Cell-matrix interactions regulate mesenchymal stem cell response to hydrostatic pressure

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Abstract

Both hydrostatic pressure (HP) and cell-matrix interactions have independently been shown to regulate the chondrogenic differentiation of mesenchymal stem cells (MSCs). The objective of this study was to test the hypothesis that the response of MSCs to hydrostatic pressure will depend on the biomaterial within which the cells are encapsulated. Bone marrow derived MSCs were seeded into either agarose or fibrin hydrogels and exposed to 10 MPa of cyclic HP (1 Hz, 4 h/day, 5 days/week for 3 weeks) in the presence of either 1 or 10 ng/ml TGF-β3. Agarose hydrogels were found to support a spherical cellular morphology, while MSCs seeded into fibrin hydrogels attached and spread, with clear stress fiber formation. Hydrogel contraction was also observed in MSC-fibrin constructs. While agarose hydrogels better supported chondrogenesis of MSCs, HP only enhanced sulphated glycosaminoglycans (sGAG) accumulation in fibrin hydrogels, which correlated with a reduction in fibrin contraction. HP also reduced alkaline phosphatase activity in the media for both agarose and fibrin constructs, suggesting that this stimulus plays a role in the maintenance of the chondrogenic phenotype. This study demonstrates that a complex relationship exists between cell-matrix interactions and hydrostatic pressure which plays a key role in regulating the chondrogenic differentiation of MSCs.

Keywords: Fibrin, agarose, hydrostatic pressure, stem cell, chondrogenesis, endochondral

1. Introduction

Environmental cues, both biochemical and biophysical, regulate chondrogenesis of mesenchymal stem cells (MSCs). Various forms of mechanical loading, such as dynamic compression, tension, hydrostatic pressure and fluid flow have been shown to play a key role in determining the differentiation pathway of MSCs [1]. This has led to increased interest in the use of hydrostatic pressure (HP) in the field of cartilage tissue engineering [2]. HP has been shown to increase both chondrogenic gene expression (e.g. Sox9, aggrecan, collagen type II) and matrix production (proteoglycan, collagen) in MSCs maintained in pellets, collagen I sponges, and synthetic scaffolds [3–8]. Furthermore, HP has been shown to play a role in the maintenance of the chondrogenic phenotype for chondrocytes and joint tissue derived stem cells by suppressing the expression of hypertrophic markers [9,10]. In contrast, other studies have demonstrated that HP has little to no effect on chondrogenic gene expression and matrix accumulation [11,12]. Therefore, uncertainty exists in the literature as to the role HP plays in regulating chondrogenic differentiation of MSCs.

Cell-matrix interactions also play a key role in the chondrogenic differentiation of MSCs [13]. Arginine-glycine-aspartic acid (RGD) is an amino acid sequence that integrins are known to bind to, and it is commonly used to allow cells to adhere to scaffolds that do not have any binding sites. Previous studies have demonstrated that when MSCs are seeded in RGD-modified alginate hydrogels chondrogenic gene expression and matrix accumulation is inhibited relative to arginine-glycine-glutamic acid (RGE)-modified controls to which MSCs cannot adhere [14]. The inhibitory effect of RGD can be blocked with the addition of soluble RGD or cytochalasin D (an F-actin cytoskeleton inhibitor), demonstrating a role for cell attachment and actin cytoskeleton formation in suppressing chondrogenic differentiation [15]. While the interplay

between MSCs and the extracellular environment may inhibit chondrogenesis, such interactions and the development of a functional actin cytoskeleton may also be necessary for mechanotransduction to occur [16–18]. For example, an intact dynamic actin cytoskeleton under tension has been shown to be necessary for fluid flow-induced changes in Sox-9 gene expression in MSCs [19]. Furthermore, chondrocytes in pellet culture respond more favorably to HP relative to cells embedded in alginate hydrogels [20], suggesting that cellular interactions with the local environment also regulate stem cell response to HP. These studies would suggest that the response of MSCs to HP might depend on cell-matrix interactions unique to the scaffold or hydrogel within which they are embedded.

The objective of this study was to examine the interplay of cell-matrix interactions and hydrostatic pressure on chondrogenesis of MSCs. We specifically sought to compare the response of MSCs to HP following encapsulation in fibrin, where cells are known to directly adhere and spread within the hydrogel [21,22], to that in agarose, where cells do not adhere and hence maintain a spherical morphology when encapsulated within the hydrogel [23–25]. Our hypothesis was that cells seeded in fibrin hydrogels would be more mechanosensitive and hence demonstrate a more robust response to HP, while cells seeded in agarose hydrogels would show little to no response to HP.

2. Materials and Methods

2.1 Cell isolation, expansion, and encapsulation

Bone marrow was harvested from the femoral diaphysis of three 4-month-old pigs (~50 kg) under sterile conditions. MSCs were isolated and expanded according to a modified method developed for human MSCs [26]. Cultures were expanded in high-glucose Dulbecco's modified

Eagle's Medium (hgDMEM GlutaMAX) supplemented with 10% fetal bovine serum (FBS), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (all GIBCO, Biosciences). After expansion (third passage) MSCs were encapsulated in either agarose (Type VII, Sigma-Aldrich) or fibrin hydrogels at a density of 15 x 10^6 cells/ml. MSCs were mixed with agarose at ~40°C to yield a final gel concentration of 2%. The agarose-cell suspension was cast in a stainless steel mold, and cored using a biopsy punch to produce cylindrical scaffolds (Ø 5 x 3 mm thickness). Fibrinogen (Sigma-Aldrich, Ireland) was dissolved in aprotinin solution (Nordic Pharma, UK). Cells were mixed with this solution and thrombin added to crosslink the gel. The solution was immediately injected into an agarose mold to yield fibrin cylindrical hydrogels (50 mg/ml fibrinogen, 5000 KIU/ml aprotinin, and 2.5 U/ml thrombin final concentrations) with the same dimensions as the agarose hydrogels. Constructs were maintained in 5 ml/construct of a chemically defined chondrogenic media consisting of hgDMEM GlutaMAX supplemented with penicillin (100 U/ml)-streptomycin (100 μg/ml) (GIBCO, Biosciences, Ireland), 100 μg/ml sodium pyruvate, 40 μg/ml $_L$-proline, 50 μg/ml $_L$-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1 x insulin-transferrin-selenium, 100 nM dexamethasone (all Sigma-Aldrich, Ireland) and either 1 or 10 ng/ml recombinant human transforming growth factor-β3 (TGF-β3; ProSpec-Tany TechnoGene Ltd, Israel). Constructs were allowed to equilibrate overnight before the initiation of hydrostatic pressure.

2.2 Application of hydrostatic pressure

Constructs were sealed into sterile bags with 2 ml of medium per construct during the loading period, and then returned to culture dishes containing 5 ml of medium per construct. Cyclic HP was applied in a custom bioreactor filled with water within a 37°C incubator as

described previously [27]. The sealed bags exposed to HP were placed into the pressure vessel while the free swelling (FS) controls were placed into an open water bath next to the pressure vessel. HP was applied at an amplitude of 10 MPa at a frequency of 1 Hz, 4 h/day, 5 days/week for 3 weeks. Half-medium exchanges were performed every 3-4 days and media samples were collected for biochemical analysis.

2.3 Biochemical Analysis

Constructs' (n=4) wet weight and diameters were measured, and digested with papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM $_{L}$-cysteine-HCl, and 0.05 M EDTA (pH 6.0, all Sigma-Aldrich) at 60°C under constant rotation for 18 hours. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as described previously [28], with a calf thymus DNA standard. Sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue dye-binding assay (DMMB; Blyscan, Biocolor Ltd., Northern Ireland) with a chondroitin sulphate standard. Collagen content was determined by measuring the hydroxyproline content. Samples were hydrolyzed at 110°C for 18 hours in 38% HCl and assayed using a chloromine-T assay with a hydroxyproline:collagen ratio of 1:7.69 [29,30]. Media samples were also analyzed using the DMMB and hydroxyproline assays, and subsequently added to that accumulated within constructs to yield the total sGAG and collagen produced. Total sGAG and hydroxyproline values were normalized to DNA values; subsequently the HP groups were normalized to the FS groups. Media samples were analyzed for alkaline phosphatase (ALP) activity using a *p*NPP ALP colorimetric assay (Cambridge Bioscience, Ltd., United Kingdom).

2.4 Confocal microscopy, histology, and immunohistochemistry

At day 21, constructs (n=2) were cut in half and fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C and rinsed with PBS. In order to examine cellular morphology and the F-actin cytoskeleton, samples were permeabilized in a 0.5% Triton-X solution, washed in PBS, incubated in a solution containing 1.5% BSA and 5 U/ml rhodamine 110-phalloidin (VWR International, Ireland) for 1 hour, and then imaged using a Zeiss 510 Meta confocal microscope at 20x magnification.

The remaining halves were dehydrated and embedded in paraffin wax. Constructs were sectioned perpendicular to the disc face yielding 5 μm thick sections. Sections were stained with either 1% alcian blue 8GX (Sigma-Aldrich, Ireland) in 0.1 M HCl for sGAG, picro-sirius red for collagen, or Fast Red TR Salt 1,5-naphthalenedisulfonate (Sigma-Aldrich, Ireland) for ALP enzyme activity. Collagen types I, II, and X were further identified through immunohistochemistry. Sections were treated with peroxidase, followed by chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37°C for one hour to permeabilize the extracellular matrix. Samples were then blocked with goat serum, and afterwards the primary antibodies for collagen types I, II, and X (mouse monoclonal, Abcam) were applied for one hour. Next, the secondary antibody (Anti-Mouse IgG biotin conjugate, Sigma-Aldrich) was added for one hour followed by incubation with ABC reagent (Vectastain PK-4000, Vector Labs) for 45 minutes. Finally the slides were developed with DAB peroxidase (Vector Labs) for 4 minutes. Samples were washed with PBS between each step, and negative and positive controls of porcine ligament (positive for type I collagen, negative for type II collagen) and cartilage (positive for type II collagen, negative for type I collagen) were also assessed.

2.5 Experimental Design

Porcine bone marrow from one donor was cultured, exposed to HP, and analyzed. In order to examine the effects of possible donor to donor variability, the experiment was independently repeated two more times with bone marrow derived MSCs from two additional donors. Data presented in figures 1-5 is from the first donor. Figure 6 is a compilation comparing key data for all three donors. Figure 7 compares ALP activity in the media from donors 1 and 3 (insufficient media prevented such an analysis for donor 2).

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.00, GraphPad Software) with 3-4 samples analyzed for each experimental group for every donor. Biochemical results, both numerical and graphical, are expressed in the form of mean ± standard deviation. Differences between HP and FS samples or between agarose and fibrin were determined using a Student's t-test. A level of $p < 0.05$ was considered significant.

3. Results

3.1 Cellular morphology, contraction, and proliferation

HP had no effect on cellular morphology, proliferation, construct contraction, sGAG or collagen production when MSCs were cultured in 10 ng/ml TGF-β3, and therefore unless otherwise stated the data shown is for MSCs cultured in the presence of 1 ng/ml TGF-β3. MSCs encapsulated in agarose hydrogels retained a spherical morphology, while MSCs in fibrin hydrogels attached and spread (Fig. 1). Actin stress fiber formation was clearly evident within MSCs embedded in fibrin hydrogels. While a few attachments did form in the agarose by day

21, this is due to the MSCs interacting with their own pericellular matrix (PCM) rather than any direct attachment between the MSCs and agarose, as these attachments were not observed if TGF- β3 was absent from the culture media (data not shown).

There was no contraction observed in any of the agarose hydrogels. HP was observed to suppress contraction of fibrin constructs (FS: 4.67±0.06 mm, HP: 4.83±0.06 mm). HP did not influence the DNA content in either agarose or fibrin hydrogels after 21 days in culture.

3.2 Extracellular matrix synthesis in fibrin and agarose hydrogels

The sum of the extracellular matrix retained within the scaffold and released into the media was used to determine the total amount of sGAG and collagen synthesized. MSCs encapsulated in agarose synthesized significantly higher amounts of sGAG (normalized to DNA) than those seeded in fibrin (fibrin: 5.23±1.03; agarose: 23.20±3.00); however, MSCs in fibrin synthesized significantly more collagen (normalized to DNA) (agarose: 81.55±11.92; fibrin: 109.02±7.03) (Fig. 2). The application of HP significantly increased sGAG synthesis in fibrin hydrogels (1.4 fold increase; $p<0.05$), however it had no effect in agarose constructs (Fig. 3). HP did not significantly influence collagen synthesis in either fibrin or agarose (Fig. 3).

Alcian blue and picro sirius red were used to qualitatively assess sGAG and collagen production and distribution within the hydrogels (Fig. 4). MSC seeded agarose constructs stained more intensely for Alcian Blue than MSC seeded fibrin constructs, particularly in the pericellular region. MSC seeded fibrin hydrogels stained more intensely and diffusively for picro sirius red, with staining in the agarose hydrogels again more intense in the pericellular region. In order to determine the specific types of collagen being produced, immunohistochemistry was performed for collagen types I and II (Fig. 5). There was strong

pericellular staining for both collagen types in the agarose constructs. Interestingly, HP appeared to decrease collagen type I production by MSCs seeded in fibrin hydrogels, while maintaining at least comparable levels of collagen II production. HP had little to no effect on the staining intensity for either type I or II collagen within the agarose hydrogels.

3.3 Donor variability in response to HP

In order to further examine the effects of HP on MSCs, this experiment was repeated using MSCs isolated from two additional donors. No significant response to loading was observed for one of the three donors (Fig. 6). Significantly less fibrin contraction was observed for donors 1 and 2 when exposed to HP, but donor 3 showed no difference due to loading. HP also significantly inhibited proliferation in donor 2. The two donors (1 and 2) in which HP inhibited fibrin contraction also demonstrated significant increases in sGAG synthesis in response to loading. Only donor 2 demonstrated a significant increase in collagen production due to HP.

3.4 Markers of Endochondral Ossification

In order to assess if HP suppressed markers of hypertrophy, ALP activity in the media was first analyzed. MSCs seeded in agarose demonstrated higher levels of ALP activity than in fibrin for constructs maintained at either 1 or 10 ng/ml TGF-β3 (data for 10ng/ml provided in Fig. 7A where activity is higher). ALP activity increased with time in agarose constructs, peaking towards the end of the 21 day culture period, while ALP activity peaked at earlier time points in the fibrin hydrogels. Also, when maintained in 10 ng/ml TGF-β3, HP acted to suppress ALP activity in both fibrin and agarose hydrogels (Fig. 7B). Evidence for reduced ALP enzyme

activity was also observed in histological sections stained for naphthol phosphate with fast red (Fig. 7C). In spite of this, there was no noticeable difference in collagen type X staining with the application of HP in either hydrogel (data not shown).

4. Discussion

Hydrostatic pressure is a key regulator of stem cell fate and has important applications in the field in cartilage tissue engineering [2]. The hypothesis under investigation in this study was that the response of MSCs to HP would depend on the hydrogel into which they were embedded. To test this hypothesis, MSCs were encapsulated into hydrogels that either prevent (agarose) or promote (fibrin) cell attachment and spreading within the construct. Since it is heavily involved with blood clots, MSC seeded fibrin hydrogels subjected to physiological levels of loading can be considered as an *in vitro* model of the spontaneous healing that occurs within osteochondral defects. Confocal microscopy revealed that MSCs embedded in fibrin hydrogels attained a spread morphology with distinct stress fiber formation, while MSCs in agarose hydrogels retained a spherical morphology. When subjected to TGF-β3 stimulation, bone marrow derived MSCs embedded in agarose hydrogels underwent more robust chondrogenesis than those in fibrin hydrogels. In spite of this, it was observed that HP only enhanced chondrogenesis of MSCs embedded in fibrin hydrogels, as evident by decreased staining for type I collagen, increased sGAG synthesis and a decrease in ALP activity. Together these results suggest that the interaction of MSCs with their local substrate regulates their response to HP.

In the current study, HP increased sGAG synthesis when MSCs were cultured in the presence of 1 ng/ml TGF-β3 but not 10 ng/ml TGF-β3. It has previously been demonstrated that MSCs exhibit a stronger chondrogenic response to mechanical cues at lower concentrations of

TGF-β [6,31]. It has been proposed that mechanical loading and TGF-β act via similar pathways, such that high concentrations of TGF-β mask the effects of mechanical loading [31]. Further work is required to help elucidate the molecular mechanisms by which HP enhances chondrogenesis of MSCs.

Cell-matrix interactions alone are known to play a key role in determining stem cell fate [13]. Fibrin better supported MSC proliferation and collagen production relative to those in agarose; however MSCs in agarose produced significantly more sGAG. The spherical cell morphology supported by agarose may help to explain this result [25]. Also, unlike agarose, fibrin facilitates integrin mediated cellular attachment through two pairs of RGD sites [32]. In a study comparing bone marrow MSCs seeded in hydrogels conjugated with RGE (no cell attachment) or RGD (cell attachment) peptides, chondrogenic gene expression and protein synthesis were significantly decreased in the RGD modified gel [14,15]. In addition, there was a significant increase in collagen I in the presence of serum [15]. This agrees with the finding of this study where cell attachment in fibrin, and subsequent cell spreading, correlates with an increase in collagen type I synthesis and a decrease in the chondrogenic phenotype of bone marrow MSCs.

While integrin mediated cell adhesion influences chondrogenesis of MSCs, it also plays a key role in mechanotransduction. As previously discussed, fibrin facilitates integrin mediated cellular attachment through two pairs of RGD sites [32]. Integrins bind both with the extracellular matrix as well as the cytoskeleton, and they help transmit mechanical signals "outside-in" and "inside-out." Therefore integrin mediated cell attachment is thought to be a prerequisite in order for a cell to respond to mechanical forces [16–18]. Previous studies where HP has been shown to enhance chondrogenesis of bone marrow derived MSCs have generally

used a culture system in which either cell-cell or cell-matrix attachments are facilitated [3–8], which may also explain the pro-chondrogenic response to HP observed in fibrin hydrogels in this study. Conversely, HP had little to no effect on cartilage specific matrix synthesis for MSCs seeded in agarose, which agrees with the findings of a previous study [12]. This supports our contention that formation of integrin mediated adhesions and subsequent focal adhesion assembly is necessary in order for HP to be sensed by the MSCs. Interestingly, previous studies in our lab have shown that the long term application of HP (6 weeks) can enhance chondrogenesis of MSCs embedded in agarose hydrogels [27], which could potentially be explained by cells attaching to their own PCM, which in turn facilitates integrin mediated mechanotransduction of HP. An alternative (although related) explanation for the substrate dependent response to mechanical loading is that HP is interfering with integrin mediated attachment of MSCs to the local extracellular matrix, which, as has already been discussed, can inhibit chondrogenesis of MSCs [33]. This hypothesis is supported by the finding that HP also suppressed MSC mediated contraction of fibrin hydrogels, which presumably is at least partially driven by integrin-mediated attachment of MSCs to their local extracellular environment.

Some donor variability in the response of MSCs to HP was observed. HP mediated suppression of fibrin contraction was greatest for donor 2, and this donor also demonstrated the largest differences in DNA, sGAG synthesis, and total collagen synthesis due to the application of HP. HP also decreased contraction for donor 1 and led to an increase in sGAG synthesis. Conversely, HP had no effect on any of the properties investigated for donor 3. This variable response could be due to batch to batch variability in fibrin properties, as well as differences between each donor. Previous studies have also shown a donor dependent response to HP when seeded in agarose hydrogels, providing evidence that there is inherent differences in the response

of MSCs from various donors to loading [27]. Further experimentation is needed to better elucidate the extent of the donor dependent response to HP.

Hypertrophy and mineralization of cartilaginous grafts engineered using bone marrow derived MSCs has been observed in an ectopic environment [34], which lacks the mechanical signals present within synovial joints. Hydrostatic pressure, a key component of the *in vivo* joint environment, has previously been shown to suppress markers of hypertrophy and to help maintain the chondrogenic phenotype of synovial membrane derived MSCs [10]. While HP did not influence the initiation of chondrogenesis in agarose, as evident by the finding that the mechanical stimulus did not influence ECM accumulation, it did appear to suppress ALP activity, a marker of endochondral ossification. Alkaline phosphatase expression by bone marrow derived MSCs *in vitro* has been shown to correlate with endochondral ossification *in vivo* [35,36]. With increasing time in culture, MSCs embedded in agarose begin to produce their own PCM (Fig. 1, Fig. 4), which may be the mechanism by which this stimulus is transduced. Therefore, the decrease in ALP activity when exposed to HP provides further support for the hypothesis that this stimulus may play a role in inhibiting hypertrophy and terminal differentiation of chondrogenically primed MSCs. Understanding how joint specific factors, such as mechanical cues and the local oxygen tension, regulate the endochondral phenotype is a key challenge for the successful translation of stem cell based therapies for articular cartilage repair [37].

5. Conclusion

In conclusion, we have demonstrated that hydrostatic pressure can enhance chondrogenesis of bone marrow derived MSCs in a biomaterial substrate dependant manner.

Furthermore, not only does HP have an effect on chondrogenic induction of MSCs, it would also appear to play a key role in the maintenance of a chondrogenic phenotype. Further understanding of the interplay between cell-matrix interactions and the extrinsic mechanical environment regulating chondrogenesis of MSCs is needed in order to improve stem cell based cartilage regeneration therapies.

6. Acknowledgements

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Figure Legends

Fig. 1. Representative confocal microscopy images of MSCs from Donor 1 seeded in either agarose (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.

Fig. 2. (A) Total sGAG/DNA of FS constructs. (B) Total collagen/DNA of FS constructs. Data is for Donor 1 (n=4). *p<0.05 indicates a significant difference between agarose and fibrin groups.

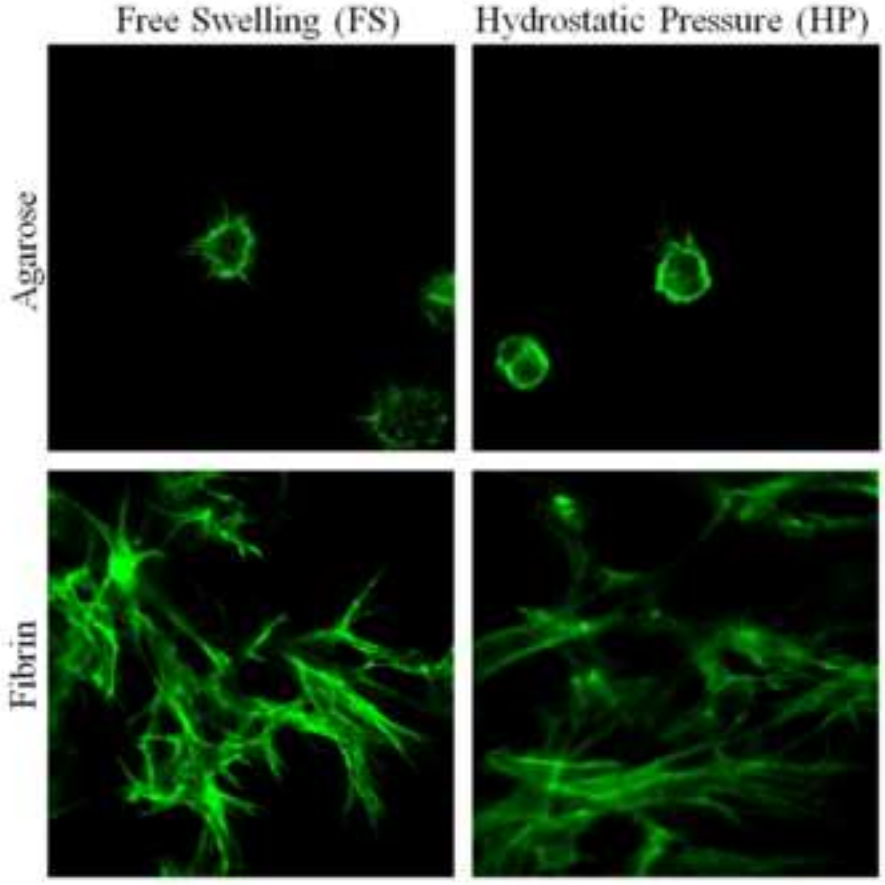
Fig. 3. (A) Total sGAG/DNA normalized to FS condition. (B) Total collagen/DNA normalized to FS condition. Data is for Donor 1 (n=4). *p<0.05 indicates a significant difference between FS and HP groups.

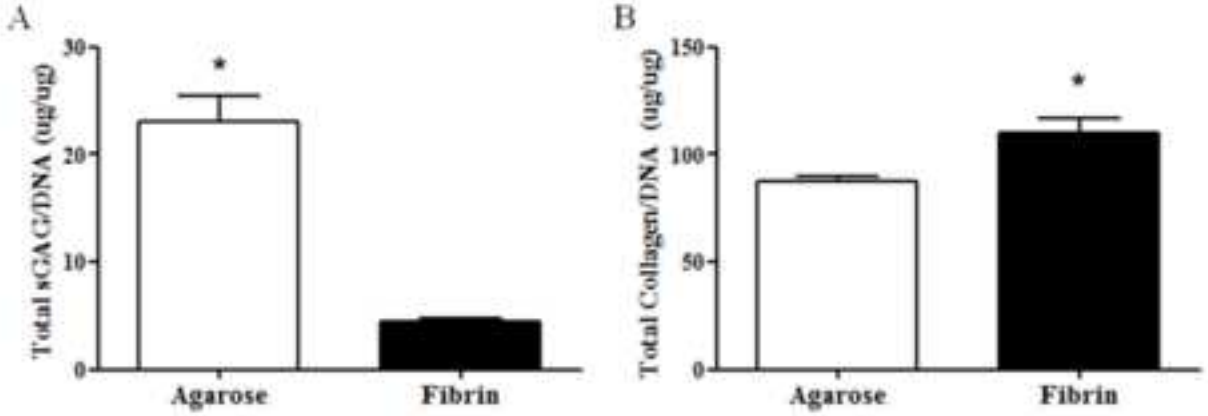
Fig. 4. Representative alcian blue and picro sirius red images of MSCs from Donor 1 seeded in either agarose (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.

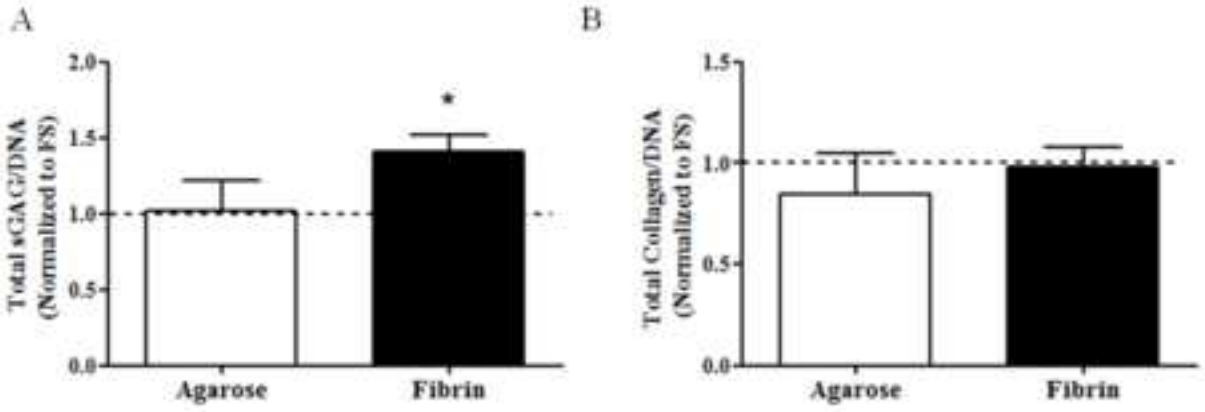
Fig. 5. Representative collagen types I and II images of MSCs from Donor 1 seeded in either agarose (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.

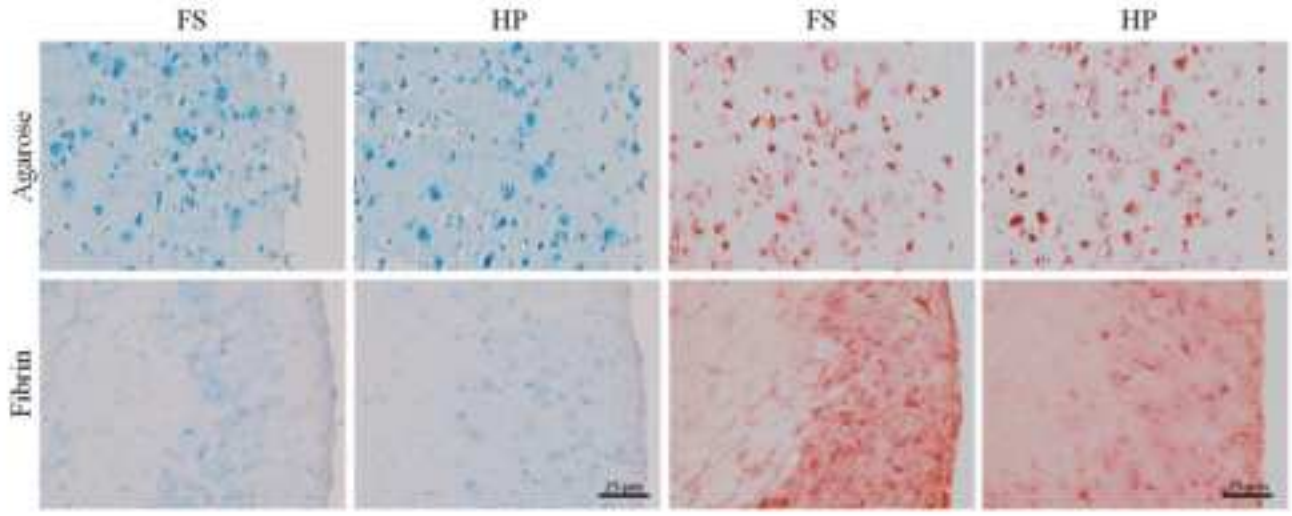
Fig. 6. (A). Diameters, (B) DNA content, (C) total sGAG produced normalized to DNA content, and (D) total collagen produced normalized to DNA content for fibrin hydrogels. Data shown for all three donors and HP groups were normalized to FS groups. *p<0.05 indicates a significant difference between FS and HP groups.

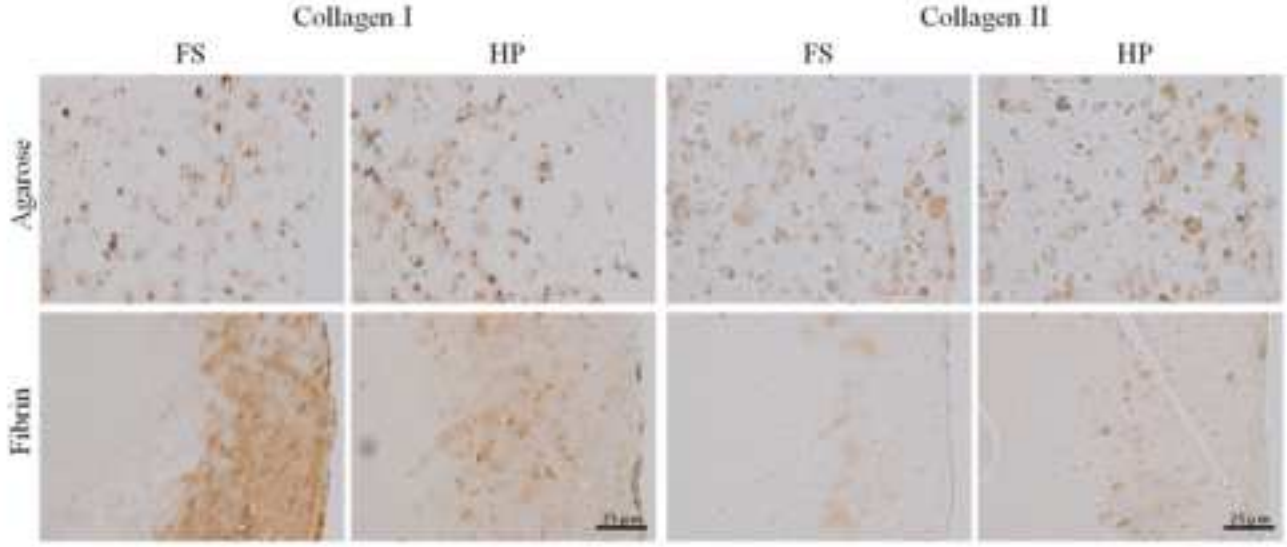
Fig. 7. (A) Quantity of ALP activity (normalized to DNA) released to the media over the three week culture period for Donors 1 and 3 in both agarose and fibrin hydrogels in 10 ng/ml TGF-β3. (B) Quantity of ALP activity (normalized to DNA) for HP groups normalized to FS groups for two replicates cultured in 1 and 10 ng/ml TGF-β3. For both Donor 1 and 3, the media was analyzed in triplicate for these assays. However, as constructs were pooled during loading, it can be argued that these triplicates are not independent and hence a statistical analysis was not performed. Insufficient media was available from donor 2 to facilitate undertaking this specific assay. (C) Representative sections stained for ALP enzyme activity for MSCs from Donor 3 seeded in either agarose (left) or fibrin (right) hydrogels at day 21 for both FS and HP groups supplemented with 10ng/ml of TGF-β3. No noticeable differences were observed for constructs supplemented with 1 ng/ml of TGF-β3.

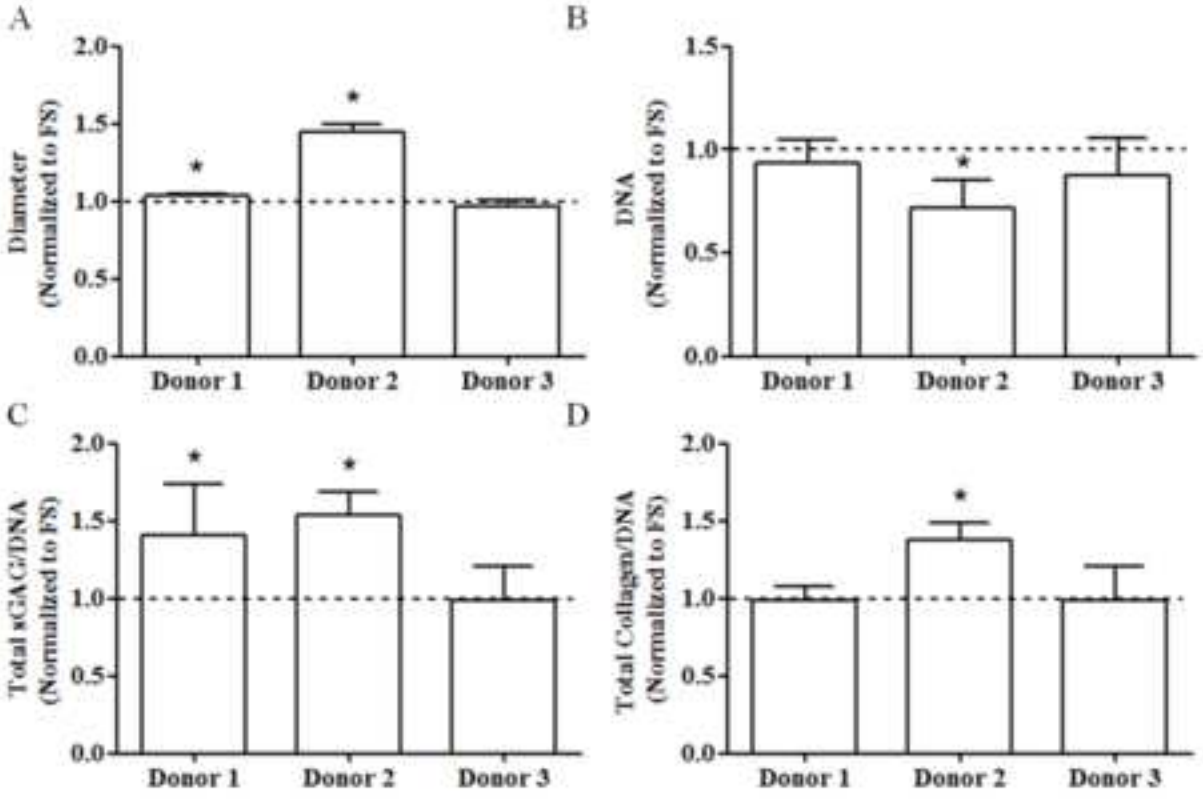

Free Swelling (FS)
Hydrostatic Pressure (HP)
Agarose
Fibrin

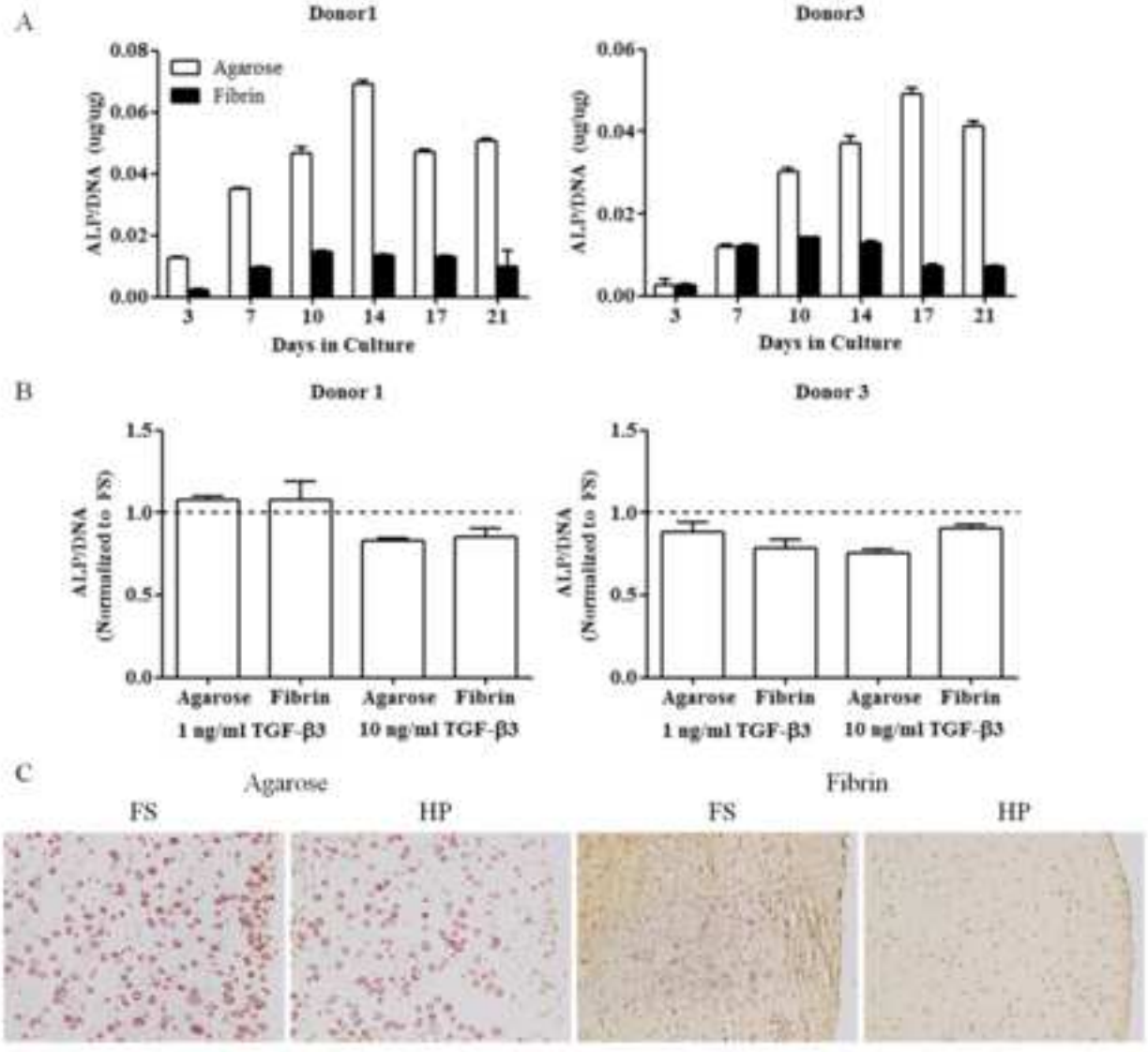

A
Total sGAG/DNA (ug/ug)
30
20
10
0
*
Agarose
Fibrin
B
Total Collagen/DNA (ug/ug)
150
100
50
0
*
Agarose
Fibrin


A
Total sGAG/DNA (Normalized to FS)
2.0
1.5
1.0
0.5
0.0
*
Agarose
Fibrin
B
Total Collagen/DNA (Normalized to FS)
1.5
1.0
0.5
0.0
Agarose
Fibrin


FS
HP
FS
HP
Agarose
Fibrin


Collagen I
Collagen II
FS
HP
FS
HP
Agarose
Fibrin


A
Diameter (Normalized to FS)
2.0
1.5
1.0
0.5
0.0
*
*
Donor 1
Donor 2
Donor 3
B
DNA (Normalized to FS)
1.5
1.0
0.5
0.0
*
Donor 1
Donor 2
Donor 3
C
Total sGAG/DNA (Normalized to FS)
2.0
1.5
1.0
0.5
0.0
*
*
Donor 1
Donor 2
Donor 3
D
Total Collagen/DNA (Normalized to FS)
2.0
1.5
1.0
0.5
0.0
*
Donor 1
Donor 2
Donor 3


A
Donor1
Donor3
Agarose
Fibrin
ALP/DNA (ug/ug)
Days in Culture
B
Donor 1
Donor 3
ALP/DNA (Normalized to FS)
Agarose
Fibrin
1 ng/ml TGF-β3
10 ng/ml TGF-β3
C
Agarose
Fibrin
FS
HP