Chondrogenesis of Infrapatellar Fat Pad Derived Adipose Stem Cells in 3D Printed Chitosan Scaffold

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Abstract

Infrapatellar fat pad adipose stem cells (IPFP-ASCs) have been shown to harbor chondrogenic potential. When combined with 3D polymeric structures, the stem cells provide a source of stem cells to engineer 3D tissues for cartilage repair. In this study, we have shown human IPFP-ASCs seeded onto 3D printed chitosan scaffolds can undergo chondrogenesis using TGFβ3 and BMP6. By week 4, a pearlenscent, cartilage-like matrix had formed that penetrated the top layers of the chitosan scaffold. Chondrocytic morphology showed typical cells encased in extracellular matrix which stained positively with toluidine blue. Immunohistochemistry demonstrated positive staining for collagen type II and cartilage proteoglycans, as well as collagen type I. Real time PCR analysis showed up-regulation of collagen type II, aggrecan and SOX9 genes when IPFP-ASCs were stimulated by TGFβ3 and BMP6. Thus, IPFP-ASCs can successfully undergo chondrogenesis using TGFβ3 and BMP6 and the cartilage-like tissue that forms on the surface of 3D-printed chitosan scaffold may prove useful as an osteochondral graft.

Introduction

Articular cartilage defects have limited capacity for self-regeneration and healing. Cartilage damage often results in pain and loss of function for the patient and often accelerates the development of osteoarthritis in the joint. Current methods of osteochondral repair aimed at improving symptoms and function include microfracture, osteochondral grafting, and autologous chondrocyte transplantation (ACT) [1,2,3,4,5,6]. However, there are inadequacies with these procedures which include the formation of fibrocartilage, donor site morbidity, hypertrophy of implant, and suboptimal long term outcomes [7,8,9,10,11].

Tissue engineering may offer treatment options that could overcome the limitations of current management options. The combination of cells, scaffold and biochemical factors may provide the possibility of true cartilage regeneration. Although the use of autologous chondrocytes has yielded some good short term results, long term results are equivocal [12,13,14,15,16]. Furthermore the use of autologous chondrocytes is limited by major factors, including donor site morbidity, and chondrocytes are limited in number comprising of only 5–10% of cartilage tissue, thus require expansion which may lead to dedifferentiation [7,17,18,19,20]. Due to these limitations, mature chondrocytes are not ideal candidate cells to use in tissue engineering constructs.

Adult mesenchymal stem cells can overcome some of the aforementioned issues. These cells can be derived from bone marrow, fat, skin, muscle, periosteum, or cord blood [21,22,23,24,25,26]. More recently, adipose tissue has become an attractive source of adipose stem cells (ASC) due to the ease of accessibility and great abundance [27,28]. Compared to bone marrow, adipose tissue is reported to give a higher yield of stem cells [29]. These cells have enormous capacity for proliferation and differentiation into chondrocytes as shown in many groups [27,28,30]. Most have used ASCs derived from the stromal vascular fraction (SVF) of liposuction [31]. However, some have used adipose stem cells derived from the infrapatellar fat pad (IPFP) during total knee arthroplasty because the removal of IPFP improves surgical access and visualization, and reduces the chance of impingement of the fat pad by the prosthesis. It may be that this autologous source of stem cells is a suitable candidate cell for repairing cartilage defects in the knee before total knee arthroplasty is required and may form part of a one-step surgical procedure for autologous stem cell transplantation in the knee [32].

Chitosan has been used widely in the tissue engineering field for cartilage engineering [33]. It shares some structural characteristics with various glycosaminoglycans and hyaluronic acid found in native cartilage. It is usually biocompatible and degradation products are often elements involved in the synthesis of cartilage,
such as chondroitin sulfate, hyaluronic acid, keratin sulfate and glycosylated collagen type II [34]. 3D structures can be designed to mimic the native cartilage environment and thus, in theory, should provide greater chance for cartilage regeneration [35]. Some studies have shown that chondrocytes require a 3D environment to avoid dedifferentiation [20]. 3D printed structures can also be engineered using computer-assisted drawing technologies (AutoCAD) and thus made to any shape or size to fill defects; this greatly enhances their potential clinical use.

In the current study we have used ASC derived from IPFP that was removed during total knee arthroplasty for osteoarthritis. Our aim was to investigate in vitro chondrogenesis of IPFP-ASCs using a 3D chitosan engineered scaffold.

**Materials and Methods**

**Ethics Statement**

Infrapatellar fat pads were obtained intraoperatively from total knee arthroplasties after informed written consent and approval from Human Research Ethics Committee at St Vincent’s Hospital (HREC-A 117/10). All necessary ethics protocols were adhered to in the process of tissue harvest and use. Only patients with primary osteoarthritis were selected. Patients with inflammatory arthritis and with a history of prior knee surgery were excluded from selection. A total of three patients (2 female, 1 male) with mean age of 69 (aged 67, 69, 71; N = 3) were included in this study.

**Materials**

Materials used for IPFP-ASC isolation, culture and differentiation are listed as the following: Dulbecco’s phosphate-buffered saline (D-PBS), fetal bovine serum (FBS), antibiotic/antimycotic solution (Amphotericin B, Penicillin, Streptomycin 100 μg/ml), glutamax, L-ascorbic acid 2-phosphate, transforming growth factor beta-3 (TGFβ3), and HEPES buffer were purchased from Gibco, Life Technologies Corporation (Carlsbad, CA, USA); Red cell lysis buffer, Dulbecco’s modified eagle medium (DMEM), insulin-transferring-selenium (ITS), dexamethasone, and 0.1% EDTA/0.25% trypsin were from Sigma-Aldrich (St. Louis, MO, USA). All culture plates, conical tubes, well inserts were from Corning Inc., (NY, USA) and cell filters from Millipore (Darmstadt, Germany). Collagenase type 1 was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Human epidermal growth factor (hEGF), human fibroblastic growth factor-2 (hFGF-2), and bone morphogenetic protein-6 (BMP6) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Histology and immunohistochemistry reagents were purchased as listed: Neutral buffered formalin (NBF), Mayer’s haematoxylin and eosin (H&E), toluidine blue were from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide was from Merck Millipore (Darmstadt, Germany); Protease K, rabbit serum, secondary antibodies (biotinylated rabbit polyclonal anti-goat and anti-mouse antibodies), and liquid DAB+ were purchased from Dako (Glostrup, Denmark); Horseradish peroxidase (HRP)-conjugated streptavidin was from the Vectastain ABC kit from Vector Laboratories (Burlingame, CA, USA); Primary antibodies included mouse monoclonal anti-human type II collagen IgG antibody (MP Biomedical, Solon, OH, USA), goat polyclonal anti-human type I collagen IgG antibody (SouthernBiotech, Birmingham, AL, USA), and mouse monoclonal anti-human cartilage proteoglycan IgG antibody (Merck Millipore, Billerica, MA, USA). Goat IgG isotype control was from SouthernBiotech (Birmingham, AL, USA) and mouse IgG isotype control was from Invitrogen, Life Technologies Corporation (Carlsbad, CA, USA).

Reagents for qPCR included Triozol (Ambion, Life Technologies, Carlsbad, CA, USA), and all other RNA extraction materials from Qiagen (Hilden, Germany). cDNA synthesis materials were...
from Promega (Madison, WI, USA). Taqman probes were used for the evaluation of collagen type I, II, SOX9, Aggrecan and GAPDH genes (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA).

**Cell Isolation and Culture**

Cell isolation and culture was based on a previously published protocol of isolating cells from liposaspirate material and adapted for the isolation of cells from the IPFP [36]. Briefly, the IPFP was immediately placed in sterile normal saline and processed within 30 minutes of harvest. Initially the tissue was washed several times with PBS, to remove contaminating blood. Fibrous material such as capsule or meniscus were dissected and discarded. The remaining fat content was diced using a scalpel and digested with 0.2% Collagenase Type 1 for three hours at 37C under constant agitation. The released cells, IPFP-ASCs, and materials were filtered through a 100 µm nylon mesh and centrifuged at 400 g at room temperature for five minutes to separate the stromal vascular fraction (SVF) from the floating adipocytes. The supernatant was discarded and the cell pellet resuspended in Red Cell Lysis Buffer and incubated at room temperature for 10 minutes. This was then filtered through a 40 µm nylon mesh before centrifugation at 400 g at room temperature for five minutes. The cells were resuspended in PBS, counted and plated in monolayer culture (75 cm² tissue culture flask) at 5000 cells/cm² in stromal media (SM) containing DMEM supplemented with 10% FBS, 1x antibiotic/antimycotic solution, 1x Glutamax, and 15 mM HEPES. Cultures were maintained 48 hours at 37C in 5% CO2. The cells were washed and media were replaced with expansion media containing stromal media with 5 ng/ml human epidermal growth factor (hEGF) and 1 ng/ml human fibroblastic growth factor (hFGF). The cells were cultured until they reached 80% confluence and then harvested with 0.1% EDTA/0.25% trypsin (hFGF). The cells were cultured until they reached 80% confluence and then harvested with 0.1% EDTA/0.25% trypsin (hFGF) and then harvested with 0.1% EDTA/0.25% trypsin (hFGF) and made into a single cell suspension for seeding onto the chitosan scaffold.

**Scaffold Preparation**

A 3% w/v medium molecular weight chitosan solution was prepared in 2% v/v acetic acid (Sigma-Aldrich, St. Louis, MO, USA). The solution was filtered and centrifuged to remove air bubbles before loading into a disposable syringe (Nordson EFD) fitted with a 200 µm diameter nozzle. The chitosan solution was extrusion printed onto a glass slide immersed in a precipitating bath of isopropyl alcohol using a custom modified computer numerical control (CNC) milling machine (Sherline Products, CA). The system was equipped with a three-axis positioning platform and designed using EMC2 software (LinuxCNC). An attachment for syringe deposition was built and connected to a controllable gas flow regulator (1–100 psi). The regulator was controlled using a Pololu SciLabs USB-to-serial microcontroller and with an in-house software interface. Solutions were extrusion printed at a flow rate of 150 mm/min, strand spacing of 0.25 mm, to a final size of 13 mm x 10 mm x 5 mm with a porosity of 250 µm. Scanning electron microscopy was used to image the scaffold to show micro-architecture of the 3D lattice structure using the Agilent 8500 FE-SEM system (Agilent Technologies Inc, Santa Clara, CA, USA) (Figure 1). The extruded 3D scaffolds were then neutralised in a dilution series of ethanol and PBS over a period of two days.

24 scaffolds were made for this experiment. Three to four 6 mm plugs were cut using a skin biopsy punch and randomly allocated. Six scaffolds were used for each time point and for each condition. Two were used for histological analysis. Four were used to harvest RNA for PCR gene analysis. A total of 72 6 mm plugs of scaffolds were used for 3 biological replicates of IPFP ASCs.

**Chondrogenic Differentiation and Culture**

Confluent IPFP-ASCs, cells at third passage, were harvested, counted and resuspended in chondrogenic medium (CM) consisting of DMEM-high glucose, 1% FBS, 1% ITS, 100 nM Dexamethasone, 50 µg/ml ascorbic acid, 1x antibiotic/antimycotic solution, 1x Glutamax, and 15 mM HEPES. Cultures were maintained 48 hours at 37C in 5% CO2. The cells were washed and media were replaced with expansion media containing stromal media with 5 ng/ml human epidermal growth factor (hEGF) and 1 ng/ml human fibroblastic growth factor (hFGF). The cells were cultured until they reached 80% confluence and then harvested with 0.1% EDTA/0.25% trypsin and made into a single cell suspension for seeding onto the chitosan scaffold.

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**Chondrogenesis IPFP-ASCs in 3D Scaffold**

A comparative image showing pellets cultured in control vs chondrogenic media at 4 weeks. A comparative image showing pellets cultured in control vs chondrogenic media at 4 weeks. A comparative image showing pellets cultured in control vs chondrogenic media at 4 weeks. A comparative image showing pellets cultured in control vs chondrogenic media at 4 weeks. A comparative image showing pellets cultured in control vs chondrogenic media at 4 weeks. A comparative image showing pellets cultured in control vs chondrogenic media at 4 weeks. A comparative image showing pellets cultured in control vs chondrogenic media at 4 weeks. A comparative image showing pellets cultured in control vs chondrogenic media at 4 weeks.
cotic, 10 ng/ml TGFβ3 and 10 ng/ml BMP6. Scaffolds were cut using a 6 mm biopsy punch (Kai Medical, Honolulu, HI, USA) and seeded with $7.5 \times 10^5$ ASCs in a 24 Transwell tissue culture plate well inserts with an internal diameter of 6.5 mm and 3.0 µm pore size (Figure 2). Cells were seeded on top of the scaffold and 1.2 ml of chondrogenic or control media was used in each well. These were incubated at 37°C in 5% CO2 for 14 and 28 days and media changed three times per week. The same protocol was used for media without growth factors, and served as the negative control. Cells were also cultured by themselves using micromass pellet culture. 250,000 cells were centrifuged at 400 g for 5 minutes in 15 ml centrifuge tubes to form cell pellets and cultured in 0.5 ml of chondrogenic or control media for the same time period as cells cultured on the chitosan scaffolds.

Histology and Immunohistochemistry

After 14 and 28 days of culture, cell pellets and cell-scaffold constructs were harvested for histological and immunohistochemical analysis using standard techniques of fixation, dehyration and paraffin embedding. Pellets and cell-scaffold constructs were fixed in 10% NBF overnight at 4°C and processed and imbedded at the histopathology laboratory, pathology department at St Vincent’s Hospital, Melbourne, sectioned into 6 µm sections and incubated overnight at 37°C. The sections were deparafinised, rehydrated through graded ethanol, and stained with haematoxylin & eosin (H&E) and toluidine blue (TB) (for glycosaminoglycans (GAGs)).

Accumulation of collagen types I and II, and cartilage-specific proteoglycan was assessed by immunohistochemistry. Sections were treated with 0.3% hydrogen peroxide ($H_2O_2$) for five minutes, antigen retrieval using Proteinase K for four minutes and were blocked using 10% normal rabbit serum (NRS) for 30 minutes at room temperature. These sections were incubated with the following primary antibodies; mouse monoclonal anti-human type II collagen antibody (1:500), goat polyclonal anti-human type I collagen (1:500), and mouse monoclonal anti-human cartilage proteoglycan antibody (1:500) for 60 minutes at 37°C. Isotype negative controls were used at the same concentration as their respective primary antibodies. Subsequently, sections were incubated using biotinylated rabbit polyclonal anti-goat and rabbit anti-mouse antibodies as secondary antibodies for 30 minutes followed by horseradish peroxidase (HRP)-conjugated streptavidin treatment according to the manufacturer’s instructions. The reaction was developed as a brown precipitation using peroxidase substrate 3,3-diaminobenzidine (DAB) for 5 minutes. Sections were counterstained with haematoxylin, dehydrated, cleared, and mounted with Pertex.

Quantitative Real Time PCR (qPCR)

Week 2 and 4 cell-scaffold constructs were pulverized in liquid nitrogen using a small mortar and pestle and then homogenized in 1 ml of Trizol solution, and RNA was extracted and purified using a combination of the Trizol method and silica membrane-based commercial extraction kit (QIAGEN; RNeasy mini kit) according to the manufacturer’s protocol. RNA from pre-differentiated cells (day 0) was also extracted as Time zero samples. The RNA concentration and purity were measured using a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany) and the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Complimentary DNA copies were reverse transcribed from
200 ng total RNA for all samples using oligo-dT primers and omniscript reverse transcriptase kit according to the recommendations of the manufacturer. qPCR was performed using standard TaqMan Probe-Based Gene Expression Analysis protocols using commercial available probes for collagen types I and II, SOX 9, and Aggrecan. The Taqman primer ID for each gene was as follows: COL1A2 (Hs00164099_m1), COL2A1 (Hs00264051_m1), SOX9 (Hs01165814_m1), and ACAN (Hs00153936_m1). GAPDH was used as housekeeping gene for relative quantification of gene expression (Hs02758991_g1). Liquid handling was performed by the CAS1200 series robot by Corbett Robotics (Corbett Life Sciences, Qiagen, Hilden, Germany). Subsequent PCR reaction was performed using the Lightcycler 480 (Roche, Basel, Switzerland).

Data Analysis
Relative quantification was derived and analyzed using the Second Derivative Maximum method through the Lightcycler 480 software version 1.3. Subsequent numerical data analysis of relative quantification of qPCR results was performed in Microsoft Excel 2010 and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) using the 2^(-ΔΔCT) method. Means, standard deviations (SD) and errors (SEM), and 95% confidence limits were calculated for each set of results. The t-test was used to assess significance between two sets of data. Friedman’s test was used to assess significance over three or more sets of data. Statistical testing was verified by a statistician.

Results

Cell Culture
IPFP-ASCs cultured for up to four weeks on a 3D chitosan scaffold in chondrogenic media (TGFβ3 and BMP6), developed a pearlescent, white and shiny cartilage-like tissue ‘cap’ (Figure 3A). Conversely, cell-scaffold constructs cultured in control media showed no visible signs of cartilage-like tissue formation (Figure 3B). Similarly, chondrogenic pellets were larger and rounder than control pellets and also had a shiny white appearance macroscopically (Figure 4).

Histology and Immunohistochemistry
H&E staining of chondrogenic pellets showed a change in cellular morphology towards a chondrocytic phenotype showing larger cells encapsulated in lacunae when compared to control pellets. Positive toluidine blue of chondrogenic pellets contrasts with a lack of toluidine blue staining in control pellets. Furthermore, in chondrogenic pellets, gradient differentiation of toluidine blue staining intensity can be seen on magnification, together with more tangential morphology of cells near the surface (Figure 5).

H&E staining of the cell-scaffold construct show a paucity of cells attached to the chitosan in the negative control, and the cells that did attach appeared to be fibroblastic in appearance and lacked features of chondrocytic morphology. In contrast, cell-scaffold constructs cultured in chondrogenic media showed cellular aggregation throughout the upper layers of the chitosan scaffold forming what can be seen macroscopically as a ‘cap’ of cartilaginous tissue. These cells exhibit features of chondrocytic morphology as seen in the chondrogenic pellets as well as staining for toluidine blue (Figure 6). The chitosan fibres were stained pink by the eosin and therefore easily recognizable in the H&E stain. The chitosan fibres did not stain for toluidine blue and therefore appeared like empty spaces in the toluidine blue stain.

Immunohistochemistry staining of chondrogenic and control pellets for the presence of collagen type II, cartilage proteoglycan, and collagen type I at week 4 is shown in Figure 7. When compared to control pellets and isotype controls, chondrogenic pellets stained strongly for collagen type II and proteoglycan in the extracellular matrix. Collagen type I was present in both the control and the chondrogenic pellets. Similar patterns of staining can be seen in the cell-scaffold constructs (Figure 8). Strong staining for collagen type II and cartilage proteoglycans is seen throughout cartilaginous ‘cap’. There was also staining of collagen type I throughout, which appears to co-localise with collagen type II in some parts. All immunohistochemistry staining show the cells are encased in their lacunae consisting of extracellular matrix consisting of predominantly collagen type II. Some non-specific staining of chitosan fibres is also present in the proteoglycan stain.
As shown in the histological stains, there is a paucity of cellular attachment in the control cell-scaffold constructs.

**Gene Expression**

There was an increase in all mRNA expression levels of chondrogenic markers in both chondrogenic pellets and cell-scaffold constructs tested from week 0 to week 4. It is important to note that there were undetectable levels of expression of collagen type II in the cells prior to plating. In contrast, collagen type II expression was present at week 2 and was increased significantly by week 4 in both pellets and cell-scaffold constructs \( (p<0.05) \) (Figure 9). However, collagen type I was present at time zero in the cells and by week 4 there was comparable expression levels of collagen type II gene with collagen type I. Collagen type I expression remained consistent over the four-week culture period and any changes across the pellets or cell-scaffold constructs were not statistically significant.

At week 4 cell-scaffold constructs and pellets cultured in control media (i.e. without TGFβ3 and BMP6) had collagen type II gene expression levels that were undetectable, and only low levels of SOX9 and aggrecan genes were expressed. All chondrogenic genes examined were expressed at a higher level when cultured in chondrogenic media. The expression of collagen type II and aggrecan at week 4 were significantly greater in the chondrogenic group compared with the control group in both pellets and cell-scaffold constructs \( (p<0.05) \). Figure 10 illustrates the chondrogenic gene expression data between of chondrogenic media and control media groups at week 4.

**Discussion and Conclusion**

This study demonstrates the infrapatellar fat pad is a reliable and abundant source of adipose stem cells with chondrogenic differentiation capacity that can readily be accessed during surgery as an autologous material. The volume of the material produced means that small harvest of autologous IPFP can yield adequate stem cell numbers for the possible repair of quite substantial areas of cartilage damage. Table 1 shows the studies that have used IPFP for chondrogenesis in the past and the combination of TGFβ3 and BMP6 is unique. In a previous study, we have characterized the chondrogenesis of IPFP-ASCs using this combination of growth factors to demonstrate their chondrogenic potential \[37\]. In this study we have demonstrated the ability for these cells to undergo chondrogenesis not only by themselves in pellet form but also attach, proliferate and differentiate on a 3D printed chitosan scaffold which may serve as a delivery mechanism for these cells into a site of cartilage repair.

In our experiments, all three samples of IPFP-ASCs did not express detectable levels of collagen type II and SOX9 genes at week 0. However, it is clear the expression of these markers increased substantially over four weeks \( (p<0.05) \). The increase in collagen type II expression was significant. Collagen type II expression remained undetectable at week 4 in the control group. This suggests the addition of TGFβ3 and BMP6 has had a profound effect on chondrogenic differentiation and the stimulation of production of collagen type II in the IPFP-ASCs. The macroscopic changes of the cell aggregates and the morphological changes in the cells also clearly demonstrated the progressive
development of a chondrogenic phenotype. Collagen type I expression remained unchanged from pre-plated cells to week 4 (under chondrogenic conditions). The expression of collagen type I by IPFP-ASCs is consistent with previous studies using these cells [32,38,39,40].

Histologically, there is evidence of some co-localization of collagen type II and type I expression which may provide evidence of early developmental progression at 4 weeks in vitro, of the cell-scaffold constructs toward a more chondrogenic phenotype. Collagen type I expression was important to investigate, not only because it is found in fibrocartilage, but because it is expressed in early chondrogenesis as part of the transformation that occurs from mesenchymal cells to chondrocytes. Therefore, collagen type I expression is also a marker of early chondrogenesis. This is consistent with the pre-natal development of the knee joint, which starts with a condensation of the mesenchyme between the two long bones prior to the distinct development of the articular surfaces of the long bones [41]. In our study, cell-matrix constructs were maintained for only four weeks and may indicate the need to extend the time period for further clarification of the in vitro development sequence. Changes to the composition and structure of the scaffold over time may also impact the production of collagen type I in the cells. This possibility was not investigated in this study, however similar observations were made by other studies of this nature [32].

SOX9 plays a significant role in chondrogenesis, and is also present in other tissues such as the notochord, otic vesicle, neural tube, brain and the developing gonads [42]. In terms of chondrogenesis, it is an important transcription factor in the activation of collagen type II gene during the process of chondrogenesis [42]. Collagen type II is predominantly found in adult articular (hyaline) cartilage and also occurs to a smaller extent in fibrocartilage tissue such as intervertebral disc and meniscus [43]. The presence of SOX 9 in our study is consistent with the chondrogenic differentiation of the IPFP-ASCs under the influence of TGFβ3 and BMP6. The concomitant increase in collagen type II gene expression, and in particular its continued increase over 4 weeks in culture is indicative of the beginning of hyaline cartilage formation. Furthermore the expression of
of these cells. These two factors, based on the observations we self-aggregation nature and extracellular matrix production ability of the IPFP-ASCs into the 3D chitosan lattice scaffold due to the porosity of the chitosan scaffold may have limited the penetration of a viable chondral graft suitable for in vivo implantation.

In conclusion, infrapatellar fat pad-derived adipose stem cells appear to provide an excellent source of cells with chondrogenic potential. Our results demonstrate the combination of TGFβ3 and BMP6 strongly promotes chondrogenesis with these cells in a 3D chitosan scaffold. This cell-scaffold construct may provide the basis for the cells to naturally aggregate, form a spheroid-like structure and expand in the framework of the scaffold. We have seen this phenomenon in micromass cultures of these cells without scaffold [38,39]. We believe this more accurately mimics the behavior of the formation of an osteochondral unit whereby chondrocytes are fully surrounded in their own matrix supported at their base by subchondral bone which provides strength and support. Whether our cell and chitosan construct behaves like a biphasic ‘implant’ in vivo remains to be seen. Further in vivo studies are required to assess the behavior of this engineered construct.

Author Contributions
Conceived and designed the experiments: KY RF KT SEM GGW PFMC DEM. Performed the experiments: KY RF KT JC. Analyzed the data: KY KT AQ DEM. Contributed reagents/materials/analysis tools: KY KT AQ DEM. Performed the experiments: KY RF KT JC. Analyzed the data: KY KT AQ DEM. Contributed reagents/materials/analysis tools: KY KT AQ DEM. Wrote the paper: KY KT AQ DEM. Contributed reagents/materials/analysis tools: KY KT AQ DEM. Wrote the paper: KY KT AQ DEM.

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| Table 1. List of studies using IPFP as the stem cell source and the growth factors used. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Study                          | Cell Source                     | Culture Type                    | Growth Factors Used             |
| Dragoo et al (2003) [29]        | IPFP-ASC                        | Micromass with fibrin           | IGF1/IGF2                       |
| Khan et al (2008) [39]          | IPFP-ASC                        | 3D cell aggregate               | IGF1/TGFβ3                     |
| Lee et al (2008) [38]           | IPFP-ASC                        | Pellet culture                  | TGFβ1/BMP7                      |
| Jurgens et al (2009) [32]       | IPFP-ASC                        | 3D PLA-CPL Scaffold             | TGFβ1                           |
| Buckley et al (2010) [40]       | IPFP-ASC (Porcine)              | Agarose hydrogel                | TGFβ3                           |

Abbreviations: IGF (insulin growth factor), TGFβ (transforming growth factor beta), BMP (bone morphogenetic protein), PLA-CPL (polylactic-co-E-caprolactone).
