

**Stem cells display a donor dependent response to escalating levels of growth factor release from extracellular matrix-derived scaffolds**

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**Abstract:** Numerous growth factor delivery systems have been developed for tissue engineering. However, little is known about how the dose of a specific protein will influence tissue regeneration, or how different patients will respond to altered levels of growth factor **presentation**. The objective of this study was to assess stem cell chondrogenesis within extracellular-matrix (ECM)-derived scaffolds **loaded** with escalating levels of TGF- $\beta$ 3. It was also sought to determine if stem cells display a donor dependent response to different doses of TGF- $\beta$ 3 (low-5 ng to high-200 ng) released from such scaffolds. **It was found that ECM-derived scaffolds possess the capacity to bind and release increasing amounts of TGF- $\beta$ 3**, with between 60-75% of this growth factor **released** into the media over the first 12 days of culture. After seeding these scaffolds with human infrapatellar fat pad-derived stem cells (FPSCs), it was found that cartilage specific ECM accumulation was greatest for the higher levels of growth factor loading. Importantly, soak-loading cartilage ECM-derived scaffolds with high levels of TGF- $\beta$ 3 always resulted in at least comparable levels of chondrogenesis as continuously supplementing the media with this growth factor. Similar results were observed for FPSCs from all donors, although the absolute level of secreted matrix did vary from donor-to-donor. Therefore while no single growth factor release profile will be optimal for all patients, the results of this study suggest that the combination of a highly porous cartilage ECM-derived scaffold, coupled with appropriate **levels of TGF- $\beta$ 3**, can consistently drive chondrogenesis of **adult** stem cells.

**Keywords:** Cartilage; Extracellular matrix (ECM); Stem cells; TGF- $\beta$ ; Donor variability; Chondrogenesis; Growth factor dosage.

## 1. Introduction

Articular cartilage regeneration still is a challenge in orthopaedic medicine. While cell based therapies have been reasonably successful clinically (Hunziker, 2002; Madeira *et al.*, 2015), they are expensive and do not consistently result in hyaline cartilage regeneration. Tissue repair can potentially be augmented by the use of porous scaffolds to provide an environment more conducive to regeneration (O'Brien, 2011; Vinatier *et al.*, 2009). Extracellular matrix (ECM)-derived scaffolds have shown particular promise in this regard, having been used in the repair of different tissues including cartilage (Almeida *et al.*, 2015; Almeida *et al.*, 2014; Benders *et al.*, 2013; Cheng *et al.*, 2014; Cheng *et al.*, 2009; Cheng *et al.*, 2011; Cheng *et al.*, 2013; Diekman *et al.*, 2010; Rowland *et al.*, 2013; Sutherland *et al.*, 2015).

Chondrogenesis is enhanced in the presence of members of the transforming growth factor (TGF)- $\beta$  family of growth factors, motivating the delivery of such proteins to articular cartilage defects to enhance regeneration (Madry *et al.*, 2014). This multifunctional cytokine not only modulates stem cell differentiation, but has also been shown to regulate chondrocyte proliferation and matrix turnover in articular cartilage (Albro *et al.*, 2013; Roberts *et al.*, 1990). Various scaffolds and hydrogels can be used to deliver cells and growth factors such as TGF- $\beta$ , thereby opening up the possibility of using such functionalized biomaterials to enhance chondrogenesis during articular cartilage repair (Almeida *et al.*, 2015; Almeida *et al.*, 2014; Benders *et al.*, 2013; Cheng *et al.*, 2014). However in the context of TGF- $\beta$  mediated articular cartilage regeneration, the optimal growth factor dose or release profile is still unclear

(Madry *et al.*, 2014; Tang *et al.*, 2009). Furthermore, it remains unclear how different patients will respond to altered levels of growth factor delivery.

A number of different strategies have been developed to control the release of growth factors to accelerate tissue repair (Madry *et al.*, 2014). TGF- $\beta$  is widely used to promote chondrogenesis *in vitro* (Madry *et al.*, 2014; Tang *et al.*, 2009), however its short half-life *in vivo* can limit its availability following delivery (Dinbergs *et al.*, 1996; Madry *et al.*, 2014; Nimni, 1997). Therefore in the context of articular cartilage regeneration, it is crucial to develop scaffolds to temporally and spatially control the release of TGF- $\beta$  to direct the differentiation of stem cells down a chondrogenic pathway (Madry *et al.*, 2014; Tang *et al.*, 2009). Established *in vitro* culture protocols that have been optimized to drive chondrogenesis can provide clues as to the optimal TGF- $\beta$  dosing and release rates required to drive chondrogenesis using growth factor delivery systems. Specifically, temporal exposure (2 weeks) to specific concentrations (10 ng/ml) of TGF- $\beta$  has been shown to promote robust chondrogenesis *in vitro* (Chung and Burdick, 2009; Fan *et al.*, 2008; Shintani and Hunziker, 2011). Furthermore, it is known that continuous stimulation with TGF- $\beta$  can promote undesirable pathologies such as fibrosis and osteophyte formation (Blaney *et al.*, 2006; Border and Ruoslahti, 1992; Elford *et al.*, 1992; Kopesky *et al.*, 2011; Madry *et al.*, 2014; Mi *et al.*, 2003; Mierisch *et al.*, 2002; Nimni, 1997; van den Berg *et al.*, 1993; van der Kraan and van den Berg, 2007; Wilson *et al.*, 2009). This motivates the development of delivery systems capable of supplying growth factor within a 2 week dosing window (Bian *et al.*, 2011; Mehlhorn *et al.*, 2006). Different approaches have been used for exogenous chondrogenic

growth factor delivery for cartilage tissue engineering, including from scaffolds (Almeida *et al.*, 2015; Almeida *et al.*, 2014; Matsiko *et al.*, 2015) and/or from microsphere delivery systems (Ahearne *et al.*, 2011; Dikina *et al.*, 2015; Solorio *et al.*, 2012; Solorio *et al.*, 2012). Delivery of TGF- $\beta$  from cartilage ECM-derived scaffolds is particularly promising in this regard, as the biomaterial itself is inherently chondrogenic, and furthermore, this growth factor is known to bind strongly to ECM (Dinbergs *et al.*, 1996). The negatively charged proteoglycans present in the pericellular matrix and ECM have been shown to bind and modulate the positively charged TGF- $\beta$ 3, thereby regulating the availability of such growth factors (Macri *et al.*, 2007).

Hence, the overall objective of this study was to assess the effect of delivering different doses of TGF- $\beta$ 3 from cartilage ECM-derived scaffolds on chondrogenesis of human infrapatellar fat pad-derived stem cells (FPSCs). Furthermore, given the well documented donor-to-donor variability that exists in stem cell populations (Koller *et al.*, 1996; Phinney *et al.*, 1999), it was also sought to determine the effects of delivering different doses of TGF- $\beta$ 3 from ECM-derived scaffold on FPSCs isolated from a range of healthy and diseased (osteoarthritic) donors.

## **2. Material and methods**

### **2.1. ECM-derived scaffold preparation**

The scaffolds were fabricated using a protocol previously developed (Almeida *et al.*, 2015). Briefly, cartilage used in the fabrication of ECM-derived scaffolds was harvested under sterile conditions from the femoral condyles of three months old female pigs. Cartilage was first broken-up into small pieces

using a scalpel. Furthermore, the scaffolds were fabricated using pulverized cartilage processed by a cryogenic mill (6770 Freezer/Mill, SPEX, UK). Then the slurry was homogenized in ultra pure water (UPW) using a homogenizer (IKAT10, IKA Works Inc, NC, USA) to create a fine cartilage slurry of 250 mg of particulated cartilage for 1 ml of UPW. The slurry was transferred to custom made moulds (containing wells 5 mm in diameter and 3 mm in height) and freeze-dried (FreeZone Triad, Labconco, KC, USA) to produce porous scaffolds, as previously described (Almeida *et al.*, 2015). Briefly, the slurry was frozen to  $-30^{\circ}\text{C}$  ( $1^{\circ}\text{C}/\text{min}$ ) and kept at that temperature for one hour. Followed by an increase to  $-10^{\circ}\text{C}$  ( $1^{\circ}\text{C}/\text{min}$ ) under vacuum for 24 hours and then finally to room temperature ( $0.5^{\circ}\text{C}/\text{min}$ ). Subsequently, two different crosslinking techniques were applied to the scaffolds. The scaffolds underwent DHT and 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) crosslinking as previously described in literature (Haugh *et al.*, 2011). The DHT process was performed in a vacuum oven (VD23, Binder, Germany), at  $115^{\circ}\text{C}$ , in 2 mbar for 24 hours. The EDAC (Sigma-Aldrich, Germany) crosslinking consisted of chemical exposure for 2 hours at a concentration of 6 mM in the presence of N-Hydroxysuccinimide (NHS) (Sigma-Aldrich, Germany), a catalyst that is commonly used with EDAC. A molar ratio of 2.5 M EDAC/M N-Hydroxysuccinimide was used (Haugh *et al.*, 2011; Olde Damink *et al.*, 1996).

## **2.2. Scanning Electron Microscopy (SEM) and Light Microscopy**

Acellular ECM-derived scaffolds were imaged using scanning electron microscopy (SEM) (Figure 1). Structures were fixed in 4% paraformaldehyde solution (PFA) overnight. Furthermore, scaffolds were dehydrated through

successive graded ethanol baths (10-100%), fixed onto aluminium stubs, coated with gold and examined under a field emission scanning electron microscope (Tescan Mira FEG-SEM XMU, Libušina, Czech Republic). Routine light microscopy was also used for morphometrical and histological analysis.

### **2.3. Cell and construct culture**

Ethical approval for the isolation of human infrapatellar fat pad (IFP) derived stem cells (FPSCs) was obtained from the institutional review board of the Mater Misericordiae University Hospital Dublin. Cells were isolated from the IFP of patients undergoing total joint arthroplasty (diseased) or anterior cruciate ligament (ACL) surgery (healthy). Cells (Donor A: Male, 26; Donor B: Male, 26; Donor C: Male, 19; Donor D: Male, 34; Donor E: Female, 67; Donor F: Female, 66) were isolated as previously described (Almeida *et al.*, 2015). Briefly, IFPs were harvested, weighed and washed thoroughly in phosphate buffered saline (PBS) (Sigma-Aldrich, Germany). Next, the tissue was diced in sterile conditions and followed by rotation at 37°C in high-glucose Dulbecco's Modified Eagle Medium (hgDMEM, GlutaMAX™)(GIBCO, Biosciences, Ireland) containing collagenase type II (750 U/ml, Worthington Biochemical, LaganBach Services, Ireland) and 1% penicillin (100 U/ml)-streptomycin (100 µg/ml) for approximately 4 hours. A ratio of 4 ml of collagenase (750 U/ml) per gram of tissue was found to be optimal based on previous work (Buckley and Kelly, 2012; Buckley *et al.*, 2010; Liu *et al.*, 2012). After digestion, cells were washed, filtered (40 µm nylon cell strainer) and centrifuged at 650 g for 5 minutes. The supernatant was removed. The remaining cells were re-suspended, counted and finally plated ( $5 \times 10^3$  cells/cm<sup>2</sup>) in T-175 flasks (Sarstedt, Wexford, Ireland).

Cells were cultured in a standard media formulation, which consisted of hgDMEM containing 10% foetal bovine serum and 1% penicillin (100 U/ml)-streptomycin (100 mg/ml) (GIBCO, Biosciences, Ireland) with the addition of fibroblast-growth factor-2 (FGF-2, 5 ng/ml; ProSpec-Tany TechnoGene Ltd, Israel). Cells were expanded to passage 2 (P2), with an initial seeding density of  $5 \times 10^3$  cells/cm<sup>2</sup> at each passage. Media changes were performed twice a week.

Each scaffold was seeded with  $0.5 \times 10^6$  human FPSCs in 40  $\mu$ l of media. Constructs were maintained in chemically defined chondrogenic medium (CDM), as previously described, for 28 days (at 5% O<sub>2</sub> and 37°C) (Almeida *et al.*, 2015; Buckley and Kelly, 2012). CDM consisted of DMEM GlutaMAX™ supplemented with penicillin (100 U/ml)-streptomycin (100  $\mu$ g/ml) (both GIBCO, Biosciences, Ireland), 100  $\mu$ g/ml sodium pyruvate, 40  $\mu$ g/ml L-proline, 50  $\mu$ g/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1x insulin-transferrin-selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human growth factor- $\beta$ 3 (TGF- $\beta$ 3; ProSpec-Tany TechnoGene Ltd, Israel). For groups described in the text as loaded with TGF- $\beta$ 3, additional TGF- $\beta$ 3 was not added to the media during the culture period. Instead, TGF- $\beta$ 3 was soak loaded into the scaffold and was not directly added to the culture media. For the first part of the study, where the dosing effects were first assessed for a single donor, the experimental groups were: Low dose (3 ng), Medium (30 ng), High (300 ng) and Media supplemented (10 ng/ml). For the second part of this study, six different cell donors were assessed with low (5 ng), medium (50 ng), high (200 ng) and media supplementation (10 ng/ml) growth factor dosage.



(Note: For a typical chondrogenic culture media - 10ng/ml of TGF- $\beta$ 3, 2.5ml of media per scaffold, 2 media changes per week - the total amount of TGF- $\beta$ 3 used per week is 50ng. This equates to 200-300ng of growth factor over a 4-6 week culture period. This motivated the choice of the dosing for the 'high' TGF- $\beta$ 3 groups). For all experiments, the scaffolds were kept in 12 well plates (1 scaffold per well) and each scaffold was placed within cylindrical agarose moulds. After cell seeding, the scaffolds were kept in the incubator for two hours. After two hours, 2.5 ml of media was added to each well. Media changes were performed twice a week. The media was stored at -85°C for further analysis.

#### **2.4. Biochemical analysis**

Constructs were biochemically analyzed at day 28, for sulphated glycosaminoglycan (sGAG) and collagen content (n=6), as previously described (Almeida *et al.*, 2015; Buckley *et al.*, 2010). Briefly, constructs were enzymatically digested by incubation in papain (125  $\mu$ 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 rotation (10 rpm) for 18 hours. The proteoglycan content was estimated by quantifying the sGAG in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), using bovine chondroitin sulphate as a standard. Collagen content was determined by measuring hydroxyproline content, after acidic hydrolysis of the samples at 110°C for 18 h in concentrated HCL (38%). Samples were assayed using a chloramine-T assay assuming a hydroxyproline/collagen ratio of 1:7.69 (Ignat'eva *et al.*, 2007).

## 2.5. Histology and immunohistochemistry

Constructs were fixed overnight at 4°C in a 4% solution of paraformaldehyde (Sigma-Aldrich, Ireland), followed by washing in PBS (Sigma-Aldrich, Ireland), dehydrated and wax embedded, as previously described (Almeida *et al.*, 2015). Wax embedded constructs were sectioned in 6 µm thick slices and mounted in microscope slides. Sections were stained with 1% Alcian blue 8GX (Sigma-Aldrich) in 0.1 M HCl for sGAG and with Picrosirius red for collagen. With the aim of monitoring the newly formed sGAG, constructs were histologically analysed (Alcian blue) at day 28. Immunohistochemical analysis was performed on 6 µm sections using monoclonal antibody to type II collagen (Abcam, UK). Samples were washed in PBS and subjected to peroxidase activity (20 min), incubated (1 hour, 37°C in a moist environment) with chondroitinase ABC (Sigma, 0.25 U/ml) with the aim of enhancing the permeability of the ECM by removing the chondroitin sulphate. Slides were rinsed with PBS and blocked with 10% goat serum (30 minutes) and incubated with mouse monoclonal anti-collagen type II diluted 1:100 (Abcam, UK; concentration 1 mg/ml; 1 hour at RT). A secondary antibody for type II collagen (Anti-Mouse IgG Biotin antibody produced in goat; concentration 1 g/L) binding was then applied (1 hour). By using Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) for 5 min in peroxidase DAB substrate kit (Vector laboratories, UK), it was possible to observe a colour alteration. Samples were dehydrated with graded ethanol and xylene and mounted with Vectamount medium (Vector Laboratories, UK).

## **2.6. Measurement of TGF- $\beta$ 3 release from ECM scaffolds**

TGF- $\beta$ 3 release from acellular scaffolds was determined via ELISA, as previously described (Albro *et al.*, 2013; Almeida *et al.*, 2014; Vonwil *et al.*, 2008). 96 well plates were coated with capture antibody (360  $\mu$ g/ml) with mouse anti-human TGF- $\beta$ 3 (R&D Systems, UK). Samples and TGF- $\beta$ 3 standards (ProSpec-Tany TechnoGene Ltd, Israel) were incubated for 2 hours. After washing and drying, detection antibody (18  $\mu$ g/ml of biotinylated goat anti-human TGF- $\beta$ 3) was added to the plate and incubated (2 hours). The next step was to wash, dry and incubate the plate in streptavidin-HRP (horseradish-peroxidase; R&D Systems, UK) for 20 minutes in the dark. Substrate solution (1:1 mixture of H<sub>2</sub>O<sub>2</sub> and tetramethylbenzidine; R&D Systems, UK) was added to each well, followed by incubation (20 minutes) avoiding direct light. Stop solution (2 N H<sub>2</sub>SO<sub>4</sub>; Sigma-Aldrich, Germany) was added and the optical density was determined immediately with a plate reader (450 nm).

## **2.7. Statistical analysis**

Results are presented as mean  $\pm$  standard deviation. Statistical analysis was performed with MINITAB 15.1 software package (Minitab Ltd., Coventry, UK). Experimental groups were analyzed for significant differences using a general linear model for analysis of variance with factors including growth factor dose and cell donors. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of  $p < 0.05$ .

### **3. Results**

#### **3.1. Cartilage ECM-derived scaffolds can bind and control the release of escalating doses of TGF- $\beta$ 3**

Three different doses (low-3 ng, medium-30 ng and high-300ng) of TGF- $\beta$ 3 were loaded onto cartilage ECM derived scaffolds, which were then seeded with human FPSCs and maintained *in vitro* for 4 weeks. Cartilage ECM-derived scaffolds possessed a consistent capacity to bind and release increasing amounts of TGF- $\beta$ 3 (Figure 2). Irrespective of the concentration of TGF- $\beta$ 3 applied, the scaffolds released between 60-75% of the loaded growth factor into the media over the first 12 days of culture. Over this time period, the low group released a total of  $2.4\pm 0.2$  ng of TGF- $\beta$ 3 (Figure 2A), the medium group released  $21.4\pm 2.3$  ng (Figure 2B) and high dose group released  $198.0\pm 25.4$  ng (Figure 2C). In terms of the total percentage of growth factor released over 12 days, this corresponded to  $74.0\pm 6.0\%$  of loaded TGF-  $\beta$ 3 released from the low group (Figure 2D),  $67.0\pm 7.0\%$  from the medium group (Figure 2E) and finally  $60.8\pm 7.0\%$  from the high group (Figure 2F).

#### **3.2. Chondrogenesis within ECM-derived scaffolds seeded with human stem cells strongly depends on the dose of TGF- $\beta$ 3 loaded into the construct**

After 4 weeks in culture, only scaffolds loaded with either medium or high doses of TGF- $\beta$ 3 resembled cartilage macroscopically (Figure 3I and M). These engineered tissues also appeared macroscopically similar to constructs were TGF- $\beta$ 3 was continuously added to the culture media (Figure 3Q; Note: a total

of 200 ng of TGF- $\beta$ 3 was added to the media of this group over the 28 day culture period). Histologically, very little cartilage matrix deposition was observed in scaffolds that were not exogenously stimulated with TGF- $\beta$ 3 (Figure 3B-D), appearing similar to day-zero scaffolds (data not shown). Alcian Blue staining suggested little sGAG accumulation within scaffolds loaded with low and medium doses of TGF- $\beta$ 3 (Figure 3F and J). Conversely, robust sGAG deposition was observed in scaffolds loaded with high levels of TGF- $\beta$ 3, or where the growth factor was directly supplemented in the culture media (Figure 3N and R). Collagen matrix staining was strong for medium dose (Figure 3K), high dose (Figure 3O) and media supplemented (Figure 3S) scaffolds. Staining for type II collagen was most intense within scaffolds loaded with high doses of TGF- $\beta$ 3 (Figure 3P).

Total sGAG content after 4 weeks of culture period was highest in scaffolds loaded with a high dose of TGF- $\beta$ 3, being at least comparable to constructs where the growth factor was continuously supplemented to the media (Figure 4A). Collagen content was significantly lower for the low dose group when compared with the other scaffolds (Figure 4B).

### **3.3. Stem cells display a donor dependent response to TGF- $\beta$ 3 delivery from ECM-derived scaffolds**

Having demonstrated that cartilage ECM derived scaffolds can be used to control the delivery of different doses of TGF- $\beta$ 3, and that chondrogenesis within these scaffolds was dependent on the amount of growth factor loaded onto the scaffold, we next sought to determine if FPSCs display a donor dependant response to the release of different doses of TGF- $\beta$ 3 from these

constructs. To this end, FPSCs from a range of healthy and diseased donors were used. After 4 weeks in culture, sGAG staining (alcian blue) was weak for all six donors (A-F) (E and F osteoarthritic - OA) when a low dose (5 ng per scaffold) of TGF- $\beta$ 3 was loaded onto the scaffolds (Figure 5). For a medium dose (50 ng) of growth factor, reasonable levels of chondrogenesis (as evidenced by Alcian Blue staining for sGAG deposition) were observed for 2 of the 6 donors (donor C and F). With the exception of donor A, robust chondrogenesis was observed in all cases when high doses of TGF- $\beta$ 3 were loaded onto the scaffolds (Figure 5). Robust collagen deposition (picro-sirius red staining) was observed for all donors in all conditions, with the exception of donor A when stimulated with a low dose of growth factor (Figure 5). In agreement with previous studies using a pellet culture system (Liu *et al.*, 2014), the disease state of the donors (either healthy or osteoarthritic) did not appear to impact chondrogenesis of FPSCs (Figure 5).

Quantification of the total sGAG content after 28 days of culture within constructs initially loaded with increasing doses of TGF- $\beta$ 3 generally agreed with the histological analysis of the engineered tissues (Figure 6). For all donors, the highest levels of sGAG accumulation were observed in scaffolds loaded with high doses of TGF- $\beta$ 3. For this high dose, sGAG accumulation was highest for donor B, reaching close to 470  $\mu$ g, in comparison to approximately 260  $\mu$ g for donor A. Again, there was no evidence of diminished sGAG deposition using FPSCs isolated from diseased (OA) donors (Figure 6).

#### 4. Discussion

The overall goal of this study was to assess the capacity of cartilage ECM-derived scaffolds to bind and release different amounts of soak loaded TGF- $\beta$ 3. Furthermore, it was also sought to determine if adult stem cells display a donor dependent response to the binding and release of different doses of TGF- $\beta$ 3 from such ECM-derived scaffolds. These scaffolds possessed a significant capacity to bind and deliver increasing amounts of TGF- $\beta$ 3. They released between 60-75% of the loaded TGF- $\beta$ 3 into the media over 12 days of culture, irrespective of the concentration of growth factor added to the scaffold, with the remainder apparently sequestered within the ECM. After a 28 day culture period, only constructs loaded with either medium or high doses of TGF- $\beta$ 3 resembled cartilage macroscopically. Histologically, very little cartilage matrix deposition was observed in scaffolds that were not stimulated with TGF- $\beta$ 3. Robust sGAG and collagen deposition was observed in scaffolds loaded with high levels of growth factor, or where the TGF- $\beta$ 3 was regularly supplemented into the media. It was also observed that staining for type II collagen, the main collagen type in articular cartilage, was most intense within scaffolds loaded with high doses of TGF- $\beta$ 3. Having demonstrated that cartilage ECM-derived scaffolds can be used to control the delivery of different doses of TGF- $\beta$ 3, and that chondrogenesis within these scaffolds was dependent on the amount of growth factor loaded onto the scaffold, we next sought to determine if FPSCs display a donor dependant response to the release of different doses of TGF- $\beta$ 3 from these constructs. To this end, FPSCs from a range of healthy and diseased donors were seeded into cartilage ECM derived scaffolds.

Chondrogenesis was observed in most of the cases when high doses of growth factor were used. The donor's disease state (healthy or osteoarthritic) did not impact chondrogenesis in these ECM derived scaffolds.

The release profile of TGF- $\beta$ 3 from cartilage ECM-derived scaffolds was relatively insensitive to the dose of growth factor loaded into the stem cell laden construct, with between 60 and 75% of the growth factor released over the first 12 days of culture. The growth factor interaction with the scaffold is possible due to known non-specific binding sites present in native cartilage ECM (Albro *et al.*, 2013). Binding sites for TGF- $\beta$  include proteoglycans, glycosaminoglycans, collagens and glycoproteins, and these interactions are known to influence growth factor availability (Albro *et al.*, 2013). **Previous studies have used gelatin (denatured collagen) microspheres to control the release of growth factors such as TGF- $\beta$  (Ahearne *et al.*, 2011; Holland *et al.*, 2003).** Positively charged growth factors can be absorbed by these lyophilized microspheres by creation of a polyion complex with gelatin (Ahearne *et al.*, 2011; Holland *et al.*, 2003). TGF- $\beta$  is released when this complex is compromised, or via material degradation due to cell mediated proteolysis. Additionally, the rate of release of such growth factor is dependent on the polymer degree of crosslinking (Ahearne *et al.*, 2011; Young *et al.*, 2005). With these gelatin microspheres, approximately 10% of loaded TGF- $\beta$ 1 is released over 15 days. Chondroitin/hyaluronic-acid scaffolds containing gelatin microspheres have also been used as TGF- $\beta$ 3 delivery systems, which showed an initial burst release of 37% of loaded growth factor, with 80% released after 18 days (Fan *et al.*, 2006a; Fan *et al.*, 2006b). Other examples include a hybrid



system consisting of type I/II collagen and TGF- $\beta$ 1-loaded fibrin (Dickhut *et al.*, 2010), and a heparin/fibrin/PCL system which supported *in situ* chondrogenesis of adipose-derived stem cells while releasing approximately 65% of the initial TGF- $\beta$ 1 loaded into the construct during the first week of culture (Jung *et al.*, 2009). The release of bound growth factor from stem cell laden scaffolds may also depend on rate of new tissue development within the scaffold, which may act as a new sink for TGF- $\beta$ 3 as it is released from the scaffold. Higher levels of new tissue is deposited within scaffolds loaded with the high doses of exogenous TGF- $\beta$ 3, which may explain why a higher total amount of growth factor appears sequestered within these construct after 12 days of culture.

In agreement with previous studies (Almeida *et al.*, 2015), exogenous growth factor (TGF- $\beta$ 3) stimulation was necessary to induce chondrogenesis of FPSCs, even in the presence of native cartilage ECM. Cartilage specific matrix accumulation within these FPSCs seeded scaffolds was greatest for the higher levels of growth factor loading. It has previously been shown that TGF- $\beta$  enhances chondrogenesis of stem cells in a dose dependent manner (Worster *et al.*, 2000). The gold standard for promoting chondrogenesis of mesenchymal stem cells *in vitro* is to supplement the media with 10 ng/ml of TGF- $\beta$ 3; however the dosage required to drive chondrogenesis *in vivo* from a growth factor delivery scaffold has yet to be elucidated. Previously reported TGF- $\beta$  dosing studies have demonstrated that a brief exposure to high concentrations of TGF- $\beta$  (100 ng/ml for 30 min) was beneficial to chondrogenesis (Miura *et al.*, 2002). This suggests that the TGF- $\beta$  burst release from the ECM-derived scaffold observed in the first three days of the current study may be advantageous for

driving robust chondrogenesis of stem cells seeded in the scaffold. There are, however, potential drawbacks associated with excessive TGF- $\beta$  release from a scaffold into the joint space that need to be considered when developing any growth factor delivery system. For example, it has previously been reported that 21 days after a single joint injection of 200 ng of TGF- $\beta$ 1, sGAG synthesis was significantly increased in a murine knee joint model (van Beuningen *et al.*, 1998). However, repeated growth factor injections can lead to TGF- $\beta$ 1-induced osteophyte formation (van Beuningen *et al.*, 1998). Therefore, there is a clear need to perform additional *in vivo* studies to identify the dose of growth factor which will accelerate articular cartilage regeneration without promoting undesirable side effects.

Human stem cells displayed a donor dependant response to escalating levels of exogenous growth factor stimulation within ECM-derived scaffolds, although importantly the greatest levels of chondrogenesis were consistently observed within constructs loaded with the higher levels of TGF- $\beta$ 3. It is known that bone marrow-derived mesenchymal stem cells from different donors may have dissimilar responses to environmental *stimuli* (Koller *et al.*, 1996; Phinney *et al.*, 1999; Pilgaard *et al.*, 2009; Solorio *et al.*, 2012). In the current study we observed that if the dose of TGF- $\beta$ 3 loaded onto cartilage ECM derive scaffolds is increased (specifically to the high dose), similar level of chondrogenesis by infrapatellar fat pad derived stem cells from a range of healthy and diseased donors was found. This suggests that the combination of an optimized chondro-inductive scaffolds (Almeida *et al.*, 2015), coupled with appropriate growth factor presentation, can minimize the donor-to-donor variability in

chondrogenesis traditionally associated with adult human stem cells. Such consistency in outcome will facilitate the development, and future implementation, of more effective treatments for human cartilage repair.

## **5. Conclusion**

This study demonstrates that by delivering a high dose of TGF- $\beta$ 3 from a cartilage ECM-derived scaffold that it is possible to minimize donor-to-donor variability in chondrogenesis of human infrapatellar fat pad-derived stem cells. Furthermore, no major difference was observed in the chondrogenic capacity of FPSCs from healthy and diseased donors when seeded onto such growth factor delivery scaffolds. Such consistency in chondrogenesis across a range of human donors should facilitate the future clinical translation of such treatments for articular cartilage regeneration.

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### Figure Captions

**Figure 1** – Scanning electron micrographs of the porous ECM-derived scaffold in low (A) and high (B) magnification.

**Figure 2** – Total cumulative TGF- $\beta$ 3 content (ELISA) of the culture media for day 3, 7 and 12 of culture for low (A), medium (B) and high (C) growth factor loaded groups (n=3). Release profile into the media for the first 12 days of culture for low (D), medium (E) and high (F) TGF- $\beta$ 3 loaded groups (n=3).

**Figure 3** – Macroscopic images of ECM-derived scaffolds seeded with infrapatellar fat pad-derived stem cells after 4 weeks in culture for no TGF- $\beta$ 3 (A), low (E), medium (I), high (M) and direct media TGF- $\beta$ 3 supplementation (Q). Alcian blue, picro-sirius red and type II collagen staining for no TGF- $\beta$ 3 (B-D), low (F-H), medium (J-L), high (N-P) and direct media TGF- $\beta$ 3 supplementation (R-T). Scale bar: 50  $\mu$ m.

**Figure 4** – (A) sGAG and (B) collagen accumulation within ECM-derived constructs seeded with infrapatellar fat pad-derived stem cells after 4 weeks of culture for media, low, medium and high TGF- $\beta$ 3 supplementation (n=5, \* $p$ <0.05). Dashed line represents day 0 values for acellular scaffolds.

**Figure 5** – Alcian blue and picro-sirius red staining for low, medium, high and media TGF- $\beta$ 3 supplementation for six different donors (A-F), healthy and diseased (osteoarthritic - OA). All micrographs are for 4 weeks in culture with human infrapatellar fat pad-derived stem cells. Scale bar: 50  $\mu$ m.

**Figure 6** – sGAG accumulation within ECM-derived constructs after 4 weeks culture period with infrapatellar fat pad-derived stem cells for media, low, medium and high TGF- $\beta$ 3 supplementation for six different donors (A-F) (n=5, \*p<0.05). Dashed line represents day 0 value for acellular scaffolds.

**Fig 1**

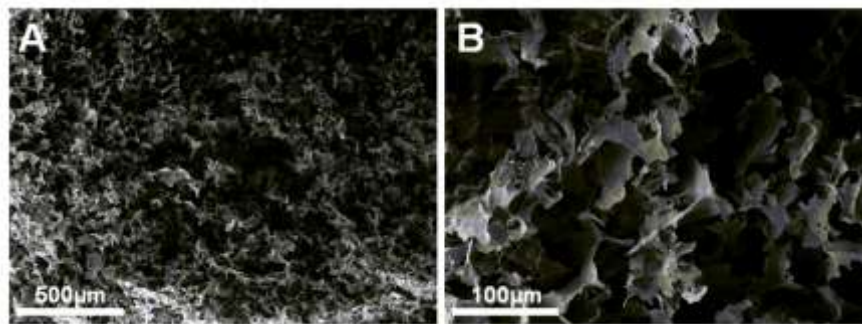


Fig 2

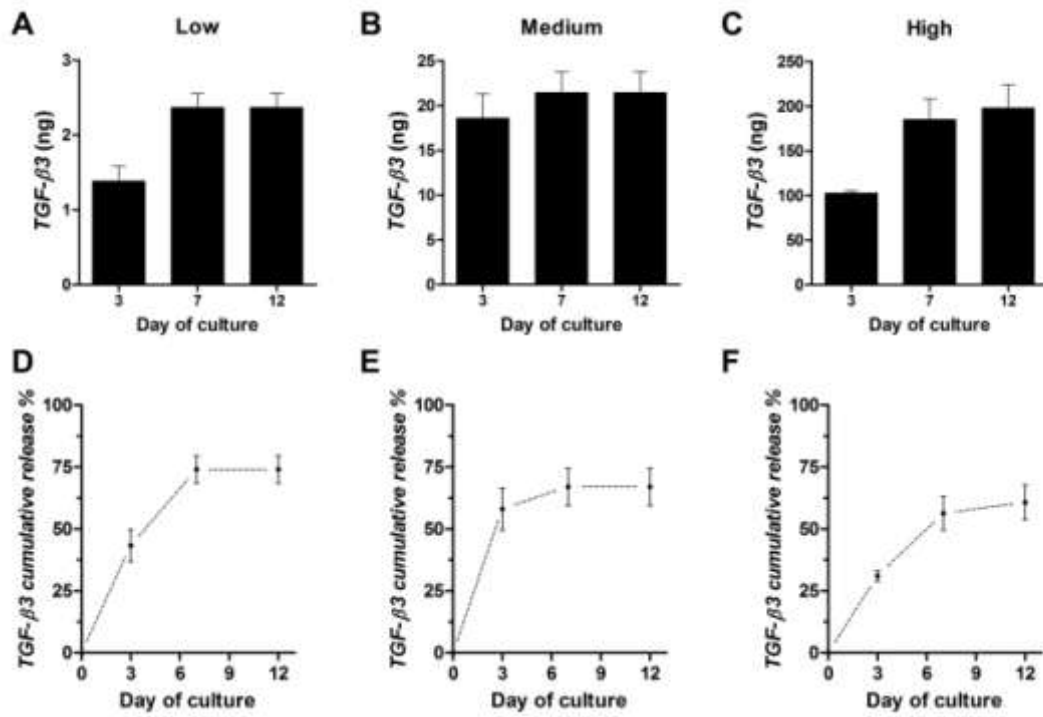


Fig 3

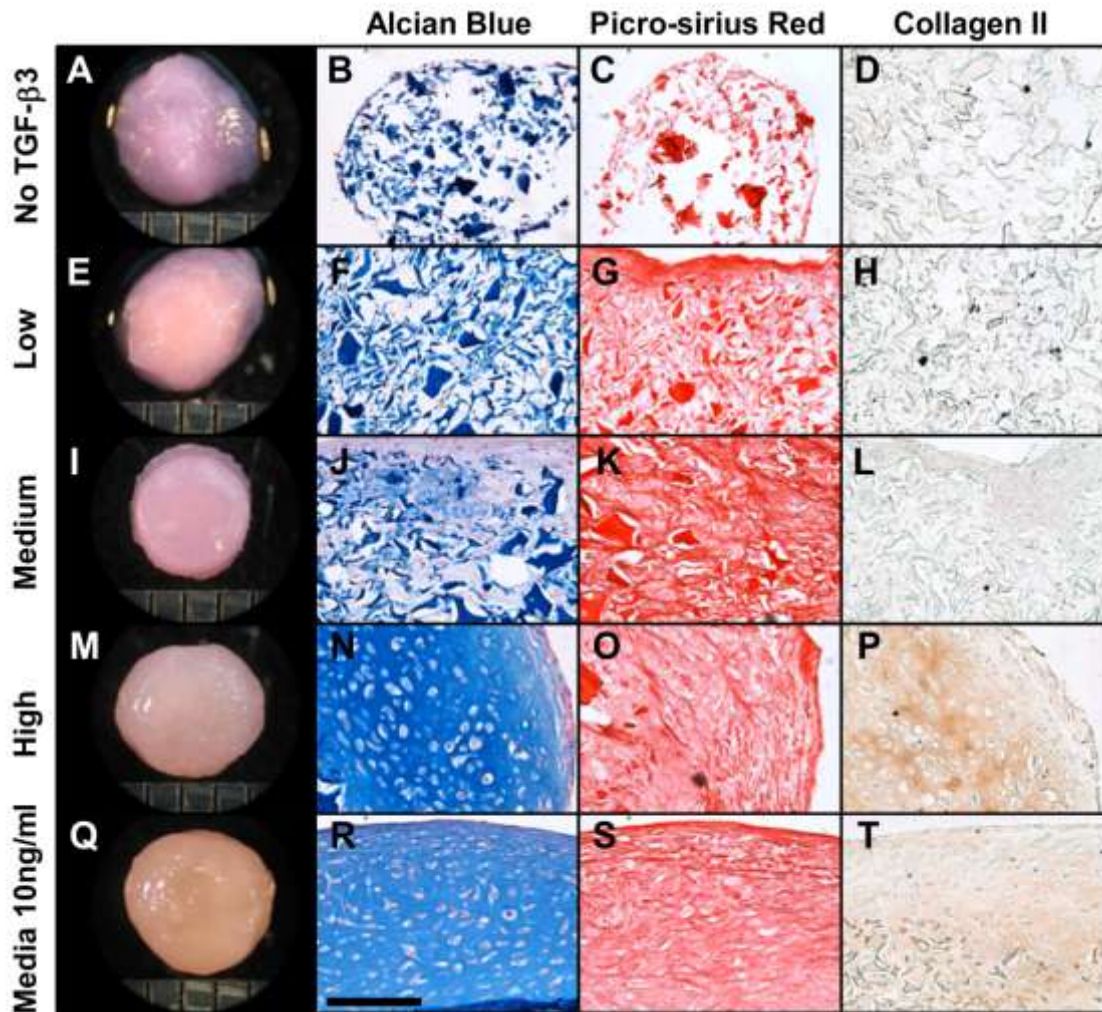


Fig 4

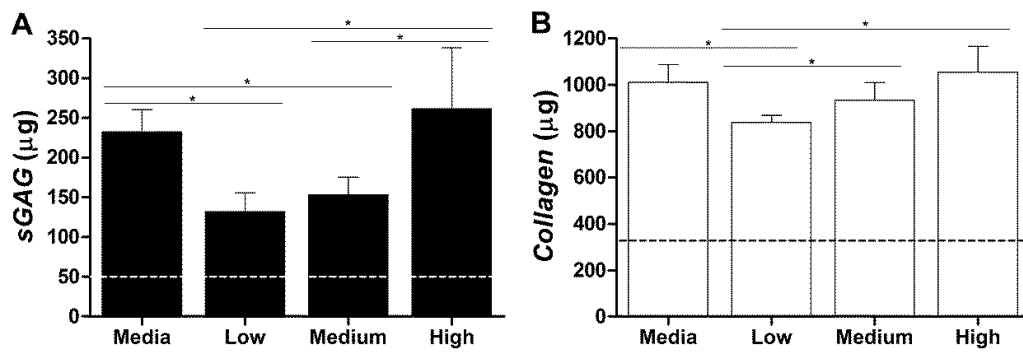




Fig 5

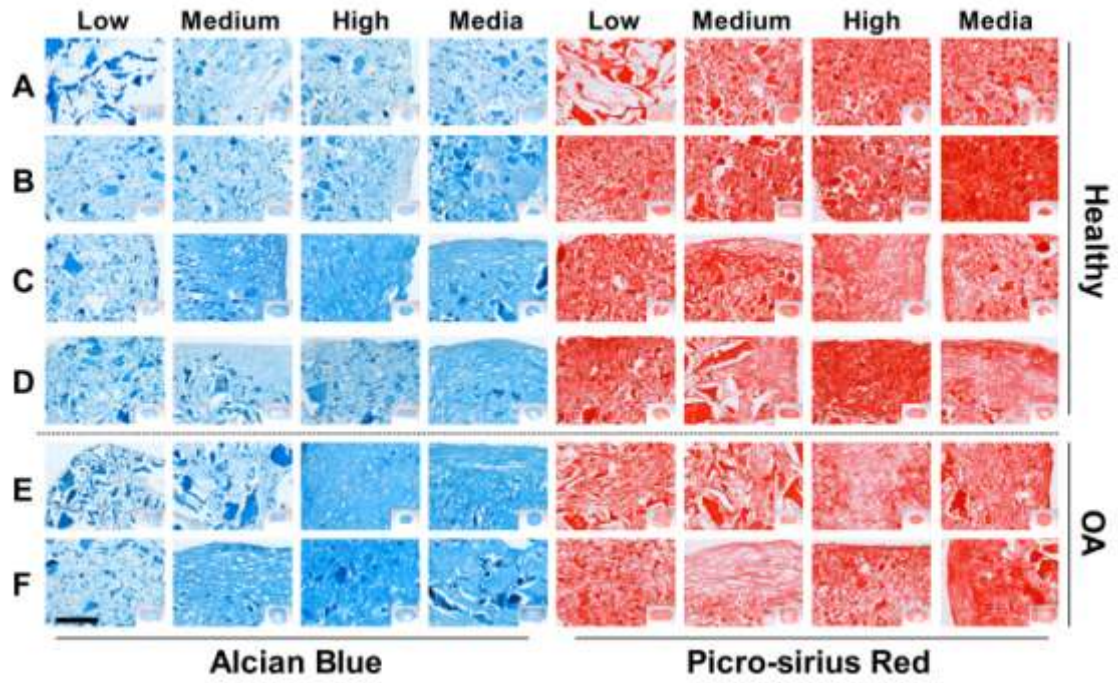


Fig 6

