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Extracellular matrix protein production in human adipose-derived mesenchymal stem cells on three-dimensional polycaprolactone (PCL) scaffolds responds to GDF5 or FGF2

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Abstract

Purpose—The poor healing potential of intra-articular ligament injuries drives a need for the development of novel, viable 'neo-ligament' alternatives. Ex vivo approaches combining stem cell engineering, 3-dimensional biocompatible scaffold design and enhancement of biological and biomechanical functionality via the introduction of key growth factors and morphogens, represent a promising solution to ligament regeneration.

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AUTHOR DISCLOSURE STATEMENT

Dr. Allan Dietz has a commercial interest in Mill Creek Life Sciences, which manufactures the clinical grade commercial platelet lysate product used for maintaining adipose tissue-derived mesenchymal stem cells.
No competing financial interests exist for the other authors.

Methods—We investigated growth, differentiation and extracellular matrix (ECM) protein production of human adipose-derived mesenchymal stem/stromal cells (MSCs), cultured in 5% human platelet lysate (PL) and seeded on three-dimensional polycaprolactone (PCL) scaffolds, in response to the connective-tissue related ligands fibroblast growth factor 2 (basic) (FGF2) and growth and differentiation factor-5 (GDF5). Phenotypic alterations of MSCs under different biological conditions were examined using cell viability assays, real time qPCR analysis of total RNA, as well as immunofluorescence microscopy.

Results—Phenotypic conversion of MSCs into ECM producing fibroblastic cells proceeds spontaneously in the presence of human platelet lysate. Administration of FGF2 and/or GDF5 enhances production of mRNAs for several ECM proteins including Collagen types I and III, as well as Tenomodulin (e.g., COL1A1, TNMD), but not Tenascin-C (TNC). Differences in the in situ deposition of ECM proteins Collagen type III and Tenascin-C were validated by immunofluorescence microscopy.

Summary—Treatment of MSCs with FGF2 and GDF5 was not synergistic and occasionally antagonistic for ECM production. Our results suggest that GDF5 alone enhances the conversion of MSCs to fibroblastic cells possessing a phenotype consistent with that of connective-tissue fibroblasts.

Keywords

Neo-ligament; polymer scaffolds; growth factors; MSC; FGF2; GDF5

INTRODUCTION

Intra-articular ligament ruptures are a common musculoskeletal injury[1] leading to painful joint instability, recurrent chondral injury, and disability, and early onset osteoarthritis [2–4]. Intracapsular ligaments, including the anterior cruciate ligament (ACL) and scapholunate (SL) ligaments, have an extremely limited intrinsic ability to heal, stemming from its envelopment by synovial fluid and low vascularity[2, 5]. Due to this poor healing capacity, ligament injuries are usually treated with complete surgical reconstruction/replacement using allograft or autograft tendons. While this technique provides a significant improvement in clinical outcomes compared with direct ligament repair, the replacement of stiff ligaments with elastic tendon grafts has a number of negative long term consequences, including donor site morbidity, incomplete ligamentization, prolonged healing times and unfavorable immunogenic response (when allograft tendons are used) [6–8]. As a result, there has been a shift in focus to create neoligament tissues which can better mimic stable, functionally normal tissue. A variety of tissue-engineering strategies have been employed to achieve the requisite biomechanical properties. These include: i) decellularization of autografts, and the use of either ii) natural polymer or iii) synthetic polymer scaffolds. A-cellular scaffolds can be designed to meet the mechanical demands within the joint, but lack cellular conductive and inductive properties [9]. The combination of these scaffold structures with progenitor cells and growth factors, however, represents a promising solution to native ligamentous regeneration[10].

Mesenchymal stromal/stem cells (MSCs) are pluripotent progenitor cells capable of differentiating into a variety of musculoskeletal tissue precursors such as osteoblasts, chondrocytes, adipocytes, and myocytes when placed under specific conditions in vitro[11]. Growth factors, hormones, and other regulatory molecules usually are incorporated into media to foster the differentiation of MSCs along specific lineages. Some physical factors including mechanical loading, electromagnetic fields, and ultrasound have been shown to play important roles in regulating the differentiation of MSCs[12–14].

Bone marrow stromal cells are among the best characterized stem cells and have been transplanted to various tissue injury sites, with enhanced tissue repair being achieved. However, there are drawbacks to their use. Bone-marrow-harvesting procedures are highly invasive, painful procedures with reported complication rates as high as 30%[15]. It is also well known that bone marrow isolations often yield a low number of stem cells[16]. Due to the similar multipotential properties, adipose-derived mesenchymal stromal/stem cells (MSCs) have become a focus of research in recent years and studies demonstrated that MSCs could differentiate into lineages of multiple mesodermal tissues, such as bone, cartilage, fat, and muscle when under the appropriate conditions and provided key environmental cues[4, 17–20]. Typically, adipose tissue is abundant in both humans and animals and can be easily harvested from subcutaneous tissue through percutaneous or limited open aspiration techniques[21, 22]. Adipose also provides a large volume of viable pluripotent stromal cells when harvested compared to that of bone marrow[23]. The ability to harvest such a high yield of stromal cells from small fractions of fat could mean that patients with low percentages of body fat could serve as autogenic sources of precursor cells for their own tissue-engineered therapies.

Fetal bovine serum (FBS) is mostly used for culture of human mesenchymal stem cells (hMSCs). However, translation of hMSC-based approaches is impeded by protracted expansion times, risk of xenogenic response, and exposure to zoonoses[24]. Human platelet lysate adherent to good manufacturing practices (GMP-hPL) provide a nonzoonotic adjuvant that enhances proliferation of hMSCs. Previous studies have shown that long-term culture in GMP-hPL maintains the multipotency of hMSCs, while protecting against clonal chromosomal instability detected in the FBS milieu[25]. Thus, we use GMP-hPL, instead of FBS, to accelerate proliferation (with no chromosomal aberrancy) of cultured hMSCs when creating neoligaments.

Polycaprolactone (PCL) is a highly biocompatible aliphatic polyester obtained by the polymerization to open-loop of ε-caprolactone with approval from the Food and Drug Administration (FDA) for use as an implantable material. It has excellent mechanical properties and exhibits slow degradation; it might therefore be a good candidate for use in in vivo tissue transplantation applications[26–30]. We thus investigated growth, differentiation and gene expression and extracellular matrix (ECM) protein production of human MSCs seeded on three-dimensional polycaprolactone (3D PCL) scaffolds, both in the presence of platelet lysate (PL) alone, as well as in response to the connective-tissue related ligands basic fibroblast growth factor 2 (FGF2) and/or growth/differentiation factor 5 (GDF5). FGF2 has been considered for enhancement of tendon healing in vivo [31–35] and proliferation of MSCs for tendon engineering applications in vitro [5, 36–41]. GDF5 is known to be

involved in tendon development in vivo [42–45] and has been investigated as a stimulatory morphogen for promoting tenogenic differentiation of mesenchymal stromal cells in culture [46–48]. We therefore set out to establish whether human MSCs are capable of undergoing ligamentous differentiation on 3D scaffolds and whether exposure to FGF2, GDF5 or a combination of these treatments augments de novo formation of ligament tissue in vitro.

Our results suggest that GDF5 does enhance the conversion of human MSCs to fibroblastic cells, resulting in a phenotype consistent with that of connective-tissue fibroblasts. When compared to FGF2, GDF5 is more effective in stimulating this phenotypic conversion of MSCs to fibroblasts capable of producing high levels of ECM proteins on 3D PCL scaffolds.

MATERIALS AND METHODS

Human MSCs isolation and culture in vitro

Adipose-derived mesenchymal stem cells (MSC) were isolated as previously described[49], in compliance with the institutional review board (IRB). Briefly, human adipose tissue was obtained from patients undergoing general surgical operations. The tissue was subsequently minced with scalpels, incubated in 0.075% collagenase type I (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37°C, centrifuged at 500g for 10 minutes and passed through a 70 μm cell strainer (BD Biosciences, San Jose, CA). Expansion medium consisted of advanced Modification of Eagle's Media (aMEM) (Invitrogen, Carlsbad, CA) with 5% human platelet lysate adherent to good manufacturing practices (GMP-hPL) (Mill Creek, Rochester, USA), 2U/ml heparin, 100 U/ml penicillin and 2mM L-glutamine. Cultured cells were maintained in a humidified incubator at 37°C and 5% CO_2. Medium was changed every 3 days, and cells were split at 70–80% confluency. All experiments were performed with cells from passage 6 and 7.

Seeding human MSCs on 3D PCL scaffolds

Three-dimensional polycaprolactone (PCL) scaffolds were acquired from Sigma-Aldrich Company (St. Louis, MO, USA). Fiber diameter is controlled by nozzle aperture while spacing between fibers is controlled by a motion control system, with a final configuration of 300 μm fiber diameter and 300 μm pore size. Cell seeding on 3D PCL scaffolds was performed according to the manufacturer 3D Insert™ cell seeding protocol, pipetting 150 μL MSC suspensions, with cell concentrations adjusted to achieve a final density of 1×10^4 cells/cm^2 onto each 3D PCL scaffold. After 3h, each scaffold was completely immersed in fresh media (Figure 1) and incubated at 37°C and 5% CO_2. 24 hours later, scaffolds seeded with MSCs were divided into four groups: i) control, with culture media (described above) ii) culture media supplemented with 10 ng/mL FGF2 (R&D, Minneapolis, USA), iii) culture media containing 100 ng/mL GDF5 (Sigma-Aldrich, St. Louis, MO, USA), and iv) culture media containing 10 ng/mL FGF2 and 100 ng/mL GDF5. Media was changed every 3 days.

Cell Viability and Cell Metabolic Activity

Cell morphology and viability of MSCs seeded on 3D PCL scaffolds was analyzed using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, Oregon) at days 1, 7 and 14 post seeding. This assay is based on simultaneous staining of viable live cells

(calcein acetoxymethyl, green) and dead or apoptotic cells (ethidium homodimer, red). Briefly, 5 μL ethidium homodimer and 5μL calcein AM were mixed in 10 mL PBS. Media was aspirated, samples were washed with PBS, and 500 uL staining solution was added to each well and kept in the dark for a 25 minute incubation. The cells were subsequently visualized using confocal microscopy (Zeiss LSM 780, Germany).

Cell metabolic activity, based on mitochondrial function, was measured in cells grown on 3D PCL scaffold using the MTS assay. This colorimetric assay monitors the activity of NADH/NADPH-dependent cellular oxidoreductases using dye conversion (Promega Corporation, Madison, WI). Briefly, scaffolds were incubated in 300 μL culture medium and 60 μL MTS at 37°C and 5% co_2 for 1 hour. Medium was then transferred into a 96-well plate, and absorbance at 490 nm was measured using a Tecan F-200 multiwell plate reader (Tecan Inc, San Jose, CA). MSCs seeded on culture plates at the same densities s were used as controls.

Total RNA extraction and RT-qPCR

RNA was collected from MSCs seeded on scaffolds at day 1 (control group; untreated), and from all four groups (control, FGF2, GDF5, FGF2 + GDF5) at days 3, 7 and 14 (Figure 2). N=1 for each group. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) and suggested protocol. Following reconstitution, 1 μg RNA samples were reverse-transcribed into cDNA using the SuperScript® III First-Strand Synthesis SuperMix Kit (Invitrogen).

Real time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed using QuantiTect SYBR® Green RT-PCR Kit (Qiagen, Valencia, CA, USA) to quantify the transcription level of tendon/ligament-related ECM genes and proliferation genes, including Collagen 1A (COL1A1), Collagen 3A1 (COL3A1), Tenascin-C (TNC), Tenomodulin (TNMD), Scleraxis (SCX), Decorin (DCN), Aggrecan (ACAN), MKI67, Lamin A (LMNA), Lamin B1 (LMNB1) and Lamin B2 (LMNB2), using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene. Forward and reverse primer sequences are listed in Table 1. Each sample was analyzed in duplicate using the iCycle (Biorad Laboratories), with the PCR threshold cycle number (C_T) for each sample calculated at the point where the fluorescence curves at 10–20 standard deviations above the average background fluorescence. C_T values of the target genes were normalized to GAPDH and relative expression levels determined using the $2^{\Delta T}$ method were compared with untreated control at day 1.

Immunofluoresence staining

The live expression of Collagen I (COL1A1), Collagen III (COL3A1) and Tenascin-C (TNC) were visualized as previously described [50]. Briefly, four treatment groups (as described previously; control, FGF2, GDF5 and FGF2 + GDF5) of MSCs were seeded and cultured on the PCL scaffolds for 14 days. The scaffolds were washed with PBS (5 min.), fixed with 4% paraformaldehyde (Wako) for 20 minutes, and then blocked with 0.3% Triton-X (Sigma-Aldrich, St. Louis, MO, USA). After PBS washing, the scaffolds were incubated in 1% BSA (10 minutes; Fisher), then 10% normal goat serum (45 minutes at room temp.;

Jackson ImmunoResearch), with PBS washing between each step. Primary antibodies (mouse; Collagen I (1:250, Sigma), Tenascin-C (1:250, Abcam), Collagen III (1:200, Abcam)) were then added to the respective wells and incubated overnight at 4°C. The secondary antibody (goat anti-mouse Cy3, Jackson ImmunoResearch) was added to each scaffold at a concentration of 1:500. Each scaffold was counterstained for 10 minutes at room temperature using DAPI (Sigma-Aldrich, St. Louis, MO, USA) at a 1:500 concentration. Images of each scaffold were then obtained using confocal microscopy (Zeiss LSM 780, Germany). Red pixels represent expression levels of each factor of interest, while blue represents the DAPI nuclear stain. Cy3 expression was quantified using Image J software (NIH).

Statistical Analysis

A two-tailed Student's t-test was used to compare the control groups in each respective assay with the individual experimental groups (FGF2, GDF5, FGF2 + GDF5) for analysis of proliferation and qPCR. The statistical significance was set at p-values <0.05.

RESULTS

Cell growth, survival and ECM production of MSCs in three-dimensional cultures with polycaprolactone scaffolds

To understand the biological properties of MSCs on three-dimensional PCL scaffolds, these cells were seeded under static conditions and cultured for two weeks in standard growth medium with platelet lysate (Figure 1A). Survival and growth of MSCs on 3D-PCL scaffolds were assessed using a cell viability assay ('live/dead cell staining') to examine the presence and morphology of live cells that adhere to the PCL scaffolds by fluorescence microscopy (Figure. 1B). At day 1 (D1), MSCs dispersed and attached to the surface of PCL, while exhibiting a round cell body with short cytoskeletal projections (Figure 1B). The density of MSCs increased with prolonged culture time. At day 7 (D7) and day 14 (D14), cells adopted a long fusiform shape and were densely distributed along the surface of the PCL filaments in a three-dimensional fashion (Figure 1B). By D7 MSCs appeared to have completed a phase of maximal proliferative expansion based on gene expression analysis of the proliferation marker MKI67, which encodes the Ki67 antigen (Figure 1C). By D14, MSCs were well-organized and arranged in parallel orientation on the scaffold surface with a further modest increase in cell density (Figure 1B), presumably due to a low residual level of proliferative activity as detected by MKI67 mRNA levels (Figure 1C). Reduced cell proliferation occurred concomitant with increased expression of a panel of extracellular matrix (ECM) proteins, including Collagen Types I and III (COL1A1 and COL3A1), as well as Decorin (DCN), Tenascin (TNC) and Tenomodulin (TNMD)(Figure 1C). The metabolic activity of MSCs on 3D PCL scaffolds, compared to cells on standard 2D plastic culture dishes, was also tested, using the MTS assay at day 1, 7 and 14. At each of these time-points a similar level of metabolic activity was observed and there was no significant difference between cells seeded on 3D PCL scaffolds and plastic dishes ($p > 0.05$) (Figure 1D). Taken together, these results show that culturing MSCs on 3D PCL scaffolds does not induce cytotoxicity. Rather, MSCs adhere, proliferate and are metabolically active on the scaffolds.

Effects of FGF2 and GDF5 on differentiation and ECM production in quiescent MSCs in three-dimensional cultures with polycaprolactone scaffolds

FGF2 and GDF5 are capable of provoking both mitogenic and metabolic cellular responses. To assess the effects of FGF2 and GDF5 on cell proliferation, we examined the biological properties of MSCs grown on 3D PCL scaffolds treated with either ligand. Cells were allowed to seed for one day and then treated with either ligand. RT-qPCR analysis of MKI67 mRNA reveals that active MSCs express the highest levels of MKI67 during the first day of culture reflecting active cell proliferation. By D3, MKI67 levels were strongly decreased in control cells and the presence of either FGF2 or GFD5 ligand does not stimulate MKI67 mRNA beyond levels observed in control cells. Furthermore, adding both FGF2 and GDF5 together did not have major effects on MKI67 levels (Figure 2). Hence, these two ligands either alone or in combination do not affect proliferation of MSCs on 3D PCL scaffolds after Day 1.

RT-qPCR assays were also performed on all four groups of MSCs grown on 3D PCL scaffolds to examine the expression of several tendon/ligament-related extracellular matrix genes (Collagen I, Collagen III, Tenascin-C, Tenomodulin, Decorin, and Aggrecan), a transcription factor gene (Scleraxis) and tissue stiffness genes (Lamin A, Lamin B1 and Lamin B2) (Figure 3).

Compared to control group, Collagen I expression decreased in cells treated with FGF2 at day 3 ($p<0.05$), and significantly increased in cells treated with GDF5 at day 14 ($p<0.01$). At day 3, Collagen III expression decreased in the FGF2, GDF5 and combination group although fold change was less than 2. The expression of collagen III was observed to increase in the GDF5 group at day 14 ($p<0.05$). At day 7, both Tenascin-C and Tenomudulin expression were significantly upregulated by FGF2 and GDF5 ($p<0.01$), but the level of Tenomudulin was decreased by FGF2 at day 14 (Figure 3). The data indicates that the main positive effects of FGF2 and GDF5 on ECM protein synthesis are evident in the late stages of the culture period, whereas combining the two ligands does not appear to be co-stimulatory.

Scleraxis expression decreased in the FGF2 samples at day 3 ($p<0.01$) and increased significantly in GDF5 at day 14 ($p<0.01$). Decorin expression increased slightly in the GDF5 and combination groups at day 14 while no obvious effects from FGF2 were observed. Aggrecan expression, however was upregulated by FGF2 at day 7 ($p<0.05$) and also by GDF5 at 14 ($p<0.05$) (Figure 3 and 4).

Finally, Lamin A expression increased at day 14 compared to day 1 for all treatment groups, with the highest difference for the control samples (fold change nearing 2) (Figure 4). Lamin B1 and B2 expression was decreased at day 3 and day 7 compared to day 1 for all groups. FGF2 upregulated Lamin B1 and B2 expression at day 7 compared to control samples, while GDF5 seemed to have no obvious effect after dropping at day 3., These results, and the similar trends observed for Collagens and Tenocyte-related ECM proteins, suggest that FGF2 and GDF5 have each distinct effects on gene expression, but they do not exhibit cooperative effects.

In situ deposition of ECM proteins on PCL scaffolds

To evaluate ECM protein accumulation of MSCs in scaffolds, immunofluoresence staining was performed using Collagen I, Collagen III and Tenascin-C antibodies. The expression of Collagen I in the ECM produced by MSCs was not obviously increased in the FGF2 or the combination group compared to control samples, but was markedly increased in the GDF5 group (Figure 5A). Compared to the control scaffolds, Collagen III expression was greatly increased in the GDF5 group and slightly increased in the combination group, while there was no observable change for FGF2 scaffolds (Figure 5B). MSCs Tenascin-C expression on both FGF2 and GDF5 scaffolds was also shown to increase (Figure 5C). Thus, GDF5 increases Collagen I, Collagen III and Tenascin-C protein production of MSCs on scaffolds, while FGF2 increases Collagen I and Tenascin-C expression but not Collagen III. No substantial synergistic effects of FGF2 and GDF5 on ECM production were observed.

DISCUSSION

The repair and regeneration of tissues for the treatment of intrasynovial ligament injuries presents a difficult challenge, due to the many complex biomechanical properties that must be met to mimic native ligament performance. The creation of stable neoligaments that combine synthetic polymer scaffold strategies with the use of clinically relevant progenitor cells and growth factors could, however, represent one promising solution to ligamentous regeneration. While various groups have had initial success in growing mesenchymal cells (including BMSCs)[51]and MSCs[9]) on scaffolds, the environment or cocktail of signals necessary to best differentiate stem cells into fibrous connective tissues is still unknown, although several growth factors, such as FGF2, GDF5, transforming growth factor-b (TGF-β), epidermal growth factor (EGF) and insulin like growth factor (IGF) have been identified as possible candidates[46].

As part of the musculoskeletal system, both ligaments and tendons are made up of fibrous connective tissues composed of dense layered collagen fibers. Although similar in composition, these tissues serve different functions. In a tendon fiber, fibroblasts (tenocytes), parallel collagen fibrils, and ECM components lend their tough, flexible elastic properties to the connecting of muscle to bone. Ligaments have criss-crossing fibers that add the strength, rigidity and stability necessary for bone-bone connections [52]. In both cases, the extracellular environment is a critical contributor to the functionality of these fibrous connective tissues. Therefore, the focus of this study was to determine whether two key growth factors, FGF2 and GDF5, could induce and enhance tendon/ligamentous differentiation of multipotent adipose-derived mesenchymal stem cells upon PCL scaffolding, with efforts made to maintain clinical relevance by seeding and treating MSCs on scaffolds composed of an FDA approved implantable biomaterial, and in human platelet lysate produced in the absence of zoonotic agents under GMP conditions.

Fibroblast growth factor 2 (FGF2) has been used previously for the stimulation of BMSC proliferation, differentiation of cells into ligament/tendon fibroblasts (tenogenesis), and enhancement of cellular collagen production, thereby increasing the mechanical strength of such constructs [39, 50, 53]. The effect of FGF2 on cultured hMSCs is less clear. One study showed that FGF2 treatment of hMSCs in vitro does not necessarily stimulate their potential

for ligament differentiation[54], whereas others have shown that in an FGF2 rich environment, MSCs deposit a collagen-rich ECM[9]. It was therefore important to ascertain whether exposure to FGF2 would impact the ligamentous differentiation of MSCs on 3D PCL scaffolds. GDF5 is also believed to play a role in a variety of musculoskeletal processes, including joint formation, endochondral ossification, and tendon and ligament maintenance and repair[55, 56]. Previous work has demonstrated that GDF5 induces neotendon and neoligamentous formation when implanted in ectopic sites, and incorporating recombinant GDF5 protein onto collagen sponges or suture materials enhances Achilles tendon healing in rodents[57]. Other studies have shown that mice deficient in the gene for GDF5 protein exhibit impaired tendon healing, which manifests as altered structural and mechanical properties of the repair tissue[58]. It has also been reported that GDF5 gene therapy increases rat Achilles tendon tensile strength, without inducing bone or cartilage formation within the healed tendon[59]. In addition, Park et al noted that rat MSCs cultured in vitro demonstrated enhanced ECM and tenogenic marker gene expression and increased proliferation in a dose- and time-dependent manner in the presence of GDF5[48]. Our results suggest that FGF2 and GDF5 both influence differentiation of MSCs on 3D scaffolds, in part by modulating cell proliferation and post-proliferative expression of ECM proteins.

Changes in the expression of a number of ECM markers for tendon and ligament differentiation were measured using RT-qPCR. We first examined Collagen I and III, primary collagens secreted by mature tenocytes that play a role in both wound healing and in endotenon and epitenon formation. In this study, GDF5 stimulated a more than fourfold increase in Collagen I and nearly twofold increase in Collagen III at day 14. In contrast, FGF2 did not increase expression of collagens I and III, a result in line with previous findings[54] (see Figure 3). These results were also confirmed with immunofluorescence staining. Addition of GDF5 significantly increased in situ deposition of collagen I and collagen III on scaffolds, while FGF2 had no obvious effects (see Figure 5). Other ECM markers tested included Tenascin-C and Tenomodulin. Tenascin-C has been implicated in tendon development and it is a relatively nonspecific marker for tendon/ligament regeneration[60]. Tenomodulin is a regulator of tenocyte proliferation and is involved in collagen fibril maturation, as it has been found in tendon primordia as well as differentiated tendon tissues[61]. Both FGF2 and GDF5 increased expression of Tenascin-C and Tenomodulin in hMSCs cultured on 3D scaffolds at day 7. By day 14, FGF2 downregulated, while GDF5 upregulated, the level of Tenomodulin. Immunofluorescence staining confirmed that both FGF2 and GDF5 could increase deposition of MSCs Tenascin-C expression on scaffolds. These results suggest that GDF5 may be more suitable than FGF2 for inducing hMSCs progression down a tenogenic lineage.

Scleraxis (Scx), a transcription factor involved in the regulation of Collagen I gene expression in cardiac fibroblasts and myofibroblasts and in demarcating the tendon-forming syndetome during development[62], has been reported by others to upregulate expression of Tenomodulin[63]. Molecular characterization of Scx knockouts in past studies has also revealed a clear decrease in the levels of Collagen I and a complete loss of Tenomodulin transcripts[64]. Our qPCR data showed that GDF5 led to an increase in Scleraxis

transcription at day 14, which was accompanied by increases in Collagen I and Tenomodulin mRNA expression.

In addition to ECM markers, RT-qPCR was collected for the proteoglycans Decorin and Aggrecan, both of which may play an important role in tendon mechanics. Decorin regulates collagen fibrillogenesis. Mice null in Decorin have previously been shown to have dysfunctional regulation of fibril assembly resulting in larger and more irregular fibril diameters than those in wild type mice, with the magnitude of these changes being tissue and age specific[65]. Aggrecan has been localized to the compressed segments of tendon tissue[66]. Slight increases in Decorin expression were observed in GDF5 and combination treatment groups in beyond day 3, while FGF2 did not seem to play a significant role. Although Aggrecan was expressed only at low levels, FGF2 and GDF5 appeared to have different effects at different stages (see Figure 3). In particular, it appears that GDF5 may be more potent than FGF2 in supporting upregulation of proteoglycan production.

Finally, we examined the impact of FGF2 and GDF5 on genes associated with tissue stiffness. Lamins are important contributors to the mechanical stiffness of nuclei. Lamin A over-expression during mesenchymal stem cell differentiation on stiff matrix enhances a high-stress, bone phenotype while Lamin-A knockdown in MSCs cultured and differentiated on soft matrix favors a low-stress, fat phenotype[67]. Our results suggest that expression levels of Lamin A, B1 and B2 are only slightly reduced by FGF2 and GDF5, consistent with the idea that FGF2 and GDF5 may act independently of lamin-mediated nuclear architecture.

CONCLUSION

In this study, we examined the biological phenotype of MSCs expanded in clinical grade human platelet lysate and cultured on biocompatible 3D polymer scaffolds in the presence of either the growth factor FGF2 or the morphogen GDF5. Our study indicates that each of these factors is capable of modulating gene expression of MSCs cultured on scaffolds. However, FGF2 and GDF5 do not appear to synergize and in some cases may perhaps counteract each other in supporting fibrous connective tissue differentiation. These studies provide an initial framework for future studies on growth factors and morphogens that control MSC differentiation and facilitate molecular strategies for MSC-based connective tissue engineering to promote surgical repair of ligaments and tendons.

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ABBREVIATIONS

3D	three-dimensional
ACL	anterior cruciate ligament
MSC	mesenchymal stem/stromal cells
calcein AM	calcein acetoxymethyl
ECM	extracellular matrix
EthD-1	ethidium homodimer
FGF2	fibroblast growth factor 2 (basic)
GDF5	growth and differentiation factor-5
MSC	mesenchymal stromal/stem cell
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
PCL	polycaprolactone
PL	platelet lysate
RT-qPCR	real time reverse transcriptase quantitative polymerase chain reaction
SL	scapholunate

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Highlights

- Because intra-articular ligament injuries heal poorly, it is necessary to develop tissue engineering approaches to generate new ligamentous tissues
- The results show that the biological signaling ligands FGF2 and GDF5 both stimulate expression of both collagenous and non-collagenous extracellular matrix proteins typical for connective tissues when administered to mesenchymal stromal cells grown on biopolymer scaffolds.
- FGF2 and GDF5 appear to activate different biological programs, because the two proteins do not cooperate and may even oppose each other.
- Treatment of mesenchymal stromal cells alone may suffice to promote expression of the fibroblastic phenotype that supports formation of ligamentous tissues.

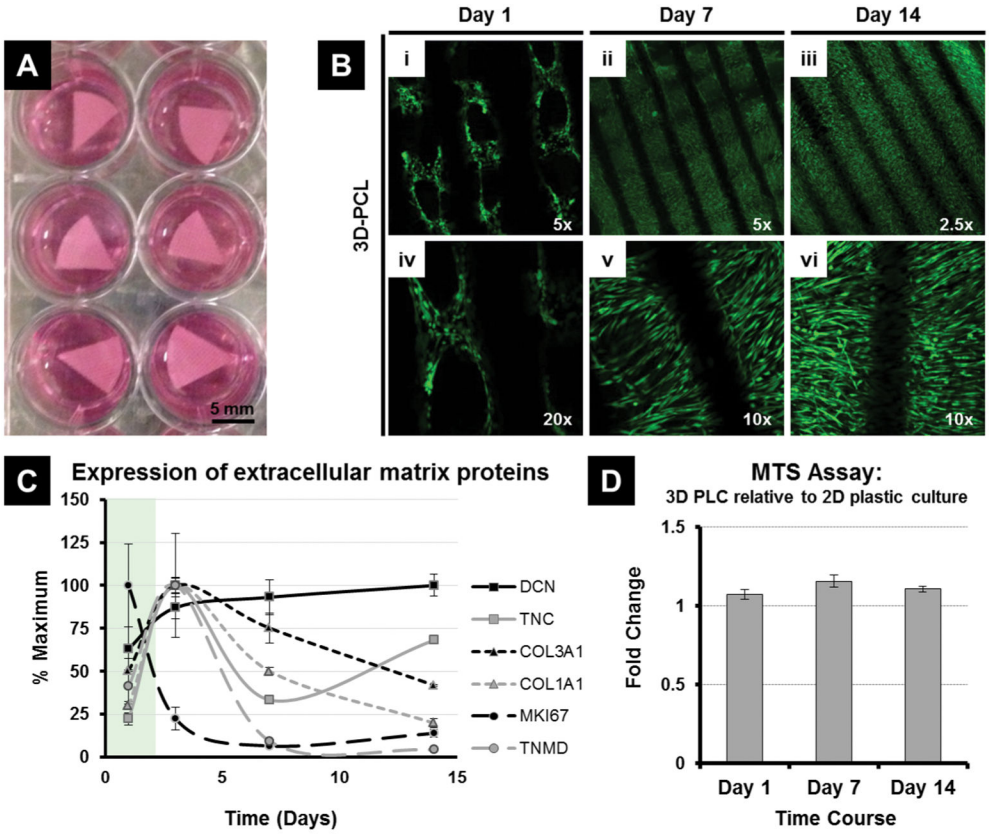

Figure 1.

Culturing Adipose Derived Mesenchymal Stem Cells (MSC) on 3D PCL Scaffolds. MSCs adhere, proliferate and are metabolically active on the scaffolds, with no indication of induced cytotoxicity. (A) PCL scaffolds seeded with MSCs and immersed in media containing 5% human platelet lysate. (B) Representative immunofluorescence microscopy images of MSCs cultured on 3D PCL scaffolds at day 1, day 7 and day 14 and stained using a cell viability assay (live cells shown in green, dead cells are stained red) to examine the survival, growth and morphology of the adherent cells. (C) Expression of key extracellular matrix proteins, as well as Decorin and the proliferation marker MKI67, in hPL cultured MSCs over 14 days. (D) Comparing the fold change in metabolic activity (via MTS assay) of MSCs cultured on 3D PCL scaffolds over 14 days relative to standard 2D plastic culture dishes. No significant difference ($p>0.05$) between treatment groups at each time point.

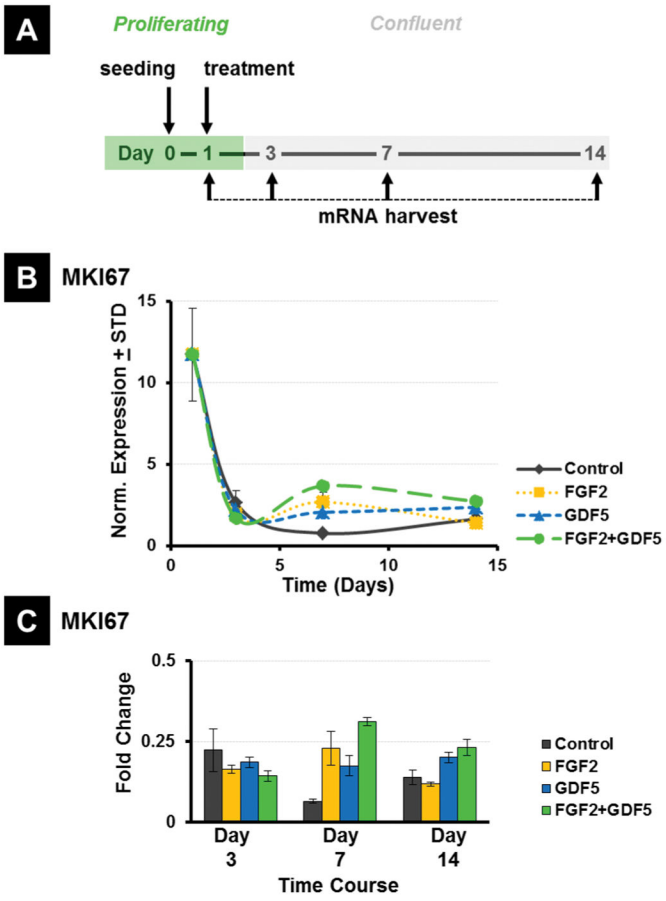

Figure 2.

(A) Experimental timeline for harvesting mRNA. (B) RT-qPCR analysis of expression of the proliferation marker MKI67, normalized to GAPDH and (C) fold change in all 4 treatment groups (Control = PL alone; FGF2, GDF5, FGF2 + GDF5) compared to untreated day 1 MSCs.

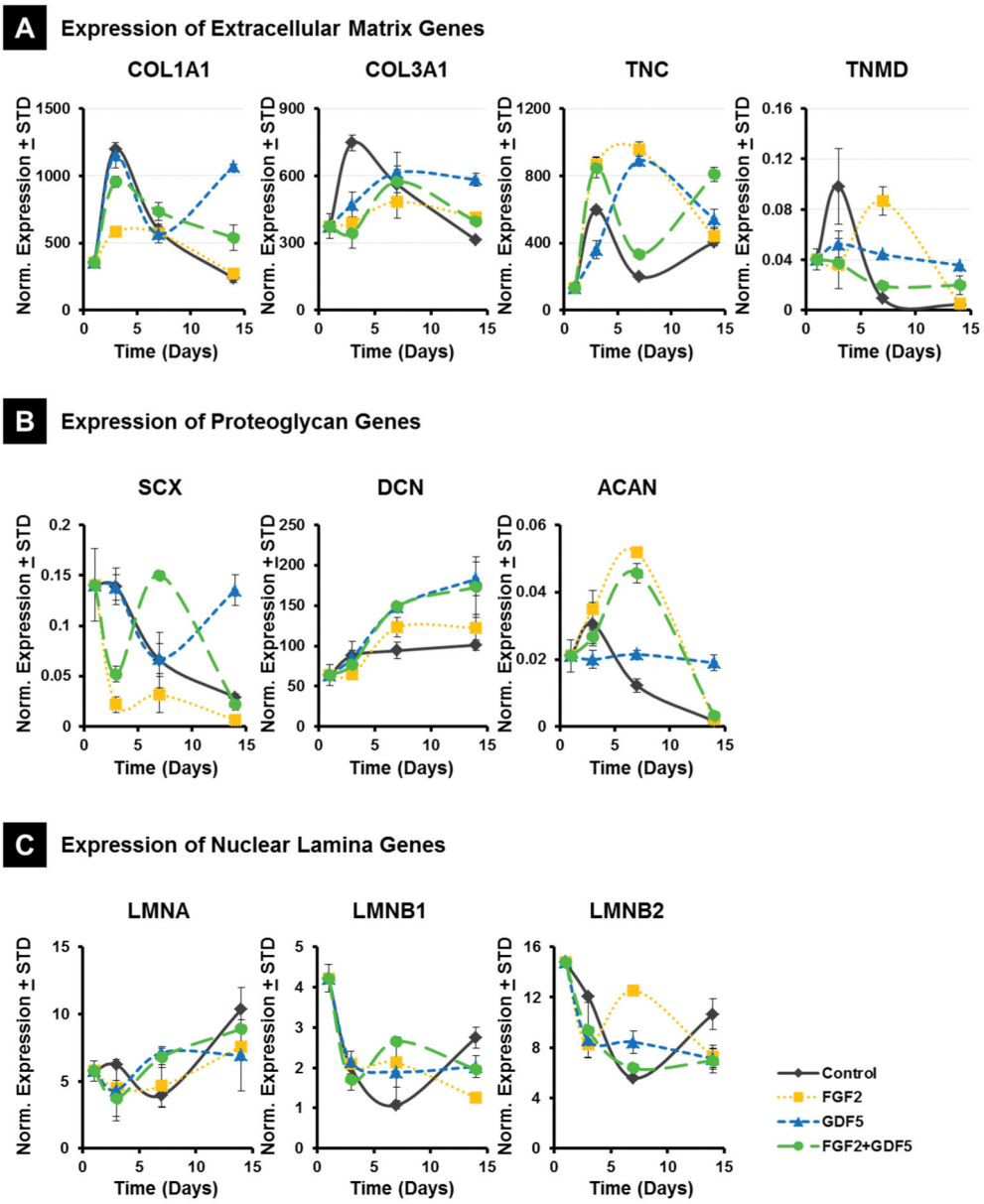

Figure 3.

Expression of tendon/ligament related extracellular matrix genes in MSCs by RT-qPCR. Cells belonging to 4 different treatment groups (Control = PL alone; FGF2, GDF5, FGF2 + GDF5) over 4 time points (Day 1, Day 3, Day 7 and Day 14) were plated and RNA isolated and processed as described in methods. X-axis indicates days of culture, with day 1 as actively dividing MSCs. Gene expression normalized to GAPDH, n=2. (A) Expression of Extracellular Matrix Genes. COL1A1, collagen type I; COL3A1, collagen type III; TNC, Tenascin-C; TNMD, Tenomodulin; (B) Expression of Proteoglycan Genes. Decorin, DCN; Aggrecan, ACAN; Scleraxis, SCX; (C) Expression of Nuclear Lamina Genes. Lamin A, LMNA; Lamin B1, LMNB1; Lamin B2, LMNB2.

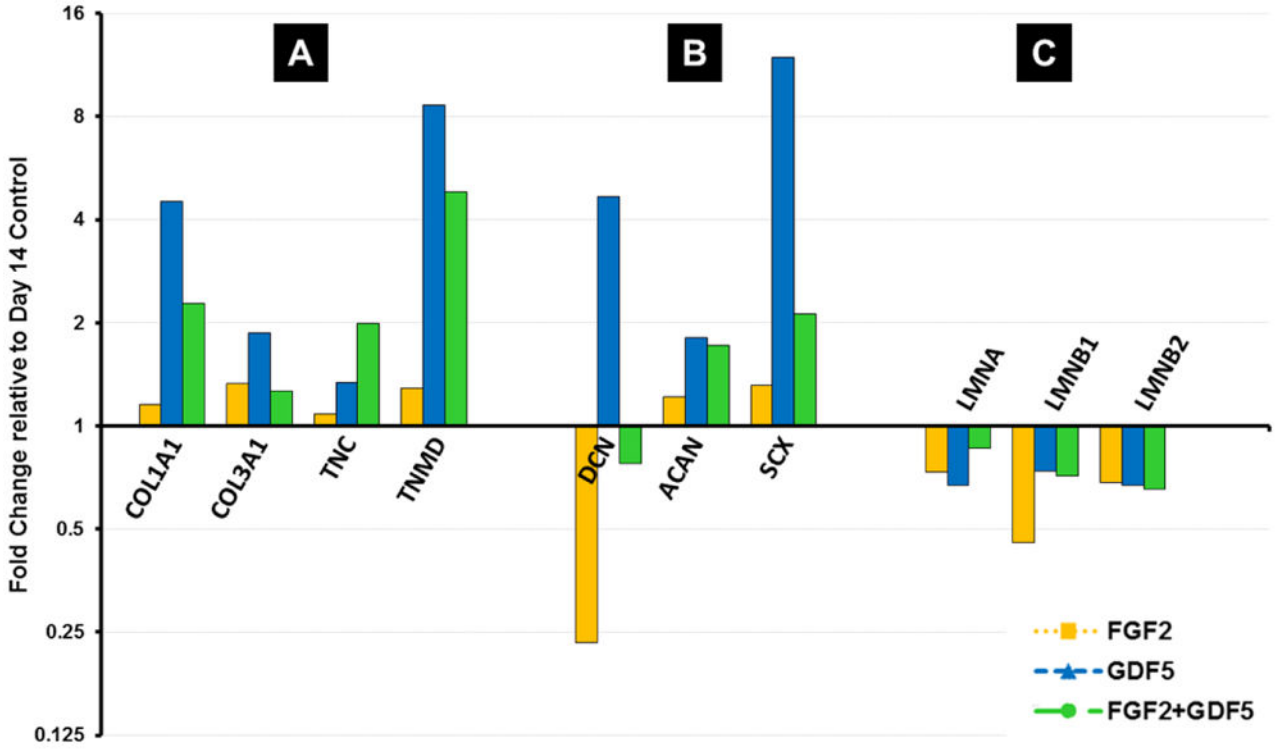

Figure 4.

Fold change values of Day 14 treatments relative to Day 14 controls. All values normalized to untreated MSC expression at day 1. (A) Expression of Extracellular Matrix Genes. COL1A1, collagen type I; COL3A1, collagen type III; TNC, Tenascin-C; TNMD, Tenomodulin; (B) Expression of Proteoglycan Genes. Decorin, DCN; Aggrecan, ACAN; Scleraxis, SCX; (C) Expression of Nuclear Lamina Genes. Lamin A, LMNA; Lamin B1, LMNB1; Lamin B2, LMNB2.

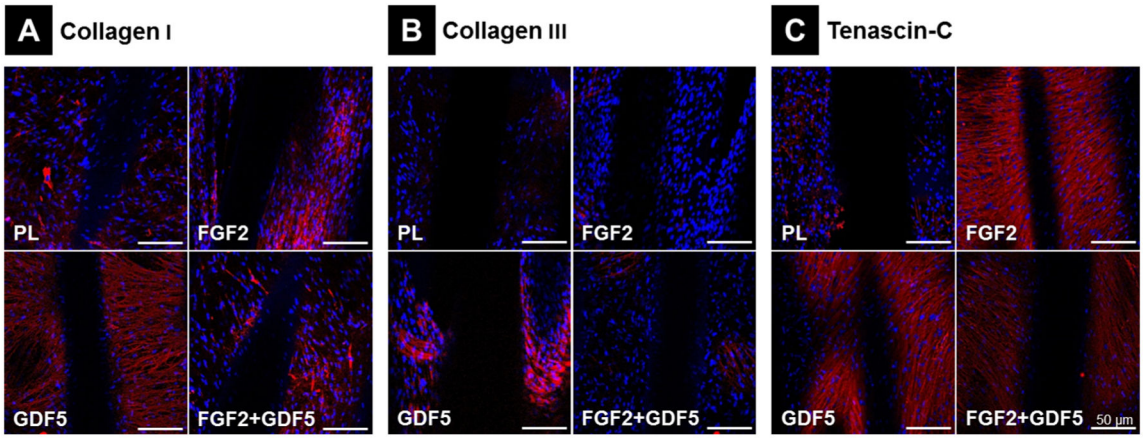

Figure 5.

Immunofluorescence Staining for ECM Cell Markers. Immunofluorescence staining (in red) of (A) Collagen 1, (B) Collagen III and (C) Tenascin-C to evaluate EMC protein accumulation in day 14 MSCs cultured on 3D PCL scaffolds according to 4 treatment groups. Blue = nuclear Dapi staining; scale bar = 50 μm.

Table 1

Forward and Reverse RT-qPCR Primer Sequences.

Primer		Forward primer sequences	Reverse primer sequences
Collagen 1A	**COL1A1**	5′-GCTACCCAACTTGCCTTCATG-3′	5′-TGCAGTGGTAGGTGATGTTCTGA-3′
Collagen 3A1	**COL3A1**	5′-TTGAAGGAGGATGTTCCCATCT-3′	5′-ACAGACACATATTTGGCATGGTT-3′
Tenascin-C	**TNC**	5′-AGGGCAAGTGCGTAAATGGAG-3′	5′-TGGGCAGATTTCACGGCTG-3′
Tenomodulin	**TNMD**	5′-CCATGCTGGATGAGAGAGGTT-3′	5′-TTGGTAGCAGTATGGATATGGGT-3′
Scleraxis	**SCX**	5′-AAACAGATCTGCACCTTCTGC-3′	5′-CGGTCCTTGCTCAACTTTCT-3′
Decorin	**DCN**	5′-ATGAAGGCCACTATCATCCTCC-3′	5′-GTCGCGGTCATCAGGAACTT-3′
Aggrecan	**ACAN**	5′-GTGCCTATCAGGACAAGGTCT-3′	5′-GATGCCTTTCACCACGACTTC-3′
MKI67	**MKI67**	5′-ACGCCTGGTTACTATCAAAAGG-3′	5′-CAGACCCATTTACTTGTGTTGGA-3′
Lamin A	**LMNA**	5′-AATGATCGCTTGGCGGTCTAC-3′	5′-CACCTCTTCAGACTCGGTGAT-3′
Lamin B1	**LMNB1**	5′-AAGCATGAAACGCGCTTGG-3′	5′-AGTTTGGCATGGTAAGTCTGC-3′
Lamin B2	**LMNB2**	5′-GTCCTGGATGAGACGGCTC-3′	5′-GCGCTCTTGTTGACCTCGT-3′