD , Myf5 and increased proliferation shows that fibronectin best supports cell proliferation.

VI. Conclusion

This research has optimized a protocol for myogenic differentiation of Hues9D cells involving ECM proteins and media components. It was concluded that cells grown in media supplemented with 5% FBS, N2 and hydrocortisone promote differentiation while laminin and collagen I support myogenic differentiation of Hues9D cells. The study shows promise of efficiently differentiating Hues9D cells but it has just looked into the early stages of myogenesis. Further studies that can be performed to fully characterize and understand the affects of ECM and media are:

- Perform a longer study to determine expression of the later myogenic transcriptional factors
- Perform PCR to determine the expressional levels of myogenic transcriptional factors
- Characterize alignment and morphological changes of Hues9D cells over time
- Measure and characterize cell secreted factors
- Observe time related changes of the ECM content through immunofluorescent staining and RT-PCR.
- Perform the study using ECM coatings consisting of a combination of the four protein used in this study

This study has shown the promise of successfully differentiating Hues9D cells into muscle cells using cell-ECM interaction and soluble factors. Further research can provide a clearer idea on how these two cues influence myogenic differentiation and can be used to cure muscle wasting diseases such as DMD. Having a defined protocol

for myogenic differentiation will provide hope for those afflicted with DMD and give an insight into curing similar diseases that afflict other tissues.

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