Regeneration of osteochondral defects using developmentally inspired cartilaginous templates

Susan Critchley^{1,2}, Gráinne Cunniffe^{1,2}, Adam O'Reilly^{1,2}, Pedro Diaz-Payno^{1,2}, Aidan McAlinden⁵, Daniel Withers⁶, Jungyoun Shin⁷, Eben Alsberg^{7,8,9}, Daniel Kelly^{1,2,3,4*}

¹Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland.

²Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin, Ireland.

³Advanced Materials and Bioengineering Research Centre, Trinity College Dublin and Royal College of Surgeons in Ireland, Dublin, Ireland.

⁴Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland.

⁵School of Veterinary Medicine, University College Dublin, Dublin, Ireland.

⁶Sports Surgery Clinic, Santry, Dublin 9, Ireland.

⁷Department of Biomedical Engineering, Case Western Reserve University, Cleveland, USA.

⁸Department of Orthopaedic Surgery, Case Western Reserve University, Cleveland, USA.

⁹National Centre for Regenerative Medicine, Case Western Reserve University, Cleveland, USA.

*Corresponding Author

Prof. Daniel J Kelly, Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland.

Email: kellyd9@tcd.ie, Tel: 353-1896-3336

Keywords

Hydrogel; Mesenchymal Stem Cell; Endochondral; Chondrogenesis; Osteochondral; Finite Element Model.

Author contact details:

Susan Critchley

Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland

E-mail: CRITCHLS@tcd.ie

Gráinne Cunniffe

Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland.

E-mail: CUNNIFG@tcd.ie

Adam O'Reilly

Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland.

E-mail: OREILLAD@ghmail.tcd.ie

Pedro Diaz-Payno

Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland.

E-mail: diazapap@tcd.ie

Aidan McAlinden

School of Veterinary Medicine, University College Dublin, Dublin, Ireland.

E-mail: mcalindenaidan@hotmail.com

Daniel Withers

Sports Surgery Clinic, Santry, Dublin 9, Ireland.

E-mail: WITHERSD@tcd.ie

Jungyoun Shin

Department of Biomedical Engineering, Case Western Reserve University, Cleveland, USA

E-mail: jungyoun86@gmail.com

Eben Alsberg

Department of Biomedical Engineering, Case Western Reserve University, Cleveland, USA

E-mail: exa46@case.edu

Daniel Kelly*

Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland.

Email: kellyd9@tcd.ie

Abstract

There is increased interest in recapitulating aspects of development when designing new tissue engineering strategies. Long bones and their epiphyses are formed through endochondral ossification, a process by which a cartilage template develops in response to genetic and environmental cues to generate a bone organ. The objective of this study was to evaluate the capacity of engineered cartilage templates to regenerate osteochondral defects created in the femoral condyle of skeletally mature rabbits. To this end, bone marrow derived mesenchymal stem cells (BMSCs) were encapsulated in RGD-functionalized, γ -irradiated alginate hydrogel and chondrogenically primed *in vitro* to engineer cartilage templates tailored for osteochondral defect regeneration. While comparable levels of healing were observed in the bony region of empty and treated groups, the quality of healing was notably different in the chondral region of these defects. Mechanical testing revealed that treatment with engineered cartilage templates promoted the development of a stiffer repair tissue at the articular surface, which correlated with histomorphometric analysis demonstrating the formation of a more hyaline cartilage-like repair tissue. Next, a computational mechanobiological model was used to better understand how local environmental cues were regulating the regenerative process *in vivo*. This model predicted that higher strains and lower levels of oxygen in the chondral region of the defect were preventing cartilage template progression along the endochondral pathway, with hyaline cartilage or fibrocartilage eventually forming depending on local strain magnitudes. In contrast, higher levels of oxygen and lower magnitudes of strain in the osseous region of the defect facilitated progression of the engineered cartilage template along an endochondral pathway. In conclusion, this study demonstrates that engineered cartilage templates can enhance osteochondral defect regeneration, pointing to the potential for developmentally inspired soft tissue templates, engineered using BMSCs, to regenerate damaged and diseased joints.

Impact Statement

Successfully treating osteochondral defects involves regenerating both the damaged articular cartilage and the underlying subchondral bone, in addition to the complex interface that separates these tissues. In this study we demonstrate that a cartilage template, engineered using bone marrow derived MSCs, can enhance the regeneration of such defects and promote the development of a more mechanically functional repair tissue. We also use a computational mechanobiological model to understand how joint specific environmental factors, specifically oxygen levels and tissue strains, regulate the conversion of the engineered template into cartilage and bone *in vivo*.

1. Introduction

Successfully treating osteochondral defects involves regenerating both the damaged articular cartilage and the underlying subchondral bone, in addition to the complex interface that separates these tissues. Current treatments such as mosaicplasty are limited by complications such as donor site morbidity, matching the topography of the damaged site and poor graft integration (1–3). This has motivated the development of tissue engineered implants to treat these clinically challenging defects (4,5). To this end there has been increased interest in recapitulating aspects of tissue or organ development when designing new regenerative strategies. All long bones, including their epiphyses, are formed, in part, by the process of endochondral ossification (6,7). Their development involves the condensation of stem cells, which differentiate into chondrocytes to form a cartilage model/template. The chondrocytes enlarge and become hypertrophic, secreting collagen type X and angiogenic factors, and their surrounding extracellular matrix (ECM) is invaded by vasculature (8). The subsequent delivery of oxygen, growth factors and other regulatory cues through this vasculature, as well as the recruitment of osteo-progenitor cells, promotes osteogenesis and bone formation (9). During postnatal development, articular cartilage also acts as a surface growth plate for the longitudinal, radial and lateral growth of the epiphyseal bone (6). Therefore, cartilage acts as the precursor tissue to the cancellous bone, subchondral bone, calcified cartilage and articular cartilage that make up a mature long bone organ. Bone marrow derived mesenchymal stem cells (BMSCs) have been used to engineer both articular cartilage (10–12) and hypertrophic cartilage templates for endochondral bone tissue engineering (7,13–15). Under appropriate environmental conditions, this suggests that cartilage tissue engineered using BMSCs could provide a template for the development of the different components of the osteochondral unit. To date, no tissue engineering strategy exists to successfully regenerate the complex bone, articular and calcified cartilage interface within damaged joints.

While it is well established that cartilaginous tissues engineered using BMSCs can progress along the endochondral pathway and mineralize (7,13,16,17), certain environmental factors such as oxygen (18–20), or the application of joint-like mechanical loading (21–23) can modulate this process and promote the development of a more stable chondrogenic phenotype. *In situ*, the oxygen tension of bone ranges from 5 - 12.5% pO₂, whereas cartilage resides in a more hypoxic environment with levels ranging from 1 - 5% pO₂ (24). BMSC-laden constructs maintained at or below 5% pO₂ undergo enhanced chondrogenesis with a suppression of hypertrophy, whereas culture at higher oxygen concentrations tends to direct BMSCs towards an osteogenic lineage (19,25,26). Physiological levels of hydrostatic pressures increases TGF- β expression, regulates ECM synthesis (21,27–29) and suppresses calcium deposition within BMSC laden constructs (22,30), however, application of levels outside the physiological range can have negative effects (27). Other mechanical cues such as dynamic compression can also enhance chondrogenesis of MSCs and suppress their tendency to progress along an endochondral pathway (21,31–33). It would be reasonable to conclude that such environmental cues will also play a central role in determining the fate of tissues engineered using BMSCs once they are implanted *in vivo*.

Cartilage and bone tissue engineering strategies often involve the encapsulation of cells into hydrogels, providing them a niche to proliferate and/or differentiate into tissue-specific cells. Alginate is a naturally derived, biocompatible hydrogel that supports cell differentiation and ECM synthesis and is often used in bone and cartilage tissue engineering (34–42). It does not contain specific ligands for cell adhesion, however introducing peptides such as the arginine-glycine-aspartic acid (RGD) sequence has been shown to facilitate cellular adhesion. This has been shown to support cellular proliferation, an osteogenic phenotype (43) and promote endochondral bone formation (14). Alginate is generally slow to degrade which can be a limitation for bone development, as vascularization and the subsequent delivery of nutrients can be impeded or inhibited (40). Strategies to accelerate degradation include altering the molecular weight (MW) through γ -irradiation and/or oxidation of the

material (43–45), which has been shown to enhance the capacity of these hydrogels to support tissue regeneration (41,46).

The first objective of this study was to tissue engineer a cartilage template using BMSCs and to then evaluate its capacity to repair a critically sized osteochondral (OC) defect in the femoral condyle of skeletally mature rabbits. To this end, BMSCs were encapsulated in a RGD-functionalized, γ -irradiated alginate hydrogel and chondrogenically primed *in vitro* to engineer cartilage templates tailored for osteochondral defect regeneration. Our hypothesis was that this cartilage template would undergo spatially defined differentiation *in vivo* in response to the unique environmental conditions within an OC defect, resulting in the development of a repair tissue consisting of hyaline articular cartilage overlying a layer of bone formed *via* endochondral ossification. A computational mechanobiological model was then employed to elucidate the environmental and mechanical conditions *in vivo* to provide further insight into the factors regulating the repair tissue phenotype (47,48).

2. Materials and Methods

2.1 Cell isolation and expansion

Bone marrow derived mesenchymal stem cells (BMSCs) were obtained from the femur of 4-6 month old lapine donors. The bone marrow was removed from the femoral shaft and washed in high-glucose Dulbecco's Modified Eagle Medium (hgDMEM, GlutaMAXTM; Biosciences, Ireland) supplemented with 8% foetal bovine serum (FBS), (Biosciences, Ireland), 2% rabbit serum (Sigma, Ireland), 1% penicillin (100 U ml⁻¹)- streptomycin (100 μ g ml⁻¹) (Biosciences, Ireland). A homogenous cell suspension was achieved by triturating with a 18G needle. The solution was centrifuged twice at 650g for 5 min, with removal of the supernatant. The resultant cell pellet was triturated and the cell suspension was filtered through a 40 μ m cell sieve before plating at a density of 5×10^3 cells/cm².

Following colony formation, cells were trypsinized, counted and re-plated for a further passage at a density of 5×10^3 cells/cm². All expansion was conducted at 5% oxygen tension and media was changed twice weekly. Cells were embedded within the alginate gel at the end of passage 2.

2.2 Engineering of the Cartilage Template

The cartilage template was prepared by dissolving RGD- γ alginate in sterile hgDMEM to make up a final concentration of 1.5% w/v. RGD- γ alginate was made as follows; low-molecular-weight sodium alginate (37,000 g/mol) was prepared by irradiating Protanal LF 20/40 (196,000 g/mol; FMC Biopolymer, Philadelphia, PA) at a gamma dose of 5 Mrad (46). RGD-modified alginates were prepared by coupling the GGGGRGDSP to the alginate by carbodiimide reaction chemistry. Alginate (10 g) was dissolved at 1% (w/v) in MES Buffer (pH 6.5). Sulfo-NHS (274 mg, Pierce, Rockford, IL), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 484 mg, Sigma), and GGGGRGDSP peptide (100 mg, AIBioTech, Richmond, VA) were then added into alginate solution. After reacting for 24 h at 48C, the reaction was stopped by addition of hydroxylamine (0.18 mg/mL; Sigma), and the solution was purified by dialysis against ultrapure deionized water (diH₂O) (MWCO 3500; Spectrum Laboratories, Rancho Dominguez, CA) for 3 days, treated with activated charcoal (0.5 mg/100 mL, 50–200 mesh; Fisher, Pittsburgh, PA) for 30 min, filtered (0.22 mm filter), and lyophilized (49–51). The constructs were formed by combining BMSCs, counted and spun to form a cell pellet, with the alginate, using a 16G needle to mix the suspension with a final density of 2×10^7 cells/mL. The alginate/cell suspension was poured into a specifically designed 4% agarose mould containing 50 mM CaCl₂, and allowed to cross-link for 30 min at 37°C to produce 4 x 4 mm cylindrical constructs. Constructs were maintained in chondrogenic medium (CDM), consisting of hgDMEM supplemented with penicillin (100 U mL⁻¹)-streptomycin (100 μ g mL⁻¹), 100 μ g mL⁻¹ sodium pyruvate, 40 μ g mL⁻¹ l-proline, 50 μ g mL⁻¹ α -ascorbic acid-2-phosphate, 1.5 mg mL⁻¹ BSA, 1 $\times$ insulin-transferrin-selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng mL⁻¹ recombinant human transforming growth factor- β 3 (TGF- β 3; ProSpec-Tany TechnoGene Ltd,

Israel). Constructs were cultured at 37°C with 5% oxygen for 32 days with medium exchange twice weekly.

2.3 Biochemical analysis

Constructs were digested in papain (125 µg mL⁻¹) in 0.1 M sodium acetate, 5 mM cysteine HCl, and 50 mM EDTA (pH 6.0) (all from Sigma-Aldrich) at 60 °C under constant rotation for 18h. Total DNA content was quantified using the Hoechst Bisbenzimidazole 33258 dye assay (Sigma-Aldrich). Proteoglycan content was estimated by quantifying the amount of sulfated glycosaminoglycan (sGAG) in constructs using the dimethylmethyle blue dye-binding assay (Blyscan, Biocolor Ltd.), with a chondroitin sulfate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolyzed at 110°C for 18 h in concentrated HCL (38%) and assayed using a chloramine-T assay with a hydroxyproline-to-collagen ratio of 1:7.69(52).

2.4 Cartilage template implantation

New Zealand white (6-8 months) rabbits were anesthetized with ketamine-medetomidine maintained using isoflurane and oxygen. The defect sites were prepared by shaving and washing with chlorhexidine surgical scrub and alcohol. Surgical drapes were used to cover the non-incision area. Defects (4 x 4 mm in diameter) were introduced in the medial femoral condyle of the hind leg of using a biopsy punch and a dental drill with a burr. The defects were cleaned with saline prior to implanting the cartilage template. Two defects were made per rabbit (one in each femur), constructs were implanted (n=6) by press-fitting and empty defects served as controls (n=6). Post-surgery, rabbits were permitted free activity with post-operative analgesia buprenorphine hydrochloride, given for three days. Animals were sacrificed after 3 months using Pentobarbital. This protocol and study was reviewed and approved by the ethics committee of Trinity College Dublin, Ireland.

2.5 Histology and immunochemistry

Constructs were fixed in 10% formalin (Sigma-Aldrich) for 48 hours. They were decalcified using 'Decalcifying Solution-Lite' (Sigma-Aldrich), then dehydrated through graded alcohols, embedded in paraffin and sliced to 10 μ m. Slices were stained with 1% alcian blue 8GX (Sigma-Aldrich) in 0.1 M HCl, pH 1 for sGAG or with aldehyde fuchsin and 1% alcian blue, pH 1. Collagen types I, II, and X were evaluated using a standard immunohistochemical technique; sections were rehydrated and treated with chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37°C to enhance permeability of the extracellular matrix. This was followed by incubation in goat serum to block non-specific sites and the relevant primary antibodies (mouse monoclonal; Abcam); collagen type I (ab90395, 1:400), collagen type II (ab3092, 1:100) or collagen type X (ab49945, 1:100) were applied overnight at 4°C. Treatment with peroxidase preceded the application of the secondary antibody (collagen type I and II, B7151, 1.5:200; collagen type X, ab49760, 1:200) at room temperature for 1 h. Thereafter, all sections were incubated with ABC reagent (Vectastain PK-400; Vector Labs) for 45 min. Finally, sections were developed with DAB peroxidase (Vector Labs) for 5 min. Positive and negative controls were included in the immunohistochemical staining protocols.

2.6 Histological Scoring

Histological and macroscopic blind scoring was conducted by 6 impartial people in groups of 2 (n=3 scores). The wax embedded sample was cut down the centre and sliced from the centre of the defect. Two histological slides (one from each side of the sample) were included in the randomly selected histological slides for scoring, and an average score was calculated. Histological and macroscopic images were scored using a modified version of the O'Driscoll score (Table 1 & 2).

2.7 Mechanical Testing

Indentation tests were performed using a single column Zwick (Zwick, Roell, Germany) with a 5 N load cell. Unconfined compression tests were carried out as previously described⁽⁵³⁾. Briefly, the repaired tissue was indented using an impermeable metal indenter of 1 mm diameter to a depth of

50µm into the tissue and held until relaxation. Subsequently, a dynamic test was performed at 1 Hz, from this the amplitude of the dynamic stress was extracted by dividing the CSA by the average force between the peak and trough of ten cycles. The mechanics of the repair tissue was assessed at the centre point, unless there was an uneven surface; in such cases preference was given to the region with the most repair tissue slightly left or right of the centre of the defect site.

2.8 Micro-computed tomography

Micro-computed tomography (µCT) scans were performed on the femoral condyle explants using a Scanco Medical 40 µCT system (Scanco Medical, Bassersdorf, Switzerland) to visualise and quantify mineral deposition. Six condyles were scanned per experimental and control group after 3 months *in vivo*. Constructs were scanned in 50% EtOH, at a voxel resolution of 30 µm, a voltage of 70 kVp, and a current of 114 µA. Reconstructed 3D images were generated to visualise the repaired bone. Quantification of mineralization within the defect site was performed by setting a threshold of 210 (corresponding to a density of 399.5 mg hydroxyapatite/cm³) and calculating the bone volume (BV) within a 3.5 x 3.5 mm cylinder, which excluded the original bone.

2.9 Computational Modelling

A previously developed computational model was used to predict BMSC differentiation and tissue development in the empty and treated defect (47,54–56). This model utilised an iterative procedure which is outlined in greater detail in a previous study (56). Briefly, a finite element (FE) model was used to determine the mechanical environment within the defect (Fig. 1 a). The dimensions and loading conditions of the condyle were obtained from the literature (57–60). Angiogenesis and BMSC migration and proliferation were modelled using a lattice based approach (61). Similar to previous studies, blood vessel growth was biased in the direction of the minimum principal strain (54,55). The results of the angiogenesis and cell migration models were then used as inputs to an oxygen diffusion model. In this case, oxygen consumption from the cells was modelled using Michealis-Menten kinetics (54,55). Cell differentiation was predicted using a previously developed algorithm which

assumed that the fate of BMSCs was dependent upon the local oxygen tension and substrate stiffness (62–64), while the fate of cartilage was influenced by the local oxygen tension and octahedral shear strain (Fig 1 b, c). Of note for this study is that cartilage can become hypertrophic if the oxygen tension is high and the mechanical strain low. Finally, based on previous studies (11,20), it was assumed that cartilage which was subjected to chondrogenic conditions (specifically a low oxygen tension) for a period of 10 weeks formed stable cartilage. This stable cartilage inhibited blood vessel growth and was resistant to hypertrophy and endochondral ossification.

In accordance with the experimental model, tissue formation was predicted over 12 weeks where each iteration of the model represented a 24-hour period. In the empty defect model, it was assumed that the defect was filled with granulation tissue which contained BMSCs. In the model of the defect treated with an engineered cartilage template, upon implantation it was assumed that the encapsulated BMSCs had undergone chondrogenic differentiation for 32 days (equivalent to pre-culture period described above). For this reason, in this model the defect was assumed filled with chondrocytes (CCs) at day 0.

2.10 Statistical Analysis

Results are presented as mean $\pm$ standard deviation. Statistical analysis was performed with GraphPad Prism 5 software package (GraphPad, USA). Experimental groups were analyzed for significant differences using either a t-test or a general linear model for analysis of variance (ANOVA). Significance was accepted at a level of $p < 0.05$.

3. Results

3.1 RGD functionalized, γ -irradiated alginate hydrogels support the development of a cartilage template in vitro

Over 32 days of *in vitro* culture the RGD functionalized and γ -irradiated alginate hydrogels supported the development of a cartilage-like tissue that stained positive for collagen type II and sGAG deposition (Fig. 2 c). sGAG and collagen accumulation were measured to be $166 \pm 28 \mu\text{g}$ and $123 \pm 10 \mu\text{g}$ respectively. There was some positive staining for collagen type X, indicating the early stages of hypertrophy in some areas of the cartilage template (Fig. 2 c), although negative staining for alizarin red (for calcium deposition) and collagen type I demonstrated that the tissue had not ossified to any extent *in vitro* (Fig. 2 c). There was a significant increase in mechanical properties of the template over time in culture, from $2.39 \pm 0.94 \text{ kPa}$ to $7.35 \pm 1.25 \text{ kPa}$ (Fig. 2 b). Live/dead analysis was conducted at the end of the culture period which showed a cell viability of 84% within the cartilage templates (data not shown).

3.2 Treatment of OC defects with an engineered cartilage template results in the development of a stiffer repair tissue

Macroscopically, there was no difference apparent between the treated group and empty control group (Fig. 3 a, b), as quantified using macroscopic scoring (Fig. 3 c, d). In both groups, there were instances of what appeared to be both complete and partial filling of the defects. However, mechanical testing identified that repair tissue was significantly stiffer in defects treated with the cartilage template compared to the empty controls (Fig 3. e, f, g).

De novo bone tissue, as measured by μCT , was observed within the osteochondral defects. The reconstructed 3D scans demonstrated mineralized tissue within the centre of the defect, with trabecular struts evident in the deeper regions of the repair tissue (Fig 4. a, b). Complete bone repair was not detected in either the empty or treated defects, and even in the best examples of repair there

was some evidence of incomplete subchondral bone regeneration at the bone-cartilage interface. Quantitative analysis of the defect area revealed no significant difference in overall levels of bone fill between empty and treated defects (Fig 4. c, d).

3.3 Engineered cartilage templates promote the development of a more hyaline cartilage-like repair tissue in osteochondral defects

All defects treated with engineered cartilage templates stained intensely with alcian blue, indicating the development of proteoglycan-rich tissue, with more variable staining observed within the empty controls (Fig. 5 a, b). The best repair observed in the empty defects was fibrocartilaginous in nature, staining positively for type I and type II collagen, whilst the corresponding cartilage template treated defects displayed a more hyaline-like tissue with only minimal type I collagen staining (Fig. 5 a, b, c, d). The repair tissue stained weakly for type X collagen, a marker for hypertrophy, in the best repair both empty and treated defects. However, there appeared to be pericellular staining for collagen type X in the empty defects that underwent poorer repair. Following blind evaluation of histological sections, a significant difference was found in the overall histological scores of empty and treated defects, with superior repair observed in treated defects (Fig. 6 a, b). Additionally, a lack of cellular alignment is observed within the empty controls when compared to native cartilage. The cartilage template treated group generally exhibited a normal cell morphology and alignment more like that of native articular cartilage (Fig. 5 d). Overall, the histomorphometric analysis demonstrated that the cartilage template trended towards improved repair in all parameters evaluated, with significant differences observed for percentage tissue that is hyaline (Fig. 6 b), integration of newly formed cartilage with surrounding cartilage (Fig. 6 f) and a lack of degenerative changes in the adjacent tissue (Fig. 6 h).

3.4 Computational predictions suggest that local levels of oxygen availability and mechanical stimuli may play a role in determining whether hyaline, fibrocartilage or hypertrophic cartilage form within osteochondral defects

The **computational mechanobiological** model predicted the same patterns of tissue development as observed experimentally in both empty and treated defects. In models of both empty and treated defects, bone and cartilage **was predicted to** initially form in the osseous phase while a mixture of cartilage and fibrocartilage formed in the chondral phase of the defect (Fig. 7). As the simulations progressed, in both groups, a bone front advanced towards the chondral phase by means of endochondral ossification (Fig. 7 b). Similar to the experimental findings, a higher quantity of cartilage was predicted at each time point in the chondral phase of defects treated with engineered templates (Fig. 7 c). Conversely, in the empty defect simulation, a higher quantity of fibrocartilage was predicted in the chondral phase at each time point. In both models, there was roughly the same quantity of bone predicted in the osseous phase of the defect at weeks 4, 8 and 12 (Fig. 7 d).

The similar patterns of bone formation predicted in the models can be attributed to the fact that there was very little difference in the spatial and temporal patterns of blood vessel formation predicted in empty and treated defects (Fig. 7 a). In both models blood vessels sprouted formed from the cancellous bone and, over time, advanced towards the surface of the defect. These similarities in the pattern of vessel formation resulted in a similar oxygen profile in empty and treated defects (Fig. 7 a), which in turn supported comparable levels of osteogenesis.

The differences in the tissues which formed in the chondral phase of the osteochondral defect can be attributed, in part, to the higher stiffness of the engineered template compared to granulation tissue that is assumed to initially fill the empty defect. The result of this was that the cells within the chondral phase of the scaffold treated defect were subjected to lower magnitudes of strain compared to in the empty defect (data not shown). This, coupled with the hypoxic environment, supported higher levels of cartilage development compared to more fibrocartilage repair in empty defects. The greater

persistence of cartilage within the chondral phase of the treated defects can be attributed to the pre-culture stage. This ensured that more stable cartilage (BMSCs chondrogenically stimulated for 32 days) had formed within the chondral phase of the treated defects compared to the empty defects. The result of this was that, as blood vessels advanced towards the chondral phase, the cartilage in the treated defect was more resistant to hypertrophy and endochondral ossification.

4. Discussion

The osteochondral unit develops postnatally from a cartilaginous precursor that undergoes endochondral ossification during skeletal maturation (6,65). Inspired by this developmental process, herein we demonstrate that cartilaginous templates engineered using BMSCs encapsulated within RGD-modified and gamma irradiated alginate hydrogels can be used to regenerate critically-sized osteochondral defects. While empty osteochondral defects are capable of undergoing spontaneous repair (66,67), more hyaline-like cartilage tissue was observed in defects treated with engineered soft tissue templates. Furthermore, the repair tissue in treated defects was stiffer than in empty controls. To provide a more mechanistic understanding of the regenerative process, we next used a computational model to simulate tissue development in both the empty and treated defects. This model provides evidence demonstrating that local levels of oxygen availability and mechanical cues direct the fate of chondrogenically primed BMSCs following implantation into an osteochondral defect.

Cartilage templates were engineered *in vitro* by encapsulating BMSCs within RGD modified and γ -irradiated alginate hydrogels and by stimulating these constructs with TGF- β 3 over 32 days *in vitro*. Such alginate hydrogels have previously been shown to support endochondral bone formation subcutaneously (14), and in this study cartilaginous tissues engineered within these gels also facilitated bone regeneration and remodeling within the osseous phase of the osteochondral defect.

Biomaterials are commonly used for bone tissue engineering; however, a key factor is tailoring the material degradation rate to synchronize with tissue formation, and to facilitate vascular invasion. We have previously shown that unmodified alginate can support the development of a hypertrophic cartilage template *in vitro* (68) and endochondral bone formation subcutaneously (16), but permitted only limited bone formation *in vivo* in a cranial defect model due to the slow degradation rate of the hydrogel (40). However, using this modified alginate, in 5 out of the 6 defects there was no evidence of any residual material 3 months post-implantation.

Defects treated with the engineered cartilage template were found to contain a more hyaline-like repair tissue as demonstrated by histological staining, superior mechanical properties and statistically significant histomorphometric scores. The inferior mechanical properties of the repair tissue within the empty control defects, coupled with positive collagen type 1 staining, suggests the development of a fibrocartilaginous tissue. The integration of the *de novo* cartilage with existing cartilage is also paramount for joint stability, and is often reported to be difficult to achieve with bioengineering strategies (69,70). For example, integration with the host tissue was one of limiting factors reported to affect complete repair of osteochondral defects using bone marrow derived stem cells in a collagen gel (70). In this study, we observe a significantly **better** integrated repair tissue in the treated group, however consistent integration with the host cartilage was not obtained. In addition, we did not observe hyaline repair in all treated defects, demonstrating that further optimization of the tissue engineering strategy is warranted.

The computational models demonstrated that both the oxygen and mechanical environment varied spatially within the defect region, with higher levels of oxygen and lower magnitudes of strain leading to the prediction of endochondral bone formation in the osseous region of the defect, which correlated closely with our *in vivo* findings. Furthermore, the model suggested that local levels of oxygen availability and mechanical stimuli play a key role in determining whether hyaline, fibrocartilage or

hypertrophic cartilage would form in the chondral region of the defect. The improvement in cartilage formation observed in the treated group compared to the empty defects can be attributed to the increased stiffness of the engineered tissue when compared to granulation tissue, as well as the chondrogenic priming of the MSCs prior to implantation. The pre-culture period ensured that stable cartilage formed within the chondral phase of the scaffold at an earlier time point compared to the empty defect. This stable cartilage was hence more resistant to vascularisation, hypertrophy and endochondral ossification.

The insight provided by the *in silico* model can also be used to improve future tissue engineering strategies targeting larger and hence more challenging chondral, osteochondral defects or whole joint **resurfacing** applications. Ideally the mechanical properties of the engineered template would more closely mimic that of the native tissue prior to implantation, or the oxygen environment within the template could be altered through the incorporation of hypoxia inducing factors (71). Potentially, a stiffer implant is required to sustain the harsh loading, however, it must not compromise the chondrogenic capacity of the implant. This could be achieved, for example, using 3D biofabrication technology to reinforce the alginate hydrogel with printed polymer fibres (72). Polycaprolactone (PCL) based scaffolds have previously been used in the repair of articular cartilage defects to some degree of success (73).

In summary, tissue engineered cartilage templates were found to undergo spatially defined differentiation in response to local environmental cues within OC defects. This engineered template prompted the development of a more hyaline-like cartilage repair tissue, pointing to the potential for developmentally inspired soft tissue templates, engineered using BMSCS, to regenerate damaged and diseased **synovial** joints.

5. Acknowledgements

Science Foundation Ireland grant number 12/IA/1554

6. Author Disclosure Statement

No competing financial interests exist

7. References

1. Nakagawa Y, Suzuki T, Kuroki H, Kobayashi M, Okamoto Y, Nakamura T. The effect of surface incongruity of grafted plugs in osteochondral grafting: A report of five cases. *Knee Surgery, Sport. Traumatol. Arthrosc.* **15**(5), 591, 2007;
2. Bowland P, Ingham E, Jennings L, Fisher J. Review of the biomechanics and biotribology of osteochondral grafts used for surgical interventions in the knee. *Proc. Inst. Mech. Eng. H.* **229**(12), 879, 2015;
3. Koh JL, Wirsing K, Lautenschlager E, Zhang L-O. The effect of graft height mismatch on contact pressure following osteochondral grafting: a biomechanical study. *Am. J. Sports Med.* **32**(2), 317, 2004;
4. Martin I, Miot S, Barbero A, Jakob M, Wendt D. Osteochondral tissue engineering. *J. Biomech.* **40**(4), 750, 2007;
5. Grässel S, Lorenz J. Tissue-Engineering Strategies to Repair Chondral and Osteochondral Tissue in Osteoarthritis: Use of Mesenchymal Stem Cells. *Curr. Rheumatol. Rep.* **16**(10), 1, 2014;
6. Hunziker EB, Kapfinger E, Geiss J. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. *Osteoarthr. Cartil.* **15**(4), 403, 2007;
7. Scotti C, Tonnarelli B, Papadimitropoulos A, Scherberich A, Schaeren S, Schauerte A, et al. Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. *Proc. Natl. Acad. Sci. U. S. A.* **107**(16), 7251, 2010;
8. Kronenberg HM. Developmental regulation of the growth plate. *Nature.* **423**(6937), 332, 2003;
9. Hu DP, Ferro F, Yang F, Taylor AJ, Chang W, Miclau T, et al. Cartilage to bone transformation during fracture healing is coordinated by the invading vasculature and induction of the core pluripotency

- genes. *Development*. **144**(2), 221, 2017;
10. Ng JJ, Wei Y, Zhou B, Bernhard J, Robinson S, Burapachaisri A, et al. Recapitulation of physiological spatiotemporal signals promotes in vitro formation of phenotypically stable human articular cartilage. *Proc. Natl. Acad. Sci.* **114**(10), 201611771, 2017;
 11. Liu K, Zhou GD, Liu W, Zhang WJ, Cui L, Liu X, et al. The dependence of in vivo stable ectopic chondrogenesis by human mesenchymal stem cells on chondrogenic differentiation in vitro. *Biomaterials*. **29**(14), 2183, 2008;
 12. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic Differentiation of Cultured Human Mesenchymal Stem Cells from Marrow. *Tissue Eng.* **4**(4), 415, 1998;
 13. Farrell E, van der Jagt OP, Koevoet W, Kops N, van Manen CJ, Hellingman C a, et al. Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? *Tissue Eng. Part C. Methods*. **15**(2), 285, 2009;
 14. Daly AC, Cunniffe GM, Sathy BN, Jeon O, Alsberg E, Kelly DJ. 3D Bioprinting of Developmentally Inspired Templates for Whole Bone Organ Engineering. *Adv. Healthc. Mater.* 2016;
 15. Dang P, Herberg S, Varghai D, Riazi H, Varghai D, McMillan A, et al. Endochondral Ossification in Critical-Sized Bone Defects via Readily Implantable Scaffold-Free Stem Cell Constructs. *Stem Cells Transl. Med.* **6**(7), 1644, 2017;
 16. Sheehy EJ, Mesallati T, Kelly L, Vinardell T, Buckley CT, Kelly DJ. Tissue Engineering Whole Bones Through Endochondral Ossification: Regenerating the Distal Phalanx. *Biores. Open Access*. **4**, 229, 2015;
 17. Vinardell T, Sheehy EJ, Buckley CT, Kelly DJ. A comparison of the functionality and in vivo phenotypic stability of cartilaginous tissues engineered from different stem cell sources. *Tissue Eng. Part A*. **18**(11–12), 1161, 2012;
 18. Chen S, Fu P, Cong R, Wu H, Pei M. Strategies to minimize hypertrophy in cartilage engineering and regeneration. *Genes Dis. Elsevier Ltd*; **2**(1), 76, 2015;
 19. Sheehy EJ, Buckley CT, Kelly DJ. Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells. *Biochem. Biophys. Res. Commun. Elsevier Inc.*; **417**(1), 305, 2012;

20. Leijten J, Georgi N, Moreira Teixeira L, van Blitterswijk C a., Post JN, Karperien M. Metabolic programming of mesenchymal stromal cells by oxygen tension directs chondrogenic cell fate. *Proc. Natl. Acad. Sci.* **111**(38), 13954, 2014;
21. Luo L, Thorpe SD, Buckley CT, Kelly DJ. The effects of dynamic compression on the development of cartilage grafts engineered using bone marrow and infrapatellar fat pad derived stem cells. *Biomed. Mater. IOP Publishing*; **10**(5), 55011, 2015;
22. Carroll SF, Buckley CT, Kelly DJ. Cyclic hydrostatic pressure promotes a stable cartilage phenotype and enhances the functional development of cartilaginous grafts engineered using multipotent stromal cells isolated from bone marrow and infrapatellar fat pad. *J. Biomech. Elsevier*; **47**(9), 2115, 2014;
23. Vinardell T, Rolfe BA, Buckley CT, Meyer EG, Ahearne M, Murphy P, et al. Hydrostatic pressure acts to stabilize a chondrogenic phenotype in porcine joint tissue derived stem cells. *Eur. Cells Mater.* **23**, 121, 2012;
24. Lafont J. Lack of oxygen in articular cartilage: Consequences for chondrocyte biology. *Int. J. Exp. Pathol.* **91**(2), 99, 2010;
25. Kanichai M, Ferguson D, Prendergast PJ, Campbell VA. Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: A role for AKT and hypoxia-inducible factor (HIF)-1 α . *J. Cell. Physiol.* **216**(3), 708, 2008;
26. Robins JC, Akeno N, Mukherjee A, Dalal RR, Aronow BJ, Koopman P, et al. Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. *Bone.* **37**(3), 313, 2005;
27. Takahashi K, Kubo T, Kobayashi K, Imanishi J, Takigawa M, Arai Y, et al. Hydrostatic pressure influences mRNA expression of transforming growth factor-beta 1 and heat shock protein 70 in chondrocyte-like cell line. *J. Orthop. Res.* **15**(1), 150, 1997;
28. Ikenoue T, Trindade MCD, Lee MS, Lin EY, Schurman DJ, Goodman SB, et al. Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. *J. Orthop. Res.* **21**(1), 110, 2003;
29. Steward AJ, Thorpe SD, Vinardell T, Buckley CT, Wagner DR, Kelly DJ. Cell-matrix interactions regulate mesenchymal stem cell response to hydrostatic pressure. *Acta Biomater. Acta Materialia Inc.*; **8**(6), 2153, 2012;

30. Thorpe SD, Nagel T, Carroll SF, Kelly DJ. Modulating Gradients in Regulatory Signals within Mesenchymal Stem Cell Seeded Hydrogels: A Novel Strategy to Engineer Zonal Articular Cartilage. *PLoS One*. **8**(4), 2013;
31. Thorpe SD, Nagel T, Carroll SF, Kelly DJ. Modulating Gradients in Regulatory Signals within Mesenchymal Stem Cell Seeded Hydrogels: A Novel Strategy to Engineer Zonal Articular Cartilage. *PLoS One*. **8**(4), 2013;
32. Yao S, Ph D, Alini M, Ph D, Stoddart MJ, Ph D, et al. Chondrogenesis of human bone marrow mesenchymal stem cells in fibrin-polyurethane composites is modulated by frequency and amplitude of dynamic compression and shear stress. *Tissue Eng. Part A*. **16**(2), 575, 2010;
33. Li Z, Kupcsik L, Yao SJ, Alini M, Stoddart MJ. Mechanical load modulates chondrogenesis of human mesenchymal stem cells through the TGF- β pathway. *J. Cell. Mol. Med*. **14**(6 A), 1338, 2010;
34. Lee KY, Mooney DJ. Alginate: Properties and biomedical applications. *Prog. Polym. Sci. Elsevier Ltd*; **37**(1), 106, 2012;
35. Wang L, Shelton RM, Cooper PR, Lawson M, Triffitt JT, Barralet JE. Evaluation of sodium alginate for bone marrow cell tissue engineering. *Biomaterials*. **24**(20), 3475, 2003;
36. Awad H a., Wickham MQ, Leddy H a., Gimple JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials*. **25**(16), 3211, 2004;
37. Daly AC, Critchley SE, Rensock EM, Kelly DJ. A Comparison of Different Bioinks for 3D Bioprinting of Fibrocartilage and Hyaline Cartilage. *Biofabrication. IOP Publishing*; **8**(4), 1, 2016;
38. Freeman FE, Allen AB, Stevens HY, Guldberg RE, McNamara LM. Effects of in vitro endochondral priming and pre-vascularisation of human MSC cellular aggregates in vivo. *Stem Cell Res. Ther.* [Internet]. *Stem Cell Research & Therapy*; **6**(1), 218, 2015; Available from: <http://stemcellres.com/content/6/1/218>
39. Freeman FE, Kelly DJ. Tuning Alginate Bioink Stiffness and Composition for Controlled Growth Factor Delivery and to Spatially Direct MSC Fate within Bioprinted Tissues. *Sci. Rep.* [Internet]. Springer US; **7**(November), 1, 2017; Available from: <http://dx.doi.org/10.1038/s41598-017-17286-1>
40. Cunniffe GM, Vinardell T, Thompson EM, Daly AC, Matsiko A, O'Brien FJ, et al. Chondrogenically primed mesenchymal stem cell-seeded alginate hydrogels promote early bone formation in critically-sized defects. *Eur. Polym. J. Elsevier Ltd*; **72**, 464, 2015;

41. Simmons CA, Alsberg E, Hsiong S, Kim WJ, Mooney DJ. Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by transplanted bone marrow stromal cells. *Bone*. **35**(2), 562, 2004;
42. Venkatesan J, Bhatnagar I, Manivasagan P, Kang KH, Kim SK. Alginate composites for bone tissue engineering: A review. *Int. J. Biol. Macromol.* [Internet]. Elsevier B.V.; **72**, 269, 2015; Available from: <http://dx.doi.org/10.1016/j.ijbiomac.2014.07.008>
43. Alsberg E, Anderson KW, Albeiruti a, Franceschi RT, Mooney DJ. Cell-interactive alginate hydrogels for bone tissue engineering. *J. Dent. Res.* [Internet]. **80**(11), 2025, 2001; Available from: <http://jdr.sagepub.com/cgi/doi/10.1177/00220345010800111501>
44. Kong HJ, Kaigler D, Kim K, Mooney DJ. Controlling rigidity and degradation of alginate hydrogels via molecular weight distribution. *Biomacromolecules*. **5**(5), 1720, 2004;
45. Boontheekul T, Kong HJ, Mooney DJ. Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. *Biomaterials*. **26**(15), 2455, 2005;
46. Alsberg E, Kong HJ, Hirano Y, Smith MK, Albeiruti A, Mooney DJ. Regulating Bone Formation via Controlled Scaffold Degradation. *J. Dent. Res.* **82**, 903, 2003;
47. O'Reilly A, Kelly DJ. Unravelling the role of mechanical stimuli in regulating cell fate during osteochondral defect repair. *Ann. Biomed. Eng.* 2016;
48. O'Reilly A, Kelly DJ. Role of oxygen as a regulator of stem cell fate during the spontaneous repair of osteochondral defects. *J. Orthop. Res.* (June), 1026, 2015;
49. Jeon O, Alsberg E. Photofunctionalization of Alginate Hydrogels to Promote Adhesion and Proliferation of Human Mesenchymal Stem Cells. *Tissue Eng. Part A*. **19**(11–12), 1424, 2013;
50. Jeon O, Powell C, Ahmed SM, Alsberg E. Biodegradable, photocrosslinked alginate hydrogels with independently tailorable physical properties and cell adhesivity. *Tissue Eng. Part A*. **16**(9), 2915, 2010;
51. Jeon O, Powell C, Solorio LD, Krebs MD, Alsberg E. Affinity-based growth factor delivery using biodegradable, photocrosslinked heparin-alginate hydrogels. *J. Control. Release. Elsevier B.V.*; **154**(3), 258, 2011;
52. Ignat'eva NY, Danilov NA, Averkiev S V., Obrezkova M V., Lunin V V., Sobol' EN. Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. *J. Anal. Chem.* **62**(1), 51, 2007;

53. Olvera D, Daly A, Kelly DJ. Mechanical Testing of Cartilage Constructs. *Methods Mol. Biol.* **1340**, 279, 2015;
54. O'Reilly A, Kelly DJ. The role of oxygen as a regulator of stem cell fate during fracture the spontaneous repair of osteochondral defect. *J. Orthop. Res.* 2015;
55. O'Reilly A, Hankenson KD, Kelly DJ. A computational model to explore the role of angiogenic impairment on endochondral ossification during fracture healing. *Biomech. Model. Mechanobiol.* 2016;
56. O'Reilly A, Kelly DJ. A Computational Model of Osteochondral Defect Repair Following Implantation of Stem Cell Laden Multiphase Scaffolds. *Tissue Eng Part A.* **1–2**, 30, 2017;
57. Grover DM, Chen A a., Hazelwood SJ. Biomechanics of the rabbit knee and ankle: Muscle, ligament, and joint contact force predictions. *J. Biomech.* **40**(12), 2816, 2007;
58. Gushue DL, Houck J, Lerner AL. Rabbit knee joint biomechanics: motion analysis and modeling of forces during hopping. *J. Orthop. Res.* **23**(4), 735, 2005;
59. Sundaramurthy S, Mao JJ. Modulation of endochondral development of the distal femoral condyle by mechanical loading. *J. Orthop. Res.* **24**(2), 229, 2006;
60. Proffen BL, McElfresh M, Fleming BC, Murray MM. A comparative anatomical study of the human knee and six animal species. *Knee. Elsevier B.V.*; **19**(4), 493, 2012;
61. Checa S, Prendergast PJ. A mechanobiological model for tissue differentiation that includes angiogenesis: a lattice-based modeling approach. *Ann. Biomed. Eng.* **37**(1), 129, 2009;
62. Burke DP, Khayyeri H, Kelly DJ. Substrate stiffness and oxygen availability as regulators of mesenchymal stem cell differentiation within a mechanically loaded bone chamber. *Biomech. Model. Mechanobiol.* **14**(1), 93, 2014;
63. Burke D, Dishowitz M, Sweetwyne M, Miedel E, Hankenson KD, Kelly DJ. The Role of Oxygen as a Regulator of Stem Cell Fate During Fracture Repair in TSP2-Null Mice. *J. Orthop. Res.* **31**(10), 1585, 2013;
64. Burke DP, Kelly DJ. Substrate Stiffness and Oxygen as Regulators of Stem Cell Differentiation during Skeletal Tissue Regeneration : A Mechanobiological Model. *PLoS One.* **7**(7), e40737, 2012;
65. Worthley DL, Churchill M, Compton JT, Tailor Y, Rao M, Si Y, et al. Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. *Cell. Elsevier Inc.*; **160**(1–2), 269, 2015;

66. Orth P, Cucchiarini M, Kaul G, Ong MF, Gräber S, Kohn DM, et al. Temporal and spatial migration pattern of the subchondral bone plate in a rabbit osteochondral defect model. *Osteoarthr. Cartil.* **20**(10), 1161, 2012;
67. Wei X, Messner K. Maturation-dependent durability of spontaneous cartilage repair in rabbit knee joint. *J. Biomed. Mater. Res.* **46**(4), 539, 1999;
68. Mesallati T, Sheehy E. ., Vinardell T, Buckley CT, Kelly DJ. Tissue engineering scaled-up anatomically shaped osteochondral constructs for joint resurfacing. *Eur. Cell. Mater.* **30**, 163, 2015;
69. Obradovic B, Martin I, Padera RF, Treppo S, Freed LE, Vunjak-Novakovic G. Integration of engineered cartilage. *J. Orthop. Res.* **19**(6), 1089, 2001;
70. Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan a I, et al. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J. Bone Joint Surg. Am.* **76**(4), 579, 1994;
71. Park KM, Gerecht S. Hypoxia-inducible hydrogels. *Nat. Commun.* **5**(410), 4075, 2014;
72. Visser J, Melchels FPW, Jeon JE, van Bussel EM, Kimpton LS, Byrne HM, et al. Reinforcement of hydrogels using three-dimensionally printed microfibrils. *Nat. Commun. Nature Publishing Group*; **6**, 6933, 2015;
73. Martinez-Diaz S, Garcia-Giralt N, Lebourg M, Gómez-Tejedor J-A, Vila G, Caceres E, et al. In vivo evaluation of 3-dimensional polycaprolactone scaffolds for cartilage repair in rabbits. *Am. J. Sports Med.* **38**(3), 509, 2010;

(a)

Cancellous Bone
 Cortical Bone
 Cartilage
 Meniscus
 Granulation Tissue

Stem Cell Source
 Angiogenic Source

(b)

MSC Differentiation Algorithm

(c)

Chondrocyte Differentiation Algorithm

(a)

Empty

Worst Repair

Best Repair

(b)

Cartilage Template

Worst Repair

Best Repair

(c)

(d)

(e)

(f)

(g)

Empty

(a)

Worst Repair

Best Repair

Cartilage Template

(b)

Worst Repair

Best Repair

(c)

(d)

(a)

Empty

Worst Repair

Best Repair

(b)

Cartilage Template

Worst Repair

Best Repair

(c)

Empty

Collagen Type II

Collagen Type I

Collagen Type X

Worst Repair

Intermediate Repair

Best Repair

(d)

Cartilage Template

Collagen Type II

Collagen Type I

Collagen Type X

Worst Repair

Intermediate Repair

Best Repair