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Expansion in the Presence of FGF-2 Enhances the Functional Development of
Cartilaginous Tissues Engineered using Infrapatellar Fat Pad Derived MSCs

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chondrogenesis.

Abstract

MSCs from non-cartilaginous knee joint tissues such as the infrapatellar fat pad (IFP) and synovium possess significant chondrogenic potential and provide a readily available and clinically feasible source of chondroprogenitor cells. Fibroblast growth factor-2 (FGF-2) has been shown to be a potent mitotic stimulator during *ex vivo* expansion of MSCs, as well as regulating their subsequent differentiation potential. The objective of this study was to investigate the longer term effects of FGF-2 expansion on the functional development of cartilaginous tissues engineered using MSCs derived from the IFP. IFP MSCs were isolated and expanded to passage 2 in a standard media formulation with or without FGF-2 (5ng/ml) supplementation. Expanded cells were encapsulated in agarose hydrogels, maintained in chondrogenic media for 42 days and analysed to determine their mechanical properties and biochemical composition. Culture media, collected at each feed, was also analysed for biochemical constituents.

MSCs expanded in the presence of FGF-2 proliferated more rapidly, with higher cell yields and lower population doubling times. FGF-2 expanded MSCs generated the most mechanically functional tissue. Matrix accumulation was dramatically higher after 21 days for FGF-2 expanded MSCs, but decreased between day 21 and 42. By day 42, FGF-2 expanded MSCs had still accumulated ~1.4 fold higher sGAG and ~1.7 fold higher collagen compared to control groups. The total amount of sGAG synthesised (retained in hydrogels and released into the media) was ~ 2.4 fold higher for FGF-2 expanded MSCs, with only ~25% of the total amount generated being retained within the constructs. Further studies are required to investigate whether IFP derived MSCs have a diminished capacity to synthesise other matrix components important in the aggregation, assembly and retention of proteoglycans. In conclusion, expanding MSCs in the presence of FGF-2 rapidly accelerates chondrogenesis in 3D agarose cultures resulting in superior mechanical functionality.

1. Introduction

MSCs derived from non-cartilaginous knee joint tissues such as the infrapatellar fat pad (IFP) (Dragoo et al. 2003; English et al. 2007; Jurgens et al. 2009; Buckley et al. 2010a; Buckley et al. 2010b) and synovium (Sakaguchi et al. 2005; Yokoyama et al. 2005; Mochizuki et al. 2006; Pei et al. 2008; Pei et al. 2009; Sampat et al. 2011) have been shown to possess significant chondrogenic potential. One of the key challenges in cartilage tissue engineering is ensuring sufficient functionality of the construct to be implanted **which is capable of withstanding the challenging mechanical environment of the joint**. MSCs and chondrocytes have been shown to be phenotypically different (Segawa et al. 2009), and one implication of this may be that they synthesise different amounts of key proteoglycans such as glycosaminoglycans (GAGs) and proteins such as collagens (predominantly Type II) that provide mechanical stability. Previous investigations using MSCs isolated from various tissues have shown they generate tissues with inferior mechanical properties compared to articular chondrocytes similarly maintained in 3D culture (Mauck et al. 2006; Huang et al. 2009; Vinardell et al. 2009; Vinardell et al. 2010). Although MSCs derived from intra-articular joint tissues are more phenotypically similar to chondrocytes (Segawa et al. 2009), we have shown that functionally they are still inferior to chondrocytes (Vinardell et al. 2010). Therefore identifying expansion and differentiation conditions that promote a more chondrogenic phenotype is critical to enhancing their utility for cartilage tissue engineering applications. Differentiation conditions that have been shown to promote the chondrogenic potential of MSCs include a low oxygen (5%) microenvironment (Khan et al. 2007; Buckley et al. 2010a; Meyer et al. 2010), various combinations of growth factors (Mastrogiacomo et al. 2001; Sakimura et al. 2006; Hennig et al. 2007; Diekman et al. 2010; Buxton et al. 2011) and mechanical signals (Huang et al. 2005; Mauck et al. 2007; Huang et al. 2010a; Huang et al. 2010b; Kelly and Jacobs 2010; Li et al. 2010; Thorpe et al. 2010; Haugh et al. 2011).

The chondrogenic capacity of MSCs can also be diminished due to the *in vitro* expansion conditions affecting stemness and accelerating senescence (Solchaga et al. 2010). Augmenting MSC expansion conditions to enhance proliferation kinetics while maintaining multipotency is a critical obstacle to overcome in order to engineer mechanically functional tissues for clinical applications. Fibroblast growth factor-2 (FGF-2, also known as basic fibroblast growth factor) has been shown to be a potent

stimulator during *ex vivo* expansion of both chondrocytes (Kato and Gospodarowicz 1985; Martin et al. 1999; Martin et al. 2001; Veilleux and Spector 2005) and MSCs (Banfi et al. 2000; Mastrogiacomo et al. 2001; Tsutsumi et al. 2001; Bianchi et al. 2003; Solchaga et al. 2005; Khan et al. 2008; Solchaga et al. 2010), as well as regulating subsequent differentiation potential. Specifically, bone marrow derived MSCs cultured in the presence of FGF-2 have been shown to be physically smaller and proliferate more rapidly during monolayer expansion (Solchaga et al. 2005). During chondrogenic differentiation, FGF-2 expanded MSCs have been shown to produce higher levels of proteoglycans with reduced deposition of collagen type I in the peripheral region of pellet cultures while promoting collagen type II formation (Akaogi et al. 2004; Solchaga et al. 2005). In addition, expansion in the presence of FGF-2 has been shown to enhance chondrogenesis of IFP derived MSCs in pellet culture models (Khan et al. 2008).

The objective of this study was to investigate the longer term effects of FGF-2 expansion on the functional development of cartilaginous tissues *in vitro* engineered using MSCs derived from the infrapatellar fat pad. Given that expansion in FGF-2 has been shown to enhance the chondrogenic potential of IFP derived MSCs in short-term pellet culture, we hypothesised that this would translate into enhanced functional properties in long-term culture after encapsulation in agarose hydrogels.

2. Materials and Methods

2.1 Cell isolation and expansion

Porcine IFPs were harvested from the knee joint capsule of three 4 month old porcine donors (~50 kg) within 3 hours of sacrifice. IFPs were weighed, washed thoroughly in phosphate buffered saline (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 collagenase type II (750U/ml, Worthington Biochemical, LanganBach Services, Ireland) and 1% penicillin (100 U/mL)-streptomycin (100 g/mL) for 4 hours. A ratio of 4ml of collagenase (750U/ml) per gram of tissue was found to be optimal based on preliminary experiments.

After tissue digestion, cells were washed, filtered through a 40 µm nylon cell strainer and centrifuged at 650g for 5mins. Supernatant was discarded to remove floating adipocytes and cells were resuspended, counted using a haemocytometer,

and trypan blue exclusion and finally plated in T-75cm² flasks (Sarstedt, Wexford, Ireland) at a density of 5×10^3 cells/cm². Cells were cultured in a standard media formulation (STD) consisting of hgDMEM containing 10% foetal bovine serum and 1% penicillin (100U/mL)–streptomycin (100 mg/mL) (all from GIBCO, Biosciences, Dublin, Ireland) with or without the addition of fibroblast-growth factor-2 (FGF-2, 5ng/ml; ProSpec-Tany TechnoGene Ltd, Israel).

Cultures were expanded to passage two (P2, ~21 days from initial isolation) at a seeding density of 5×10^3 cells/cm² at each passage. Complete medium exchanges were performed twice weekly.

2.2 Calculation of population doubling times

Population doubling (PD) times were determined during passages 1 and 2 based on the initial seeding density and the total cell number harvested, and assuming exponential growth [EQN 1].

$$\text{Population Doubling Time} = \left[\frac{\text{Culture period in days}}{\log_2 \left(\frac{\text{Total cell yield}}{\text{Initial number of cells seeded}} \right)} \right] \quad \text{EQN 1}$$

2.3 Hydrogel construct fabrication and culturing

IFP MSCs (P2) were encapsulated in agarose (Type VII) at ~40°C, to yield a final gel concentration of 2% and a cell density of 15×10^6 cells/mL. The agarose-cell suspension was cast in a stainless steel mould and cored using a biopsy punch to produce cylindrical discs (Ø 5mm×3mm thickness). Constructs were maintained in a chemically defined chondrogenic medium (CDM) consisting of DMEM GlutaMAX™ supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL) (both 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, $1 \times$ insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human transforming growth factor-β3 (TGF-β3; ProSpec-Tany TechnoGene Ltd, Israel). Hydrogel constructs were cultured in standard 12 well plates with one construct per well in a low oxygen (5%) environment which has been previously shown to enhance subsequent chondrogenic differentiation (Khan et al. 2007; Buckley et al. 2010a;

Meyer et al. 2010). Each construct was maintained in 2.5mL of complete medium with complete medium exchanges performed twice weekly for the total culture duration of 42 days.

2.4 Assessment of functional properties

Constructs were mechanically tested in unconfined compression at days 0, 21 and 42 using a standard materials testing machine with a 5N load cell (Zwick Z005, Roell, Germany). A preload of 0.01N was applied to ensure that the surfaces of the gel constructs were in direct contact with the impermeable loading platens. Stress relaxation tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 1 mm/s until 10% strain was obtained and maintained until equilibrium was reached. Dynamic tests were performed immediately after the stress relaxation cycle. A cyclic strain of 1% was applied at a frequency of 1Hz. Further details of the mechanical testing protocol are available elsewhere (Buckley et al. 2009). After mechanical testing, constructs were weighed and the total wet mass recorded. Constructs were cored using a 3mm biopsy punch and separated from the annulus; the wet mass of both the core and annulus was recorded and frozen for subsequent biochemical analyses.

2.5 Quantitative biochemical analysis

Samples were digested at days 0, 21 and 42 in papain (125 g/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 DNA content was measured using a Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes, Biosciences) with a lambda DNA standard. The 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 18hrs in concentrated HCL (38%) and assayed using a chloramine-T assay (Kafienah and Sims 2004) with a hydroxyproline-to-collagen ratio of 1:7.69 (Ignat'eva et al. 2007).

2.6 Histology and Immunohistochemistry

Constructs were fixed in 4% paraformaldehyde, wax embedded and sectioned at 5 μ m to produce a cross section perpendicular to the disc face. 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 immunohistochemistry. Briefly sections were rinsed with PBS and quenched of peroxidase activity for 20 mins and treated with chondroitinase ABC (Sigma, 0.25 units/ml) in a humidified environment at 37°C for 1 hr to enhance permeability of the extracellular matrix by removal of chondroitin sulphate. Slides were again rinsed with PBS and blocked with 10% goat serum for 30 mins. Sections were incubated with mouse monoclonal collagen type I diluted 1:400 (Abcam, UK) (concentration 5.4 mg/ml) or mouse monoclonal anti-collagen type II diluted 1:100 (Abcam, UK) (concentration 1 mg/ml) for 1 hr at room temperature. After washing in PBS, the secondary antibody for type I and type II collagen (Anti-Mouse IgG Biotin antibody produced in goat) (concentration 1 g/L) binding was applied for 1 hr. Colour was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) for 45 min and 5 min exposure to peroxidase DAB substrate kit (Vector laboratories, UK). Slides were dehydrated through ethanol and xyelene and mounted with Vectamount medium (Vector Laboratories, UK). Positive and negative controls (porcine cartilage and ligament) were included in the immunohistochemistry staining protocol for each batch.

2.7 *Sampled media analysis*

Cell culture medium was sampled for each construct at each media exchange (n = 3) and subsequently analyzed for sGAG and collagen secreted to the media. Total media volume was accounted for and the data is presented as the mean of the component released into the media per construct at each time point, and compared to the amount retained in the hydrogel.

2.8 *Statistical analysis*

Numerical and graphical results are presented as mean $\pm$ standard deviation. Statistics were performed using MINITAB 15.1 software package (Minitab Ltd., Coventry, UK). Groups were analyzed for significant differences using a general linear model for analysis of variance with factors of time, growth factor supplementation, construct

region, and interactions between these factors examined. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p < 0.05$. The experiment was replicated a further two times using MSCs isolated from different donors.

3. Results

IFP MSCs expanded in FGF-2 supplemented medium proliferated more rapidly than the control media formulation (STD), with significant differences observed in cell yields and population doubling times at each passage (Fig. 1). MSCs expanded with FGF-2 had a reduced population doubling time of 1.7 ± 0.2 days compared to STD expanded cells of 4.8 ± 1.3 days ($P < 0.05$).

FGF-2 expanded MSCs embedded in agarose hydrogels generated cartilaginous tissues with equilibrium moduli that were 1.9 fold and 1.5 fold higher than MSCs expanded in STD conditions at day 21 and 42 respectively (Fig. 2A). Similarly, the dynamic modulus was 2.9 and 1.9 fold higher for FGF-2 expanded MSC constructs at day 21 and 42 respectively (Fig. 2B). Similar results were observed in two replicate studies using MSCs isolated from different donors (see supplementary Fig. S1). Withdrawal of TGF- β 3 from culture after 21 days was shown to have no beneficial effect on the functional properties of the engineered constructs (see supplementary Fig. S2).

Significant cell proliferation occurred from day 0 to day 21 for both STD and FGF-2 expanded groups following agarose encapsulation, with decreases occurring from day 21 to day 42 (Fig. 3A). By day 42 DNA levels were 1.4 fold and 1.5 fold higher than day 0 levels in STD and FGF-2 groups respectively. No statistical differences in DNA content were observed between STD and FGF-2 expanded MSC constructs at any of the time points investigated.

As a percentage of wet weight, FGF-2 expanded MSCs accumulated 2.5 fold higher total sGAG and 4.7 fold higher collagen content by day 21 compared to STD expanded MSCs. From day 21 to 42 there was a reduction in accumulated sGAG and collagen for the FGF-2 group (Fig 3B&D), although the levels were still higher than STD expanded MSCs. By day 42 sGAG and collagen accumulation was 1.4 fold higher and 1.7 fold higher respectively for FGF-2 expanded cell constructs. Similar differences were observed when normalised by DNA content (Fig. 3C&E).

To quantify the spatial accumulation of extracellular matrix components,

constructs were separated into core (a 3mm full thickness cylindrical biopsy taken from the centre of the engineered tissue) and annulus regions. Higher DNA levels were observed in the annular regions for both expansion formulations at day 21 compared to day 0. A decrease in DNA content occurred in this annular region from day 21 to day 42 for both expansion formulations, although there was no significant change in DNA content within the core (Fig. 4A).

In terms of percentage wet weight, at day 21 no significant difference in annular and core sGAG accumulation existed for either STD or FGF-2 expanded MSC constructs (Fig. 4B). However when normalised by DNA, higher levels were found in the core region for both STD and FGF-2 constructs (Fig. 4C). At day 42 higher sGAG/DNA core levels also existed. For FGF-2 expanded MSC constructs, there was a decline in sGAG levels in the annular region at day 42 compared to day 21.

The annular region of FGF-2 MSC constructs contained the highest level of collagen at 4.3 ± 0.07 % wet weight by day 21 and was 5.6 fold higher than the annulus of STD MSC constructs (Fig. 4D). This difference was also observed when normalised by DNA content. For FGF-2 constructs there was a decline in both annulus and core collagen levels at day 42 compared to day 21 (Fig. 4E).

A significant amount of sGAG was released into the media for both STD and FGF-2 expanded MSC constructs. The release from FGF-2 expanded groups was more pronounced peaking between day 21 and 28 and was 3.3 fold higher than STD expanded MSC constructs at this time point (Fig 5A). By day 42 the total amount of sGAG generated was 2.4 fold higher for FGF-2 expanded MSC constructs. STD constructs retained ~38% of the total amount of sGAG generated with FGF-2 expanded MSC constructs retaining ~25%, although the absolute amount retained was still higher for the FGF-2 group (Fig. 5B). Similar release profiles were observed for collagen, with the peak again occurring between day 21 and 28, although no significant difference was observed between STD and FGF-2 constructs (Fig. 5C). By day 42 the total amount of collagen generated was 1.7 fold higher for FGF-2 compared to STD expanded MSC constructs. STD expanded constructs retained ~72% of the total amount of collagen generated with FGF-2 expanded MSC constructs retaining ~77% (Fig. 5D).

Histological evaluation revealed that constructs stained positively for sulphated proteoglycan and collagen for both culture conditions and the distribution correlated well with biochemical results (Fig. 6). A small region staining weaker for cartilage

specific matrix was observed to develop within FGF-2 expanded constructs by day 42. Minimal collagen Type I staining was observed for both expansion treatment groups (results not shown).

4. Discussion

We have previously shown that MSCs isolated from infrapatellar fat pad tissue are a viable alternative cell source for cartilage tissue engineering (Buckley et al. 2010a; Buckley et al. 2010b; Vinardell et al. 2010). It is evident from the literature that stem cell expansion conditions can significantly influence their subsequent chondrogenic capacity (Tsutsumi et al. 2001; Bianchi et al. 2003; Solchaga et al. 2005; Khan et al. 2008; Solchaga et al. 2010). This study develops upon previous work to ascertain if such expansion conditions, specifically expansion in the presence of FGF-2, could further enhance the mechanical functionality of cartilaginous constructs engineered using IFP derived MSCs. The observed beneficial effects of expanding in the presence of FGF-2 is consistent with previous findings for other sources of stem cells in terms of more rapid proliferation and improved chondrogenic potential evident by increased levels of matrix synthesis (Solchaga et al. 2005; Khan et al. 2008; Solchaga et al. 2010). After 3 weeks of *in vitro* culture, IFP derived MSCs expanded in the presence of FGF-2 generated cartilaginous grafts whose mechanical functionality was at least comparable to that which we have previously reported using other cell types from the same species at the same time point (Thorpe et al. 2008; Buckley et al. 2010a; Meyer et al. 2010; Thorpe et al. 2010; Vinardell et al. 2010). The ability to achieve high cell yields with substantial chondrogenic potency within a short time frame has significant implications for the development of more rapid cell based therapies.

We observed that expansion in the presence of FGF-2 rapidly accelerated subsequent chondrogenesis in 3D agarose cultures up to day 21. These findings are in agreement with pellet culture models using human infrapatellar fat pad derived MSCs expanded in the presence of FGF-2 (Khan et al. 2008). Normalisation of sGAG and collagen contents by DNA suggests that the higher levels of these matrix components retained in constructs at the specific timepoints investigated is due to greater synthetic activity and not due to higher numbers of cells. The decline in total sGAG and DNA content from day 21 to 42 in the FGF-2 expansion group was somewhat unexpected. Rapid cell proliferation in the annular region of the constructs, in conjunction with

significant levels of matrix accumulation, may be leading to oxygen and nutrient transport limitations that affect cell viability within the constructs. Contrary evidence for this hypothesis can be found in the annulus-core data which demonstrates greater reductions in sGAG and DNA levels in the annular region of the constructs compared to the core. It should also be noted, however, that a small region staining weakly for cartilage-specific matrix was observed in the very bottom core of FGF-2 expanded constructs, indicating that separation of constructs into just core and annulus does not provide sufficient spatial detail on DNA levels and matrix accumulation, and furthermore, that nutrient transfer limitations may be negatively impacting matrix accumulation in the centre of the engineered tissue.

As expected, the reduction in sGAG and collagen content from day 21 to day 42 in FGF-2 expanded constructs was accompanied by a decrease in the equilibrium modulus, but no significant change in the dynamic modulus was observed. The relationship between construct dynamic modulus and biochemical composition is complex, and will depend not only on the sGAG and total collagen content, but also on the types of collagen present, cross-linking of the collagen network and the organization of the engineered tissue (Kelly and Prendergast 2004; Kelly et al. 2007; Yan et al. 2009; Nagel and Kelly 2011). Changes in any of these parameters, which were not measured as part of this study, could potentially explain why the dynamic modulus did not decrease with reducing levels sGAG and total collagen accumulation.

A significant release of sGAG from constructs into the media was observed during the 42 day culture period. We have previously observed significant levels of sGAG release from agarose hydrogels seeded with bone marrow derived MSCs (Thorpe et al. 2010). By day 42, only about 25% of the total sGAG generated had been retained within the construct for FGF-2 expanded MSCs. One explanation for the failure to retain high levels of proteoglycan within a tissue engineered construct could be the failure to develop a collagenous network capable of retaining these key matrix components. Generation of sufficient levels of collagen is a well documented challenge in the field of cartilage tissue engineering, however this work demonstrates that IFP derived MSCs are capable of generating high levels of collagen in a relatively short time frame (~3.5% w/w by day 21). Future work comparing IFP derived stem cells to chondrocytes will help to elucidate whether such high levels of sGAG release is a characteristic feature of a chondrocyte-like cell type embedded in agarose hydrogels, or whether IFP derived MSCs have a diminished capacity to synthesise

other matrix components important in the aggregation, assembly and retention of proteoglycans, such as link protein and hyaluronan. Investigating the synthesis of collagenases and aggrecanases is also worthy of future investigation, as this could potentially be affected by FGF-2 expansion. More advanced biomaterials may also aid in retaining or binding of the synthesised matrix components thereby increasing the accumulation of generated matrix components and further enhancing construct mechanical properties.

The temporal profiles of sGAG and collagen release to the media suggest that matrix synthesis peaked shortly before or around day 28 of culture. Cartilage tissue engineering studies using chondrocytes (Byers et al. 2008), and more recently synovium derived stem cells (Sampat et al. 2011), have demonstrated that withdrawal of TGF- β from the media after 2-3 weeks of culture enhances long-term matrix accumulation. In this study the withdrawal of TGF- β after 21 days did not enhance the subsequent functional development of the engineered construct, consistent with our previous findings (Buckley et al. 2010b). A strategy of withdrawing TGF- β from the culture media may eventually overcome the problem of diminishing matrix synthesis in long-term culture, but may depend, among other factors, on further optimization of stem cell isolation and expansion conditions (Sampat et al. 2011) and the cell seeding density (Huang et al. 2009). Another concern is that the long-term culture data presented here could be indicative of some inherent inability of cartilaginous constructs engineered using IFP derived stem cells to generate a fully functional tissue once implanted into a defect, or whether these results are merely a result of suboptimal *in vitro* culture conditions. Clearly the microenvironment of a joint is very different to that within a petri dish, and it may provide the necessary biochemical and biomechanical signals to maintain matrix synthesis following implantation of a construct seeded with IFP derived MSCs. Future *in vivo* studies are required to investigate this critical question.

5. Conclusions

In conclusion, expansion in the presence of FGF-2 rapidly accelerates chondrogenesis of IFP derived MSCs embedded in 3D agarose cultures resulting in superior functional properties. Future studies will focus on strategies to promote the synthesis and assembly of matrix components into fully functional cartilaginous grafts.

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Figure Legends

Fig. 1 – Proliferation kinetics with or without FGF-2 supplementation (5ng/ml) during expansion for all 3 donors combined (A) Cell yield/cm² (B) Population doubling time. Significance compared to MSCs expanded in STD media formulation: * (P< 0.05), ** (P< 0.01).

Fig. 2 – Functional properties of cartilage constructs engineered using infrapatellar fat pad (IFP) derived MSCs at different time points for donor 1. (A) Equilibrium modulus (B) 1Hz dynamic modulus. * significance compared to MSCs expanded in STD media formulation (P< 0.001) at the same timepoint.

Fig. 3 – Total biochemical composition of cell seeded constructs containing STD or FGF-2 expanded MSCs for donor 1. (A) DNA content (ng/mg w/w) (B) GAG content (% w/w) (C) GAG/DNA content (D) collagen content (% w/w) and (E) collagen/DNA content. * significance compared to previous time point within the same expansion group (P<0.05). [†] compared to MSCs expanded in STD media at the same time point (P<0.05).

Fig. 4 – Regional biochemical composition of cell seeded constructs containing STD or FGF-2 expanded MSCs for donor 1. Constructs were separated into core (Ø3mm) and annular regions (Ø5mm). (A) DNA content (ng/mg w/w) (B) GAG content (% w/w) (C) GAG/DNA content (D) collagen content (% w/w) and (E) collagen/DNA content. * significance compared to corresponding annulus within the same expansion group at the same time point (P<0.05). [†] compared to the same region at day 21 for the same expansion group (P<0.05).

Fig. 5 – (A) Weekly release of sGAG into media (B) Total sGAG content generated, released into media and retained by the hydrogel at day 42. (C) Weekly release of collagen into media (D) Total collagen content generated, released into media and retained by the hydrogel at day 42. Data presented is for donor 1. * significance compared to MSCs expanded in STD media at the same time point (P<0.01), [†] compared to previous time point for the same expansion media formulation (P<0.01). ^{\$} compared to amount released for the same expansion media formulation (P<0.0001). [#] compared to STD media formulation (P<0.0001).

Fig. 6 – Histological evaluation at day 21 and 42 for donor 1. Alcian blue staining for sulphated proteoglycan (sGAG), picro-sirius red for total collagen and immunohistological staining for collagen type II (Col II). Scale bar is 1mm.

Supplementary Fig. S1 –Functional properties of cartilage constructs engineered using infrapatellar fat pad (IFP) derived MSCs for three different donors at day 21 and 42. (A) & (C) Equilibrium modulus (B) & (D) 1Hz dynamic modulus. Significance compared to MSCs expanded in STD media formulation: * (P< 0.05), ** (P< 0.01), *** (P< 0.001).

Supplementary Fig. S2 – The effect of removal of TGF- β 3 from culture after 21 days on (A) equilibrium modulus and (B) 1Hz dynamic modulus at day 42 for donor 3. ^s significance compared to day 21 for the same expansion media formulation and TGF- β 3 supplementation. [#] significance of continued supplementation with TGF- β 3 (TGF+) compared to withdrawal after 21 days (TGF-), ($P < 0.05$). Dashed line indicates mean day 21 level.

Figure 1

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