Abstract:

Three-dimensional tissue engineered matrices, such as microthreads, can be used to grow new myofibers that will reduce scar formation and integrate easily into native myofibers. The goal of this study was to investigate whether adsorbing growth factors to the surface of braided collagen scaffolds using crosslinking strategies will promote muscle derived fibroblastic cell (MDFC) attachment and growth, which will serve as a platform for delivering cells to large muscle defects for muscle regeneration. To test this hypothesis, self-assembled type I collagen threads were braided and crosslinked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), with and without heparin, and with different concentrations of fibroblast growth factor (FGF-2) bound to the surface. To determine the effect of FGF-2 on MDFC attachment, growth, and alignment, scaffolds were seeded with a MDFC cell suspension for 4 hours using a PDMS mold with a sealed 1 mm by 12 mm channel and cultured for 1, 5, or 7 days. Our findings indicate that after 7 days in culture, the MDFCs responded to FGF-2 with a positive linear correlation between growth rate and concentration of FGF-2 on the surface. These **results** show braided collagen scaffolds crosslinked with EDC/NHS with heparin, and a controlled quantity of FGF-2 can support MDFC attachment and growth and may serve as a novel approach to facilitate the delivery of cells to large defects in muscle regeneration.

Introduction

The controlled expression of certain trophic factors, such as the families of fibroblast growth factors (FGFs), transforming growth factors-β (TGFs-β), insulin-like growth factors (IGFs), hepatocyte growth factor (HGF), and interleukin-6 (IL-6), regulate the activation, proliferation, and differentiation of satellite cells, [which are undifferentiated progenitor cells located beneath the basal lamina of the myofibers] as well as the initiation of vascularization and reinnervation.[1] During tissue remodeling, fibrosis within the defect area causes the formation of scar tissue due to an excessive amount of type I collagen present.[6] Muscle defects caused by trauma are classified as ranging from first-degree defects (minimal myofiber damage) to third-degree defects (spanning the entire depth of the muscle). During the regeneration of minor injuries, capillaries and myofibers are able to regenerate with limited scar tissue formation allowing for almost complete tissue regeneration. However, in full thickness defects, the increased scar formation and lack of satellite cell availability limits the regeneration and revascularization of the skeletal muscle resulting in the loss of some or all of the muscle function.[7]

The major concern with substantial muscle loss related to large muscle defects is that it causes deformities, persistent muscle weakness, and loss of function. Skeletal muscle degradation associated with innate genetic diseases causes the muscles eventually to become completely atrophied leading to extensive long-term care and death of the afflicted (tissue?).[2,6,14-16] Clinically, first and second-degree muscle defects are treated using a combination of rest, ice, compression, and elevation, but this method does not eliminate the formation of scar tissue and is sometimes associated with injury reoccurrence.[6,7,17,18] Plastic and reconstructive surgeries, such as the transplantation of autologous muscle grafts or myogenic cell injections, are performed as treatments for third degree injuries to bridge the gap and help initiate muscle regeneration.[18] Limitations associated with surgical procedures are scar formation, limited cell incorporation, weakness, morbidity, and mortality.[19,20]

In order to overcome limitations associated with the sub-optimal clinical treatments, research is being performed on implanting biomaterials into muscle defects. Three-dimensional-tissue-engineered muscle needs to be biocompatible, biodegradable, biologically and mechanically stable, and induce host cell migration, regeneration, and revascularization.[1,8,16] *In vitro* and *in vivo* studies using synthetic materials and natural polymers, like polyglycolic acid (PGA), poly(ε-caprolactone) (PCL), hyaluronic acid, alginate, fibrin, and acellular matrix, have shown to support myoblast migration, differentiation, fusion, and in some cases promote revascularization.[21-24] However, due to limitations in scaffold design including biodegradability (which affects myofiber maturity, density, and population homogeneity) and stiffness (which affects myofiber length and the force generated) these biomaterials do not meet the conditions needed for clinical studies.[8]

Another biomaterial investigated for muscle regeneration is collagen because it possesses strong mechanical properties, suitable cell-matrix binding efficiencies, weak immunogenic responses, and high biodegradability.[25-29] Another advantage of using collagen is that it can be manipulated into different structures, such as gels, microthreads, and porous sponges, making it a good candidate for tissue-engineering applications. By incorporating chemical, mechanical, or extracellular matrix modifications on the surface of collagen scaffolds, myoblasts can be differentiated to form mature myofibers.[30-34] Cornwell et al. has shown that collagen microthreads seeded with fibroblast cells promoted cell migration and alignment, which suggests the potential for microthreads to be used for muscle regeneration.[35,36] In addition, by manipulating the structure of microthreads through bundling, twisting, weaving, or braiding, the scaffolds show structurally similar properties as native myofibers, as well as increased surface area and mechanical strength.[37] Chemical crosslinking can increase the biostability and mechanical strength of the scaffold and promote cell migration, viability, differentiation, and myofiber formation.[28,38-40]

Another way to optimize collagen scaffolds for muscle regeneration is by binding heparin and FGF-2 to the surface. Studies show that in the presence of FGF-2, cellular proliferation and migration increase, and that FGF-2 is stabilized and protected from denaturation and proteolytic degradation when it is bound to heparin.[41-43]

In addition to the studies of different biomaterials for muscle regeneration, the benefits of using certain cell types, such as myoblasts and satellite cells, have been investigated to show that myoblasts and satellite cells remain viable and active on three dimensional scaffolds.[44,45] The limitations associated with using these cells clinically are loss of myogenicity, low cell survival and incorporation, and the fact that they are not self renewing satellite cells.[11] A potential way to overcome these limitations is by using fibroblast cells that have been programmed to express a stem cell phenotype. Research shows that exposing fibroblasts to FGF-2 in a low oxygen environment induces them to express stem cell related genes, suggesting this treatment may lead to cell dedifferentiation.[46]

The aim of this project was to chemically conjugate heparin to the surface of braided collagen scaffolds to serve as an effective method to load heparan sulfate binding growth factors, such as FGF-2, in order to promote cell attachment and growth. Using immunohistochemistry, braided collagen scaffolds showed the presence of FGF-2 on the surface, and braiding the microthreads increased the mechanical properties when compared to single threads. To determine the effect of FGF-2 on MDFC attachment, growth, and alignment, scaffolds were seeded with a MDFC cell suspension for 4 hours using a PDMS mold with a sealed 1 mm by 12 mm channel and cultured for 1, 5, or 7 days. After 1 day of culture, the results showed a significant increase in cell attachment on braids crosslinked with EDC/NHS with heparin and no significant difference in

attachment between the different concentrations of FGF-2 and EDC/NHS crosslinked scaffolds. After 7 days in culture, the MDFCs responded to FGF-2 with a positive linear correlation between growth rate and concentration of FGF-2 on the surface. Additionally, all control scaffolds showed cellular alignment after 7 days, while MDFCs on FGF-2 modified scaffolds showed limited alignment. These results show braided collagen scaffolds crosslinked with EDC/NHS with heparin, and a controlled quantity of FGF-2 can support MDFC attachment and growth and may serve as a novel approach to facilitate the delivery of cells to large defects in muscle regeneration. This technology could serve as a platform for delivering cells to promote regeneration of large muscle defects.

Materials and Methods
Type I Collagen Extraction from Rat Tendon

Acid-soluble type I collagen was extracted from rat tail tendons as previously described.[36,144] Briefly, tendons were removed from 13 Sprague-Dawley rat tails with a hemostat, rinsed in phosphate buffered saline (PBS, pH 7.4), and dissolved in 1600 mL of 3% (vol/ vol) acetic acid overnight at 4°C. The collagen solution was filtered through layered cheesecloth and centrifuged for 2 hours at 8500 rpm at 4°C. Discarding the pellet, a salt precipitation was performed where 320 mL of 30% NaCl (wt/ vol) solution was dripped into the supernatant. The solution was allowed to sit overnight at 4°C. The entire solution was then centrifuged at 4°C for 40 minutes at 4900 rpm, and the resulting pellet was resuspended on a stir plate in 400 mL of 0.6% (vol/ vol) acetic acid at 4°C until the pellet had dissolved completely. The solution was placed in dialysis membranes (Spectrum Laboratories, Inc., Rancho Dominguez, CA) and dialyzed at room temperature in 1 mM HCl changing the dialysate every 4 hours until the solution was clear. The type I collagen solution was lyophilized and stored at 4°C. Prior to use, the lyophilized collagen fleece is dissolved in 5 mM HCl at a concentration of 10 mg/mL.

Self Assembled Collagen Thread Extrusion

Self-assembled collagen threads were produced from acid soluble type I collagen using methods described previously.[35] Briefly, type I collagen (10 mg/mL in 5 mM HCl) was placed in a 5 mL syringe connected to a polyethylene tube with an inner diameter of 0.86 mm (Becton Dickinson, Franklin, NJ). Using a syringe pump, the solution was extruded through the tubing at a rate of 0.255 mm/min into a 37°C bath of fiber formation buffer (pH 7.4, 135 mM NaCl, 30 mM Tris Base, 30 mM Tris HCl, and 5 mM NaPO$_4$ dibasic; Sigma, St. Louis, MO) and incubated for 24 hours (*Figure 5*). The formed threads were transferred to a 37°C bath consisting of fiber incubation buffer (pH 7.4, 135 mM NaCl, 10 mM Tris Base, 10 mM Tris HCl, and 30 mM NaPO$_4$ dibasic; Sigma) for an additional 24 hours. The threads were then washed in a 37°C bath of distilled water for 24 hours to remove the salt, air dried, and stored at room temperature in a dessicator until use.

Braided Scaffold Preparation

To create braided collagen scaffolds, type I collagen microthreads were attached to a single point and split into three groups [specified in Figure 6A.] Afterwards, the procedure found in Figure 6 was followed to produce the final braided scaffolds containing 18 self-assembled threads.

These were attached to PDMS (Dow Corning, Midland, MI) rings with an inner diameter of 14 mm using Silastic Silicone Medical Adhesive Type A (Dow Corning, Midland, MI) in order to easy fit inside a 12 well tissue culture plate (*Figure 6E*).
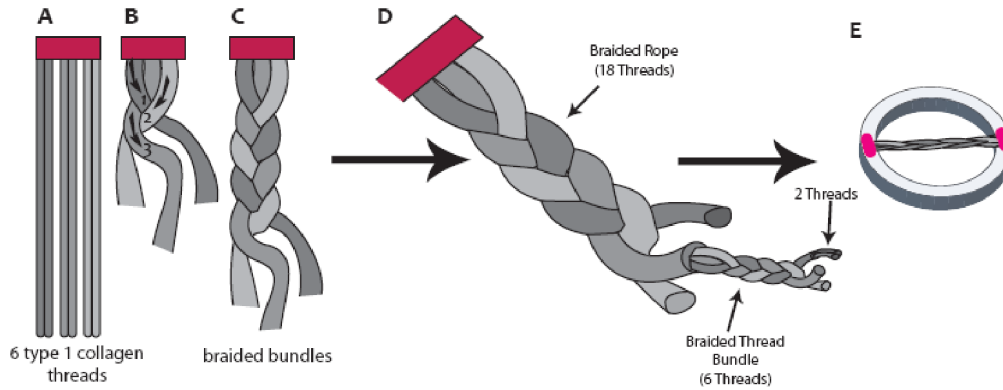

Figure 6: Braided collagen scaffold preparation
To create braided collagen scaffolds, first six type I collagen microthreads were attached to a single point and split into three groups of two threads each (A). The grouped threads were braided together with 28 crossovers per centimeter (B and C). The final braided scaffold was produced by attaching three six-thread braids to a single point and braiding them together with 26 crossovers per centimeter (D). The final braided scaffolds were attached to PDMS rings using Silastic Silicone Medical Adhesive Type (E).

The phase images and table below compare the size of single threads, 6 thread braids, and 18 thread braids both dry and hydrated in PBS (*Figure 7*). Phase images were obtained using an Olympus IX81 motorized inverted microscope coupled to a 12-bit Hamamatzu CCD camera and processed using Slidebook®.
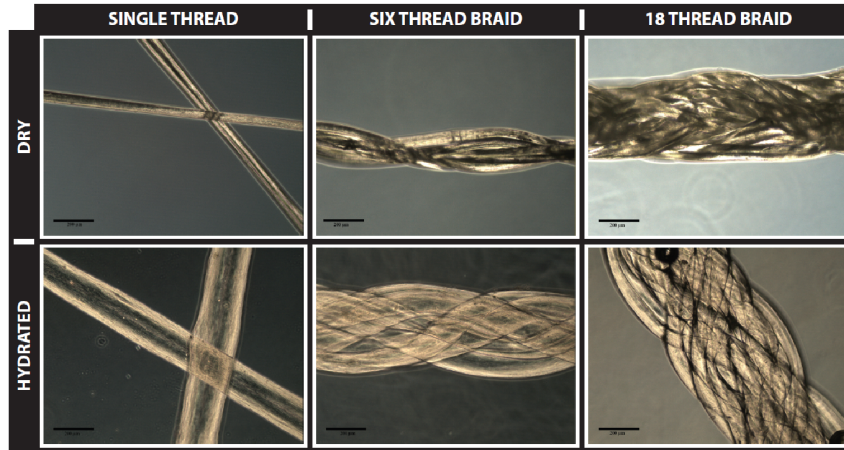

Figure 7: Phase images of single threads, six thread braids, and 18 thread braids
Phase images comparing the size of single threads, six thread braids, and 18 thread braids both dry (top row) and hydrated in PBS (bottom row). Scale bar = 200 μm.

Microthread Crosslinking with Heparin

Braided collagen scaffolds were crosslinked using the chemical crosslinker 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC; Sigma) and N-hydroxysuccinimide (NHS; Sigma) with and without heparin sodium salt (Calbiochem, Gibbstown, NJ) (*Figure 8*).[40,133,144] In a sterile field, braided collagen scaffolds were inverted and inserted into a 12 well plate with one scaffold per well, and they were washed with 70% (vol/ vol) ethanol 4 times for 30 minutes each and 40% (vol/ vol) ethanol 5 times for 15 minutes each to sterilize. Subsequently, the scaffolds were

submerged in 3 mL of sterile filtered 40% (vol/ vol) ethanol including 50 mM 2-morpholinoethane sulphonic acid (MES, pH 5.0; Sigma) for 30 minutes at room temperature. Next, the scaffolds were incubated in 2 mL of sterile filtered 40% (vol/ vol) ethanol including 50 mM MES, 14 mM EDC, 8 mM NHS, with and without 100 µg/mL heparin for 4 hours at room temperature. The scaffolds were washed in 70% (vol/ vol) ethanol 5 times for 10 minutes each with a final overnight wash at 4ºC.

FGF-2 Binding through Passive Adsorption

Fibroblast growth factor (FGF-2; Chemicon, Temecula, CA) in varying concentrations was passively adsorbed to the surface of braided collagen scaffolds and crosslinked with EDC/NHS and heparin using methods previously described.[38] Due to material limitations, passively adsorbing FGF-2 to the surface of uncrosslinked and EDC/NHS crosslinked scaffolds without heparin was not tested, but in the future, it will be beneficial to study these interactions as well. Briefly, scaffolds were washed 5 times for 10 minutes with sterile Dulbecco's phosphate buffered saline (DPBS, pH 7.4) without calcium and magnesium at room temperature. Subsequently, the chamber walls, PDMS ring, silicone adhesive and nonspecific binding sites on the braided collagen scaffolds were blocked using a blocking solution of 3 mL of sterile filtered DPBS containing 0.25% (wt/ vol) bovine serum albumin (BSA; Sigma) for 1 hour at room temperature. Next the blocking solution was aspirated from each well, and replaced with 2 mL of sterile DPBS containing 0.25% (wt/ vol) BSA with FGF-2 at a concentration of either 5 ng/mL, 10 ng/mL, or 50 ng/mL. The scaffolds were incubated for 2 hours at room temperature. The braided collagen scaffolds were washed in DPBS 5 times for 10 minutes each and stored at 4ºC in DPBS until use.

Braided Collagen Scaffold Structural Characterization
Characterization of Bound FGF-2

To verify that FGF-2 has bound to the surface of the scaffolds, immunocytochemistry was performed. Due to imaging limitations of the geometry of braided collagen scaffolds, single collagen threads were used to characterize the localization of FGF-2 on the surfaces. Single collagen threads were EDC/NHS crosslinked in the presence of heparin and then loaded with FGF-2 at the concentrations mentioned above. Single collagen threads that were EDC/NHS crosslinked in the presence of heparin were used as negative controls. DPBS was removed from the wells by aspiration, and the threads were incubated at room temperature in 300 µL of 1 µg/mL FGF-2 goat polyclonal IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS with 0.05% Tween-20 (Promega Corporation, Madison, WI) for 30 minutes. The threads were washed with 500 µL of PBS with 0.05% Tween-20 for 5 minutes three times. The threads were then incubated in 300 µL of 5 µg/mL Alexa Fluor 647 donkey anti-goat IgG (Invitrogen, Carlsbad, CA) in PBS with 0.05% Tween-20 for 30 minutes. They were then washed in 500 µL of PBS with 0.05% Tween-20 for 5 minutes twice.   Single collagen threads were imaged using fluorescence microscopy on an Olympus IX81 motorized inverted microscope coupled to a 12-bit Hamamatzu CCD camera and processed using Slidebook®.

Mechanical Testing of Braided Collagen Scaffolds

In order to determine the effect of surface modifications on mechanical strength, EDC/NHS crosslinked and uncrosslinked braided collagen threads were analyzed by mechanically loading the hydrated samples in uniaxial tension. Braided collagen scaffolds that were crosslinked with heparin and exposed to FGF-2 were not tested in this study. Braided collagen threads were cut to

a sample length of 30 mm with the last 5 mm of each end bound and sealed using Silastic Silicone Medical Adhesive Type A (Dow Corning, Midland, MI). For tensile testing, the samples were secured horizontally with 2711 Series Lever Action Fiber Grips (Instron, Norwood, MA) on an E1000 ElectroPuls mechanical testing system (Instron, Norwood, MA) with a fixed 50 kN Dynacell dynamic load cell (Instron, Norwood, MA). The mechanical testing system and data acquisition were controlled using Bluehill 2 Materials Testing software (Instron, Norwood, MA). The samples were secured insuring that the silicone adhesive remained outside of the outer grip boundary. An initial gauge length of 7.0 mm was defined as the distance between the inner grip boundaries, and the braids were loaded to failure at a 50% strain rate (3.5 mm/min).

To calculate the ultimate tensile strength, the cross-sectional area of the samples was approximated using histological sections of hematoxylin and eosin stained unseeded braided collagen threads at five different locations. Bright field images were obtained using an Olympus IX81 motorized inverted microscope coupled to a 12-bit Hamamatzu CCD camera and processed using Slidebook®, and analyzed using Image J software (U.S. National Institutes of Health, Bethesda, MD). The outer edge of the braided collagen threads was traced to measure the cross-sectional area. The stress-strain curve, the load at failure, ultimate tensile strength (UTS), stain at failure (SAF), and maximum tangent modulus or stiffness (MTM) were calculated from the data obtained during testing.

In post processing of the data, a strain of zero was defined as the point where the braided collagen scaffolds were minimally loaded to a threshold of 0.01 grams, or less than 1% the ultimate load of the weakest uncrosslinked scaffold. In addition, load-elongation curves were truncated when the load fell by 20% of the ultimate load, or the point of the initial break (*Figure 11A*). After this point, as each individual thread within the braided collagen scaffold broke, they created peaks lower than the ultimate load until each thread in the scaffold failed. For the purpose of this analysis, only the ultimate load was considered (*Figure 11B*). The stiffness was defined as the maximum value for a tangent to the stress-strain curve over an incremental strain of 0.03.
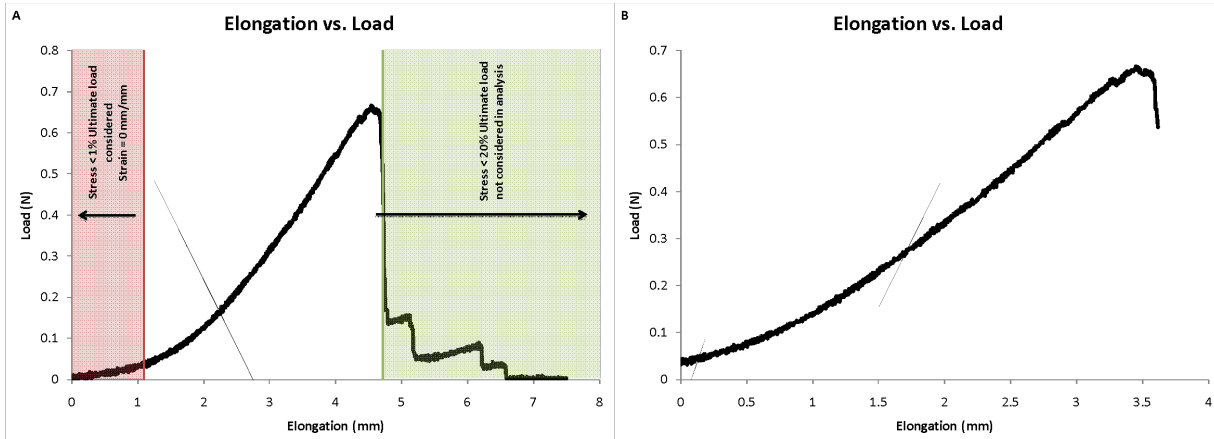

Figure 11: Post processing of mechanical data
Load-elongation curves were truncated when the load fell by 20% of the ultimate load, or the point of the initial break (A). Only the ultimate load of the load-elongation curve was considered (B).

MDFC Seeding to Braided Collagen Scaffolds
MDFC Culture and Braided Collagen Scaffold Sterilization

The MDFCs were extracted from the calf flexor muscle of a human adult male through methods described previously.[46] The MDFCs were grown in culture media (40% DMEM, 40% F-12, 20% FC III serum; Mediatech, Inc, Manassas, VA and Hyclone, Logan, UT) supplemented with 10 ng/mL epidermal growth factor (EGF, Chemicon, Temecula, CA) at ambient conditions (20% O_2 and 5% CO_2) until culture flasks were confluent. Passages 7-8 were used for all cell-seeding experiments. Prior to MDFC seeding, the braided collagen scaffolds were incubated at room temperature with 3% penicillin/streptomycin (Pen/ Strep; Gibco BRL, Gaithersburg, MD) in DPBS (vol/ vol) for one week changing the antibiotic solution every 2 days to sterilize scaffolds.

The Cell Seeding Method

To overcome the limitations associated with using Mitotracker Green prior to seeding on the braided collagen scaffold, MDFCs were incubated with 5 µg/mL Hoechst dye (Invitrogen, Carlsbad, CA) in culture medium for 15 minutes at 37ºC on the day of initial seeding. The cells were washed twice with DPBS and placed back into 37ºC incubation with fresh medium until seeding. To determine the optimal environment for uniform and reproducible seeding, the channel within the PDMS mold designed with dimensions included in *Figure 13A*. A smaller channel width was used to eliminate the void space around the scaffold when inserted into the channel. The sterile braided collagen scaffolds were inverted and inserted into the vacuum grease [as seen in] *Figure 13C*. The MDFCs were seeded on the scaffolds by adding a cell suspension in serum free medium (50% DMEM, 50% F-12) to the channel containing the braided scaffolds and incubated for 4 hours at 37ºC (*Figure 13D*). A cell suspension of 200,000 cells in 90 µL was used for the 2.0 mm by 12 mm channel, and a suspension of 150,000 cells in 30 µL was used for the 1.0 mm by 12 mm channel. Different seeding volumes were used since 90 µL of solution exceeded the volume of the smaller channel. The seeded braided scaffolds were removed from the PDMS molds and placed in a 12-well plate containing culture medium (45% DMEM, 45% F-12, 10% FC III serum) supplemented with 1% pen/strep (vol/ vol) (*Figure 13E*). The seeded scaffolds were incubated at 37ºC. Uncrosslinked and EDC/NHS crosslinked braided collagen scaffolds were seeded, incubated for 24 hours, and then fixed in 4% paraformaldehyde solution in PBS (USB, Cleveland, OH) for 20 minutes at room temperature. The scaffolds were imaged using fluorescence microscopy with an Olympus IX81 motorized inverted microscope coupled to a 12-bit Hamamatzu CCD camera and processed using Slidebook®. Due to better seeding uniformity and more reproducible data (*Results: Optimizing the Cell Seeding Method*), the PDMS mold with a channel dimension of 1.0 mm by 12 mm was used in all subsequent experiments.
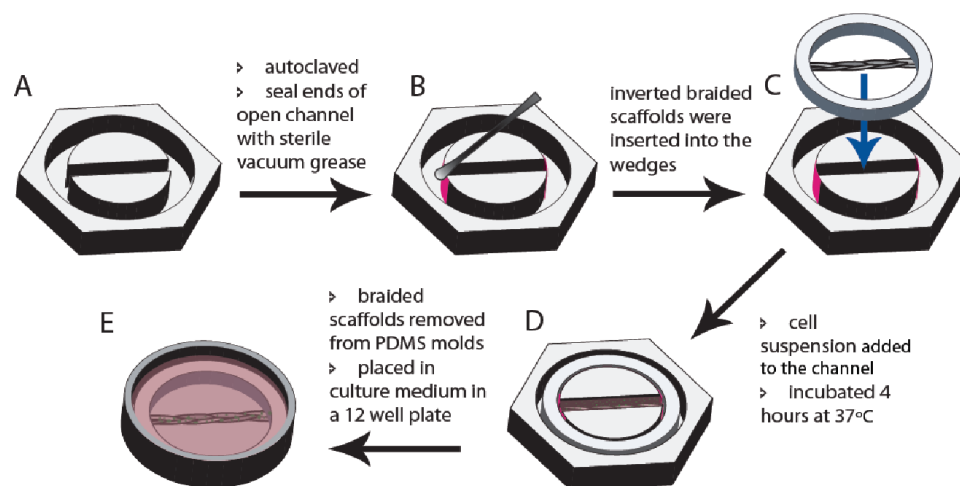
Figure 13: Seeding MDFCs onto a braided collagen scaffold
The channel within the PDMS mold designed with dimensions of either 2.0 mm by 12 mm or 1.0 mm by 12 mm (A). The molds and the channels were sealed at the ends using a thin layer of sterile vacuum grease (B). The sterile braided collagen scaffolds were inverted and inserted into the vacuum grease such that the braid lies on the bottom of the channel (C). The MDFCs were seeded on the scaffolds (D). The seeded braided scaffolds were removed from the PDMS molds and placed in a 12-well plate containing culture medium (E).

Quantification of Cell Number on Braided Collagen Threads
Cell Attachment

To determine the effects of different surface modifications on attachment of MDFCs to braided collagen scaffolds, MDFCs were seeded as described above onto uncrosslinked scaffolds and scaffolds treated with EDC/NHS, EDC/NHS with heparin, or EDC/NHS with heparin coated with FGF-2 at concentrations of either 5 ng/mL, 10 ng/mL, or 50 ng/mL. Unseeded braided collagen scaffolds were used as controls. Seeded braided collagen scaffolds were cultured at 37ºC for 24 hours before fixing in 4% paraformaldehyde solution in PBS (USB, Cleveland, OH) for 20 minutes at room temperature. Scaffolds were washed twice for 5 minutes in PBS, and stored in PBS at 4ºC until imaging. In order to image the braided scaffolds, they were removed from the PDMS ring and placed on a glass slide covered with enough PBS to maintain hydration throughout the imaging process. For each condition, 8 to 14 scaffolds were imaged from 4 separate experiments by fluorescence microscopy on an Olympus IX81 motorized inverted microscope coupled to a 12-bit Hamamatzu CCD camera and processed using Slidebook®. Imaging locations were chosen for cell quantification at 10X magnification in nonoverlapping focal regions across the entire length of the scaffold by placing it on the slide parallel to the x-axis. This resulted in 5 to 18 images per scaffold depending on the number of focal regions in the z-direction.

The images were analyzed using Image J software with the grid and cell counter plug-in for cell attachment and cell distribution across the length of the scaffold. A grid was placed on each image with an area of 10,000 μm^2 (1.55 pixels/μm) between each grid line. Using the cell counter plug-in, raw data was collected from each image as the average number of Hoechst dye stained nuclei counted in four separate regions. Not all of the cells are in the focal plane of each image because of the limited focal depth when imaging three-dimensional scaffolds. As such, the data was normalized by reporting it as the number of cells within an area of 10,000 μm^2. To determine whether there was an equal cell distribution across length of the braided collagen

scaffold, cells were counted using the procedure described above for images taken every 900 μm along the length of the scaffold. Although the majority of the scaffolds were seeded over the entire surface area, only one side of the braided scaffold was analyzed. There was no difference in seeding throughout the surface area of the scaffold.

Cell Growth

To determine the effects of different surface biochemistries on the growth of MDFCs on braided collagen scaffolds, MDFCs preloaded with Hoechst dye were seeded as described above onto uncrosslinked scaffolds and scaffolds treated with EDC/NHS, EDC/NHS with heparin, and EDC/NHS with heparin coated with FGF-2 at concentrations of either 5 ng/mL, 10 ng/mL, or 50 ng/mL. Unseeded scaffolds were used as controls. Seeded braided collagen scaffolds were cultured at 37°C moving the scaffolds to a new sterile 12 well plate with fresh medium every other day to prevent contamination during extended culture periods. Scaffolds were cultured for 5 days and 7 days before fixing in 4% paraformaldehyde solution in PBS for 20 minutes at room temperature. Scaffolds were washed twice for 5 minutes in PBS, and stored in PBS at 4°C until imaging. Seeded scaffolds cultured for 5 and 7 days were analyzed for cell growth in the same manner as described previously for cell attachment and cell distribution.

Estimation of Total Cell Attachment and Growth

To compare the cell attachment and growth to results found in literature, the total number of cells attached to the braided collagen at each time point needed to be approximated. The cross sectional perimeter was established using the histological sections of three hematoxylin and eosin stained unseeded braided collagen threads. The outer edge of the scaffolds was traced using Image J software in order to obtain an approximate surface perimeter. To account for the differences in surface topography on the scaffold, sections were measured at four different locations along the length of the scaffold and averaged together. When the braided collagen scaffold is placed inside the PDMS mold, the ends of the scaffold are exposed to sterile vacuum grease, which prevents MDFCs from attaching beyond this boundary. Using the cell distribution data, the length of the seeded area of the braided collagen scaffold can be determined. Using the assumption that all sides have been seeded with MDFCs, the total surface area of the braided collagen scaffold can be determined by multiplying the cross sectional perimeter by the length of the seeded area of the braid. Using this information, the total number of MDFCs attached to the surface at each time point can be extrapolated by multiplying the number of cells counted per 10,000 μm^2 region by the total seeded surface area. In addition to total cell attachment and growth calculations, the percentage of the cells seeded that attached to the surface and the fold increases of the cells over time was calculated. The increase in cell number over the number of cells that attached, T_d, was calculated using the following equation, where q_1 is the average number of cells attached for each surface modification and q_2 is the number of cells at counted at 5 and 7 days.

$$T_d = \frac{q_2}{q_1}$$

Fluorescence Microscopic Analysis of Cell Density and Cellular Alignment

To determine MDFC alignment and orientation on braided collagen scaffolds, scaffolds were seeded with MDFCs, incubated and stained to illuminate the f-actin filaments. Braided collagen scaffolds of each type were assembled and seeded as described previously and incubated for 1, 5, or 7 days. After incubation for the designated period, scaffolds were rinsed twice in PBS and fixed with 4% paraformaldehyde solution in PBS for 20 minutes at room temperature. Scaffolds were then rinsed twice in PBS for 5 minutes and stained with 165 mM Alexafluor 488

phalloidin (Molecular Probes, Eugene, OR) for 45 minutes. To image the braided scaffolds, they were removed from PDMS rings and placed on a glass slide covered with enough PBS to maintain hydration throughout the imaging process. To analyze cell density, scaffolds were imaged by fluorescence microscopy on an Olympus IX81 motorized inverted microscope coupled to a 12-bit Hamamatzu CCD camera and processed using Slidebook® under 4X magnification to visualize the Hoechst stained nuclei. Cellular alignment was determined by removing scaffolds incubated for 1 or 7 days from PDMS rings and placing them into 35 mm diameter glass bottom culture dishes with a 10 mm diameter cover slip in the middle with a thickness of 0.19 mm (MatTek Corporation, Ashland, MA). The braids were held flat against the cover glass surface using vacuum grease and covered with enough PBS to maintain hydration throughout the imaging process. The scaffolds were imaged using fluorescence microscopy on a Leica TCS SP5 II point scanning confocal microscope (Leica Microsystems Inc., Bannockburn, IL) under an oil immersion 20X magnification lens to visualize the nuclei and f-actin filaments. Images were taken along the z-axis at a depth of 100 to 150 µm of the braided collagen scaffold. Cellular alignment was qualitatively analyzed by determining if the cells aligned with the curvature of the braids or parallel to the x-axis after 7 days in culture.

Statistics

Statistical analyses were executed using SigmaPlot 11.0 (Systat Software, Inc, Point Richmond, CA). For the mechanical data analysis, statistical differences between the uncrosslinked and crosslinked samples were evaluated using a Student's T-test or the Mann Whitney Rank Sum test for cases of unequal variance. For all cell-based assay experiments, statistical difference between scaffolds was analyzed using one-way analysis of variance (ANOVA) with Holm-Sidak post hoc testing. In cases where data failed the normality test an ANOVA on Ranks followed by a Dunn's post hoc test was used since the group sizes were unequal. Significance was established for $p < 0.05$.

Results
Braided Collagen Scaffold Material Characterization
Characterization of Localized FGF-2

To verify and characterize the localization of FGF-2 on braided collagen scaffolds, different concentrations of FGF-2 were bound to single collagen threads that were crosslinked with EDC/NHS in the presence of heparin. The threads were immunostained and the results are shown in Figure 18. Threads crosslinked with EDC/NHS and heparin in the presence of FGF-2 showed FGF-2 on the surfaces of the threads when compared to the control braids that were crosslinked with EDC/NHS and heparin, but not exposed to FGF-2 (*Figure 18A*). Immunocytochemical analysis of threads exposed to 5 ng/mL FGF-2 showed FGF-2 having inconsistent coverage on the surface (*Figure 18B*). Although it is apparent that FGF-2 was present on the surface, it is not uniform along the length of the thread. Threads exposed to 10 ng/mL FGF-2 (*Figure 18C*) and 50 ng/mL FGF-2 (*Figure 18D*) showed similar localization and uniform coverage of FGF-2 along the length of the thread. Threads exposed to 50 ng/mL FGF-2 seem to have a more FGF-2 bound to the surface due to the higher fluorescence intensity across the whole surface. However, these differences were not evaluated quantitatively.
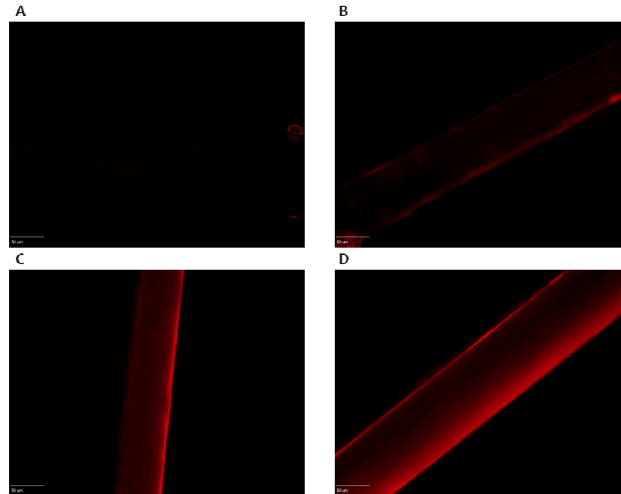
Figure 18: Immunocytochemistry verifying the presence of FGF-2
EDC/NHS crosslinked with heparin (A), and EDC/NHS crosslinked with heparin and passively adsorbed with 5 ng/mL (B), 10 ng/mL (C), or 50 ng/mL FGF-2 (D). Scale bar = 200 μm

Mechanical Testing of Braided Collagen Scaffolds

To characterize the mechanical properties of braided collagen scaffolds, uncrosslinked and EDC/NHS crosslinked braids were loaded under uniaxial tension until failure. The results of this analysis are summarized in Table 5. Characteristic load-elongation curves for each of the individual uncrosslinked and crosslinked braided collagen scaffolds showed a generally linear shape with the scaffold failure occurring as the first of the three internal braids failed (*Figure 19*). After the point of ultimate failure, the load drops in an incremental manner until each of threads has broken. In addition, each individual braid shows similar curves demonstrating that the production of the braided collagen scaffold from self-assembled type I collagen microthread extrusion to braid development is consistent and reproducible.

	Sample Size	Cross-sectional Area (mm² ± SD)	Ultimate Load (N ± SD)	UTS (MPa ± SD)	Strain at Failure (mm/mm ± SD)	Maximum Tangent Modulus (MPa ± SD)
Uncrosslinked	16	0.115 ± 0.025	0.591 ± 0.076	5.130 ± 0.662	0.420 ± 0.064	13.60 ± 2.668
Crosslinked	16	0.072 ± 0.013	1.979 ± 0.237	26.97 ± 2.835	0.516 ± 0.118	68.52 ± 8.242

Table 5: Mechanical properties summary table for braided collagen microthreads
Indicates statistically significant differences between uncrosslinked and crosslinked braided collagen scaffolds with $p < 0.05$ using Mann-Whitney Rank Sum Test.
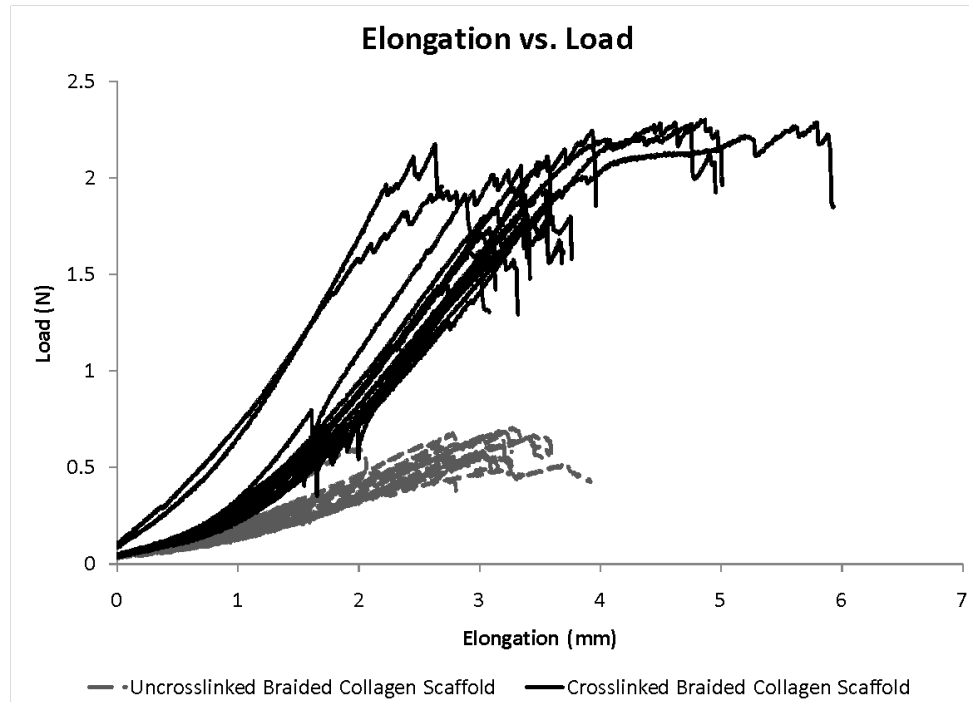
Figure 19: Characteristic load-elongation relationship for braided collagen microthreads

Characteristic load-elongation curves for each of the individual uncrosslinked and EDC/NHS crosslinked braided collagen scaffolds showed a generally linear shape with the scaffold failure occurring as the first of the three internal braids fails (n = 16)

In order to calculate the stress-strain curves of the braids, the cross-section area was calculated using the histological cross-sections of the braid. The average cross-sectional area of an uncrosslinked and crosslinked braided collagen scaffold was calculated to be 0.115 ± 0.025 mm^2 and 0.072 ± 0.013 mm^2 respectively. The representative stress-strain curves comparing uncrosslinked to crosslinked braids show they are roughly linear in shape with crosslinked threads withstanding a greater amount of stress per unit strain (*Figure 21*). The curve measurements allow for the calculation of the maximum tangent modulus (MTM) of each sample to be calculated.

$$MTM = \text{max slope of the stress-strain curve}$$

In this equation, σ represents the tensile stress, MTM stands for the maximum tangent modulus of elasticity, and ε represents the tensile strain. Relative to uncrosslinked braided collagen scaffolds, the ultimate load and ultimate tensile strength of crosslinked scaffolds were increased significantly by crosslinking using EDC/NHS (*Figure 22A* and *Figure 22B*). The crosslinked braids were able withstand an ultimate load almost three times that of uncrosslinked scaffolds. Similarly, the strain at failure and maximum tangent modulus of the crosslinked scaffolds were significantly higher relative to uncrosslinked collagen scaffolds (*Figure 23A* and *Figure 23B*). Even though the crosslinked braids are approximately five times stiffer than uncrosslinked scaffolds, the crosslinked braids have a significantly increased strain at failure.
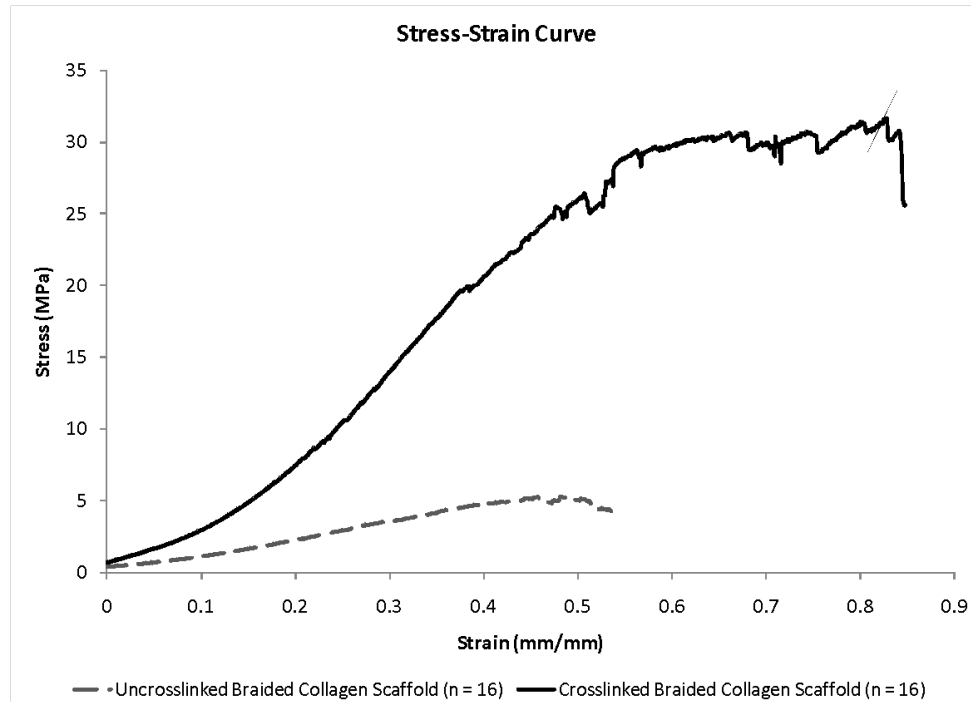
Figure 21: Characteristic stress-strain curve relationship for braided collagen microthreads
The representative stress-strain curves comparing uncrosslinked to crosslinked braids also shows they are roughly linear in shape with crosslinked threads withstanding a greater amount of stress per unit strain (n = 16).
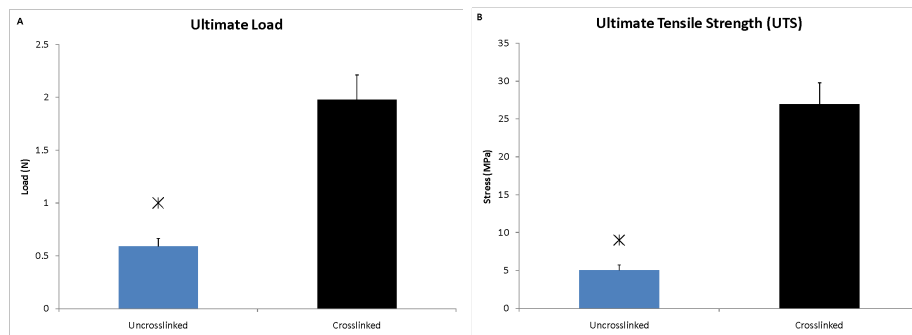

Figure 22: Ultimate Load and UTS at failure for braided collagen microthreads
Ultimate Load (A) and UTS (B).   Indicates $p < 0.05$ using Mann-Whitney Rank Sum Test. Bars indicate mean ± SD (each representing the data in Table 5 having n = 16).
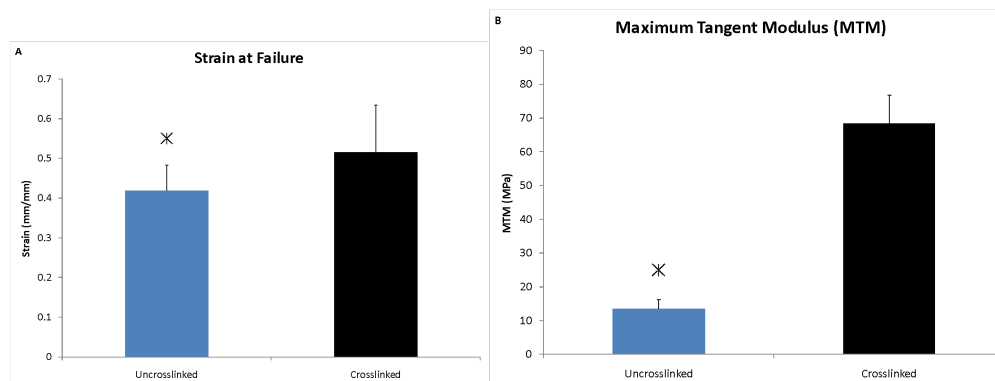
Figure 23: Strain at failure and young's modulus for braided collagen microthreads

Strain at failure (A) and MTM (B).　　Indicates p < 0.05 using Mann-Whitney Rank Sum
Test. Bars indicate mean ± SD (each representing the data in Table 5 having n = 16).

MDFC Seeding to Braided Collagen Scaffold

MDFCs were loaded with Hoechst dye prior to seeding onto braided collagen scaffolds instead of Mitotracker green. MDFCs were seeded onto uncrosslinked and crosslinked braided collagen scaffolds using PDMS molds with channel widths of either 2.0 mm or 1.0 mm, and the results are summarized in Table 6. Using a channel sealed with sterile vacuum grease resulted in elimination of scaffold breakage, but there was still a risk of the cell suspension leaking out of the ends of the channel. Since more than fifty percent of the braided collagen scaffolds seeded in both PDMS mold channel types, this was not considered a significant problem.

Hoechst stained images of uncrosslinked and crosslinked braided collagen scaffolds seeding using the two channel widths are shown in Figure 25. These images do not show a large visual difference between the surface treatments due to limitations in imaging a three-dimension scaffold, but in contrast to scaffolds preloaded with Mitotracker green, individual cells can be distinguished from one another enabling quantitative analysis. It is apparent that the MDFCs attached onto the scaffolds seeded in the 1.0 mm wide channel more uniformly with a clear increase in cell number when compared to the scaffolds seeded using the wider channel.

		Uncrosslinked	EDC/NHS
2.0 mm x 12.0 mm	Sample Size	3	3
	Total Scaffolds Successfully Seeded (%)	66.7	100
	MDFC Attachment (# of cells/10,000 μm^2 ± SEM)	23.4 ± 0.70	29.9 ± 1.2
	Total MDFC attachment (# cells ± SEM)	23,501 ± 704.6	30,087 ± 1,230
	Total MDFCs Successfully Seeded (% ± SEM)	11.8 ± 0.4	15.0 ± 0.6
1.0 mm x 12.0 mm	Sample Size	4	4
	Total Scaffolds Successfully Seeded (%)	100	75
	MDFC Attachment (# of cells/10,000 μm^2 ± SEM)	31.1 ±1.12	36.1 ± 0.91
	Total MDFC attachment (# cells ± SEM)	31,277 ± 1,038	36,356 ± 914.8
	Total MDFCs Successfully Seeded (% ± SEM)	20.9 ± 0.7	24.2 ± 0.6

Table 6: Cell seeding optimization summary table comparing different seeding channel dimensions

Indicates statistically significant differences between 2.0 mm wide channels and 1.0 mm wide channels with p < 0.05 using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method.
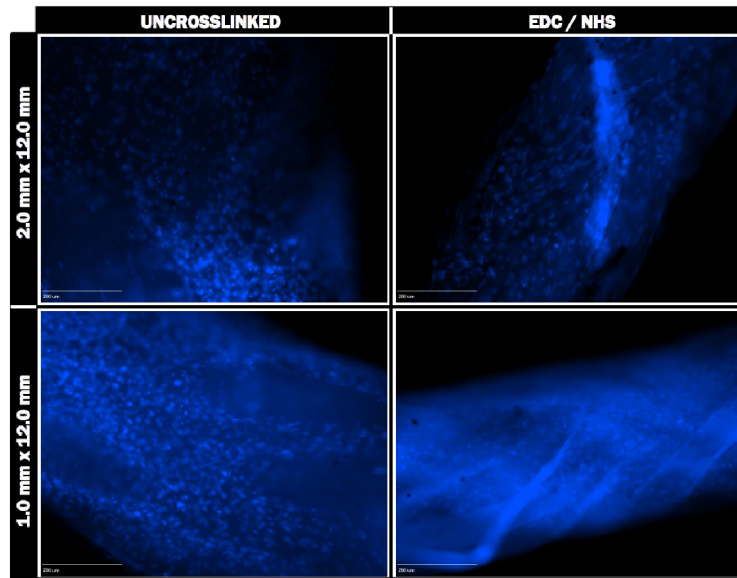

Figure 25: Hoechst stained MDFCs seeded onto braided collagen scaffolds using different methods
Fluorescence images comparing attachment of MDFCs on uncrosslinked and EDC/NHS crosslinked braided collagen scaffolds using 2.0 mm wide channels (top row) or 1.0 mm channels (bottom row) for seeding. MDFCs were preloaded with Hoechst dye. Scale bar = 200 μm.

The density of cells that attached to the braided collagen scaffolds in an area of 10,000 μm^2 were counted visually to compare the two different seeding methods. Seeding with either the 2.0 mm wide channel or the 1.0 mm wide channel showed a significant increase in cell density between uncrosslinked and crosslinked scaffolds (*Figure 26*). The results show seeding using the narrower channel significantly increased the seeding density on both uncrosslinked and crosslinked scaffolds compared to the wider channel.
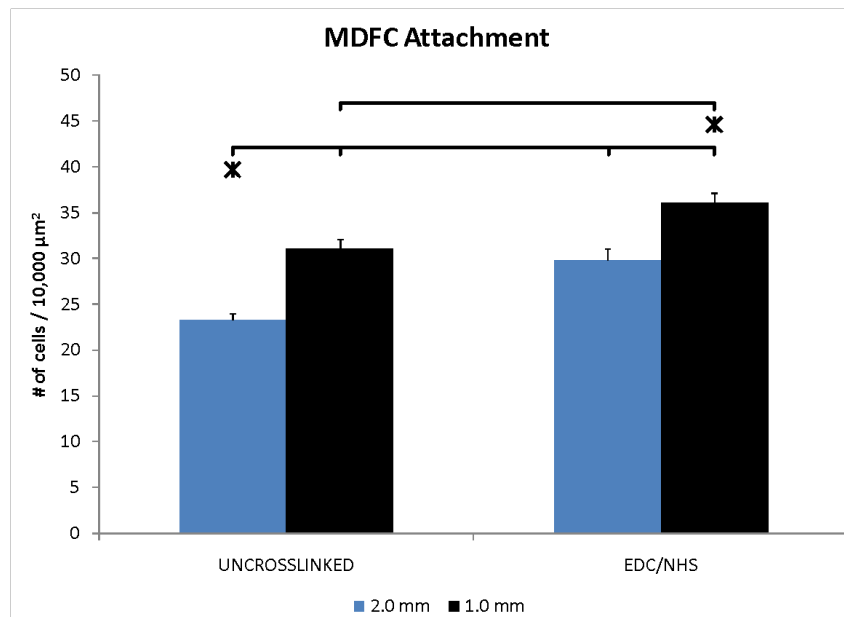
Since the channels were seeded with different cell suspension concentrations to adjust for the different channel volumes, the total cell attachment was normalized by calculating the percentage of MDFCs used in the cell suspension that actually seeded (*Figure 28*). Using the narrower channel to seed MDFCs resulted in 20-25% of the cells in the suspension attaching to the braided collagen scaffold, which was significantly higher than the wider channel, which resulted in less than 15% attachment on each surface modification.
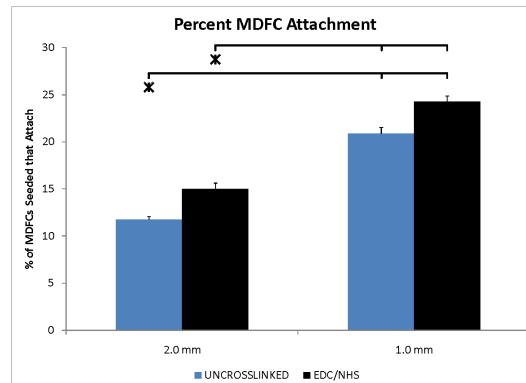
Figure 28: Percentage of MDFCs seeded that attached to the braided collagen scaffold
Since the channels were seeded with different cell suspension concentrations to adjust for
the different channel volumes, the total cell attachment was normalized by calculating the
percentage of MDFCs used in the cell suspension that actually seeded.   Indicates p <
0.05 using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method. Bars
indicate mean ± SEM (total n numbers indicated in Table 6).

Quantification of Cell Number on Different Surface Modifications
Cell Attachment

In order to determine the effects of surface modifications on MDFC attachment, braided collagen scaffolds with different surface modifications were seeded with MDFCs and incubated for 24 hours.[1] Fluorescence images of the Hoechst dye stained braided collagen scaffolds are shown in Figure 29. The MDFCs seeded uniformly spread over the entire surface of the braided collagen scaffold. The images show a clear increase in cell attachment from the uncrosslinked scaffold surface (*Figure 29A*) to the crosslinked and FGF-2 bound scaffold surfaces. The EDC/NHS HEP (*Figure 29C*) braided collagen scaffold appears to have a higher density of cells compared to the EDC/NHS (*Figure 29B*), 5 ng/mL FGF-2 (*Figure 29D*), 10 ng/mL FGF-2 (*Figure 29E*), and 50 ng/mL FGF-2 (*Figure 29F*) braided collagen scaffolds.

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[1] Based on the results of the development of the cell seeding protocol, all further cell attachment and growth experiments, MDFCs were loaded with Hoechst dye prior to seeding to allow for quantitative analysis. In addition, due to the higher seeding efficiency, the narrower channels were used for all future attachment and growth experiments.
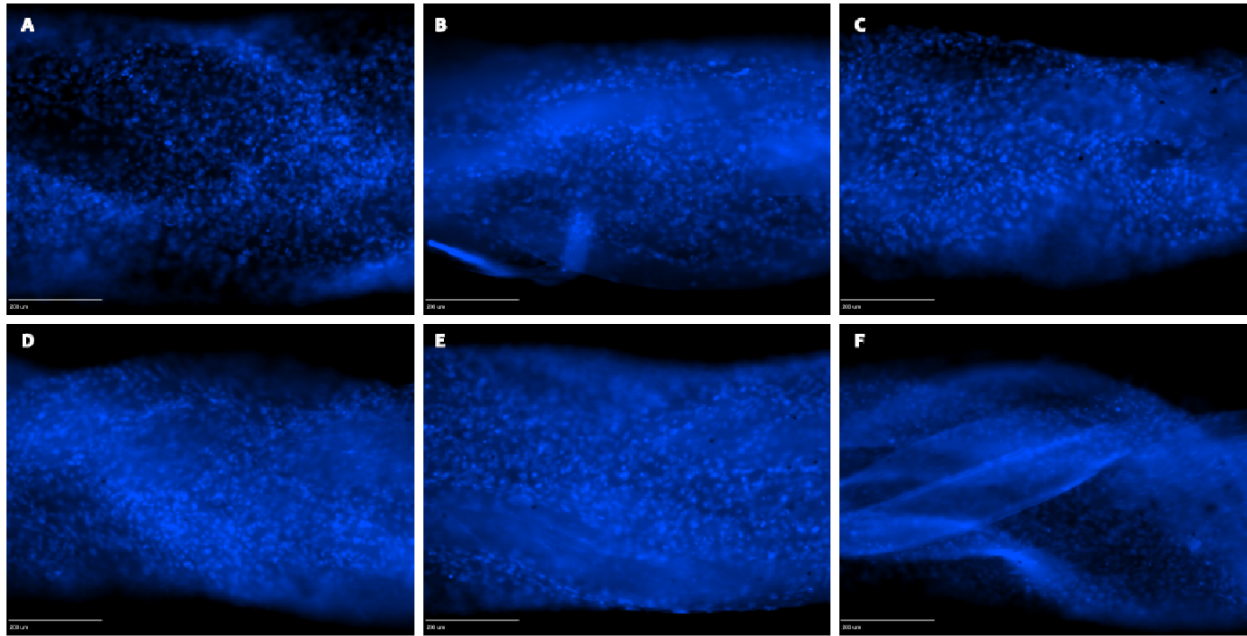
Figure 29: Hoechst stained MDFCs on braided collagen scaffolds on day 1
Uncrosslinked (A), EDC/NHS (B), EDC/NHS HEP (C), 5 ng/mL FGF-2 (D), 10 ng/mL
FGF-2 (E), and 50 ng/mL FGF-2 (F); Scale bar = 200 µm.

The density of cells that attached to the braided collagen scaffolds in an area of 10,000 µm² were counted visually to compare how surface modifications affected cell attachment (*Figure 30*). There was a significant increase in cell attachment from uncrosslinked braided collagen scaffolds to braids with surface modifications. Braided collagen scaffolds that were EDC/NHS crosslinked with heparin promoted a significantly higher cell attachment to its surface than all other scaffold surfaces. Increasing the amount of FGF-2 bound to the surface does not significantly affect cell attachment. There was not a significant difference in cell attachment between EDC/NHS crosslinked scaffolds and all scaffolds with FGF-2 bound to the surface.
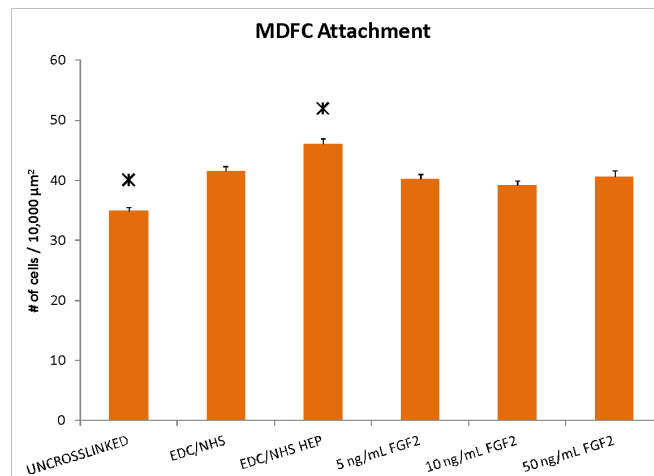

Figure 30: MDFC attachment for different surface modifications
The density of cells that attached to the braided collagen scaffolds in an area of 10,000 µm²
were counted visually to compare how surface modifications affected cell attachment.
Indicates $p < 0.05$ using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method.
Bars indicate mean ± SEM (total n numbers indicated in Table 7).

To determine if seeding using the PDMS mold distributes the MDFCs uniformly across the entire length of the scaffold, images were taken in adjacent regions across the entire length of the braids and cells were counted. The results are reported as the average number of cells per 10,000 μm^2 for every 900 μm across the scaffolds on the x-axis (*Figure 31*). These results show that using the PDMS mold to seed the braided collagen scaffolds resulted in uniform distribution across the length of the braid, with approximately $7{,}392 \pm 1{,}669$ μm of the scaffold being exposed to cells on average. Although only one side of the scaffold was analyzed for cell distribution, it was apparent from visual analysis that the majority of the scaffolds seeded uniformly across the entire surface area.
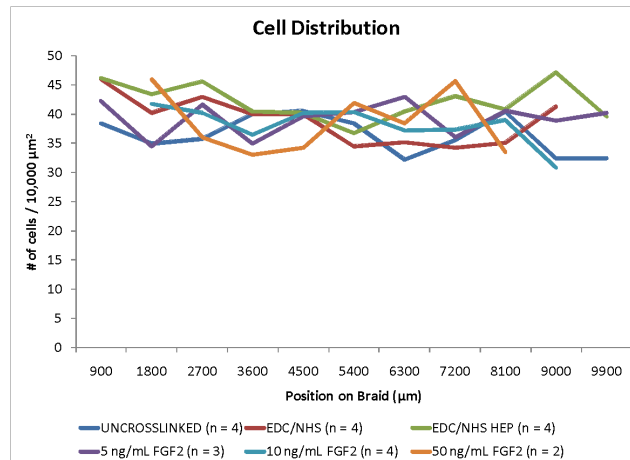

Figure 31: Cell distribution on braided collagen scaffolds after 1 day
Images were taken in adjacent regions across the entire length of the braids and cells were counted to determine cell distribution. The results are reported as the average number of cells per 10,000 μm^2 for every 900 μm across the scaffolds on the x-axis.

Cell Growth

The effect of binding FGF-2 on MDFC growth and proliferation was determined by seeding MDFCs and incubating them on the braided collagen scaffolds for 1 day, 5 days, or 7 days. During incubation, MDFCs seeded uniformly showing a minor increase in cell concentration in the grooves of the braid topography, and by the seventh day, cells had completely spread out to cover the surface of the braid. All braided scaffolds showed an increase in cell density from 1 day to 7 days showing most of the growth happening between 5 and 7 days. The greatest overall cellular growth appears to occur within the scaffolds with FGF-2 bound to the surface.

The concentration of MDFCs on the surface of the braids increased on both control and modified braids between day 1 and day 7. After 5 days in culture, the number of cells on each of the braid types did not increase significantly except for cells attached to braids modified with EDC/NHS crosslinking and 5 ng/mL and 10 ng/mL of FGF2. Uncrosslinked scaffolds had significantly fewer cells on the surface than all other scaffold types, and scaffolds modified with 5 ng/mL FGF-2 had a significantly higher cell densities than all other braids except types modified with EDC/NHS and heparin and 50 ng/mL FGF-2. By day 7, all braided collagen scaffolds showed a significant increase in cell concentration compared to day 1. In addition, between day 5 and day 7, scaffolds modified with different concentrations of FGF-2 showed a significant increase in cell growth compared to the controls with increasing levels of FGF-2 (*Figure 34*).
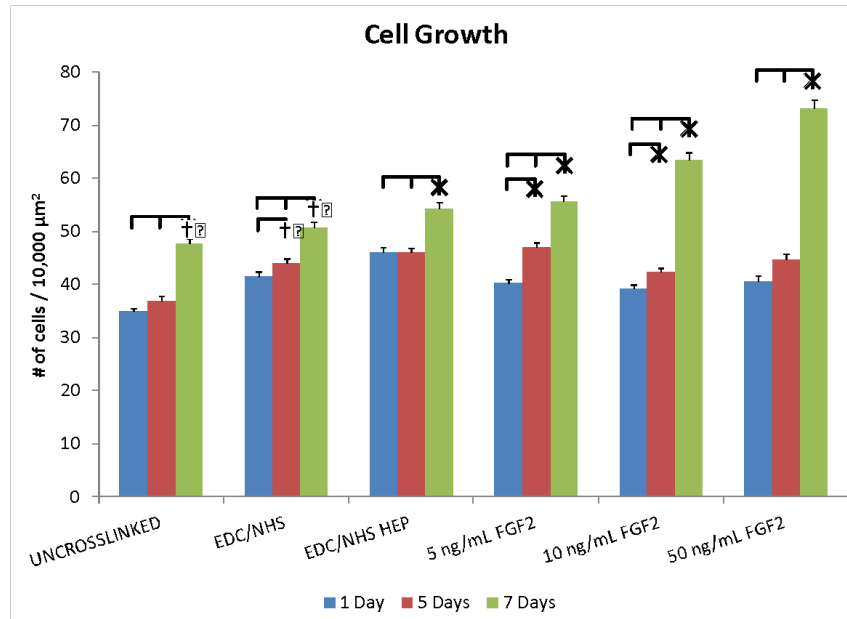
Figure 34: Cell growth comparison after 7 days in culture

Indicates p < 0.05 using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's

Method, and † indicates p < 0.05 using One Way ANOVA with Holm-Sidak method. Bars

indicate mean ± SEM (total n numbers indicated in Table 8).

The cell distribution data for the cell growth over 7 days on the different surface modifications shows the cells grew evenly along the length of the scaffold (*Figure 35*). The trend of the distribution lines (solid) fluctuated around the average cell growth (dashed) for each braided scaffold with minimal changes between 1 and 5 days. Scaffolds loaded with 10 ng/mL and 50 ng/mL FGF-2 showed a statistically significant difference in growth from 5 to 7 days.
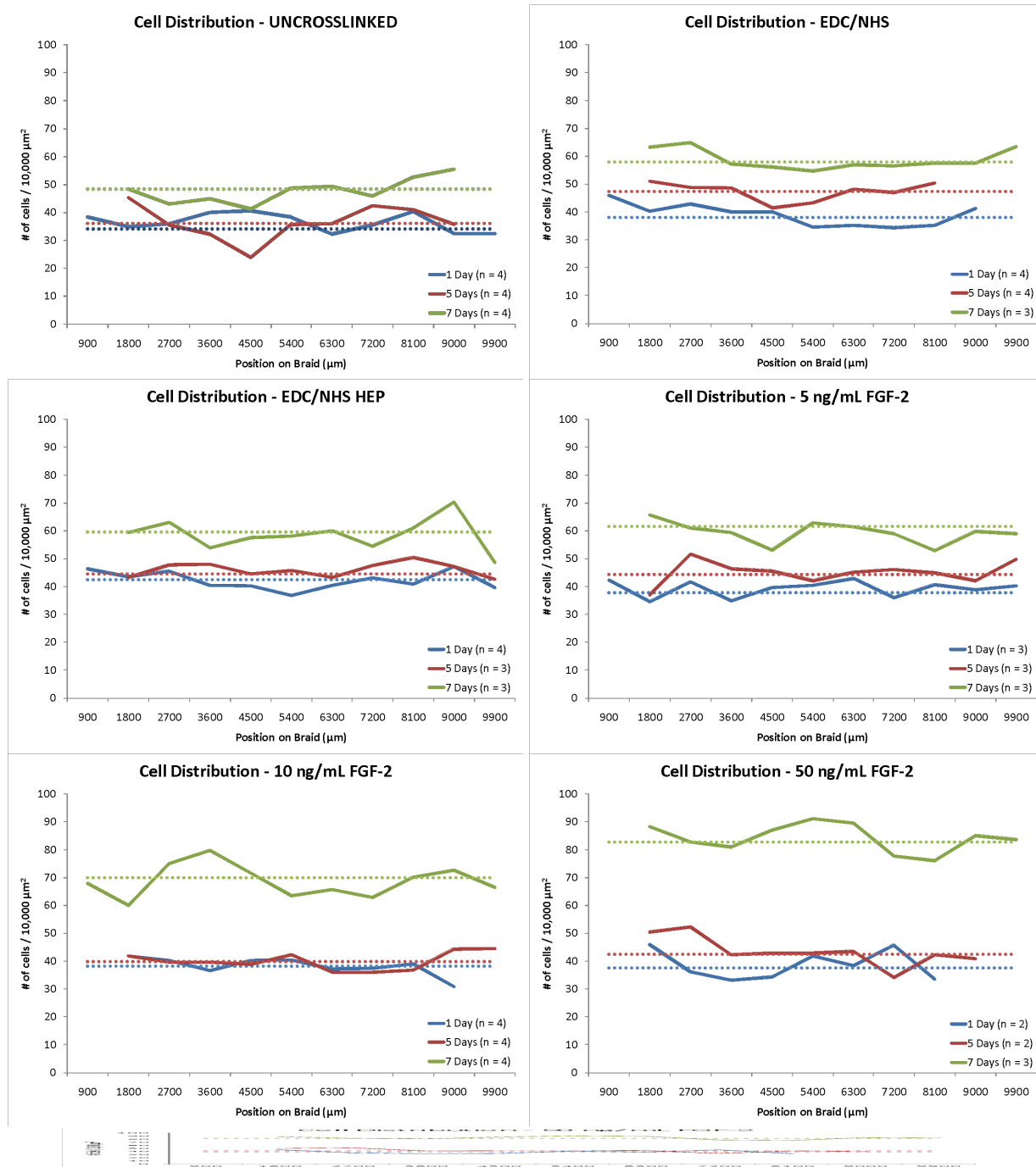
Figure 35: Cell Distribution for braided collagen scaffolds over 7 days

Solid lines indicate average cell distributions for representative scaffolds in each group at 1, 5, and 7 days (n numbers indicated within each legend). Dashed lines indicate the average attachment of all scaffolds analyzed for each group (total n numbers for 1 Day – Average, 5 Days – Average, and 7 Days – Average indicated in Table 8).

Estimation of Total Cell Attachment and Growth

The total number of cells that attached to the braided collagen scaffolds was determined by multiplying the results in Table 7 by the surface area of an unseeded braided collagen scaffold calculated from histological cross-sections. The cross-sectional perimeter of a braid containing 18

collagen microthreads, which was not significantly different between each surface modification, was found to be $1,361 \pm 278$ μm. The cross-sectional perimeter was then multiplied by the length of the seeded portion of the braid, which was determined using the cell distribution data, to get a total surface area of $10,059,532 \pm 2,058,025$ μm^2. In order to calculate the total attachment, the results in Table 7 were multiplied by $1,006 \pm 205.8$, which is the surface area divided by 10,000 μm^2. The results of the total cell attachment are summarized in Table 9.

	Total MDFC Attachment (# of cells ± SEM)	Percentage of Attached Cells (% of 150,000 cells seeded ± SEM)
UNCROSSLINKED	$35,139 \pm 693$	23.4 ± 0.5
EDC/NHS	$41,893 \pm 765$	27.9 ± 0.5
EDC/NHS HEP	$46,386 \pm 913†$	$30.9 \pm 0.6†$
5 ng/mL FGF2	$40,511 \pm 736$	27.0 ± 0.5
10 ng/mL FGF2	$39,414 \pm 700$	26.3 ± 0.5
50 ng/mL FGF2	$40,912 \pm 1,000$	27.3 ± 0.7

Table 9: Total cell attachment summary table on different surface modifications
Indicates statistically significant differences between uncrosslinked and all other conditions with $p < 0.05$ using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method. † Indicates statistically significant differences between uncrosslinked and all other conditions with $p < 0.05$ using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method.

The total number of MDFCs that attached to the braided collagen scaffolds was approximated to determine which surface treatment promoted more cellular attachment. As expected from the regional cell attachment counts, uncrosslinked braided collagen scaffolds promoted significantly less cell attachment than the other braided collagen scaffolds while EDC/NHS with heparin scaffolds promoted significantly more cells to attach. In order to determine which scaffold had the best seeding efficiency, the percentage of the total amount of cell in the suspension that actually attached to the scaffolds was calculated (*Figure 37*). Uncrosslinked braided collagen scaffolds resulted in a significantly lower seeding percentage compared to the other surface modifications with approximately 23% attachment. EDC/NHS with heparin scaffolds resulted in a significantly higher seeding percentage with approximately 31% attachment.
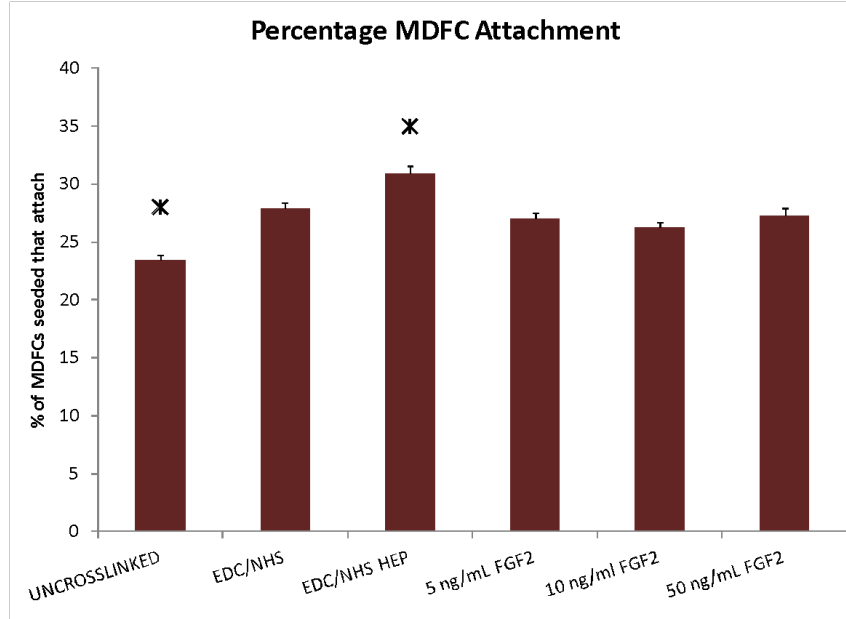
Figure 37: Percentage of MDFC seeded that attached to the braided collagen scaffolds
In order to determine which scaffold had the best seeding efficiency, the percentage of the total amount of cell in the suspension that was actually attached to the scaffolds was calculated by dividing the number of cells attached by the initial number of cells seeded.

Indicates $p < 0.05$ using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method. Bars indicate mean ± SEM (total n numbers indicated in Table 8).

The effect the surface modifications have on the rate of MDFCs growth was determined by extrapolating the doubling time from the total MDFC growth after 5 and 7 days. The results of the total cell growth are summarized in Table 10.

	5 DAYS		7 DAYS	
	Total MDFC Growth (# of cells ± SEM)	Fold Increase (increase ± SEM)	Total MDFC Growth (# of cells ± SEM)	Fold Increase (increase ± SEM)
UNCROSSLINKED	37,205 ± 918	1.06 ± 0.026	47,978 ± 893	1.37 ± 0.025
EDC/NHS	44,337 ± 794	1.06 ± 0.019	51,123 ± 1,080	1.22 ± 0.026
EDC/NHS HEP	46,670 ± 753	1.00 ± 0.017	54,670 ± 1,265	1.18 ± 0.027
5 ng/mL FGF-2	47,399 ± 770	1.17 ± 0.019	55,977 ± 1,187	1.38 ± 0.029
10 ng/mL FGF-2	42,742 ± 712	1.08 ± 0.018	63,907 ± 1,371	1.62 ± 0.035
50 ng/mL FGF-2	45,094 ± 916	1.10 ± 0.022	73,610 ± 1,639	1.80 ± 0.040

Table 10: Total cell growth summary table on different surface modifications
Indicates statistically significant differences between the growth / increase over attachment of MDFCs at 7 days for 10 ng/mL and 50 ng/mL FGF-2 and all other surface

The total number of MDFCs present on the braided collagen scaffold after 5 and 7 days in culture was approximated to determine which surface modification promoted the highest growth rate. As expected from the regional cell growth counts, after 5 days in culture, none of the modified braid types promoted significant cell growth relative to the day 1 attachment data expect braids with 5 ng/mL FGF-2 bound to the surface. However, there was significant growth after 7 days, with surfaces modified with 50 ng/mL FGF-2 having approximately 74,000 cells present, which was significantly higher than all other conditions (*Figure 38*). To determine which scaffold promoted the highest growth rate, the fold increase in cell number normalized to the average cell attachment for each condition was calculated (*Figure 39*). After 5 days, all control surface modifications as well as scaffolds modified with 10 ng/mL FGF-2 had significantly less growth than surfaces modified with 5 ng/mL FGF-2. After 7 days, the growth rate on EDC/NHS crosslinked scaffolds and EDC/NHS crosslinked with heparin scaffolds was significantly less than all other conditions, and scaffolds modified with 10 and 50 ng/mL FGF-2 had significantly higher growth rates than all other scaffold types.
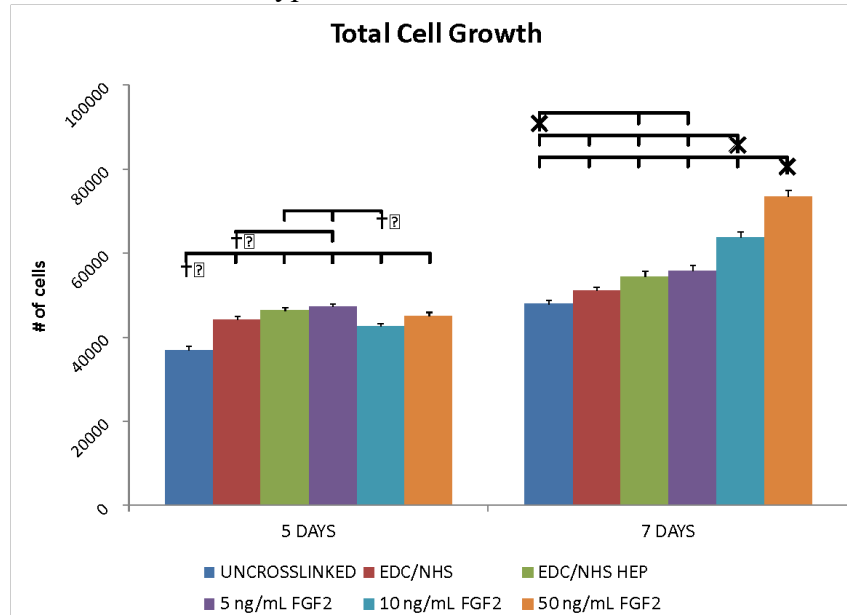

Figure 38: Total cell growth after 7 days in culture

Indicates $p < 0.05$ using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method, and † indicates $p < 0.05$ using One Way ANOVA with Holm-Sidak method. Bars indicate mean ± SEM (total n numbers indicated in Table 8).
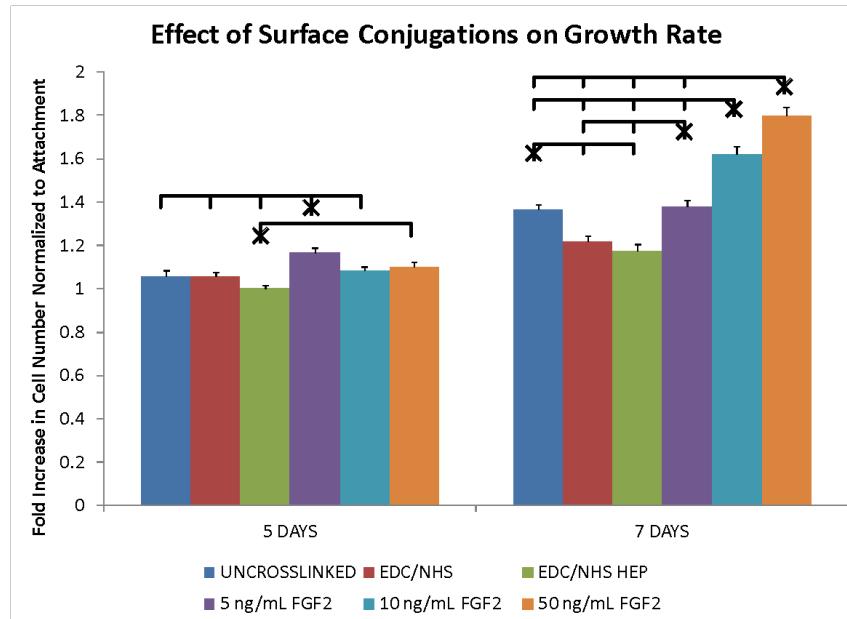
Effect of Surface Conjugations on Growth Rate

Figure 39: Effect of surface modifications on growth rate

◆◆ Indicates p < 0.05 using Kruskal-Wallis One Way ANOVA on Ranks with Dunn's Method, and † indicates p < 0.05 using One Way ANOVA with Holm-Sidak method. Bars indicate mean ± SEM (total n numbers indicated in Table 8).

Qualitative Analysis of Cell Density and Cellular Alignment
Fluorescence Microscopic Analysis of Cell Density and Cellular Alignment

The effect of FGF-2 surface modifications on MDFC cell density and cellular alignment was determined by seeding MDFCs and incubating them on braided collagen scaffolds for 1, 5, and 7 days. The scaffolds were imaged either to determine cell density using Hoechst dye fluorescence microscopy, or to determine cellular alignment using phalloidin confocal fluorescence microscopy.

The uniform concentration of cells indicates that imaging a small subsection of the braid would be a satisfactory representation of the alignment over the seeded area. Since the working distance of the confocal microscope was not large enough to image through the entire scaffold, only a small fraction of the braid could be analyzed per image. Confocal images of phalloidin stained braids at 1 day and 7 days, showed a distinct difference in the f-actin configurations between the two time points (*Figure 42*). At 1 day, all scaffolds exhibit a lack of cellular alignment, with f-actin filaments spread out with no specific orientation. At 7 days, the cells began to orient themselves along the linear axis, meaning the direction of the threads not accounting for the curvature of the braids, on uncrosslinked braids and braids crosslinked with and without heparin. The f-actin filaments appeared to be aligned parallel to each other over the braid structure with some following the curvature of the individual braided threads. Braided collagen scaffolds modified with different concentrations of FGF-2 showed limited alignment resembling the 1 day scaffolds as opposed to the 7 day uncrosslinked and crosslinked with and without heparin scaffolds.
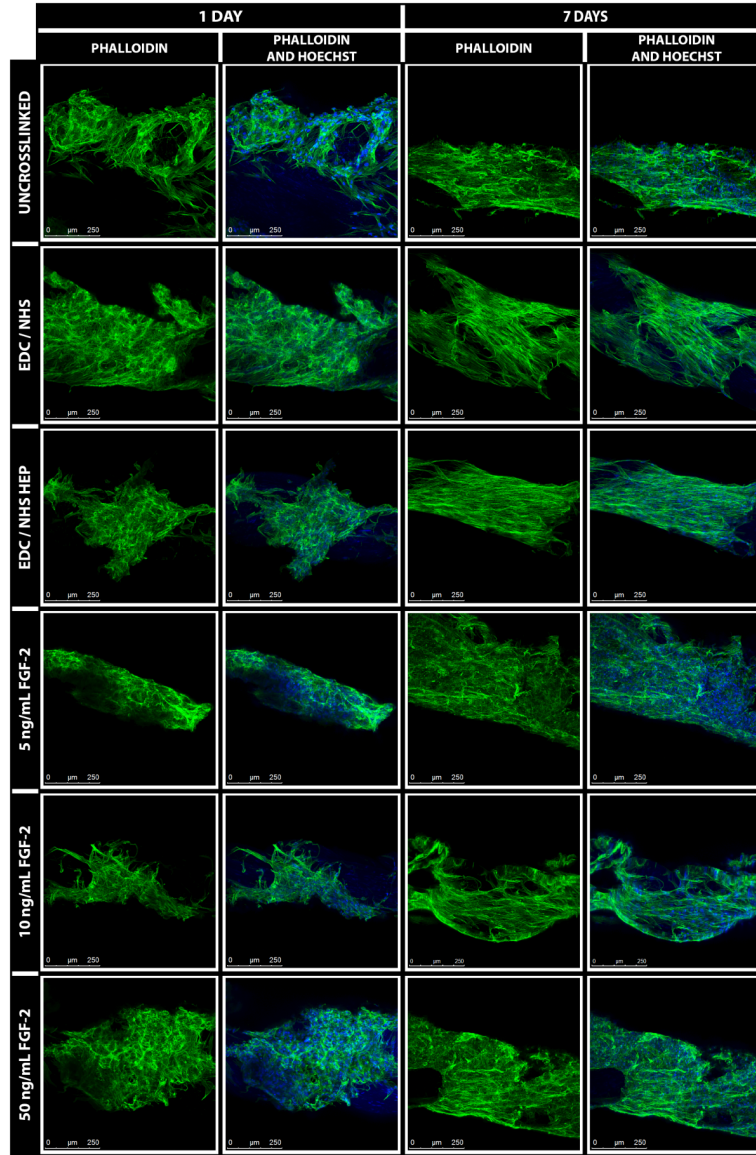
Figure 42: Analysis of alignment using phalloidin staining

Representative images for alignment analysis of braided collagen scaffolds with different surface modifications (rows) at 1 day and 7 days (columns) using phalloidin staining (green) and Hoechst stained nuclei (blue). Scale bar = 250 µm .

Discussion

The goal of this study was to investigate a novel method to attach and grow muscle derived fibroblastic cells on collagen microthreads. It has been proposed that such a method could be used to regenerate skeletal muscle with large defects such as those sustained by injury or trauma. This study has succeeded in characterizing the mechanical properties of the braids, developing a novel seeding method to attach MDFCs to braided collagen scaffolds, quantifying the number of cells that attached and grew on the braids in culture, and verifying the ability of the braided collagen threads to aid in the alignment of the MDFCs.

The braided collagen microthread scaffold provides an organized structure to facilitate the growth of MDFCs, and using heparin to bind FGF-2 to the surface could induce MDFCs to exhibit a stem cell phenotype. After 7 days in culture, the seeded MDFCs had begun to align on the braided collagen scaffold, but it is not completely clear if the MDFCs are aligning with the curvature of the collagen threads or with the x-axis. Further studies will need to be conducted in order to determine how the MDFCs are aligning, and if this is important when implanting stem-like cells into a large muscle defect. Another parameter that needs attention in the future is the mechanical properties associated with collagen microthreads. Since the stiffness is significantly higher than native skeletal muscle, other methods, such as with growth factors will need to be utilized to attempt to mimic the environment. In future *in vivo* studies, delivering stem-like cells to a large muscle defect will promote the migration of surrounding satellite cells to the wound area as well as differentiate into myoblasts. The braided collagen muscle construct will integrate into the native muscle and help restore function by mimicking cells and responses of native muscle regeneration. However, before the braided collagen scaffold can be used for *in vivo* experimentation, further studies need to be performed to characterize and modify the scaffold and cellular stimuli to optimize the muscle delivery system.

The seeded scaffolds were not cultured for a sufficient period to express stem cell markers and phenotype in the MDFCs. In order to show that the release of FGF-2 from the scaffold is sufficient to stimulate dedifferentiation of MDFCs into stem like cells, seeded scaffolds will have to be cultured for longer than 7 days and express the stem cell markers, OCT4, SOX2, and NANOG.[46,137] Once the stem cell phenotype is verified, *in vitro* studies into the maintenance of the stemness of these cells will have to be explored to ensure that the cells do not spontaneously differentiate before implantation into a wound site. Once the stemness can be controlled for extended periods and programmed to differentiate into myoblasts at the desired point, the braided collagen scaffolds will be optimal for the promotion of skeletal muscle regeneration.

In this study, FGF-2 was electrostatically bound to the surface of the braided collagen scaffold for the application of delivering stem-like cells to a large muscle defects to induce native skeletal muscle regeneration as well as satellite cell migration, differentiation, and myofiber maturation. In future studies, it would be important to investigate the effects of binding other growth factors important to regeneration in combination with and without FGF-2 to attempt to mimic the native environment. *In vivo*, IGF-I has been found to stimulate proliferation and differentiation of myoblasts into functional myotubes faster than native muscle regeneration with decreased fibrosis.[49,114, 107] By incorporating IGF-I into the braided collagen scaffold, there could be increased regulation of myofiber formation upon initiation of differentiation iPS cells to better resemble native skeletal muscle. Other studies have incorporated HGF, which is responsible for the activation and differentiation of satellite cells during wound healing, into the scaffolds for muscle regeneration.[70,163] Studies show that incorporating HGF into a scaffold alongside FGF-2 enhances the regenerative properties of FGF-2 in the presence of myoblast cells.[163]

The braided collagen scaffolds are meant to be used to repair large muscle defects, which are unable to repair naturally since the entire basal lamina of the myotube has been destroyed eliminating the satellite cells needed for regeneration.[17] By delivering stem-like cells to the muscle defect, the satellite cell population within the wound is replenished, which will allow for new muscle formation. This method would be an improvement over current attempts, since it would not only fill the defect, but would make it functional. Future research still needs to be explored into the *in vivo* response of native tissue to the braided collagen scaffold.