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The role of environmental factors in regulating the development of cartilaginous grafts engineered using osteoarthritic human infrapatellar fat pad derived stem cells

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The role of environmental factors in regulating the development of cartilaginous grafts engineered using osteoarthritic human infrapatellar fat pad derived stem cells

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Abstract

Engineering functional cartilaginous grafts using stem cells isolated from osteoarthritic human tissue is of fundamental importance if autologous tissue engineering strategies are to be used in the treatment of diseased articular cartilage. It has previously been demonstrated that human infrapatellar fat pad (IFP) derived stem cells undergo chondrogenesis in pellet culture; however the ability of such cells to generate functional cartilaginous grafts has not been adequately addressed. The objective of this study was to explore how environmental conditions regulate the functional development of cartilaginous constructs engineered using diseased human IFP derived stem cells (FPSCs). FPSCs were observed to display a diminished chondrogenic potential upon encapsulation in a three dimensional hydrogel compared to pellet culture, synthesising significantly lower levels of glycosaminoglycan and collagen on a per cell basis. In order to engineer more functional cartilaginous grafts, we next explored if additional biochemical and biophysical stimulation would enhance chondrogenesis within the hydrogels. Serum stimulation was observed to partially recover the diminished chondrogenic potential within hydrogel culture. Over 42 days, stem cells that had first been expanded in a low oxygen environment proliferated extensively on the outer surface of the hydrogel in response to serum stimulation, assembling a dense type II collagen positive cartilaginous tissue resembling that formed in pellet culture. The application of hydrostatic pressure did not further enhance extracellular matrix synthesis within the hydrogels, but did appear to alter the spatial accumulation of extracellular matrix leading to the formation of a more compact tissue with superior mechanically functionality. Further work is required in order to recapitulate the environmental conditions present during pellet culture within scaffolds or hydrogels in order to engineer more functional cartilaginous grafts using human osteoarthritic FPSCs.

Introduction

Critically sized defects to the articular surface of synovial joints generally do not heal and if left untreated lead to the development of osteoarthritis.[1] This has led to increased interest in cell based therapies for articular cartilage regeneration. Autologous chondrocyte implantation (ACI) has been used with reasonable success to treat focal lesions in the knee joint,[2, 3] however it is still unclear if ACI is superior to other treatment strategies.[4-6] Furthermore, the ACI technique suffers from a number of technical disadvantages, including the need to biopsy regions of the undamaged joint surface to isolate chondrocytes and the de-differentiation of these chondrocytes during monolayer expansion.[7-9] This has led to increased interest in the use of mesenchymal stem cells (MSCs) for the treatment of damaged and diseased articular cartilage.[10] MSCs have been isolated from multiple different tissues, including bone marrow,[11-13] adipose tissue,[14-17] synovium[18-21] and infrapatellar fat pad[22-26] among others. These cells have been incorporated into different types of scaffolds[27, 28] and hydrogels[29-38] in attempts to tissue engineer cartilaginous grafts, with different combinations of growth factors[39, 40] and biophysical cues such as compression[41] and hydrostatic pressure[42] used to improve the functionality of the construct.

MSCs are beginning to be used clinically in the treatment of focal cartilage defects.[43, 44] While the use of MSCs has been shown to improve repair compared to untreated controls, critically normal hyaline cartilage does not regenerate, rather a hyaline-like or fibro-cartilagenous tissue forms.[45-47] Chondrogenic pre-differentiation of MSCs within collagen gels, leading to improved functionality of the engineered graft prior to implantation, has been shown to improve the outcome in long-term sheep studies,[48] resulting in superior repair compared to undifferentiated MSCs or chondrocytes.[49] While promising, the quality of repair

observed in these animal models studies using pre-differentiated MSCs is still variable,[48, 49] suggesting that engineering a more functional graft may be required prior to implantation. Furthermore, it remains to be elucidated if such pre-differentiated cartilaginous grafts can be engineered using stem cells isolated from diseased human donors. If autologous MSCs are to be ultimately used for regeneration of damaged or diseased articular cartilage in osteoarthritic joints, it will be necessary to first identify the optimal culture conditions that lead to the development of functional cartilaginous tissues using such cell sources.

Human infrapatellar fat pad (IFP) contains multipotent stem cells (FPSCs) which can be easily harvested arthroscopically,[23] and which possess at least comparable chondrogenic capacity to chondrocytes isolated from adult articular cartilage.[24] We have previously demonstrated that functional cartilage tissue can be engineered using immature porcine FPSCs,[32-34] however it remains unclear if such grafts can be engineered using stem cells isolated from human osteoarthritic IFP tissue. The infrapatellar fat pad of patients with osteoarthritis has been shown to have an inflammatory phenotype,[50] which may impact the ability of stem cells isolated from this tissue to generate a functional cartilaginous tissue. The objective of this study was to firstly compare chondrogenesis of FPSCs in pellet culture to that in agarose hydrogels which are commonly used to engineer functional cartilaginous grafts. The influence of the oxygen tension during monolayer expansion of FPSCs on the subsequent properties of these cartilaginous grafts was assessed. We then explored how biochemical (serum) and biophysical (hydrostatic pressure) cues would influence the functional development of cartilage tissues engineered using human FPSCs.

Materials and Methods

Cell isolation and expansion

Ethical approval for the study was obtained from the institutional review board of the Mater Misericordiae University Hospital with infrapatellar fat pad tissue being obtained from 5 patients with knee osteoarthritis (OA) at joint arthroplasty (1 Male, 4 Females, 50-79 Years old). Fat pad was maintained in sterile phosphate buffer solution (PBS) and transferred immediately to Trinity Centre For Bioengineering for further processing. Fibrous tissue was carefully removed from the fat pad. Remaining tissue was weighed, washed thoroughly in PBS and diced followed by incubation under constant rotation at 37°C with high-glucose Dulbecco's Modified Eagle Medium (hgDMEM, GlutaMAX™) (GIBCO, Biosciences, Ireland) containing 1% penicillin (100 U/ml)-streptomycin (100 µg/ml) and collagenase type II (4 ml solution/g tissue, 750 U/ml, Worthington Biochemical, LanganBach Services, Ireland) for 4 hours. Cells were filtered through serial cell sieves (Falcon, Sarstedt, Ireland) with pore size from 100 µm, 70 µm to 40 µm. The isolated FPSCs were seeded at the density of 5000 cells/cm^2 and cultured in expansion medium [hgDMEM GlutaMax supplemented with 10% v/v fetal bovine serum (FBS), penicillin (100 U/ml)-streptomycin (100 µg/ml) (all from Gibco, Biosciences, Ireland)]. The expansion medium was also supplemented with 5 ng/ml fibroblast growth factor-2 (FGF-2) (Prospect-Tany TechnoGene Ltd., Israel) which has previously been shown to enhance proliferation and chondrogenesis of porcine FPSCs.[51] Cells were expanded to passage two (P2) and the oxygen level during cell expansion was maintained at either 20% or 5% O_2. All cultures in this study were kept at 37°C. For the hydrostatic pressure study (described below), a superlot of FPSCs pooled from 3 different donors was used. For all other studies, FPSCs isolated from individual donors was used, with

replicate studies undertaken using FPSCs isolated from separate donors undertaken as described in the results section.

Colony-forming unit-fibroblast (CFU-F) assay

For the CFU-F assay freshly isolated cells were plated in 58 cm^2 petri dishes at a density of 135 cells/cm^2 growth with or without FGF-2 at either 20 % O_2 or 5 % O_2. Triplicate dishes were plated for all conditions. After 12-14 days, cells were fixed with 2% paraformaldehyde (PFA), stained with 1 % crystal violet (Sigma-Aldrich, Arklow, Ireland) and colony numbers (> 50 cells) counted. Total colony number and colony diameter was determined using ImageJ software (Rasband, W.S., Image J, U.S National Institutes of Health, Bethesda, Maryland, USA). For the purposes of comparison between groups, colony size was defined as the average diameter of the 10 largest colonies formed.

Chondrogenesis in pellet culture

Pellets were formed from culture-expanded cells by centrifuging 250,000 cells (P2) in 1.5 ml conical microtubes at 650 g for 5 min. The pellets were maintained in chondrogenic medium (CM): hgDMEM GlutaMax supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml) (Invitrogen, Paisley, UK), 100 ug/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 4.7 µg/ml linoleic acid, 1.5 mg/ml bovine serum albumin, 1x insulin-transferrin-selenium, 100 nM dexamethasone (all from Sigma-Aldrich). The chondrogenic medium were additionally supplemented with 10 ng/ml recombinant human transforming growth factor-3 (TGF-beta3; Prospect-Tany TechnoGene Ltd., Israel) or 10 ng/ml TGF-beta3 + 10 % FBS (Lot no. 10270, Gibco). Each pellet was cultured in 1 ml

medium and the medium was changed twice a week. The oxygen level for pellet culture was maintained at either 20 % O_2 or 5 % O_2. All pellets were cultured for 3 weeks with pellets assessed at days 0 and 21.

Chondrogenesis in agarose hydrogels

FPSCs (P2) were suspended in 2% agarose hydrogels (type VII; Sigma-Aldrich). All hydrogels were seeded at a density of $10x10^6$ cells/ml, except for the agarose gels subjected to hydrostatic pressure (both free swelling controls and loaded constructs), which were seeded at a higher density of $20x10^6$ cells/ml in an attempt to improve the overall functionality of the construct. The agarose cell suspension was cast in a stainless steel mould and cored using a biopsy punch to produce construct cylinders (Ø5mm x 1.5mm). Constructs were maintained in chondrogenic medium at 5 % O_2 for a period of 42 days with medium exchanges performed twice weekly. Gel samples were assessed at day 0, 21 and 42.

Hydrostatic pressure loading

Gel constructs were subjected to hydrostatic pressure at a magnitude of 10 MPa and a frequency of 1 Hz for 2 hours/day and 5 days/week for the final 4 weeks of the 6-week differentiation period in a custom hydrostatic pressure (HP) bioreactor.[42] Six cell-seeded gels were transferred to sterilized, heat-sealed bags filled with 15 ml of medium for the 2 hour loading period. The bags for HP loading were placed into a water-filled pressure vessel while those bags as free swelling (FS) control groups were placed into open water bath, both maintained at 37 ^oC. After 2 hours, all constructs were removed from heat-sealed bags and cultured in tissue culture polysterene plate at 37 ^oC and 5 % O_2.

Mechanical analysis

Constructs were mechanically assessed using a protocol described previously.[52] Briefly, constructs from each group were tested in unconfined compression between impermeable platens using standard Zwick testing machine with a 5 N load cell (Zwick Z005, Roell, Germany). A ramp and hold cycle with a ramp displacement of 1 μm/s until 10 % strain was applied and maintained until equilibrium was reached (30 min). At this point, dynamic tests were performed with a cyclic strain amplitude of 1 % at 1 Hz. Dynamic moduli were calculated as the ratio of the determined stress amplitude to the applied strain amplitude.

Biochemical analysis

Pellet and gel samples were digested in papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich, Ireland) at 60°C under constant rotation for 18 hours. Total pellet DNA content was measured using a Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes, Biosciences, Ireland) with a lambda DNA standard and the DNA content of gels was quantified using the Hoechst Bisbenzimide 33258 dye assay (Sigma-Aldrich, Ireland). Proteoglycan content was estimated by quantifying the amount of sulfated glycosaminoglycan (sGAG) in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulfate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110 °C for 18 h in concentrated HCL (38 %) and assayed using a chloramine-T assay[53] with a hydroxyproline-to-collagen ratio of 1:7.69[54].

Histology and Immunohistochemistry

Pellets and gels were fixed in 4 % PFA, embedded in paraffin and sectioned (5 μm). Sections were stained with 1 % alcian blue 8GX (Sigma-Aldrich) in 0.1 M HCl for sGAG, and picro-sirius red for collagen. The deposition of collagen types I and II were identified through imuno-histochemistry[42, 55]. Briefly sections were quenched of peroxidase activity for 20 min (PBS were used to rinse sections between steps) and treated with 0.25 U/ml chondroitinase ABC (Sigma) in a humidified environment at 37 °C for 1 h to enhance permeability of the extracellular matrix by removal of chondroitin sulfate. After incubation with 10 % goat serum to block non-specific sites, the primary antibody of mouse monoclonal anti-collagen type I diluted 1:400 or mouse monoclonal anti-collagen type II diluted 1:100 (Abcam, Cambridge, UK) was applied for 1 h at room temperature. Then the secondary antibody (Anti-Mouse IgG biotin conjugate, Sigma-Aldrich) was added for another hour. Colour was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) for 45 min and exposure to peroxidase DAB substrate kit (Vector laboratories, UK) for 5 min. Slides were dehydrated through ethanol and xyelene and mounted with Vectamount medium (Vector Laboratories, UK). Human ligament and cartilage were included as controls for collagen type I and collagen type II respectively.

Statistics

Numerical and graphical results are presented as mean ± standard deviation (3-4 samples). Statistics were performed using R (The R Foundation for Statistical Computing, Vienna, Austria). Groups were analyzed for significant differences using a linear model for analysis of variance with multiple factors and interactions between these factors were also examed.

Tukey HSD's test for multiple comparisons was used as post-tests. Significance was accepted at a level of $p < 0.05$.

Results

The chondrogenic potential of FPSCs is diminished in hydrogel culture in comparison to pellet culture

As we have reported previously,[56] expansion of FPSCs in the presence of FGF-2 significantly reduced the CFU-F colony number and increased the colony diameter (Fig. S1 A, B). Furthermore, the colony diameter in FGF-2 expanded stem cells was significantly larger when maintained at 5 % O_2 compared to 20 % O_2. Following expansion to passage 2, stem cells expanded in the presence of FGF-2 were differentiated at either 5 % O_2 or 20 % O_2 using a pellet culture model. Stem cells expanded at 5 % O_2 formed larger pellets (Fig. 1A) and were more proliferative during chondrogenesis, as evident by a higher DNA content at day 21 (Fig. 1B), with total sGAG and collagen accumulation greatest for cells expanded and differentiated at 5 % O_2 (Fig. 1C). When normalised to DNA content the levels of sGAG and collagen within the pellets were comparable for stem cells expanded at either 5 % O_2 or 20 % O_2, but were always greatest for differentiation at 5 % O_2 (Fig. 1D). These general results were observed for stem cells isolated from other donors, with the only exception being that for some donors differentiation at 5 % O_2 was found to suppress total collagen production (see Fig. S2).

In an attempt to engineer a cartilaginous graft, FPSCs were next seeded into agarose hydrogels and the levels of matrix accumulation (on a per cell basis) after 21 days in culture at 5 % O_2 were compared to that in pellets. Both sGAG and collagen synthesis were lower in

hydrogel culture in comparison to pellet culture, with noticeably weak staining for both alcian blue and type II collagen, despite the attainment of a spherical cellular morphology within the hydrogel system (Fig. 2). This was observed for stem cells expanded at either 5 % O_2 or 20 % O_2. Furthermore, the greater proliferative potential of stem cells expanded at 5 % O_2 that was observed in pellet culture was diminished in hydrogel culture.

Serum stimulation promotes cell proliferation in both pellets and hydrogels and enhances matrix accumulation within hydrogels

In an attempt to provide a further stimulus to FPSCs embedded in agarose hydrogels to synthesise cartilage-specific extracellular matrix, constructs were additionally stimulated with 10 % serum. In pellet culture this led to greater cell proliferation but no increase in matrix synthesis (Fig. 3A). In contrast, the presence of serum significantly enhanced sGAG synthesis within cell seeded hydrogels, although synthesis levels were still lower than that observed in pellets. Collagen synthesis within hydrogels, when normalised to DNA content, was not affected by serum supplementation. Again, oxygen tension during expansion did not appear to affect levels of matrix accumulation within cell seeded hydrogels over 21 days of *in vitro* culture.

Oxygen conditions during the expansion of FPSCs influences the long-term development of cartilaginous grafts

We next explored the development of cartilaginous grafts engineered using FPSCs embedded in agarose hydrogels over a longer period of 42 days of *in vitro* culture. Unlike the findings for constructs cultured for 21 days, the DNA content within hydrogels dramatically increased

for stem cells that were first expanded at 5 % O_2 and differentiated in the presence of serum (Fig. 4A), mirroring the findings first observed in pellet culture (Fig. 3). Expansion at 5 % O_2 also lead to noticeably higher levels of total GAG and collagen accumulation within constructs supplemented with serum. Histological analysis of these constructs revealed that these higher levels of extracellular matrix accumulation were not due to dramatically greater synthesis within the hydrogels, but rather due to the formation of a dense layer of highly cellular cartilaginous tissue that formed on the surface of these constructs (Fig. 4B). This self assembled tissue that formed on top of the hydrogels stained positive for type II collagen and weakly for type I collagen (Fig. 4C).

The application of hydrostatic pressure enhances the mechanical functionality of cartilaginous grafts by generating a more compact tissue

In an attempt to further enhance chondrogenesis, cell seeded hydrogels were subjected to 10 MPa of cyclic hydrostatic pressure (1 Hz for 2 hours per day, 5 days per week). Hydrostatic pressure did not influence total DNA, sGAG or collagen content within the hydrogels, although it did appear to lead to a more homogeneous matrix distribution in gel constructs, with staining within the hydrogel appearing slightly more intense in constructs subjected to hydrostatic pressure (Fig. 5B). Significant cartilaginous tissue outgrowth is observed on the surface of the hydrogels. In the absence of serum, hydrostatic pressure resulted in constructs of lower height (Fig. 5C). When mechanically evaluated, these constructs were stiffer than controls maintained in free swelling conditions (Fig. 5D).

Discussion

Chondrogenic priming of MSCs to generate a more functional cartilage-like graft prior to implantation has been shown to improve the quality of repair in animal model studies of cartilage regeneration.[48, 49] Translating such therapies to treat damaged and diseased articular cartilage in man will most likely necessitate the development of tissue engineering strategies to generate functional cartilaginous grafts using autologous stem cells. The objective of this study was to explore how environmental factors during both the expansion and differentiation of diseased human FPSCs would influence their chondrogenic capacity following encapsulation into three dimensional hydrogels. Despite previous findings that agarose hydrogels support robust chondrogenesis of stem cells isolated from skeletally immature animals,[32-34] we found that the chondrogenesis of diseased human FPSCs was diminished in these hydrogels compared to pellet culture. Stimulation of the cell seeded hydrogels with potent biochemical (serum) or biophysical (hydrostatic pressure) stimuli did lead to improvements in cartilaginous matrix synthesis and construct functionality respectively, although the inherent chondrogenic capacity observed in the pellet environment was never recapitulated.

When expanded in the presence of FGF-2, FPSCs maintained at 5 % pO_2 formed larger colonies, suggesting a greater proliferative potential at this lower oxygen tension. Enhanced proliferation has previously been reported for MSCs maintained at a low oxygen tension.[57-60] This proliferative potential during monolayer expansion was maintained during chondrogenic differentiation, both in pellet culture, but also in hydrogel culture once FPSCs were stimulated with serum. FPSCs did not appear to proliferate within the hydrogel, but rather on the construct surface. Cells cannot directly adhere to agarose, which coupled with the fact that the FPSCs are completely encapsulated within the hydrogel, leads to limited proliferation. However, FPSCs can migrate towards the hydrogel surface, adhere to newly

synthesised extracellular matrix, and then proliferate as they are no longer encapsulated in agarose, a process that is accelerated in the presence of serum. Similar cellular outgrowth and capsule formation has been observed in chondrocyte-seeded hydrogels supplemented with serum.[61, 62] Interestingly, previous studies have reported that these layers are rich in type I collagen and therefore termed fibrous capsules, although the layers formed by the FPSCs stain positively for type II collagen, suggesting that specific experimental factors such as the presence of TGF-β3 in the medium, or maintenance at a low oxygen tension during differentiation, promoted a more cartilaginous phenotype.

Encapsulation of FPSCs in a three dimensional hydrogel led to a diminished chondrogenic potential compared to pellet culture. It is well established that agarose can help support a chondrogenic phenotype for culture expanded chondrocytes,[63] and has been widely used as a hydrogel for cartilage tissue engineering using chondrocytes,[64-66] bone marrow derived MSCs,[67, 68] adipose derived stem cells[69] and FPSCs[70-73], although many of these studies used cells isolated from young animals. A key difference between the high density pellet culture system,[11] and hydrogel encapsulation in agarose are the differences in cell-cell and cell-matrix interactions. Cellular condensation and expression of proteins associated with cell-cell interactions, such as N-cadherin, are known to be important at the onset of chondrogenesis.[74, 75] The pellet culture system also supports paracrine signalling important for chondrogenesis. The absence of ligands within the agarose hydrogel to facilitate integrin mediated binding of FPSCs to their local substrate may also play a role in diminishing their chondrogenic potential. Alternative approaches to engineering functional cartilaginous grafts that recapitulate aspects of the pellets culture system, such as the self assembly process,[76] may represent promising approaches when using diseased human FPSCs.

Serum has previously been shown to influence the growth and development engineered cartilaginous constructs.[61, 77-81] Supplementing the chondrogenic medium with

serum was observed to enhance cartilaginous extracellular matrix accumulation in FPSC seeded hydrogels, although synthesis levels on a per cell basis were still lower than that in pellet culture. The abundance of growth factors present in serum (such as transforming growth factor-β (TGF-β) superfamily, insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF)), may be playing a role in this enhanced biosynthetic activity. Serum also contains adhesive glycoproteins such as fibronectin [82, 83], which has numerous biological activities, including mediating cell-cell adhesion and enhancing cell-substrate anchoring and spreading.[84] Furthermore, by facilitating the proliferation of FPSCs on the surface of the hydrogels, serum may also be indirectly enhancing chondrogenesis by allowing greater cell-cell interactions in this region of the engineered tissue, thereby mimicking aspects of the pellet culture system know to support more robust chondrogenensis of diseased human FPSCs.

The application of hydrostatic pressure did not enhance overall levels of matrix accumulation within FPSCs seeded constructs. This is in contrast to the results of previous studies demonstrating that cyclic hydrostatic pressure can enhance chondrogenesis of bone marrow[42, 85-90] and adipose-derived[91] stem cells. These discrepancies could potentially be explained by differences in phenotype between stem cells isolated from different tissues, or alternatively due to differences in cell-cell and cell-scaffold interactions between different studies that may play a central role in mechano-transduction of biophysical signals such as hydrostatic pressure. The cell seeding density utilized in this study may also impact the response to biophysical cues.[92, 93] It should be noted, however, that while the application of hydrostatic pressure did not increase overall levels of matrix accumulation, it did appear to influence the spatial distribution of matrix within the construct. Hydrostatic pressure has previously been shown to result in the formation of more compact pellets generated using bone marrow derived MSCs,[89] and influence the spatial accumulation of matrix in MSC

seeded hydrogels.[42] Histological analysis suggests that the application of hydrostatic pressure preferentially supports the accumulation of matrix components within the hydrogel as opposed to on the construct surface. In hydrogels not additionally stimulated with serum, the application of hydrostatic pressure was also observed to suppress outgrowth and/or swelling of the construct, as evident by a lower construct thickness. This may explain why these constructs were stiffer than their free swelling counterparts, as compaction of an engineered cartilaginous tissue can lead to improvements in biomechanical functionality.[94] Therefore the application of cues such as hydrostatic pressure may play a key role in mechanically priming cartilaginous constructs engineered using FPSCs prior to implantation. Further optimization of these environmental conditions is required as the mechanical properties of these engineered grafts are still two orders of magnitude lower than normal articular cartilage.

In conclusion, we have demonstrated that encapsulation of diseased human FPSCs into agarose hydrogels in an attempt to engineer a functional cartilaginous graft can suppress their inherent chondrogenic potential. While this can be partially recovered through the application of biochemical or biophysical stimuli, their chondrogenic capacity is still diminished, and the resulting mechanical properties of the engineered grafts are still noticeably lower than native articular cartilage. Further work is required in order to recapitulate the environmental conditions present during pellet culture within hydrogels or scaffolds in order to engineer fully functional cartilaginous grafts using stem cells derived from human osteoarthritic IFP tissue.

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Author Disclosure Statement

The authors have nothing to disclose.

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Figure legends

Fig. 1. The effect of oxygen tension during expansion and differentiation on chondrogenesis of FPSCs in pellet culture for 21 days. DNA content (A), sGAG and Collagen content (B), pellet morphology (C), GAG/DNA and Collagen/DNA (D). Scale bar, 1 mm. '*' indicates a significant difference between FPSCs undergoing chondrogenesis at either 5% or 20% O_2, where all other culture conditions during differentiation were identical. 'a' indicates a significant difference between FPSCs expanded at either 20% or 5% O_2, where all other culture conditions during differentiation were identical.

Fig. 2. A comparison of the chondrogenic potential of FPSCs maintained in pellets or in agarose hydrogels (10 million cells/ml) for 21 days. (A) DNA content, GAG/DNA and Collagen/DNA. (B) Alcian blue staining, picro sirius red staining and collagen II immunostaining of the pellets and hydrogels expanded at 20% O_2. Scale bar, 0.5 mm. '*' indicates a significant difference between FPSCs expanded at either 20% or 5% O_2, where all other culture conditions during differentiation were identical. 'b' indicates a significant differences between pellets and hydrogels maintained in otherwise identical environmental conditions.

Fig. 3. Effect of serum on FPSCs maintained in pellets or in agarose hydrogels (10 million cells/ml) for 21 days. (A) DNA content, sGAG content, Collagen content, GAG/DNA and Collagen/DNA. (B) Alcian blue staining and picro sirius red staining . Scale bar, 0.5 mm. '*' indicates a significant difference between cultures maintained in the presence or absence of serum, where all other culture conditions during differentiation were identical. 'a' indicates a

significant difference between FPSCs expanded at either 20% or 5% O_2, where all other culture conditions during differentiation were identical. 'b' indicates a significant differences between chondrogenesis in pellets and hydrogels for FPSCs maintained in otherwise identical environmental conditions.

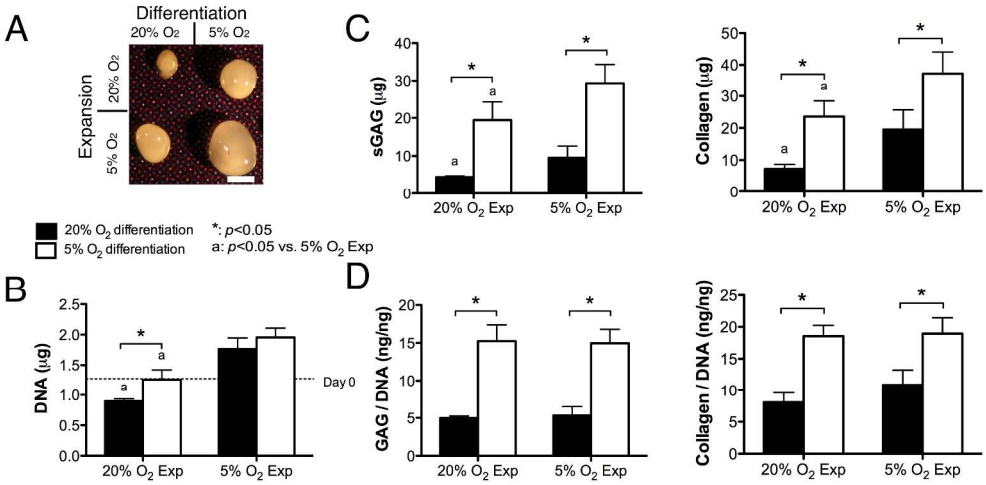
Fig. 4. Effect of serum on the FPSCs maintained in agarose hydrogels (10 million cells/ml) for 42 days. (A) DNA, sGAG, Collagen content, GAG/DNA and Collagen/DNA. (B) Alcian blue staining and picro sirius red staining. (C) A high magnification view of a region (dashed-box) of a hydrogel seeded with FPSCs expanded at 5% O_2 and differentiated in the presence of serum demonstrating the formation of a dense layer of cartilaginous tissue on the surface of the hydrogel. Solid scale bar, 0.5 mm. Dash scale bar, 0.1 mm. '*' indicates a significant difference between cultures maintained in the presence or absence of serum, where all other culture conditions during differentiation were identical. 'a' indicates a significant difference between FPSCs expanded at either 20% or 5% O_2, where all other culture conditions during differentiation were identical.

Fig. 5. Effect of hydrostatic pressure and serum on chondrogenesis of FPSCs maintained in agarose hydrogels (20 million cells/ml) for 6 weeks. Hydrostatic pressure loading was applied for the final 4 weeks of culture. (A) DNA, sGAG and Collagen content. (B) Alcian blue staining, picro sirius red staining and collagen II immunostaining. Hydrogels subject to hydrostatic pressure had a lower thickness (C) and higher dynamic modulus (D). Scale bar, 0.5 mm. The average dynamic modulus of acellular agarose hydrogels is 39 kPa. 'c' indicates a significant difference between constructs maintained in the presence or absence of serum,

where all other culture conditions during differentiation were identical. '*' indicates a significant difference between constructs subjected to HP or maintained in FS conditions.

Fig. S1. (A) The effect of FGF-2 and oxygen tension on the CFU-F potential of human FPSCs as demonstrated by colonies stained with crystal violet. (B) Colony number and diameter. The colony diameter was defined as the average diameter of the 10 largest colonies in each dish.

Fig. S2. The effect of oxygen tension during expansion and differentiation on chondrogenesis of FPSCs from a separate donor cultured for 21 days.



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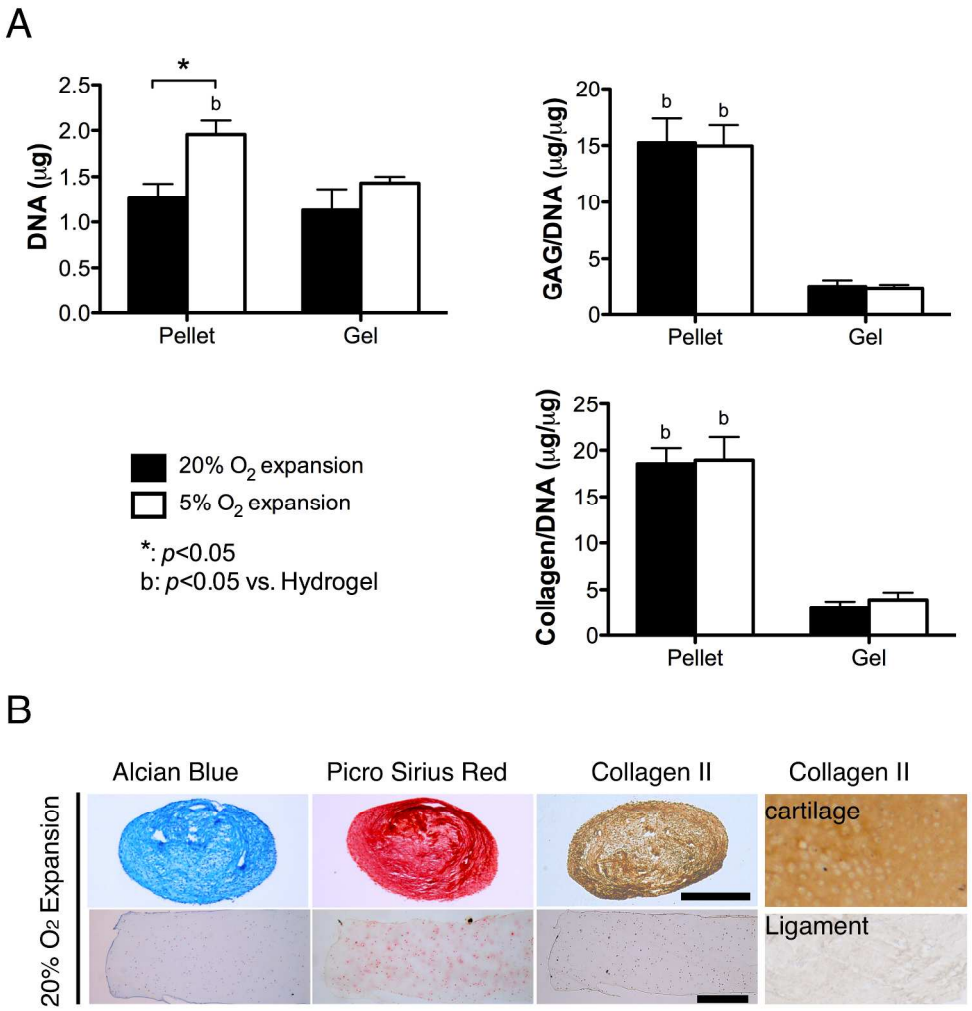

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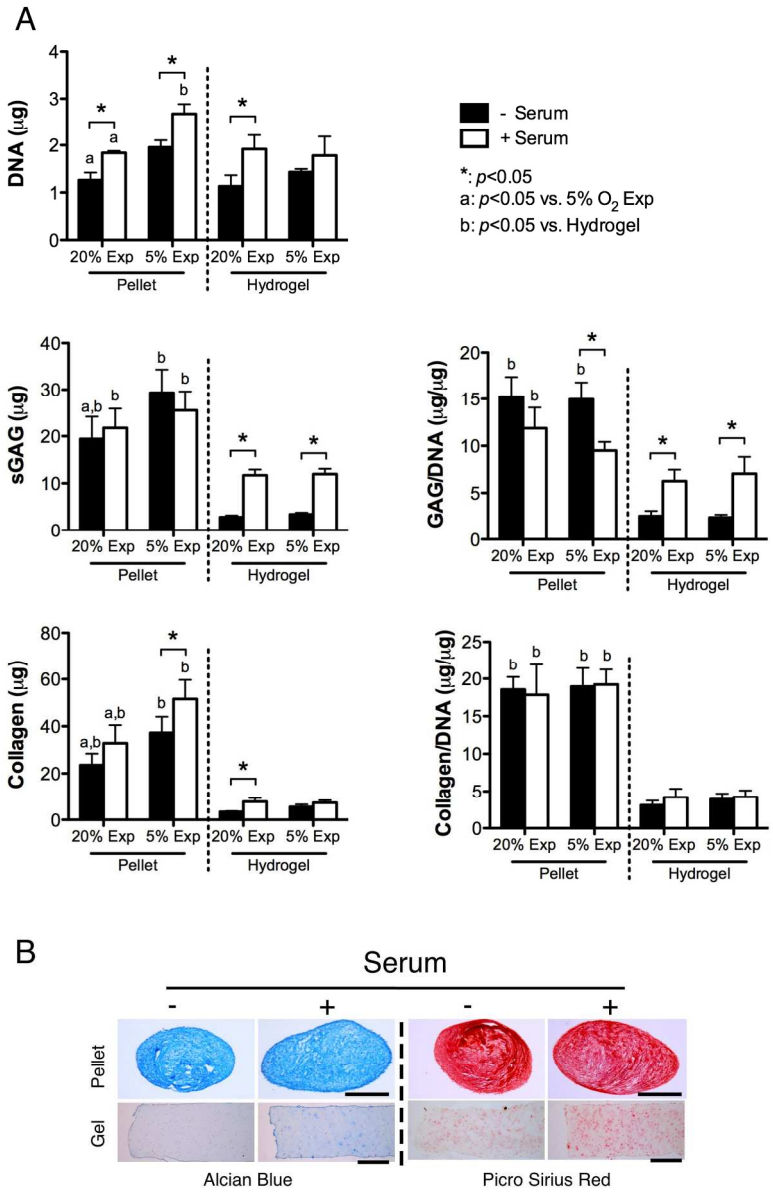

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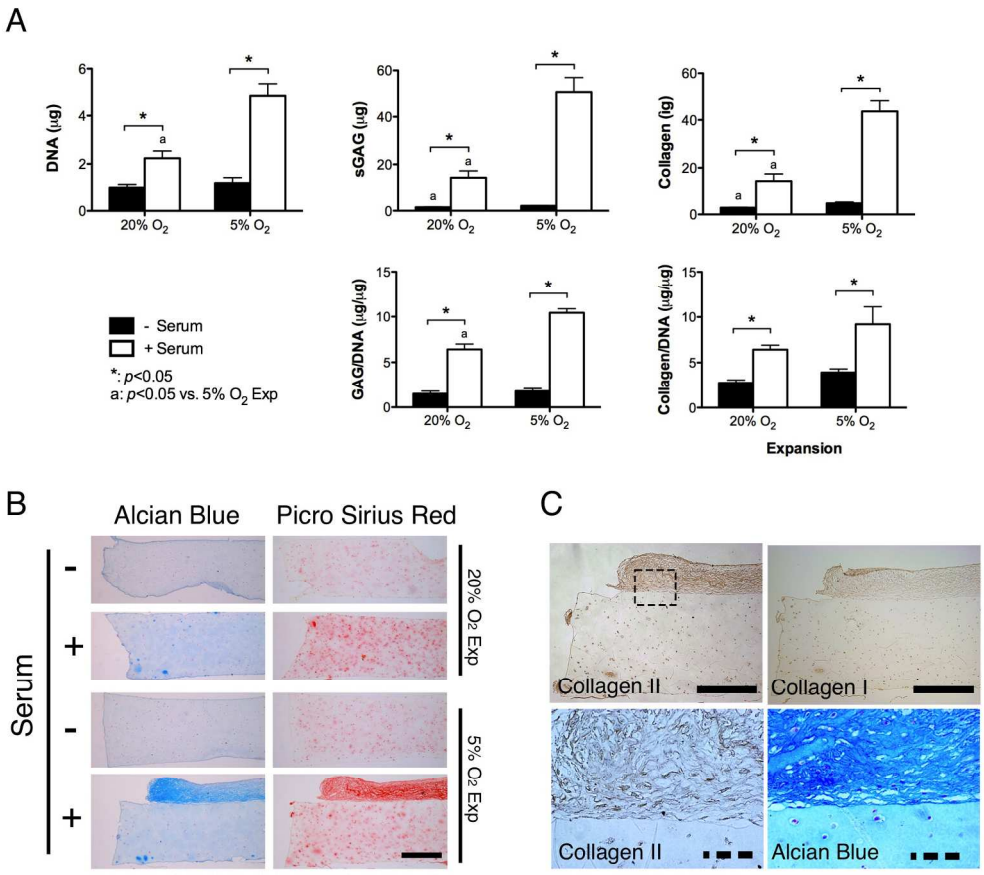

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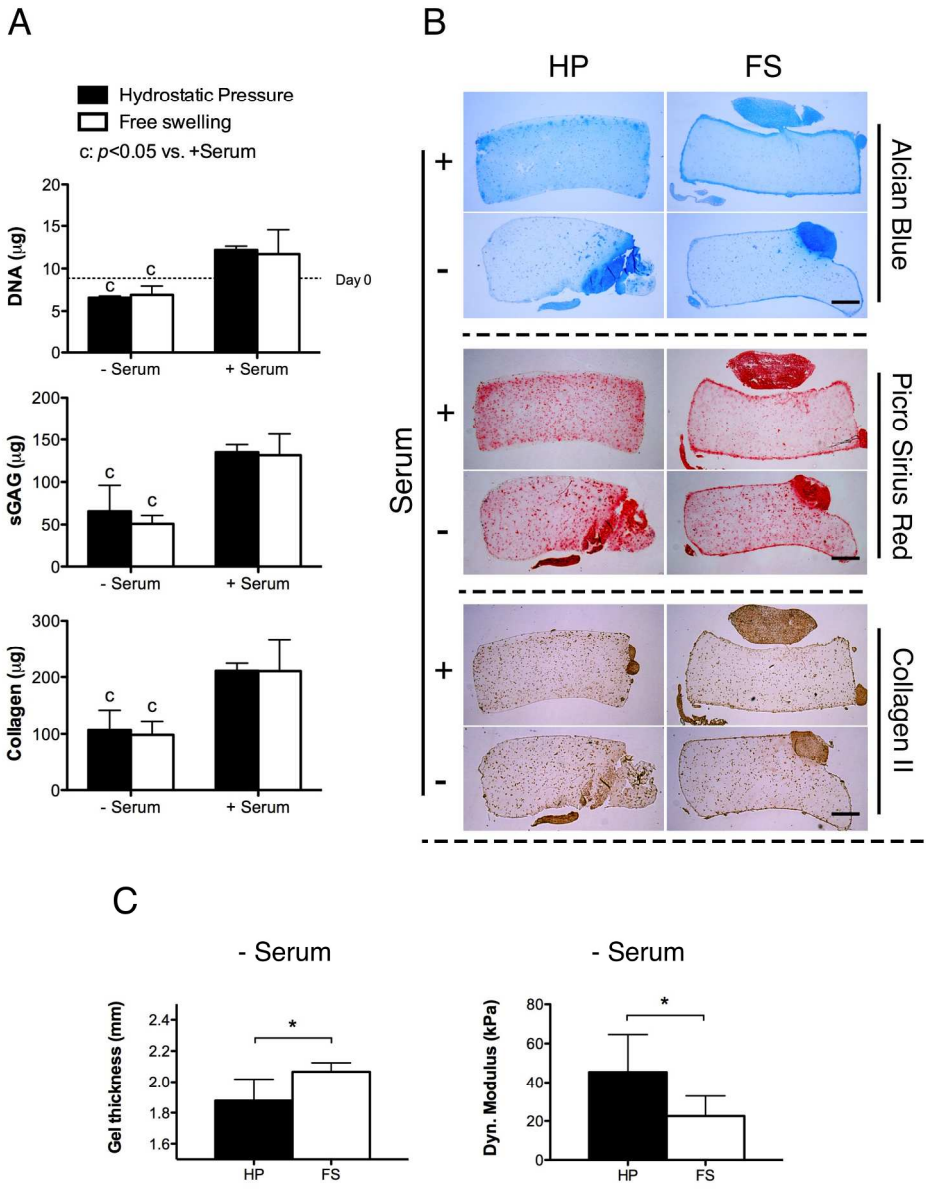

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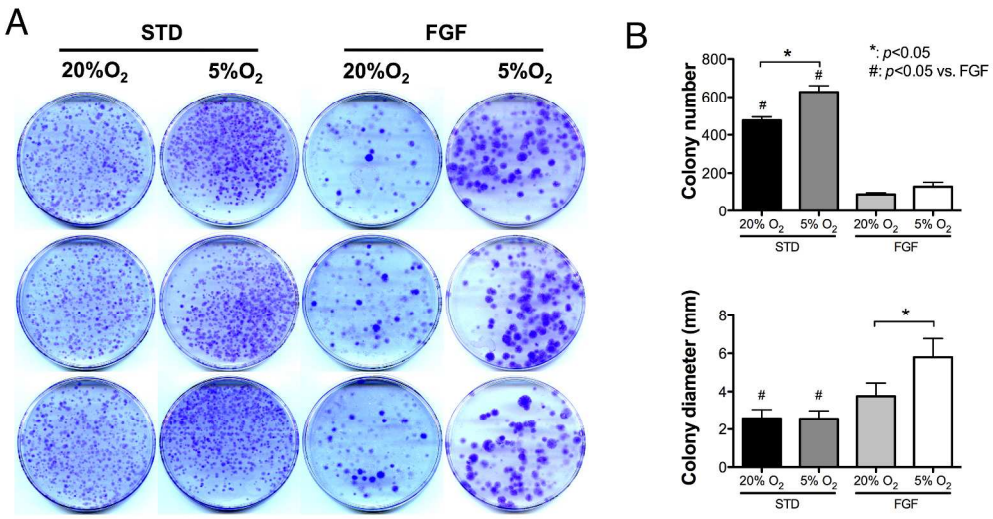

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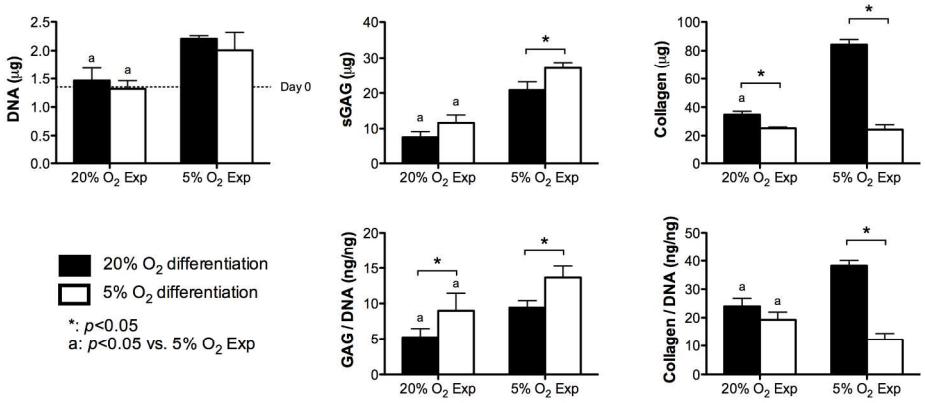

Fig. S2. The effect of oxygen tension during expansion and differentiation on chondrogenesis of FPSCs from a separate donor cultured for 21 days.
208x95mm (300 x 300 DPI)