The Development of Cell-Mediated Tissue Engineered Collagen-Based Strategies for Osteochondral Defect Repair

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Citation

Moran C. The Development of Cell-Mediated Tissue Engineered Collagen-Based Strategies for Osteochondral Defect Repair [MD Thesis]. Dublin: Royal College of Surgeons in Ireland; 2018.
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The Development of Cell-mediated Tissue Engineered Collagen-based Strategies for Osteochondral Defect Repair

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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of Doctor of Medicine

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2018
Declaration
I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Medicine (MD), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed:

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Date: 14/01/18
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Nomenclature

°C  Degrees Celsius
*  Registered Trade Mark
ACI  Autologous chondrocyte implantation
ACL  Anterior cruciate ligament
ADL  Activities of daily living
ADSC  Adipose derived stem cells
AL  Antero-lateral
AMIC  Autologous matrix induced chondrogenesis
ANOVA  Analysis of Variance
BMPs  Bone Morphogenetic Proteins
BMSC  Bone marrow mesenchymal stem cells
BV  Bone Volume
CE  Conformité Européene
CFU  Colony forming unit
CNOH  Cappagh National Orthopaedic Hospital
Col1  Collagen Type I Slurry
DCU  Dublin City University
DHT  Dehydrothermal treatment
DMEM  Dulbecco’s modified Eagle’s medium
DNA  Deoxyribonucleic Acid
ECM  Extracellular matrix
EDAC  1-ethyl-3-(3dimethylaminopropyl) carbodiimide
EDTA  Ethylenediaminetetraacetic acid
FBS  Foetal bovine serum
FBS  Foetal Bovine Serum
FDA  Food and Drug Administration
FGF  Fibroblast Growth Factor
FPMSC  Fat pad mesenchymal stem cells
g  Grams
GAG  Glycosaminoglycan
H&E  Haematoxylin and Eosin
HA  Hydroxyapatite
HTO  High tibial osteotomy
HyA  Hyaluronic Acid
ICRS  International Cartilage Repair Society
IGF  Insulin Growth Factor
Kg  Kilograms
LAST  Laboratory animal science and training
MACI  Matrix-induced Autologous Chondrocyte Implantation
MACI  Matrix Associated Autologous Chondrocyte Implantation
MFC  Medial femoral condyle
MFX  Microfracture
mg  Milligram
micro-CT  micro-Computed Tomography
min  Minute
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Abstract

Smooth articular cartilage is vital for the pain free movement of the joint, therefore the formation of osteochondral defects remains a challenge for orthopaedic surgeons. The hyaline cartilage, however is avascular and is poorly supplied with cells roughly 1.7% cell volume density in the medial femoral condyle (Hunziker, 1999). Therefore, any injury to the joint surface does not heal easily. The latest focus is on tissue engineering techniques to design scaffolds that can be implanted to stimulate the body’s own healing. In the design of these scaffolds the gradient nature of the tissue at the joint surface must be considered. One such scaffold has been designed in the Tissue Engineering Research Group of the Royal College of Surgeons in Ireland. This collagen based scaffold has been developed using a novel iterative freeze-drying technique with three seamlessly integrated layers representing the bone layer, the intermediate, or tidemark layer, and the cartilage layers which include the gliding, transitional and radial zones (Levingstone et al., 2014). The overall objective for this thesis is to develop and optimise a tissue engineered biomaterial scaffold based method of osteochondral defect repair that facilitates cell-seeding onto the scaffold as a single-stage procedure, with the possibility of implantation via arthroscopic technique. In order to achieve this, the specific aims of the thesis were to assess the modalities through which the scaffold directs cell differentiation in the different layers, to identify the optimal cell seeding regimen that can be used for the scaffold on the defect to produce best outcome of hyaline cartilage generation, to perform a large animal in vivo trial to assess the outcomes at 3, 6 and 12 months, and finally to develop an arthroscopic delivery device that can allow the scaffold be implanted through a minimally invasive procedure that will further decrease patient morbidity.

In Chapter 2 of this thesis the intrinsic properties of an RCSI developed, collagen-based tri-layered scaffold, to direct stem cell differentiation as required within each layer were examined. The bone layer was found to have osteoinductive as well as osteoconductive abilities when seeded with rat mesenchymal stem cells. It was shown that the intermediate layer was chondroconductive and would not allow the encroachment of mineralised tissue into the cartilage layer protecting the tidemark, even when the scaffold is cultured all together in osteochondrogenic media. The
cartilage layer proved itself to be chondroconductive as well as mildly chondroinductive. It was also shown that there was not a significant amount of contraction in the tri-layered scaffold in vitro. These results taken together show that the scaffold is suitable for use in press-fit technique into osteochondral defects and should ensure good outcomes of bone and cartilage formation in the relevant layers.

Chapter 3 of this thesis focused on establishing an optimal cell-seeding regime for this multi-layered collagen-based scaffold. Currently cell based approaches require an in vitro expansion phase of roughly six weeks (Brittberg et al., 1994), however it has been shown here that the use of a co-culture of rapidly isolated primary chondrocytes (CC) and fat pad derived mesenchymal stem cells (FPMSCs) in a ratio of 1:3 is a valid alternative, and the co-culture of FPMSCs:CC was also shown to have a synergistic effect that augmented the results of using merely chondrocytes alone. These FPMSCs and CCs can be harvested on the morning of surgery, seeded back onto the scaffold after isolation and re-implanted without the expensive in vitro expansion phase. This eliminates a large cost element of the procedure and simplifies the procedure for both patient and surgeon.

In Chapter 4 a large caprine in vivo study was undertaken to assess the cell-seeded and cell free version of the scaffold implanted in a critically sized defect of a caprine stifle joint and compare it to a commercially available biomaterial for osteochondral repair. Results demonstrated that the cartilage was satisfactorily repaired both macroscopically and microscopically, and that the tri-layered scaffold was able to generate cartilage and subchondral bone that was of a similar standard to the commercial product both as a cell-free, off-the-shelf-product and as a cell-seeded scaffold. There was no significant difference between the cell-seeded scaffold group and the cell-free scaffold group in this study.

Chapter 5 focusses on the development and testing of an arthroscopic delivery device for implanting the multi-layered scaffold into a human knee via a minimally invasive arthroscopic technique. A device was developed that involved a reusable trigger handle and a single-use windowed cassette in various sizes that would allow safe delivery of the scaffold to the osteochondral defect inside the joint. Benchtop testing of the arthroscopic delivery device showed that the delivery device successfully
implanted the scaffolds in a consistent manner without overly compressing them and causing the cell suspension to be extruded.

Collectively the results from this thesis have shown that the tri-layered scaffold has intrinsic properties to optimally repair osteochondral defects by directing cell differentiation appropriately for the individual layers. Cartilage repair can be augmented by pre-seeding the scaffold with cells prior to implantation, and best results for this are obtained using a co-culture of FPMSCs:CC, which can be harvested and isolated using a rapid isolation technique. These tri-layered scaffolds have been proven in in vivo trials to generate hyaline-like cartilage in a large animal model, and a device has been designed to enable the implantation of this device by arthroscopic technique.
Acknowledgements

Firstly, I would like to thank my supervisor Professor Fergal O’Brien for encouraging my interest in the future of osteochondral defect repair, for his enthusiasm for what could be achieved within the Tissue Engineering Research Group, and for allowing me to become a member of that team. I would also like to thank my clinical supervisor Professor John O’Byrne who has always been there throughout my ongoing education to advise me when I needed it, from my first day as a medical student through my postgraduate achievements and on to my specialist orthopaedic training.

A special note of thanks to Dr. Tanya Levingstone who gave a significant amount of time and energy to teaching me and guiding me through my exciting time in the lab and throughout my time writing up. She offered words of wisdom and experience at every corner and helped me out of every conundrum I got myself into.

None of this body of work, however, would have been possible without the assistance of each and every member of the Tissue Engineering Research Group in RCSI and the Trinity Centre for Bioengineering. These scientists have really shown me what it is to lend expertise from many different backgrounds and disciplines freely to help each other. I hope that I have been able to give back some expertise from my background in the way that they have given to me. I also have to thank all the friends that I have made during my time as a postgraduate research student for supporting me and making long hours in the lab go a little quicker.

I have to mention my thanks to Mr. Paul Moroney and Mr. Dylan Murray and their teams in the Mater Hospital who, during my time working on this research project were able to cobble me back together when I was encouraged to make a speedy dismount from my motorcycle, and allowed me to experience life on the other end of the orthopaedic scalpel.

I would also like to thank my family, my parents who, through their interests in education and the world around them have set me on a path with a never-ending thirst for knowledge, my brothers who are very good at reminding me that I may not have all the answers and that perhaps I should review my position, and my
Uncle John who has always been so approachable and enthusiastic about my career path.

Most importantly I must acknowledge my funding sources Science Foundation Ireland and the Health Research Board (SFI/HRB Translational Research Award funding (TRA/2011/19)) for the grant support to allow me to complete this work.

Thank you all

Dedication

This thesis is dedicated to my wife, Anne-Sophie. It was only at the beginning of my time in the lab that Anne-Sophie made the brave move of marrying me, but for the last thirteen years she has been right there next to me through thick and thin supporting me when I needed her the most and sharing my most memorable moments and successes. I would never have reached this point without you love, and words cannot express how grateful I am for everything you do.
Publications, Prizes, Presentations

Publications


Accepted for publication


Peer-reviewed Conference Abstracts Published


Presentations

Oral Presentations:

TJ Levingstone, C Moran, RT Brady, GM Cunniffe, HV Almeida, PAJ Brama, D Kelly, JM O'Byrne, FJ O'Brien (2016) Directed osteogenesis and chondrogenesis in a multi-layered osteochondral scaffold facilitates joint regeneration in a goat model using both cell-free and cell-seeded approaches. Proceedings of the 22th Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland, M. Walsh, D. Hoey and E. deBarra (Eds.), Galway, Ireland


Poster Presentations


Patents
EU and PCT Patent (Pending): Application number: EP14164455.9 (Priority Date, April 11, 2014) A device for delivering an implant to a desired location within the body provided in the form of a kit. Inventors: Levingstone, T.; O’Brien, F.J.; Hitchin, N.; Moran, C.; O’Byrne, J.M.; Lyons, F.

Prizes
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1.8  Thesis Objectives
1.1 Overview

Articular cartilage damage is a growing problem worldwide, arthritis affects 9.6% of men and 18% of women over the age of 60 in Europe (Woolf and Pfleger, 2003). An intact smooth cartilage layer is vital for pain-free movement of a synovial joint. However, if this cartilage surface becomes damaged, its ability to self-repair is limited. It has been seen for hundreds of years that damage to the cartilaginous surface of the joint can cause significant morbidity (Hunter, 1995), and yet, successful repair remains a challenge for clinicians despite considerable research currently ongoing in the area. Defects in the cartilage surface are classified clinically as chondral defects if damage is limited to the articular cartilage layer, or osteochondral defects if the subchondral bone is involved (Figure 1.1, Figure 1.2). Although both defect types lead to similar symptoms for the patient, they require different treatment methods and have different associated issues and challenges surrounding them. For chondral defects, although only one tissue type is involved, repair is limited due to the avascularity of the articular cartilage. Osteochondral defects require the subchondral bone to be repaired to support a chondral layer that is flush with the surface of the remaining cartilage. Osteochondral defects thus have a greater complexity as they require a gradient of cell types from osteocytes at the bony layer to chondrocytes at the cartilage surface with the relevant extracellular matrices surrounding them. However, when the subchondral bone layer is involved, there is more access to regenerative stem cells in the underlying marrow than the cartilage surface alone. Although there are differences, without appropriate treatment both defect types have a final common treatment pathway of total joint arthroplasty. Traditionally, orthopaedic surgeons try to stave off this significant invasive operation with symptomatic management, and in recent years, more advanced cell-based methods, that aim to repair the joint surface rather than merely manage the decline, are gradually becoming available.

The difficulty in cartilage regeneration is due in part to its highly specialised composition. Cartilage is predominantly made up of a collagenous extracellular matrix (ECM) composed of aggrecan bound to hyaluronan to form a supramolecular aggregate. This creates a large osmotic draw of water into the cartilage, placing the
collagen under tension, giving it compressive resilience and making it an effective shock absorber (Hardingham, 2010) that can maintain a lubricated joint surface. The ECM is made by, and contains, small numbers of chondrocytes which rely on diffusion for nutrients (Oldershaw, 2012). Chondrocytes by nature have low mitotic activity and undergo cell cycle arrest. Due to the rigidity of the cartilage matrix they are unable to migrate. This means that an isolated chondral defect has minimal access to stem cells for regeneration and repair unless the subchondral bone is involved. In that case there is increased access to mesenchymal stem cells (MSCs) from the bone marrow which can be recruited to the defect site to achieve healing. The difficulty in the treatment of these osteochondral defects is that the MSCs must differentiate into different highly specialised cell types at different layers within the defect site for the joint to function optimally. The cartilage at the joint surface is divided into four distinct zones (Figure 1.2), the superficial or gliding zone, middle or transitional zone, deep or radial zone, and the calcified zone. The gliding layer is the thinnest layer and contains flattened chondrocytes. These chondrocytes are arranged within collagen fibres that align tangential to the joint plane and help to resist shear forces. The transitional zone fibres are organised in an oblique pattern to the joint surface. This layer, along with the vertically orientated fibres or the radial zone, act as the spring mechanism of the joint and act against compression forces. There is a tidemark between the radial zone and the calcified cartilage below, which is only 5 µm thick with tangentially organised fibres. This layer resists shear of the cartilage above from the calcified cartilage and bony layers below. The calcified cartilage layer contains hydroxyapatite crystals and acts as an anchor to the bone (Miller, 2008, Matsiko et al., 2013). To optimally repair or regenerate these osteochondral defects and stave off osteoarthritic changes in the joint, each of these layers must be recreated.
There are several methods currently in use for osteochondral defect repair, including bone marrow stimulation techniques such as microfracture, mosaicplasty, and cell-based treatments such as autologous chondrocyte implantation (ACI). However, equivocal results for these treatment types has led to significant research into tissue
engineering (TE) techniques for the repair of osteochondral defects. There is significant research into the optimal biomaterials to use along with variations in growth factors and whether or not these scaffolds should be pre-seeded with cells or used as off-the-shelf implants (Hunziker, 2002). Although seeding the scaffolds can augment their potential, one of the main difficulties currently is the requirement for two operative procedures. The patient must have one initial procedure to harvest the chondrocytes, then, due to the low density of the cells in the harvested tissue, these cells must be expanded in vitro prior to re-implantation in a second procedure. To improve this situation a method of harvesting, isolating the cells, seeding them onto a bioengineered scaffold and re-implanting the scaffold as a “one-stage” procedure could be devised (Almeida et al., 2015). This method would dramatically decrease the financial cost of the procedure as well as reducing the morbidities associated with multiple operations (Samuelson and Brown, 2012).

1.2 Current surgical approaches for cartilage and osteochondral repair

Current strategies in use by surgeons for the treatment of cartilage and osteochondral defects focus on stimulation of the subchondral bone marrow, implanting cells directly into the defect as with Autologous Chondrocyte Implantation (ACI), or using a tissue graft, as with mosaicplasty.

1.2.1 Bone marrow stimulation techniques

Currently, the most straightforward chondral defect repair techniques are bone marrow stimulating techniques, including microfracture and Pridie drilling. Microfracture is used most frequently, and involves accessing the bone marrow mesenchymal stem cells (BMSCs) by creating small micro holes that breach the subchondral bone below the defect, using an awl, to cause bleeding (Figure 1.3). A fibrin clot is then formed with the proliferation of multipotent MSCs. At the most superficial layer a fibrocartilage matrix is produced in 4-8 weeks. In the deeper zones bone remodelling continues, and goes on to form the subchondral bone layer (Chen
et al., 2009). Pridie drilling uses a drill rather than an awl, and has the disadvantage that the drill temperatures can cause thermal osteonecrosis. With either of these approaches, however, the fibrocartilage matrix formed is mechanically inferior to the surrounding hyaline cartilage and frequently breaks down over time.

Microfracture has several advantages, including the ability to treat defects in a single procedure which is easy and quick to perform. At present, there is insufficient evidence to show that more complex regenerative processes are cost-effective in terms of quality adjusted life years (QALYs) in comparison to microfracture in the short term (Clar et al., 2005, Mithoefer et al., 2005). There have, however, been suggestions that using microfracture initially leads to worse outcomes for subsequent treatments, as breaching the subchondral bone leads to increased incidence of osseous overgrowth and intraluesional osteophytes, which can cause advancement of the tidemark and thinning of the cartilage, predisposing it to degeneration (Minas et al., 2009, Mithoefer et al., 2005, Steadman et al., 2003, Chen et al., 2009).

![Figure 1.3: Isolated debrided chondral lesion undergoing microfracture – the bleeding and release of bone marrow cells from the subchondral bone can be seen from the drill holes. Image courtesy of 3D4Medical (Cairnduff, 2014)](image-url)
1.2.2 Mosaicplasty

Mosaicplasty is a method of joint surface defect repair whereby small osteochondral plugs are transplanted from a non-weight bearing portion of the joint to the defect site in a mosaic like pattern (Figure 1.4). Thus, the defect area is filled with autologous chondrocytes and extracellular matrix. It has been shown that the hyaline cartilage and the chondrocytes contained at the centre of the plug consistently survive with greater than 99% cell viability (Hangody et al., 1997, Kock et al., 2011, Huntley et al., 2005, Nam et al., 2010). The benefits of this technique include superior hyaline cartilage and a smooth gliding surface at the weight bearing portion of the joint, where the defects predominantly first appear (Hangody et al., 2001, Buckwalter, 1998). An important benefit is the gradient of tissues from subchondral bone through calcified cartilage and the cartilaginous joint surface is not lost in the osteochondral defect repair. Generally, activity levels are improved over microfracture after ten years, due to the integration of the hyaline cartilage (Hangody et al., 2008, Lyon et al., 2013, Krych et al., 2012a, Jakob et al., 2002, Gudas et al., 2012). Although autograft is often seen as the gold standard in tissue regeneration (Janicki and Schmidmaier, 2011), the disadvantages of this technique relate to the donor site, which heals with poorly organised fibrocartilage, and operative difficulties such as the graft not being flush with the joint surface, and integration issues between the graft and the surrounding tissue (Hangody et al., 2008, Gudas et al., 2012, Bentley et al., 2012, El Sayed et al., 2010). While mosaicplasty is popular among surgeons, its use is limited to small and medium sized defects up to 4-5 cm² due to both donor site morbidity and availability, and the reduced stability of many small adjacent plugs in a large defect, (Bentley et al., 2013, Agneskirchner et al., 2002). To achieve repair in larger defects without equivalent donor site morbidity, more advanced therapeutic methods must be used. (Bentley et al., 2013, Sherman et al., 2014).
1.2.3 Autologous Chondrocyte Implantation
More advanced cell-based techniques have been developed to improve on the results from grafting and microfracture procedures. Autologous Chondrocyte Implantation (ACI) was introduced to maximise the cells available for repair and decrease the amount of tissue harvested (Peterson et al., 2000). The technique involves taking a cartilage biopsy from a non-weight bearing portion of the joint and isolating and expanding chondrocytes *ex vivo* in autologous serum. In a second procedure 6 weeks later, chondrocytes are then re-implanted into the defect under a membrane of periosteum harvested from the proximal tibia (Figure 1.5) (Brittberg et al., 1994). ACI has been used in the clinical setting for a number of years and although most data shows similar short term results to microfracture, ACI has been shown to have better long term results, and a superiority of characterised chondrocytes in ACI over microfracture has been described (Bentley et al., 2012, Niemeyer et al., 2012).

While ACI has shown some promising results, there are several disadvantages relating to these techniques. The need for two separate procedures doubles the recovery time for the operation. The high cost of *ex vivo* expansion also poses a challenge. Chondrocytes have a propensity to dedifferentiate in culture making them
a sub-optimal cell for harvesting and expansion (Beane and Darling, 2012, Bekkers et al., 2013). There is some evidence of graft hypertrophy and ossification of the covering periosteal membrane occurring in ACI, which led to the development of a collagen membrane composed of porcine type I and III collagen as an alternative. Similar overall outcomes have been reported for the use of collagen membranes as compared to periosteal membranes in ACI procedures, however the operating time is shorter and there is a lower incidence of graft hypertrophy (Batty et al., 2011, Bartlett et al., 2005).

Expanding on the idea of collagen membranes used in ACI, the idea of a cell-free collagen scaffold that could be implanted off the shelf and allow the body's own stem cells infiltrate into the scaffold to heal the defect has also been examined, in an effort to reduce the operating time and the cost of the procedure (Crawford et al., 2012, Benthien and Behrens, 2010).
1.3 Tissue engineering approaches to osteochondral defect repair

Several of the disadvantages of ACI, including unequal distribution of the chondrocytes and long operating time, have led researchers to look towards the use of tissue engineering (TE) solutions in the form of biomaterials scaffolds. TE has been defined as, ‘the creation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells, through a systematic combination of molecular and mechanical signals’ (Williams, 2006). TE seeks to regenerate human tissues requiring repair due to dysfunction, disease, injury, age or congenital disorders (Sheyn et al., 2010) and can be broadly divided into three chief strategies used to guide tissue regeneration and repair. These are: 1) the direct delivery of cells, 2) the implantation of an acellular matrix (i.e. a scaffold which may be a synthetic material) comprising structural, biological and morphological cues or 3) a combination of the former i.e. a cell-seeded matrix (Figure 1.6). This technique involves the harvesting of a biopsy from normal tissue in the body, from which the desired cell type is isolated. In cartilage repair currently, this cell type is predominantly chondrocytes, but other tissue engineering techniques use endothelial cells, or MSCs. The cells are then cultured and expanded ex vivo until sufficient numbers are available. The cells are then seeded onto a biomaterial scaffold which may use a form of mechanical stimulation such as flow or pressure, or growth factors to encourage tissue formation. The cell-loaded scaffold is then re-implanted into the body in the defect site where healing is required.
1.3.1 Scaffold and hydrogel based cell delivery
Using TE techniques to combat uneven cellular distribution and cell leakage in ACI (Sohn et al., 2002) has led to a focus on using biodegradable biomaterial scaffolds that can maintain the cells within the defect site, such as Matrix-induced Autologous Chondrocyte Implantation (MACI®), where expanded cells are seeded onto a porcine collagen scaffold that is directly implanted into the defect (Vascellari et al., 2014)(Figure 1.7). Although the clinical outcomes for ACI and MACI® have been found to be broadly similar (Bartlett et al., 2005), the use of a scaffold offers shorter operating times than ACI, and some randomized controlled trials have reported improved functional results (Bartlett et al., 2005, Basad et al., 2010, Crawford et al., 2012, Saris et al., 2014). Building on this, recent scaffold developments include optimisation of the scaffold composition, structural and mechanical properties.
aimed at directing chondrogenesis of host stem cells rather than relying solely on the implantation of chondrocytes.

Off-the-shelf cell-free scaffolds, or scaffolds combined with growth factors such as bone morphogenic protein (BMP), have also shown promise in the repair of osteochondral defects (Kon et al., 2010a, Kon et al., 2011, Kon et al., 2014, Kon et al., 2015). These scaffolds typically have a three-dimensional porous architecture and act as templates for tissue formation. There are a wide variety of these scaffolds and hydrogels on the market currently or about to come on the market (Table 1.1). They are composed of a range of materials, including human allograft derived materials, such as particulate juvenile cartilage allograft (deNOVO NT) or cryopreserved osteochondral allograft (Cartiform), animal matrix derived scaffolds, such as those fabricated from porcine or bovine collagen, plant derived scaffolds, such as the Cartipatch derived from agarose-alginate hydrogel scaffold, or those including biopolymer chitosan solution, or hydrogels composed of polyethylene glycol.
diacrylate (PEG-DA). The micro-structural and bio-chemical cues provided by the new generation scaffolds are typically proposed to stimulate either chondroinduction or osteoinduction, of the host’s own stem cells (Niemeyer et al., 2012, Levingstone et al., 2014, Murphy et al., 2010, O’Brien et al., 2005, Matsiko et al., 2012, Ho et al., 2010, Carroll et al., 2014, de Vries-van Melle et al., 2014). These scaffolds can thus potentially be combined with the microfracture technique to cover and stabilise the clot forming in the defect site from the bone marrow in a technique known as autologous matrix induced chondrogenesis (AMIC) or scaffold enhanced microfracture technique (SEMT).

For osteochondral defect repair, the use of cell-free scaffolds has shown promise in early trials and several devices have already received regulatory approval. The examples given in Table 1.1 are those that are currently available or are seeking regulatory approval (Levingstone et al., 2014, Matsiko et al., 2012). Within the Tissue Engineering Research Group (TERG) in the Royal College of Surgeons in Ireland (RCSI), a novel biomimetic tri-layered scaffold designed to replicate anatomical structures has been developed (Levingstone et al., 2014, Gleeson JP, 2009). This scaffold has shown success as an off-the-shelf cell free product in the treatment of focal defects in several animal models including rabbits and goats (Levingstone et al., 2016a, Levingstone et al., 2016b). It consists of a collagen type I - hydroxyapatite base layer, with a collagen type I hyaluronic acid intermediate layer and a collagen type I/collagen type II top layer (Figure 1.8, Figure 1.9). The scaffold was developed through optimisation of the collagen content, freeze drying processes and crosslinking protocols (Levingstone et al., 2014). Repair of osteochondral defects following scaffold implantation has been shown at three months in a rabbit medial femoral condyle model, and in a pilot goat study with the formation of mature hyaline cartilage and specifically, the cartilage, tidemark and subchondral bone visible on histological analysis of the regenerated tissue at twelve months.
Figure 1.8: Schematic diagrams showing (a) the superficial, intermediate and deep layers of the osteochondral region and (b) the multi-layered scaffold. This scaffold is fabricated using three distinct collagen-based slurries sequentially freeze-dried to produce a highly porous, seamlessly integrated multi-layered scaffold, as demonstrated by scanning electron microscopy (SEM) (extreme right), designed to mimic the composition and microstructural properties of the osteochondral region (Levingstone et al., 2014).

Figure 1.9: Close up of SEM micrographs of a tri-layer osteochondral defect repair scaffold showing the integration of the component layers. These biomimetic layers allow the implanted cells to differentiate into the required cells within each layer (Levingstone et al., 2014)
Currently there is a large amount of research focussed on tissue engineering and the use of different cell types such as chondrocytes and stem cells derived from bone marrow and infrapatellar fat pad to produce tissue types that can be implanted or inserted into the body to fill a defect. One of the complexities faced when developing a tissue engineered osteochondral defect graft is the gradient nature of the tissue and consequently the need to stimulate the cells to differentiate along a chondrogenic or osteogenic lineage depending on the different layers in the scaffold. The MSCs in the bone layer must differentiate into osteocytes and the cells in the cartilage layer into chondrocytes. They must also, crucially, maintain a tidemark between the two surfaces to prevent hypertrophy and the ossification of the cartilage layer. To do this the scaffold ideally should provide sufficient stimuli to cause osteoinduction/osteoconduction and chondrogenesis within the respective layers. Within current treatment methods, cell differentiation is thought to be stimulated by circulating factors from adjacent tissue and mechanical stimuli. However, in an ideal tissue engineered scaffold reliance would not only be on external stimuli, but from within the implant itself.
Table 1.1: There are a variety of products currently on the market that are designed to be used in conjunction with cells to repair chondral and osteochondral defects

<table>
<thead>
<tr>
<th>Product and Composition</th>
<th>Regulatory Status</th>
<th>Description</th>
<th>Material Properties</th>
<th>Cell Source</th>
<th>Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>deNOVO NT (Zimmer, Inc, Warsaw, IN)</td>
<td>FDA approved</td>
<td>Particulate juvenile cartilage allograft</td>
<td>✓ ✓</td>
<td>cartilage particulate inserted through needle</td>
<td></td>
</tr>
<tr>
<td>Cartiform (Osiris Therapeutics, Columbia, Maryland, USA)</td>
<td>FDA approved</td>
<td>Cryopreserved viable osteochondral allograft with pores and a reduced bone portion</td>
<td>✓ ✓</td>
<td>sheet</td>
<td></td>
</tr>
<tr>
<td>Chondro-Gide™ (Geistlich, Switzerland)</td>
<td></td>
<td>Bilayer structure of porcine type I/III collagen membrane</td>
<td>✓ ✓</td>
<td>sheet</td>
<td></td>
</tr>
<tr>
<td>CaReS® MACT (Arthrokinetics, Esslingen, Germany)</td>
<td>CE marked</td>
<td>Rat-tail type I collagen matrix seeded with chondrocytes</td>
<td>✓ ✓</td>
<td>3D disc</td>
<td></td>
</tr>
<tr>
<td>Novocart 3D (B. Braun-Tetec, Germany)</td>
<td>phase III</td>
<td>Bovine collagen membrane cover and a collagen sponge lying underneath</td>
<td>✓ ✓</td>
<td>3D disc</td>
<td></td>
</tr>
<tr>
<td>NeoCart™ (Histogenics, Waltham, MA)</td>
<td>Phase III</td>
<td>Bovine type I collagen scaffold</td>
<td>✓ ✓</td>
<td>sheet</td>
<td></td>
</tr>
<tr>
<td>Cartipatch; (Tissue Bank of France, France)</td>
<td>Phase III</td>
<td>Agarose-alginate hydrogel scaffold</td>
<td>✓ ✓</td>
<td>3D disc</td>
<td></td>
</tr>
<tr>
<td>ChondroCelect (Tigenix, Leuven, Belgium)</td>
<td>CE marked</td>
<td>Characterised expanded Chondrocytes</td>
<td>✓</td>
<td>suspension</td>
<td></td>
</tr>
<tr>
<td>Carticel (Sanofi, New Jersey, United States)</td>
<td>FDA approved</td>
<td>Autologous cultured chondrocytes</td>
<td>✓</td>
<td>suspension</td>
<td></td>
</tr>
<tr>
<td>Product and Composition</td>
<td>Regulatory Status</td>
<td>Description</td>
<td>Material Properties</td>
<td>Cell Source</td>
<td>Form</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------</td>
<td>-------------</td>
<td>---------------------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td><strong>BioSeed®-C</strong>&lt;br&gt;(BioTissue Technologies, Freiburg, Germany)</td>
<td></td>
<td>Resorbable polymers consisting of pure polyglycolic acid or Polyglactin 910 and Poly-p-iodoxanone on a polyglycolic acid basis combined with Fibrin glue (Tisseel) to hold cells in place</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>CaReS®-1S MACT</strong>&lt;br&gt;(Arthrokinetics, Esslingen, Germany)</td>
<td>CE marked</td>
<td>Rat-tail type I collagen matrix</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Novocart Basic</strong>&lt;br&gt;(B. Braun-Tetec, Germany)</td>
<td>CE marked</td>
<td>Bovine collagen membrane cover and a collagen sponge lying underneath</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>MeRG®</strong>&lt;br&gt;(Bioteck, Torino, Italy)</td>
<td>CE marked</td>
<td>Type I lyophilised collagen from equine achilles' tendon (Type I &gt;95%, Type III &lt;5%, glucosaminoglycans 2.75 mg/g)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>BST-Cargel</strong>&lt;br&gt;(Piramal Life Sciences, Laval, Quebec, Canada)</td>
<td>CE marked</td>
<td>Biopolymer chitosan solution and a buffer</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Gelrin C</strong>&lt;br&gt;(Regentis Biomaterials, Israel)</td>
<td>CE marked</td>
<td>Hydrogel composed of polyethylene glycol diacrylate (PEG-DA)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Chondrotissue</strong>&lt;br&gt;(BioTissue AG, Switzerland)</td>
<td></td>
<td>Polyglycolic acid textile treated with hyaluronic acid</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Hyalofast</strong>&lt;br&gt;(Anika therapeutics, Massachusetts USA)</td>
<td>CE marked</td>
<td>Benzyl ester of hyaluronic acid (HA)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Product and Composition</td>
<td>Regulatory Status</td>
<td>Description</td>
<td>Material Properties</td>
<td>Cell Source</td>
<td>Form</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------</td>
<td>-------------</td>
<td>---------------------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td><strong>Trufit</strong> (Smith &amp; Nephew, Andover, MA, USA)</td>
<td>CE marked</td>
<td>Cartilage Layer: PGA, PLGA, surfactant. Bone layer: calcium-sulfate</td>
<td>✓ ✓ ✔</td>
<td>autologous expanded</td>
<td>plugs</td>
</tr>
<tr>
<td><strong>MaioRegen</strong> (Fin-Ceramica SpA, Faenza, Italy)</td>
<td>CE marked</td>
<td>Cartilage layer: deantigenated type I equine collagen. Bone layer: magnesium-enriched hydroxyapatite (Mg-HA). Intermediate layer: Mg-HA and collagen</td>
<td>✓ ✓ ✔</td>
<td>autologous expanded</td>
<td>sheet</td>
</tr>
<tr>
<td><strong>Agili-C</strong> (Cartiheal, Kfar Saba, Israel)</td>
<td>Phase II/III</td>
<td>Bone layer: aragonite crystalline calcium carbonate. Cartilage layer: modified aragonite and hyaluronic acid</td>
<td>✓ ✓ ✔</td>
<td>autologous expanded</td>
<td>plugs</td>
</tr>
</tbody>
</table>
1.4 Different Cells for use in cell mediated osteochondral defect repair

1.4.1 Chondrocytes

There are many types of cells under investigation for the regeneration of the cartilage surface of the joint. Traditionally chondrocytes used in cell-based cartilage repair have been harvested from the articular surface of the knee joint; however, as hyaline cartilage found in joints has few cells that are widely dispersed, harvesting large numbers causes significant morbidity. Therefore, in the search for better outcomes, investigations are ongoing into heterogeneous sources of chondrocytes. Hyaline cartilage is also available in the costal cartilage, the xiphisternal cartilage, and nasoseptal cartilage. The use of chondrocytes harvested from elastic cartilage, which is found in auricular cartilage, has also been investigated for articular surface repair. A number of studies have investigated the regenerative potential of chondrocytes sourced from extra-articular cartilage (El Sayed et al., 2010, Kato and Gospodarowicz, 1985, Lohan et al., 2011).

1.4.1.1 Articular chondrocytes

The use of articular chondrocytes in cell defect repair, such as in ACI and MACI procedures, offers the advantage of using cells that are committed to the appropriate phenotype, and are naturally resistant to vascular invasion, mineralisation and ossification (Brittberg et al., 1994). There are several commercially available treatment options for articular cartilage defects using these cells (Table 1). However, harvesting articular chondrocytes causes a further trauma to the joint in the form of the first surgery and donor site where the numbers of healthy cells are limited. Articular chondrocytes expanded in vitro are known to have an increased expression of collagen X which is a marker for dedifferentiation of the chondrocyte, meaning they lose their chondrocytic phenotype and can become mineral forming thereby exacerbating the pathology (Sabatino et al., 2015).
1.4.1.2 Nasoseptal chondrocytes

The hyaline cartilage at the nasal septum originates from the neural crest during embryonic development. It has a higher cell content than articular cartilage and the proliferation capacity of the chondrocytes is maintained with proliferation rates reported to be four times faster for nasoseptal chondrocytes than articular chondrocytes (El Sayed et al., 2010, Kafienah et al., 2002). Harvesting of the chondrocytes from the nasal septum is a straightforward procedure performed frequently by otolaryngologists or plastic surgeons (Kafienah et al., 2002, Mumme et al., 2016). Research into human and porcine nasoseptal chondrocytes has shown that they dedifferentiate in vitro and, after 35 days, produce a small amount of collagen type X – a marker for chondrocyte hypertrophy not seen in native nasoseptal cartilage. However, no mineralisation was found when these expanded cells were implanted into a subcutaneous mouse model and a proteoglycan-rich matrix was seen (Lohan et al., 2011, Hellingman et al., 2011). Although it has been shown that like articular cartilage chondrocytes, dedifferentiation takes place during in vitro in 2D culture, when nasoseptal chondrocytes are loaded onto a 3D scaffold there is recovery of aggrecan and type II collagen at transcription level and redifferentiation, with no further production of elastin, making these cells a viable option for chondral defect repair (Vinatier et al., 2009, Kafienah et al., 2002, Hellingman et al., 2011). First in man trials have been performed by Mumme et al (Mumme et al., 2016) and shown good results at early stages of cartilage formation with expanded chondrocytes on a collagen type I/III membrane (Chondro-Gide; Geistlich Pharma AG).

1.4.1.3 Auricular chondrocytes

Another source of chondrocytes that has been investigated for use in reconstructive surgery is the auricular cartilage in the pinna of the ear. This cartilage, however, bears more similarity to the nasoseptal cartilage than the articular cartilage due to the elastin expressed, and the reliance on the perichondrium for nutrition. These auricular chondrocytes have been shown to proliferate at a slower rate than nasoseptal chondrocytes but to give a higher yield of chondrocytes (Hellingman et
al., 2011). Like articular and nasal chondrocytes these cells have a tendency to dedifferentiate in vitro, but are reported to be less likely to ossify when reimplanted than nasal and costal chondrocytes (Leijten et al., 2013, El Sayed et al., 2010). Hellingman notes that when the expanded auricular chondrocytes were reimplanted subcutaneously in a mouse model they took on their stable native cartilage type with the production of elastin and collagen type X but without mineralisation (Hellingman et al., 2011). Although the early in vitro trials using auricular chondrocytes seem promising, results in vivo have been mixed. In one in vivo study, scaffolds seeded with articular chondrocytes and auricular chondrocytes implanted into osteochondral defects in New Zealand rabbits showed that on histological analysis at 6 and 12 weeks the auricular chondrocytes performed worse than even the empty defect (Lohan et al., 2014). However, in a mini-pig chondral defect model the auricular chondrocytes were found to perform far better than the empty defect, and the histological structure was superior even to articular chondrocyte based implants, although in terms of macroscopic defect appearance and biomechanical stiffness the articular chondrocytes were superior (Lohan et al., 2013). In humans there are few accounts of auricular cartilage being used in joints; however, in a case series of auricular cartilage sheet grafts used in arthritic temporomandibular joints for pain relief, 16/30 patients reported improvement, with 30% requiring repeat surgery to remove the graft as pain persisted (Svensson et al., 2010).

1.4.1.4 Costal chondrocytes

Costal cartilage, a hyaline cartilage that connects the rib ends to the sternum, represents another potential source of chondrocytes. It is particularly rigid and has a tendency for ossification in older individuals. Although cell yield is less than for auricular and nasoseptal cartilage, it remains higher than articular, cartilage with a threefold greater proliferation than articular chondrocytes and can be harvested with less morbidity (El Sayed et al., 2010, Gelse et al., 2009, Sato et al., 2008). Experimental studies using osteochondral plugs from the bone cartilage interface in the ribs to fill articular surface defects have shown that there is survival of the chondrocytes and extracellular matrix; however, most samples show incomplete
integration at the level of the cartilage (Sato et al., 2012, Sato et al., 2008). Further studies, using cell spheroids isolated from the costal cartilage, found that costal chondrocytes retained their chondrocytic phenotype with collagen type II expression and a proteoglycan rich matrix, when implanted in full or partial thickness defects (Gelse et al., 2009).

While chondrocytes are an obvious first choice as a cell source in any tissue engineered approach to defect repair, the low numbers of cells available and dedifferentiation when expanded *in vitro* in two dimensional culture represent significant limitations (Benya and Shaffer, 1982, El Sayed et al., 2010, Kato and Gospodarowicz, 1985). This loss of phenotypic traits could reflect a progressive loss of potential to form stable cartilage *in vivo*, thereby putting the long-term outcome at risk (Peterson et al., 2000). As a result of the inherent limitations associated with chondrocytes, the use of stem cells has been investigated in order to achieve repair of chondral and osteochondral defects (Wakitani et al., 1994, Nakahara et al., 1990).

### 1.4.2 Mesenchymal stem cells

Mesenchymal Stem Cells (MSCs) are multipotent adult stem cells that can be derived from many different tissues in the body. These cells can be isolated from other cells by their plastic adherence *in vitro*. They are defined by their ability to differentiate into adipocytes, chondrocytes or osteocytes and their expression of certain surface molecules (Dominici et al., 2006). Current research aims to identify the optimal source of MSCs for joint surface defect repair, and determine whether MSCs derived from a particular tissue or a specific subgroup of MSCs can provide an enhanced regenerative response (Almeida et al., 2015). To this end MSCs from various tissue types are being examined to assess their potential for use in chondral and osteochondral defect repair applications.
1.4.2.1 Bone marrow derived mesenchymal stem cells

MSCs can be easily harvested from the bone marrow (BMSCs) from the iliac crest. These are also among the cell types released when microfracture is performed on the subchondral bone beneath the defect. When BMSCs are cultured in vitro, chondrogenic and osteogenic differentiation can be achieved by the addition of several bioactive agents (Heng et al., 2004). To achieve repair of chondral and osteochondral defects in vivo, they must differentiate into the required cell type in each region of the defect. BMSCs have several advantages over chondrocytes for the treatment of joint surface defects, including their ability to expand easily in vitro and their ability to differentiate as required. They have also been shown to have an immunomodulatory effect which is of additional benefit (Glennie et al., 2005, Baghaban Eslaminejad and Malakooty Poor, 2014). This immunosuppressive activity can help to modulate the inflammatory processes occurring with the site of injury, and can also enable the body to accept the foreign graft being inserted (Leijten et al., 2013). A disadvantage of using BMSCs is the difficulty preventing their hypertrophic differentiation. The normal endochondral ossification pathway allows for the differentiation of MSCs into cartilage which then undergoes ossification by hypertrophy. While the ease with which MSCs can be differentiated into chondrocytes presents a major advantage of this cell type, it is necessary to avoid eventual hypertrophy (Sheehy et al., 2014). Thus, current research focus is aimed at determining the optimal source of MSCs for use in cartilage repair, and evaluating whether stem cells can achieve equivalent or improved repair when compared to chondrocytes.

1.4.2.2 Adipose derived stem cells

Adipose tissue has also emerged recently as a valuable source of adipose derived stem cells (ADSCs) which can be persuaded towards one cell type or other depending on the culture medium (Sakaguchi et al., 2005). The large supply means that they present an excellent option for defect repair without any donor site implications (Jurgens et al., 2013, Guilak et al., 2010). Autologous ADSCs have previously been used to repair bony defects with success (Lendeckel et al., 2004, Jurgens et al., 2008).
Apart from ADSCs the adipose tissue also has a stromal vascular fraction (SVF) which consists of a heterogeneous mixture of cells, including endothelial cells, smooth muscle cells, pericytes, leucocytes, mast cells, and pre-adipocytes. A pilot caprine in vivo comparison of expanded ADSCs, freshly isolated SVF cells harvested at the time of implantation, and an empty defect, showed little significant difference between the groups at 4 weeks. However, at 4 months there was significant improvement in the cell based groups over the empty defect, in terms of both macroscopic and microscopic results and higher glycosaminoglycan formation, with the freshly isolated SVF group having the best results overall (Jurgens et al., 2013). The high availability of the SVF and ADSCs mean that it is possible to harvest sufficient numbers to implant without expanding the cells ex vivo.

ADSCs can also be harvested in high yields from the fat pad within the joint. The infrapatellar fat pad is one of the structures which arises from the interzone, an area with increased cell density formed during embryonic development at the future position of the joint (Khan et al., 2007). Interzone cells then differentiate and go on to produce hyaluronan and an enlarged cavity consisting of articular cartilage and synovium, from this, all intra-articular tissues are developed. It has been shown that stem cells derived from these interzone tissues have more in common with each other than stem cells derived from extra-articular tissues such as bone marrow (Segawa et al., 2009). The chondrogenic potential of infrapatellar fat pad cells (FPMSCs) and their functional properties has been widely investigated, with reported advantages including a short population doubling time and ease in manipulating cells to differentiate (Ahearne et al., 2011, Ahearne et al., 2014, Liu et al., 2014, Mesallati et al., 2014). Furthermore, FPMSCs have also been shown to maintain a comparable chondrogenic capacity when the individual involved has developed signs of osteoarthritis which means that they remain viable as a cell source for cartilage repair in osteoarthritic patients (Liu et al., 2014).

1.4.2.3 Synovium and synovial fluid derived stem cells

The synovium has been shown to have isolatable MSCs and has advantages as a cell source as it is also easy to harvest at arthroscopy and demonstrates full healing leaving no donor site morbidity (Sakaguchi et al., 2005, Ando et al., 2008). Several in
vitro studies have been performed using MSCs isolated from synovial tissue in the
defect with promising results, and an in vivo trial using rabbits showed superior
histological results when treated with synovium derived MSCs compared to empty
defects (Koga et al., 2008, Ando et al., 2008, Ando et al., 2007). Synovial fluid also
contains an inherent MSC and mesenchymal progenitor cell (MPCs) population with
an increased propensity toward chondrogenesis rather than osteogenesis (Sakaguchi
et al., 2005, Khan et al., 2007). The degree of processing involved is less for the
synovial fluid than other tissues and does not require the use of collagenase (Ando
et al., 2014). Limitations to the use of synovial fluid MSCs remain in the very small
volumes that can be aspirated from the joint. This means that a large number of
population doublings are required before the cells can be used clinically (Ando et al.,
2014). The synovial fluid itself also becomes contaminated with inflammatory
markers and inhibitory factors to chondrogenesis as a cartilage defect becomes
chronic, which may impact on tissue regeneration (Rodrigo et al., 1995, Saris et al.,
2003).

1.4.2.4 Peripheral blood MSCs
Peripheral blood (PB) can be harvested from a routine blood test and thus offer
significant advantages as an easily accessible alternative source of MSCs, in
comparison to other stem cell sources where an invasive harvesting procedure is
required prior to cell implantation. The major limitation for this cell type is the low
numbers of MSCs present (Lazarus et al., 1997). However, recently several authors
have shown success in mobilising MSCs from the bone marrow into the blood and
thus increasing the concentration of MSCs available (Wise et al., 2012, Fu et al.,
2014b). For example, using this method, Fu et al. went on to implant MSCs cultured
from peripheral blood into cartilage defects in a rabbit model (Fu et al., 2014b). They
found that although there were differences in differentiation and proliferation
between MSCs harvested from PB and MSCs harvested directly from the bone
marrow, they both had the same ability to repair cartilage defects in vivo (Fu et al.,
2014b). A recent clinical case study demonstrated the use of peripheral blood MSCs
in the treatment of an athlete with a large osteochondral defect. Good results
following the utilisation of these cells were reported with well healed cartilage observed on second look arthroscopy and significantly improved pain scores reported (Fu et al., 2014a).

1.4.2.5 Pluripotent stem cells

Pluripotent stem cells are cells that can differentiate into several different cell types. These cells are attractive as they can be sourced outside of the injury site, and they can have the ability to differentiate into osteocytes and chondrocytes as required in the requisite layers of the defect site. Recent advances within the field of tissue engineering have led to the development of induced pluripotent stem cells (iPSCs), where adult cells are reprogrammed into pluripotent stem cells. Examples include adult dermal fibroblasts from a skin biopsy and circulating lymphocytes taken from a blood sample (Takahashi et al., 2007, Seki et al., 2011). By the transfection of certain genes through retroviruses these cells take on an appearance similar to human embryonic cells and can, from there, be guided to differentiate into multiple tissue types. iPS cells have been successfully reprogrammed to produce cartilage in vitro and thus they may have potential for use in cartilage defect repair (Yamashita et al., 2015, Lach et al., 2014). One major disadvantage of iPSCs is reported teratoma formation due to the increased proliferation rate and the reprogramming process (Uto et al., 2013). While some studies have demonstrated the safe use of these cells the risk of teratoma formation remains a concern (Yamashita et al., 2015).

1.4.3 Co-culture systems in cartilage repair

Although stem cells from various sources have shown potential for the repair of cartilage defects, the native cells to a chondral defect are chondrocytes. As discussed, the major challenge in the use of chondrocytes in cartilage repair procedures is the lack of adequate numbers of cells available for harvest and the associated donor site morbidity. To overcome these limitations, co-culture strategies, where chondrocytes are combined with another cell type in order to achieve enhanced tissue regeneration, have been investigated. The multipotency of MSCs can also lead to
some difficulties, as their differentiation cannot be fully controlled \textit{in vivo}, and differentiation of MSCs prior to implantation can cause them to lose their anti-inflammatory properties (Ryan et al., 2014). A co-culture of primary chondrocytes and MSCs maintains the immunomodulatory effect, but also induces MSC chondrogenesis. Chondrocytes have previously been shown to gradually secrete a wide variety of protein molecules and it has been demonstrated that they provide a chondrogenic environment for ADSCs in co-culture (Lv et al., 2012). It has been shown that with an increasing ratio of chondrocytes to MSCs up to 100:1 there is increased expression of type II collagen, and decreased expression of type I collagen, an osteogenic marker compared to MSC controls (Yueh-Hsun Yang et al., 2012). Co-culture in a hydrogel was also shown to decrease the amount of collagen X expression, compared to MSCs alone (Bian et al., 2011). These results show that the presence of a co-culture with chondrocytes improves the cartilage production, and decreases hypertrophy.

\section*{1.5 Single surgery cell isolation and implantation procedures}

Despite advances in cell based chondral and osteochondral repair, the development of a method for harvesting, isolating and implanting cells in a one-stage surgical procedure, remains a major clinical goal in the regeneration of hyaline cartilage. This would eliminate the need for a second operation and the need for and expense of \textit{ex vivo} expansion. Potential one-stage procedures that have been investigated include the use of minced cartilage, which utilises cartilage that is harvested and cut up into small pieces. The material is then \textit{re}-implanted into the defect during the same procedure. Short term \textit{in vivo} studies with goats and rabbits have had good results compared to empty defects or cell-free scaffolds (Lu et al., 2006, Marmotti et al., 2012, Marmotti et al., 2013). Rapid cartilage digestion techniques whereby the tissue is diced, washed and then left in digestive enzyme for 45 minutes rather than several hours, have also been investigated. These methods can retain some of the pericellular matrix (Lee et al., 1997, Bekkers et al., 2013). This pericellular matrix is made
up predominantly of type VI collagen which plays an important role in the metabolic activity of the chondrocyte. It has been proposed that the inclusion of the pericellular matrix could enhance regeneration (Bekkers et al., 2013). To use these rapidly isolated cells would enable the tissue to be harvested at the beginning of the procedure, and the cells to be isolated while the surgeon is debriding the diseased cartilage and cleaning the defect site. The surgeon can then re-implant the cells within the timeframe of a single operation. Jurgens et al. investigated rapid digestion (60mins with a digestion enzyme), filtration, and pelleting, in order to quickly isolate the stromal vascular fraction ADSCs in a caprine model. They showed the feasibility of harvesting and implanting in a single surgery and showed that the rapidly isolated cells were at least equal to expanded cells in terms of cartilage formation at 4 weeks (Jurgens et al., 2013, Ahearne et al., 2014, Lu et al., 2007). These early studies show that a single surgery cell-based method of osteochondral and chondral defect repair is possible and offers a potential avenue for further investigation.

1.6 In vivo investigation of osteochondral defect repairs

While in vitro testing and biomechanical analysis of biomaterials can provide much information about their safety, efficacy and potential for repair, in order to truly assess their regenerative capabilities, and the immune response associated with implantation, the use of animal models is required (Chu, 2001, Hoemann et al., 2011, Hurtig et al., 2011, Moran et al., 2016). However, the principles of the three R’s, Reduction, Replacement and Refinement, must be borne in mind when planning studies (Russell and Burch, 1959). When planning an in vivo trial, the correct animal model must be chosen to produce worthwhile results. Small animal models include rodents such as mice, rats and rabbits; while large animal models include dogs, goats, sheep, pigs and horses. For the purposes of knee articular cartilage regeneration, the following important considerations must be taken into account before deciding the appropriate animal model for in vivo cartilage repair studies. This includes, the size of the joint, the cartilage thickness, the depth and critical size of the defect (critical
size implies a defect which will not heal spontaneously without any intervention), the maturity of the cartilage (better results in young patients regardless of treatment type), load distribution of the joint, affordability and ease of animal handling. Guidelines have been set out in ASTM F 2451-05 for animal models suitable for the assessment of cartilage repair. These are detailed in Table 1.2

![Table 1.2: Various models used in preclinical models and their comparison to the human knee joint (ASTM, 2010)](image)

<table>
<thead>
<tr>
<th>Species</th>
<th>Breed</th>
<th>Age of skeletal maturity</th>
<th>Adult weight</th>
<th>Cartilage Thickness at femoral condyle</th>
<th>Critical Size defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td>18 - 22 years</td>
<td>60 - 90kg</td>
<td>2.4 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td>Rabbit</td>
<td>New Zealand White</td>
<td>9 months</td>
<td>3 - 4kg</td>
<td>0.25 - 0.75 mm</td>
<td>3 mm</td>
</tr>
<tr>
<td>Dog</td>
<td>Mongrel, Beagle</td>
<td>1 - 2 years</td>
<td>15 - 30kg</td>
<td>0.95 - 1.3 mm</td>
<td>4 mm</td>
</tr>
<tr>
<td>Mini-pig</td>
<td>Gottingen Mini-pig</td>
<td>10 months - 1 year</td>
<td>20 - 40kg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pig</td>
<td>Large White,</td>
<td>2 years</td>
<td>250kg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Goat</td>
<td>Spanish, Dairy, Boer Cross, Saanan</td>
<td>2 - 3 years</td>
<td>40 - 70kg</td>
<td>0.8 - 2 mm</td>
<td>6 mm</td>
</tr>
<tr>
<td>Sheep</td>
<td>Suffolk, Texel</td>
<td>2 - 3 years</td>
<td>35 - 80kg</td>
<td>1.7 mm</td>
<td>7 mm</td>
</tr>
<tr>
<td>Horse</td>
<td>Mixed, Thoroughbred, Quarter Horse</td>
<td>2 - 4 years</td>
<td>500 - 600kg</td>
<td>2.0 - 3.0 mm</td>
<td>9 mm</td>
</tr>
</tbody>
</table>

1.6.1 Small animal models

1.6.1.1 Rodent models

Small animal models can be very useful to give information about the residence time of an implant, and also to determine the type of repair tissue formed (ASTM-F2451-
05, 2010). The availability of athymic, transgenic and knockout strains of both rats and mice means these models can be used to assess a multitude of factors, including the use of strains of mice (DBA/1) in which osteoarthritis occurs spontaneously (Nordling et al., 1992) or athymic strains, which can be used to assess allogenic and xenogeneic cells and tissues (Chu et al., 2010). Rodents such as mice and rats have the advantage of being purpose bred to reduce biological variation, affordable, and easy to breed and maintain in-house. They act as a good bridge between *in vitro* and *in vivo* experiments to provide proof of concept data. However, their joints are small with very thin cartilage consisting of only a few cell layers (Chu et al., 2010). Rodents are therefore very limited in their potential to be used as a translational model for cartilage surface repair in humans. Rodent models can provide useful subcutaneous models and intramuscular models for the assessment of the degradation rate and safety profile of biomaterials and implants with studies of 6 – 8 weeks in duration generally employed (Chu et al., 2010).

### 1.6.1.2 Rabbit model

The rabbit model is the only small animal model suitable for the assessment of cartilage repair, as they have larger joints that are amenable to surgical intervention and specimen handling with a cartilage thickness of 0.25 mm – 0.75 mm (Figure 1.10) (ASTM-F2451-05, 2010). They have been widely used for cartilage regeneration in studies lasting up to 16 weeks, although some 1 year rabbit studies have been performed (Maruyama, 1979, Brittberg et al., 1997, Fragonas et al., 2000, Yanai et al., 2005, Funayama et al., 2008, Luengo Gimeno et al., 2006, Chu et al., 1997). Rabbits offer many advantages as they are cost effective, easy to handle and to house. The femoral condyle is the most often used defect site for weight bearing models, especially the inferoposterior region (Janson, 1999). Intercondylar groove defects can be used as partial weight bearing defects. There is some debate about the rabbit model, as rabbits are capable of intrinsic healing and thus a greater facility to repair chondral defects without intervention (ASTM-F2451-05, 2010, Chu et al., 1997). The greater levels of repair observed in rabbit articular cartilage models compared to other species may be due to higher metabolic activity and density of
pluripotent stem cells near the defect site (Figure 1.10) (ASTM-F2451-05, 2010). In addition, while the size of chondrocytes in human and rabbit articular cartilage do not differ significantly from each other (Hunziker, 1999), the overall cell volume density is approximately 1.7% in cartilage from the human medial femoral condyle (MFC) as opposed to 12.2% in the adult rabbit. These amount to cell densities of 1800 and 7500 per mm$^3$ in humans and rabbits respectively (Hunziker, 1999). The low cellularity of human hyaline cartilage thus contributes to the poor levels of repair observed while the increased density of chondrocytes in rabbits means there are more cells abutting the defect site for repair. Rabbit stifle joints have different load characteristics and cartilage thickness compared to humans, making it difficult to investigate translation potential in this model.

Figure 1.10: H&E stained histology specimens of the distal femur of (A) rabbit (B) goat and (C) horse. These images demonstrate the histological similarity between the different models, but also the vast differences in the thickness of the cartilage at the joint surface. The chondrocyte distribution differences are also evident, with the rabbit cartilage being much more densely packed with chondrocytes than either goat or horse which could explain some better intrinsic healing of cartilage defects in rabbits. (Moran et al., 2016)
1.6.2 Large animal models

In large animals, short (8-12 weeks) studies can be used to provide information regarding the biocompatibility, early cellular responsiveness and persistence and condition of the implant within the defect. Longer studies (6-12 months) are necessary to gain confidence in extent of success in the repair and regeneration of articular cartilage, including interface with adjacent cartilage and subchondral bone as well as the opposing articular surface (ASTM-F2451-05, 2010). A range of large animal models suitable for the investigation of cartilage repair have been explored, including dogs (Engkvist, 1979, Igarashi et al., 2012, Breinan et al., 2000), pigs (Hunziker et al., 2001, Lohan et al., 2013, Boopalan et al., 2011, Klein et al., 2009), sheep (Kon et al., 2010a, Milano et al., 2010, Erggelet et al., 2009), goats (Getgood et al., 2012, Jurgens et al., 2013, Lu et al., 2006, Wang et al., 2007) and horses (Kon et al., 2010b, Frisbie et al., 2009, Hendrickson et al., 1994). When using any large animal model, it is important to determine where on the joint the defect should be created based on the biomechanics of the joint. In humans, since most cartilage defects occur on the weight bearing medial condyle, this is the most common defect site used in cartilage repair studies (Janson, 1999). If using this defect type, the following should be considered in order to meet ASTM-F2451-05 (2010).

1. The defect size should not exceed 15 to 20% of the articulating surface or 50 – 60 % of the condylar width.
2. Due to the convex curvature of the defect sites the defect can differ from the centre to the margins.
3. It is necessary to consider the impact of articulation with both the meniscus and the tibial plateau.

1.6.2.1 Canine model

Dogs, like humans, do not have an intrinsic healing capacity when defects are larger than the critical size. They also suffer from cartilage pathologies found in humans including osteochondritis dissecans and osteoarthritis (Shortkroff et al., 1996), and veterinary surgeons regularly perform arthroscopies on canine stifles. As such, canine
models are considered to be a good choice for cartilage repair studies (Janson, 1999). They accept rehabilitation regimens, cope well with immobilising the joint, and can be trained to walk on treadmills, and can co-operate in swimming and controlled weight bearing rehabilitation (Hurtig et al., 2011). The cartilage thickness, however, is significantly thinner than human cartilage even in medium to large dogs (range: 0.95–1.3 mm) and the critical size defect diameters even in the largest dogs are considerably smaller at 4 mm (Ahern et al., 2009). Another issue to contend with is that due to their longstanding status as companion and family pet, ethical issues also prevent their widespread use. In the UK and Ireland, cats, dogs, horses, non-human primates and endangered species require a special justification for use to show no other species is suitable for the specified programme of work. It is therefore easier and more practicable to get ethical approval for agricultural animals such as pigs, goats and sheep, than canine models (Janson, 1999, Wolfensohn and Lloyd, 2003, Hollands, 1986)

1.6.2.2 Porcine model

Pigs (porcine) models are advantageous in the terms of their joint size, joint loading mechanics, weight (an adult sow can weigh up to 250 kg) (Wolfensohn and Lloyd, 2003), lack of spontaneous healing of any significant defects, bone trabecular thickness and the arrangement of collagen network which resemble a human joint (Chu et al., 2010). Nevertheless, these are large animals and require specialised husbandry and can be expensive to maintain in a research facility.

Mini-pigs are significantly smaller than full sized swine, weighing roughly 50 – 70 kg as adults (Wolfensohn and Lloyd, 2003), and can thus provide some of the advantages of the pig model while overcoming some of the limitations (Schneider et al., 2011, Ebihara et al., 2012). The physiological parameters such as blood count, blood clotting, electrolytes and liver enzymes have been shown to be similar to values for humans (Chu et al., 2010). Histomorphometric analysis of peripheral bone in mini-pigs has also shown the bone apposition rate and trabecular thickness in the Gottingen Mini-pig resembles human bone (Chu et al., 2010), which is a significant
factor when measuring the inflammatory response and toxicity of any implanted biomaterials. Mini-pigs of a defined type and known health status can be sourced from specialist laboratory suppliers in the United Kingdom, but they are not skeletally mature until they reach 18 - 22 months of age (Chu et al., 2010) and require specialist housing (Wolfensohn and Lloyd, 2003).

1.6.2.3 Ovine model

The sheep (ovine) model is commonly used for in vivo trials of materials for cartilage repair (Milano et al., 2010, Kon et al., 2010a, Guo et al., 2004). Weighing between 35-80 kg when skeletally mature at 2-3 years, they transmit a scientifically relevant amount of weight through their femorotibial joint (Wolfensohn and Lloyd, 2003). They have a cartilage thickness of roughly 0.4 - 1.7 mm at the medial condyle (ASTM-F2451-05, 2010, Ahern et al., 2009). Sheep are also readily available, as they are commonly bred in agriculture, and are relatively placid, tolerate stifle surgery well and are easily housed and maintained. They have, however, been reported to have a very variable articular cartilage thickness, between 0.4 and 1.7 mm on the MFC (Ahern et al., 2009), and this variability can cause issues with study design and results. Contact pressures generated in sheep are largely comparable to those in humans, although while humans can reach a mean peak contact force of 5.4 times body weight ascending stairs (Taylor et al., 2004), the maximum in vivo contact force measured in sheep is 2.25 times body weight (Taylor et al., 2006, Patil et al., 2014).

Disadvantages of the goat and sheep model include reports of increased formation of cysts in the subchondral bone of both goats and sheep models (von Rechenberg et al., 2003, Orth et al., 2012b) after osteochondral allograft placement, either through fluid intrusion or bony contusion. This cyst formation can hamper subchondral bone repair (Pallante-Kichura et al., 2013, von Rechenberg et al., 2003). Other disadvantages include, the more labour intensive husbandry practices required for animal handling (Wolfensohn and Lloyd, 2003) and the acquisition of animals from an agricultural background, instead of bred for purpose, meaning their health status and genetic background will be less uniform. The use of goats and sheep
in research is much less common than the use of rodents and thus specialised commercial products, such as antibodies, available to researchers utilising rodent models are not readily available for ruminants.

1.6.2.4 Equine model

Horse (equine) models offer several advantages in the investigation of cartilage repair strategies. Horses are primarily bred and kept for their athletic performance and, as a result, suffer regularly from cartilage injuries and joint diseases such as osteoarthritis and osteochondrosis (Malda et al., 2012). Due to the large joint surface, arthroscopies are routine and can be used both for cartilage defect creation and repair, and longitudinal follow up on the process of cartilage repair at different time points. Critical size defects in the equine stifle joint model are 9 mm, cartilage mean thickness of 2.0 – 3.0 mm and horses maintain a vertically loaded stifle joint during gait, and so the model is beneficial for translatable cartilage studies and especially examining partial thickness defects which are the most relevant to human therapy (Ahern et al., 2009, Malda et al., 2012, ASTM-F2451-05, 2010). The horse is the largest animal model in use as a model for cartilage repair, commonly weighing around 500-600 kg. The joint is, therefore, adapted to withstand elevated loads, with a hardened subchondral bone and efficient joint force distribution. While the horse is an appealing model in terms of cartilage thickness and joint morphology, a highly specialized centre with well-trained personnel is required to carry out equine surgeries (Hollands, 1986, Hurtig et al., 2011).

1.6.2.5 Caprine model

Goats are among the earliest animals domesticated by humans. They are farmed throughout the world and are used for a variety of products, including milk, meat and coat fibres (mohair and cashmere). They are, as a result, relatively easy to obtain when skeletally mature. The caprine (goat) femoral condyle and trochlear defect models have been used successfully for evaluation of new implants for treatment of partial thickness and osteochondral defects (Jurgens et al., 2013, Klein et al., 2009,
Nukavarapu and Dorcemus, 2013). Such models offer the advantages of joint size, cartilage thickness (although the ASTM F 2451-05 reports thicknesses of 1.5-2.0 mm, there are reports in the literature ranging from 0.8 mm (ASTM-F2451-05, 2010, Chu et al., 2010, Brehm et al., 2006)), critical defect size (6 mm is the most commonly reported and will not spontaneously heal at 6 months) (Ahern et al., 2009, Getgood et al., 2012, Shahgaldi, 1998, Jackson et al., 2001) and proportion of cartilage to bone and subchondral bone thickness being close to humans. The caprine stifle joint, like the human knee, consists of tibiofemoral and patellofemoral articulations. In a direct comparative study of the stifle joint of cows, sheep, goats, dogs, pigs, and rabbits, the goat stifle was found to have the closest anatomy to the human knee (Proffen et al., 2012). However, the femur has a deep long trochlear groove with prominent medial and lateral ridges (Figure 1.11). The femoral condyles are also distinct with a large intercondylar notch. The tibial plateau is convex and sloped posterolaterally with a prominent fibular styloid laterally, roughly correlating to the fibular head and styloid process in humans (LaPrade et al., 2006). Additionally, the soft tissue structures which prevent abnormal joint movement specifically in the lateral compartment of the goat knee are similar to those in the human knee joint (Gollehon et al., 1987, Patil et al., 2014). In the human knee flexion is limited to less than 30° in normal walking and stance phase however the goat stifle joint is flexed between 50° and 70° (Patil et al., 2014) meaning weightbearing contact areas are different and must be considered.
Goats are also relatively inexpensive to maintain, easy to handle, and the cartilage thickness allows for the creation of both chondral and osteochondral defects (Chu et al., 2010). If adequate facilities are available to house them, then this model is feasible to conduct large animal studies to evaluate biological responses, durability, toxicology, lesion size and location analogous to human studies (Cook et al., 2014). Caprine models thus represent a good option for in vivo assessment of chondral and osteochondral defect repair.

### 1.6.3 Operative factors influencing model selection

There are many reasons to choose one in vivo model over another (Table 1.3); however, surgical technical limitations of certain models play a large part in the selection of the appropriate group. The available surface area of the joint and the critical defect size is a significant factor, and it is necessary that the area is of sufficient size to allow implantation of the biomaterial. For example the knee joint of a rat has a cartilage thickness of 0.1 mm on the MFC, and in the mouse cartilage is only a few cell layers thick (Chu et al., 2010) compared to between 0.8 and 2.0 mm on the MFC of the caprine stifle (Chu et al., 2010, ASTM-F2451-05, 2010). To create a defect this small presents a technical difficulty for the operator, meaning purely chondral defects are almost impossible and can lead to large inter-animal variation even when producing osteochondral defects, meaning a much greater sample size is
required to achieve a statistically significant outcome contravening the three R’s (Russell and Burch, 1959).

Table 1.3: Advantages and disadvantages of various in vivo models commonly used in cartilage defect repair

<table>
<thead>
<tr>
<th>Species</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low cost, manageable easily available</td>
<td>Very small joints – in situ examination impossible</td>
</tr>
<tr>
<td></td>
<td>Transgenic and athymic strains available</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Can be used in subcutaneous and intramuscular model for degradation rate and safety profile</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low cost, easily available</td>
<td>Permanently open growth plates accelerating intrinsic healing</td>
</tr>
<tr>
<td></td>
<td>Athymic strains available</td>
<td>Increased density of cells in cartilage causing more efficient healing</td>
</tr>
<tr>
<td></td>
<td>Maintain in-house</td>
<td>Partial thickness defects impossible</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low cost</td>
<td>Increased intrinsic healing due to increased cell density</td>
</tr>
<tr>
<td></td>
<td>Maintain in-house</td>
<td>Very different load characteristics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Consistent partial thickness defects very difficult to achieve</td>
</tr>
<tr>
<td>Dog</td>
<td>Naturally occurring disease state</td>
<td>Thin cartilage</td>
</tr>
<tr>
<td></td>
<td>Co-operate with rehabilitation regime</td>
<td>Small critical size defect (4mm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complex ethical approval process</td>
</tr>
<tr>
<td>Pig</td>
<td>Biochemistry similar to humans</td>
<td>Expensive</td>
</tr>
<tr>
<td></td>
<td>Bone apposition rate and trabecular thickness similar to human</td>
<td>Difficult to obtain at skeletal maturity</td>
</tr>
<tr>
<td></td>
<td>Partial thickness defects possible</td>
<td>Specialised habitat</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Temperament</td>
</tr>
<tr>
<td>Goat</td>
<td>Anatomy and biomechanics similar to humans</td>
<td>Subchondral cyst formation</td>
</tr>
<tr>
<td></td>
<td>Partial thickness defects possible</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Easily available</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low maintenance</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Anatomy similar to humans</td>
<td>Subchondral cyst formation</td>
</tr>
<tr>
<td></td>
<td>Partial thickness defects possible</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Easily available</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low maintenance</td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>Large defects similar to humans</td>
<td>Expensive to acquire and maintain – specialised centre required</td>
</tr>
<tr>
<td></td>
<td>Partial thickness large diameter defects possible</td>
<td>Cannot avoid weight bearing on the joint during rehab phase if required</td>
</tr>
<tr>
<td></td>
<td>Naturally occurring defects</td>
<td>Very dense subchondral bone</td>
</tr>
<tr>
<td></td>
<td>Similar biomechanics in trochlear groove</td>
<td>MRI/CT impossible due to size</td>
</tr>
<tr>
<td></td>
<td>Second look arthroscopy possible</td>
<td></td>
</tr>
</tbody>
</table>
1.6.4 Ex vivo factors influencing model selection
When planning an in vivo study, the scientific question will determine the model chosen. For example, in smaller animals, it is possible to do in situ microcomputed tomography (µCT) or magnetic resonance imaging on live animals, allowing good radiological scoring. Due to limited equipment availability, this is more challenging for large animals. In large animals, the larger joint size allows for second look arthroscopy to be carried out at multiple timepoints during of the study prior to euthanasia. The use of large animal models results in larger tissue specimens for analysis. For example, in the horse model, the critical size defect is 9 mm. This poses some technical disadvantages as the dense subchondral bone requires longer decalcification times prior to histological staining. However, division of samples is possible without much difficulty, allowing for example, mechanical testing to be performed on one half and histological staining on the other. This doubles the amount of information collected a single sample. Dividing smaller samples from 6 mm (caprine) or 7 mm (ovine) critical size defects can be a more daunting prospect and can leave artefact obscuring true results. These factors must be carefully considered at the outset of the study and the appropriate model chosen for the results and analyses required. There is a significant pre-clinical gap to be bridged in the development of a device to ease suffering and halt joint degeneration before it can be used as a therapeutic clinically. Therefore, the selection of an appropriate pre-clinical in vivo model is important in ensuring successful translation to the clinic. The financial and labour costs involved in a large animal study can be prohibitive, and so for a proof of concept or degradation and safety profile it can be appropriate to use a small animal or rodent model prior to confirming effectiveness in a large animal study.

1.7 Operative techniques in the implantation of osteochondral defect repair scaffolds
With such a large amount of research going into the development of the optimal articular cartilage regeneration system, it is important to remember the surgical
approach involved in accessing the damaged region of articular cartilage. The surgical approaches for assessment and treatment of damage within the joint are constantly evolving, with the objective of achieving improved patient outcomes. Such procedures can be carried out using traditional open surgical techniques, less invasive mini open procedures, termed mini-arthrotomies, and more recently using minimally invasive arthroscopic procedures. (Steadman et al., 2003, Hangody et al., 1997, Brittberg et al., 1994, Bentley et al., 2013, Karthikeyan et al., 2012, Koulalis et al., 2015, Rodriguez-Merchan, 2012, Kon et al., 2014, Ochs et al., 2011, Piontek et al., 2012).

1.7.1 Open surgical approaches to the knee joint

The standard open surgical approach to the knee joint for procedures requiring access to the entire cartilage surface or retropatellar cartilage, such as a total knee replacement, involves a longitudinal incision with a medial parapatellar approach into the joint capsule itself (Figure 1.12). The patella is everted laterally if access to the retropatellar cartilage is required and the entire joint surface is visible to the operating surgeons (Siebold et al., 2011, Brittberg et al., 1994). Although this open technique although allows greater access for the surgeon, it prolongs rehabilitation for the individual, has a greater chance of wound infection, stiffness and a poorer cosmetic result than less invasive procedures (Jungmann et al., 2012).

Figure 1.12: Longitudinal incision with medial parapatellar approach to knee joint exposes cartilage surface for procedures requiring access to the entire joint surface such as total knee arthroplasty (Wheeless, 2014)
The more common mini-arthrotomy involves a significantly smaller incision usually only 4-5 cm, can avoid dislocation of the patella, and allows the operator to directly visualise and palpate the chondral surface to verify that any implant is flush with the native cartilage surface. The mini-arthrotomy, however, offers very limited exposure and access to the joint surface and does not allow access to the retropatellar cartilage (Siebold et al., 2011), while still exposing the joint to the risks of open surgery and leaving the patient with a weakened joint capsule and a large superficial scar (Siebold et al., 2011, Marcacci et al., 2002). Greater patient benefits can thus be achieved through the use of arthroscopic procedures.

1.7.2 Arthroscopic approaches to the knee joint

Arthroscopic techniques are now used for a wide range of surgical procedures in the joint as highlighted in Table 1.4. Arthroscopy offers a range of benefits over standard open procedures in terms of minimised trauma to surrounding tissues and decreased muscular deficits, decreased chances of wound infection, reduced stiffness and improved cosmetic outcome (Jameson et al., 2011). Edwards et al. (2014) compared open and arthroscopic surgical methods and found significantly better postoperative active knee flexion achieved as early as 8 weeks in the arthroscopic group, which was maintained up until 12 months post-surgery. Whereas they found a mild but persistent fixed flexion deformity existed in the open surgery group, with full knee extension not regained until 3 to 6 months postoperatively (Philippon et al., 2010, Bizzini et al., 2007, Edwards et al., 2014). An additional benefit of arthroscopic surgery is that complications tend to be relatively rare. A prospective study by Small reports complications in just 1.68% of 10,262 cases (Small, 1993). Unfortunately, access to particular areas within the joint can be limited with arthroscopy, in particular in the treatment of patellar lesions and posterior portions of femoral condyles or the tibial plateau. (Hernandez-Vaquero et al., 2012, Marcacci et al., 2005). Surgical tools and equipment for arthroscopic surgery require additional improvements and advancement (Hodgins et al., 2014). Despite these limitations, arthroscopy has gone from being used only for specific cases, to being the gold
standard for joint surgeries, and most of the joints in the body are now accessed by arthroscopy.

Table 1.4: Indications for use of arthroscopy in the knee with an emphasis on cartilage defect repair (Adapted from Macmull et al. 2015) (196)

<table>
<thead>
<tr>
<th>Category</th>
<th>Indications</th>
</tr>
</thead>
</table>
| Diagnostic                                    | • Diagnosis of knee pathology  
• Assessment and Classification of cartilage injuries and defects  
• Assessing knee joint for suitability prior performing a unicompartmental knee replacement (UKR) or high tibial osteotomy (HTO) |
| Therapeutic                                   | • Removal of loose bodies causing impingement, pain locking or giving way.  
• Acute arthroscopic lavage of infected knees and painful crystal arthropathy  
• Lateral retinacular release for lateral patellar overload/pressure syndrome |
| Treatment of Articular cartilage injuries and defects | • Abrasion chondroplasty  
• Mosaicplasty  
• Autologous cartilage implantation (ACI)  
• Osteochondral defect or osteochondritis dissecans fixation |
| Synovectomy in cases of:                     | • Rheumatoid arthritis  
• Infections (septic arthritis etc.)  
• Pigmented villonodular synovitis (PVNS)  
• Synovial chondromatosis (multiple loose bodies) |
| Reconstruction and Fracture Repair            | • Repair or resection of meniscal tears that are symptomatic of locking, pain or swelling.  
• Anterior cruciate ligament (ACL) ligament reconstruction  
• Posterior cruciate ligament (PCL) reconstruction.  
• Arthroscopic-assisted fixation of tibial plateau fractures |
1.7.3 Current arthroscopic procedures in treatment of cartilage damage

1.7.3.1 Arthroscopic assessment and debridement

Articular cartilage damage is one of the most commonly seen pathologies at arthroscopy. The arthroscopic procedure for assessment and treatment of cartilage defects of the knee currently involves access of the defect through carefully positioned arthroscopic ports (Figure 1.13). The surgeon can then use arthroscopic probes to examine the quality of the cartilage surface and use a shaver to debride back to healthy tissue and remove any loose material that can be causing pain.

![Arthroscopic portal positioning](image)

**Figure 1.13:** Arthroscopic portal positioning. The surgeon marks the patella position and the location of the tibial tubercle. Horizontal lines are drawn just below the patella and at two fingerbreadths above the patella. Lines are then drawn from each side of the tibial tubercle, along the edge of the patellar circle to cross over the horizontal lines. The main ports are marked where the lines cross below the patella (antero-medial (AM) and antero-lateral (AL) and above the patella (medial supra-patellar (SM) and lateral supra-patellar (SL) portals). Additional ports can be placed below the patellar mid-line or at the side of the patella if required.

1.7.3.2 Arthroscopic Microfracture

Along with cartilage surface assessment some of the more straightforward treatment methods for cartilage defects are already carried out arthroscopically. Bone marrow stimulation techniques, such as microfracture and subchondral drilling, are often performed in the treatment of cartilage lesions in the knee as well as other joints such as the hip, shoulder and ankle (El Bitar et al., 2014, Karthikeyan et al., 2012). Microfracture has long since been performed arthroscopically with general
arthroscopic instruments including a pick, a Kirshner wire or a drill bit (Chen et al., 2011, Pascarella et al., 2010, Steadman et al., 2003, Chen et al., 2009). The fact that microfracture can be performed in such a straightforward manner with good results (Steadman et al., 2003) means that many surgeons will be reluctant to perform more complex procedures where an arthrotomy is required (Basad et al., 2010, Van Assche et al., 2010, Lim et al., 2012). It is important to any surgeon that the benefits of the operation outweigh the risks, and if the operation can be performed arthroscopically the risks decrease.

1.7.3.3 Arthroscopic Osteochondral Grafting

There have been many reports of osteochondral grafting procedures being performed via arthroscopic ports. One such example is mosaicplasty, a hyaline cartilage plug transplantation procedure that can be carried out arthroscopically. This technique involves placing a superior portal and using a tube chisel to harvest the graft from the non-weight bearing edges of the lateral trochlea. An appropriately sized drill guide and drill bit are used to allow the correct depth and positioning of the recipient holes in the defect base. The graft is then placed through the drill guide and pushed into place using a tamp. Arthroscopic osteochondral grafting procedures can be technically difficult to perform, in particular the harvesting and placement of arthroscopic plugs can cause challenges for surgeons. However, it has been shown that so long as the defects are in an accessible location there is no difference in plug placement between open and arthroscopic technique and similar clinical outcomes are reported (Koulalis et al., 2015, Ahearne et al., 2011, Ahearne et al., 2014, Ahlers, 2008). In addition, Marcacci et al. report on the limitations of existing surgical instruments and report that improvements in surgical instruments are required (Ahlers, 2008). These hurdles still mean that the majority of osteochondral grafting procedures are still being performed via mini arthrotomy.
1.7.3.4 Arthroscopic Autologous Chondrocyte Implantation

While ACI is usually performed through an arthrotomy or mini-arthrotomy, it can also be performed arthroscopically. One major technical challenge is that the periosteal patch, required to maintain cells within the defect site, needs to be held in place with sutures which can be technically difficult. A recent multi-centre study investigating the clinical outcomes after ACI has shown that more than 26% of the procedure-related complications of ACI can be contributed to the arthrotomy itself (Erggelet et al., 2000). Ferruzzi et al. carried out a direct comparison of open and arthroscopic techniques for ACI. They report reduced surgical trauma, a better cosmetic result, and a faster recovery time in the arthroscopic ACI group with the achievement of stability six months sooner than in the open surgery group (Ferruzzi et al., 2008). The advantages of developing techniques that enable a move towards arthroscopic ACI are clear.

There are significant advances being made in the field of bioengineering toward an acceptable long-term cartilage repair technique, and all types of surgery, orthopaedic and otherwise, are moving toward less and less invasive techniques in order to reduce patient morbidity. It is important then, when developing biomaterials and scaffolds for cartilage repair, to recognise the significant impact an open surgery can have on the overall treatment and wellbeing of the patient. As such, although biomaterials should be developed with optimal repair as the primary goal, a secondary goal should be the ability for them to be used in a minimally invasive arthroscopic fashion, which would increase their benefit to patients and their uptake by surgeons. An arthroscopically deliverable cartilage repair scaffold could allow the expansion of use of cartilage repair techniques to other large joints such as the hip or ankle, with significant gains to the patient, and give relief to more individuals.
1.8 Thesis Objectives

The overall objective for this thesis is to develop and optimise a tissue engineered biomaterial scaffold-based method of osteochondral defect repair that facilitates cell-seeding onto the scaffold as a single-stage procedure, with the possibility of implantation via arthroscopic technique.

To achieve this objective the specific aims of the thesis were to:

1. Examine the intrinsic properties of a collagen-based tri-layered scaffold, developed within the RCSI, to direct stem cell differentiation as required in each layer to achieve osteochondral defect repair
2. Establish an optimal cell-seeding regime for this multi-layered collagen-based scaffold
3. Investigate the in vivo repair potential of a FPMSC and chondrocyte co-culture seeded scaffold in comparison to a cell-free scaffold and a commercially available biomaterial for osteochondral repair in a critically sized surgically created osteochondral defect in the stifle joint of a caprine model.
4. Develop and test an arthroscopic delivery device for implanting the multi-layered scaffold into a human knee via a minimally invasive arthroscopic technique.
Chapter 2: Assessment of stem cell differentiation within a tri-layered collagen-based scaffold designed for osteochondral defect repair

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2.1 Introduction

In tissue engineering, the implanted biomaterial must be carefully suited to the environment in order to achieve successful tissue repair. It should not evoke a host immune response, should have sufficient porosity to allow the infiltration and proliferation of cells, while maintaining mechanical properties that tolerate the biological and physical demands of the host tissue. The implant should degrade over time to allow the body to repair the defect with the appropriate native tissue (Henson and Getgood, 2011). Apart from providing these properties when the scaffold is in situ, it must also be sterilisable, easy to handle and manipulate using surgical instruments, and consistently reproducible (Henson and Getgood, 2011). A range of materials have been investigated for use for bone and cartilage repair applications. Natural biomaterials have shown particular promise in this regard, and collagen, the most abundant protein in the human body, has been used with good effect in TE for bone and cartilage regeneration. Specifically, collagen has been used extensively in bone graft substitute devices, and as part of second generation autologous chondrocyte implantation (ACI) (Gooding et al., 2006) and matrix-assisted chondrocyte implantation (MACI™) procedures (Bartlett et al., 2005, Basad et al., 2010, Saris et al., 2014). Collagen has also shown an ability to co-polymerise with other biological materials such as hydroxyapatite or hyaluronic acid to enhance the biofunctionality of collagen-based scaffolds and improve bone or cartilage repair (Matsiko et al., 2012, Murphy et al., 2010, Gleeson et al., 2010). To mimic the osteochondral tissue, the challenge of the gradient nature of the tissue must be overcome. While developing a bone layer and overlying cartilage layer, the tidemark between the two must be protected in order to prevent the bone layer advancing and thinning the cartilage. One method to do this is to develop different scaffolds designed to repair each region of the osteochondral defect, i.e. bone and cartilage, and to fuse these individual scaffolds together using either sutures or biological glues (Schaefer et al., 2000, Kreklau et al., 1999, Levingstone et al., 2014). Success of such materials has been limited due to poor cellular infiltration through the layers of the structure. An increasing number of multi-layered scaffolds have emerged onto the market in recent years including TruFit (Smith & Nephew, MA, USA), a bi-layered scaffold composed of poly-(DL-lactide-co-glycolide) (PLGA) and calcium sulphate, and
MaioRegen (Finceramica, Italy), consisting of a type I collagen cartilage layer, a magnesium-enriched hydroxyapatite (Mg-HA) bone layer and a Mg-HA and collagen intermediate layer.

The Tissue Engineering Research Group (TERG) in the RCSI has developed a tri-layered matrix designed to mimic the biochemical and biophysical properties of the different layers of the native joint. The scaffold consists of three distinct but seamlessly integrated layers, designed to replicate the bone layer, intermediate layer, and a cartilage layer of an osteochondral defect, and is fabricated using a novel iterative freeze-drying process. The bone layer is composed of hydroxyapatite, the main mineral component of bone, and collagen type I the primary organic component of bone (Miller, 2008, Gleeson et al., 2010). The intermediate layer is composed of type I collagen (0.5%w/v) and hyaluronic acid. The cartilage layer is composed of type I collagen (0.25%w/v), type II collagen (0.25%w/v), which is present in articular cartilage, and hyaluronic acid (HyA) (0.05%w/v) (Levingstone et al., 2014, Gleeson JP, 2009). The scaffold was developed through optimisation of collagen content; freeze drying processes, crosslinking protocols and addition of HA to alter the mechanical stiffness (Levingstone et al., 2014, O’Brien FJ, 2008).

This tri-layered scaffold has shown success in vivo as an off-the shelf cell-free scaffold in focal defects in a rabbit medial femoral condyle model at 3 months post implantation (Levingstone et al., 2016b) and in goat medial femoral condyle and lateral trochlear ridge models at 12 months post implantation (Levingstone et al., 2016a), by recruiting host cells from the bone marrow and directing them to form bone and cartilage in the requisite layers, with restoration of the anatomical tidemark, resulting in joint regeneration. However, the intrinsic properties of the scaffold to direct stem cell differentiation in each layer and the osteogenic properties of the bone layer and chondrogenic properties of the cartilage layer have not yet been demonstrated.

While separate osteogenic and chondrogenic culture of rMSCs has been well established within the literature (Sartori et al., 2017), the in vitro culture of osteochondral repair scaffolds with both a cartilage layer and a bone layer has presented a challenge. Previous studies have shown some success using techniques
such as mixed media types (Dorcemus et al., 2017) or specifically designed wells containing a septum to divide the media types (Chen et al., 2016). However, the optimal supplemented media, capable of inducing the differentiation of MSCs down separate lineages dependant on their position in the scaffold, must be specific to the composition of the scaffold under investigation and dependant on its inherent chondrogenic and osteogenic properties.

2.2 Aims and objectives
The overall aim of this chapter was to examine the intrinsic properties of the tri-layered scaffold to direct stem cell differentiation as required in each layer to achieve osteochondral defect repair. In order to achieve this aim, the specific objectives were as follows:

1. To assess the osteoinductive properties of the Col1/HA bone layer of the tri-layered scaffold both with and without the presence of osteogenic stimulation
2. To explore osteogenic differentiation of MSCs within the Col1/HyA intermediate layer with and without supplementation
3. To assess the chondrogenic differentiation of MSCs within the Col1/Col2/HyA cartilage layer of the tri-layered scaffold both with and without the presence of chondrogenic stimulation
4. To characterise the dimensional stability of the tri-layered scaffold during in vitro culture
5. To assess the infiltration and proliferation of MSCs within the tri-layered scaffold
6. To determine the optimal osteochondrogenic media type, capable of supporting chondrogenesis in the cartilage layer and osteogenesis in the bone layer of the scaffold
2.3 Methods

2.3.1 Fabrication of individual layer and tri-layered scaffolds

To determine the properties of each region of the tri-layered scaffold, each layer of the scaffold was fabricated as an individual scaffold. The bone layer scaffold consisting of 0.5 % (w/v) type I microfibrillar bovine tendon collagen (Col1, Collagen Matrix Inc., NJ, USA) and 1 % (w/v) hydroxyapatite (HA, Plasma Biotal, CAS No. P288R, Capital R) was fabricated as follows: 1.8 g of microfibrillar bovine tendon collagen was added to 320 ml of 0.5 M acetic acid solution (Fisher Scientific, CAS No. 64-19-7) and blended (15,000 rpm, 4 °C, 90 mins) using an overhead blender (Ultra Turrax T18 Overhead Blended, IKA Works Inc., USA). A HA suspension was then made by adding 3.6 g of HA to 40 ml of 0.5 M acetic acid solution. This suspension was added to the blended collagen in 10 ml aliquots every twenty minutes until it had all been added. The entire suspension was then blended for a subsequent hour with repeated visual inspections to ensure that a homogenous suspension was obtained. This led to a total blending time of five and a half hours. Prior to freeze-drying, degassing was carried out in a vacuum chamber (50 mTorr) to removed air introduced during blending. 15.6 ml of the bone layer suspension was pipetted slowly and evenly into each custom made stainless steel tray (internal dimensions, 60 mm x 60 mm) and the trays were then placed on the shelves within the freeze dryer (Virtis Genesis 25EL, Biopharma, Winchester, UK) and freeze-dried at a constant cooling rate of 1 °C/min to a final freezing temperature of -40 °C and dried under a vacuum pressure of 200 mTorr.

The intermediate layer scaffold consisting of Col1 [0.5% (w/v)], and hyaluronic acid sodium salt derived from streptococcus equi. (HyA) [0.05% (w/v), Contipro] was fabricated as follows: a hyaluronic acid solution was then made up by adding 0.11 g of hyaluronic acid (hyaluronic acid sodium salt derived from streptococcus equi) (Sigma-Aldrich, CAS No. 53747-10G) to 40 ml of the 0.5 M acetic acid and stirring for 60 mins. 1.2 g of Col1 was then blended with 200 ml of the 0.5 M acetic acid solution (15,000 rpm, 4 °C). The hyaluronic acid solution was added dropwise to the blending collagen to avoid clumping. The slurry was left to blend for an additional 60 minutes with repeated visual inspection to avoid any clumping. Prior to freeze-drying, final
degassing was carried out in a vacuum chamber (50 mTorr) and 15.6 ml of the suspension was pipetted slowly and evenly into a stainless-steel tray (internal diameter 60 mm x 60 mm) and freeze-dried at a constant cooling rate of 1 °C/min to a final freezing temperature of -10 °C and dried under a vacuum pressure of 200 mTorr.

For the manufacture of the cartilage layer, consisting of Col1 [0.25 % (w/v)], type II collagen (Col2) [0.25 % (w/v) porcine Col2, Symatese, France] and hyaluronic acid (HyA) [hyaluronic acid sodium salt derived from streptococcus equi, Contipro, Czech Republic] [0.05 % (w/v)]. 0.6 g of Col1 collagen and 0.6 g of Col2 were added to 300 ml of 0.5 M acetic acid solution. This was blended using an overhead blender (Ultra Turrax T18 overhead blender, IKA Works Inc., USA) at 15,000 rpm at 4 °C for 90 minutes with repeated visual inspection for clumping and to ensure all collagen was homogenously distributed throughout. 0.11 g of HyA was solubilised in 40 ml of 0.5 M acetic acid and then added dropwise to the blending type I/type II collagen suspension. The blender was maintained at 15,000 rpm for a further 90 minutes. Prior to freeze-drying, degassing was carried out in a vacuum chamber (50 mTorr) and 15.6 ml of the suspension was pipetted slowly and evenly into a stainless-steel tray and freeze-dried at a constant cooling rate of 1 °C/min to a final freezing temperature of -10 °C and dried under a vacuum pressure of 200 mTorr.

The combined tri-layered scaffolds were required for use in the osteochondral media experiments. These scaffolds were fabricated as previously described (Levingstone et al., 2014). Briefly, the first step involved the fabrication of the bone scaffold as above. Following freeze drying the scaffold was cross-linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)/N-hydroxysuccinimide (NHS) (Sigma–Aldrich, Arklow, Ireland) at a concentration of 6 mM EDAC/g of collagen, and a 5:2 M ratio of EDAC:NHS for 2 hours at room temperature (Haugh et al., 2011). The scaffold was then slowly hydrated with 0.025 M acetic acid solution in a stainless-steel tray to provide a support for the bone layer scaffold during addition of the next layer. The second step was to add 7.8 ml intermediate layer suspension on top of the hydrated bone layer scaffold with freeze drying repeated as before. The last step involved adding 15.6 ml of the cartilage layer suspension. The process of freeze-drying was
repeated as described previously, incorporating prolonged freezing and drying steps to ensure optimal freeze-drying of the tri-layered construct (Figure 2.1) (Levingstone et al., 2014). Following freeze-drying, the scaffolds were dehydrothermally (DHT) cross-linked in a vacuum oven (VacuCell, MMM, Germany) for 24 hours. The DHT cross-linking was carried out at a pressure of 50 mTorr, and a temperature of 105°C to generate cross-links through a condensation reaction which also sterilises the scaffolds. Finally, cylindrical scaffold plugs 6 mm in depth were cut from the tri-layered scaffold sheet using a punch of the required diameter. The entire construct was then chemically cross-linked again under sterile conditions with EDAC as described previously.

**Figure 2.1:** Iterative layering fabrication process diagram. This is a three-step process that allows the material composition and scaffold micro-architecture in each region of the scaffold to be specifically tailored while producing a resultant scaffold with a seamlessly integrated layer structure. (Levingstone et al., 2014)
2.3.2 Stem cell isolation and expansion
MSCs were isolated from 8-week-old female Fischer 344 rats (338) with the approval of the Research Ethics Committee of the RCSI (REC Approval No. 237). After euthanasia, marrow was flushed from the tibiae and femora with phosphate buffered saline (PBS; Sigma-Aldrich, Ireland) and a single cell suspension was recovered. After centrifugation (600 g, 10 minutes), cells were plated at 120 x 10^6 cells/cm^2, in standard rat MSC growth medium [10 % foetal bovine serum (FBS; Hyclone, Fisher Scientific, Ireland), 45 % F12-Ham and 45 % α-MEM (Biosciences, Ireland) supplemented with antibiotics (100U/ml penicillin G and 100 μg/ml streptomycin sulphate; Gibco)]. Flasks were incubated at 37 °C in 5 % CO_2/90 % humidity. After 8 days colonies became compact and cells were detached with 0.25 % trypsin/EDTA and re-plated at 2000 cells/cm^2. Subsequently, cultures were passaged at 5-7 day intervals and expanded to passage 5 for experimentation. The MSCs were cultured in Dulbecco’s Modified Eagles Medium (Sigma-Aldrich, Ireland) supplemented with 2 % penicillin/streptomycin (Sigma-Aldrich, Ireland), 1 % L-glutamine (Sigma-Aldrich, Ireland), 10 % FBS (BioSera, UK), 1 % glutamax (Biosciences, Ireland) and 1 % non-essential amino acids (Biosciences, Ireland).

2.3.3 Scaffold seeding
Scaffolds were hydrated in PBS until saturated, then moved onto a dry plastic surface to allow excess PBS to drain off. They were then placed into non-adherent 24 well plate. MSCs were detached from their plates with 0.25 % trypsin/EDTA and counted. Cells were suspended at a density 1x10^6 cells/34 μl. 17 μl of media was then placed on the surface of each scaffold and scaffolds were incubated at 37 °C for 15 minutes to allow the cells to attach. Each scaffold was then carefully turned over so that the seeded side now faced downward and the other surface was seeded with a further 17 μl of media. Scaffolds were again incubated at 37 °C for 15 minutes prior to the addition of 2 ml of complete MSC growth medium (described above) to each well of the 24 well plate. The plates were then returned to the incubator at 37 °C in 5 % CO_2/90 % humidity. The seeded scaffolds were fed by partial feeding with complete
MSC growth medium every 2 days for a period of seven days to allow the cells attach prior to transferring to standard media, osteogenic media [(Sigma; Dulbecco Modified Eagles Media 5671 Media, 10 % FBS (Biosera; S1900/500), 100nM Dexamethasone (Sigma; CAS No. 50-02-2), 50 µM Ascorbic acid 2-Phosphate, (Sigma; CAS No. 66170-10-3), 10 mM β-glycerophosphate (Sigma; CAS No. 13408-09-8), 10000 U/mL penicillin and 10 mg/mL streptomycin (Sigma; P4333-100 ml)] or chondrogenic media [(Sigma; Dulbecco Modified Eagles Medium D5671), 10% Foetal Bovine Serum (FBS) (Gibco), 2 % 10000 U/mL penicillin and 10 mg/mL streptomycin (Sigma; P4333-100 ml), 0.5 % 1-Glutamine (Gibco), 0.5 % Glutamax 100x (Gibco), 1 % non-essential amino acids (NEAA)(Gibco), 20 ng/ml Human TGFβ-3 (Prospec), 50 µg/ml Ascorbic Acid, 40 µg/ml Proline (Sigma), 100 nM Dexamethasone (Sigma), 1x ITS supplement (Insulin, transferrin, sodium selenite) (BD), 0.11 mg/ml Sodium Pyruvate (Sigma)]. This timepoint was labelled as day zero.

The bone layer group was divided into two groups and cultured in either standard MSC growth media (described above) or osteogenic growth media (n=4 per timepoint). The intermediate layer group was divided into three groups, an osteogenic media group, a standard media group and a chondrogenic media (n=4 per timepoint). The cartilage layer was divided into two groups; a standard media sample group and a chondrogenic group (n=4 per timepoint) (Figure 2.2).
Figure 2.2: The three individual layers were fabricated and seeded with MSCs. Each layer scaffold was cultured in the relevant media types as shown above.

Samples were harvested at timepoints of 0, 7, 14, 21 and 28 days. On the day of harvest, media was removed from the plate and each scaffold placed in a 1.5 ml eppendorf tube. Scaffolds were flash frozen using liquid nitrogen and stored at -80°C until all timepoints were ready for analysis. One sample from each group was fixed in 10% formalin solution and wax embedded for histological analysis.
2.3.4 Assessment of calcium production
A quantitative calcium assay was performed to assess mineral production in the bone layer and intermediate layer scaffolds cultured in standard and osteogenic medium. This assay was performed using a StanBio Calcium Liquicolour Kit (StanBio 0150). Scaffolds were placed on a shaker in 0.5 M HCl over 24-48 hours at 4 °C until digested. The assay was then carried out according to the manufacturer’s instructions. Briefly, triplicates of 10 μl of each sample were added to 200 μl of working solution (1:1 binding reagent and working dye) in a 96 well plate and absorbance was measured using a photometric plate reader (Wallac 1420 Victor 2 D, Perkin Elmer, MA, USA) based on absorbance at 595 nm against a standard curve of known values.

2.3.5 Assessment of sulphated GAG production
A Blyscan sulphated glycosaminoglycan (sGAG) assay (Biocolour Ltd, UK) was used to measure the quantity of sGAG laid down in the cartilage layer and the intermediate layer scaffolds. Samples were digested in papain enzyme solution (10 mg of papain with 10 ml of papain buffer made of PBS, 1 % 0.5M EDTA and 0.79 mg/ml of cysteine-HCl). Then 100 μl of each sample was added to 500 μl of Blyscan dye reagent. Tubes were then placed in a mechanical shaker for 30 minutes to allow sulphated glycosaminoglycan-dye complex to form and precipitate out of the soluble unbound dye. Tubes were then spun at 12,000 rpm for a period of 10 minutes to firmly pack the complex at the bottom of the tube. The tubes were then inverted removing any excess dye. The Blyscan dissociation agent was then added (500 μl per tube) to release the bound dye back into solution. Once the bound dye was dissolved back into solution duplicates of 200 μl of each sample was placed in individual wells of a 96 well plate and absorbance was measured using a photometric plate reader (Wallac 1420 Victor 2 D, Perkin Elmer, MA, USA) based on absorbance at 656 nm was measured against a standard curve of known values including a blank (distilled water), and sample test values.
2.3.6 Histological analysis
At each time point, one scaffold from each group was fixed in 10% formalin for 1 hour for histological analysis and then processed in an automatic tissue processor (ASP300, Leica, Wetzlar, Germany). Scaffolds were then embedded in paraffin wax and sectioned transversely at a thickness of 10 µm using a rotary microtome (RM2255, Leica microtome, Leica). Sections were stained with alizarin red to stain for calcium, Toludine Blue, or stained using immunohistochemical staining techniques specifically looking for type II collagen present in hyaline cartilage – the optimal cartilage for joint surface repair. Collagen II is not present in fibrocartilage. Digital images were obtained using a microscope (Nikon 90i, Nikon, Japan) and attached camera unit (Nikon DS Camera control unit, Nikon, Japan) to evaluate the calcium deposition, the cartilage generation and the type II collagen formation through the scaffold.

2.3.7 Assessment of dimensional stability of the scaffold
At each timepoint photographs were taken of each scaffold and ImageJ software (public domain software developed by Wayne Rasband in National Institutes of Health, USA) was used to assess change in diameter of the scaffolds over the culture period and determine levels of scaffold contraction.

2.3.8 Assessment of proliferation in the tri-layered scaffold
Cell number was assessed on the tri-layered scaffold by using a Quant-iT™ PicoGreen® dsDNA kit (Invitrogen, UK) (n=4 at each timepoint). Each scaffold was seeded as above with MSCs. The cells were allowed 7 days of attachment time and after this, samples were taken off at 24 hrs, 48 hrs and 7 days and flash frozen prior to analysis. Each scaffold was digested using a Qiazol Lysis Reagent (Qiagen, Germany), by adding 1 ml of Qiazol lysis reagent to the scaffold in a 3.5 ml tube, and then homogenised three times (5-10 s, 5,000-10,000 rpm) using a handheld homogeniser (X120, Ingenieurbüro CAT, Germany). The lysates were then placed on the benchtop to allow nucleoprotein complexes to dissociate for 5 minutes. The PicoGreen reagent was then prepared in accordance with the manufacturer’s
protocol. Briefly, 14 μl of the sample was diluted with 336 μl of 1X Tris-EDTA Buffer. The Quant-iT PicoGreen dsDNA reagent was then prepared by adding 100 μl to 19.9 ml of 1X Tris-EDTA buffer. 100 μl of each solution was plated in triplicate in a black 96 well plate and fluorescence of the samples was measured (excitation 485 nm, emission 538 nm). The DNA concentration was deduced using a standard curve.

2.3.9 Optimisation of an osteochondrogenic in vitro culture environment
In order to identify a suitable osteochondrogenic culture media, MSCs were seeded on tri-layered osteochondral defect repair scaffolds cultured in a number of different media compositions. The MSCs were expanded in standard growth media alone until confluent, and at passage 4 they were seeded onto 9.5 mm diameter cylindrical scaffolds at a seeding density of 0.5x10⁶ cells per scaffold using the seeding technique outlined above. The media was changed every 2-3 days for one week. The media was then completely removed and replaced with supplemented media, dependant on the group, either standard, chondrogenic, osteogenic (Figure 2.2) or osteochondrogenic media as outlined in Table 2.1. This was marked as Day 0 and the constructs were cultured to time points of 14 and 28 days (n=4) with partial media exchange of appropriate supplemented media every 2-3 days.

2.3.10 Histological assessment of mineral deposition in the tri-layered scaffold
Histological analysis was performed at 28 days to qualitatively examine the mineralisation in the tri-layered scaffolds in all media types. It was important to ensure that the osteogenesis was occurring in the bone layer, and that the intermediate layer had the ability to protect the tidemark. Histological analysis of the tri-layered scaffold was carried out as described in Section 2.3.6, with scaffolds sectioned longitudinally to demonstrate all layers within a single slice.
Table 2.1: MSCs were seeded onto tri-layered scaffolds and supplemented with four different media types in order to determine the optimal media for osteogenesis and chondrogenesis within the one scaffold. The four media types were the osteogenic, chondrogenic and standard media as used previously (Figure 2.2) and the osteochondrogenic media outlined below.

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Supplement</th>
<th>Supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dulbecco’s Modified Eagles Medium</td>
<td>DMEM; Sigma D5671</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Foetal Bovine Serum</td>
<td>FBS; Gibco</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>penicillin/streptomycin</td>
<td>Gibco 15070-063</td>
<td>2%*</td>
</tr>
<tr>
<td></td>
<td>I-Glutamine 200mM</td>
<td>Gibco</td>
<td>0.5%*</td>
</tr>
<tr>
<td></td>
<td>Glutamix “100x”</td>
<td>Gibco</td>
<td>0.5%*</td>
</tr>
<tr>
<td></td>
<td>non essential amino acids (NAA)</td>
<td>NAA; Gibco</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Human TGFβ-3</td>
<td>Prospec</td>
<td>20 ng/ml</td>
</tr>
<tr>
<td></td>
<td>Ascorbic Acid</td>
<td>Sigma</td>
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<tr>
<td></td>
<td>Proline</td>
<td>Sigma</td>
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<td></td>
<td>Dexamethasone</td>
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<td>ITS supplement</td>
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<td>1x</td>
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<tr>
<td></td>
<td>Sodium Pyruvate</td>
<td>Sigma</td>
<td>0.11mg/ml</td>
</tr>
<tr>
<td></td>
<td>β glycerophosphate 1M</td>
<td>5ml</td>
<td>10mM</td>
</tr>
</tbody>
</table>

2.3.11 Statistical analysis
All statistical analyses were performed using statistics software Prism 7 GraphPad (GraphPad Software, California USA). For analysis of two variables a paired student’s T test was used and where three variables were present a repeated measure analysis of variance (ANOVA) test was performed.
2.4 Results

2.4.1 Assessment of osteogenesis within the bone layer scaffold

Osteogenesis in the bone layer scaffold in osteogenic medium was quantified using a calcium assay at day 0, 7, 14, 21 and 28. The results demonstrated a significant increase in the amount of calcium laid down by rat MSCs in the bone layer of the scaffold over the 28-day duration of the study when cultured in osteogenic media (p=0.0008) (Figure 2.3). The 2.48-fold increase in the mineralisation in the scaffold between day 0 and day 28 demonstrates its osteoconductive properties.

![Graph showing calcium deposition over time](image)

Figure 2.3: Mineralisation of the bone layer scaffold in osteogenic media over time. Calcium assay of bone layer scaffold in osteogenic media demonstrated a significant increase in mineral deposition between the day 0 and day 28 timepoints (***p=0.0008). Please note that throughout all graphs green represents cultured in osteogenic media, red standard media and yellow chondrogenic media.

Having demonstrated the ability of the bone layer scaffold to support the osteogenic differentiation of MSCs in osteogenic media, the osteoinductive properties were then assessed. Osteoinduction refers to the ability to induce differentiation of osteogenic cell sources such as MSCs into osteogenic lineage and therefore induce
matrix deposition and mineralisation (Chan et al., 2010, Mishra and Kumar, 2014, Wang et al., 2012). This was done by culturing the bone layer scaffolds in standard growth media for 0, 7, 14, 21 and 28 days (Figure 2.4). Calcium levels were seen to have a 1.74-fold increase over the 28 day study (p<0.05) indicating that mineral is being deposited by the MSC cells within the scaffold, without any exogenous osteogenic properties. This demonstrates that the scaffold has osteoinductive properties.

Calcium deposition over time within both media types was further demonstrated through histological analysis (Figure 2.5). Alizarin red staining was positive at all time points due to the presence of HA, however intensity was observed to increase over the 28-day time frame and mineral was observed to be uniformly deposited throughout the scaffold.
2.4.2 Assessment of osteogenesis within the intermediate layer

Mineral deposition in MSC seeded intermediate layer scaffolds in standard media and osteogenic media at timepoints up to 28 days showed an increase in the calcium deposition in the osteogenic media group over the 28-day culture period, whereas, there were no significant levels of calcium observed in the standard growth media group (Figure 2.6). One of the most common problems in the design of biomaterials for osteochondral defect repair is the hypertrophy and ossification of the superficial layers and the advancement of the bony tidemark with thinning of the overlying cartilage often exasperating the original pathology. This demonstrates that while the intermediate layer scaffold can support osteogenesis in osteogenic supplemented media, it does not have intrinsic osteoinductive properties.
2.4.3 Assessment of chondrogenesis in the cartilage layer scaffold

Following the assessment of osteogenesis within the bone layer and intermediate layer scaffolds, the chondrogenic properties of the cartilage layer scaffold was then examined using a sulphated GAG assay. Culture of MSCs within the cartilage layer scaffold in chondrogenic media containing TGF-β resulted in a significant increase (p=0.0073) in sGAG content over the 28-days. This demonstrates the ability of the scaffold to support MSC chondrogenesis (Figure 2.7). Culture of this scaffold withMSCs in standard growth medium demonstrated a significant increase in chondrogenesis at day 14 (p=0.0318), demonstrating the ability of the scaffold to direct stem cells toward a chondrogenic lineage without exogenous stimulus. There was, however, no significant difference between day 14, 21 and 28 (Figure 2.8). In order to qualify our quantitative data, immunohistochemistry for type II collagen (Figure 2.9) and toluidine blue for sulphated GAG deposition were performed (Figure
The results of the staining show an increase over time in the amount of collagen II and GAG present in the cartilage layer scaffolds cultured in both media types, with higher levels observed in the chondrogenic media group as expected.

\[ \text{Figure 2.7: sGAG assay of cartilage layer in chondrogenic media. These results show an increase in the cartilage formation over time (*** p} < 0.0001 ** p} < 0.001 * p} < 0.05) \]
Figure 2.8: sGAG assay of cartilage layer in standard growth media. These results show a significant increase at day 14 (* p = 0.0318). However, there is no significant difference between day 14, day 21 or day 28.

Figure 2.9 Collagen II immunohistochemical staining of cartilage layer scaffold. This staining shows an increase over time in collagen II in both the chondrogenic media and in standard media (Scale = 500 µm)
Figure 2.10: Toluidine Blue staining of representative slides from cartilage layer of scaffold cultured in chondrogenic media or standard media. These slides taken at each time point show a slight increase in sGAG production over time in both the chondrogenic and the standard media (Scale = 1000µm)
2.4.4 Dimensional stability of individual layer scaffolds and tri-layered scaffolds *in vitro*

There are a number of suggested techniques for implanting scaffolds into defects; however, the most popular is the press-fit technique whereby the scaffold is deliberately oversized and pressed into the defect. Often this is used in conjunction with fibrin glue for added stability. It is therefore crucial that the scaffold maintains its size, and does not contract over time and fall out of the defect. Measurement of changes in diameter of the individual layer scaffolds over the course of the 28-day study demonstrated that the bone layer scaffold underwent the least contraction of the three separate layers, followed by the cartilage layer scaffold. Significant contraction of the intermediate layer scaffold was observed during the study (Figure 2.11). Statistical analysis demonstrated significant differences between the scaffold groups; however, no significant difference was observed between the different media types investigated within each group. It is evident that the increased stiffness of the bone layer scaffold ensured that it was better able to resist the tensile forces occurring as a result of cell-mediated contraction, whereas the lower stiffness intermediate layer underwent a greater degree of contraction (mean 38.1%; range 22.3%-54.4%).
Within the contraction study, change in diameter of the tri-layered scaffold was assessed in standard growth media. The results demonstrate lower levels of contraction over the 7-day culture period assessed than the individual layer scaffolds (Figure 2.12). There was a 2.7% contraction in the tri-layered group as opposed to a 6.6% and 6.7% contraction in the bone and cartilage groups respectively in standard media at day 7. This demonstrates that the bonds between the individual layers achieved through the iterative layering fabrication process were capable of withstanding cell-mediated tensile forces occurring during the culture period. In addition, the increased stiffness of the bone layer scaffold was able to provide support in resistance to contraction to the intermediate and cartilage layers.

Figure 2.11: % contraction of scaffolds measured by percentage diameter change over 28 days in culture in different media types. This graph shows that the bone and cartilage layer of the tri-layered scaffold maintain their size contracting by less than 10% and 10-20% respectively. The intermediate layer however contracts by a mean value of 38.86%. As seen above though, when cultured as a complete scaffold the top and bottom layers are able to prevent the contraction of the intermediate layer. There was no significant change in size in the bone layer either in standard or osteogenic media over the 28 days. There was a significant change in size \( p<0.0001 \) for the intermediate layer in all media types. There was also a significant change in size in the cartilage layer \( p<0.0001 \) although the percentage contraction was less than the intermediate layer.
Figure 2.12: Diameter of the tri-layered scaffold seeded with rat MSCs in standard growth media culture over 7 days showing less than 10% contraction. Similar to the bone layer in Figure 2.11 there was no statistically significant change in size over the course of the study.

2.4.5 Cell proliferation on the tri-layered scaffold
Initial assessment of cell proliferation in the tri-layered scaffolds, at 24hr, 48hr and 7 days demonstrated a significant increase in the number of cells present at day 7 compared to the 24-hour timepoint (p<0.05) demonstrating the biocompatibility of the scaffold (Figure 2.13).
2.4.6 Establishing the optimal medium to support osteogenesis and chondrogenesis

Having demonstrated the osteoinductive properties of the bone layer and chondrogenic properties of the cartilage layer, this study aimed to determine the ideal osteochondral medium for in vitro culture of the tri-layered scaffold. Osteogenesis and chondrogenesis in the tri-layered scaffolds in standard, osteogenic, chondrogenic and osteochondrogenic medium were compared at timepoints of 0, 14, 21 and 28 days. Assessment of osteogenesis in the tri-layered scaffold showed a significant increase in calcium production at day 28 compared to day 0 in osteogenic media. An increase in calcium production in the osteochondrogenic media and chondrogenic media groups was also observed (Figure 2.14). However, no significant difference was found between the values. It is possible that, had the experiment continued beyond the 28-day timepoint, significance would have emerged. In order to qualify the results generated, and to assess the distribution of deposited mineral, histological analysis of the tri-layered scaffold was carried out. Alizarin-Red staining demonstrated that in all media types calcium deposition was confined to the bone.
layer of the scaffold with no positive mineral staining observed in the cartilage layer (Figure 2.15). Sulphated GAG quantification also demonstrated an increase in sulphated GAG content over time, with a trend emerging toward highest production in both the chondrogenic media and the osteochondrogenic media (Figure 2.16). Toludine blue staining of the tri-layered scaffold confirms cartilage matrix formation predominantly within the cartilage layer (Figure 2.17).

![Graph of calcium deposition in the tri-layered scaffold over time.](image)

*Figure 2.14: Graph of calcium deposition in the tri-layered scaffold over time. There is a significant difference in the amount of calcium laid down between day 0 and day 28 in the osteogenic media (green) (p=<0.001) and significant difference between the osteogenic media and all other media types (* = p<0.05) at day 28. There was also a trend toward significance in the osteochondrogenic media (blue) and compared to those cultured in chondrogenic media (yellow).*

![Alizarin Red staining for calcium of representative samples of the scaffolds at day 28 as cultured in](image)

*Figure 2.15: Alizarin Red staining for calcium of representative samples of the scaffolds at day 28 as cultured in (A) osteogenic media (B) chondrogenic media (C) osteochondrogenic media. These images show the calcium deposition in the bony layer of the scaffold with a protected tidemark and very little calcium deposition in the cartilage layers. (Black = bone layer, green = intermediate layer, yellow = cartilage layer)*
Figure 2.16: Graph of sGAG deposition over time in culture of the tri-layered scaffold over 28 days in various media types. Although there was no significant difference in deposition, it is clear that a trend is emerging. The chondrogenic and osteochondrogenic media depositing the most sGAG with the osteogenic media maintaining a consistently low level.

Figure 2.17: Toludine Blue staining of representative samples of the scaffolds at day 28 as cultured in (A) osteogenic media (B) chondrogenic media (C) osteochondrogenic media. (cartilage layer = yellow green = intermediate layer, black = bone layer)
2.5 Discussion
The chondrogenic and osteogenic properties are important considerations in scaffolds developed for osteochondral defect repair. This study aimed to examine the intrinsic properties of the tri-layered scaffold developed in our group to direct stem cell differentiation in each layer in order to successfully achieve osteochondral defect repair. The results show that the tri-layered scaffold has the capability to direct the differentiation of MSCs down an osteogenic route leading to bone formation in the bone layer and down a chondrogenic route leading to cartilage formation in the uppermost cartilage layer with a clear tidemark between. The results also demonstrate that the scaffold is capable of avoiding contraction during in vitro culture – an important consideration for in vivo implantation.

Quantification of mineral deposition within the bone layer of the tri-layer scaffold demonstrates the scaffolds ability to support osteogenesis and to direct the osteogenic differentiation of MSCs. MSCs alone in a plastic plate given standard growth media are known to continue to culture MSCs until a differentiation stimulus is applied. Therefore, the scaffold was used in both arms of this experiment with the only difference being in the medium. The results show a 2.48-fold increase in the mineral deposition within the scaffold when cultured in osteogenic media, but they also show a 1.74-fold increase in the mineral deposition in standard growth media without any exogenous osteogenic growth factors. This demonstrates that the bone layer scaffold has the ability to direct MSCs down an osteogenic lineage without exogenous growth factors proving the osteoinductive as well as osteoconductive properties of the scaffold. These osteoinductive properties (Wang et al., 2012, Mishra and Kumar, 2014, Chan et al., 2010) are a direct result of the presence of HA in the scaffold, which has been shown previously to have osteoinductive properties (Gleeson et al., 2010, Lyons et al., 2010), and the stiffness of the scaffold, which is a factor previously shown to influence the differentiation of MSCs (Engler et al., 2006).

The addition of the ceramic (HA) phase to the biodegradable polymer phase has led to the production of a composite structure which possesses all the prerequisite biological, morphological and mechanical characteristics necessary to facilitate the body’s own natural bone regenerative process in vivo. The bone layer of this tri-
layered scaffold is fabricated in the same way, and using the same components, as HydroxyColl™ (SurgaColl Technologies, Dublin, Ireland), a bone graft substitute material designed to be used off-the-shelf to fill bony defects. HydroxyColl™ was brought to market by an RCSI spinout company and is currently in clinical use. This scaffold has shown success in vivo as a cell-free scaffold in rat calvarial defects (Lyons et al., 2010, Lyons et al., 2014), in rabbit radial defects (Lyons et al., 2014, Murphy et al., 2014) and in an equine clinical case (David et al., 2015). This in vitro study provides further evidence for these positive in vivo results by demonstrating both the osteoconductive and osteoinductive properties of this scaffold.

The intermediate layer consisting of type I collagen and hyaluronic acid was shown to support stem-cell mediated chondrogenesis when cultured in the chondrogenic media, as evident by the highly significant increase in the amount of sGAG over the period of the study. There was also an increase in the sGAG when the scaffold was cultured, without any exogenous chondrogenic stimuli, in standard growth media. This finding is likely due to the hyaluronic acid content of the scaffold (Amann et al., 2017). The intermediate layer models the calcified cartilage layer of the native joint and is a protective tidemark against the vascularisation and encroachment of the subchondral bone into the cartilage layer. This encroachment causes thinning of the overlying cartilage and can even exacerbate the initial morbidity. It is often seen in repair techniques, such as microfracture, and in scaffold-free repair (Orth et al., 2012a, Qiu et al., 2003). Therefore, one of the most important findings relating to the intermediate layer was that a significant increase in mineral deposition in standard growth media was not demonstrated. The Alizarin Red staining of the tri-layered scaffold above (Figure 2.15) has shown no mineralisation advancing beyond the tidemark and into the cartilage layer over the course of the study. It can therefore be surmised that when implanted in the joint and exposed to the stimulating differentiation factors, the intermediate layer will resist mineralisation and protect the overlying cartilage from hypertrophy and vascularisation which would lead to ossification and thinning of the gliding surface (Levingstone et al., 2016a, Minas et al., 2009).
Chondrogenesis was observed within the cartilage layer when it was exposed to the chondrogenic factors, such as TGF-β, by the demonstrated increase in the levels of sGAG. Chondrogenesis was also observed in standard growth media without exogenous chondrogenic supplements, albeit to a lesser extent. This does indicate that the scaffold has some intrinsic ability to guide differentiation of MSCs in a chondrogenic route. Matrix produced within the scaffold was shown, using immunohistochemistry, to contain collagen type II consistent with hyaline cartilage. Hyaline cartilage repair is preferable to fibrocartilage repair, found with bone marrow stimulating repair techniques, as fibrocartilage is a mechanically inferior scar tissue unable to deal with the shear stresses and strains of the articulation (Kreuz et al., 2006, Furukawa et al., 1980, Nukavarapu and Dorcemus, 2013). The increasing collagen II observed within the cartilage layer scaffold is consistent with the increased sGAG and stimulation of matrix production seen in other scaffolds fabricated using type II collagen (Rutgers et al., 2013, Tamaddon et al., 2017) and in scaffolds fabricated with hyaluronic acid (Amann et al., 2017). Therefore, it follows that the presence of both type II collagen and hyaluronic acid in this scaffold are important factors in directing stem cell differentiation down the chondrogenic route allowing the surface layer of the tri-layered scaffold to produce hyaline cartilage when it is implanted in vivo.

While significant levels of contraction were observed in the individual layer scaffolds, the tri-layered scaffold when seeded with MSCs does not undergo significant contraction over time. A common problem is that, as the cells proliferate and form strong cell-cell bonds, cell mediated contraction of the entire scaffold can occur. If excess scaffold contraction occurs, gaps left between the implanted scaffold and the defect rim will lead to difficulty in integration between the scaffold and surrounding tissues and a persistent defect. Due to the crosslinking of the scaffold using an EDAC crosslinking protocol previously developed within TERG (Haugh et al., 2011) and the interfacial adhesion strength (Levingstone et al., 2014) no significant contraction was seen when examining the tri-layered scaffold. Therefore, at the time of surgery the scaffold can be implanted in a press-fit technique without risk of cell-mediated contraction. The press-fit technique utilised for scaffold implantation is a
straightforward technique and does not require the use of glues or sutures that may impact on the repair, complicate the surgery, and increase both the surgical time and the learning curve for surgeons.

Investigation of a suitable media type for *in vitro* culture of osteochondral scaffolds demonstrated that a combination media containing both chondrogenic and osteogenic supplementation, i.e. osteochondrogenic media, enabled chondrogenic and osteogenic differentiation of rat MSCs within the relevant regions of the tri-layered scaffold. The results show that the tri-layered scaffold, when cultured as a whole in an osteochondrogenic media, has the ability to cause an increase in sGAG in the cartilage layer over the tri-layered scaffold cultured in the standard growth media and a simultaneous increase in the mineralisation in the calcium layer. This is important in advancing research in osteochondral defect repair tissue engineering, as a common difficulty faced is the gradient nature of the tissue. This gradient nature requires different lineages of cells to differentiate from the initially seeded cells and also from the MSCs infiltrating from the subchondral bone marrow dependant on their position in the defect. Attempting to recreate an environment *in vitro* to replicate the gradient nature of the osteochondral defect is a challenge that has not been explored greatly in the literature. There have been some attempts at a minimum common differentiate media (Li et al., 2009) mixed media (Dorcemus et al., 2017) or using custom wells with a membrane around the layers of scaffold separating the media types (Chen et al., 2016). The medium that was developed in this instance containing both osteogenic and chondrogenic supplements allowed the tri-layered scaffold to lay down calcium in the bone layer and cartilage in the cartilage layer while maintaining the tidemark in between. These findings reinforce the results of the experiments assessing the inductive properties of the scaffold and cell differentiation (Sections 2.4.1, - 2.4.3). As all layers were cultured together in similar media, dissimilar differentiation was reliant primarily on intrinsic scaffold factors. Strengths of this series of investigations are that each layer was able to produce the correct tissue type for its layer using only the scaffold as its stimulating factor and no other exogenous stimulation. Limitations of this study however are in its *in vitro* nature. Ideally, the cell seeded scaffold would be implanted subdermally or
intramuscularly in an *in vivo* model and allowed to form tissue in an area distal to the joint. Presence of bone or cartilage on retrieval would further confirm the osteoinductive nature of the bone layer or the chondrogenic nature of the cartilage layer. The size of the scaffold and its rate of contraction will also depend on the surrounding tissues and the mechanical forces acting upon it in the *in vivo* setting, these forces of both tension and compression are not present in the *in vitro* setting and their absence is a limitation of this kind of study.

Taken together, the above findings in this study explain the healing potential seen with the tri-layered scaffold and demonstrate that it has layer specific osteoinductive and chondrogenic properties due to the macromolecules present in each layer of the scaffold. Therefore, when used as an off-the-shelf cell-free treatment for osteochondral defect repair, the scaffold has the capability to direct the differentiation of infiltrating bone marrow mesenchymal stem cells. The results of this study explain the mechanisms by which this scaffold successfully repaired both the bony defect visible on micro computed tomography (µCT) and simultaneously the overlying cartilage surface at 12 months in an *in vivo* setting (Levingstone et al., 2016a, Levingstone et al., 2016b). It was also noted that levels of chondrogenesis observed within the tri-layered scaffold were lower than the levels of osteogenesis observed. This may indicate that more rapid tissue repair could be achieved through the incorporation of cells or growth factors within the cartilage layer of the scaffold. Additionally, this study developed a methodology for the *in vitro* culture of this osteochondral defect repair scaffold, through the use of a combined osteochondrogenic media. This important finding can enable further development in osteochondral tissue engineering research in the future.

### 2.6 Conclusions

The intrinsic properties of the tri-layered scaffold were shown to direct stem cell differentiation as desired in each layer. The bone layer was found have both osteoconductive and osteoinductive properties and the cartilage layer was shown to have chondroconductive properties while the intermediate layer resisted hypertrophy and mineralisation, therefore protecting the tidemark between the
subchondral bone and overlying cartilage. The scaffold was also found to be dimensionally stable and allowed for the infiltration and proliferation of cells in each layer. Taken together, these results show that the tri-layered scaffold has the capability to direct stem cell differentiation in the appropriate layers and therefore has potential for regenerating hyaline cartilage at the joint surface and subchondral bone below. It was therefore taken forward to the next stage of development including in vivo testing in a large animal model (Chapter 4).

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3.1 Introduction

The findings from Chapter 2 of this thesis have demonstrated the intrinsic osteoinductive properties of the bone layer, and the chondrogenic properties of the cartilage layer of the tri-layered osteochondral scaffold. Therefore, this scaffold has potential as an off-the-shelf device, dependent on infiltration of cells from the surrounding tissues (Crawford et al., 2012, Murphy et al., 2010). Successful use of this cell-free scaffold in the repair of osteochondral defects has been demonstrated in vivo; however, further stimulus may be required in the case of very large defects. For example, pre-seeding a scaffold with cells prior to implantation could potentially lead to improved clinical outcomes.

This chapter aims to investigate the potential of incorporating cells into the cartilage layer of this scaffold to further enhance its regenerative capacity and potentially provide new treatment options for larger or more complex cartilage and osteochondral injuries. Cell-seeding approaches are used in existing procedures, such as MACI™ and second generation ACI (Bartlett et al., 2005, Benthien and Behrens, 2011). The benefit of using a cell-seeded method is that the desired cell type is established on the scaffold from the outset (Sabatino et al., 2015). As a result of this, the defect is more likely to heal with hyaline cartilage. Current bone marrow stimulating treatments rely on fibrocartilage (Gudas et al., 2006) - a mechanically inferior cartilage type. Pre-seeding the scaffold with cells also means that there is reduced lag time for the cells to infiltrate and proliferate on the scaffold, leading to a better tissue outcome and better patient recovery.

One of the major issues with cell-based osteochondral defect repair is the low density of the cells present within the native cartilage. Only 1.7% of the cartilage matrix is cellular, with a corresponding numerical density of roughly 1800 per mm$^3$. This suggests that over 98% of articular cartilage is occupied by the extracellular matrix (Hunziker, 1999, Matsiko et al., 2013). The low density indicates that implanting a large number of phenotypically identical cells (i.e. articular cartilage chondrocytes) would require harvesting cells from an expansive donor area or fewer cells from a small donor area. Fewer cells would require costly in vitro expansion prior to re-implantation in a second stage procedure (Samuelson and Brown, 2012, Brittberg et
In order to circumvent this issue, and harvest sufficient cells at the initial surgery for immediate scaffold seeding and re-implantation, a wide range of other cell types have been investigated in TE. As discussed in Chapter 1, these cells include nasoseptal chondrocytes (Kafienah et al., 2002), auricular chondrocytes (Kafienah et al., 2002, Vinatier et al., 2009), costal chondrocytes (Gelse et al., 2009), and MSCs derived from bone marrow (Leijten et al., 2013), adipose tissue (Jurgens et al., 2013), synovium (Ando et al., 2007, Koga et al., 2008) and peripheral blood (Fu et al., 2014b). Despite this research, the optimal cell source has yet to be established. Infrapatellar fat pad MSCs (FPMSCs) and bone marrow MSCs (BMMSCs) have shown particular potential for cartilage repair and are easily available in large numbers. Therefore, these cells will form the focus of this study (Mesallati et al., 2014, Mesallati et al., 2017, Almeida et al., 2015, Buckley et al., 2010, Liu et al., 2014, Liu et al., 2012, Vinardell et al., 2012b, Thorpe et al., 2010). Benefits of BMMSCs and FPMSCs include ready availability, in addition to a demonstrated immunomodulatory effect which can enhance tissue repair (Ryan et al., 2014). In particular, FPMSCs have been proven to be an abundant source of cells that are often harvested at diagnostic arthroscopy without causing morbidity to the patient (Doner and Noyes, 2014). FPMSCs have been shown to retain their chondrogenic potential, even in the diseased state. This is clinically important for patients who may present late with osteochondral defects that already show evidence of osteoarthritic changes (Liu et al., 2014). BMMSCs are commonly used in defect repair. They can be accessed by drilling into the subchondral bone to stimulate BMMSC release, such as during microfracture. When larger numbers are required, BMMSCs are accessed from a secondary location, such as the iliac crest. This second surgical site, however, can add a further risk of infection and elongate the operation (Steadman et al., 2003). Articular chondrocytes have previously been shown to gradually secrete a wide variety of protein molecules to provide a chondrogenic environment for adipose derived stem cells in co-culture (Lv et al., 2012, Bian et al., 2011). Co-culture of MSCs with primary CC therefore has potential to achieve successful cartilage repair. Work done in TCD and elsewhere (Mesallati et al., 2017, Bian et al., 2011) has shown that a close ratio of FPMSCs and CC leads to enhanced chondrogenesis over wider spaced ratios.
Identification of a source of cells that could be harvested and seeded directly onto the scaffold in a ‘one-stage’ surgical procedure, (i.e. an approach that can be carried out within one procedure or at least within one day), without any *in vitro* expansion, would reduce significantly reduce the cost of osteochondral defect repair (Samuelson and Brown, 2012). Additionally, the cost to the patient in terms of morbidity and recovery time would be reduced. A rapid isolation technique has been developed in TCD based on the adherence of cells to plastic with this in mind (Ahearne et al., 2014). Traditional isolation methods take far longer with steps requiring 6-8 hours of digestion time in collagenase along with multiple steps for washing and centrifugation to obtain the cells required. (Lopez-Ruiz et al., 2013, Goldring, 2005, Ali and Alman, 2012).

### 3.2 Aims

The overall aim of this chapter is to identify the optimal chondrogenic cell source for use with the tri-layered scaffold in order to enhance its regenerative capacity in a one-stage procedure.

The specific aims of this chapter are:

1. To assess the harvesting and isolation protocols of caprine articular chondrocytes, BMMSCs and FPMSCs and determine suitability of a rapid isolation technique.
2. To assess the tri-potentiality of the isolated MSCs and their suitability for use as a further regenerative stimulus on the tri-layered scaffold.
3. To determine the influence of cell seeding density on sulphated GAG production within the cartilage layer of the tri-layered scaffold.
4. To compare CCs, FPMSCs and a co-culture of FPMSCs:CC on the scaffolds in order to determine the optimal cell source for cartilage repair.
3.3 Methods

3.3.1 Harvesting of Chondrocytes, FPMSCs and BMMSCs from the goat leg

Euthanasia of a skeletally mature male goat was performed at a licensed abattoir, and chondrocytes, FPMSCs and BMMSCs were harvested in Trinity Centre for Bioengineering (TCBE) under Ethics licence approved by the Animal Research Ethics Subcommittee (AREC-P-12-71-Brama). Both hind legs were disarticulated at the level of the hip, without exposing the bone marrow. The flayed leg was then brought into the open sterile hood. The stifle joint was carefully opened, and the infra patellar fat pad removed in its entirety, placed in Phosphate Buffered Saline (PBS; Sigma-Aldrich, Ireland) and stored at 4 °C. The knee joint was then disarticulated and the cartilage surface exposed. The cartilage was scraped off the distal femur with a 6 mm punch biopsy and the cartilage strips placed in PBS with antibiotic supplementation (100 U/ml penicillin G and 100 μg/ml streptomycin sulphate; Gibco and 50 μl of Amphotericin B). Once the entire cartilage surface was removed the distal femur was opened and the bone marrow was removed using a sterile spatula and placed directly into a 50 ml falcon tube, 5 ml/tube with 10 ml of pre-warmed expansion medium [Gibco DMEM + GlutaMAX (61965, Gibco) + 10 % FBS (Foetal Bovine Serum, (Labtech, UK) + 10 ml (100 U/ml penicillin G and 100 μg/ml streptomycin sulphate)].

3.3.2 Isolation of the chondrocytes

The chondrocytes were isolated from the harvested cartilage tissue using a novel rapid-isolation technique protocol optimised in TCD using collagenase and plastic adherence (Almeida et al., 2014, Almeida et al., 2015, Ahearne et al., 2014). The cartilage pieces were removed from the antibiotic supplemented PBS and the cartilage was then sliced to pieces less than 1 mm in size, prior to rinsing again with antibiotic supplemented PBS and placing in sterile 50 ml tubes. Collagenase (Worthington, LS004176 Collagenase Type CLS-2) was dissolved in media [Gibco DMEM + GlutaMAX (61965, Gibco)] (350 U/ml) (8 ml/g cartilage), and sterile filtered prior to adding to the 50 ml tubes. These tubes were then placed in a tube rotator (Stuart, UK) and rotated in the collagenase solution (2 hours, 37 °C). The tissue was then passed through a 40 μm cell strainer into a new sterile 50 ml tube. Collected
cartilage particles were then crushed using a pipette tip and added to 8ml/g collagenase solution rotating for a further one hour (37 °C). The sieved media was added to stopping medium [Gibco DMEM + GlutaMAX (61965, Gibco) + 10 % FBS (Foetal Bovine Serum, (Labtech, UK))] of equal volume and mixed. The crushed cartilage was then removed from the rotator and stopping medium of equal volume added and mixed. This was again strained in a 40 μm cell strainer and centrifuged at 650 g for 10 minutes. The supernatant was discarded, and the pellet re-suspended in standard expansion medium [Gibco DMEM + GlutaMAX (61965, Gibco) + 10% FBS (Foetal Bovine Serum, (Labtech, UK) + 10 ml (100 U/ml penicillin G and 100 μg/ml streptomycin sulphate)] and counted using a trypan blue exclusion test. This is carried out by taking equal volumes of cell suspension (10 μl) and trypan blue (10 μl) (Sigma-Aldrich, USA), mixing them in an eppendorf tube and then taking 11 μl of the mixture to count on the haemocytometer. The chondrocytes were then plated into T175 flasks at a density of 875x10^3 cells/ flask and expanded to 90 % confluency.

3.3.3 Isolation of the FPMSCs
The FPMSCs were isolated also using collagenase as per previously published protocols (Almeida et al., 2014, Almeida et al., 2015, Ahearne et al., 2014). The fat pad digestion was also carried out using collagenase dissolved in media (750 U/ml, 4 ml/gram of tissue). As previously, the tissue was minced and placed into sterile 50 ml falcon tubes. 4 ml of collagenase solution was added per gram of tissue ensuring that the tubes were only 50 % filled to allow thorough mixing. The tubes were rotated in a tube rotator at 37 °C for 3-4 hours. Two volumes of stopping medium was added to the tissue collagenase mixture. The solution was then passed through a sieve (150 μm) into fresh sterile tubes. Once sieved the tubes were placed into the centrifuge for 10 minutes at 650 g. The floating fat fraction containing the adipocytes was aspirated off and discarded. The supernatant was also discarded, and the pellet resuspended in fresh standard expansion media. The new suspension was then passed through a 40 μm strainer into a fresh tube. Further media was added, and the suspension centrifuged once more at 650 g for 5 minutes. The supernatant was again discarded and the pellet resuspended. The pellet was counted using a trypan blue exclusion stain as described above. FPMSCs were then seeded onto
colony forming unit fibroblast (CFU-F) plates at a density of 25 cells/cm² and the remaining cells were seeded into T175 plates at a density of 1x10⁶ cells per flask. The CFU-F plates were then incubated and analysed after 10 days. At 10 days, the media was removed, and the plates were washed twice with PBS. The cells were then fixed in a 2 % paraformaldehyde solution and stained with crystal violet (Sigma-Aldrich, USA). The plates were then examined the colonies formed counted.

### 3.3.4 Isolation of the Bone Marrow MSCs
The BMMSCs were isolated as per the protocols developed by TCBE (Ahearne et al., 2014). Initially the media/marrow solution was titrated with a 16 G needle to break up any clumps, then the volume was made up to 40 ml using expansion media and mixed to form a homogenous solution. The tube then underwent centrifugation twice at 650 g for 5 minutes, with the supernatant removed and discarded each time. The cell pellet was then resuspended in 10 ml and once again triturated using a 16 G needle. The suspension was then sieved into a fresh Falcon tube using a 40 μm nylon mesh and then topped up to 20 ml. In order to separate out the mononuclear cells a separating agent was used. 20 ml of Lymphoprep™ (Axis-Shield, Norway) was placed in a 50 ml falcon tube and the 20 ml suspension added gently on top. The solution then underwent centrifugation for 20 mins at 650 g with deceleration set to 0 and acceleration set to 1. When the centrifugation was complete the mononuclear cell layer resided at the interface of the lymphoprep and the cell suspension. This was removed using a Pasteur pipette. A cell count was performed using 6% acetic acid and trypan blue as described above. CFU-F plates were seeded for BMMSCs at a density of 2.5x10⁶ cells/10 cm (Figure 3.1). The remaining cells were seeded into T175 flasks at a density of 20x10⁶ cells/ T175 flask. The CFU-F plates were incubated and analysed after 10 days. At 10 days, the media was removed, and the plates were washed twice with PBS. The cells were then fixed in a 2 % paraformaldehyde solution and stained with crystal violet (Sigma-Aldrich, USA). The plates were then examined as before and the colonies formed counted.
3.3.5 Tri-potentiality assessment of the isolated BMMSCs and FPMSCs

According to the International Society for Cellular Therapy (Dominici et al., 2006), the minimum criteria for defining multipotent mesenchymal stromal cells is that they must be plastic adherent, develop colony forming units and demonstrate tripotentiality. Tripotentiality is the ability to successfully differentiate into adipocytes, chondrocytes or osteocytes given the right conditions. Assessment of tripotentiality was performed on FPMSCs and BMMSCs. For this the cells were cultured in normal expansion media until confluent and divided into three groups per cell source. Each group was designated osteogenic, adipogenic or chondrogenic. The chondrogenic groups of cells were made up to a density of 0.5x10^6 cells per ml of expansion media. A 250,000 cell pellet was then created with centrifugation and the cells were cultured in pellet form in a chondrogenic media as per protocols from the TCBE. This media consisted of 10 % foetal bovine serum (FBS; Hyclone, Fisher Scientific, Ireland), 90 % DMEM + GlutaMAX (61965 Gibco) supplemented with antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulphate; Gibco) with 10 ng/ml of TGF-β3 (R&D Systems) Dexamethasone 0.4 μg/ml (Sigma), ascorbic acid 0.5 mg/ml (Sigma), linoleic acid 47 μg/ml (Sigma), and ITS 1X; (Sigma). The pellets were cultured with a media change every 2-3 days, and three samples of each were taken off at day 0 and day 7. These samples were then assessed for sulphated GAG levels using Blyscan Sulphated Glycosaminoglycan (sGAG) assay (Biocolour Ltd, UK) as previously described in Chapter 2.

The osteogenic potential of the FPMSCs and BMMSCs were assessed by culturing in osteogenic media. Each cell type was seeded at a density of 1x10^5 cell per well in 6 well plates (n=6) and then cultured in osteogenic media as developed in TCBE [10% foetal bovine serum (FBS; Hyclone, Fisher Scientific, Ireland), 90 % DMEM + GlutaMAX (61965 Gibco) supplemented with antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulphate; Gibco) dexamethasone 0.4 μg/ml (Sigma), β-glycerol phosphate 0.01M (Sigma) and ascorbic acid 6.9 mg/ml (Sigma)] opposite negative controls in standard expansion media for a total of 21 days. After the 21 days in culture all wells of the 6 well plate (i.e. three osteogenic media wells and
three standard media wells) were stained with alizarin red (Sigma, CAS No. 130 22 3) staining for calcium. The excess stain was removed from the plates and the plates examined. Results were binary based on whether the orange-red Alizarin Red S-calcium complexes were present or not.

Lastly, the analysis of the adipogenic ability was carried out. The cells were divided into two groups, the BMMSCs and the FPMSCs. Each cell type was seeded at a density of 1x10^5 cell per well in 6 well plates (n=6) and then cultured in adipogenic media [10% foetal bovine serum (FBS; Hyclone, Fisher Scientific, Ireland), 90 % DMEM + GlutaMAX (61965 Gibco) supplemented with antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulphate; Gibco) dexamethasone 20 μg/ml, (Sigma) 0.5 mM IBMX (Sigma) 50 μM indomethacin (Sigma)] opposite negative controls in standard expansion media for a total of 21 days. After 21 days in culture the plates were removed and imaged under an optical microscope (Nikon 90i, Nikon, Japan) and attached camera unit (Nikon DS Camera control unit, Nikon, Japan). In this instance, again the results were binary, in that the presence of vacuoles indicated adipogenesis.

3.3.6 To compare sGAG production in the cartilage layer using FPMSCs and a FPMSC:CC co-culture
To compare chondrogenesis between the cell types it was only necessary to use the cartilage layer (Col 1/Col 2) of the tri-layered scaffold, therefore this was manufactured alone as described in Chapter 2. The scaffolds were then cut into 7 mm diameter discs to mimic the size of the scaffold that would be used in the in vivo model. Scaffolds were sterilised using DHT sterilisation and EDAC crosslinked as outlined in Chapter 2. To determine the ideal cell type to seed onto the cartilage layer of the scaffold to augment cartilage regeneration, but without necessitating in vitro expansion of cells, the cartilage layer scaffold was seeded with a number of different cell groups and cell seeding densities as described in Table 3.1. Chondrocytes at two seeding densities were investigated, the low seeding density chondrocyte group (CCLD) was seeded at 125,000 cells per scaffold, and the high-density chondrocyte group (CCHD) seeded at 500,000 cells per scaffold. The low-density group (CCLD) was
selected to mimic a clinical situation by providing a relevant number of chondrocytes to that available clinically from a subcritical size defect, likely to self-heal. The high-density group (CCHD) was designed to mimic the use of expanded cells as currently used in ACI and MACI. FPMSCs were also seeded on the scaffold at a low seeding density (FPMSCs LD) and a high seeding density (FPMSCs HD). The final group consisted of a co-culture of FPMSCs and chondrocytes at a ratio of 3:1 of FPMSC to CC and seeding density of 500,000 cells per scaffold (FPMSCs:CC). This group reflects the number of CC that can be harvested from a subcritical defect without expansion (125,000, i.e. the CC LD group), augmented with FPMSCs (Bian et al., 2011, Mesallati et al., 2017). BMMSCs were not taken forward to this stage of testing due to associated limitations of BMMSCs in a clinical situation. Harvesting BMMSCs requires either lengthening the wound and performing a tibial osteotomy to harvest cells (Brittberg et al., 1994), or harvesting them from a second surgical site at a different location, i.e. the iliac crest. The reason for the development of a one-stage procedure is to reduce the impact on the patient. In order to further reduce the potential morbidities, efforts must be made to limit the surgical insult to the patient. The use of FPMSCs which can be harvested through same arthroscopic incision as the CC limits the insult to only one surgical site. FPMSCs have additionally been shown to maintain their chondrogenic capacity in osteoarthritic donors (Liu et al., 2014), which is crucial in order to be able to treat those who present late with the defect. Therefore, only MSCs derived from the fat pad were taken forward.

Table 3.1: Cell types comparison groups and cell seeding density. Note that in the FPMSC:CC group, the number of chondrocytes used is equal to that in the CCLD group i.e. a clinically relevant number of chondrocytes

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Density</th>
<th>Abbreviation</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat Pad MSCs</td>
<td>Low Density</td>
<td>FPMSCs LD</td>
<td>125,000</td>
</tr>
<tr>
<td>Fat Pad MSCs</td>
<td>High Density</td>
<td>FPMSCs HD</td>
<td>500,000</td>
</tr>
<tr>
<td>Chondrocytes</td>
<td>Low density</td>
<td>CCLD</td>
<td>125,000</td>
</tr>
<tr>
<td>Chondrocytes</td>
<td>High density</td>
<td>CCHD</td>
<td>500,000</td>
</tr>
<tr>
<td>Fat Pad MSCs: Chondrocytes</td>
<td>High Density</td>
<td>FPMSCs:CC (375,000:125,000)</td>
<td>500,000</td>
</tr>
</tbody>
</table>
The scaffolds were cultured in standard growth media and allowed to attach to the scaffold for a period of 3 days. Seeded scaffolds were then cultured in chondrogenic media up to a total of 28 days, with feeding by partial media exchange every 2-3 days. Samples were removed from culture at 0, 14, 21 and 28 days. 4 scaffolds from each group were flash frozen in liquid nitrogen and stored at -80°C until ready to be analysed. Once all timepoints were available the scaffolds underwent papain digestion and a sGAG assay was performed using the Blyscan sGAG assay kit (Biocolour Ltd, UK), as described in Chapter 2.

3.3.7 Histological analysis of the cartilage layer scaffolds
At each time point one scaffold was taken off and fixed in 10% formalin for 1 hour for histological analysis. Scaffolds fixed in formalin were then processed in an automatic tissue processor (ASP300, Leica, Wetzlar, Germany). Constructs were embedded in paraffin wax and sectioned at a thickness of 10µm using a rotary microtome (RM2255, Leica microtome, Leica). Sections were stained with haematoxylin & eosin staining (H&E) to assess the cell proliferation and infiltration, (haematoxylin stains the DNA and RNA rich cell nuclei purple, eosin stains the extracellular matrix pink). Sections were also stained with safranin-O to evaluate the cartilage generation (safranin-O stains sGAG red). Digital images were obtained using a microscope (Nikon 90i, Nikon, Japan) and attached camera unit (Nikon DS Camera control unit, Nikon, Japan) with NIS, Elements software (Nikon Instruments Europe, The Netherlands).

3.3.8 Statistical analysis
All statistical analyses were performed using statistics software Prism 7 GraphPad (GraphPad Software, California USA). For analysis of two variables a paired student’s t-test was used and where three variables were present a repeated measures analysis of variance (ANOVA) test was performed.
3.4 Results

3.4.1 Tripotentiality assessment of harvested FPMSCs and BMMSCs

Assessment of the multipotency of the BMMSCs and FPMSCs demonstrated that they were plastic adherent and had the ability to generate colony forming units (CFU) when initially plated out onto 10 cm$^2$ plates, as shown in Figure 3.1.

Culture of the FPMSCs and BMMSCs in chondrogenic media was shown to have an increase in sGAG deposition over time as seen in Figure 3.2. Although less chondrogenesis is visible in the BMMSCs than the FPMSCs, it is clear that there is an increase over the course of the study in both groups. This increase in sGAG during the study demonstrates that the harvested cells are MSCs with chondrogenic potential.
Alizarin Red staining of the BMMSCs and the FPMSCs that had been cultured with osteogenic media versus standard growth media were examined (Figure 3.3). The chelation of the orange-red Alizarin Red S-calcium complexes seen in the wells cultured in osteogenic media shows the osteogenic potential of MSCs originating from the fat pad and from the bone marrow.

Figure 3.3: Alizarin Red stained 6 well plates after 21 days in culture. Plate A is seeded with BMMSCs and plate B is seeded with FPMSCs. The upper row wells of both A and B are cultured in osteogenic media for a duration of 21 days. The lower row wells of both A and B are cultured in standard growth media. The difference in the uptake of the stain can be seen clearly in the upper plates over the lower plates showing increased calcium deposition and mineralisation of MSCs cultured in osteogenic media, this shows the osteogenic ability of the cells.

Both the FPMSCs and the BMMSCs cultured in adipogenic medium were visualised under a stereomicroscope and compared to those cultured in standard medium. The
presence of vacuoles is consistent with adipogenesis of the MSCs (Figure 3.4). From the assessments of plastic adherence, colony formation chondrogenesis, osteogenesis and adipogenesis in the fat pad and bone marrow cells harvested from the goat leg, it has been demonstrated that this method of harvesting and isolating cells results in the isolation of MSCs from both the fat pad (FPMSCs) and from the bone marrow (BMMSCs).
Figure 3.4: BMMSCs and FPMSCs cultured for a total of 21 days. The upper rows A and C are cultured in adipogenic media and the lower rows, B and D, in standard growth media to visualise the adipogenic potential of the harvested cells. The presence of vacuoles in the groups cultured in adipogenic media (A, C) demonstrate the adipogenic potential of the harvested cells.
3.4.2 Assessment of the effect of cell type and cell seeding density on chondrogenesis

The optimal cell type and cell seeding density were then investigated by quantifying sGAG deposition within the scaffold. Comparing seeding density for each cell type demonstrated that higher levels of sGAG deposition were observed in the scaffolds of the high seeding density groups than the low seeding density groups (Figure 3.5). There was no significance found between the FPMSCs LD and FPMSCs HD at any of the four timepoints. Significant differences were found between the CC LD and the CC HD only by day 28 (p= <0.05 CI -23.95 to -0.5011). Significantly higher levels of sGAG were found between the FPMSC:CC group and the CC LD group. At day 28 significance was p=<0.001 (CI -30.30 to -6.851).

Current cell based techniques from chondral defect repair such as ACI utilise expanded chondrocytes in high numbers represented by the CC HD group; importantly, the results here show that to bypass the expansion phase and use only the small numbers of freshly harvested CC available leads to significantly poorer results (CC LD group). However, the addition of FPMSCs to the CC LD group in the form of the FPMSC:CC group led to a 7.8-fold increase in sGAG over the CC LD group and a 1.4-fold increase in sGAG over the CC HD group.
3.4.3 Histological analysis of the cartilage layer scaffolds

The H&E staining (Figure 3.6) shows that there is far less cell proliferation in the low-density scaffolds at day 28 than in high density groups at this timepoint. The Safranin-O staining (Figure 3.7) confirm the findings of the quantitative analysis with increased uptake of red/purple stain demonstrating higher levels of cartilage generation in the FPMSC:CC co-culture group over either the CC HD or the FPMSC HD groups.
Figure 3.6: H&E staining of representative samples from Day 14, 21 and 28 of each of the different cell groups A= FPMSCs LD, B= FPMSCs HD, C = CC LD, D= CC HD and E= FPMSCs:CC. (Scale = 500μm) Black arrows = areas of high proliferation particularly visible in images B, D and E of the day 28 group showing that there is increased cell proliferation with the increased initial seeding density. Green arrow = small area of proliferation in the FPMSC LD group, which is not as widespread as the high-density group. No similar areas of proliferation are visible in group C the CC LD group.
Figure 3.7: Safranin-O staining of representative samples of scaffolds seeded with the different cell groups at Day 14, day 21 and day 28. A= FPMSCs LD, B= FPMSCs HD, C = CC LD, D= CC HD and E= FPMSCs:CC. The black arrows at day 28 demonstrate areas (purple/red) with increased levels of sGAG deposition. These are most visible especially in the high-density groups (i.e. B, D and E) but particularly in group E, the FPMSC:CC group. There are no comparable areas of sGAG deposition in the low-density groups, either A, FPMSC LD or C CC LD. (Scale = 500µm)
3.5 Discussion

The tri-layered osteochondral defect repair scaffold under investigation in this thesis has shown promise as an off-the-shelf device for the repair focal osteochondral defects in rabbit and goat models (Levingstone et al., 2016a, Levingstone et al., 2016b). Chapter 2 of this thesis demonstrated the inherent osteogenic and chondrogenic properties of this scaffold which contributed to the positive in vivo results. However, the treatment of larger osteochondral defects might require the use of added stimulation such as pre-seeding the scaffolds with cells. The overall goal of this study was to examine FPMSCs and chondrocytes and determine the optimal cell type and cell seeding density required to use in a one-stage procedure without in vitro expansion of cells. The study involved initially harvesting and isolating CC, FPMSCs and BMMSCs using rapid isolation techniques. Chondrogenic potential was then assessed by quantifying sGAG production within the cartilage layer scaffold seeded with either low density CC and FPMSCs, high density CC or FPMSCs, or when seeded with a FPMSC:CC co-culture group. It was demonstrated that cells harvested using rapid isolation methods from the infrapatellar fat pad of a goat knee joint and from bone marrow from the goat femur demonstrated the properties required to achieve cartilage repair. It was also demonstrated that higher cell seeding densities resulted in increased sGAG production, and it was determined that the optimal cell seeding regime is a co-culture of FPMSCs and chondrocytes.

Bone marrow, fat pad and articular cartilage were harvested from a goat leg, and the rapid isolation technique was successfully used to isolate bone marrow mesenchymal stem cells, and fat pad mesenchymal stem cells from the femur, and infrapatellar fat pad respectively of the knee joint of goats. The MSCs isolated demonstrated tripotentiality in terms of chondrogenesis, osteogenesis and adipogenesis (Dominici et al., 2006). This rapid isolation technique thus shows the potential for use within a ‘one stage’ surgical procedure for cell-based cartilage repair. Currently patients require two operations, with a gap of roughly six weeks in order to harvest and expand the chondrocytes in vitro prior to re-implantation (Brittberg et al., 1994, Brittberg and Winalski, 2003, Peterson et al., 2010). This means that the patient has to rehabilitate twice and is out of work far longer, as well as the increased financial
costs of the surgery and the \textit{in vitro} expansion (Samuelson and Brown, 2012). Rapid isolation and immediate re-implantation of cells seeded onto the scaffold would reduce the procedure down to a single operation, and allow the patient a single recovery time, as well as remove the expensive \textit{in vitro} aspect of the procedure. While the ability to harvest and isolate BMMSCs from the goat femur was demonstrated in this study, BMMSCs were not taken further. This is due to associated limitations of BMMSCs in a clinical situation. Harvesting BMMSCs requires either lengthening the wound and performing an osteotomy to harvest cells from the proximal tibia (Brittberg et al., 1994), or harvesting them from a second surgical site at a different location, i.e. the iliac crest. The reason for the development of a one-stage procedure is to reduce the impact on the patient. In order to further reduce the potential morbidities, efforts must be made to limit the surgical insult to the patient. The use of FPMSCs which can be harvested through same arthroscopic incision as the CC limits the insult to only one surgical site. FPMSCs have additionally been shown to maintain their chondrogenic capacity in osteoarthritic donors (Liu et al., 2014), which is crucial in order to be able to treat those who present late with the defect. Therefore, only MSCs derived from the fat pad were taken forward.

When examining the cartilage formation, the high seeding density groups demonstrated increased cartilage development compared to the low seeding density groups (Huang et al., 2016, Foldager et al., 2012, Talukdar et al., 2011). This, therefore, reinforces the point that, for a one-stage procedure, sufficient cells must be harvested to adequately seed the scaffold (Ahearne et al., 2014, Almeida et al., 2015). Overall, at both high and low seeding densities CC resulted in higher sGAG production than FPMSCs, which is consistent with both their phenotype, and previous cell based cartilage defect repair mechanisms (ACI/MACI) which are based on \textit{in vitro} expanded chondrocytes (Brittberg et al., 1994). However, high levels of sGAG were also seen in both FPMSC LD and FPMSC HD groups, showing their potential for use in cartilage defect repair. The highest levels of chondrogenesis was observed in the co-culture group, where FPMSCs and CC were combined in a 3:1 ratio. (Bian et al., 2011, Mesallati et al., 2017). The FPMSC:CC group investigated here uses the same overall number of cells as both the CC HD and the FPMSC HD groups.
This important point shows that there is a synergistic effect of the co-culture, whereby the FPMSCs and the CC together can achieve a higher level of sGAG production than either can separately. It is also important to note that the number of CC used in the FPMSC:CC group is the same as the clinically relevant number used in the CC LD group. This shows that, avoiding the in vitro expansion phase by adding FPMSCs to a relatively small number of chondrocytes, can lead to a 7.8-fold increase in the sGAG produced over just the chondrocytes alone. The fact that the sGAG production can be optimised by avoiding the in vitro expansion phase of the cartilage repair procedure using the novel rapid isolation techniques developed in TCD for the isolation of the FPMSCs means that this system is appropriate to move toward a one-stage procedure.

3.6 Conclusion
The overall aim of this chapter was to determine the optimal cell type for seeding onto the tri-layered scaffold prior to implantation in order to augment osteochondral defect repair in a one-stage surgical technique. The rapid isolation technique was found to successfully isolate BMMSCs and FPMSCs. These cells were found to have the tripotential MSC characteristics and are thus suitable for use as a further regeneration stimulus within the tri-layered scaffold. It was demonstrated that greater levels of chondrogenesis occur with an increased seeding density of cells on the scaffold, regardless of cell type. The greatest level of chondrogenesis was in the FPMSC:CC co-culture group, proving that there is a synergistic effect between these two cell types. We conclude therefore that FPMSC:CC co-culture is a viable method to maximise cartilage production in the single-stage procedure setting without compromising the density of the seeded cells. With the ultimate aim of using a cell-seeded tri-layered scaffold in osteochondral defect, it has been shown here that the co-culture model of FPMSCs and chondrocytes is an appropriate model to bring forward to in vivo trials.
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<td>Conor Moran and Tanya Levingstone</td>
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<td>Tripotentiality assessment</td>
<td>Conor Moran</td>
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<td>Scaffold production</td>
<td>Conor Moran</td>
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<td>Cell expansion, seeding, culture and analysis</td>
<td>Conor Moran</td>
</tr>
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<td>Histological slide preparation</td>
<td>Conor Moran</td>
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<td>Result analysis and interpretation</td>
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Chapter 4: The in vivo repair potential of a cell-free and a cell-seeded tri-layered scaffold in comparison to a commercially available product

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4.1 Introduction

The osteogenic and the chondrogenic properties of the different layers of the tri-layered scaffold, and their ability to direct the differentiation of MSCs have been examined in Chapter 2. Following this, the optimal chondrogenic cell source for use in enhancing the regenerative capacity of this scaffold was then explored in the results presented in Chapter 3. This chapter focuses on the assessment of both the cell-free and the cell-seeded scaffolds in vivo when implanted into defects created on the medial condyle and the trochlear ridge of a large animal model, in this case a goat stifle joint. The in vivo study also assessed the handleability of the scaffold and the ease of implantation during the surgery. Such studies have previously been done in a small animal (rabbit) model, which demonstrated improved levels of bone and cartilage repair over an empty defect (Levingstone et al., 2016b). Additionally, a small pilot large animal (goat) study was performed, comparing the cell-free tri-layered scaffold to an empty defect and the leading commercial comparator (Levingstone et al., 2016a).

Bringing forward the results from Chapter 3, the co-culture group, which consisted of a 3:1 ratio of fat pad mesenchymal stem cells (FPMSCs) and chondrocytes, resulted in the highest levels of chondrogenesis when seeded on the tri-layered scaffold. This was therefore selected for the cell-seeded tri-layered scaffold. The commercial comparator used in the original goat pilot study was the market leading comparator at the time, TruFit (Smith & Nephew, MA, USA). However, since completion of the pilot study there have been negative reports relating to the TruFit™ scaffold (Quarch et al., 2014, Gelber et al., 2014, Hindle et al., 2013, Dhollander et al., 2012). As a result of these findings it was decided to re-examine the available products to find the current market leading comparator for use in this study and these are summarised in Table 4.1.
Table 4.1: Characteristics of osteochondral defect repair scaffolds available at time of study design

<table>
<thead>
<tr>
<th>Name</th>
<th>Trilayered scaffold</th>
<th>TruFit CB™ Smith &amp; Nephew</th>
<th>Agili-C™ CartiHeal</th>
<th>MaioRegen™ Finceramica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of layers</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Bone Phase</td>
<td>Collagen Hydroxyapatite</td>
<td>Calcium Sulphate</td>
<td>Aragonite</td>
<td>70 % Hydroxyapatite 30 % type I collagen</td>
</tr>
<tr>
<td>Intermediate Phase</td>
<td>Collagen Hyaluronic Acid</td>
<td>-</td>
<td>-</td>
<td>40 % Hydroxyapatite 60 % type I collagen</td>
</tr>
<tr>
<td>Cartilage Phase</td>
<td>Type I Collagen, Type II Collagen, Hyaluronic Acid</td>
<td>Hydrophillic polymer (PGA, PLGA, surfactant)</td>
<td>Hyaluronate-impregnated aragonite</td>
<td>100% type I collagen</td>
</tr>
<tr>
<td>Plug Size</td>
<td>⌀ 6-12 mm</td>
<td>⌀ 5 – 11 mm</td>
<td>⌀ 6 – 18 mm</td>
<td>Manual sizing 35 x 35 mm 50 x 50 mm</td>
</tr>
<tr>
<td>Plug Depth</td>
<td>Max 12 mm</td>
<td>Max 18 mm</td>
<td>15 or 20 mm</td>
<td>6 mm</td>
</tr>
<tr>
<td>Porosity</td>
<td>&gt;90 %</td>
<td>70 %</td>
<td>?</td>
<td>&gt;60 %</td>
</tr>
<tr>
<td>Surgical Implantation</td>
<td>Delivery Device in development</td>
<td>Delivery Device supplied</td>
<td>Agili-Kit™ surgical toolset</td>
<td>No delivery device</td>
</tr>
</tbody>
</table>

Although Agili-C™ was commercially available within Europe, it was not possible to purchase it in Ireland at the time of setting up the study. Following assessment of the commercially available scaffolds for osteochondral defect repair, MaioRegen™ was selected as the most suitable market leading comparator. This scaffold is supplied sterile (25 kGy gamma rays) in sheets and is designed to be custom cut to size by the surgeon within the operating theatre. It consists of a bone layer of 70 % hydroxyapatite:30 % type I collagen, an intermediate layer of 40 % hydroxyapatite:60 % type I collagen and a cartilage layer of 100 % type I collagen.
4.2 Aims of this chapter
The overall aim of this chapter was to determine the regenerative capacity of the tri-layered scaffold, both alone and in combination with cells, in a critical sized defect in a long term large animal model.

The specific objectives were as follows:

1. To macroscopically assess regeneration and integration of cartilage in the articular surface at 6 months and 12 months in the cell-free and cell-seeded tri-layered scaffold groups compared to the commercial comparator group.

2. To assess subchondral bone repair achieved at 6 months and 12 months in the cell-free and cell-seeded scaffold groups compared to the commercial comparator groups.

3. To assess histologically, the capacity of the cell-free and cell-seeded tri-layered collagen scaffold to lead to regeneration of tissue within each region of the osteochondral defect i.e. superficial articular cartilage, intermediate calcified cartilage and deep subchondral bone in comparison to the commercial comparator group.
4.3 Materials and Methods

4.3.1 Scaffold preparation for the in vivo study
The tri-layered scaffold used in the in vivo study were prepared in SurgaColl’s GMP (Good Manufacturing Practices) manufacturing facility based in Dublin City University (DCU), using the iterative freeze-drying technique developed within TERG, as described in Chapter 2. This enabled bioburden levels in the scaffold to be minimised and thus the safety of the scaffolds for implantation in vivo to be ensured. Following fabrication, the scaffolds were cut to 7 mm diameter plugs, double packaged and sterilised using gamma irradiation using the using VD\textsubscript{max} 25 kGy method by Synergy Healthcare, Tullamore.

4.3.2 Cell isolation
As a step toward one step procedures, the research presented in this chapter used freshly isolated cells seeded directly onto the scaffold and re-implanted into the joint without ex vivo expansion. However, due to limitations of equipment and facilities being off site, it was necessary to use an allogenic cell-seeded scaffold construct rather than autogenous cells, i.e. cells harvested from a goat on day 1 were isolated overnight in an off-site facility (TCBE) and re-implanted to a second goat on day 2. Numerous studies have reported the use of allogenic chondral transplants in clinical practice (Giannini et al., 2017, Tschon et al., 2017). This is possible without risk of immunogenic response due to the immunoprivileged nature of articular cartilage and its avascular nature. After harvesting, the tissues were kept in sterile saline at 4°C prior to transfer to TCBE for isolation of FPMSCs and chondrocytes. FPMSCs were isolated using the procedure described in Chapter 3. The fat pad MSCs were also isolated using collagenase as per previously published protocols (Almeida et al., 2015, Almeida et al., 2014). The fat pad digestion also required collagenase dissolved in media (750 U/ml, 4 ml/gram of tissue). As in Chapter 3 the tissue was minced and placed into sterile falcon tubes with collagenase solution. The tubes were rotated at 37 °C for 3-4 hours. The solution was then passed through a sieve (150 µm) and placed into the centrifuge for 10 minutes at 650 g. The resulting pellet was
resuspended in fresh media and passed through a 40 µm strainer. Further media was added, and the suspension centrifuged once more at 650 g for 5 minutes. The pellet was resuspended, and the cells counted using a trypan blue exclusion test.

Chondrocytes were isolated from the cartilage tissue using a protocol optimised in TCD (Almeida et al., 2014, Almeida et al., 2015) and previously described in Chapter 3. Briefly, the cartilage was sliced to less than 1 mm at its thickest section. The cartilage was rotated in a collagenase solution (2 hours, 37 °C). The tissue was then passed through a 40 µm cell strainer. Collected cartilage particles were then crushed and added to 8 ml/g collagenase solution rotating for a further one hour (37 °C). The crushed cartilage was then removed from the rotator and stopping medium of equal volume added and mixed. This was again strained in a 40 µm cell strainer and centrifuged at 650 g for 10 minutes. The pellet was re-suspended in standard medium and cells counted using a trypan blue exclusion test. The chondrocytes are then ready to be used as primary cells. They were then stored in a falcon tube at 37 °C until ready to seed onto the scaffolds.

4.3.3 Cell seeding
Tubes containing the chondrocytes and the FPMSCs were placed in the centrifuge at 650 g for 5 minutes. The supernatant was removed, and the cells were resuspended at a 3:1 ratio of FPMSCs to chondrocytes in the required amount of media to give a cell seeding density of 500,000 cells/scaffold. Cells were seeded onto the cartilage surface of the tri-layered scaffolds using a dropwise technique. The seeded scaffolds were placed in petri dishes, doubly wrapped under sterile conditions, and transported out to UCD’s Lyons Research Facility in a heated transportation box. Upon arrival, they were placed in an incubator at 37 °C and 5 % CO₂.

4.3.4 Implantation of the scaffolds in the caprine stifle
In vivo assessment of the tri-layered scaffold was carried out within the surgical facilities on UCD’s Lyons Research Facility led by collaborator Prof. Pieter Brama (designated veterinary surgeon, UCD School of Veterinary Medicine). Ethics approval
was sought from and approved by the Animal Research Ethics Subcommittee (AREC-P-12-71-Brama). A total of 70 skeletally mature adult female goats (2-3 years old) were randomly divided into 6 and 12-month groups. A power analysis was carried out for this study based on the results of a previous pilot study in a similar goat model (Levingstone et al., 2016a) to determine the minimum number of goats that would be required for the purposes of the experiment, primary outcomes used were significant difference values in the micro CT imaging and were calculated using G*Power software (Faul et al., 2007) The individuals involved in the in vivo study attended the Laboratory Animal Science and Training (LAST) course and achieved the certificate required to perform an in vivo study. The handling portion of this training was completed on the farm with the goats, once they were acclimatising to the location, under the guidance of an accredited demonstrator and evaluator.

Pre-operative sedation was provided by an intravenous injection of 0.2-0.5 mg/kg Diazepam (Wockhardt UK Ltd, UK). General anaesthesia was achieved using Propofol (Aspen, Ireland) (2-6 mg/kg titrated to effect) lidocaine spray (Thornton & Ross Ltd., UK) (1 mg/kg) was used on the larynx prior to intubation. Maintenance was achieved with an isoflurane vaporiser (AbbVie Ltd., United Kingdom) at 2.5-3 % in 100% oxygen at a flow of 1-2 L/min. Intra-operative analgesia was maintained with an epidural of lidocaine (Concordia International, UK) (2 mg/kg) and bupivacaine (Concordia International, UK) (0.5-0.75 mg/kg). Intraoperatively the heart rate, blood pressure and end titre CO₂ were monitored by qualified veterinary anaesthetists. If hypotensive, compound sodium lactate (Baxter Healthcare, United Kingdom) was administered intravenously at 5-10 ml/kg/hr. Once the goat was under general anaesthetic it was moved to the operating theatre and placed in the dorsal recumbency position. There the area over the stifle joint was shaved and the skin was disinfected using chlorhexidine gluconate solution 4 % (w/v) (HiBiSCRUB, Regent Medical, Manchester, UK). The leg was then draped with disposable sterile drapes.

A mini-arthrotomy was performed on each hind leg. Briefly, the operation involved a longitudinal superficial incision lateral to the patellar ligament and extending upwards along the lateral border of the patella. The joint capsule was opened, and the patella dislocated medially. Approximately ¼ of fat pad present was harvested.
(roughly 1 g of tissue) at this point and immediately stored in sterile PBS (Sigma-Aldrich, Ireland) at 4°C. The medial condyle was exposed by flexion and internal rotation of the stifle joint. Two defects were then created on the chondral surface using a 6 mm biopsy punch. These defects were positioned on the apex of the medial femoral condyle (MFC) and on the lateral trochlear ridge (TR) (Figure 4.1). The cartilage was harvested from the defect site using a fresh scalpel blade and a sharp curette. The cartilage tissue was immediately placed into sterile PBS (Sigma-Aldrich, Ireland) and also stored at 4°C. Once the subchondral bone was exposed the defect was drilled initially using a standard 6mm drill bit in a custom-made drill guide with a 6 mm stop, and then using a flat-bottomed drill bit in a similar guide. This created 6 mm x 6 mm cylindrical flat-bottomed critically sized defects (Jackson et al., 2001). The joint was then washed out with normal sterile saline.

![Figure 4.1: 6mm x 6mm osteochondral defects created on the joint surface a) the lateral trochlear ridge (TR) b) the medial femoral condyle (MFC)](image)

Each joint was then assigned to one of three groups. 1) Cell-seeded tri-layered scaffold 2) Cell-free tri-layered scaffold 3) Commercial comparator (MaioRegen, Finceramica, Italy (Figure 4.2)) and an n of at least 7 was used for each group. To increase statistical power internal controls for each tri-layered scaffold group were required. For each animal one joint was assigned one group, and the contralateral joint was to a separate group.
Each tri-layered scaffold was implanted into the defect using the standard press-fit technique which is also the recommended technique in the manufacturer’s instructions for the MaioRegen product. The scaffold position was checked, and the surface was palpated to ensure it was flush with the native cartilage tissue (Koh et al., 2004). The patella was then relocated, and the capsule sutured closed using an appropriate suture material, 2-0 Vicryl (Ethicon, MA, USA). A layer of subcuticular sutures was also placed to ensure good closure followed by interrupted skin sutures. A stent or wound pad was then placed over the wound and sutured in situ to protect the healing wound from contamination and from interference by the goats themselves. The goats were then allowed to wake up while on the operating table and once they were sufficiently awake and self-ventilating they were moved to a heated recovery room where they were closely monitored until they were sufficiently
alert. The endotracheal tube was removed in the separate recovery room once the
goat was able to maintain its own airway. The animal was then allowed to ambulate
immediately and eat as per desire.

The goats were allowed to mobilise immediately post-operatively. They were kept in
a small (3m x 3m) high sided (2m) pen for the first 2-4 weeks to limit ambulation and
monitored closely. Each goat received a 5-day course of antibiotics intramuscularly
(8.75mg/kg Noroclav, Amoxicillin Trihydrate 140mg/ml and Potassium Clavulanate
35mg/ml) Norbrook UK) and non-steroidal anti-inflammatory analgesic (1.4mg/kg
Carprieve (Carprofen) Norbrook, UK) twice daily for the first three days and as
required thereafter based on gait and signs of discomfort. After 5 days, the stents
were removed, and the joints examined. After 14 days the skin sutures were
removed.

4.3.5 Macroscopic assessment of repair tissue
Tissue repair was assessed at timepoints of 6 and 12 months post-surgery. Euthanasia
was performed at Lyons Research Farm using an overdose of sodium pentobarbital
by qualified personnel. Following euthanasia, the goats were transported to UCD
Veterinary Hospital in order to harvest tissue for further evaluation. Upon opening
the joints, the defect sites were photographed and macroscopic scoring was carried
out using the ICRS (International Cartilage Repair Society) cartilage repair assessment
tool (Table 4.2) to evaluate the quality of the repair and integration of the scaffolds
into the surrounding tissues (Levingstone et al., 2016b). Assessors were blinded to
the treatment groups until after the macroscopic scoring had been completed.
Osteochondral segments containing the defect site were then resected and the
samples were fixed in 10% formalin prior to micro-CT and histological analysis.
Table 4.2: International Cartilage Repair Society (ICRS) cartilage repair assessment tool. This tool is used to evaluate the macroscopic appearance of cartilage repair tissue following interventions such as ACI, subchondral drilling and microfracture. (Levingstone et al., 2016b, Hoemann et al., 2011)

<table>
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<th>CRITERIA</th>
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<tr>
<td><strong>DEGREE OF DEFECT REPAIR</strong></td>
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<tr>
<td>Level with surrounding cartilage</td>
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</tr>
<tr>
<td>75% repair of defect depth</td>
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<td>50% repair of defect depth</td>
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<tr>
<td>0% repair of defect depth</td>
<td>0</td>
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<tr>
<td><strong>INTEGRATION TO BORDER ZONE</strong></td>
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<tr>
<td>Complete integration with surrounding cartilage</td>
<td>4</td>
</tr>
<tr>
<td>Demarcating border &lt;1mm</td>
<td>3</td>
</tr>
<tr>
<td>3/4 of graft integrated, 1/4 with a notable border &gt;1mm width</td>
<td>2</td>
</tr>
<tr>
<td>1/2 of graft integrated with surrounding cartilage, 1/2 with a notable border &gt;1mm</td>
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</tr>
<tr>
<td>From no contact to 1/4 of graft integrated with surrounding cartilage</td>
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<tr>
<td><strong>MACROSCOPIC APPEARANCE</strong></td>
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<td>Intact smooth surface</td>
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</tr>
<tr>
<td>Fibrillated surface</td>
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<tr>
<td>Small, scattered fissures or cracks</td>
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<td>Grade IV severely abnormal</td>
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4.3.6 Micro computed tomography
Micro computed tomography (µCT) was performed on the 10 % formalin fixed samples using a Scanco UCT40 MicroCT Scanner (Scanco, Bassersdorf, Switzerland) with 70 kVP X-ray source at medium resolution of 36 µm. The individual micro-CT sections were reconstructed to create a 3D reconstruction of the defect site using the standard Scanco software package. A global threshold of 150 in a 0-1000 range was applied corresponding to a density of 254.59 mg hydroxyapatite/cm³ for all reconstructions. A volume of interest (VOI) was defined within the subchondral bone region of the defect site and repair was expressed as percentage bone volume over the total volume (% BV/TV).

4.3.7 Histological analysis
Samples for histological analysis were then decalcified using a hydrochloric acid based decalcification solution (Decalcifying Solution-Lite, Sigma-Aldrich, Arklow, Ireland). The decalcification process was monitored with periodic micro-CT scans, and once complete the specimens were dehydrated in a graded series of ethanol solutions using an automated tissue processor (ASP300, Leica, Germany) and embedded in a single paraffin wax block. Subsequently, 10 µm sections were then cut using a rotary microtome (Microsystems GmbH, Germany), and the sections were mounted on l-polylysine coated glass slides (Thermo Scientific, MenzelGmnh & Co KG, Germany) using a water bath. When dry, slides were placed in an oven at 60 °C overnight. Prior to staining, the samples on the glass slides were deparaffinised and hydrated using established protocols.

The slides were then stained using haematoxylin and eosin staining (haematoxylin stains the DNA and RNA rich cell nuclei purple, eosin stains the extracellular matrix pink), to examine repair tissue morphology, composition and arrangement, cell infiltration, extracellular matrix (ECM) production and scaffold degradation (Levingstone et al., 2016b) and Safranin-O with a fast green counterstain (Safranin-O stains sulphated GAG red) (Rosenberg, 1971). Once the slides were stained, DPX (Sigma-Aldrich, Arklow, Ireland) mounting agent was used to secure the cover slides and digital images were obtained using a microscope (Nikon 90i, Nikon, Japan) and
attached camera unit (Nikon DS Camera control unit, Nikon, Japan) with NIS Elements software (Nikon Instruments Europe, The Netherlands).

### 4.3.8 Statistical Analysis

The initial sample size was determined based on results of an initial caprine pilot study performed by in the Tissue Engineering group of RCSI (Levingstone et al., 2016a). It was determined that in order to achieve significance in the outcome a sample size of n=7 was required for each group using both joints in each animal (Orth et al., 2013). All statistical analyses were performed using statistics software Prism 7 GraphPad (GraphPad Software, California USA). For analysis of two variables a paired student’s t-test was used. Where three variables were present, a repeated measure analysis of variance (ANOVA) test and Bonferroni *post hoc* analysis for multiple comparisons, to look for any statistical differences among three groups. An alpha value was set at *p*<0.05. Statistical analysis for nonparametric values such as the macroscopic assessment was done with a Kruskal-Wallis test for nonparametric with a Dunn’s post test.
4.4 Results

4.4.1 Clinical observations after scaffold implantation
There were no complications relating directly to the surgery or the scaffold during the course of the study. Assessment of the scaffolds intraoperatively showed that the tri-layered scaffold was easier to handle and more robust than the MaioRegen scaffold, and some delamination of the MaioRegen scaffold was noted. However, all scaffolds were successfully implanted, and all incisions closed with ease. There were no postoperative infections or patellofemoral dislocations, and the recovered animals mobilised with ease prior to leaving the recovery area and return to the pen.

4.4.2 Macroscopic assessment of the level of repair at defect sites
In the macroscopic assessment the tri-layered scaffold (cell-free) had a median score of 8.5 out of 12 (range 5.5 – 10) at 12 months, consistent with nearly normal cartilage (Figure 4.7). The animals treated with the cell-seeded tri-layered scaffold had a median score of 7.75 (range 6-11) at 12 months. Defects treated with the commercial comparator had the lowest overall median score at 12 months of 7.5 (range 4-9) classified as abnormal (grade III) cartilage. There was however no significant difference found in the macroscopic appearance of the cartilage generated (Figure 4.3 Figure 4.4) between the treatment types. An improvement in the macroscopic appearance of the cartilage was noted between the 6 and 12 month mark on the TR (Figure 4.4), however, the results were not statistically significant (p=0.08). Although the macroscopic scoring is a crude method of assessing the cartilage formed it is interesting to note that there was a significant difference in the cartilage generated in the MFC and the TR at the 12 month timepoint (Figure 4.5). This is highly relevant because the vast majority of clinically relevant osteochondral defects occur on the weightbearing portion of the joint and this should be considered when analysing research findings. Representative macroscopic images, showing the cartilage surface upon opening the joint and the bisected samples prior to histological analysis, are shown in Figure 4.6 and Figure 4.7.
Figure 4.3: ICRS scoring of the macroscopic appearance of the cartilage generated on the MFC at 6 and 12 months. There was no significant difference seen in the appearance of the regenerated cartilage between the treatment modalities.

Figure 4.4: ICRS scoring of the macroscopic appearance of the cartilage generated on the TR at 6 and 12 months. There was no significant difference seen in the appearance of the regenerated cartilage between the scaffold groups.
Figure 4.5: ICRS scoring of the macroscopic appearance of the cartilage generated on the TR and the MFC at 12 months. The results were broadly superior in the TR over the MFC, however they only reached significance ($p = 0.02$) between the macroscopic appearance of the cartilage at the trilayered cell-seeded TR group and the market product in the MFC with improved repair in the TR.

Figure 4.6: Macroscopic images of representative joints at 6 months both in situ and in cross-section post excision. These images demonstrate the high quality 'nearly normal' cartilage generated on macroscopic assessment when using the tri-layered scaffold, either cell free or cell-seeded and the commercial comparator.
4.4.3 Micro-computed tomography evaluation of subchondral bone formation

When the samples were analysed using the µCT techniques it was noted that there was no difference in the bone volume formed at the defect repair site at each individual time point between the experimental groups. However, it was noted that with the weightbearing MFC defect site there was a significant (p<0.001) increase in the volume of bone formed between 6 and 12 months in all three groups (Figure 4.8). There was also a difference over time in the volume of bone formed at the TR, however this did not reach significance (p=0.072) (Figure 4.9). Although the difference between the bone volume at 6 months and 12 months on the TR was not quite significant, there was no significant difference between the bone formed at the MFC and the TR at the 12 month time point (Figure 4.10). The tri-layered scaffold both cell-seeded and cell-free was able to generate similar bone volumes to the commercial comparator in all defect sites and at all time points.
Figure 4.8: Percentage bone volume over total volume in the subchondral bone portion of the MFC defect repair site. Although there is no difference at each timepoint in the bone formed dependant on the treatment type, there is a significant (p=0.0001) difference in the bone formed over time regardless of treatment type.

Figure 4.9: Percentage bone volume over total volume in the subchondral bone portion of the TR defect repair site. Although there is no statistical difference at each timepoint in the bone formed dependant on the treatment type, there is an increase in the bone formed over time with all the scaffolds.
4.4.4 Microscopic assessment of the level of repair at defect sites

Microscopic assessment at the 6 month and 12 month timepoints (Figure 4.11 and Figure 4.12) showed an improvement over time with all three groups. The medial condyle samples especially showed consistent regeneration of the cartilage layer at the surface, consistent in thickness with the surrounding tissue. The trochlear ridge samples, which are not weightbearing to the same extent as the medial condyle, have a thinner cartilage layer consistent with the surrounding cartilage. Analysis also showed that over the course of the 12 month there was increased integration into the subchondral bone below and, in the majority of the tri-layered scaffold implanted defect sites, the integrated bony scaffold is indistinguishable from the surrounding bone. The intermediate layer of the tri-layer scaffold successfully maintained the tidemark between the bone and cartilage layers with a layer of calcified cartilage. The commercial comparator also had the ability to generate a layer of quality cartilage at the joint surface; however, it was slightly thinner than the surrounding cartilage at the trochlear ridge. At both 6 and 12 months this group did not maintain
as clear a tidemark between the subchondral bone and the cartilage as was seen in the tri-layered scaffold.

Both the cell free tri-layered scaffold and the cell-seeded tri-layered scaffold demonstrated an ability to fill the osteochondral defects created in the joint surface. Both groups were able to stimulate the regeneration of bone and good quality cartilage with adequate thickness and maintain the tidemark between cartilage and bone by the 6 month timepoint (Figure 4.11). They continued to improve over time, as is visible in the 12 month timepoint histology image in Figure 4.12. The quality of the regenerated tissues visible is roughly equal across the three experimental groups, showing that the tri-layered scaffold both cell-seeded and cell-free has comparable regenerative potential to the commercially available product.

<table>
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<th>Medial Condyle</th>
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<tr>
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<td>H&amp;E</td>
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*Figure 4.11 Representative slides taken from the 6 month group. Left column medial condyle, right column trochlear ridge. In each column Left is Safranin–O showing the surface cartilage formation (red) at the top of each slide and Right is H&E staining which shows the cell infiltration and architecture of each sample. (scale = 1000µm)*
Figure 4.12: Representative slides taken from the 12 month group. Left column medial condyle, right column trochlear ridge. In each column Left is Safranin–O showing the surface cartilage formation (red) at the top of each slide and Right is H&E staining which shows cell infiltration and architecture of each sample. (scale = 1000µm)

### 4.5 Discussion

For clinical translation, the ability of the tri-layered scaffold to generate good quality cartilage and integrate with the underlying bone in a large animal in vivo model is crucial. Although the scaffold might stimulate mineralisation and cartilage formation in vitro there are many other factors in an in vivo situation, such as biomechanical effects due to weightbearing, as well as circulating stimulating and inflammatory factors, that may help or hinder the result. Therefore, the scaffold must be tested in a setting that models the clinical scenario as close as possible. The tri-layered scaffold was implanted both cell-free and when seeded with a co-culture of FPMSCs and CC in a ratio of 3:1 into an in vivo goat model into two defect sites, on the trochlear ridge and the medial femoral condyle. It was compared against a positive control in the
form of the market leading commercial product. Macroscopic assessment of the cartilage generated after one year showed high quality cartilage formation at the joint surface with grade II, nearly normal cartilage, formed in the cell free tri-layered scaffold group on the MFC and TR. The median score of the commercial comparator group and the cell seeded tri-layered scaffold however, showed grade III, abnormal cartilage, at 12 months on the MFC. Although, it was found that there was no statistical difference in the macroscopic appearance of the scaffold between the cell-free tri-layered scaffold, the cell-seeded tri-layered scaffold and the commercial comparator at any timepoint. Micro CT analysis of the subchondral bone showed excellent bone regeneration in the subchondral layer in all three groups, with a continued improvement over time seen between 6 and 12 months. The tri-layered scaffold both cell-seeded and cell free was able to generate similar bone volume as the commercial comparator, as no significant difference in the subchondral bone healing at either timepoint was seen between the scaffold groups. Histologically it was seen that the scaffolds universally had the ability to regenerate the cartilage layer. The bone layer of the tri-layer scaffold showed satisfactory integration into the surrounding bone tissue, to the point that the scaffold is indistinguishable from the surrounding bone at the 12 month timepoint in both the cell-free and the cell-seeded groups.

Macroscopically, at both the 6 and 12 month timepoints, the tri-layered scaffold demonstrated the ability to regenerate a joint surface that appeared “nearly normal” and scored between 8-11 out of 12 based on the ICRS cartilage assessment tool scoring system on the TR. The median score of the cell-seeded tri-layered cartilage group scored just outside of this category into the abnormal cartilage category (4-7) with a median score of 7.75 on the MFC, although high scores were reflected in all the subcategories of assessment of both groups, showing that the tri-layered scaffold led to good defect repair, good cartilage integration, and macroscopically, the regenerated cartilage appeared to be smooth. Similar macroscopic score results were observed in the commercial comparator group on the TR, but the MFC group at 12 months also showed grade III abnormal cartilage with a median score of 7.5/12. It was noted that there was a significantly better appearance of the cartilage formed
across all groups on the trochlear ridge versus the medial femoral condyle. This reflects differences between the MFC and the TR that have been seen previously (Jurgens et al., 2013). This finding is important because the vast majority of clinically important osteochondral defects are found on the medial femoral condyle or other weightbearing portions of the joint (Knutsen et al., 2007, Hangody et al., 2008, Basad et al., 2010). Therefore, research examining the regeneration of cartilage on the non-weightbearing portions (TR) of the joint should be mindful of this phenomenon.

Analysis of the micro CT results for subchondral bone formation demonstrated that our scaffold was able to stimulate bone regeneration to a similar extend to the commercial comparator. It also showed that there was a clear increase over time in the volume of the bone regenerated in both the medial condyle and the trochlear ridge. The increase in the bone regeneration over time at the MFC was greater than the increase at the TR. This is likely due to the weightbearing at the MFC area and Wolff’s law, which dictates that the bone will adapt to the loads under which it is placed. It is an important finding that the tri-layered scaffold has the capability to develop a solid subchondral bone base, as it was designed for use in larger defects that may take time to heal. This solid subchondral bone base, coupled with the interfacial strength of the tri-layered scaffold (Levingstone et al., 2014), will give a stable foundation to the cartilage layer that is regenerated, without the concern of mechanical instability of the scaffold.

Histological assessment of the scaffold demonstrated that the three groups (tri-layered cell-free, tri-layered cell-seeded and the commercial comparator scaffold) allowed the infiltration and proliferation of cells. They were also able to generate a cartilage layer of thickness similar to that of the surrounding tissue with the expected cell distribution and alignment for the tissue type. This cartilage layer was seen to seamlessly integrate into the surrounding tissue in all three groups. The intensity of the staining of the Safranin-O was greater in both the cell-free and the cell-seeded tri-layered scaffolds than in the commercial comparator scaffold, indicating the presence of greater levels of sGAG. This was noted especially in the thicker MFC cartilage tissue. The histological appearance of chondrocytes present in
the cartilage layer and the cellular alignment was also similar to normal hyaline cartilage surrounding cartilage tissue.

No significant benefit was seen by the addition of cells to the tri-layered scaffold at the timepoints investigated. It is possible that the cell numbers used in the cell-seeded tri-layered scaffold was too small to be influential in the in vivo scenario, or that the high levels of healing with the scaffold alone masked the effect of the cells. The intermediate layer beneath the cartilage was able to maintain the tidemark in the in vivo situation and prevented the advancement of the bone layer into the cartilage layer. On examination of the histology of the bone layer, it was shown that the bone formed was well integrated with, and indistinguishable from the surrounding subchondral bone. These findings are consistent with the findings of the micro CT analysis. A limitation of this study is that histomorphometry was not performed. It is possible that this may have revealed improved tissue quality in the tri-layer cell-seeded group over the cell free group. It is also worth considering the improvement seen over time in both the macroscopic appearance of the cartilage and the bone volume, examining longer timepoints may also demonstrate differences in longer term stability of cartilage tissue and benefits of the cell-seeded tri-layered scaffold over the cell-free tri-layered scaffold. Of note, the use of allogenic cells from a different goat didn’t cause any evidence of negative inflammatory reaction, such as giant multi-nucleated macrophage type cells, at the time points investigated. Although this is an important finding for the continued use of allogenic cells in an in vivo model for the investigation of cell-seeded scaffolds for osteochondral defect repair, perhaps autologous cells would have led to a more noticeable benefit.

4.6 Conclusion
The capacity of the tri-layered collagen scaffold to regenerate cartilage, bone and maintain the tidemark between tissue within each region of an osteochondral defect was shown at both a macroscopic and a microscopic level. Although no difference was seen between the cell-free tri-layered scaffold group and the cell-seeded tri-layered scaffold group, histological findings demonstrated a cartilage layer of
adequate thickness and stability superficial to a well-integrated bone layer and a satisfactory tidemark between. Both the cell-free and cell-seeded scaffolds had comparable outcomes to the commercial comparator scaffold.

Table 4.3 Acknowledgement of credit for work done as part of Chapter 4

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<td>Conor Moran</td>
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<tr>
<td>Operating theatre design, set up, and fitting</td>
<td>Prof. Peter Brama, Tanya Levingstone, Conor Moran, Grainne Cunniffe, Robert Brady</td>
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<td>Caprine surgery</td>
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Chapter 5: Design and testing of an arthroscopic delivery device

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5.1 Introduction
There is a significant focus on the optimal outcome for cartilage regeneration; however, it is important not to overlook the surgical approach for assessment and treatment of damage within the joint. The traditional open surgical technique, although it allows substantive access to the surgeon, prolongs rehabilitation for the individual, has a greater chance of wound infection, stiffness and poor cosmetic result, than a less invasive arthroscopic technique (Jameson et al., 2011, Ferruzzi et al., 2008). These factors are less acceptable outcomes for patients seeking treatment for osteochondral or chondral defects who tend to be of a younger, more active cohort (Jungmann et al., 2012, Siebold et al., 2011, Marcacci et al., 2002). The idea of minimally invasive surgery is founded on the principle that surgeons aim to give the patient the maximum benefit by causing the minimum amount of tissue damage, thus allowing them complete recovery with minimal pain (Hernandez-Vaquero et al., 2012).

The benefits of arthroscopic surgery are that complications tend to be relatively rare (Small, 1993) and post-operative pain is reduced (Jameson et al., 2011, Edwards et al., 2014). There are however, a number of limitations associated with arthroscopic techniques. During the procedure, access to desired areas within the knee joint can be difficult to attain - for example in the treatment of patellar lesions and posterior portions of femoral condyles or the tibial plateau (Hernandez-Vaquero et al., 2012, Marcacci et al., 2005). Surgical tools and equipment for arthroscopic surgery also require additional improvements and advancement (Hodgins et al., 2014). Despite these limitations, arthroscopy has gone from being used only for specific diagnostic cases in knees and shoulders, to being the standard for many joint surgeries including ankle, wrist and hip surgeries. (El Bitar et al., 2014, Karthikeyan et al., 2012). In terms of cartilage repair, a recent multicentre study investigating the clinical outcomes after ACI has shown that more than 26% of the procedure-related complications of ACI can be contributed to the arthrotomy itself (Erggelet et al., 2000). There is therefore a move toward less invasive surgery for cartilage repair (Cortese et al., 2012). Arthroscopic access to other joints such as the hip joint can further decrease morbidity. Complex cartilage regeneration techniques such as AMIC still require a
surgical dislocation of the hip including a trochanteric osteotomy, which can be very painful, and requires protected weight bearing up to eight weeks (Leunig et al., 2012). Similarly, the ankle joint, a source of many osteochondral defects, requires an osteotomy to access many parts of the joint (Wajsfisz et al., 2014). An arthroscopically deliverable cartilage repair scaffold would expand the potential benefits of the osteochondral defect repair scaffold to those patients suffering from defects in other large joints including the hip or ankle.

In order to minimise patient morbidity during a procedure designed to use the tri-layered scaffold to repair osteochondral defects, development of an arthroscopic device to implant the scaffold should be examined. The process of designing a medical device is a complex undertaking. After initial conceptual design of the device a working prototype must be manufactured. Then bench top testing and validation can be performed. A final prototype design freeze must happen prior to submission of invention disclosure. This is the first step for the protection of intellectual property (IP) and patent filing. To be marketable in the European Union (EU) the device must achieve CE marking (Conformité Européene) and conform with European regulatory approval requirements. An arthroscopic delivery device would be classed as a sterile Class IIa device (i.e. one that is surgically invasive, but only for transient use) and would be assessed as per the protocols in Figure 5.1. (Council Directive, 1993/42/EEC). These steps, although arduous, maintain a high standard of safety and integrity.
Figure 5.1: Options for pathways of regulations a Class IIa device must undergo to receive CE marking for marketing within the European Union. A sterile Class IIa device (i.e. one that is surgically invasive, but only for transient use) requires: conformity assessment, compilation of the Technical File, quality assurance audit by a Notified Body, declaration of conformity, and vigilant post market surveillance.

5.2 Aims of this study

The overall aim of this chapter was to design and test a delivery device capable of implantation of the tri-layered scaffold using an arthroscopic surgical technique, while ensuring rapid and repeatable implantation of the matrix to the defect site.

In order to achieve this, the specific aims of the chapter were:

1. To design, in conjunction with a device design team and surgical feedback, an arthroscopic delivery device for the implantation of a scaffold for osteochondral defect repair
2. To examine market leading competitor delivery devices suitable for comparison with the designed delivery device
3. To examine scaffold distortion during ejection from the device
4. To assess the ability of the device to deliver cell-seeded or hydrated scaffolds
5.3 Materials and Methods

5.3.1 Design Input

Significant planning must go into the design of a medical device or instrument for it to meet end user requirements and achieve regulatory approval (Council Directive, 1993/42/EEC). In order to achieve this satisfactorily, a design team was convened in RCSI. This team included Nicolas Hitchins (an engineer with experience in medical device design), Dr. Tanya Levingstone (a bioengineer with a special interest in osteochondral defect repair and the use of implantable scaffolds for same), Professor John O’Byrne (Consultant Trauma and orthopaedic surgeon with an interest in bioengineering and tissue regeneration) and Mr. Frank Lyons (trainee orthopaedic surgeon with an interest in tissue engineering). The first step of the design process involved reviewing the literature and existing patents for similar devices, or devices that would perform the task of delivering a bioengineered implant to a defect site in an endoscopic fashion. The relevant regulatory requirements, including the European Directive concerning medical devices (Council Directive, 1993/42/EEC), were also examined to identify the regulatory requirements that must be met within the device design.

A crucial aspect of product design is to determine the end user requirements and expectations. In order to achieve this, a meeting was organised in Cappagh National Orthopaedic Hospital (CNOH) with two separate Trauma and Orthopaedic surgeons not associated with the design team. This included the Director of Training in Orthopaedics in Ireland, Mr. Keith Synnott, and Senior Specialist Registrar in Trauma and Orthopaedics, Mr. Alan Molloy. Design inputs from these surgeons related to basic shape and size characteristics, as well as ease of use and their expectations of a device that would be used to deliver a scaffold into the specific defect through standard arthroscopic ports.

Further to this face to face meeting, a questionnaire was devised (Appendix A) using open ended questions to gain an insight into a range of practitioners’ experiences with devices currently available and methodologies of cartilage repair currently employed and where they saw the future of cartilage repair. A gatekeeper was recruited to reduce selection bias, this gatekeeper was an administrator in RCSI.
based in CNOH who has access to all the orthopaedic consultants in the Republic of Ireland. The questionnaire was disseminated by e-mail to 13 consultants from Dublin and Louth, and one Senior Specialist Registrar with a special interest in cartilage repair and tissue engineering, chosen by the gatekeeper based on their interest in cartilage repair and the procedures that they regularly perform. Responses from this questionnaire were compiled into an amalgamated response and reviewed as part of the design inputs. Based on the design input received, a delivery device was designed, and a prototype created.

5.3.2 Market leading competitor devices.
As part of the design process, existing commercial devices used for the delivery of cartilage repair devices were assessed. The market leading competitor devices were identified as the TruFit™ delivery device (Smith and Nephew MA, USA) (Figure 5.2) and the Orthomimetic™ (Dublin, Ireland) (Figure 5.3) delivery device. The TruFit device acts as a tamp in that the scaffold is loaded into the ejector to the appropriate depth and then cut to size. The device is then placed against the cartilage surface and the scaffold pushed into the defect. The Orthomimetic device has a different mode of operation in that it is designed to sit into the defect and push the scaffold out as the device withdraws and the scaffold expands to fill the gap (Figure 5.4). These devices were also used in comparative benchtop testing using the delivery device prototype developed within this study.
Figure 5.2: TruFit™ CB Plug Kits (Smith & Nephew) containing a) TruFit Plug, Delivery Device, Trimming Knife b) TruFit™ CB Plug Delivery Device components c) TruFit™ CB Plug loaded into delivery device d) The TruFit delivery device itself. This is a small tube structure with a tamp and two hydration ports at the implant end. The operator uses the back of the device as a measure to ascertain the depth of the defect and then using a knife supplied with the device cuts the scaffold to the correct depth based on the measurements taken. The device is then applied to the defect and the protruding handle is depressed pushing the implant into the defect.

Figure 5.3: The Orthomimetic device. This device comes with the scaffold preloaded into the delivery device and the defect is modified with the included drill bit to fit the intended scaffold. There are also ports on the distal end of the device should the operator wish to hydrate the scaffold with saline or seed the scaffold with a cell suspension. This device is pushed into the defect so that the tip of the device is resting against the base of the defect. The button is then pushed at the same time as the device is withdrawn. The implant then springs out and is press-fit in the defect. (TeamConsultingLtd, 2011)
5.3.3 To assess scaffold distortion during deployment of the device using a custom built jig.

The shape and microstructure of scaffolds used in tissue engineering are designed to provide the ideal environment for tissue repair. In order to achieve the best possible outcome in terms of cartilage regeneration, it is critically important that they are implanted correctly without damage to the scaffold during insertion. In order to combat this, it is important to ascertain if the scaffold is protected and inserted correctly by the device. To examine this the RCSI device, the TruFit device and the Orthomimetics device were each used to implant an RCSI tri-layered scaffold into a custom jig designed to simulate an osteochondral defect. Briefly, a fresh scaffold was prepared in accordance with the defect size and was then loaded into the delivery device in question. The device was then deployed by hand based on the manufacturer’s instructions to insert the tri-layered scaffold into the defect on the jig. The delivery device was then removed, and the contact of the scaffold with the surrounding jig was assessed. A four-point scoring system was used based on the percentage of the scaffold circumference in direct contact with the edges of the defect (Figure 5.5). This scoring system is a modified portion of an assessment element within the ICRS macroscopic scoring system (Table 5.1) that assesses integration of an implant with its border zone (Hoemann et al., 2011, Levingstone et al., 2016b). The process was repeated for each delivery device using a fresh tri-layered scaffold each time (n=6 per delivery device). The RCSI tri-layered scaffold was
used for all testing as the purpose of these tests was to find the optimal delivery device to deliver this particular scaffold.

Figure 5.5: Graphic representation of scaffolds (black) in the jig (brown) for testing edge contact post insertion of the scaffold into the jig using each delivery device A= 75-100% in contact with surrounding jig B=50-75% in contact, C= 25-50% in contact D= 0-25% in contact with the surrounding jig

Table 5.1: Section of the International Cartilage Repair Society (ICRS) cartilage repair assessment tool. This tool is used to evaluate the macroscopic appearance of cartilage repair tissue following interventions such as ACI, subchondral drilling and microfracture. (Levingstone et al., 2016b, Hoemann et al., 2011)

<table>
<thead>
<tr>
<th>CRITERIA</th>
<th>POINTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTEGRATION TO BORDER ZONE</td>
<td></td>
</tr>
<tr>
<td>Complete integration with surrounding cartilage</td>
<td>4</td>
</tr>
<tr>
<td>Demarcating border &lt;1mm</td>
<td>3</td>
</tr>
<tr>
<td>3/4 of graft integrated, 1/4 with a notable border &gt;1mm width</td>
<td>2</td>
</tr>
<tr>
<td>1/2 of graft integrated with surrounding cartilage, 1/2 with a notable border &gt;1mm</td>
<td>1</td>
</tr>
<tr>
<td>From no contact to 1/4 of graft integrated with surrounding cartilage</td>
<td>0</td>
</tr>
</tbody>
</table>
Scaffold distortion was also measured in terms of height difference pre and post ejection from the delivery device. The height of each scaffold was noted prior to insertion into the jig. The scaffold was loaded into the delivery device under examination and inserted into the jig using the manufacturer’s instructions for use of the delivery device. The distance between the scaffold and the edge of the jig on both the top and the bottom was measured against the known height of the jig to give an in-situ value for the height of the scaffold (Figure 5.6) (n=6 per delivery device).

![Figure 5.6: Graphic representation of the side-view cross section of a scaffold post insertion into the jig. Measurement of height change of the scaffold (black) was done after insertion to the jig (brown) with each delivery device. The known height of the scaffold pre-ejection from the delivery device was compared to the post ejection height, which was calculated by the formula C - (A+B) on the above diagram](image)

### 5.3.4 Assessing the ability of the delivery device to deliver cell-seeded or hydrated scaffolds

The tri-layered scaffold can be implanted as an off-the-shelf cell-free scaffold, or, as shown in Chapters 2-4, as a cell-seeded scaffold. Some surgeons may wish to pre-hydrate the cell-free scaffold with saline. Alternatively, they may wish to include Plasma Rich Plasma (PRP) or bone marrow aspirate to stimulate healing and to encourage integration of the scaffold. In order to facilitate this, it was important to assess the impact that the ejection action of the delivery device would have on the fluid retention of the scaffold during implantation. To perform this test, scaffolds that were pre-cut to the size of the defects within the test jig (as used in Section 5.3.3) and hydrated with PBS (Dulbecco’s Phosphate Buffered Saline, Sigma-Aldrich,
Ireland). When each scaffold was hydrated it was placed on a dry plastic surface in order to allow any excess PBS to drain off. The scaffold was then weighed and loaded into the delivery device and implanted into the defect site using the device as per the manufacturer’s instructions. Once complete, the scaffold was carefully removed from the jig, without squeezing it and weighed immediately. In this fashion, the pre and post-implantation weights were measured to estimate the amount of liquid that had been lost from the scaffold by the delivery device (n=6 per delivery device).
5.4 Results
5.4.1 Design input
The responses of the questionnaire were amalgamated (Appendix B) and taken as a qualitative assessment of what methods are being used in current practice, as well as current clinical opinion on limitations of surgical approaches and techniques and user requirements for delivery device design. The questionnaire amalgamated results revealed information relating to current practice and where the participants saw the future of osteochondral defect repair. Predominantly the respondents (75 %) said that to treat an osteochondral defect they would use an open or mini-arthrotomy technique using mosaicplasty. 100 % of respondents said they performed debridement or microfracture via an arthroscopic approach. In terms of limitations to surgical techniques for treatment of damage to articular surface, all respondents commented that the limiting factor was that available treatment modalities were expensive and that there was no conclusive long-term evidence to back up their use. In terms of cell based techniques for the treatment of damage to the articular surface, the respondents unanimously replied that the only technique they used in their own practice is microfracture. The main reasons they were not using any newer techniques was the lack of evidence to back them up and also the prohibitive cost involved.

The questionnaire showed participants were interested in the possibility of an arthroscopic approach, but apprehensive when dealing with a prolonged procedure (Table 5.2, Lines 82-83, 85-86 88-89); however, it was emphasised that arthroscopy would not be a suitable approach for mosaicplasty due to the difficulty in matching the convexity of the joint surface when projected onto a 2-dimensional screen (Table 5.2, Lines 83-85, 86-87, 90). In terms of the device itself only one participant specified particular parameters and emphasised the need for options in terms of angulation of the device neck for access to all parts of the joint and flexibility in terms of sizes of available implant (Table 5.2 Lines 98-100).
The face to face interview allowed for more specific questioning and discussion of the points raised. This revealed again, that the surgeons were keen on the idea of a device that would be easy to use and quick to deliver an implant into the defect, and they felt it would be a useful instrument to have in their armoury. The angle of the neck was again identified as an important feature in the design input feedback. This confirmed findings in the literature that $0^\circ$, $30^\circ$ and $70^\circ$ angled instruments (Figure 5.7) are in common use in arthroscopic and endoscopic surgeries as it allows the operator to see or work around a corner to defects that are perhaps not accessible or visible to a viewer looking straight down a camera (Hoshino et al., 2016).

Figure 5.7: Close-up of the camera angles of arthroscopes, left to right $0^\circ$, $30^\circ$ and $70^\circ$ (Spight et al., 2015) the face to face discussion with surgeons about delivery devices revealed the importance of having angled options such as these for manipulation of the tri-layered scaffold inside the joint.
Based on the review of the literature, assessment of existing commercial devices, review of the clinical requirements and input from the surgeons in both the face to face meeting and the questionnaire, a concept for the device was developed in conjunction with the delivery device design team (Table 5.3).

Table 5.3: Examples of key design features of the delivery device and the source of the design inputs

<table>
<thead>
<tr>
<th>Key Design Features</th>
<th>Input Source</th>
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<tbody>
<tr>
<td>Optional angulation of delivery device neck 0° and 30°</td>
<td>Questionnaire</td>
</tr>
<tr>
<td></td>
<td>Face to face meeting</td>
</tr>
<tr>
<td>Ergonomic gun-stock handle to allow manipulation of the approach angle by pronation</td>
<td>Face to face meeting</td>
</tr>
<tr>
<td>and supination of the forearm</td>
<td></td>
</tr>
<tr>
<td>Trigger action</td>
<td>Face to face meeting</td>
</tr>
<tr>
<td>Reusable handle</td>
<td>Design team</td>
</tr>
<tr>
<td>Disposable cassette for scaffold to protect from excess manipulation and contamination</td>
<td>Design team</td>
</tr>
<tr>
<td>Windows in cassette to allow for hydration/cell-seeding</td>
<td>Literature review</td>
</tr>
<tr>
<td>Modifiable scaffold depth by trimming the excess bone layer</td>
<td>Design team</td>
</tr>
<tr>
<td>Multiple sizes of scaffold available</td>
<td>Questionnaire</td>
</tr>
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Based on the key design features given in Table 5.3, 3D models of the delivery device (Figure 5.8) were then created by project engineer Nicholas Hitchins, using 3D CAD drawing software package Solidworks (MA, USA) (Figure 5.8). The design had a “gun-stock” handle which was revealed as the preference during the face-to-face interview. This allows manipulation of the device easily by supination and pronation of the forearm. The design allowed options of a 0° or a 30° shaft neck angulation to allow for access to areas of the joint that may not be accessible with a completely straight device, such as the posterior condyles, the tibial plateau or the retropatellar surface. The device was made of three primary components: 1) the reusable handle, which was proposed to be autoclavable, 2) the shaft, which can be straight or angled, and 3) the delivery cassette (Figure 5.9). This design proposed that the surgeon would decide what diameter implant would be required. The appropriate cassette, pre-
packed with the scaffold inside, would then be opened into the surgical field, minimising the need for manipulation of the scaffold prior to insertion into the defect. Windows in the cassette (Figure 5.9) allowed the scaffold to be hydrated or seeded with cells without removing it from the protective cassette. The windows in the delivery device are similar to the window in both the Orthomimetics delivery device and the TruFit device; however, the Orthomimetics device has only one window, restricting seeding to one surface of the implant. The design of the RCSI delivery device also allows the depth of the scaffold to be altered by the operator. The scaffold protrudes from the end of the cassette and can be trimmed. In this instance, the tri-layered scaffold would have a fixed predetermined depth of cartilage and intermediate layers, but the protruding bone layer would be alterable in order to ensure correct fit of the scaffold in the defect. Although this is similar in design to the TruFit device, the Orthomimetics device does not allow for this as the scaffold is held up inside the device until the point of delivery.

Once the 3D models of the RCSI delivery device were completed, two prototypes were then created on a rapid prototype machine (Eden 250 Rapid Prototyper, Computer Aided Technology, Illinois United States of America) using a polymer (FullCure® 830 Vero White Resin for Rapid Prototyping, Objet, Israel). One prototype had the straight 0° neck, and the other the angled 30° neck. This polymer prototype was used for prototype testing. This gives a good representation of how the final device will function; however, elements of the final product would be fabricated with different materials in order to meet the medical device regulations (Council Directive, 1993/42/EEC). For example, the reusable handle would be fabricated from stainless steel (ASTM-F899-12b, 2012) in order to withstand repeated autoclaving. The disposable cassette would be a single use portion of the device and therefore be made from cheaper plastics similar to those used in the rapid prototyper. From this regard, bench top testing can be performed with the prototype as the change in materials will not affect the size or shape of the cassette or the scaffold cassette interface.
Figure 5.8: Computer render of the prototype for the RCSI tri-layered scaffold delivery device. Above is the device with the 30° neck angulation. This angulation allows the surgeon to insert the scaffold into parts of the joint inaccessible with the straight device. Below is the straight device with a close up of the delivery cassette. There are windows in the cassette for either pre-hydration of the scaffold, or seeding cells onto the scaffold prior to insertion.
The regulatory requirements of the delivery device are determined in the EU by the European Council in Council Directive (1993/42/EEC) concerning medical devices. These regulations categorise different classes of medical devices based on their design complexity, their use characteristics, and their potential for harm if misused. The delivery device is categorised as a Class IIa device, i.e. one that would be used in a surgically invasive manner in the body, albeit only for a transient period. The pathways for receiving CE marking for such a device are laid out in Figure 5.1. In order to prepare the initial technical documentation and testing of the device, the prototype device was brought forward for benchtop testing.

5.4.2 Distortion of the dry scaffold by the delivery device
To assess the distortion of the scaffold by the action of the delivery device, the integration to the border zone was examined. Each device was loaded with an RCSI tri-layered scaffold pre-cut to match the defect in the jig, and each device was used to implant the scaffold. The Orthomimetic device performed poorly. No tri-layered scaffolds ejected from this device had greater than 50% contact with the edge of the defect, post insertion of the scaffold into the jig. The TruFit device had a broad spread...
of results, with 33% of scaffolds having less than 25% contact with the defect rim and 66% of scaffolds having greater than 50% contact with the defect rim. The device designed in RCSI performed better than either of the other devices, and tri-layered scaffolds ejected from the RCSI device had uniformly greater than 75% contact with the surrounds of the jig (Figure 5.10). The amount of the scaffold touching the defect rim is a marker for the distortion of the scaffold by the individual delivery devices. Each scaffold was initially cut to fit the pre-made defects with 100% contact. Therefore, any loss of shape of the scaffold through use of the delivery device will lead to increased gapping between the scaffold and the rim. These are significant findings as the microstructure of the scaffolds must be optimised to achieve best results in cartilage regeneration and distortion can hinder regeneration. The defect rim is also the source of the invading cells and stimuli for cell differentiation from the surrounding cartilage tissue. Gapping can therefore lead to delayed integration of the scaffold and delayed healing with sub-optimal outcomes.

![Graph](image)

**Figure 5.10:** Graphic representation of the scaffold contact with the rim of the defect grouped per delivery device used for insertion. Consistently the tri-layered scaffolds ejected from the RCSI device were between 75-100% in contact with the surrounds of the defect, in contrast, the Orthomimetics device was never greater than 50%. The TruFit device was between 50%-100% in the majority of cases, however in 33% of cases it was less than 25% in contact.
The mechanism of action of the three delivery devices under investigation is to gently push the scaffold into the defect from above. This tamping action can lead to a compressive force being applied to the scaffolds. Therefore, there is a risk of loss of height, and further distortion of the microstructure, of the scaffolds as they are impacted into place. This was assessed by measuring the height of the scaffolds pre and post ejection from the device into the jig. Although there was a change in height in all the scaffolds as they were inserted, there was no significant difference from one device to the other. Comparing each scaffold to its pre-insertion height, only the Orthomimetics delivery device led to a significant \((p<0.05)\) loss of height in the scaffolds (Figure 5.11). This poor performance in both height and edge contact means that the Orthomimetics delivery device causes significant distortion to the tri-layered scaffold during implantation of the scaffold into the defect.

![Figure 5.11: Percentage change in height of the scaffolds from before they were ejected from the device into the defect. Only the Orthomimetics device had a significant difference between pre and post-delivery \((p<0.05)\) however, there was no significant difference between the devices.](image)
5.4.3 Assessment of the ability of the delivery device to deliver cell-seeded or hydrated scaffolds

All of the devices for comparison push the scaffold out of the delivery device and into the defect using a tamp. The pushing action of the tamp leads to a compressive force on the scaffold as the device forces it into the defect. This compressive force can act to squeeze out any fluid that has been added to the scaffold. When dealing with a scaffold that has been expressly hydrated with an active ingredient such as a cell-suspension, PRP or bone marrow aspirate, it is important to maintain as much of this fluid as possible within the scaffold during delivery. Therefore, the amount of fluid lost during delivery is important to assess. The percentage change in weight was used as a marker for the amount of fluid lost from the scaffold during ejection from the delivery device. This is demonstrated in Figure 5.12. The scaffolds ejected from the RCSI device led to a loss of 9.05 % (range 5.58 % - 17.82 %) of the fluid added; however, there was no significant difference between post ejection weight and the pre-ejection weight. The TruFit device led to a significant loss of fluid (p=0.02), losing 18.11 % (range 1.08 % - 37.72 %) of the fluid. The Orthomimetics device had the worst performance, with a highly significant (p<0.0001) change in weight, losing a mean of 40.54 % (range 35.57 % - 47.22 %). When the devices were compared directly to each other, significantly more fluid was lost when the tri-layered scaffold was ejected from the Orthomimetics device than when it was ejected from the RCSI device.
Figure 5.12: The percentage change in weight of the hydrated scaffold before and after ejection from the delivery device into the custom jig. When compared to the pre-ejection weight there was a significant loss of fluid caused by the TruFit device (** p=0.02) and a highly significant loss of fluid caused by the Orthomimetics device (*** p<0.0001). There was a significantly higher amount of fluid lost by the Orthomimetic device than the RCSI device *** p=0.0001
5.5 Discussion
This study has led to the development of a device designed to enable arthroscopic delivery of the tri-layered scaffold described in the previous chapters. Initially the design team reviewed the literature as well as the regulations pertaining to the device. Input was sought from the end user and the market leading competitor products were examined. This process led to particular traits being included in the prototype design. These traits included an ergonomic gun-stock handle with trigger action to deploy the scaffold. Surveyed surgeons were keen on this to allow for easy manipulation of the device intra-operatively. Design input from surgeons also included the desire for an optional angulation of the neck to reach less accessible parts of the joint. The neck angulation particularly, is not seen in other commercially available scaffold delivery devices but is widely discussed in the literature (Hoshino et al., 2016, Spight et al., 2015). Surgeons stressed too the availability of multiple sizes of scaffold in order to match the variety of defects that may be found. The delivery device was composed of three parts, a reusable autoclavable handle which the design team felt would decrease the cost per unit, an interchangeable shaft with the optional angles, and the final piece being a novel disposable cassette to hold and protect the scaffold. This cassette would be separately packaged, pre-loaded with the tri-layered scaffold, ready for attachment to the handle. Examination of the literature showed that the cassette should feature multiple windows to allow pre-hydration with a cell suspension, or other fluids such as PRP, bone marrow aspirate or saline, without excess manipulation of the scaffold.

Examination of the distortion of the scaffold by the delivery device tests showed that the tri-layered scaffold was least distorted by the RCSI device compared to the other devices assessed. Minimal scaffold distortion is crucial, as the shape and microstructure of scaffolds used in tissue engineering are carefully designed to provide the ideal environment for tissue repair (Gleeson et al., 2010, Murphy et al., 2010, O'Brien et al., 2005). It is important to note that the other devices were designed for the delivery of other scaffolds that have different properties to the RCSI tri-layered scaffold. The TruFit scaffold for instance is far more rigid in all dimensions than the collagen based tri-layered scaffold and therefore the forces applied by the
TruFit delivery device may have been greater than the tolerances of the tri-layered scaffold. The Orthomimetics device performed poorly in this study primarily because of its design, in that the device was intended to sit into the defect and be withdrawn as the scaffold was ejected (Figure 5.4) rather than sit against the intact joint surface and push the scaffold into the defect. This involves the scaffold being squeezed laterally in order to fit down through the device mouth as well as the compressive force from the tamp above. As shown here these forces have caused significant permanent changes in the shape and size of the scaffold, which will impact on the outcomes of the cartilage repair.

Assessment of the change in the weight of the scaffold was performed to reflect a scaffold that had been pre-hydrated with either an active component such as a cell suspension, PRP, bone marrow aspirate or merely saline pre-hydration. It was shown that the Orthomimetics device caused the most significant loss of weight in the hydrated scaffold, indicating that a large amount of fluid had been lost. This is once again due to the lateral compression that the scaffold undergoes as it passes through the mouth of the device squeezing out the fluid that had been added to the scaffold. This decrease in the weight meant that a mean of 40.5% of the hydration fluid was lost during the delivery of the scaffold. This design would thus be unsuitable for use for the delivery of a cell seeded construct as the expanded cells have a huge associated cost both financial and labour costs. The TruFit delivery device led to a significant change in scaffold weight before and after deployment, although the results had a very wide range. This indicates that the force applied to the scaffold by the TruFit device is not a dependable repeatable force, and therefore unreliable when dealing with a carefully designed scaffold loaded with cells that have been especially harvested and isolated. The RCSI designed delivery device did not cause a significant change in weight between pre and post ejection of the hydrated scaffold and the results were in a tighter range than the TruFit device. Therefore, it was demonstrated that the RCSI designed delivery device can be used dependably to deliver the hydrated or cell-loaded tri-layered scaffold successfully to an osteochondral defect.
A limitation to this study is that the testing was performed with the prototype delivery device made using rapid prototyping. The final prototype would be made of slightly different materials as per the standards for use of materials for medical devices (Council Directive, 1993/42/EEC). The reusable handle will be fabricated with stainless steel (ASTM-F899-12b, 2012) in order to withstand the repeated sterilisation it will require, and the disposable cassette feature will be made of cheaper plastics. This change from the entirely polymer fabricated prototype may have slight impact on the ejection process; however, the shape and size of the cassette should remain the same and therefore should not impact on the distortion of the scaffold. Overall the benchtop testing of the scaffold demonstrated that, of the currently available designs for delivery devices for osteochondral defect repair plugs, the RCSI design is the most acceptable in terms of scaffold protection and fluid retention throughout the transfer process.

A patent has been filed on this device and it remains under option to be licenced to RCSI spinout company SurgaColl Technologies Ltd (Dublin, Ireland) for use in combination with the tri-layered scaffold.

5.6 Conclusions
A prototype device was developed in conjunction with the device design team based on: surgical input, review of the literature and the regulatory requirements. This device has several novel features including angulation of the neck of the device to access hard-to-reach areas of the joint and a disposable windowed cassette to hold and protect the scaffold while still allowing hydration or the addition of a cell suspension while avoiding excess manipulation of the carefully designed tri-layered scaffold. This RCSI designed delivery device outperformed other commercially available delivery devices for scaffold implantation in terms of avoiding scaffold distortion and maintaining fluid within the scaffold throughout deployment of the device.
Table 5.4 Acknowledge of credit for work done in Chapter 5

<table>
<thead>
<tr>
<th>Study Design</th>
<th>Prof. Fergal O’Brien and Tanya Levingston</th>
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<tr>
<td>Design Input Team</td>
<td>Nicolas Hitchins, Tanya Levingstone, Prof. John O’Byrne, Frank Lyons, Conor Moran</td>
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<tr>
<td>Questionnaire design and circulation</td>
<td>Conor Moran</td>
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<tr>
<td>Device simulation and printing</td>
<td>Nicholas Hitchins</td>
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<td>Benchtop testing of device</td>
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Chapter 6 General Thesis Discussion

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6.1 Introduction

The repair of articular cartilage continues to present a difficulty in the field of orthopaedic surgery. Articular cartilage is avascular, aneural and has limited access to stem cells. It therefore has limited healing potential. Articular cartilage defects, if untreated, continue to abrade the joint surface and wear away the smooth gliding surface that is required for pain free movement. This abrasion leads to osteoarthritis for which the only management options are symptomatic, or highly invasive joint replacement (Murphy and Helmick, 2012). Attempts to find a treatment to halt the progression of isolated defects have resulted in several treatment modalities. Unfortunately, these treatments have equivocal results and none of them lead to long-lasting cartilage repair. Management options which are focused on the regeneration of the cartilage by direct bone marrow stimulation, such as microfracture (Steadman et al., 2003), primarily generate weaker fibrocartilage to replace the damaged hyaline cartilage. Other more complex treatments such as mosaicplasty, aim to replace the missing hyaline cartilage with transplanted plugs from a non-weightbearing portion of the joint (Bentley et al., 2013, Bentley et al., 2012). Unfortunately, this also has its disadvantages in terms of limitations of size and donor site morbidity (Koh et al., 2004). The introduction of cell-based treatments such as ACI (Brittberg et al., 1994) have led to improved short-term outcomes. However, difficulties in maintaining the cells within the defect and hypertrophy of the periosteal flap (Gooding et al., 2006) has led to the development of collagen matrices, upon which the cells are seeded. This subsequently led to tissue engineered treatment options for cartilage and osteochondral defect repair. Both cell-free and cell-based tissue engineering approaches for cartilage and osteochondral defect are in development (Table 1.1). Cell-based techniques, such as ACI, require two operations, the first to harvest cells from the donor site prior to in vitro expansion, and a second, post expansion, re-implantation procedure. This second procedure is scheduled roughly six weeks later and means a significantly added cost in terms of the in vitro expansion, as well as repeated surgical and anaesthetic risks, and second rehabilitation time for the individual involved (Samuelson and Brown, 2012).
Apart from the decreased cellularity and avascularity, the regeneration of cartilage tissue is further complicated by the gradient nature of the tissue. The joint surface is stratified from the surface cartilaginous layer, to calcified cartilage layer and then bone deep to this. In order to achieve regeneration of the joint surface, each of these individual layers must be regenerated. If tissue engineering is to be used, a stratified scaffold mimicking the native tissue is required (Jiang et al., 2010, Stoddart et al., 2009, Frenkel et al., 2005, Kon et al., 2014), and it must facilitate the differentiation of cells into the different lineages that are required. In this context, the focus of Chapter 2 was to investigate the ability of a novel tri-layered scaffold, developed within Prof O’Brien Tissue Engineering Research Group in the Royal College of Surgeons in Ireland (Levingstone et al., 2014, Levingstone et al., 2016a), to direct the differentiation of cells into chondrocytes in the cartilaginous layer and into osteoblast in the bone layer, while simultaneously protecting the cartilage layer from hypertrophy and mineralisation.

Although there has been a significant amount of research into the use of cell-free “off-the-shelf” scaffolds for osteochondral defect repair (Bentley et al., 2013, Crawford et al., 2012, Levingstone et al., 2016a, Kon et al., 2014), the promising results with ACI have shown that the use of cells in the defect site can stimulate repair in larger defects. Therefore, numerous researchers have investigated the use of autologous cells to augment repair. Cells can be used in a self-assembled matrix model (Mesallati et al., 2014), or more commonly, they can be seeded onto a bioengineered scaffold. While articular chondrocytes represent the ideal cell type in order to form hyaline cartilage repair tissue at the joint surface, the density of the cells available is extremely low (Hunziker, 1999). Therefore, the cells must be cultured in vitro and expanded to reach adequate numbers. There has been significant research into different cells sources in order to avoid harvesting from the joint surface. The most commonly used cells are MSCs derived from bone marrow. The disadvantage of these cells is the requirement for a second operative site as well as their ability to hypertrophy and cause unwanted bone formation. In this context, the primary focus of the research presented in Chapter 3, was to determine the optimal source of cells for use in seeding onto the tri-layered scaffold. Additionally,
we aimed to validate the rapid isolation technique developed by Prof. Danny Kelly’s lab in TCD for the isolation of chondrocytes, and MSCs harvested from goat bone marrow and the fat pad. The overall focus of this chapter was thus to identify a method of harvesting cells, isolating them and seeding them onto the scaffold prior to re-implantation. without the use of *in vitro* expansion.

The artificial *in vitro* setting is different to the *in vivo* setting. The exogenous stimulants are strictly controllable when the scaffold is in culture and the biomechanics of the joint are not accounted for when the scaffold is *in vitro*. The majority of osteochondral defects occur on the weightbearing portion of the knee joint (Gudas et al., 2012), and the pressure that is placed on the implanted tissue has an effect on the generated cartilage (Vinardell et al., 2012a). Therefore, it is important during pre-clinical work to investigate a large animal *in vivo* model with equivalent biomechanics and tissue sizes to humans. A pilot study was previously completed with the implantation of the scaffold into cartilage defects that were created on the medial condyle and the trochlear ridge of goats (Levingstone et al., 2016a). Results from this study suggested better outcomes than the market leader at the time. Chapter 4 of this thesis focused on expanding this previous work into a full scale *in vivo* study with an updated market leading scaffold as a comparator.

To increase the benefit of the intervention, a move toward arthroscopic intervention needs to be assessed to decrease recovery time and risk of infection (Edwards et al., 2014). Another major advantage to arthroscopic treatment of osteochondral defects is the increased scope of the treatment modality. Joints, such as the hip and ankle, are prone to osteochondral defects, but require osteotomies to access the entire joint (Wajsfisz et al., 2014, Krych et al., 2012b). Arthroscopy of these joints has recently become widely available (Jennings and Bark, 2011, Fickert et al., 2014). Methods of treating osteochondral defects quickly and easily, without having to perform an osteotomy, would benefit a huge number of individuals and postpone the need for hip replacement and ankle fusion/replacement. The focus of Chapter 5 of this body of work was therefore to develop a device that can be used to implant a seeded scaffold into the joint arthroscopically.
6.2 Stem cell differentiation in a tri-layered collagen-based scaffold designed for osteochondral defect repair

Previous work in the RCSI Tissue Engineering Research Group led to the development of the tri-layered collagen based scaffold that mimics the gradient nature of the osteochondral tissue (Levingstone et al., 2014). The tri-layered scaffold is produced using a unique iterative layering fabrication method and consists of three layers. Each layer of the scaffold is specially designed to regenerate the individual cartilage layer, tidemark and bone layer of osteochondral tissue. To test the biocompatibility of the scaffold in vitro, and its ability to direct the differentiation of cells down the required lineages, the scaffold was tested mechanically and was seeded with rat BMMSCs.

It was shown that there was a 2.48-fold increase in mineral deposition in the bone layer of the scaffold over 28 days when cultured in osteogenic media, and a 1.74-fold increase even when cultured in standard growth media. This mirrors previous findings that show that the addition of the ceramic (HA) phase to the biodegradable polymer phase has led to the production of a composite structure which possesses all the prerequisite biological, morphological and mechanical characteristics necessary to facilitate the body’s own natural bone regenerative process in vivo (Lyons et al., 2014). The study also crucially demonstrated, through histological analysis, that mineralisation within the tri-layered scaffold did not advance past the tidemark into the cartilage layer. This protection of the tidemark is necessary to retain the gradient nature of the tissue and prevent advancement of the bony layer and early onset osteoarthritis. When the intermediate layer of the tri-layered scaffold was examined alone, it was found to be chondroconductive, but no mineral deposition was observed.

When the cartilage layer of the tri-layered scaffold was examined, chondrogenesis was observed in standard growth media. While the overall levels of sulphated GAG deposition were low, there was a significant increase in sulphated GAG deposition when the scaffold was cultured in media with chondrogenic stimulants such as TGF-β. This chondroconductive nature of the scaffold is suitable to the in vivo environment,
particularly as the cartilage formed was confirmed to be hyaline cartilage by immunohistochemical staining of Collagen type II.

The mechanical aspects of the scaffold were also examined in this study. When examined separately, it was found that the bone layer underwent less than 10% contraction during in vitro culture due to its improved stiffness resulting from the presence of HA. The intermediate layer contracted by 38.86%, and the cartilage layer contracted by less than 20%. When the entire tri-layered scaffold was examined, contraction was found to be less than 10% due to the bone and cartilage layer having the ability to support the intermediate layer. The fact that there is very little contraction is clinically important as it allows the surgeon to use a press-fit technique with the scaffold without concern that it will contract and fall out of the defect, it also means that the cells from the surrounding tissues will be able to immediately invade the adjacent scaffold.

6.3 Assessment of the optimal cell source for the cell-seeded multi-layered scaffold
For cartilage repair, one of the major current research topics is the identification of the optimal cell seeding regimen to enhance chondrogenesis. In particular, some success has been achieved through the use of cell-seeded scaffolds for larger defects and there is great interest in one-step approaches to cartilage repair (Almeida et al., 2015, Bekkers et al., 2013). This study showed that the rapid isolation technique developed in TCD could be successfully used to harvest BMMSCs, FPMSCs and chondrocytes from the femur, and infrapatellar fat pad and articular surface respectively of the knee joint of goats. This rapid isolation technique thus shows the potential for use within a ‘one stage’ surgical procedure for cell-based cartilage repair. This would eliminate the need for two operations, and the six weeks delay required to isolate and expand the chondrocytes in vitro prior to re-implantation (Brittberg et al., 1994, Brittberg and Winalski, 2003, Peterson et al., 2010). Rapid isolation of sufficient cells would reduce the procedure down to a single operation and allow the patient a single recovery time, half the time away from work, and other
activities of daily living (ADLs), as well as remove the expensive \textit{in vitro} aspect of the procedure.

The CC density in the articular cartilage is roughly $1800 \text{CC/mm}^3$ (Hunziker, 1999). This is far too sparse to harvest sufficient chondrocytes alone from the cartilage surface to seed straight onto the scaffold without \textit{ex vivo} expansion (Mesallati et al., 2017). This study showed that the low scaffold seeding density group (CC LD), which contained an equivalent number of cells to those freshly harvested from a subcritical defect, resulted in inferior results compared to the CC HD group, which represented the numbers of cells that would be used in procedures where \textit{in vitro} expanded cells are used. Therefore, a source of cells that can be harvested in abundance for seeding directly onto the scaffold at the time of the initial procedure needs to be found. FPMSCs maintain their chondrogenic capacity in the diseased state (Liu et al., 2014), and significant amounts can be debrided without morbidity to the patient. The fat pad is also accessible at arthroscopy and does not require a second surgical approach, other than the arthroscopic ports used to inspect and debride the cartilage defect. Consequently, these cells represent an obvious choice for further examination.

As anticipated, a high seeding density demonstrated increased cartilage formation compared to the low seeding density (Huang et al., 2016, Foldager et al., 2012, Talukdar et al., 2011). Current literature proposes a co-culture model (Bian et al., 2011, Mesallati et al., 2017) and it was seen here that the addition of FPMSCs to the CC LD group led to a better result over any other group. There was a 7.8-fold increase in the amount of sGAG deposited between the FPMSC:CC group and the CC LD group. There was also a 1.4-fold increase sGAG production between the co-culture group and the CC HD group. The data shows that, for a one-stage procedure, sufficient cells must be harvested to adequately seed the scaffold (Ahearne et al., 2014, Almeida et al., 2015). It has also been shown that the synergistic effect of the FPMSCs combined with the CC is more beneficial than CC alone even when high densities are used.
6.4 Cell seeded scaffold *in vivo* using a goat model

Continuing the research performed in Chapters 2 and 3, cell-free and cell-seeded versions of the tri-layered scaffold were brought forward to be examined in standardised osteochondral defects created on the medial femoral condyle and the trochlear ridge of a large animal model. The scaffolds were examined against a comparator in the form of a commercially available hydroxyapatite and collagen I scaffold designed for osteochondral defect repair, MaioRegen.

Both scaffolds demonstrated the ability to completely regenerate a full thickness osteochondral defect. It was shown that the tri-layered scaffold had the ability to generate a good quality cartilage layer that was of the same quality as the current commercial leader. It was also seen that the subchondral bone volume continued to increase over time allowing the scaffold to integrate fully with the surrounding tissue.

Histological assessment of the scaffold demonstrated that the three scaffolds (tri-layered cell-free, tri-layered cell-seeded and the MaioRegen scaffold) allowed the infiltration and proliferation of cells. They were also able to generate a cartilage layer of thickness similar to that of the surrounding tissue with the expected cell distribution and alignment for the tissue type. This cartilage layer was also able to seamlessly integrate into the surrounding tissue in all three groups. The intermediate layer beneath the cartilage was able to maintain the tidemark in the *in vivo* situation and prevented the advancement of the bone layer into the cartilage layer. No significant improvement was seen by the addition of cells to the tri-layered scaffold at the timepoints investigated. This may be due to the fact that the healing potential of the scaffold alone masked the additional beneficial effects of the cells, it is also possible that the cell numbers used were too few to be influential in the *in vivo* situation. As allogenic cells were used in this study, although there was no evidence of negative inflammatory reaction it is possible that a better outcome may have been achieved with autologous cells. A limitation of this study is that histomorphometry was not carried out. This may reveal differences in the tissue quality in the cell seeded tri-layered scaffold group.
These findings indicate that the tri-layered scaffold is suitable for use in osteochondral defect repair treatments. The scaffold can be used as a cell-free off-the-shelf product and, if further stimulation is necessary to induce healing, it can also be used as a cell-seeded scaffold loaded with freshly isolated FPMSC:CC cells.

6.5 Arthroscopic device for use in cell-seeded scaffold implantation

In order to minimise morbidity during surgical implantation of the tri-layered scaffold, an arthroscopic device was developed. Inputs from a design team, end-user canvassing, and review of the literature and medical device regulations led to the development of a prototype delivery device that could be used to implant the tri-layered scaffold in a minimally invasive technique. It was made of three primary components. The first component is a reusable handle with a gun-stock grip. The second component involves an option of either a straight neck, or a 30° neck angle. These components are autoclavable and reusable. The third component is a single-use delivery cassette which would come pre-packed with the tri-layered scaffold inside. During surgery, the surgeon would measure the defect and decide what size implant would be used, and the appropriate sized cassette would be opened into the surgical field for attachment to the handle. This encapsulation of the scaffold inside the windowed cassette minimises the manipulation of the scaffold prior to insertion while allowing the scaffold to be seeded with cells or hydrated with fluids such as saline, PRP or bone marrow aspirate.

Bench-top testing demonstrated that our delivery device design was able to deliver the scaffold to the defect site with minimal distortion of the implant. Maintaining the implant shape is crucial to maintain its carefully developed microstructure and shape to optimise cartilage repair. Our delivery device also has the capabilities to deliver a hydrated tri-layered scaffold with minimal loss of fluid. This is an important feature when considering the labour and financial costs involved in creating a cell suspension to seed onto the scaffold. The device was found to significantly outperform the Orthomimetics device, in terms of both scaffold distortion and ability to deliver hydrated scaffolds. The TruFit device was found to be inconsistent, and to have a
wide spread of results when examining distortion of the scaffold, and also to have a significant loss of fluid during delivery. This indicated that the TruFit delivery device is not reliably suitable for use with the RCSI tri-layered scaffold, especially if prehydrated or seeded with cells. These findings show that the RCSI-designed arthroscopic delivery device is a viable option for the implantation of the tri-layered scaffold in order to reduce the impact and morbidity of the procedure on the patient.

6.6 Future work
Having demonstrated the significant potential of the scaffold to stimulate osteogenesis in the bone layer and chondrogenesis in the cartilage layer (Chapter 2), the next obvious step is for the tri-layered scaffold to be used in osteochondral defect repair as a cell-free scaffold for clinical cases in humans. A first-in-man clinical trial is scheduled to begin in January 2018. Ethics approval has been granted and first patients recruited. This initial pilot study will focus on the use of the scaffold as backfill in the donor sites of mosaicplasty procedures. Follow-up will include pain and movement scoring and Magnetic Resonance Imaging (MRI) scanning to assess the bone and cartilage formed. The clinical trial will be carried out under the care of Prof. John O’Byrne in Cappagh National Orthopaedic Hospital in Finglas, and under Prof. Cathal Moran in the Sports Surgery Clinic in Santry. The tri-layered scaffold has also been licenced to RCSI spinout company, SurgaColl Technologies, who are working towards obtaining CE mark approval for the product to bring it to market.

Chapter 3 demonstrated that the optimal cell-seeded construct for cartilage generation using the tri-layered scaffold is a co-culture of FPMSCs:CC. It was also seen that there was potential to harvest, isolate and seed the scaffold in a rapid fashion that would facilitate a single-stage surgery for an osteochondral defect repair. Future work might streamline the isolation protocol and equipment to a single kit. This would mean that, during a single operation, the tissue could be harvested by the surgeon and handed off to a technician in the corner of the operating theatre. The technician would then isolate the FPMSCs and the CC and seed the scaffold while
the surgeon prepares the defect, prior to re-implantation of the seeded scaffold all within the one procedure.

Having shown successful repair of cartilage, bone and tidemark layers using a cell-seeded tri-layered scaffold as well as the cell-free tri-layered scaffold in a caprine osteochondral defect model (Chapter 4), the next step may be to examine a longer timepoint and see if the improvements in the bone and cartilage layers continue. If the cell-free model performs with sufficient success as backfill for mosaicplasty donor sites in the first-in-man trial referenced above, then, seeding FPMSC:CC cells onto the tri-layered scaffold should be examined in a similar study design. Acceptable results with this approach will lead to development of a cell-seeded model to augment the results especially in larger area defects.

The focus of Chapter 5 was to develop an arthroscopic instrument that could be used to implant the cell-seeded scaffold without the use of a mini-arthrotomy. A patent has been filed on this device and it is currently under option to be licenced to spinout company SurgaColl for use with the tri-layered scaffold. Future work in this area will involve taking these plans forward to a GMP facility to commence work on large scale production of a functional version delivery device using regulatory approved materials, and then proceeding to arthroscopic simulator trials before using the device in an in vivo situation.
6.7 Thesis conclusion
Chapter 2 investigated the directed stem cell differentiation in a tri-layered scaffold developed in the RCSI Tissue Engineering Research Group. It was demonstrated that the bone layer of the scaffold had osteoinductive as well as osteoconductive properties. It was also demonstrated that the cartilage layer was conducive to chondrogenesis. The intermediate layer had properties such that it was able to maintain the tidemark between the bone layer and the cartilage layer and saw no calcium deposition when cultured in standard growth media. It was also shown that the tri-layered scaffold could resist cell mediated contraction due to the stiffness and crosslinking techniques used. Taken together this shows the scaffold to be a suitable implant for osteochondral defect repair using a press-fit technique.

Chapter 3 saw the assessment of the optimal cell-seeding regimen for the cartilage layer of the scaffold in order to determine the best option for a single stage-cell-mediated osteochondral defect repair technique. A technique previously developed by Prof. Danny Kelly’s lab in TCD for rapidly isolating the stem cells from the fat pad was assessed and validated. Most importantly, it was demonstrated that after 28 days in culture, the co-culture group of fat pad derived mesenchymal stem cells and freshly isolated chondrocytes produced significantly more sGAG than chondrocytes or FPMSCs alone. This finding means that the synergistic effects of rapidly isolated FPMSCs and chondrocytes taken on the morning of surgery, can be harnessed for use in generating optimal results in cell-mediated scaffold based repair of osteochondral defects with optimal results.

In Chapter 4, the in vivo potential of the cell-free and cell-seeded tri-layered scaffold to repair osteochondral defects was assessed in comparison to the commercial comparator. It was shown that the RCSI tri-layered scaffold was able to integrate seamlessly into the bone layer, and that the regenerated hyaline-like cartilage was of similar thickness and morphology to the surrounding tissue both macroscopically and microscopically. A defined tidemark between the two layers was maintained. These findings mean that the tri-layered scaffold, encourages regeneration and reparation of osteochondral defects, with long lasting and biomechanically appropriate hyaline-
like cartilage. It was also seen that the tri-layered scaffold generated tissue comparable to the current market leading comparator.

Considering open surgery is a large insult to the joint, Chapter 5 investigated the potential of using an arthroscopic device to minimise this insult during implantation of the cell-seeded scaffold. It has been shown that the RCSI delivery device does not overtly distort or cause the loss of significant amounts of seeded cells or hydration fluid from the scaffold during implantation. This delivery device performed better than the commercially available competitor devices and shows that there is potential to use this arthroscopic device for implantation of the scaffold and expand the scope of its use beyond the knee to other joints such as the hip and ankle.

Taken together, the results from this thesis have shown that the tri-layered scaffold has intrinsic properties to optimally repair osteochondral defects by directing cell differentiation appropriately for the individual layers. Cartilage repair can be augmented by pre-seeding the scaffold with cells prior to implantation, and best results are obtained using a co-culture of FPMSCs:CC, which can be harvested and isolated using a rapid isolation technique. These tri-layered scaffolds have been proven in in vivo trials to generate hyaline-like cartilage in a large animal model, and a device has been designed to enable the implantation of this device by arthroscopic technique.
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Appendix A

Surgeon Questionnaire

1.0 Scope:

The purpose of this questionnaire is to gauge surgical opinion on the following:
• Current options available for the treatment of damage to the articular surface
• Future advances in the treatment of such defects
• The use of arthroscopic procedures in articular surface repair

2.0 Questionnaire Details

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3.0 General Questions:

1. What is a typical articular surface defect (size, chondral or osteochondral, depth, location) that you see clinically?

2. What surgical techniques do you typically use in the treatment of such defects? Give details of open techniques, arthroscopic techniques and the types of devices used if any.

   Open:

   Arthroscopic:

   Devices used (list brand names):

3. In your opinion what are the biggest limitations relating to current surgical techniques for the treatment of damage to the articular surface?
4.0 Questions on cell and/or biomaterial scaffold based techniques for treatment of damage to the articular surface

4. Do you currently use cell based (eg: ACI, MACI, microfracture/drilling) or biomaterial scaffold based techniques in your clinic?
   a. If yes, which treatments are you using? What would you say are their strengths and weaknesses?
   b. If not, what are your reservations?

5. What do you see as the limitations of cell based therapies such as ACI? What is impeding the widespread clinical adoption of cell and tissue engineering based techniques?

5.0 Questions on open surgery vs. arthroscopic techniques in the treatment of articular surface injury

6. In what situations would you choose to carry out an open procedure rather than an arthroscopic procedure? Describe the limitations of arthroscopic procedures in terms of a) patient factors, b) surgeon factors and c) instrumentation factors.
Patient Factors:

Surgeon Factors:

Instrumentation Factors:

7. Do you feel that there is scope to carry out more complex/advanced procedures arthroscopically? What do you feel would be the biggest challenges for the surgeon?

8. What would be the benefits to a) the surgeon and b) the patient resulting from improvements to arthroscopic cartilage repair procedures?

5.0 Related Questions

9. What is your vision for future developments in the field?
Appendix B

Surgeon questionnaire responses

We made a questionnaire in relation to cartilaginous defects in joints and treatments used for these and we circulated it to several influential surgeons in the field. The scope of the questionnaire was to gauge surgical opinion on current options available for the treatment of damage to the articular surface, future advances in the treatment of such defects and the use of arthroscopic procedures in articular surface repair. We received 4 responses. W, an arthroplasty surgeon working in an elective hospital with a special interest in knees. X, an arthroplasty surgeon working in both an elective hospital and a trauma centre. Y, a trauma and orthopaedic surgeon working in an elective centre and a trauma centre, and Z a specialist registrar in orthopaedics with a special interest in cartilage defect repair and tissue engineering.

The first Category was General Questions:

1. What is a typical articular surface defect (size, chondral or osteochondral, depth, location) that you see clinically?
   All of the surgeons were in agreement that there is huge variability in the size in terms of depth and location of the defects, although there may be an increased incidence over the medial femoral condyle and patellar under surface.
   Y – “Irregular usually in shape and depth, size and location vary, all parts of all three surfaces” (i.e. the distal femur, tibial plateau and under surface of patella)
   X – “Variable – all combinations of sizes and depths, chondral > osteochondral, medial femoral condyle and patellofemoral most commonly”
   W – Arthritic Joints frequently have large areas of damage – 50-60% destruction of the joint surface with areas of partial thickness damage and damage progressing to the bare bone. Osteochondral defects are present in 1 in 50 arthritic joints
   Z – Lateral femoral condyle associated with patellar dislocation, medial femoral condyle associated with ACL rupture or other internal rotation event. Each 2-30mm

2. What surgical techniques do you typically use in the treatment of such defects? Give details of open techniques, arthroscopic techniques and the types of devices used if any.
   W – Open: Mosaicplasty carried out through a mini-arthrotomy – not very commonly
   Arthroscopic: Debridement using a shaver, Microfracture using a k-wire or microfracture pick
   X – Open Oats, total knee arthroplasty
   Arthroscopic: Debridement, microfracture, hyaluronic acid injections
   Y – Open rare
   Arthroscopic drilling with drill or k-wire
Z - **Open** TruFit (Smith & Nephew)

**Arthroscopic:** microfracture (abrasion)

**Devices used:** TruFit, MiTek

3. In your opinion what are the biggest limitations relating to current surgical techniques for the treatment of damage to the articular surface?

**Y** – Experimentally unproven and I believe expensive

**X** – Focussing on cartilage too much and not subchondral bone, treating effect rather than cause, incomplete understanding of aetiology and pathogenesis of cartilage regeneration

**W** – Dependent on the presence of intact cartilage around the lesion for good results from treatment options, no current treatment for wide spread cartilage damage, joint replacements are non-biological and have a limited life.

**Z** – Abrasion microfracture may allow fibrocartilage healing. Other devices never worked and have even caused mechanical knee problems upon becoming dislodged

The next category of questions was on cell and/or biomaterial scaffold based techniques for treatment of damage to the articular surface

4. Do you currently use cell based (e.g.: ACI, MACI, microfracture/drilling) or biomaterial scaffold based techniques in your practice?

   a. If yes, which treatments are you using? What would you say are their strengths and weaknesses?

   b. If not, what are your reservations?

For this question, the respondents unanimously replied that the only technique they used in their own practice is microfracture. The main reasons they weren’t using any newer techniques was the lack of evidence to back them up and also the prohibitive cost involved.

**W** – Microfracture/drilling is the main technique used. ACI isn’t used frequently in this country, possibly by a single consultant based in a private hospital

**X** – Microfracture – strengths = simplicity, weakness = delayed rehab, early post op pain, poor success rate. ACI/MACI – probably only appropriate in specialist centres, high cost, delayed rehab, patient acceptance/compliance, not sure re robustness of evidence and long term outcomes. Learning curve for surgeons.

**Y** – not used in my current practice, experimentally unproven and expensive

**Z** – I have never used to date

5. What do you see as the limitation of cell based therapies such as ACI? What is impeding the widespread clinical adoption of cell and tissue engineering based techniques?

**Y** – not focussed enough, everyone doing a little.

**X** – Only appropriate in specialist centres, high cost, delayed rehab, patient acceptance/compliance, unsure about robustness of evidence and long term outcomes, learning curve for surgeons. Also fears of uncontrolled cell proliferation?
The limitations of ACI are the requirement for 2 procedures, currently samples have to be sent abroad (to Denmark) for cell isolation and the cost of the procedure versus the success rate.

Time involved, multiple procedures, conflicting data supporting use, expense

The next set of questions compared open versus arthroscopic techniques in the treatment of articular surface injuries

6. In what situations would you choose to carry out an open procedure rather than an arthroscopic procedure? Describe the limitations of arthroscopic procedures in terms of a) patient factors, b) surgeon factors and c) instrument factors

All debridement and microfracture techniques are carried out arthroscopically as patient outcomes are better when the surgical site is minimised. Mosaicplasty is carried out using an open surgical technique as it is difficult to match the convexity of the surface of a mosaicplasty plug to the 3D joint convexity when visualising the joint space arthroscopically. Arthroscopic techniques lead to improved patient recovery and 3D visualisation can be challenging during arthroscopic surgery.

In terms of patient factors the duration of surgery, the increased risk of thrombosis, infection and fluid extravasation with prolonged arthroscopic procedures. In terms of surgeon factors there is a lack of tactile feedback in arthroscopic work and there can be a lack of expertise of the surgeon the theatre nursing staff and the surgical trainee assistants in arthroscopic techniques. For instrumentation there is a lack of adequate inventory/quality of arthroscopic instruments and the cost is also a factor

I have very limited ideas other than drilling via scope and hope for the best.

The patient should be a young Patient with large defect would suggest opening for an optimal attempt at repair. Arthroscopic is best suited to a defect <35mm and in experienced hands. The surgeon should have experience with arthroscopic techniques and devices and carry out large numbers of procedures regularly. The instrument should be a flexible or angled device to allow ease of access arthroscopically. The instrument should also have multiple sizes available and angle options or a flexible/telescopic device.

Do you feel that there is scope to carry out more complex/advanced procedures arthroscopically? What do you feel would be the biggest challenges for the surgeon?

Evidence base for intermediate/long term efficacy

Scope is there. Challenges are learning curve and training support staff

Yes

Yes, hip arthroscopy is a good example, very steep learning curve for surgeons, now widely accepted as a valuable procedure.

What would be the benefits to a) the surgeon and b) the patient resulting from improvements to arthroscopic cartilage repair procedures

A reliable, easy to use delivery device is required. This will allow more repeatable results.
Y – Patient benefits immense. This is an exciting area for a small number of researchers and surgical centres

X – Having an option for patients who are in a therapeutic no-man’s land, avoiding total knee arthroplasty for weaker indications

Z – The advantages of improvements in articular cartilage repair include definitive repair of lesions, deferred knee replacement or HTO surgery and decreased pain.

The final category of questions was related opinion questions

9. What is your vision for future developments in the field
X – Prevention may be better than cure. The aging process is hard to halt/control/reverse. Some kind of biocompatible adhesive may be the ultimate answer to chondral replacement with accelerated rehab. Long Term clinical outcomes and survival of focal surface replacement devices e.g. hemi cap may influence need/desire for the future development of cartilage regeneration techniques.

Y – None for me personally

W – Larger area joint surface repair for all joints, frequently patients are young e.g. 25 yrs. There are large numbers of patients presenting with signs of degenerative changes in the shoulders and hips. Treatments need to be suitable for use in different joints. Mechanical stability of the implanted device is important for repair

Z – Marrow aspiration, cell culture and transplantation.