Effect of Matrix Architecture on Myoblast Migration

By

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ABSTRACT

Muscle regeneration, an essential homoeostatic phenomenon, entails the process of quiescent muscle stem cells (MuSCs) undergoing activation, proliferation, migration, differentiation, and fusion to myotubes [1]–[3]. MuSCs can sense and respond to the mechanical microenvironment of the tissue while migrating through the interstitial matrix [4], [5]. The regeneration of muscle fibers is impaired in fibrotic diseases where the interstitial matrix volume increases, such as Duchenne muscular dystrophy (DMD). In fibrosis the mechanical microenvironment of the tissue is modified [6]–[9]. The extracellular matrix (ECM) has an increased stiffness and decreased porosity [10]–[12]. This constrained environment has been found to modulate the differentiation ability as well as nuclear integrity of MuSCs [13]. The migration patterns and frequency of nuclear rupture/DNA damage of MuSCs through a constrained environment are not well understood. I propose that satellite cells are blocked by the constrictions made by fibrotic tissue. In this thesis, we demonstrate that the capacity of human myoblasts to undergo constricted migration through small rigid pores is hindered and only those cells that are highly migratory can cross them.

In normal tissue, myoblasts undergo morphological changes and may increase collagen cross-links and ECM remodeling to propel themselves forward. However, in fibrotic conditions, the ECM architecture is already modified, and it is characterized by excessive collagen production and an increase in cross-linking [11]. ECM architecture modulates stem cell differentiation and proliferation, but the effect of migration is unknown [14]. I hypothesized that in ECM architecture mimicking fibrosis myoblast migration will be compromised, leading to reduced speed. In this thesis, we demonstrate the differential effects of different 2D and 3D matrices on myoblast migration.

From our results, we can look at the constrictive environment with modified ECM architecture presented by muscle fibrosis, as a potential biomechanical target, and a critical aspect in defining treatment approaches for fibrotic skeletal muscle diseases such as DMD.

CHAPTER 1: INTRODUCTION

Myoblasts, the precursor cells for skeletal muscle fibers, play a crucial role in the regeneration of damaged muscle tissue. Muscle Stem Cells (MuSCs) remain in between the basal lamina and sarcolemma of muscle fibers in their quiescent state [15], [16]. Upon activation due to biochemical or mechanical stimuli, they proliferate to generate more MuSCs to participate in the regenerative process, migrate through the extracellular matrix (ECM) to reach the site of regeneration, differentiate and fuse to themselves or existing myofibers [17]. Migration of MuSCs through the ECM is an essential component of muscle regeneration, as it enables the cells to reach the site of injury and differentiate into functional muscle fibers, yet comparatively little is known regarding ECM impacts on myoblast migration.

In vitro, MuSCs or satellite cells have been found to use mesenchymal migration patterns; however, the other mechanisms are not well understood[18]. Upon receiving chemical cues from cytokines, growth factors, and other signaling molecules, MuSCs get activated and polarize forming a front-rear asymmetry. This occurs due to the localization of intracellular proteins such as Rho-GTPases that reorganize the cytoplasm. This uneven cell morphology helps the cells probe with leading-edge structures called lamellipodia that cells use to move in the direction of the signal [19].

The cells also form physical connections with the ECM by protein-protein interactions predominantly with integrins – transmembrane proteins that bind to collagen, laminins, and fibronectin – producing the force necessary for movement. Integrins are mechanotransducers meaning they transmit signals from the ECM to the cell which further employs focal adhesion complexes and localizes Rho-GTPases that are involved in cell migration [20]. Syndecan-4, an MuSC marker and a transmembrane heparan sulfate protein that binds to ECM, regulates myoblast

migration through the regulation of Tiam-1 an intracellular GEF protein that activates Rac-1, which in turn drives cell polarization [21].

Cells also facilitate migration by remodeling ECM by the secretion of proteolytic enzymes called matrix metalloproteinases (MMPs) creating "pores" or spaces cells migrate through. MMPs cleave ECM components and release ECM-bound growth factors. Release of these sequestered growth factors allows them to bind to their specific receptors on cells and trigger downstream signaling events. The controlled release and activation of growth factors regulate the process of muscle cell migration. Previous studies have shown that MMP-13 is modulated in C2C12s and is correlated with their ability to migrate in transwell based migration assays [22]. MMP-14 is necessary for the migration of human myoblasts through 3D collagen gels [23]. MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) to maintain ECM homeostasis and both MMPs and TIMPs are dysregulated during pathological conditions [24].

In normal tissue, myoblasts undergo morphological changes and utilize collagen adhesions and ECM remodeling to propel themselves forward. Upon injury or in diseased states, muscle regeneration is directly impacted. A common manifestation of impaired regenerative processes is fibrosis.

In fibrotic conditions, the ECM architecture is modified, and it is characterized by excessive collagen production, stiffness, and an increase in cross-linking [11], [25]–[28]. This leads to a constricted environment for myoblasts to migrate through and regenerate. Smith et al. have shown that constricted migration of C2C12 cells results in impaired differentiation *in vitro* and *in vivo* [13]. They also demonstrated that constricted migration of MSC's caused increased nuclear damage and promoted osteogenic differentiation. Mechanical properties of a matrix

modulate the fate of stem cells [29], altering gene expression, and steering them towards a different cell fate.

It remains unclear whether these constrictions themselves restrict/block cell migration to the regenerative site or whether the damage caused by restricted migration has a greater effect on regeneration. We hypothesize that constricted migration blocks migration and the cells that migrate through will have impaired regenerative capacity.

Satellite cells from patients with fibrotic disease Duchenne muscular dystrophy (DMD) have impaired regenerative capabilities as a result of the pathological tissue microenvironment [6], [8], [30]. Previous work from our group has shown that ECM architecture modulates stem cell differentiation and proliferation [14], but the effect of migration is unknown. We hypothesize that in ECM architecture mimicking fibrosis myoblast migration will be compromised, leading to reduced migration speed.

During fibrosis, the matrix does indeed have a microenvironment with decreased porosity, increased collagen content and crosslinking that is not conducive to regeneration. Understanding how the modification in matrix architecture affects MuSC migration is a critical aspect in defining treatment approaches for fibrotic skeletal muscle diseases such as DMD. This thesis explores the effect of the matrix architecture on human and mouse muscle progenitor migration.

CHAPTER 2: CONSTRICTED MIGRATION IMPAIRS MOBILITY OF HUMAN MYOBLASTS

Introduction

During development, skeletal muscle forms multinucleated myotubes through the fusion of myoblasts. During secondary myogenesis, myoblasts adhere to an existing myotube, differentiate and fuse to promote maturation. Over time, myotubes mature into myofibers which are responsible for the contractile forces generated by adult muscle. This process is known as myogenesis and occurs during embryonic development and is recapitulated during regenerative processes in response to injury or exercise [31].

The nuclei within myotubes are post-mitotic and they are dependent on satellite cells for secondary growth or regeneration following injury. Satellite cells or MuSCs are inherently migratory. They reside in a quiescent state between the basal lamina and the plasma membrane of mature muscle fibers [15], [16]. Upon activation, they tend to move along a single fiber, but there is evidence that they may also travel between adjacent muscles. After reaching the niche, they adhere to the periphery of the myofiber [32].

The endomysium is the ECM layer around each myofiber. It is estimated to have a thickness of between 0.2 – 1μm [33]. This could mean that satellite cells migrate through tight interstitial spaces even in normal physiology. Potential damage during migration is compounded in pathological conditions when the matrix architecture becomes denser, stiffer, or more heavily crosslinked.

Our group has investigated the effect of constricted migration using transwell systems on human myoblasts (HuMB) to determine whether they behave similar to mouse myoblasts. HuMB

were found to have impaired nuclear integrity characterized by nuclear blebbing and DNA damage following constricted migration through 3μm-diameter pores. Additionally, constricted migration also caused deficits in adhesion and differentiation.

Given these observations, we were interested in the effect of constricted migration on human myoblasts migration rates. It was evident that constricted migration of HuMB led to impaired differentiation which could contribute to impeded regeneration, but it was unclear if this resulted from damage during constricted migration or the inability of HuMB to migrate.

Using transwell cell culture inserts with porous membranes to model constricted migration in an immortalized human skeletal muscle myoblast cell line that expresses tdTomato globally and blue fluorescent protein (BFP) with a nuclear localization signal (NLS). Using live cell imaging, we were able to track migration patterns over a period of 24 hours and the different reporters allowed us to track the nuclear and cytoplasmic migration patterns separately. Since nuclei are the stiffest organelle and localize to the trailing edge of the cell, it is highly likely that they would migrate last and require a modification of nuclear mechanics [34]. Using this method to determine how constrictive pores impact myoblast nuclear migration and cytoplasmic protrusions allowed me to test the hypothesis that constrictions block cell migration.

Methods

Cell culture of HuMB

hTERT/cdk4 immortalized human myoblasts were obtained from Center for Research in Myology (Paris, France). They were cultured in growth medium containing DMEM and Medium 199 at a 4:1 volume ratio, supplemented with 20% fetal bovine serum, 25μg/mL fetuin, 5ng/mL human epidermal growth factor, 0.5 ng/mL basic fibroblast growth factor, 5 μg/mL insulin, 0.2

μg/mL dexamethasone, and 1% penicillin streptomycin [35]. Cells were cultured in tissue culture, treated flasks and incubated at 37°C and 5% carbon dioxide. Cells were passaged at 70 - 80% confluency and were used from passage 3 to passage 10 [36].

Transfection of cell lines

Cells were plated at 5120 cell/cm^2. tdTomato containing lentiviral vector (Addgene #62733) was added at a multiplicity of infection ratios from 2 to 40, which are the ratios of the number of lentivectors to the number of cells. Lentivector number was determined by a "p24 ELISA" test by the manufacturer. C2C12/HuMB cells and lentivector were co-incubated for 24 h, unless otherwise specified. At 24 h post transfection, cells were assayed for reporter gene expression by fluorescence microscopy. Total cell number was determined by DNA stain (Hoechst 33342 Invitrogen). Colony selection and expansion were performed by routinely checking for fluorescence intensity. The differentiation potential of the transfected cells was checked to confirm that the cells retained myogenic potential.

tdTomato HuMB cells were transfected additionally with pLV-EBFP2-nuc (a gift from P. Tsoulfas; Addgene plasmid no. 36085) with a protocol similar to that described above but without colony selection. Transfected cells were immediately trypsinized and used for our experiments.

Transwell Migration

To analyze cell migration patterns, we studied cell migration through permeable Falcon FluoroBlok 24-Well (Fisher Scientific) cell culture inserts (Corning) with membrane pore diameters of either 3μm or 8μm with $<1.70 \times 10^6$ and $<0.85 \times 10^5$ pores per cm^2, respectively [36]. The tops of 24- well inserts were seeded with 5×10^4 cells. (Fig 1 A). Cell culture medium was

added to the top and bottom of the insert so that no nutrient or chemical gradient existed across the membrane. The transwell inserts were incubated for 36 h for HuMB tdTomato cells to migrate to the bottom of the transwell membrane (Fig 1A). Following the incubation period, they were imaged using live cell imaging for the next 24 h. Cells were also cultured in wells without inserts as a control.

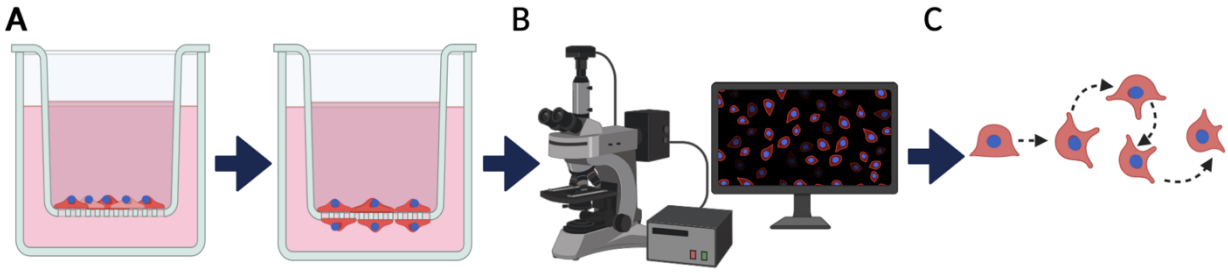


Figure 1: Diagram of transwell assays. (A) Cells are seeded on the top of the transwell inserts and left for 36 hours, during which they migrate through the pores (3μm or 8μm) to the bottom of the transwell. (B) To look at cell migration rates, they were live cell imaged for 24 hours from hour 36-60 every 7 minutes. (C) To track cells, imaged were analysed on Fiji using TrackMate plugin to generate files with calculated parameters. (Created with Biorender.com)

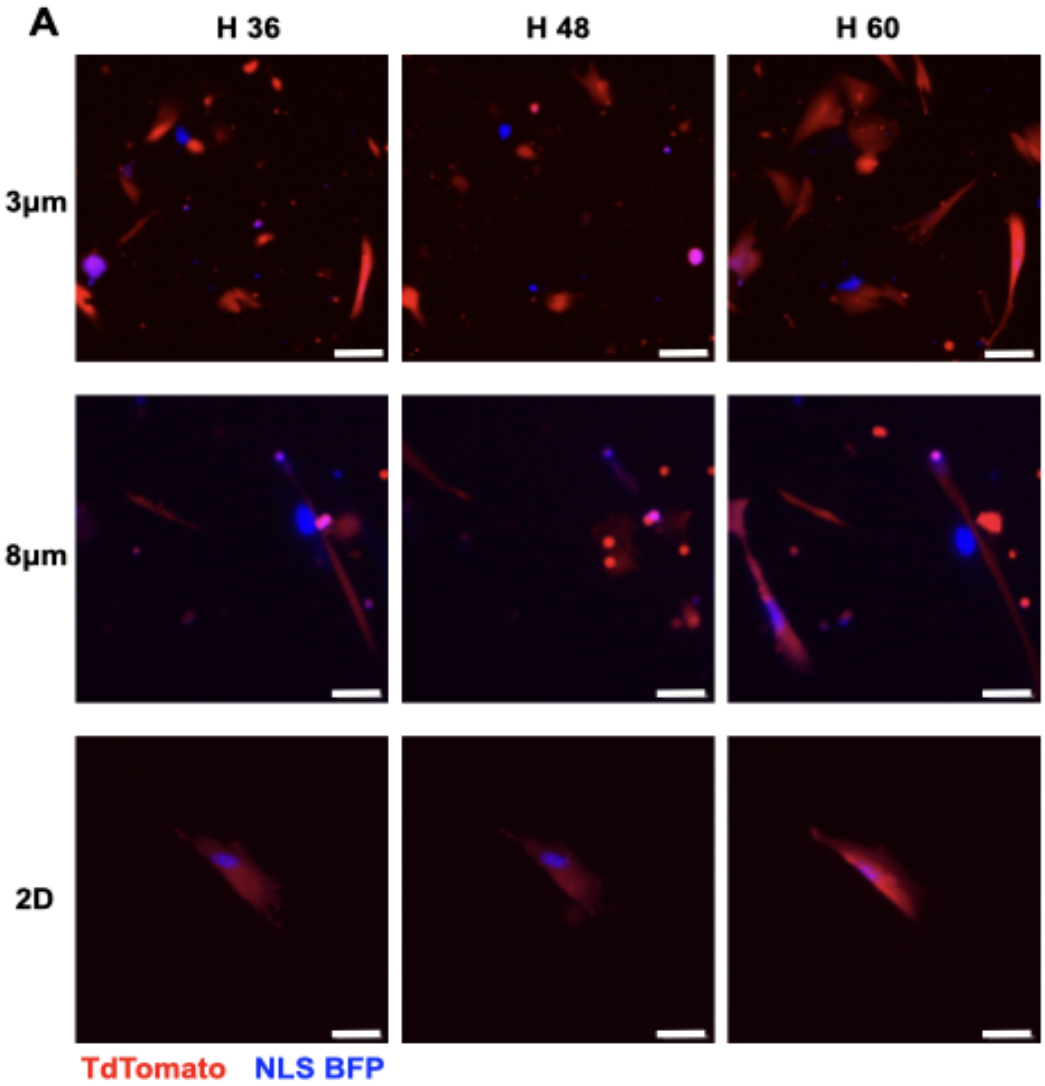
Live Cell imaging

Live-cell imaging was conducted using a Leica DMi8 with an environmental chamber at 37°C and 5% carbon dioxide using a 20× objective. Live images were taken every 7 min using Fluoroblok 24-well inserts. (Fig 1B).

Image analysis was performed using FIJI/ImageJ. All analyses were performed on images with background subtraction applied using an appropriate radius and equal smoothening of the images [36]. Cells were tracked using TrackMate plugin on FIJI using the thresholding detector. (Fig 1C). Thresholding values differed based on image conditions. But the representative images were brightened and sharpened after quantification to make features clearer.

Statistical Analysis

When quantifying migration %, the number of protrusions on the bottom of the transwell was divided by total protrusions through each pore of the transwell, and the mean was calculated. At least 60 cells were analyzed for each condition, and the experiment was repeated three times. Cell speeds were calculated for protrusions on the bottom of the transwell, they were normalized with cells migrating on plastic and averaged for each condition. One-way ANOVA with Tukey's multiple comparisons test was used for both migration % as well as speed.



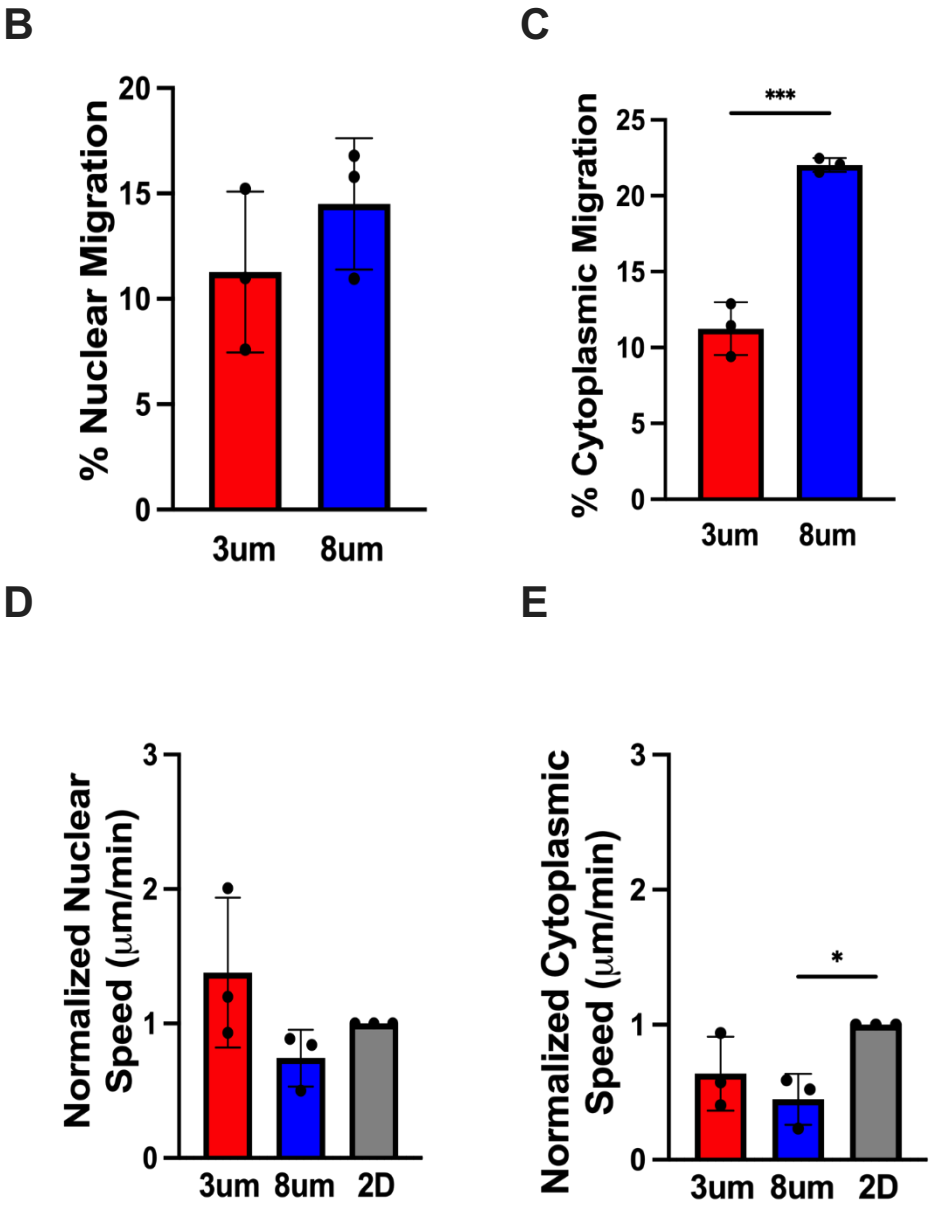


Figure 2: Representative images of nuclei (NLS BFP) and cytoplasm (tdTomato) HuMB that have migrated through either 3- or 8-μm pores over the course of 36 - 60 h. Images show migrated cells on the bottoms of nontransparent transwell pore filters at 36, 48, 60 h. 2D migration on plastic was used as control. Scale bar = 50μm (B) Quantification of Nuclear migration rate through 36-60h (C) Quantification of Cytoplasmic migration rate through 36-60h ***$p<0.0005$ (D) Nuclear migration speeds normalized to 2D migration. (E) Cytoplasmic migration speeds normalized to 2D migration. *$p<0.05$

Results

Constricted migration restricts cytoplasmic probing but has no effect on post migration cell speed

To determine the effect of constricted migration on human myoblasts migration rate, immortalized HuMB expressing tdTomato and NLS BFP proteins were seeded on FluoroBlok inserts and allowed to migrate through pores that were either 3μm or 8μm in diameter for 36 hours (Fig 1 A). Myoblasts typically have nuclei ranging in size from 5 - 10μm, resulting in significant constriction in the smaller (3μm) pores and limited constriction by the 8μm pores [37].

We were able to track the cytoplasm (tdTomato) and nuclei (NLS BFP) of the migrating cells independently. Tracking nuclei and cytoplasm independently was advantageous since it allowed quantification of the % of nuclei that migrated to the bottom of the transwell. Migration of nuclei through 3μm pores has been implicated in nuclear rupture and DNA damage. We wanted to see whether the nuclear migration or cytoplasmic probing was restricted in smaller pores, and we hypothesized that smaller constrictions would restrict cell migration.

Cytoplasm and nuclei with protrusions through the pores were considered as a migration attempts and nuclear and cytoplasmic spreading above a set area greater than area of the 8μm pore was considered migration at the bottom of the transwell. % of actual migration to total attempts were calculated. Nuclear migration rates were comparable in both pore sizes at around 11% successful migration rate. (Fig 2B). Whereas the cytoplasmic migration rates were significantly higher in 8μm pores compared to 3μm pores with almost double the number of cytoplasmic probing and migration occurring at 8μm pores compared to 3μm pores (Fig 2C).

We also wanted to see how the speeds of the cells on the bottom of the transwells are affected post migration through constricted pores. Speed of migration of individual cells were

obtained from Trackmate data files in Fiji and averaged for each cell across each condition. They were then normalized with the average migration speed of cells on the 2D plastic control. We found that there were no significant differences in cell (nuclear and cytoplasmic) speed post migration regardless of pore size (Fig 2D & E).

Discussion

Many studies have demonstrated the migratory properties of stem cells and myoblasts, and some of them have even demonstrated the compromise in nuclear integrity and differentiation post migration through constricted matrices [13]. The constrictions cause self-inflicted cellular damage resulting from nuclear rupture and DNA damage. Previous research from our group indicates that HuMB migration through 3μm pores causes impairment to nuclear integrity, cell adhesion and differentiation.

Damaged nuclei may cause impaired differentiation, but cellular differentiation is associated with DNA damage repair as means of preserving genetic integrity of cells [38]. Previous research indicated that DNA damage results in delayed differentiation of mouse myoblasts by 2 days [13]. Prolonged constricted migration could also mean that the degree of DNA damage and recovery time would be greater and increase the degree of impaired differentiation. Nuclear rupture in addition to causing DNA damage, as noted from our previous work, can also cause mislocalization of transcription factors between the cytoplasm and the nucleus. MyoD, a myogenic regulatory factor, and KU80, a DNA damage repair factor, have been noted to be mislocalized following constricted migration [13], [39], [40].

While we do understand constricted migration affects cellular processes to an extent, we however do not know if reduction in the interstitial pore sizes during fibrosis reduces the degree

of migration. It is unclear whether *in vivo*, there is low regeneration happening ensuing from the DNA damage or it is because there are not enough cells migrating to the site of regeneration or it is a combination of both.

Our findings indicate that transwell pore sizes did no impact nuclear migration potential, but smaller pores did restrict cytoplasmic probing and migration. This could be due to the fact MuSCs have been shown to exhibit mesenchymal migration making cytoplasmic protrusions called lamellipodia on the leading edge and shifting the nucleus to the trailing edge. The cytoplasmic probing leads to cell migration because of cytoskeleton rearrangement due to actomyosin contractility. However, we noted that cytoplasmic probing rate was significantly higher in larger 8μm pores with almost 22% of probing cells migrating to the bottom of the transwell (Fig 2C).

Our results are in line with a previously published report that looked at primary mouse myoblast migration through a microfluidic device with 100μm long constriction channels with larger channels promoting more cell migration. While transwell studies indicate the effect of constrictions on HuMB cell migration, it is difficult to visualize and track cells as they are moving through these constrictions.

The speed of myoblast migration through transwells have not previously been reported. It was unclear whether only the highly migratory cells migrate through the pores and maintain their speeds or show impaired mobility and reduced speed. When we quantified the speed of cells that migrated to the bottom of the transwell, we did not see a significant difference in migration speeds across different pore sizes. (Fig 2D&E). This could mean that constrictions do not slow the cells, or that constricted migration restricts passage to more migratory cells. Our findings that cell migration speeds remain constant post migration through constricted pores is in line with a

previous study that looked at how confinement induces amoeboid mode of migration allowing the mesenchymal stem cells to move faster [41]. A better understanding of single cell migration patterns could result from microfluidic studies where we are able to track the speeds pre and post migration of cells.

We were able to make an addition to our existing findings on constricted migration of HuMB. Future work may consist of coating the transwells with different ECM proteins to better mimic the *in vivo* ECM. But to visualize and quantify the effect of constricted migration on cell motility, usage of microfluidic devices that allow for single cell assessment over a greater distance and time could be beneficial. Microfluidic devices would also help test efficacy of therapeutics that prevent or rescue nuclear rupture or DNA damage that results from constricted migration. These would be helpful to conduct more informed research to develop new therapeutic interventions for fibrosis.

CHAPTER 3: EFFECT OF COLLAGEN ARCHITECTURE ON MYOBLAST MIGRATION

Introduction

Fibrosis is the accumulation of excess ECM leading to impaired muscle function as in chronic or severe injury or diseases such as DMD [6]. Satellite cells are depleted in DMD with reduced cell number and function in mdx mice [42]. This combinatorial effect of low cell number and dysfunction further decreases muscle regeneration. Therefore, the fibrotic environment inhibits muscle regeneration [6]. However, the role of migration in the pathological mechanisms are not very well understood.

Deposition of excess inelastic scar tissue stiffens the muscle resulting in a rigid, constricted, environment for cells [43]. Stem cells are mechanosensitive and substrate stiffness and alignment regulate stem cell proliferation and differentiation [29], [44]. Collagen I, the most abundant ECM protein, has been found to be upregulated in stiffer tissues [45].

Collagen also undergoes post translational modifications which may contribute to the stiff microenvironment in the tissue. However, the amount of collagen has not been found to be correlated with stiffness in muscles from mice [12]. This could be indicative of the fact that other factors pertaining to ECM deposition/architecture could have a greater contribution to tissue stiffness.

Fibrotic ECM is related to passive muscle stiffness in the mouse model of DMD [46]. Other features of ECM architecture such as alignment have been found to be altered in fibrosis. Fibrotic muscle exhibits increased collagen crosslinking and altered fibril size [47]–[49]. Cross-linking is also associated with collagen stiffness. Some of these architectural features of ECM such as fibril

size and crosslinking have been found to have an impact on the C2C12 and MuSC proliferation and differentiation [14].

Limited research has attempted to understand the effect of ECM architecture on myoblast migration. Migration is a pivotal step in the regenerative process of muscle, but is poorly understood in the context of fibrosis and DMD. Certain ECM proteins such as fibronectin have been found to promote migration of C2C12 *in vitro* [50]. Further, intact acellular muscle matrices direct myoblast migration even under serum free conditions [51].

In normal tissue, myoblasts undergo cytoplasmic rearrangements utilizing cell-matrix interactions to remodel ECM to migrate. However, in fibrotic conditions, ECM alteration as characterized by excessive collagen production and an increase in cross-linking could be factors impacting migration of myoblasts is significantly impacted. We hypothesized that in ECM architecture mimicking fibrosis, myoblast migration will be compromised, leading to reduced migration speeds in particularly confined 3D environments.

We engineered collagen hydrogels with various properties and used them in 2D and 3D culturing systems to study the effect of collagen architecture on myoblast migration. HuMB and C2C12 myoblast cell lines that expressed tdTomato constitutively were used in this study. Using live cell imaging, we were able to track migration patterns over a period of 6 hours. These tools allowed testing of the hypothesis that fibrotic like architecture inhibits myoblast migration.

Methods

Cell Culture

C2C12 cells (ATCC CRL-1772) were cultured in a standard growth media (high-glucose DMEM, 10% FBS, and 1% penicillin-streptomycin) in cell culture flasks in a cell culture incubator at 37℃ and 5% CO2. Cells were passaged when the culture reached 60%–70% confluence. C2C12 cells from passage 3 to passage 20 were used for experiments [14].

hTERT/cdk4 immortalized human myoblasts (Center for Research in Myology, Paris, France) were cultured in growth medium containing DMEM and Medium 199 at a 4:1 volume ratio, supplemented with20% fetal bovine serum, 25μg/mL fetuin, 5ng/mL human epidermal growth factor, 0.5 ng/mL basic fibroblast growth factor, 5 μg/mL insulin, 0.2 μg/mL dexamethasone, and 1% penicillin streptomycin [35]. Cells were cultured in tissue culture, treated flasks and incubated at 37°C and 5% carbon dioxide. Cells were passaged at 70 - 80% confluency and were used from passage 3 to passage 10 [36].

Transfection of cell lines

Cells were plated at 5120 cell/cm^2. tdTomato containing lentiviral vector (Addgene #62733) was added at a multiplicity of infection ratios from 2 to 40, which are the ratios of the number of lentivectors to the number of cells. Lentivector number was determined by a "p24 ELISA" test by the manufacturer [36]. C2C12/HuMB cells and lentivector were co-incubated for 24 h, unless otherwise specified. At 24 h post transfection, cells were assayed for reporter gene expression by fluorescence microscopy. Total cell number was determined by DNA stain (Hoechst

33342 Invitrogen). Colony selection and expansion were performed by routinely checking for fluorescence intensity. The differentiation potential of the transfected cells was checked to confirm that the cells retained myogenic potential.

2D Collagen Gels

Collagen solutions [telocollagen (Telocol 10) and atelocollagen (Fibricol), Advanced Biomatrix] were diluted to the desired concentrations with sterile deionized water and 10X PBS and neutralized to pH 7.0 with 1 N NaOH [14]. 400 µL of collagen gel was pipetted into wells in a 24 well plate (Corning Life Sciences) with surface area 1.9 cm^2. (Fig 3A). The collagen plates were either incubated at 37°C for 60 – 90 min for gels with different concentrations - Telocol, crosslinking - Telocol and Fibricol, and smaller fibrils (Telocol 10) or left at room temperature for 4 h to ensure collagen polymerization for gels with larger fibrils (Telocol 10) [14].

3D Collagen Gels

Collagen solutions [telocollagen (Telocol 10) and atelocollagen (Fibricol), Advanced Biomatrix] were diluted to the desired concentrations with 10X DMEM (Gibco™ 1X DMEM diluted in $1/10^{th}$ PBS) and neutralized to pH 7.0 with 1 N NaOH [14]. A mixture of HuMB tdTomato cells in HuMB Growth media - 10,000 cells /400 µL gel prepared was added and mixed in with the gel before polymerizing. (Fig 3A). 400 µL of collagen gel was pipetted into wells in a 24 well plate (Corning Life Sciences) with surface area 1.9 cm^2. The collagen plates were either incubated at 37°C for 60 – 90 min for gels with different concentrations - Telocol, crosslinking -

Telocol and Fibricol, and smaller fibrils (Telocol 10) or left at room temperature for 4 h to ensure collagen polymerization for gels with larger fibrils (Telocol 10).

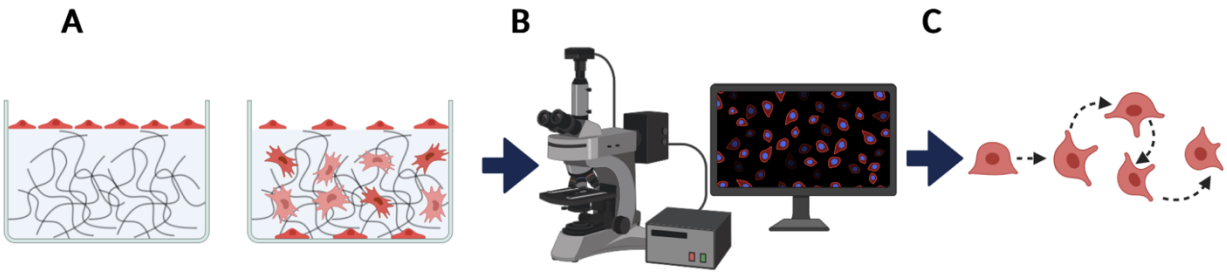


Figure 3: Diagram of Collagen Gel migration assay. (A) Cells are seeded on the top of 2D Collagen gels or into 3D Collagen gels (B) To look at cell migration rates, they were live cell imaged for 6 hours from hour 24 - 30 every 30 minutes. (C) To track cells, imaged were analysed on Fiji using TrackMate plugin to generate files with calculated parameters. (Created with Biorender.com)

Live Cell imaging

Live-cell imaging was conducted using a Leica DMi8 with an environmental chamber using a 20× objective. Live images were taken every 30 min using Fluoroblok 24-well inserts over 6 hours. (Fig 3B). Image analysis was performed using FIJI/ImageJ. All analyses were performed on images that had 4-dimensional data x, y, z, t. The images were resliced from x, y, z, t to x, z, y, t and a maximum projection along xz was taken for further analysis to distinguish between horizontal and vertical migration. Images were further analyzed with background subtraction applied using an appropriate radius and equal smoothening of the images. Cells were tracked using TrackMate plugin on FIJI using the thresholding detector. Thresholding values differed based on image conditions. But the representative images were brightened and sharpened after quantification to make features clearer. (Fig 3C). When quantifying migration speeds and cell area, means across frame was averaged for each condition. For cell directional persistance, the vectorial distance was divided by the maximum distance was each track to obtain a value between 0 - 1, 0 meaning random migration and 1 meaning directional and averaged for each condition.

Statistical Analysis

At least 30 cells were analyzed for each condition, and the experiment was repeated three times. T-tests were performed comparing the means of area, migration speed and persistence for the 3D conditions of crosslinking and fibril size. It was also used to compare C2C12 area on 2D telocollagen and atelocollagen gels. One-way ANOVA with Tukey's multiple comparisons test was used for migration speed, cell spread area and persistance for all 2D and collagen concentration 3D experiments.

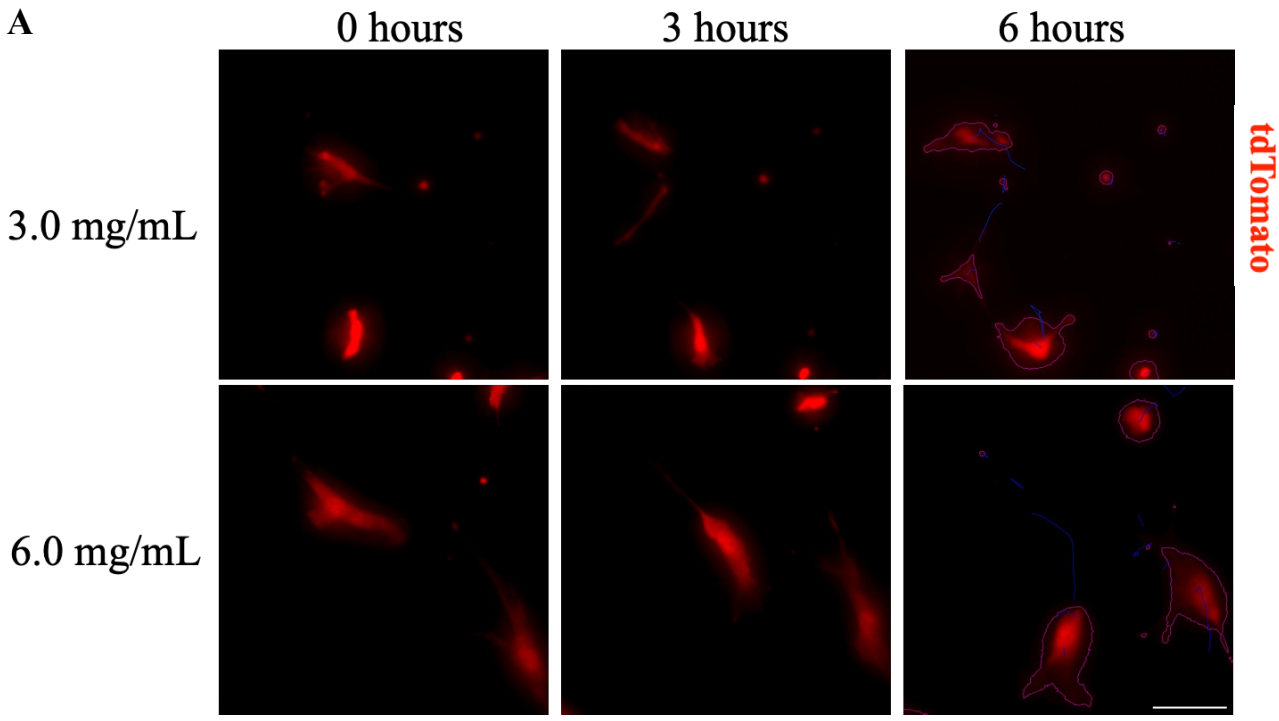
Results

Collagen Concentration does not affect myoblast migration in 2D but slows it down in 3D

To study the effect of collagen concentration on myoblast migration, we examined cell behavior on telocollagen gels at increasing concentrations (1.5, 3, 4.5, and 6 mg/mL). In 2D collagen gels, cells were found to spread more, exhibiting greater area with increasing collagen concentration (Fig 4B). However, cell migration speeds were not impacted by concentration of collagen for either HuMB or C2C12 cells (Fig 4C & Fig 5C). The concentration of collagen also did not influence directional persistence (defined as the ability of the cell to maintain a straight path of motion) of both HuMB and C2C12 cells in 2D (Fig 4D & Fig 5D).

In 3D, the increasing concentration of collagen resulted in decreased overall cell speeds for HuMB (Fig 6B). Speeds in individual directions of X and Z tended to decrease with increased collagen concentration (Fig 6 C&D). The cells in 6 mg/ml collagen migrated the slowest compared to 1.5 mg/ml. C2C12 in 3D had a clear negative effect on their speeds in X direction with increasing collagen concentration but this did not impact overall speed or speed in the Z direction (Fig 7 C&D). Thus, these results indicate that effects of collagen concentration are different in 2D

vs 3D environments and cell migration speeds decrease with increased collagen concentration in 3D.



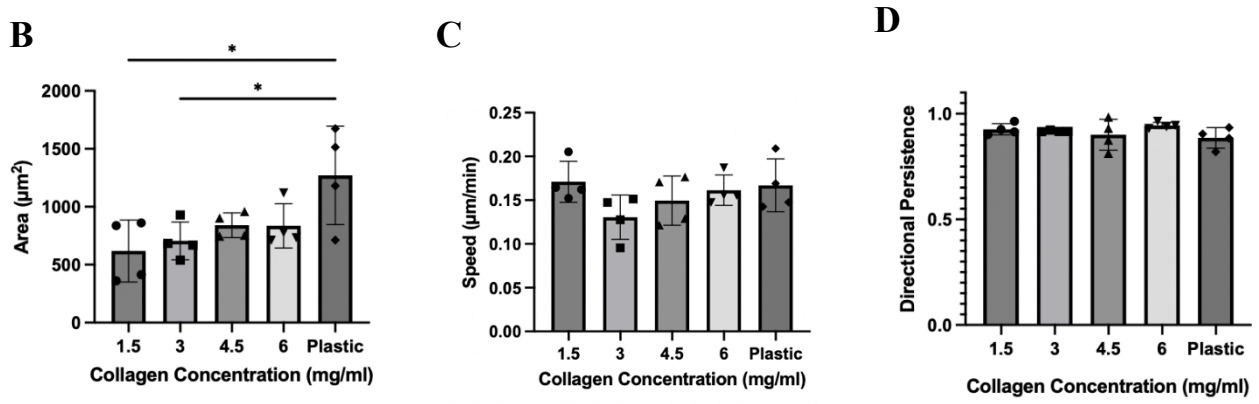


Figure 4: Cell migration on 2D collagen gels of 1.5-6 mg/mL. (A) Time lapse images of HuMB tdTomato on 3 mg/mL and 6mg/mL gels at hour 0, 3, 6. (B) Quantification of the cell area on collagen gels of different concentrations. (C) Quantification of cell speed on collagen gels of different concentrations (B) Quantification of the directional persistance of cells on collagen gels of different concentrations. Scale bar = 50µm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

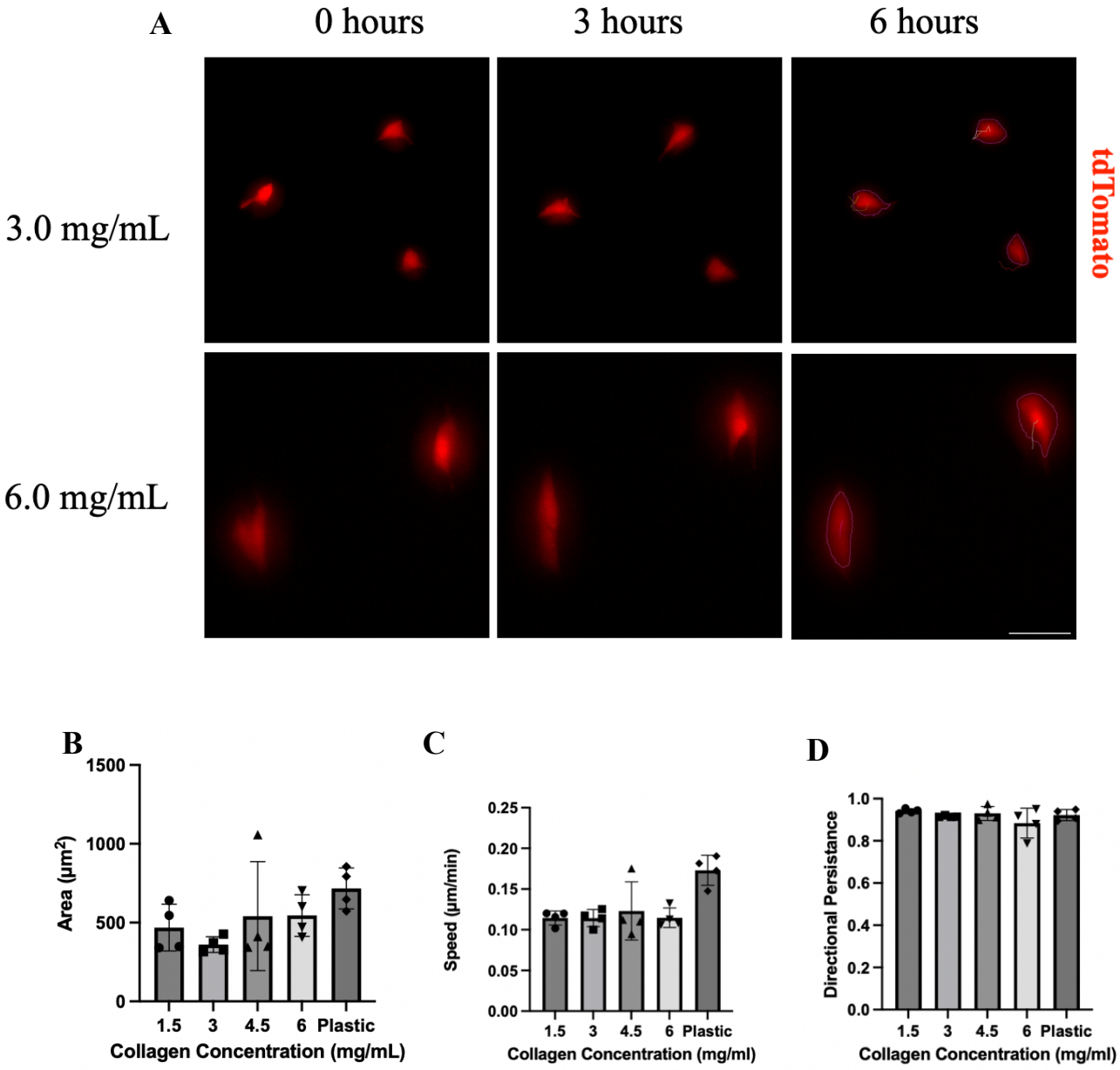


Figure 5: Cell migration on 2D collagen gels of 1.5-6 mg/mL. (A) Time lapse images of C2C12 tdTomato on 3 mg/mL and 6mg/mL gels at hour 0, 3, 6. (B) Quantification of the cell area on collagen gels of different concentrations. (C) Quantification of cell speed on collagen gels of different concentrations (B) Quantification of the directional persistance of cells on collagen gels of different concentrations. Scale bar = 50µm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

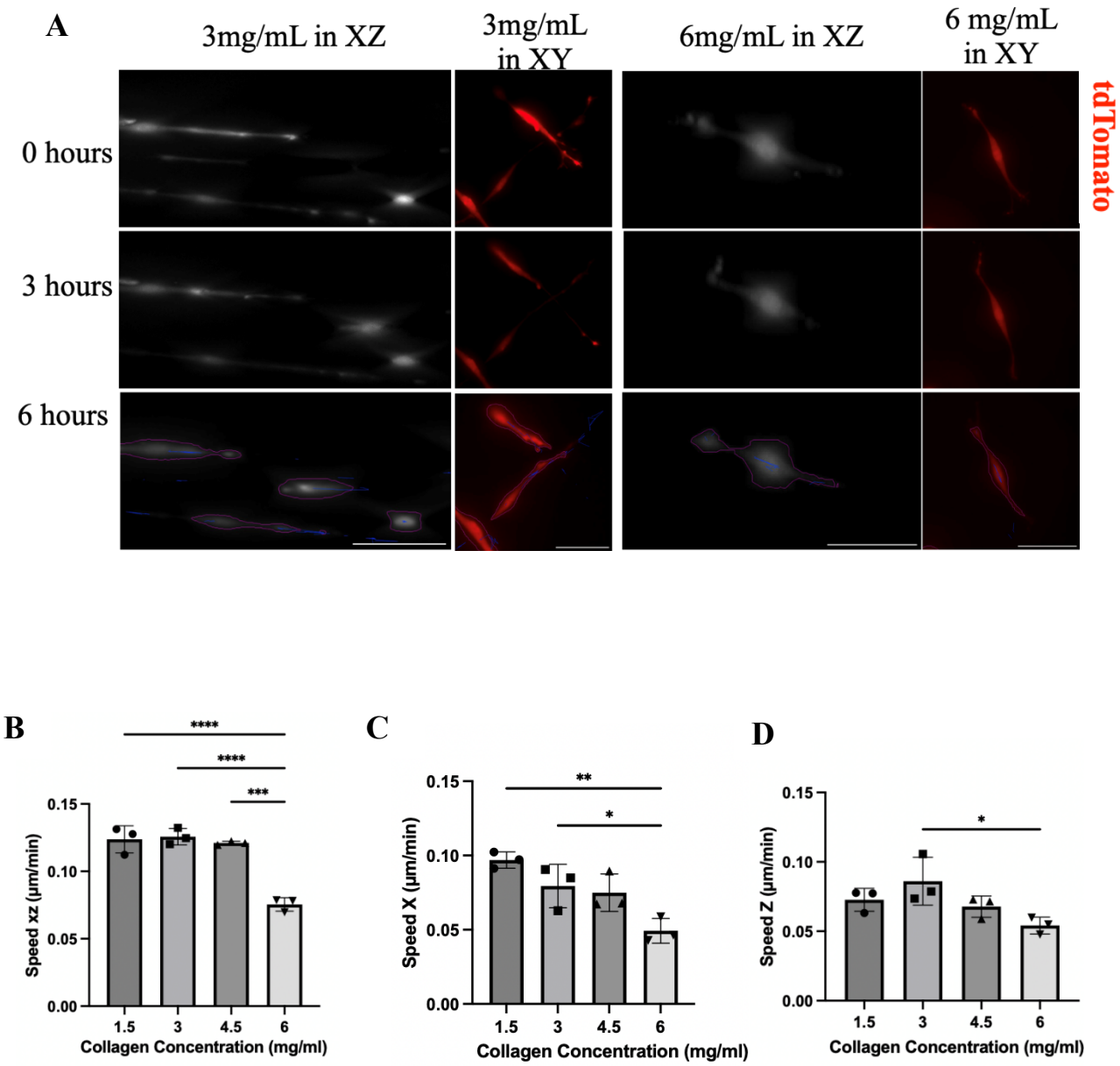


Figure 6: Cell migration on 3D collagen gels of 1.5-6 mg/mL. (A) Time lapse images of HuMB tdTomato on 3 mg/mL and 6mg/mL gels at hour 0, 3, 6 in XZ and XY dimensions. (B) Quantification of cell speed in XZ dimension on collagen gels of different concentrations. (C) Quantification of cell speed in X direction on collagen gels of different concentrations. (B) Quantification of cell speed in Z direction on collagen gels of different concentrations. Scale bar = 50µm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

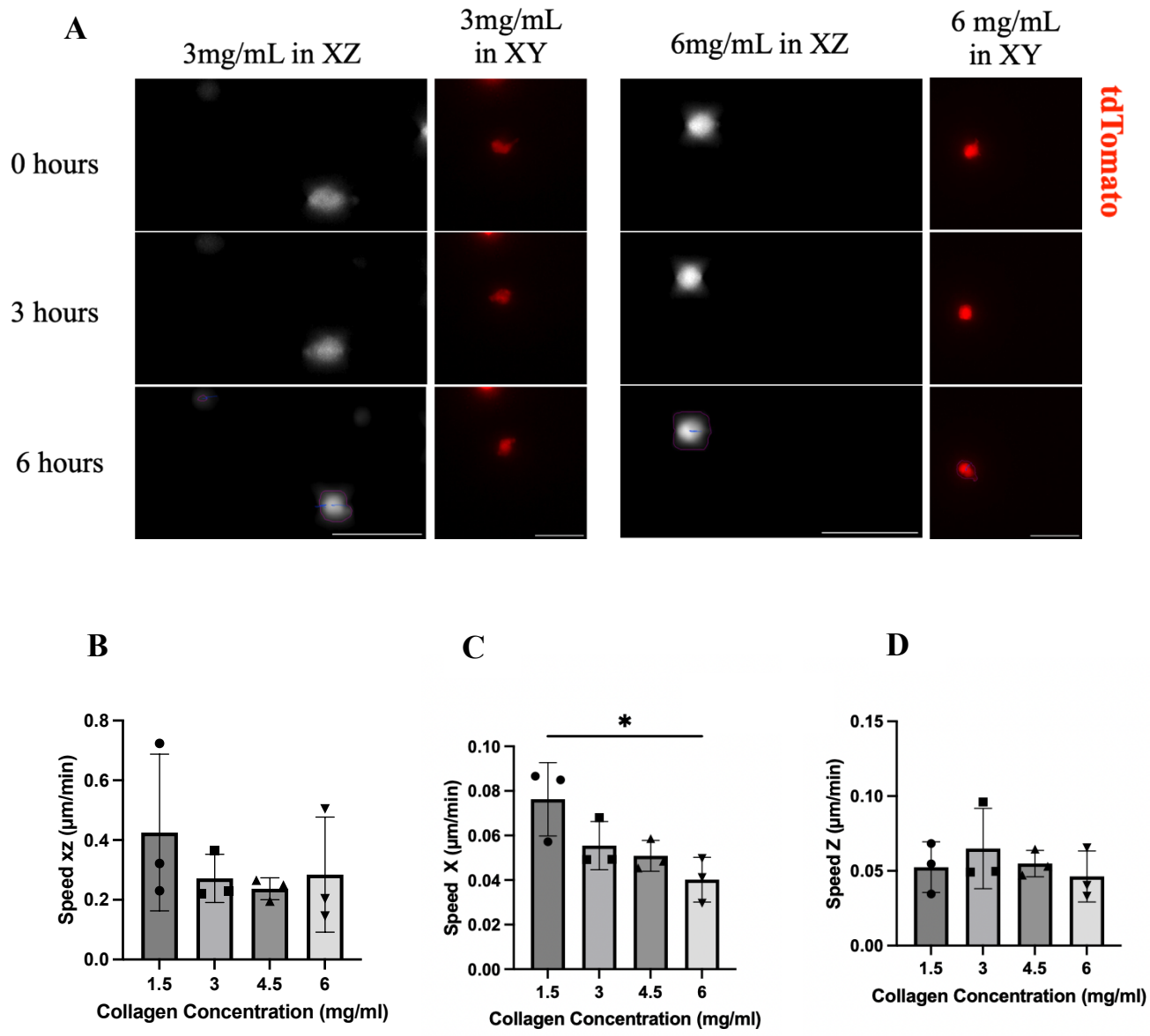


Figure 7: Cell migration on 3D collagen gels of 1.5-6 mg/mL. (A) Time lapse images of C2C12 tdTomato on 3 mg/mL and 6mg/mL gels at hour 0, 3, 6 in XZ and XY dimensions. (B) Quantification of cell speed in XZ dimension on collagen gels of different concentrations. (C) Quantification of cell speed in X direction on collagen gels of different concentrations. (B) Quantification of cell speed in Z direction on collagen gels of different concentrations. Scale bar = 50µm. * $p < 0.05$

Collagen Crosslinking does not impact HuMB Migration but Impairs C2C12 Migration

Collagen molecules form intramolecular pyridinoline cross links within the telopeptide region of collagen. Atellocollagen, where the N and C terminal ends of the collagen peptide are cleaved to block crosslinking, was used as a the non-crosslinked substrate for myogenic cells to assess the influence of collagen cross linking. Collagen cross linking had no bearing on the area, speed or the directional persistence of HuMB in 2D. (Fig 8 B, C, D).

There was a significant difference in cell area of C2C12 as a function of crosslinking, with cells on crosslinked collagen showing greater cell spreading (Fig 9 B). C2C12 on atelocollagen also migrated at a significantly faster rate compared to C2C12 on telocollagen gels. (Fig 9 C). There was no effect of collagen cross linking on directional persistence (Fig 9 D).

In 3D, HuMB migration speed was not different between crosslinked and non-crosslinked (Fig 10 B, C, D). C2C12 in non-crosslinked 3D gels showed enhanced migration rates. (Fig 11 B, C, D). These data demonstrate that collagen cross linking impairs C2C12 migration in 2D and 3D, but the same effect was not detected in HuMB.

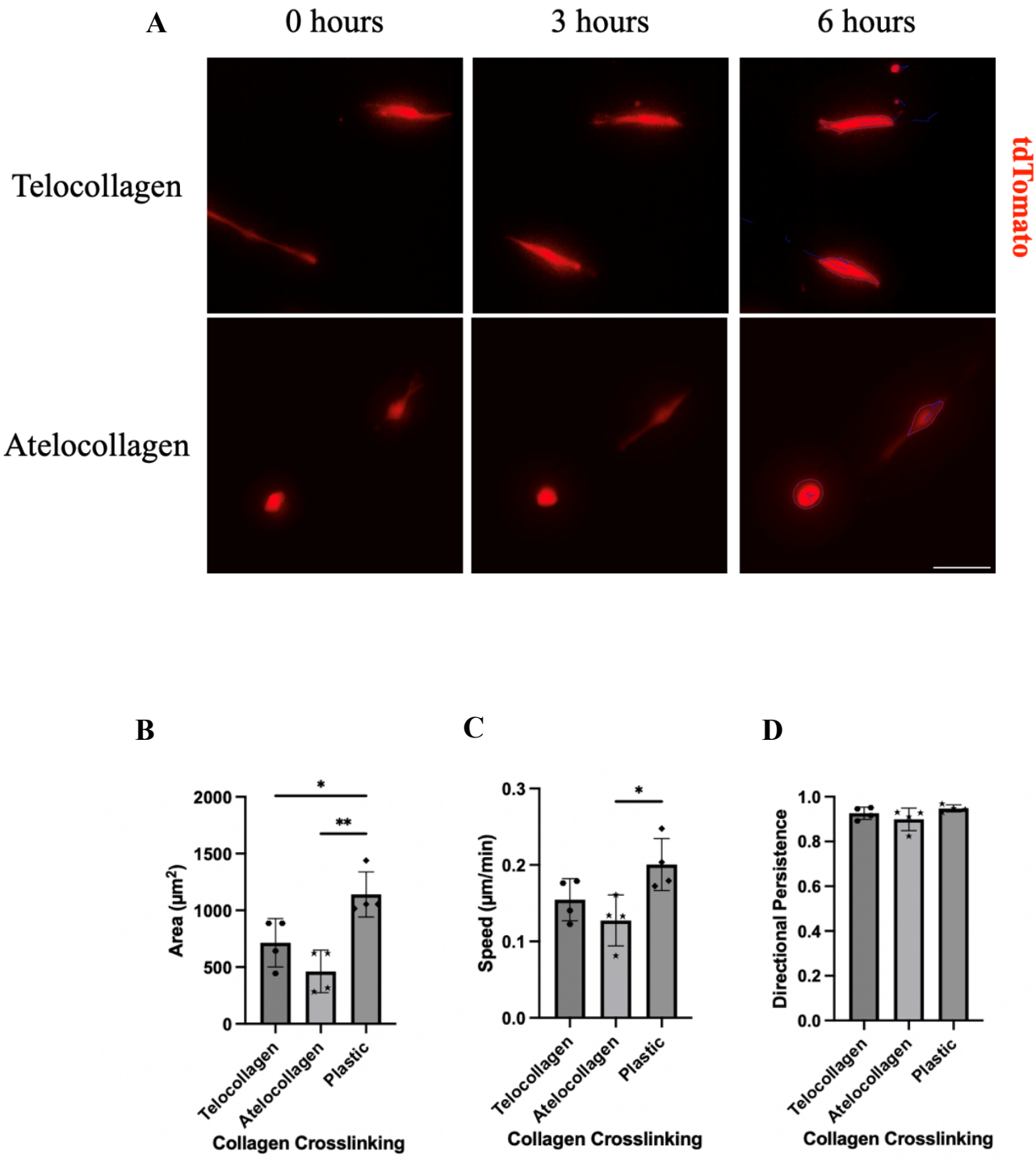


Figure 8: Cell migration on 2D telocollagen and atelocollagen gels of 3 mg/mL. (A) Time lapse images of HuMB tdTomato on 3 mg/mL telocollagen and atelocollagen gels at hour 0, 3, 6. (B) Quantification of the cell area on collagen gels of different crosslinking. (C) Quantification of cell speed on collagen gels of different crosslinking (B) Quantification of the directional persistance of cells on collagen gels of different crosslinking. Scale bar = 50μm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

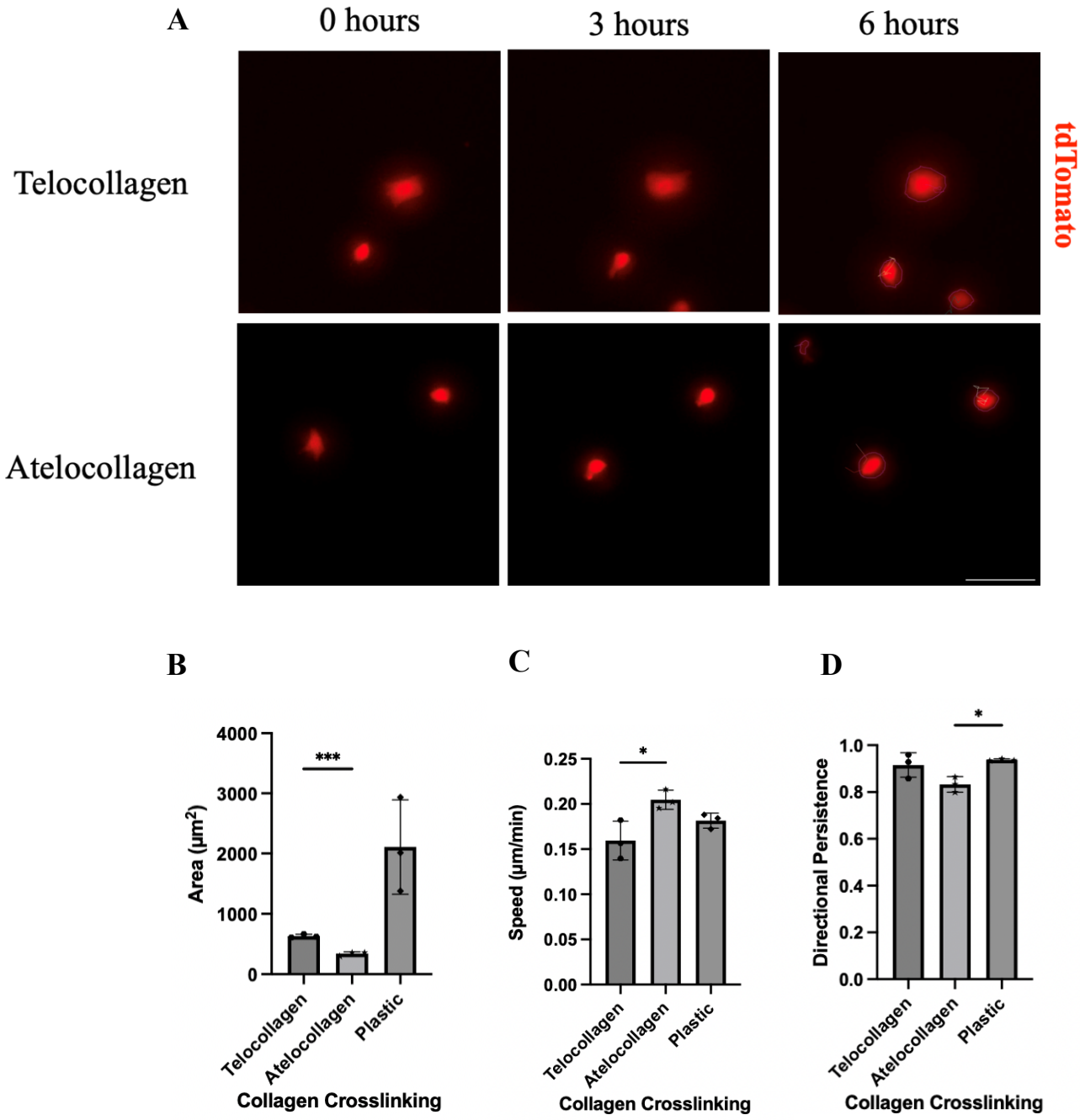


Figure 9: Cell migration on 2D telocollagen and atelocollagen gels of 3 mg/mL. (A) Time lapse images of C2C12 tdTomato on 3 mg/mL telocollagen and atelocollagen gels at hour 0, 3, 6. (B) Quantification of the cell area on collagen gels of different crosslinking. (C) Quantification of cell speed on collagen gels of different crosslinking (B) Quantification of the directional persistance of cells on collagen gels of different crosslinking. Scale bar = 50μm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

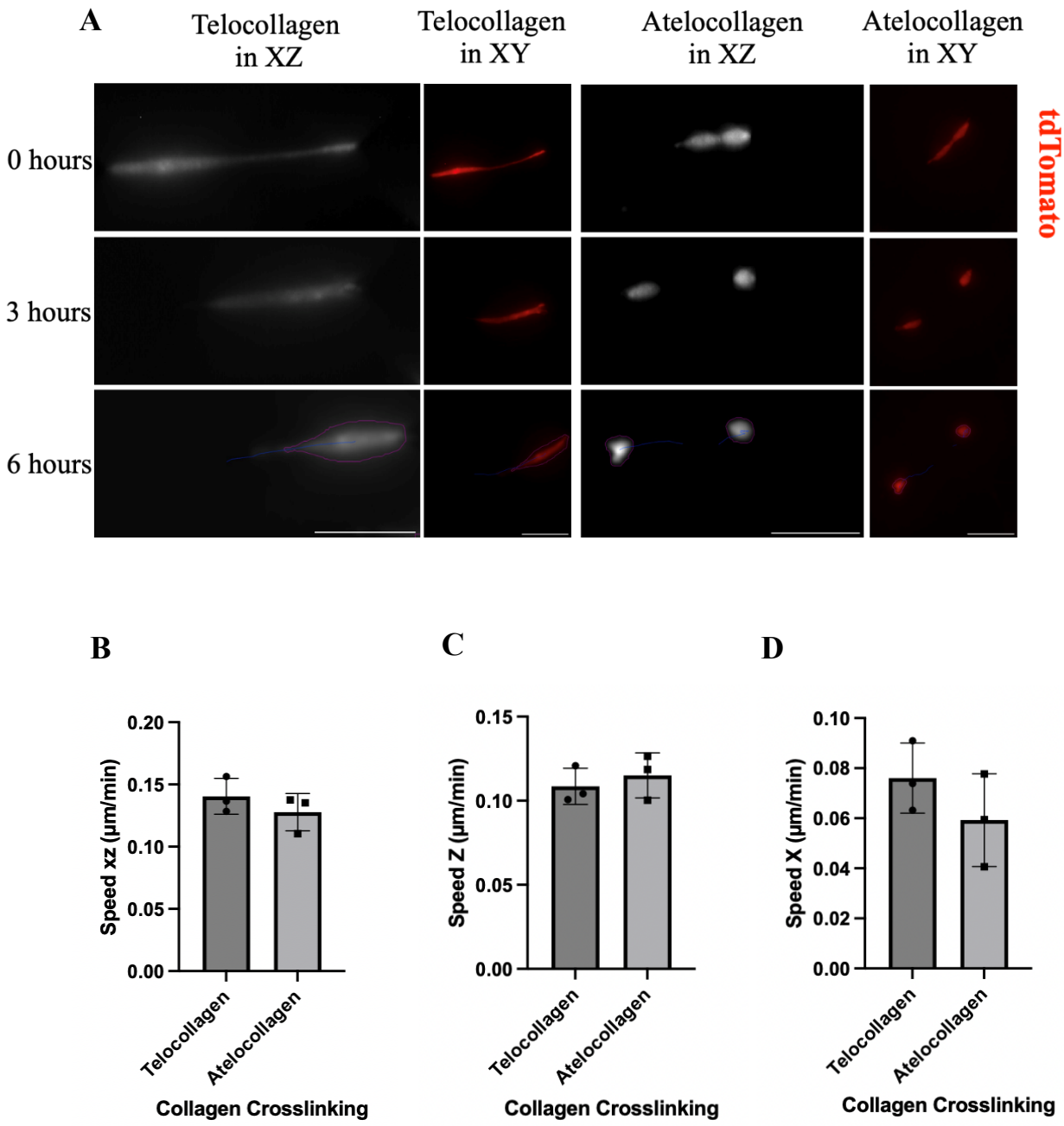


Figure 10: Cell migration on 3D telocollagen and atelocollagen gels of 3 mg/mL. (A) Time lapse images of HuMB tdTomato on 3 mg/mL telocollagen and atelocollagen gels at hour 0, 3, 6 in XZ and XY dimensions. (B) Quantification of cell speed in XZ dimension on collagen gels of different crosslinking. (C) Quantification of cell speed in X direction on collagen gels of different crosslinking. (B) Quantification of cell speed in Z direction on collagen gels of different crosslinking. Scale bar = 50µm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

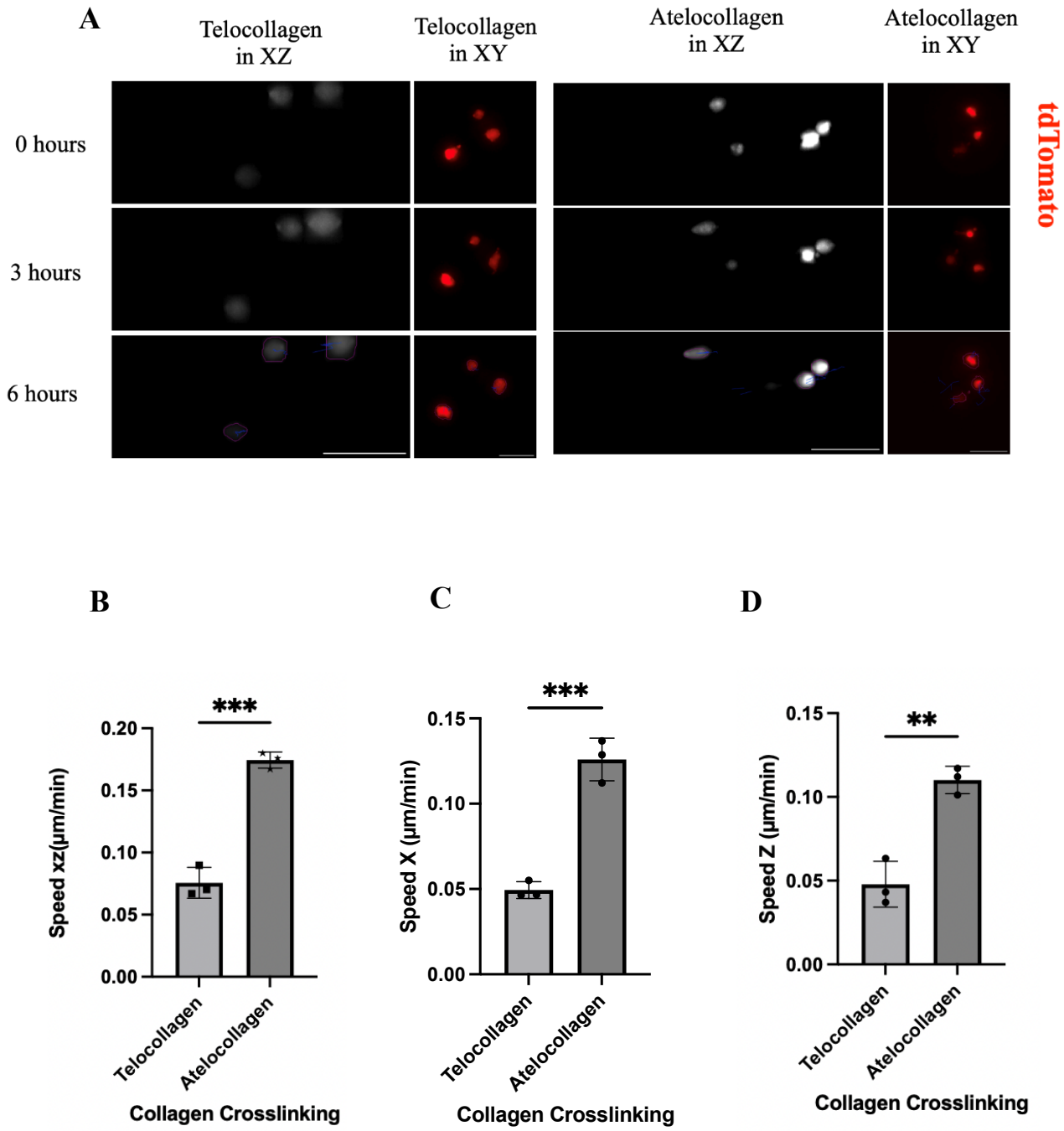


Figure 11: Cell migration on 3D telocollagen and atelocollagen gels of 3 mg/mL. (A) Time lapse images of C2C12 tdTomato on 3 mg/mL telocollagen and atelocollagen gels at hour 0, 3, 6 in XZ and XY dimensions. (B) Quantification of cell speed in XZ dimension on collagen gels of different crosslinking. (C) Quantification of cell speed in X direction on collagen gels of different crosslinking. (B) Quantification of cell speed in Z direction on collagen gels of different crosslinking. Scale bar = 50µm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

Collagen Fibril Size does not impact HuMB migration in 2D and 3D but affects C2C12 directional persistance in 2D

Fibrosis can be associated with larger collagen fibrils. Although the actual data for that is less prevalent. Polymerization of collagen molecules at relatively high temperatures (37°C) results in small fibrils compared to polymerization at lower temperatures (22°C) [14]. Telocollagen gels polymerized at 37°C or 22°C did not alter cell migration behaviour of HuMB or C2C12 in 2D. (Fig 12 B, C, D & Fig 13 B, C). HuMB had similar cell spread area, migration speeds. (Fig 12 B, C). Directional persistence of C2C12 was affected by fibril size; with cells on 22°C gels having significantly higher persistence compared to cells on 37°C gels (Fig 13 D).

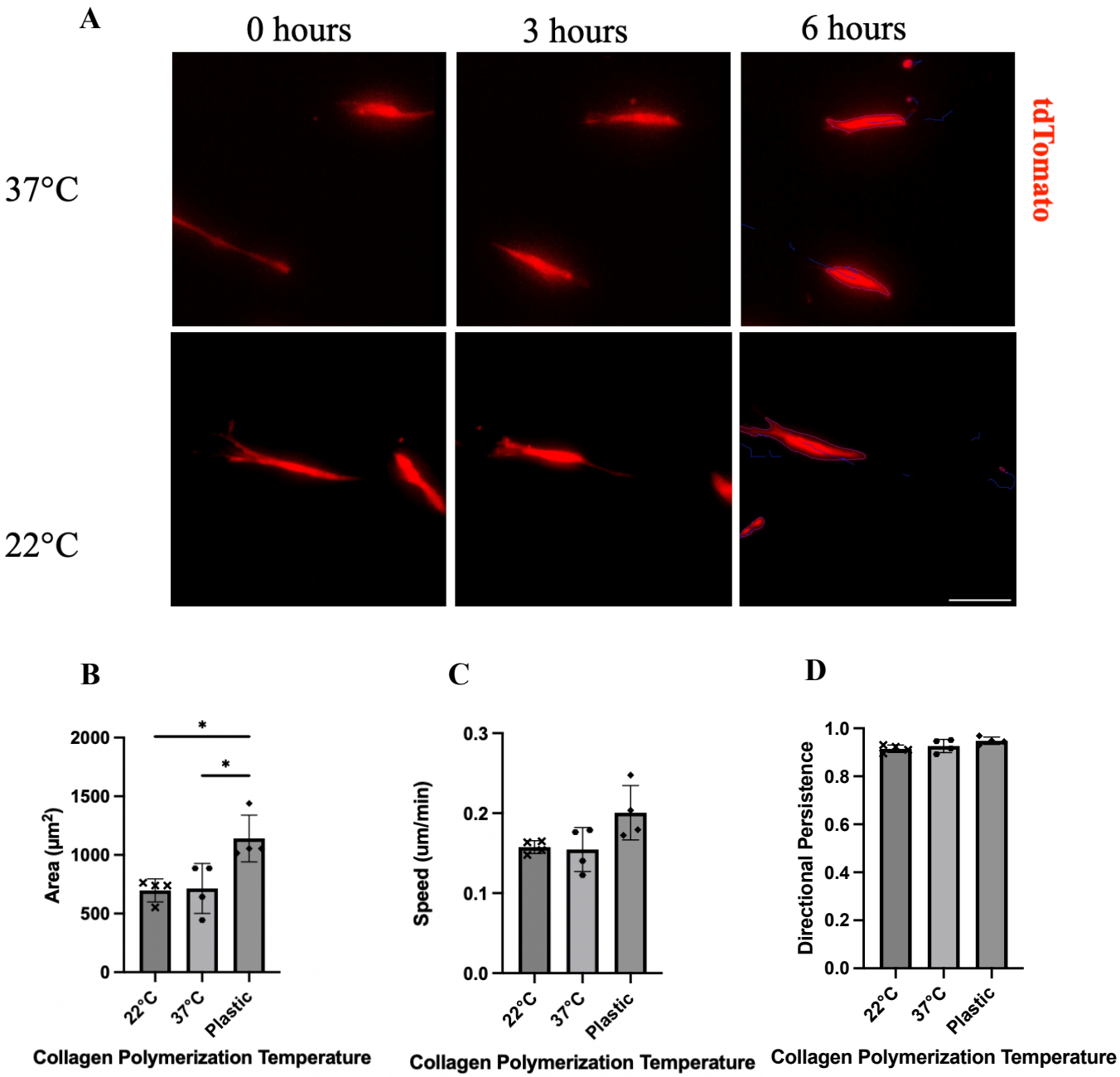


Figure 12: Cell migration on 2D telocollagen gels of 3 mg/mL polymerized at 22°*C* and 37°*C*. (A) Time lapse images of HuMB tdTomato on 3 mg/mL telocollagen gel polymerized at 22°*C* and 37°*C* at hour 0, 3, 6. (B) Quantification of the cell area on collagen gels of different fibril sizes. (C) Quantification of cell speed on collagen gels of different fibril sizes. (B) Quantification of the directional persistance of cells on collagen gels of different fibril sizes. Scale bar = 50µm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

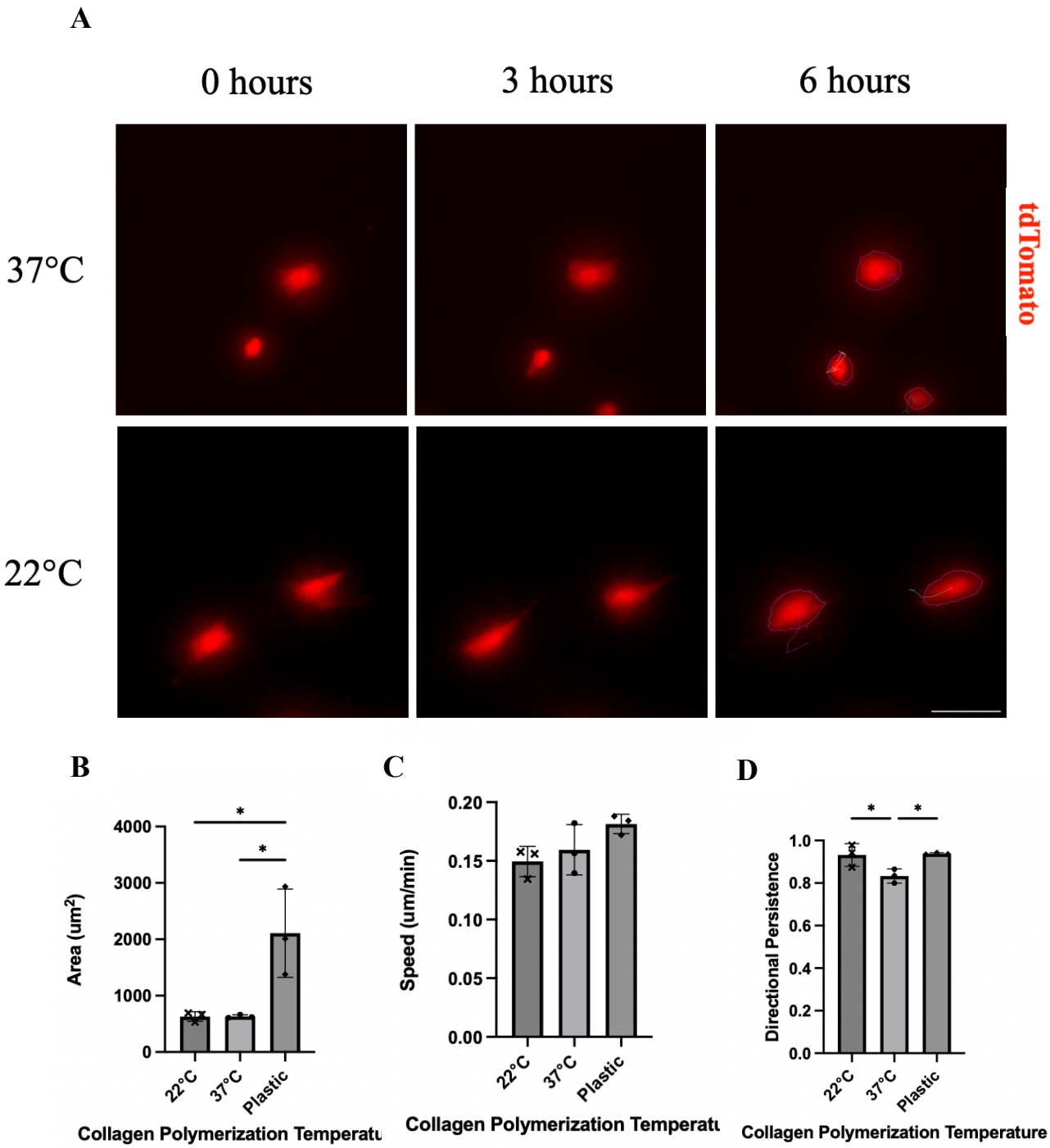


Figure 13: Cell migration on 2D telocollagen gels of 3 mg/mL polymerized at 22°*C* and 37°*C*. (A) Time lapse images of C2C12 tdTomato on 3 mg/mL telocollagen gel polymerized at 22°*C* and 37°*C* at hour 0, 3, 6. (B) Quantification of the cell area on collagen gels of different fibril sizes. (C) Quantification of cell speed on collagen gels of different fibril sizes. (B) Quantification of the directional persistance of cells on collagen gels of different fibril sizes. Scale bar = 50μm. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$

Discussion

Myoblasts migrate through a three-dimensional matrix to reach the site of muscle regeneration. Cell-matrix interaction is essential in cell migration as cells make physical connections necessary for migration. The cells make protein-protein contacts between transmembrane proteins such as integrin to ECM proteins, such as collagen or fibronectin. These contact points facilitate intracellular signaling pathways that regulate cytoskeletal rearrangements and the formation of focal adhesions [52]. Focal adhesions are structures that are needed to generate tractional forces. Cells detach and attach in repeating cycles of cytoplasmic rearrangement, focal adhesion formation, and the development of traction force necessary to move. Cellular scaffolds thus play an important role in offering physical substratum cells adhere to allowing them to migrate effectively [53].

In vivo, cells migrate through a 3D matrix which is mostly comprised of collagen I, remodeling the ECM as they move along. Previous research has shown that cell migration is affected by matrix properties. We were able to study how different matrix properties modulated myoblast migration in 2D and 3D with our engineered matrices.

Cells migrate toward stiffer regions when cultured on a 2D matrix with gradient stiffness [54]. This process by which cells undergo stiffness guided migration is termed as durotaxis. There have been several other topological features of the matrix that have been found to impact cellular behavior such as fiber size, alignment, and viscoelasticity. Fibril size has been found to be positively correlated with stiffness which is also said to be related with collagen concentration [49], [55]. Previous work from our group found that matrix architecture modulates MuSC proliferation and differentiation. Collagen crosslinks were found to inhibit mouse primary MuSC

differentiation and larger fibrils impaired both mouse primary MuSC as well as C2C12 differentiation [14].

Most studies looking at migration have been performed in 2D identifying spatio-temporal data. Myoblast migration in 2D has been found to be affected by Syndecan-4 expression a transmembrane protein [21]. Another study demonstrated the necessity of MMP-14 which is a matrix metalloproteinase that helps remodeling the matrix in the invasion by human muscle satellite cells through 3D collagen matrix [23]. Although a few studies have investigated some of these aspects for migrations of other cell types. It is unclear how the matrix architecture impacts myoblast migration.

The goal of this study was to investigate the effect of collagen architecture on myoblast migration. We looked at the effects on both human immortalized myoblasts as well as C2C12. From literature it is indicative that human and mouse myoblasts have varying cellular behaviors with a particular example of human myoblasts not regenerating as well as mouse myoblasts in vitro [56].

This thesis investigated the effect of matrix architecture on myoblast migration using both 2D and 3D collagen cultures. In 2D cultures, myoblast migration is restricted to a single plane, and this may not accurately reflect the complex fibrillar collagen 3D environment in vivo. In contrast, 3D cultures allow myoblasts to migrate in multiple directions, which may more accurately reflect the in vivo environment. 2D collagen gels were engineered according to our previously established protocols as described in the methods section and the 3D gels were engineered with a similar protocol with a slight variation with the cells being seeded within the gels. The 3D gels were not characterized but should be analyzed in the future using second harmonic generation imaging.

Neither HuMB myoblasts nor C2C12 migration speeds were impacted by collagen content in 2D but were reduce with increased collagen concentration in 3D. This could be due to the fact that cells need to remodel the ECM and the more concentrated it gets, the remodelling mechanisms could be affected. In a pervious study, migration behaviour of pre-osteoblastic cells were seen to be strongly dependent on matrix stiffness controlled by polymer concentration with proteolytic migration reducing with increasing concentrations [57]. Our study is line with previously published results of how concentration affects cell migration in 3D. However in 2D there have been studies where it was found that Neutrophils migrating on polyacrylamide exhibited a biphasic speed profile with varying with substrate stiffness as well as fibronectin concentration [58]. Therefore, both concentration and substrate stiffness even though correlated, may have a combinatory effect on migration speed.

HuMB migration was not impacted by fibril crosslinking but C2C12 migration was inhibited by the presence of crosslinks in 2D as well as 3D. Matrix crosslinking can improve cell adhesion [59]. Mesenchymal migration requires cell matrix interactions. Absence of crosslinks may have reduced cellular adhesion and the cells may exhibit a more amoeboid migration pattern. Low adhesion amoeboid migration enables cells to move at a faster rate [41]. Cells have been found to have an enhanced ability of reorganize smaller fibrils [60] and this may cause a more random migration pattern.

Invasive cancer cells exhibit structures known as invadopodia which are protrusions that are highly dependent on ECM cross-linking [61]. Other in vivo studies demonstrated that invadopodium are present on cells migrating in an MMP-dependent manner [62]. Cancer cells can

also migrate using varied mechanisms including amoeboid migration. Such migration mechanisms have not been studied in the context of myoblasts yet.

Immortalized human myoblasts are reported to move using lamellipodia. In our study, we looked at single cell migration patterns of immortalized human myoblasts. A previous study using isolated myofibers indicated the ability of satellite cells to move in groups [32]. Another study reported that satellite cells migrate on single isolated fibers employing ameboid migration [63]. A limitation of the current study is that we are unable to study the migration of satellite cells in the context of moving on a native environment on a myofibers for regeneration. There is growing evidence that myoblasts may have different mechanisms for migration.

Another limitation of this study is that characterization of 3D gels is not performed and is unable to have conclusive remarks about how myoblasts may interact and modify the 3D ECM. 3D collagen gels with different fibril sizes were particularly challenging to make. The gels at lower temperature 22°C polymerised slowly and leaving the cells at such a temperature for long periods of time is not recommended. Currently, higher temperatures are being tested and the gels would be characterised to determine if they do indeed have different fibril sizes before proceeding to polymerise gels at that particular temperature. This would be included in our future work going forward.

Future works could also investigate the effect of MMP inhibitors on migration. The interaction between cell-ECM is essential for cells to making physical connections and remodel the matrix using proteolytic proteins called MMPs. Inhibitor studies of MMP might reveal more information on how the cell modulates the matrix to maneuver through. This would help understand whether they could be useful targets in improving cell migration for regeneration.

Overall, this thesis demonstrates that myoblasts are sensitive to the architecture of their

substrate and specifically identifies collagen content and crosslinking as potential targets to explore further. This would allow the manipulation of ECM properties and enhance the migratory capacity of MuSC for regeneration.

CHAPTER 4: CONCLUSION

This thesis concludes that immortalized human myoblasts are blocked from migrating by constrictive pores. These cells probe through constricted pores and migrate if they can deform their nuclei for them to migrate. Prior work demonstrated the high levels of nuclear rupture that were found to occur following migration through 3μm-diameter pores, causing increased DNA damage. We noted no change in cell speeds post migration which could mean that only the most migratory cells are able to cross constrictions. We suggest that lack of cells present at the regenerative site due to constricted blocking could be partially attributed the impaired regenerative function of muscle satellite cells that is observed in fibrotic diseases such as Duchenne muscular dystrophy.

There is however a limitation of this study that should be explored in future work. First, the migration of primary human and mouse myoblasts must be evaluated in vitro. There is increasing evidence that immortalized human myoblasts used in many studies do not exhibit similar migration patterns as primary cells. Isolating MuSCs from mouse or human muscle biopsies using MACS and running migration assays on them could be an approach that might elucidate more native MuSC behavior.

This thesis also explores the impact of the matrix architecture on human and mouse muscle progenitor migration. We were able to discover differential effects of 2D and 3D matrices on cell migration. C2C12 and HuMB were modulated differently by different matrix properties. While HuMB migration speed decreased with increased collagen concentration in 3D, C2C12 migration speed decreased with both increased collagen concentration and crosslinking. Future work also involves studying the effect of MMP inhibitors on myoblast migration to better understand how ECM remodeling helps in migration. This would provide valuable insight into the mechanisms underlying impaired muscle regeneration in pathological conditions.

Together, these results motivate and demonstrate the potential for further studies of matrix architecture on myoblast migration and the effect of a fibrotic environment on myoblast behavior. The work in this thesis together with further studies can contribute to developing new approaches in regenerative medicine by improving stem cell migration. Better replication of in vivo muscular niche can help understand complexities of muscle and to improve research toward therapeutic solutions to combatting fibrotic diseases such as DMD.

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