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# **Effects of in vitro endochondral priming and pre-vascularization of human MSC cellular aggregates in vivo.**

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## Abstract

**Introduction:** During endochondral ossification, both the production of a cartilage template and the subsequent vascularization of that template are essential precursors to bone tissue formation. Recent studies have found the application of both chondrogenic and vascular priming of MSCs enhanced the mineralisation potential of MSCs in vitro whilst also allowing for immature vessel formation. However, the in vivo viability, vascularisation and mineralisation potential of MSC aggregates that have been pre-conditioned in vitro by a combination of chondrogenic and vascular priming, has yet to be established. In this study, we test the hypothesis that a tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage constructs, by co-culture with Human Umbilical Vein Endothelial cells (HUVECs) in vitro, will improve vessel infiltration and thus mineral formation once implanted in vivo.

**Methods:** Human MSCs were chondrogenically primed for 21 days, after which they were co-cultured with MSCs and HUVECs and cultured in endothelial growth medium for another 21 days. These aggregates were then implanted subcutaneously in nude rats for 4 weeks. We used a combination of bioluminescent imaging (BLI), microcomputed tomography ( $\mu$ CT), histology (Masson's trichrome and Alizarin Red) and immunohistochemistry (CD31, CD146, and  $\alpha$ -smooth actin) to assess the vascularisation and mineralisation potential of these MSC aggregates in vivo.

**Results:** Prevascularised cartilaginous aggregates were found to have mature endogenous vessels (indicated by  $\alpha$ -smooth muscle actin walls and erythrocytes), after 4 weeks subcutaneous implantation, and also viable human MSCs (detected by BLI imaging), 21 days after subcutaneous implantation. In contrast aggregates that were not prevascularised had no vessels within the aggregate interior and human MSCs did not remain viable beyond 14 days. Interestingly, the prevascularised cartilaginous aggregates were also the only group to have

mineralised nodules within the cellular aggregates, whereas mineralisation occurred in the alginate surrounding the aggregates for all other groups.

**Conclusions:** Taken together these results indicate that a combined chondrogenic priming and prevascularisation approach for in vitro culture of MSC aggregates shows enhanced vessel formation and increased mineralisation within the cellular aggregate when implanted subcutaneously in vivo.

## **Keywords**

Tissue Engineering, Endochondral Ossification, Endothelial Cells, Mesenchymal Cells, Vasculogenesis, Osteogenesis, Cell Viability.

## **Introduction**

Tissue engineering and regenerative medicine have significant potential to treat bone pathologies by exploiting the capacity for bone progenitors to grow and produce tissue constituents under specific biochemical and physical conditions (1-19). However, the regenerated bone tissue produced through such approaches is limited, due to the degradation occurring in the centre of the constructs and loss of cell viability due to hypoxia occurring within the constructs, which arise from lack of vascularisation (13, 20-26), and a lack of mechanical integrity of the regenerated tissue. As a result such strategies are not yet widely used for clinical treatment of large bone defects.

Endochondral ossification is the process by which all long bones are formed during early fetal development. It relies on the production of a cartilage template, which is followed by vessel invasion. This occurs once the cartilage template has formed; endothelial cells invade through the cartilage canals already present in the developing bone tissue (27-30), and this process typically occurs between 14 and 18 days of embryogenesis (30, 31). Both cartilage template formation and vascularisation must occur before bone tissue can be formed. Recent findings have suggested that mimicking the cartilage template formation phase of the endochondral ossification process, by chondrogenically priming MSCs, may be an effective approach to overcome issues such as poor oxygen and nutrient supply in bone tissue engineered constructs (26, 32-34), as chondrocytes are physiologically functional even at reduced oxygen tension (35). However, even with chondrogenic priming, construct degradation and an uneven distribution of bone mineral have been reported throughout the construct after implantation (25, 26, 33). In a recent study we found that chondrogenic priming of BALBc mice MSCs and human MSCs in vitro for specific durations (14, 21 days) can influence their mineralization capacity and produce a construct that is mineralized throughout the core, to a greater degree than culturing the cells in osteogenic growth factors

alone (34). In vivo studies demonstrated that chondrogenically primed constructs seeded with embryonic stem cells (36), chick embryonic stem cells (37) and human MSCs (25, 26, 33, 38) subsequently mineralised and in some cases formed bone marrow cavities (37, 38) following subcutaneous implantation in rodent animal models. Chondrogenically primed rat MSCs cultured on a PLGA scaffold were found to have increased bone healing in both a 5mm and 15mm rat femur defect (39). Similar rapid healing was also reported when chondrogenically primed human MSC cellular aggregates were implanted in a 6mm rat femur defect (40).

Without a suitable vascular supply, cells within tissue engineered constructs lack the necessary requirements to regenerate bone tissue and readily perish when implanted in vivo (41, 42). In vitro co-culture studies have investigated whether pre-vascularising 3D tissue engineered constructs, such as trabecular bone (43), PCL (44), poly(LLA-co-DXO) (45), collagen GAG (46, 47), and hydroxyapatite (48) scaffolds in vitro, through the co-culture of MSCs and HUVECs, would allow faster host integration post-implantation (43-48). It has been shown that pre-vascular networks can be formed in a subcutaneous animal model in vivo when human MSCs (43, 45, 46, 48) are first co-cultured with HUVECs in vitro. Moreover in vitro co-culture studies of HUVECs and MSCs have detected an upregulation of the early osteogenic growth factor alkaline phosphatase (ALP) in both 2D and 3D culture (49-53).

While current bone regeneration strategies have sought to incorporate either the production of the cartilage template or the vascularisation of the construct, no strategy has sought to incorporate both events simultaneously, even though both are crucial precursors for bone formation during endochondral ossification in vivo. In a recent in vitro study, we found that chondrogenic priming (for 21 days) together with co-culture of human MSCs and HUVECs significantly increased the osteogenic potential of the culture compared to chondrogenic priming alone (54). This study also reported that both MSCs and HUVECs

must be added to the formed cartilage template for the formation of rudimentary vessels to occur in vitro. We found that the application of both chondrogenic and vascular priming of MSCs enhanced the mineralisation potential of MSCs in vitro whilst also allowing for immature vessel formation. However, the in vivo viability, vascularisation and mineralisation potential of MSC aggregates that have been pre-conditioned in vitro by a combination of chondrogenic and vascular priming, has yet to be established.

In this study, we test the hypothesis that a tissue regeneration approach that incorporates both chondrogenic priming of MSC aggregates, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage constructs, through the co-culture of HUVECs in vitro, will improve cell survival, vessel infiltration and thus mineral formation once implanted in vivo. The specific objectives of this study are to assess these outcome measures within a subcutaneous implantation nude rat model.

## **Materials and Methods**

### **Cell Culture**

#### ***Human Donor MSCs***

Bone marrow-derived human mesenchymal stem cells (MSCs) harvested from two male donors 20-25 years old, with established multi-potency, were commercially available and purchased from the Texas A&M University Health Science Centre (Temple, TX). As the human MSCs were bought from Texas A&M University Health Science Centre, all ethical approval was conducted by them. The human MSCs were expanded in Minimum Essential Medium alpha ( $\alpha$ MEM, Invitrogen, Carlsbad, CA) containing 16.7% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and 100 units/mL penicillin/100 $\mu$ g/mL streptomycin/2 mM L-glutamine (PSL, Invitrogen) at 37°C and 5% CO<sub>2</sub>. For all cell culture



performed in this study, cell culture medium was changed twice weekly unless stated otherwise. At passage 2, cells from each donor were detached using 0.25% trypsin-EDTA (Invitrogen) and combined 1:1 to produce a pooled human MSC population. MSCs were further cultured to passage 3-4.

### ***Cell Labelling***

Human MSCs were co-transduced using lentiviral vector containing green fluorescent protein (GFP) and firefly luciferase (Luc) downstream of the ubiquitin promoter as previously described (55-57). Briefly, human MSCs were suspended in polybrene and a viral vector at a multiplicity of infection (MOI) of 20 and incubated in flasks at a density of 10,000 cells/cm<sup>2</sup> overnight (Sigma, St. Louis, MO). Medium was changed daily for 3 days, after which the labelling efficiency of GFP/Luc was determined using fluorescent microscopy. GFP/Luc labelled human MSCs were replated at a seeding density of 500 cells/cm<sup>2</sup> and were further cultured to passage 3-4. These GFP/Luc labelled human MSCs were only used in the constructs tested for BLI.

### ***HUVECs Culture***

Human umbilical vein endothelial cells (HUVECs) were commercially available and purchased from Lonza (Maryland, USA) and cultured in Clonetics Endothelial Growth Medium (EGM) SingleQuotes (Lonza). As the HUVECs were purchased from Lonza, all necessary ethical approval was conducted by them. Media was replaced every 3 days and, upon reaching 80-90% confluency, cells were passaged using 0.25% trypsin-EDTA (Invitrogen). HUVECs were further cultured to passage 3.

### **Aggregate Formation**

Once the human MSCs (labelled and unlabelled) reached a confluency of ~80% the cells were trypsinized, counted, and centrifuged at 650 g at a temperature of 22°C for 5 minutes.

The cells were then resuspended in expansion media at a density of  $0.25 \times 10^6$  cells/mL. This cell suspension was divided into 1.5 mL tubes so that there were 250,000 cells in each tube, and these were then centrifuged for 5 mins (Eppendorf Centrifuge 5430R; Vashaw Scientific, Norcross, GA) at 400 g to create cell aggregates. The media was removed from the tube carefully, so as to avoid the newly formed aggregate, and 0.5 mL of Chondrogenic Media was added. Chondrogenic medium consisted of a chemically defined medium, which contained high glucose Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX™ (Invitrogen), 10 ng/mL TGF- $\beta$ 3 (Invitrogen), 50  $\mu$ g/mL Ascorbic Acid (Sigma Aldrich), 4.7  $\mu$ g/mL Linoleic Acid-Oleic Acid (Sigma Aldrich), 100 nM Dexamethasone (Sigma Aldrich) and 1x insulin-transferrin-selenium (ITS, Invitrogen). For all experiments aggregate cultures were fed twice per week by performing a 50% medium exchange. During each feed the aggregates were agitated, so as to prevent them from adhering to the micro-tube. This was achieved through aspirating the media beneath the aggregate with a micro-pipette.

After 21 days, the aggregates were separated into 3 different experimental conditions. **(1) CP21-HUVECs:** Aggregates were chondrogenically primed for a period of 21 days and then cultured in EGM media for a further 21 days (in the Results section this group will be hereafter known as the **Cartilage Template group**). **(2) CP21+HUVECs:** Aggregates were chondrogenically primed for 21 days after which 250,000 suspended HUVECs in EGM were added to the cellular aggregate and cultured in EGM for further 21 days (hereafter known as the **Co-Culture Cartilage Template group**). **(3) CP21+HUVECs:MSCs:** Aggregates were chondrogenically primed for 21 days after which 250,000 suspended HUVECs and MSCs at a ratio of 1:1 (125,000:125,000 cells) in EGM were added and further cultured in EGM for 21 days (hereafter known as the **Prevascularised Cartilage Template group**), see Figure 1.

For the co-culture groups, confluent layers of HUVECs/MSCs were trypsinized and counted. Cells were suspended depending on experimental conditions so that there was  $0.5 \times 10^6$

cells/mL. In the case of the CP21+HUVECs:MSCs, the ratio of cells was 1:1 HUVECs:MSCs. Both the HUVECs and the MSCs added were at passage 3, which was the same passage of the MSCs used to form the original cellular aggregate. The cells were suspended in EGM media containing osteogenic growth factors and 20% methocel; from a stock solution that was generated by dissolving 6 g of carboxymethylcellulose (Sigma Aldrich) in 500 mL of DMEM as previously described (58). The addition of the methocel to the media increases the viscosity of the media and promotes the attachment of the cells to the already formed aggregate. After 24 hours the medium that contained methocel was removed and was replaced with EGM media alone and this EGM media alone was used for the further 20 days of culture.

### **Construct Preparation**

After 42 days of in vitro culture, the primed aggregates were prepared for implantation. A dual syringe approach, previously described by Kolambkar et al. (59), was adapted to imbed the cellular aggregates within hydrogels. Briefly functionalised alginate (FMC Biopolymer; Sandvik, Norway) containing BMP-2 (Pfizer, MA, USA) at a concentration of 1.6 µg/100µL was cross-linked by adding calcium sulphate (Sigma) to a final concentration of 8.4 mg/mL. Constructs were prepared by injecting 100 µL of cross-linked alginate into an electrospun, polycaprolactone (PCL) nanofiber mesh tube (59), and two cellular aggregates from each group were placed within each alginate/mesh construct, see Figure 1. One group, which contained no aggregates within the mesh, was used as an acellular group (known as the **Alginate group**). These constructs were then incubated in culture medium within a 24-well ultralow-attachment plate (Corning, Lowell, MA, USA) for 2-6 hours prior to implantation.

## **Surgical Procedures**

All animal procedures were ethically approved and conducted in accordance with the Georgia Institute of Technology Institutional Animal Care and Use Committee (IACUC) protocol (#A13023). Ten 11-week-old female, athymic nude rats (Charles River Labs; Wilmington, MA, USA) were anesthetized using isoflurane. Two incisions were made in the skin slightly lateral to the spine of each animal and a custom made tunnelling device was used to create four subcutaneous pockets. One construct (from each of the four groups) was placed in each pocket. Construct were implanted in a balanced manner, such that each group contained an implant placed at each of the subcutaneous locations and samples were randomly distributed across the operated animals. Once the four constructs were implanted, incisions were closed using suture and wound clips.

## **Bioluminescent imaging (BLI)**

Two rats received constructs with aggregates formed from GFP-Luc labelled human MSCs (as discussed above) and were maintained under anaesthesia to perform Day 0 BLI.

BLI was performed on the animals at Day 0, 7, 14, and 21, following a previously developed approach (57). Briefly rats were anesthetized using isoflurane and 300 $\mu$ L luciferin was injected subcutaneously in close proximity to the construct site. After 30 minutes animals were positioned with their lateral side facing up and scanned using an IVIS Lumina machine (Caliper Life Sciences, Hopkinton, MA, USA). The animals were then repositioned so that their other side could be scanned. BLI images were evaluated by demarcation of a 4cm<sup>2</sup> elliptical region of interest (ROI) centred on each construct using Living Image software version 3.2 (Caliper Life Sciences). BLI counts were normalised by exposure time and region of interest (ROI) for each sample.

## **Microcomputed Tomography Imaging**

At 4 weeks post-surgery, eight rats (each rat contained the four groups) underwent a vascular perfusion protocol modified from that developed by Duvall et al. (57, 60). Briefly, the rats were put under anaesthesia and maintained at 4% isoflurane. Once anesthetized, the thoracic cavity was opened to insert an 18 gauge catheter (SURFLO Teflon IV catheter; Terumuo Medical; Somerset, NJ) through the left ventricle of the heart into the ascending aorta. The inferior cava was cut and 0.9% saline was perfused through the vasculature using a peristaltic pump (Masterflex, Cole Parmer, Vernon Hills, IL) until the vasculature system was completely flushed clear. A solution of 0.9% saline containing 0.4% (w/v) papaverin hydrochloride was then perfused followed by 10% Neutral Buffered Formalin (NBF) for 5 min. Animals received a final perfusion of 20-25 mL of radiopaque contrast agent Microfil (Flow Tech, Carver, MA) and were left at 4°C overnight. In this way, animals were euthanized by the combined effects of isoflurane overdose and exsanguination. Explants were extracted and incubated in NBF for 24 hours before being imaged via micro-computed tomography ( $\mu$ CT) scans on a MicroCT42 (Scanco Medical, Brüttisellen, Switzerland) at 55kVp, 145 $\mu$ A, and a 12 $\mu$ m voxel size. The volume of interest was defined as the construct and the minimal tissue surrounding the construct. Microfil has the same threshold as bone mineral and therefore to segment perfused vasculature from mineralised tissue within each construct two scans were analysed; calcified construct versus decalcified construct. The calcified constructs were scanned and post-processed using a threshold value that accurately depicted both the mineral content and the vessel volume by visual inspection of the 2D grayscale tomograms (Scanco Medical MicroCT42). Noise was removed using a low-pass Gaussian filter (sigma=1.2, support=2). Next samples were decalcified in Immunocal (Formic acid bone Decalcifier, Decal Chemical Corporation) for 1 week with the decalcification solution replaced every day (decalcified constructs). After one week these decalcified

constructs were scanned using the same settings, and post-processed at the same threshold as the calcified constructs to determine mineral content. Mineralised tissue content was determined by subtracting the bone volume of the decalcified scans from the calcified scans. Next the decalcified scans were post-processed at a threshold value that accurately depicted just the vessel volume upon visual inspection of the 2D grayscale tomograms.

### **Histochemical analysis**

Following  $\mu$ CT scanning the samples were dehydrated and embedded in paraffin using an automatic tissue processor (Excelsior ES tissue processor, Thermo Scientific; Austin, TX). All samples were sectioned with a thickness of 8  $\mu$ m using a rotary microtome (Leica Microtome RM2235, Leica). Sections were stained with Masson's Trichrome and Alizarin Red (all Sigma Aldrich).

### **Immunohistochemical Analysis**

Immunohistochemical analysis was used to detect CD31, CD146 and  $\alpha$ -smooth muscle actin. Sections were deparaffinised overnight before a series of rehydration steps through varying ethanol grades (100%-50%). The samples were then treated with 40 $\mu$ g/mL of proteinase K for 20 minutes at 37°C (Sigma Aldrich), rinsed with phosphate buffered saline (PBS)-Tween and blocked with PBS with 1% w/v Bovine Serum Albumin (BSA) and 3% w/v Normal Goat serum (NGS) (Sigma Aldrich) for 60 mins. Sections were then incubated overnight at 4°C with either rabbit polyclonal anti-CD31 (ab28364 Abcam, 1:50) or rabbit monoclonal anti-CD146 (ab75769 Abcam, 1:250). After three washing steps with PBS containing 1% w/v BSA the sections were incubated with Dylight488 goat anti-Rabbit secondary antibody (Jackson ImmunoResearch, 115-485-209, 1/200), for one hour at room temperature in the dark. The samples were washed three times in PBS with 1% w/v BSA, and the slides were then incubated overnight at 4°C with mouse monoclonal anti-alpha smooth muscle actin

antibody (ab7817 Abcam, 1:50). After three washing steps with PBS with 1% w/v BSA the sections were incubated with Dylight549 goat anti-mouse secondary antibody (Jackson ImmunoResearch, 115-505-062, 1/200), for one hour at room temperature in the dark. Finally samples were washed three times with PBS with 1% w/v BSA and the sections were mounted using 4',6-diamidino-2-phenylindole (DAPI) mounting media (Sigma Aldrich).

### **Statistical Analysis**

Results are expressed as mean  $\pm$  standard error. All  $\mu$ CT quantitative analyses were examined using one-way analyses of variance (ANOVA) with the addition of Tukey's correction for multiple comparisons testing. BLI quantitative analysis was examined using two-way analyses of variance (ANOVA) with the addition of Tukey's correction for multiple comparisons testing. All analyses were performed using GraphPad. For all comparisons, the level of significance was  $p \leq 0.05$ .

## **Results**

### **Construct Morphology**

#### ***Prior to implantation***

All three experimental groups, stained positive blue for sGAG and Alizarin Red prior to implantation after 42 days of culture (see Figure 2 (A, B)). There was no significant difference in sGAG or calcium production after the 6 weeks of culture.

There was no positive (green) staining for CD31+ in the CP21-HUVECs group cultured, as there was no endothelial cells present (see Figure 2 (C)). However, for both the CP21+HUVECs and the CP21+HUVECs:MSCs groups there was positive (green) staining seen around the periphery (indicated by arrows in Figure 2 (C)) and after 3 weeks of co-culture, both groups had positive green staining present within the centre of the aggregates. However, the CP21+HUVECs:MSCs group was the only group to have structures characterised by a circular CD31+ positive wall with irregularly shaped nuclei present within the lumen (indicated by arrows in Figure 2 (C)).

#### ***Post implantation***

All aggregates were identifiable as a clear circular bundle of cells present within the nanofiber mesh (as indicated by the letter A in Figure 3) after 4 weeks in vivo. The aggregates are predominately surrounded by alginate and host cells (see Figure 3). As expected there was evidence of degradation in the Cartilage Template and the Co-culture Cartilage Template groups, as indicated by the channels present within the centre of the aggregates (indicated by the letter D), along with the build-up of fibrous collagen tissue surrounding the aggregate (indicated by the letter C). However, in the Prevascularised Cartilage Template group the degradation was minimal (Figure 3). There was also positive collagen staining present in all of the aggregates.



## **Mineral Formation**

Quantitative mineralisation of the constructs and the surrounding tissue in the hydrogel was analysed from the reconstructed  $\mu$ CT data to determine Mineral Volume. All groups produced mineral volume between 0.5-0.8 mm<sup>3</sup>, however there was no significant difference between any of the groups after 4 weeks of implantation (See Figure 4).

Positive Alizarin Red staining was present in all of the groups, however the location of the mineral differed by group. The only group to have mineralisation nodules present within the aggregate itself was the Prevascularised Cartilage Template group. All of the other groups only had mineralisation nodules present in the surrounding alginate, as seen in Figure 5. Some of these mineralisation nodules were present in close proximity to mature blood vessels, as indicated in Figure 5 by the arrows.

## **Cell Viability**

BLI data obtained over the course of the study showed that the live cell number from the original cellular aggregate decreased in all groups from the day of surgery to 2 weeks after implantation. However, there was significantly higher BLI signal in the Prevascularised Cartilage Template group and Co-culture Cartilage Template group ( $p < 0.05$ ) compared to the Alginate group at both Day 0 and Day 7. The Prevascularised Cartilage Template group also retained more cells compared to the other groups at Day 7 (88% vs. 82-20.5%) and Day 14 (27.4% vs. 18.3-1.7%) and by Day 21, there was more viable human MSCs present in the Prevascularised Cartilage Template group compared to both the Alginate ( $p = 0.1$ ) and Co-culture Cartilage Template groups ( $p = 0.13$ ) (see Figure 6(B)). BLI imaging was performed on Day 28 however, no detectable signal was found.

## **Vessel Infiltration**

$\mu$ CT reconstruction of the explant vasculature illustrated the presence of host blood vessels surrounding the construct and infiltrating the construct through the holes present within the nanofiber mesh (Figure 8 (A)). Vessel volume was quantified in two ways; (1) Total Vessel Volume and (2) Average Vessel Diameter. After four weeks in vivo there was no significant difference in Total Vessel Volume or Average Vessel Diameter between any of the groups (Figure 7 (A, B)). To further analyse the data a vessel diameter of 0.15mm (150  $\mu$ m) was chosen as a threshold to distinguish between thick and thin vessels, and preclude smaller structures (that were unlikely to be mature vessels) from obscuring the results of the analysis. The threshold was chosen on the basis of our immuno-staining (described in detail below), which revealed that positively stained  $\alpha$ -smooth actin and CD31 and structures with a visible lumen had diameters in the range of 150  $\mu$ m (see Figure 9, 10, described in detail below). Moreover, the majority of vessels in a Sprague-Dawley rat femora are in the range of 120–150  $\mu$ m (61) and the average vessel diameter achieved within a bone tissue engineering scaffold implanted in a rabbit alveolar bone defect was 152  $\mu$ m by 4 weeks (62). For both the Cartilage Template and the Prevascularised Cartilage template groups a large proportion of the vessels were thicker than 0.15 mm. When only vessels with a diameter greater than 0.15 mm are considered, in both the alginate and the Co-culture Cartilage Template group only 3 out of 8 rats had vessels greater than 0.15mm. These vessels only accounted for 7% of the overall vessels within the construct. However, in both the Cartilage Template and Prevascularised Cartilage Template group 5 out of 8 rats had vessels present with a greater diameter than 0.15mm and these vessels accounted for up to 14% of the overall vessels seen within the construct (see Figure 8 (B)).

Histological staining revealed that there was little vessel formation present within the Cartilage Template and Co-culture Cartilage Template aggregates, but most of the vessels

were found outside the aggregates within the alginate (see Figure 3). However, the Prevascularised Cartilage Template group was the only group to contain vessels within the aggregate itself complete with red blood cells (see Figure 3 denoted by the arrows).

Immunohistochemical analysis confirmed that mature vessels were present in the surrounding alginate in all of the groups, as indicated by the  $\alpha$ -smooth actin staining (Figure 9, 10). In contrast the Prevascularised Cartilage Template group had mature vessels present within the centre of the aggregates, rather than around the periphery in the surrounding alginate. CD146 and CD31 staining, both endothelial cell markers, revealed that, for both the Co-culture Cartilage Template and the Prevascularised Cartilage Template groups, endothelial cells were involved in the formation of these vessels. Moreover, CD31 positive stained cells were present within the structure of some of the vessels (Figure 9, 10) indicating that the implanted human endothelial stem cells were involved in the formation of these vessels as CD31 is only present in human endothelial cells. The staining also showed CD31 positive stained cells were not only present on the periphery of the aggregates but were also present within the surrounding alginate.

## Discussion

This study investigated whether a tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template, and subsequent pre-vascularisation of the cartilage constructs, through the co-culture of HUVECs in vitro, would improve the survival of implanted cells, leading to vessel infiltration and thus mineral formation once implanted subcutaneously in vivo. Our results showed that the prevascularised cartilaginous aggregates successfully developed mature vessels (as indicated by  $\alpha$ -smooth muscle actin walls and red blood vessels) within the aggregates and retained viable cells from the original aggregate (as indicated by BLI signalling) 21 days after subcutaneous implantation. The prevascularised cartilaginous aggregates were also the only aggregates to have mineralisation nodules present within the aggregates. In contrast chondrogenically primed aggregates, with and without HUVECS, did not have viable cells remaining from the original aggregate after 14 days, had a high vessel volume, but these vessels were not present within the aggregate, and only had mineralisation nodules present in the alginate surrounding the aggregates. Collectively, these results indicate that prevascularised cartilaginous aggregates survive for a longer duration following subcutaneous implantation in vivo compared to all other groups, and also that these cellular aggregates contribute to the formation of vessels, with  $\alpha$ -smooth muscle actin walls and red blood cells, and increased mineralisation deposition within the construct, which was not observed in aggregates that were not subjected to a combination of chondrogenic priming and pre-vascularisation.

A possible limitation of the study is that MSCs from two male donors were pooled and we did not directly explore whether the human MSCs displayed a donor dependent response to mineral formation. Previous studies have seen donor variability in the expression of osteogenic growth factors both in vitro (63) and mineral formation in vivo (64). However

the control groups also contained pooled cells, so the differences observed between the groups cannot be explained by donor variability. A second limitation is that we used MSCs and HUVECs from different donors rather than acquiring both cell types from the same donor. However, it was not feasible to obtain the necessary cell numbers to perform the entire experiment, involving multiple aggregates and priming groups, using cells sourced from the same donors. Future studies could investigate the *in vivo* potential of one of these groups (i.e. the Prevascularised cartilage template group) using cells from the same donor source to fully understand the clinical relevance of the approach. Another potential limitation was the length of time the samples were examined for mineral formation (4 weeks). Previous ectopic bone formation models indicate that little to no bone formation will occur until approximately 8 weeks (25, 26, 33, 36-38). The choice of the 4 week time point allowed us to see both early mineral formation and vessel infiltration and was able to distinguish differences at this early time point. Future studies should investigate the long term effect of subcutaneous implantation of the prevascularised cartilaginous aggregates in order to fully understand their mineralisation potential. Finally, the nanofiber mesh/alginate delivery system with osteogenic growth factors (BMP-2) was used to ensure the retrieval of the aggregates after 4 weeks *in vivo*, which has proved challenging in other subcutaneous implantation studies (25, 26, 33). However, as we included an acellular control group, we clearly showed that the results obtained could not be explained by the addition of BMP-2 alone, but that the addition of the cells leads to the differences seen between the groups.

Current bone tissue engineering strategies are limited by challenges arising due to lack of nutrient delivery and waste removal arising from the lack of vasculature (13, 20-26). Our recent *in vitro* study reported that a combination of chondrogenic priming and co-culture of human MSCs and HUVECs can lead to the formation of rudimentary vessels and significantly increased the *in vitro* osteogenic potential of MSC aggregates (54). Other

studies have investigated whether pre-vascularising trabecular bone (43), PCL (44), poly(LLA-co-DXO) (45), collagen GAG (46, 47), and hydroxyapatite (48) scaffolds in vitro would allow faster host integration post-implantation and reported that microvascular networks established in vitro can be maintained when implanted in vivo (43-48). In this study we investigated whether prevascularisation of chondrogenically primed constructs in vitro prior to implantation could overcome limitations of vascularisation and thus degradation of the implanted constructs and uneven mineral distribution. The results from this study show that vessel formation was achieved within the constructs of all groups after 4 weeks implantation (as indicated by  $\mu$ CT angiography, Masson's Trichrome and  $\alpha$ -smooth actin staining). However, the only group to have vessel formation within the aggregates, and not just in the surrounding alginate or in the periphery of the aggregate, was the prevascularised cartilaginous aggregates. This may be due to the fact it was also the only group to have rudimentary vessels present prior to implantation (as indicated by CD31 staining). Moreover, it was also the only group to have mature vessels complete with a smooth muscle lining (as indicated by  $\alpha$ -smooth actin staining) and red blood cells (as indicated by Masson's Trichrome). The prevascularised cartilaginous aggregates also had the thickest vessel diameters present within the constructs as a whole (as indicated by  $\mu$ CT angiography), with 5 out of 8 of the rats having vessels with diameters between 0.15 – 0.35 mm and these vessels accounted for up to 14% of the overall vessels present within the constructs. Moreover, the prevascularised cartilaginous aggregates were the only group to have viable cells 21 days after implantation than other groups. Previous studies have only shown maintenance of viable MSCs to 7 days (57). Taken together, these results indicate that prevascularisation of the cartilaginous aggregates prior to implantation exerts a positive effect on maintenance of the viability of implanted human stem cells in aggregates implanted for 4 weeks in vivo and this

is directly associated with the formation of mature vessels present within the centre of the aggregates.

MSCs are a perivascular cell type (65-67), and have been shown to have pro-angiographic effects on endothelial cells when co-cultured in vitro (54, 68). Our results show both the perivascular role of MSCs in vivo and the pro-angiographic effects on endothelial cells, as the only group to form vessels within the cellular aggregates were the prevascularised cartilaginous group, which had both MSCs and HUVECs added to the cartilage template. Interestingly, immunohistochemical staining also revealed that the HUVEC cells that were added to the already formed cartilage template (in the Co-culture Cartilage Template group and the Prevascularised Cartilage Template group) were not just present around the periphery of the aggregates but were also present within the surrounding alginate. Moreover these HUVECs were shown to play a role in the formation of the mature vessels and integrate with the host cells to form vessels (as indicated by CD31 staining). However, whether it is the human MSCs added during the co-culture or the human MSCs used to form the cartilage template, or the host MSCs that are involved in the formation of these vessels is still unknown. The CD31 stain used was specific for human cells and the persistent staining by 4 weeks after implantation confirms that human cells did persist and may be involved in the formation of the vascular networks. Further studies are needed to elucidate which cells types are involved in forming the vessels observed here.

Unlike other studies (43-48) this study did not use a scaffold. One of the major limitations to current scaffold tissue engineering studies is the inhomogeneous distribution of cells within the construct (69). This uneven distribution can then lead to heterogeneous properties, fibrous tissue encapsulation (13), and degradation within the centre of the construct, which ultimately leads to the degradation of the scaffold itself (13, 20-26). Our approach allows the cells to form their own scaffold, mimicking native endochondral

ossification, therefore ameliorating the distribution of cells. Previously we have shown that chondrogenically priming MSCs in vitro, to form a cartilage template, provides a suitable scaffold for HUVEC and MSC cells to attach, proliferate, infiltrate, and ultimately form rudimentary vessels (54). This study not only verifies the benefits of this scaffoldless setup but also shows that, even after being implanted for 4 weeks, there was minimal degradation of the centre of the aggregates in the cartilage template of the prevascularised cartilaginous aggregates. In contrast, the non-prevascularised groups had fibrous tissue present surrounding the aggregates, which can lead to hypertrophy of the cells in the centre of the aggregate and hence the degradation of the centre of the aggregates (seen in the Masson's Trichrome).

During endochondral ossification angiogenesis occurs once the cartilage template has formed. This process involves endothelial cells invading through the cartilage canals already present in the developing bone tissue (27-30), and typically occurs between 14 and 18 days of embryogenesis (30, 31). Therefore in order for mineralisation to occur, the cartilage template must be formed, and vessel infiltration must then occur. Previous studies, which have looked at just the formation of the cartilage template through the subcutaneous implantation of either chondrogenically primed construct (25, 26, 36, 37) or hypertrophic constructs (33, 38), found little to no mineral formation before 8 weeks in vivo. This study found that there was mineralisation present in all of the groups after 4 weeks in this ectopic bone model. Alizarin Red staining of the groups also shows that mineralisation nodules were present predominately in the surrounding Alginate. As the alginate contained BMP-2 this was to be expected. However, the only group that had mineralisation nodules present within the centre of the aggregates was the prevascularised cartilaginous aggregates. Our previous in vitro study found that when both MSCs and HUVECs were added to a chondrogenically primed aggregate, mineralisation was reduced, compared to the addition of HUVECs alone (54). Furthermore this mineral was characterised by the formation of discrete mineralised nodules



rather than homogenous mineralisation throughout the construct, similar to those seen in this study. Researchers have postulated that, in order to mimic bone formation that occurs naturally during the early fetal development, vasculogenesis should be induced prior to osteogenesis in vitro in order to obtain functional bone tissue when implanted in vivo (30, 43, 54). The results from this study are in agreement with such theories, as the only aggregates to have mineralisation nodules present within the aggregates were also the only group to have mature vessels present within the aggregate. We propose that mineralisation deposition does not occur until after vessel formation, and that this was a possible explanation for why mineralisation was not seen in the chondrogenically primed aggregates (without and with HUVECS alone) as vascularisation within the centre of the aggregate had not occurred. However, mineralisation nodules were beginning to form in the prevascularised group but only once mature vessels had formed within the aggregates. Moreover mineralisation only occurred within close proximity of these vessels. Therefore, it is possible that culturing this group in vivo for longer than 4 weeks will ultimately allow for enhanced mineralisation, but this cannot be verified from the results of the current study and future in vivo investigations are required.

## **Conclusions**

This study shows for the first time that a tissue regeneration approach that incorporates both chondrogenic priming of MSCs, to first form a cartilage template and subsequent pre-vascularisation of the cartilage constructs, through the co-culture of HUVECs and MSCs in vitro, improves implanted stem cell viability, vessel formation (as indicated by  $\alpha$ -smooth muscle actin walls and red blood vessels) and mineral formation once implanted in vivo. Specifically the results from this study show that the only group to have mature vessels present within the aggregates after 4 weeks in vivo was the prevascularised cartilaginous aggregates. We propose that this vascularisation exerted a positive effect on the viability of implanted stem cells and mineralisation potential of the aggregate, as it was also the only group to have both viable cells 21 days after implantation and mineralisation nodules present within the aggregates. Taken together, these results indicate that, endochondral priming of MSC aggregates can increase the survivability of implanted cells, which then contribute to vascularisation of the aggregate and mineral deposition of tissue engineering constructs once implanted in vivo. Future bone tissue engineering strategies could be designed with these conditions in mind such that the factors needed to mimic the endochondral ossification process are incorporated to the point where the constructs themselves can autonomously progress to engraftment, remodelling and ultimately tissue regeneration.

## **Abbreviations**

Mesenchymal Stem Cells (MSCs), Human Umbilical Vein Endothelial cells (HUVECs), Bioluminescent imaging (BLI), microcomputed tomography ( $\mu$ CT), alkaline phosphatase (ALP), glycosaminoglycan (GAG), Minimum Essential Medium alpha ( $\alpha$ MEM), fetal bovine serum (FBS), Ethylenediaminetetraacetic acid (EDTA), green fluorescent protein (GFP), firefly luciferase (Luc), multiplicity of infection (MOI), Endothelial Growth Medium (EGM),

Dulbecco's Modified Eagle Medium (DMEM), Transforming growth factor beta 3 (TGF- $\beta$ 3), insulin-transferrin-selenium (ITS), Chondrogenically Primed (CP), polycaprolactone (PCL), region of interest (ROI), Neutral Buffered Formalin (NBF), Bovine Serum Albumin (BSA), phosphate buffered saline (PBS).

## **Competing Interests**

The authors declare they have no competing interests.

## **Authors' Contributions**

FEF, HYS, ROG, and LMM conceived and designed the experiments. ABA and FEF performed the operations and the vascular perfusion. FEF performed the experimental characterisation, obtained the data and performed the statistical analysis. FEF, ABA, HYS, ROG and LMM analysed the data and drafted the manuscript. FEF, ABA, HYS, ROG, and LMM read and approved the final manuscript.

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## Figure Legends

**Figure 1:** Schematic of the (A) cellular groups and hydrogel methodology, (B) experimental setup and (C) Timeline of the experiment.

**Figure 2:** (A) Alcian Blue and (B) Alizarin Red staining of all three groups on the day of implantation (following 3 weeks of in vitro priming). Images were taken at a magnification of 10X and 40X. (C) Representative images of CD31+ (green) stained sections in the CP21+HUVECs: MSCs group prior to implantation. Each section is 8  $\mu\text{m}$  thick and each of the images was taken at a magnification of 20X and 60X. Nuclear counterstain: DAPI (blue).

**Figure 3:** Masson's Trichrome of the groups after 4 weeks implantation. A denotes nanofiber mesh, B denotes islands of alginate, C denotes sGAG rich encapsulation, D denotes area of degradation and arrows denote vessels complete with red blood cells. Images were taken at 5X 20X and 40X. Schematic of the plane in which the section was taken in in the top right corner.

**Figure 4:** Total Mineral Volume. Error bars denote standard error ( $n=8$ ).

**Figure 5:** Alizarin Red staining of the groups after 4 weeks implantation. Red Staining denotes mineralisation nodules present, A denotes aggregates present within the alginate, and arrows denote vessels present. Images were taken at 4X and 40X. Schematic of the plane in which the section was taken in in the top right corner.

**Figure 6:** (A) Representative BLI heat-maps for representative rat over the time course of the study (B) Total BLI count of all the groups over the course of the study.

**Figure 7:** (A) Total Vessel volume, and (B) Average Vessel Diameter demonstrating the level of vessel formation within the implanted constructs after 4 weeks.

**Figure 8:** (A) Microcomputed tomography angiography representative images of vessel diameter and (B) Histograms of Vessel Diameters from all planes of the construct for each group, demonstrating the varying vessel thickness of the vessels present after 4 weeks.

**Figure 9:** Immunohistochemical staining of the groups after 4 weeks implantation. CD31 stained in green, Nucleus stained in blue, smooth actin stained in red. Boxes denote area of magnification. Arrows denote presence of CD31 (green) within vessel formation. Images were taken at 10X and 60X. Schematic of the plane in which the section was taken in in the middle of the image.

**Figure 10:** Immunohistochemical staining of the groups after 4 weeks implantation. CD146 stained in green, Nucleus stained in blue, smooth actin stained in red. Boxes denote area of magnification. Arrows denote presence of CD146 within vessel formation. Images were taken at 10X and 60X. Schematic of the plane in which the section was taken in in the middle of the image.