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Fibrin Microthreads Support Mesenchymal Stem Cell Growth While Maintaining Differentiation Potential

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Abstract

We developed a method to produce discrete fibrin microthreads, which can be seeded with human mesenchymal stem cells (hMSCs) and used as a suture to enhance the efficiency and localization of cell delivery. To assess the efficacy of fibrin microthreads to support hMSC attachment, proliferation and survival, microthreads (100 μ m diameter per microthread) were bundled together, seeded with 50,000 hMSCs for 2 hours, and cultured for 5 days. Cell density on microthread bundles increased over time in culture, to a maximum average density of 731 ± 101 cells/mm² after 5 days. A LIVE/DEAD assay confirmed that the cells were viable and Ki-67 staining verified hMSC proliferation. Additionally, functional differentiation assays demonstrated that hMSCs cultured on microthreads retained their ability to differentiate into adipocytes and osteocytes. The results of this study demonstrate that fibrin microthreads support hMSC viability and proliferation, while maintaining their multipotency. We anticipate that these cell-seeded fibrin microthreads will serve a platform technology to improve localized delivery and engraftment of viable cells to damaged tissue.

Introduction

Cellular therapy is a promising method for regenerating new tissue for many different organ systems. However, one of the technical issues limiting cell therapy is the lack of a delivery vehicle to efficiently deliver cells to well defined regions. For example, current methods for delivering progenitor cells to the heart, which include intravascular (IV),^{1–2} intracoronary (IC)³ and intramyocardial (IM) injection,³ are inefficient due to low cell retention and a lack of targeted localization. While IV delivery of cells is the least invasive, most of the delivered cells are trapped in the lungs¹, with less than 1% of the cells residing in the infarcted heart. During angioplasty, cells can be delivered by IC infusion directly to the region of interest. However, upon restoration of blood flow the majority of cells are washed away from the region of interest and only 3% of the delivered cells are engrafted into the heart.³ By comparison, direct IM injection of cells into the heart wall resulted in a modest increase in the number of cells delivered to the myocardium, with 11% of the cells engrafting.³

Recent research efforts have attempted to overcome these limitations by utilizing biomaterial scaffolds for more efficient delivery of cells to the heart. Materials such as collagen,⁴ fibrin,⁵ gelatin,⁶ alginate,⁷ and Matrigel™⁸ have been studied for this application. For example, Simpson and colleagues demonstrated 23% cell engraftment in the

heart by using a collagen scaffold-based delivery vehicle applied to the epicardium.⁹ However, stem cells delivered by these scaffolds appear to be retained within the scaffold or restricted to the epicardial space; rarely traversing the myocardial wall to reach the endocardium,⁹ where most clinical myocardial infarctions reside.

While promising, the results of these studies illustrate the continued need for biomaterial scaffolds that more efficiently facilitate targeted delivery of stem cells to defined regions, while allowing stem cell growth and maintaining cell viability. Ideally this scaffold should persist long enough to guide the integration of cells, but not so long as to interfere with cell coupling essential to myocardial function.¹⁰

Fibrin, a natural provisional matrix scaffold for cell attachment and migration during wound healing, has been used in the form of gels to deliver cells to infarcted myocardium.^{5,11–12} Fibrin gels have also been shown to support hMSC viability and growth.^{5,11–12} Extrusion of fibrin gel materials into discrete biopolymer microthreads¹³ combines the desirable biological properties of fibrin with unique structural properties and increased mechanical strength, resulting in a substrate material conducive to both cell support and delivery. Cornwell et al. demonstrated that fibrin microthreads were significantly higher in tensile strength than fibrin gels and supported fibroblast viability, alignment, growth, and migration.¹³ Additional benefits of fibrin microthreads as biomaterial scaffolds for cardiac applications include: the possibility of using autologous fibrinogen and thrombin, the presence of growth factors in the matrix, its Food and Drug Administration (FDA) approval for clinical use, and its angiogenic characteristics.^{5,14–15}

Herein, we propose to use fibrin microthreads as a matrix to anchor cells during delivery. The goals of this study were to establish methods for seeding hMSCs onto bundled fibrin microthreads, and to assess the efficacy of fibrin microthreads as a scaffold to support hMSC proliferation and survival, while allowing hMSCs to maintain their ability to differentiate into multiple cell types. In addition, the number of hMSCs attached to each bundle of fibrin microthreads at each time point was quantified to assess the cell-loading “capacity” of fibrin microthread scaffolds.

Methods

Fibrin Microthread Production and Seeding

Fibrin microthreads were produced according to a previously published protocol.¹³ Briefly, fibrinogen and thrombin from bovine plasma (Sigma Aldrich, St. Louis, MO) were placed into separate 1 mL syringes. The solutions were combined by a blending applicator tip (Micromedics, St. Paul, MN) and extruded through polyethylene tubing (0.38mm inner diameter, Beckton Dickinson, Franklin Lakes, NJ) into a bath of 10mM HEPES, pH 7.4 at room temperature. After 15 minutes, the microthreads were removed from the bath and hung to dry overnight. This process produced individual fibrin microthreads with an average hydrated diameter of approximately 100 μ m.¹³

In order to facilitate cell attachment, the individual microthreads were grouped together to form a bundle of fibrin microthreads. Specifically, bundles of microthreads were formed by placing four microthreads adjacent to each other and hydrating them with a droplet of phosphate buffered saline (PBS) along the length of the microthreads, which allowed them to adhere to each other (Figure 1 A). To prepare microthreads for seeding, dried bundles were glued to 3.0 cm outer diameter stainless steel washers (Seastrom Manufacturing, Twin Falls, ID) with Silastic Silicone Medical Adhesive Type A (Dow Corning, Midland, MI). Individual washers were placed in wells of a standard 6-well plate over a 13 mm diameter circular ThermanoxTM coverslip (Nalge Nunc International, Rochester, NY) as seen in

Figure 1B. Prior to seeding, the washers with attached bundles were rehydrated with PBS for 15 minutes, then sterilized using 70% isopropyl alcohol for 1 hour, rinsed with three washes in sterile PBS for 15 minutes, and air dried in a laminar flow hood overnight.

Human mesenchymal stem cells (hMSCs; Lonza, Walkersville, MD; passage 4–9) were cultured in Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza, Walkersville, MD) according to the manufacturer's instructions. For visualization experiments, hMSCs were preloaded with quantum dots (QDs, Qdot 655 ITK Carboxyl Quantum Dots, Invitrogen Cat. No. Q21321MP) as previously described.¹⁶ Briefly, QDs were added to complete MSCGM and vortexed for 60 seconds. Cells were washed once in phosphate-buffered saline (PBS) and incubated in the QD solution for up to 24 hours at 37°C. To prepare hMSCs for fibrin microthread seeding, cells were trypsinized, centrifuged, and re-suspended at a concentration of 500,000 cells/mL in MSCGM. A 100 μ L drop of cell suspension (50,000 cells) was placed in the center of the coverslip and the plate was placed in a 37°C, 5% CO₂ incubator. After 2 hours of incubation, the washers with attached microthreads were rinsed with fresh PBS to remove unattached cells, transferred to new 6-well plates containing 2 mL of fresh media, and media was changed every three days (Figure 1C).

Visualization and Quantification of hMSCs on Fibrin Microthreads

After 5h, 1, 2, 3, 4, and 5 days in culture, washers with attached microthread bundles were removed from the 6-well plate and rinsed with PBS. The microthreads were cut from the washers, placed on glass slides, fixed in 4% paraformaldehyde and permeabilized with 0.25% Triton-X100. Microthreads were blocked with 10% bovine serum albumin in PBS, stained with phalloidin (conjugated to AlexaFluor 488; Invitrogen, Carlsbad, CA) at 1:40 dilution for 30 minutes to illuminate the f-actin filaments in the cytoskeleton, and counterstained with Hoechst dye (Cambrex Bio Science, Charles City, IO) at 1:6000 dilution for 5 minutes to visualize cell nuclei.

Cell density was determined by counting the number of cells per square millimeter of microthread from fluorescent images captured with a Leica DM LB2 microscope. A total of six microthread bundles from two separate experiments were imaged per time point under 10 $\times$ magnification. Images were taken successively along the cell-populated region of the microthreads until the entire length was viewed, this required anywhere from 5 to 13 images. Counts and area measurements were performed using Image J software with the Cell Counter plug-in. Raw data was reported as the number of cells per square millimeter of microthread bundle.

Evaluation of Cell Proliferation

To determine whether the increase in cell number over time in culture on fibrin microthreads was due to cellular proliferation, microthreads were fixed and stained with an antibody against Ki-67; a protein expressed during G1, S, G2, and M phases of the cell cycle.¹⁷ Microthread bundles were seeded with hMSCs as described previously. At 1, 3, and 5 days, bundles were removed from the washers, placed on glass slides, fixed with 4% paraformaldehyde, and permeabilized with 0.25% Triton-X100. Following two PBS rinses, microthreads were exposed to epitope retrieval for 40 minutes in 10% Dako Cytomation Target Retrieval Solution (Dako, Carpinteria, CA) in diH₂O, blocked with 5% normal rabbit serum and incubated in anti-Ki-67 mouse IgG1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 1 hour. Microthreads were then rinsed with PBS, incubated with Alexa 488 rabbit anti-mouse IgG (Invitrogen, Carlsbad, CA) for 1 hour, and counterstained with Hoechst dye. As negative controls, cell-seeded microthreads were incubated in mouse IgG (Vector Labs, Burlingame, CA, I-2000) instead of the Ki-67 primary antibody. Fluorescent images were captured at 20 $\times$ magnification with a Leica DM LB2 microscope from four bundles stained

in two separate experiments. Images were taken randomly along the cell-populated regions of microthread bundles. From these, ten images were randomly selected and the percentage of cells in the cell cycle was calculated by counting the number of Ki-67 positive cells and dividing by the total number of cells per image.

Cell Viability

In order to confirm viability of hMSCs cultured on microthreads, the cell-seeded bundles were analyzed with a LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen L-3244, Carlsbad, CA). This was done by preparing and seeding microthread bundles as described previously. After 5 days in culture, the microthread bundles were cut from the washers and placed on a glass slide to incubate in 8 μ M ethidium, 4 μ M calcein solution for 30 minutes. Fluorescent images were captured with a Leica DM LB2 microscope. Dead positive control cells were prepared by incubating hMSCs in 30% methanol for 30 minutes.

Differentiation

To confirm that hMSCs maintain their multipotency after being cultured on fibrin microthreads, cells were released from microthreads by trypsinization, re-plated, and exposed to established hMSC differentiation protocols to induce adipogenesis (Adipogenic Differentiation Medium, PT-3004, Lonza) or osteogenesis (StemPro Osteogenesis Differentiation Kit, A10072-01, Invitrogen). Mesenchymal stem cells that had not been cultured on threads served as controls. Both differentiation protocols were repeated with treated hMSC control (n = 6), untreated hMSC control (n = 6), and hMSCs from threads (n = 4) for each experiment.

Microthreads were bundled, sterilized, seeded, and cultured for 5 days. Subsequently, bundles were digested in 0.5 mL of 0.25% trypsin in Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA) at 37° C for 5 minutes. An equivalent amount of MSCGM was added to inactivate the trypsin and the suspension was centrifuged for 5 minutes at 1,000 RPM. For adipogenic differentiation, cells were re-plated at 2.1×10^4 cells per cm² tissue culture surface area and fed every 2–3 days with MSCGM until cultures reached 100% confluence, approximately 5–13 days. Cells were then fed for three cycles of 3 days with Adipogenic Induction Medium followed by 1–3 days with Adipogenic Maintenance Medium. Negative control hMSCs were fed with Adipogenic Maintenance Medium only. After the three cycles, all cells were cultured for an additional week in Adipogenic Maintenance Medium. At the end of each week, cells were visually inspected using light microscopy for characteristic lipid vacuole formation. At the conclusion of the feeding cycle adipogenic cultures were fixed in 4% paraformaldehyde, rinsed in distilled water, dehydrated in 60% isopropanol, stained with 0.3% Oil Red O (MP Biomedical, Solon, OH) in distilled water, rinsed in 60% isopropanol, counterstained with hematoxylin (Sigma Aldrich, St. Louis, MO), rinsed with distilled water, and viewed under a light microscope.

For osteogenic differentiation, cells were re-plated at 5×10^3 cells per cm² tissue culture surface area and cultured overnight in MSCGM. Cells were then fed with Complete Osteogenesis Differentiation Medium which was replaced every 3–4 days for 2–3 weeks. Negative control cells were fed with MSCGM on the same schedule. At the end of each week, cells were visually inspected using light microscopy for characteristic cobblestone appearance. At the conclusion of the feeding schedule, cells were stained with Alizarin Red S to assess calcium deposition. Cells were removed from culture, rinsed in PBS containing calcium and magnesium (Invitrogen, Carlsbad, CA), fixed in 4% paraformaldehyde, rinsed with one wash of PBS containing calcium and magnesium followed by one wash of distilled water, stained with 2% Alizarin Red S solution at pH 4.3 for 5 minutes, rinsed three times with distilled water and viewed under a light microscope.

Suturing hMSC seeded Microthreads

Four fibrin microthreads were bundled together and cut to 4 cm lengths. Bundles were then hydrated with PBS, threaded through a size-20 surgical needle, folded onto themselves to yield a suture length of 2 cm, and then sterilized. The bundles were then seeded with passage 5 hMSCs for 24 hrs as described above. Collagen gels were formed by mixing 0.8 mL of 10 mg/mL soluble rat tail collagen, 200 μ L of 5 $\times$ DMEM / 0.22 M sodium bicarbonate solution (3.7 g NaHCO₃ and 13.48 g DMEM into 200 mL of deionized H₂O), and 40 μ L of 0.1 M sodium hydroxide. The mixtures were allowed to polymerize for 24 hours in an incubator at 37°C and 10% CO₂. After 24 hours, microthread bundles and collagen gels were removed from the respective incubators, and microthread bundles were randomly segregated into 3 groups: unsutured LIVE stain positive controls (n=5), unsutured DEAD stain positive controls (n=5), and sutured samples (n=5). To assess cell viability, the bundles were assayed using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells as described above. The unsutured LIVE stain control group was immediately stained for viable cells, while the unsutured DEAD stain control group was submersed in cold methanol for 20 minutes before staining. Each bundle from the sutured microthread group was pulled through a collagen gel for an approximate length of 5 mm, and then stained for viability with LIVE/DEAD. After the staining procedure, microthread bundles were immediately evaluated with fluorescence microscopy.

Statistics

Statistical difference between two groups was analyzed using Student's t-test or the Mann Whitney Rank Sum test for cases of unequal variance. Statistical differences between groups were analyzed using ANOVA with Holm-Sidak post hoc testing. In cases where data was non-normal an ANOVA on Ranks followed by a Tukey post hoc test was used since data was not transformed to accommodate the non normality. Significance was established for $p < 0.05$.

Results

Human MSCs attach to fibrin microthreads and increase in number with culture time

Microthreads were bundled to provide increased surface area for cell growth as compared to a single microthread. An interesting result of microthread bundling was that "grooves" form in the spaces where two microthreads attach to each other. Cells appear to settle in the grooves of the thread bundle at day 1 (Figure 2), and then proceed to spread out and cover the surface of the microthreads by day 3. Phalloidin staining showed that hMSCs appear to align with the long axis of the microthread, with the percentage of cells aligning along the microthread increasing over time. There was a statistically significant increase in cell number by day 3 (Table 1), reaching a maximum cell concentration of 731 ± 101 cells/mm² after 5 days (the latest time point evaluated). By day 5 of incubation, the cells appear to be nearly confluent, based on the phalloidin staining (data not shown).

Human MSCs were pre-loaded with quantum dots (QD) prior to cell seeding, to evaluate the effect of QDs on hMSC attachment to fibrin microthreads, and to determine whether the QD signal could be detected and retained in hMSCs cultured on fibrin microthreads. The QD signal was maintained when the hMSCs were cultured on microthreads and did not appear to interfere with cell to microthread binding (Figure 2).

Human MSCs proliferate when cultured on fibrin microthreads

Ki-67 staining of bundles showed that hMSCs entered the cell cycle over 5 days in culture on microthreads (Figure 3). One day after incubation on microthreads, hMSCs show positive staining for Ki-67 in the nucleus. The percentage of cells expressing Ki-67 significantly

increased after 3 days of culturing (4.9 ± 5.4 vs. 22.6 ± 5.9 ; Day 1 vs. Day 3; $p < 0.05$). Ki-67-positive cells were still detected at day 5; however, there is a statistically significant decrease in percentage of positive cells from day 3 (6.8 ± 5.7 ; Day 5; $p < 0.05$ vs. Day 3). Table 2 shows a summary of the percentage of hMSCs in the cell cycle at each time point.

Fibrin microthreads support hMSC survival

To determine whether the hMSCs were still viable after 5 days of incubation on the microthreads, a LIVE/DEAD assay was performed. The assay showed cells were viable after 5 days in culture on microthread bundles with minimal cell death (dead cells indicated by white arrows in Figure 4). The viability staining was predominately in the cytoplasm, as expected. The control dead cells showed red staining in the nucleus, with no green fluorescence (live stain) in the methanol-fixed microthread bundle, suggesting that the green signal was due to live hMSCs on the fibrin microthreads and not microthread autofluorescence.

hMSCs maintain their multipotency on fibrin microthreads

Functional differentiation assays demonstrated that hMSCs retained their ability to differentiate into adipocytes and osteocytes after being cultured on microthread bundles for 5 days. After 3 weeks in culture, treated hMSCs began to form lipid vacuoles, which are characteristics of adipocytes, as seen in Figure 5. Oil Red O staining after 5 weeks of culture confirmed the presence of lipid vacuoles (Figure 6). As expected, no vacuoles were seen in hMSCs not exposed to adipogenic culture conditions.

After 4 weeks in culture, Alizarin Red S staining of osteogenic cultures showed extensive calcium deposition in both groups of treated hMSCs (with or without culturing on microthreads), indicating their differentiation into osteocytes (Figure 7). Many cells in these groups also exhibited a cobblestone morphology. Again, as expected, no calcium deposition was seen in hMSCs that were not exposed to the differentiation protocol.

hMSCs remain viable after microthread suturing through a collagen gel

To demonstrate that hMSC-seeded microthreads can be used as a platform technology for targeted delivery of viable stem cells to tissue, hMSC-seeded fibrin microthreads were sutured through collagen gels and then stained for cell viability. Bundles of four fibrin microthreads seeded with hMSCs were mechanically strong enough to be pulled through collagen gels with no signs of mechanical failure. A viability assay demonstrated that hMSCs seeded on fibrin microthreads remained viable during the procedure (Figure 8). An occasional cell was found with DEAD (red staining) in the nucleus, suggesting some cells were not viable. LIVE-stained control microthreads clearly exhibited viability staining with no DEAD staining, whereas DEAD-stained controls exhibited no cells with green (LIVE) stain in the cytoplasm.

Discussion

Using current methods, it is difficult to efficiently deliver a large number of stem cells to a well defined region of tissue, *in vivo*. To overcome these limitations, we have developed novel fibrin microthreads which we hypothesize will facilitate targeted localization of hMSCs. The results of the present study demonstrate that hMSCs can be successfully seeded and cultured on fibrin microthreads, while retaining their ability to proliferate and remain pluripotent (determined by successful induction of adipogenesis and osteogenesis in hMSCs that had been cultured on fibrin microthreads). These microthreads can be attached to a surgical needle and pulled through a collagen gel while maintaining seeded cell viability,

thereby suggesting that this method may be used to deliver viable cells to various tissues in the body.

The Ki-67 staining illustrated that hMSCs cultured on microthreads enter the cell cycle. Between 1 and 3 days of culture there is an increase in Ki-67 expression. However, the expression prior to day 5 in culture drops. This may be due to hMSCs approaching confluence on the microthread bundle. Between day 2 and day 3, there is a 76% increase in cell number (Table 1). However, the increase drops to 11% and 19% from days 3 to 4 and days 4 to 5, respectively, which is also in agreement with the cells being contact inhibited as they become confluent on the microthreads.

Functional differentiation assays demonstrated that hMSCs retain their ability to differentiate down adipogenic and osteogenic lineages after 5 days of culture on fibrin microthreads. This is consistent with previous research, which demonstrated that hMSCs can differentiate down both lineages when cultured in a fibrin gel.^{18–19} Catelas et al. investigated hMSC morphology, proliferation and osteogenesis in different formulations of fibrin gels. Cells entered into the early stages of osteogenic differentiation as measured by alkaline phosphatase assay, von Kossa staining, and real-time reverse transcriptase-polymerase chain reaction.¹⁸ Additionally, Gerard et al. demonstrated adipogenic differentiation of hMSCs cultured in a fibrin gel as documented by Oil Red staining.¹⁹ While we have investigated adipogenesis and osteogenesis here, these are only two of the many different cell lineages hMSCs have been shown to differentiate into. Research suggests hMSCs are also capable of chondrogenic, myogenic, neurogenic and endothelial differentiation thus future characterization of their multipotency may be expanded to include some of these assays.²⁰

Although the mechanism by which hMSCs offer myocardial protection is currently debated,²¹ retention of differentiation capability may be important. Future studies may expand the differentiation analysis to include differentiation on the surface of the microthread. However, the fibrin microthreads are likely to degrade within days of being delivered to most tissues and therefore may not have a prolonged impact on the hMSCs post implantation. The osteo- and adipogenic differentiation in this work were performed to show whether or not the hMSCs remained multipotent. Although they may not be directly applicable to cardiac applications, these results demonstrate that hMSCs retain multipotency (i.e. culture on fibrin microthreads did not impact the ability of hMSCs to differentiate into cells of different lineages). This may be important when applying fibrin microthread-mediated delivery of hMSCs for tissue repair to treat other diseases or organs. However, the next step in the application of microthread-mediated hMSC delivery for cardiac repair would be to conduct more specific differentiation and paracrine factor synthesis experiments that are directly relevant to the ability of delivered hMSCs to benefit myocardial repair.

An attractive feature of using fibrin microthreads is inherent in their combination of form factor, cell signaling and structural properties. Their cylindrical structure and small diameter may facilitate orientation of cells, based on the organization of the cell's actin microfilament bundles.²² By guiding alignment of hMSCs on the fibrin microthreads, we may provide an aligned environment for regeneration. Additionally, the microthread scaffold can be tailored to control cell function by incorporating therapeutic molecules such as growth factors into the matrix. By strategic selection of such factors, the microthreads will ultimately define the biochemical cues in the cellular microenvironment or dictate stem cell differentiation. Finally, the tunable degradation time and tensile strength of fibrin microthreads¹³ suggest that these materials can be tailored for cell delivery to many different tissues, including neurologic, orthopedic, cardiovascular and urinary applications where initial cell therapy has

shown promise for repair. Ultimately, this technology may be tailored for application in semi- or minimally invasive surgical procedure.

One potential application for fibrin microthreads is for delivery of hMSCs to the heart. In the clinic, delivery of hMSCs to the heart has been shown to improve mechanical function, although the exact mechanism remains elusive.²³ To be clinically useful, the number of hMSCs attached to the microthreads must be at least as many as the number of cells that must engraft into the heart to induce improved myocardial function. Five days of incubation resulted in an average density of 731 ± 101 cells/mm² of microthread. By extrapolating this data to the total number of cells per microthread bundle (which has a surface area of approximately 18.8 mm²), we find that it is theoretically possible to deliver approximately 10,000 cells with one 2 cm long bundle. This coincides well with previous research reporting the number of engrafted cells that also resulted in an improvement in cardiac function (Table 3). For example, engraftment of 10–30,000 cells per kg body weight into a pig heart corresponds to 3–12,000 cells for a rat heart with a body weight of 300–440 g, which could be delivered on a 2 cm microthread bundle. While a single microthread bundle can deliver 10,000 cells, 4 bundles will deliver approximately 40,000 cells. Delivering 1 to 4 microthread bundles to the rat heart is feasible, as their diameter is only a fraction of the wall thickness of infarcted rat hearts (< 250 μ m bundle diameter, compared to 1.5 to 2mm wall thickness). Thus, to increase the number of cells delivered, more microthreads bundles can be implanted.

Human MSCs were pre-loaded with quantum dots (QDs) to determine whether the QD signal could be detected and retained in hMSCs cultured on fibrin microthreads. Our results demonstrate that it was retained when the hMSCs were cultured on microthreads and that QDs did not appear to interfere with cell attachment and culture on microthreads (Figure 2). Quantum dots will be used to label cells for future in vivo studies to distinguish implanted MSCs from native cardiac cells. Autofluorescence is a significant concern when tracking cells, especially in the heart.²⁴ The QD signal is easy to detect, does not require any secondary staining, and we have previously used QDs to track hMSCs delivered to hearts for up to 8 weeks.¹⁶ However the uptake of QDs by other cells, leading to a false positive signal, must be ruled out prior to their use as tracking agents in tissue.

Future work in our laboratory will utilize a rat model to study microthread-mediated cell delivery within the heart. It is still vital to investigate how many cells are lost in the microthread implantation process and how this would affect engraftment rate. We expect a much higher engraftment rate than systemic delivery because the cells are delivered locally and are attached to the microthread scaffold. This should minimize, or even eliminate, the wash out phenomenon that has limited cell retention when direct intramyocardial injection is used as a delivery method. However, since the hMSCs are cultured on the exterior of the microthread bundle, shear stress will be exerted on them during delivery. To demonstrate feasibility for delivering cells to tissue via microthreads, we sutured hMSC seeded fibrin microthread bundles through a collagen gel. Although preliminary results indicated that most hMSCs remained viable, a more quantitative analysis is warranted. There is a distinct possibility that the shear stress these cells encounter while the microthread bundle is being sutured into the myocardium could detrimentally impact cell viability and adhesion. Should this be identified as a problem, there are several design modifications that could be implemented to protect the hMSCs from shear stress during implantation.

In summary, we have begun to validate the use of fibrin microthreads as a platform technology for delivery of hMSCs. This innovative cellular delivery strategy may permit localization to a precise region within a tissue by attaching the cell seeded microthreads to a surgical needle. No additional training or knowledge of special implantation techniques is

required since it is utilized in a manner similar to a suture. The approach would allow the surgeon to customize the treatment by controlling the location of implantation, the size and number of microthread bundles delivered (thereby controlling the number of cells delivered), and the region of tissue that should be treated. While our work was done with a bundle of four fibrin microthreads, this technology has the capability for scale-up to accommodate increased number of cells.

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Figure 1. Set up for loading fibrin microthreads

(A) Schematic drawing demonstrating how four microthreads are bundled together. (B) Image showing microthreads (white horizontal lines). (C) Schematic drawing describing how cells are loaded and cultured on microthreads. Microthread bundles are glued to washer. Cells are seeded on microthreads with a coverslip placed under the seeding region. Washers are sized to fit into a well in a 6 well plate format. After seeding period, the washers, with microthread attached, are moved to a new 6 well plate where they are cultured for various periods of time.

Figure 2. Human mesenchymal stem cells (hMSCs) attached to fibrin microthread bundles
Quantum dot-loaded hMSCS on fibrin microthreads after 1 day (a–f) and 3 days (g–l) of culture. Low magnification images (a,b,g,h; scale bar = 250 μ m) demonstrate that the cells are located throughout the length of the microthread. High magnification images (c–f, i–l; scale bar = 50 μ m) document the increase in cell density of the microthreads between 1 and 3 days of incubation. The actin (green) appears to align along the long axis with increased incubation time. Cells are stained with Hoechst (blue) and loaded with quantum dots (red). Panels b & h are unseeded control threads exposed to the same staining protocol as the other panels. White dotted lines (a, b, g, h) denote the outline of the microthread bundle.

Figure 3. Cells on microthreads stain positive for cell cycle marker Ki-67

Human mesenchymal stem cells incubated on microthread bundles are positive for Ki-67 (green). The staining localizes with cell nuclei (Hoechst staining, blue). Positive staining was noted in cells incubated on microthread bundles for 1, 3 and 5 days. The control microthreads were exposed to the staining process without the addition of a primary antibody. White arrows denote some of the Ki-67 positive cells. White dotted lines denote the outline of the microthread bundle.

Figure 4. Human mesenchymal stem cells remain viable when incubated on microthread bundles
Cells incubated on microthread bundles were stained with calcein (green) to discriminate live cells and ethidium (red) to identify dead cells. After five days of incubation, very few cells stained positive for ethidium. Control dead cells on microthreads, fixed in methanol, exhibited ethidium in the nuclei and no calcein in the cytoplasm. White dotted lines denote the outline of the microthread bundle.

Figure 5. Human mesenchymal stem cells maintain the ability to form vacuoles after incubation on microthreads

Cells incubated on microthreads (Treated hMSCs – threads) or incubated on conventional cell culture plates (Treated hMSCs) form vacuoles, characteristic of adipocytes, 3 and 5 weeks after exposure to adipogenic differentiation conditions. Cell not exposed to adipogenic differentiation (Non Treated hMSCs) did not demonstrate vacuole formation.

Figure 6. Cells maintain the ability to differentiate into adipocytes after culturing on microthreads

Human mesenchymal stem cells incubated on microthreads (Treated hMSCs – microthreads) stained positive with Oil Red O, confirming adipocyte differentiation. Cells incubated on conventional cell culture plates and exposed to adipocyte differentiation (Treated hMSCs) also stained positive with Oil Red O, whereas cells not exposed to adipocyte differentiation (Non Treated hMSCs) did not demonstrate any positive staining for Oil Red O.

Figure 7. Osteogenic differentiation of cells incubated on microthread bundles

Human mesenchymal stem cells incubated on microthreads (Treated hMSCs – threads) stained positive with Alizarin Red S, confirming osteocyte differentiation. Cells incubated on conventional cell culture plates and exposed to osteogenic differentiation (Treated hMSCs) also stained positive with Alizarin Red S, whereas cells not exposed to osteogenic differentiation (Non Treated hMSCs) did not demonstrate any positive staining for Alizarin Red S.

Figure 8. hMSC seeded microthreads pulled through collagen gel

(A) To mimic microthread-based cell delivery, hMSC-seeded fibrin microthreads were attached to a needle and pulled through a collagen gel. (B) hMSC-seeded microthreads after suturing stained with LIVE (green) stain to determine viable cells. The inset shows a control unsutured hMSC-seeded microthread. (C) hMSC-seeded microthreads were also stained with DEAD (red) stain to determine dead cells. The inset shows DEAD positive control unsutured hMSC-seeded microthreads. (D) Overlay of panels B and C. All scale bars are 100 μm .

Table 1Average number of cells/mm² on microthread bundles (n = 6)

Time Point	Average #cells/mm ² ± SEM
5 hours	93 ± 21
1 day	253 ± 57
2 day	315 ± 84
3 day	555 ± 49 [*]
4 day	616 ± 83 ^{*†}
5 day	731 ± 101 ^{*†}

(* indicates statistically significant difference between 5 hour, 1 day and 3, 4, 5 day for p<0.05, there is no statistical difference between 5 hour and 1 day as well as between 3, 4, and 5 days; † indicates statistically significant difference between 2 day and 4, 5 day for p<0.05, there is no statistical difference between 4 day and 5 day)

Table 2Percent of Ki-67 positive hMSCs on microthreads (Average $\pm$ Stdev)

Time Point	% hMSCs in cell cycle
1 Day	4.9 $\pm$ 5.4
3 Day	22.6 $\pm$ 5.9 [*]
5 Day	6.8 $\pm$ 5.7 [†]

(* indicates statistically significant differences between day 1 and day 3 $p < 0.05$, † indicates statistically significant difference between day 3 and day 5)

Table 3

Total number of cells engrafted by species

Species	Number of Cells Delivered	Engraftment	Number of Cells Engrafted	# Cells Engrafted Normalized to Rat	Source
Mouse	5×10 ⁵	0.44%	2,200	17,600	(4)
Mouse	5×10 ⁵	0.2%	1,000	8,000	(29)
Rat	4×10 ⁶	< 1%	<40,000	<40,000	(8)
Pig	50×10 ⁶	0–6%	<3×10 ⁶	<36,900	(30)
Pig	1×10 ⁵ to 1×10 ⁶ per kg bodyweight	1–3%	1,000–30,000 per kg bodyweight	400–12,000	(31)