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<td>Author(s)</td>
<td>Neary, Martin</td>
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<td>Publication Date</td>
<td>2018-02-14</td>
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<td>Item record</td>
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Cartilage repair in a rabbit model: Development of a novel subchondral defect and assessment of early cartilage repair using a rabbit mesenchymal stem cell seeded scaffold

Dr Martin Neary, MB, BCh, BAO, MRCS, LFOM

Regenerative Medicine Institute, National University of Ireland, Galway
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Declaration

I declare that this dissertation is all my own work. Where I have relied on the work of other people, I have acknowledged this according to the normal academic conventions. I understand that my dissertation may be subject to electronic scrutiny. I consent to the publication of an abstract or abstracts on the Faculty's website.

Signed:

Name in print: Martin Neary

Date: 20th May 2017
Acknowledgements

I would like to acknowledge the following people without whom this thesis wouldn’t have been possible:

My research supervisors Professor Frank Barry, Dr Mary Murphy and Dr Valerie Barron of the Regenerative Medicine Institute (REMedI), National University of Ireland, Galway.

My clinical supervisor Mr Fintan Shannon, Consultant Orthopaedic Surgeon, Galway University Hospital and Bon Secours Hospital, Galway.

My family, in particular my wife Meabh, my son Féilim, and my Mum and Dad who have supported me in getting this work completed.
Chapter 1

Introduction
1.1 Background

Articular cartilage acts to provide smooth, frictionless movement at the interface between two articulating bones, as well as functioning as a weight-bearing and shock-absorbing medium. It is a specialised connective tissue consisting of chondrocytes and collagen (mostly Type II) suspended in an extracellular matrix. Articular cartilage isn’t a uniform tissue, but has discrete functional layers, with different collagen orientation and cell morphology (Fig1.1). Avascular in nature, the chondrocytes rely on diffusion to receive the materials required to produce and maintain the structural components of the cartilage. This poor supply mechanism, compounded by a paucity of chondrocytes, results in a very limited ability for cartilage repair and regeneration, if any at all. This fact underpins the end-stage process in many common orthopaedic conditions, namely cartilage loss e.g. osteoarthritis. Acute injuries can result in chondral or osteochondral defects (Fig 1.2&1.3), clinically manifested as pain, stiffness, and progressive loss of function. The incidence of these lesions vary with age, but are quite common, occurring in approximately 11% of young patients, and up to 60% of older patients (>40 years) \(^1\). Treatment modalities run the gamut from weight loss right up to joint arthroplasty. Established techniques include those classified as Marrow Stimulation Techniques (MSTs). The aim of these modalities is to deliver primitive mesenchymal cells to the site of injury, be it by abrasion arthroplasty, microfracturing (Fig 1.4) or subchondral drilling. The hope is that these cells will differentiate into chondrocytes and facilitate cartilage repair and regeneration. In reality, the best case scenario is that repair with fibrocartilage is achieved. This differs from normal articular cartilage in both its collagen make-up,
being predominantly Type I collagen, and also in its biomechanical properties. Pain relief and restoration of joint function are variable, but good outcomes are seen in selected patients. Problems identified resulting in poor outcomes and failure include poor integration of graft, and transformation of the multipotent stem cells into osteoblasts, resulting in bone formation. Integration is seen in only about 63% of cases.

An area of ongoing interest and development is Autologous Chondrocyte Implantation (ACI). This is a two-stage procedure aimed at repairing a chondral defect. The initial stage is arthroscopy to evaluate the lesion and to harvest pieces of healthy cartilage tissue from less weight-bearing parts of the joint (Fig 1.5). This tissue is then processed in the lab to harvest chondrocytes, which are then cultured in the laboratory to undergo replication and to increase the quantity of cells. A second stage procedure is undertaken to debride the defect and to implant the cultured chondrocytes. The cells are then held in place using a periosteal patch (1st Generation ACI); manufactured cell carriers (2nd Generation ACI); or 3D scaffolds (3rd Generation ACI or Matrix-induced Autologous Chondrocyte Implantation MACI). ACI has been further modified to improve the capacity for hyaline cartilage formation by identifying chondrocytes expressing a cell marker associated with chondrogenesis, Characterised Chondrocyte Implantation (CCI). Surgical technique is identical to ACI, but only chondrocytes exhibiting the desired marker are implanted. Despite these developments, a recent Cochrane review found that there was “insufficient evidence to draw conclusions on the use of ACI for treating full thickness articular cartilage defects in the knee.”
The next area of research interest is the manipulation of stem cells to induce repair with hyaline-like cartilage, as opposed to the less desirable fibrocartilage. Research has looked at stem cell types, cell induction, and cell anchorage. Current models require a scaffold for anchorage of the cells in the defect to facilitate repair. This has been extensively studied in defects using various scaffolds, most commonly a biphasic scaffold to mimic the bone/cartilage layers in an osteochondral defect\(^8\text{--}^{14}\). Research in the area of chondral defects is also promising, showing an improved outcome with stem cell coated scaffolds compared to controls\(^{15,16}\).

---

Fig 1.1 Structure of Articular Cartilage ([http://articularcartilage.yolasite.com](http://articularcartilage.yolasite.com))

Fig 1.2 Chondral Defect
Fig 1.3 Osteochondral Defect

Fig 1.4 Microfracture at Arthroscopy

Fig 1.5 Autologous Chondrocyte Implantation
1.2 Scaffold Design

As mentioned before, articular cartilage isn’t a uniform tissue, but has discrete functional layers, with different collagen orientation and cell morphology. The scaffold studied has a unique manufacturing process, using laser micro-machining technology in combination with thermal crimping methods, that results in a multiphasic composition, designed to replicate the multilayer morphology of native articular cartilage. It is fabricated from polylactic acid poly-ε-caprolactone (PLCL), a synthetic polymer. It provides a support structure for cells to adhere to, migrate through, and lay down repair tissue. It also has a biomechanical role, in that it acts as a weight-bearing material during the repair process.

A biomimetic architecture was created by a combination of laser machining and precise offsetting of the various layers resulted in an open pore structure, with microtunnels visible from the top surface through to the bottom Using scanning electron microscopy SEM (Fig. 1.6), it can be seen that the pore size increases from 180µm in diameter at the top (Fig. 1.6 i) to 200µm x 600µm at the bottom surface of the construct (Fig. 1.6 ii) creating an open tunnel through the structure (Fig. 1.6 iii). The dimension of the completed scaffold were 3mm radius x 1mm depth.

Biomechanical testing of the scaffold post-fabrication demonstrated a mean compressive modulus of 9.98 ± 1.91 MPa, which is in the ideal range previously elucidated to be optimal for cartilage repair\textsuperscript{17}. It also is a slowly degrading material, which improves the scaffold’s biocompatibility\textsuperscript{18}, and minimises the effect on
cartilage regeneration from by-products of degradation, particularly in the first 28 days\textsuperscript{19}.

Fig 1.6. SEM imaging showing structure of scaffold

1.3 Study Aims

The overall aim of my research was to evaluate the suitability and efficacy of a novel scaffold as a treatment modality for cartilage injuries and defects \textit{in vivo}. My study was conducted in tandem with a number of researchers who looked at other important characteristics of the scaffold, such as the biomechanical properties, and the chondrogenic potential of the scaffold \textit{in vitro}. My role in the overarching project was to assess the bio-compatibility of the scaffold and its effects on early cartilage repair.

1.4 Study Design

The study was designed to allow stepwise progression from the pure \textit{in vitro} phase, to the pre-clinical \textit{in vivo} phase. The \textit{in vitro} phase looked at manipulating the methods of mesenchymal stem cell seeding onto the scaffold, in order to provide the optimal
implantable treatment that would be conducive to chondrogenesis when introduced to the pre-clinical defect model. The next stage aimed to take the scaffold closer to the in vivo environment by looking at fixation methods and effects of the scaffold in an explant. Finally, once the scaffold preparation and defect creation protocols had been refined, the pre-clinical phase would assess the bio-compatibility of the scaffold, and the resultant effects on cartilage repair tissue.

1.5 Ethical Declaration

All animal procedures were carried out by suitably qualified personnel, following approval by the National University of Ireland, Galway’s Animal Care and Ethical Research Committee, and were licensed by the Department of Health. All animals were euthanised humanely.

1.6 Financial Disclosure

This research was conducted as a result of funding received from the following sources: European Union’s 7th Framework Programme under grant agreement no. HEALTH-2007-B-223298 (PurStem); Science Foundation Ireland (grant number 09/SRC/B1794); and Wellcome Trust Biomedical Vacation Scholarships grant number WTD004448.

1.7 Author Disclosure

The author has no competing financial interests.
Chapter 2

Scaffold Seeding Optimisation
2.1 Introduction

The initial phase of our study involved *in vitro* work to establish a protocol for scaffold preparation, which would then be used as the standard for future experiments. Previous work on the scaffold had been performed in the institution, and this allowed us to focus the cell seeding concentrations to the range outlined below. This work also identified the optimal volume of medium for delivering the cells onto the scaffold, again outlined below. Human mesenchymal stem cells (hMSCs) were used for this experiment, as the scaffold is designed for human cartilage.

2.2 Aims

- To assess the effect of varying cell seeding density on cell attachment, distribution and migration throughout the scaffold
- To quantify the optimal cell seeding density for use in future experiments
- To assess the effect of fibronectin pretreatment on cell attachment, distribution and migration

2.3 Study Groups

6 groups were defined, with varying seeding cell concentrations, and with or without fibronectin pre-treatment, with n=3 in each group

**Group A:** $0.6 \times 10^6$ cells per scaffold, no fibronectin pretreatment (n=3)

**Group B:** $0.6 \times 10^6$ cells per scaffold, with fibronectin pretreatment (n=3)

**Group C:** $0.9 \times 10^6$ cells per scaffold, no fibronectin pretreatment (n=3)
Group D: 0.9 x 10^6 cells per scaffold, with fibronectin pretreatment (n=3)

Group E: 1.2 x 10^6 cells per scaffold, no fibronectin pretreatment (n=3)

Group F: 1.2 x 10^6 cells per scaffold, with fibronectin pretreatment (n=3)

2.4 Timepoint

24 hours from time of seeding and start of incubation

2.5 Endpoints

- Cell attachment and distribution throughout the scaffold, as visualised using:
  - Fluorescent Microscopy (FM)
  - Scanning Electron Microscopy (SEM)

- Cell attachment, as assessed using Pico Green Assay, which detects double stranded DNA

- Cell viability, as assessed using Live/Dead® Assay (Molecular Probes®, Thermo Fisher, Dublin)

2.6 Methodology

Preparation for scaffold seeding commenced one week prior to the experiment. This time was spent culturing hMSCs (see section 2.7) to yield the quantity required. Once a sufficient number were cultured, solutions with the required cell concentrations were formulated. At the same time, half the scaffolds underwent fibronectin pretreatment (see section 2.8). Fibronectin is a molecule that promotes cell adherence
and migration (Fig 2.1). All scaffolds were seeded, and placed in an incubator for 24 hours. The samples for SEM were fixed in 10% Formalin for a period of 7 days, before undergoing the appropriate preparation protocols for imaging. The samples for Live/Dead Assay were used fresh. The detailed methodology of each aspect of the experiment is described below.

![Fibronectin molecule and interaction with cell](www.ks.uiuc.edu)

### 2.7 Culturing of hMSCs

4 x 10^6 hMSCs Passage 2 (P2) cells were thawed following storage at -80°C as follows. Passage refers to the amount of times a cell has undergone subculture, with P2 indicating that the cells have undergone subculture twice. Prior to thawing the hMSC sample, 10 ml complete hMSC medium was pipetted into a 15 ml centrifuge tube in an operating Biological Safety Cabinet. The vial of cells was held in a water bath preheated to 37°C without letting the water level go above the level of the thread of the vial. The vial was gently agitated while holding in the water bath for a maximum of two minutes. Once the hMSC were thawed, the vial was removed from
the water bath. They were not allowed to reach 37°C. This was ensured by allowing a small crystal of frozen liquid to remain in the vial. The outside of the vial was wiped with an alcohol soaked tissue and placed in the Biological Safety Cabinet. Using a sterile serological pipette, the contents of the vial were transferred into the pre-prepared 15 ml centrifuge tube containing 10 ml complete hMSC medium. The suspension was pipetted up and down to mix the tube contents and then approximately 1 ml of the suspension was pipetted into the vial in order to rinse it. This 1ml was then pipetted back into the centrifuge tube. The hMSC cell suspension was centrifuged at 400 g for 5 minutes. The supernatant was aspirated and the pellet resuspended in 15ml of hMSC medium (Alpha Minimum Essential Medium (MEM), 10% Foetal Bovine Serum, 1% Penicillin/Streptomycin, Fibroblast Growth Factor (FGF) 1ng/ml) and plated in T175 flasks. The flasks were incubated at 37°C and medium was changed every 2-3 days. The cells were examined by light microscopy at each medium change for confluency. Once 90% confluence was reached (Fig 2.2), the cells were trypsinised off the plates as follows.

The medium from the flask was aspirated using a 30 ml sterile serological pipette, without disturbing the monolayer, and discarded in a container of bleach. 10 ml Dulbecco’s Phosphate Buffered Saline (D-PBS) was added to the flask using a 10 ml sterile serological pipette to wash the hMSC. The D-PBS was re-aspirated using a 10 ml sterile serological pipette, again without disturbing the monolayer, and discarded in the container of bleach. 5 ml 0.25% Trypsin – 1mM Ethylenediaminetetraacetic acid (EDTA) mixture was added to each flask using a 5ml sterile serological pipette. The flask was incubated at room temperature for 6 minutes. Using an inverted light
microscope, the cells were observed to ensure that the hMSC had dislodged from the surface of the flask. Inactivation of the 0.25% Trypsin – 1mM EDTA mixture was performed by adding an equal volume of prepared complete hMSC medium to each flask using a sterile serological pipette. Pipetting of the suspension over the bottom surface of the vessel several times to fully dislodge the hMSC was done, and then the cell suspension was transferred to a fresh sterile 15 ml centrifuge tube. This was centrifuged for 5 minutes at 400 g. The supernatant was aspirated and discarded in bleach. The cells were counted using a manual haemocytometer. A yield of 18 x 10^6 cells was noted. The cell pellet was then reconstituted in 600µL of the serum containing medium, giving a concentration of 30 x 10^6 cells per mL. From this stock concentration, two further concentrations were made by dilution: 22.5 x 10^6 cells per mL, and 15 x 10^6 cells per mL. Previous work in our facility identified 40µL per scaffold as the optimum volume for seeding the scaffold. Therefore, 40 µL of each concentration would give 1.2 x 10^6 cells, 0.9 x 10^6 cells, and 0.6 x 10^6 cells per scaffold respectively.

Fig 2.2 hMSC cultures pre-confluency (Pic A); 90% Confluency (Pic B)
2.8 Fibronectin Pre-Treatment

Fibronectin solution was made to a concentration of 10µg/mL by adding 10µg of fibronectin to 1ml of D-PBS. 9 scaffolds were placed in individual wells of a 48-well plate. 100µL of the fibronectin solution was added to each scaffold-containing well. After 1 hour of soaking in the fibronectin solution, the scaffolds were removed and rinsed with D-PBS. This rendered them pretreated and ready for cell seeding.

2.9 Cell Seeding

The 18 scaffolds were placed in a 24-well plate. The above mentioned concentrations of hMSCs were used to seed 3 scaffolds each. To do this, 20µL of cells was carefully pipette onto each scaffold, raising a droplet on the top (Fig 2.3). The plate was returned to an incubator at 37°C. After 30 minutes, the scaffolds were turned over using a non-toothed surgical forceps and a further 20µL of cells was pipetted onto the other side. Again, the plate was returned to the incubator for 30 minutes. Following this period, 1ml of complete medium was added to each well. The scaffolds were incubated at 37°C for 24 hours.

Fig 2.3 Raised droplet of cells on scaffold
2.10 Processing and Analysis of Samples

The scaffolds were removed from the incubator after 24 hours. The complete medium was pipetted off and discarded in bleach. The scaffolds and wells were rinsed with D-PBS to remove any remaining medium, and this was also discarded in bleach. The scaffolds were removed from the wells. 2 scaffolds from each group were placed into 10% Formalin for 7 days for fixation. The remaining 1 scaffold from each group was held fresh, to immediately undergo a Live/Dead Assay. 200µL of cell culture grade water were added to each well. The plate was then stored at -20°C to allow later analysis of DNA content using a Pico Green Assay.

2.11 Processing for Live/Dead Assay

Live/Dead® Assay solution from Molecular Probes® was made up as follows, in accordance with the manufacturer’s instructions. The Live/Dead® reagent stock solutions were removed from the freezer and allowed to warm to room temperature. 20 µL of the supplied 2 mM Ethidium Homodimer 1 (EthD-1) stock solution (Component B) was added to 10 mL of sterile, tissue culture–grade D-PBS, vortexing to ensure thorough mixing. This gave a 4 µM EthD-1 solution. The reagents were combined by transferring 5 µL of the supplied 4 mM calcein AM stock solution (Component A) to the 10 mL EthD-1 solution. The resulting solution was vortexed to ensure thorough mixing. This 2 µM calcein AM and 4 µM EthD-1 working solution was then added directly to cells. The final concentration of DMSO is ≤ 0.1%, a level generally innocuous to most cells. The aqueous solutions of calcein AM are susceptible to hydrolysis and therefore were used within one day. To prepare the
samples, the following steps were followed. 100–150 µL of the combined Live/Dead® assay reagents were added to each scaffold to ensure all the cells and material were covered with solution. The scaffolds were incubated for 30–45 minutes at room temperature. The samples were wrapped in tin foil during transfer to the imaging room. Following incubation, 10 µL of the fresh Live/Dead® reagent solution was added to a clean microscope slide. Using fine-tipped forceps, the scaffolds were placed on the microscope slide. The scaffolds were then imaged using a confocal microscope.

**2.12 Processing for Scanning Electron Microscopy**

Scaffolds for scanning electron microscopy were fixed in 10% Formalin for 7 days. Progressive dehydration using increasing concentrations of ethanol (50%, 75%, 80%, 90%, 100%) was carried out. The scaffolds were left in each concentration for 5 minutes at a temperature of 4°C. After the dehydration with alcohol, each scaffold was placed into hexamethyldisilazane (HMDS) for 30 minutes at room temperature. They were allowed to air dry, before being mounted and gold plated.

**2.13 Processing for Cryosectioning**

Scaffolds for cryosectioning were fixed in 10% Formalin for 7 days. They were then flash frozen in OCT. This was performed by suspending a beaker of isopentane in a larger container of liquid nitrogen. The scaffolds were placed on the plunger of a syringe and covered in OCT. When crystals were forming at the bottom of the isopentane beaker, the sample was lowered into the beaker. The sample was held
there for 30 seconds to ensure complete freezing. The samples were stored at -80°C until sectioning.

2.14 Pico Green Assay

A 1xTE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) solution was prepared from the 20x stock provided in the Quant-iT Kit. Dilute PicoGreen solution was prepared using a 200-fold dilution of DMSO stock in 1xTE. Next, dilute DNA stock (100µg/ml) 20µl was added to 980µl 1xTE to get DNA working stock concentration of 800ng/ml. DNA standards as follows were prepared:

<table>
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<tr>
<th>DNA Working Stock</th>
<th>1xTE</th>
<th>Final Concentration of DNA per ml</th>
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<tr>
<td>400µL</td>
<td>0µL</td>
<td>2000ng</td>
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<tr>
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<td>200µL</td>
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<td>390µL</td>
<td>50ng</td>
</tr>
<tr>
<td>4µL</td>
<td>396µL</td>
<td>20ng</td>
</tr>
<tr>
<td>0µL</td>
<td>400µL</td>
<td>0ng</td>
</tr>
</tbody>
</table>

Fig 2.4 DNA Standards

The samples for analysis were diluted 1:20 (20µl sample + 380µl 1xTE). To the wells of a 96-well plate 100µl of the appropriate standards and samples to be assayed in triplicate were added. 100µl of PicoGreen solution was added to each well and the plate was then incubated at room temperature for 2-3 min in the dark. The plate was read on a fluorescent plate reader using Pico DNA protocol (Fig 2.5).
Protocol description

Protocol name .................. Fluorescein (485nm/535nm, 0.1s)

Name of the plate type .......... Generic 8x12 size plate

Number of repeats .............. 1

Delay between repeats .......... 0 s

Measurement height ............. 8.00 mm

Name of the label ............... Fluorescein (0.1s)

Label technology ............... Prompt fluorometry

CW-lamp filter name .......... F485

CW-lamp filter slot .......... A5

Emission filter name .......... F535

Emission filter slot .......... A5

Measurement time ............... 0.1 s

Emission aperture ............. Small

CW-lamp energy ................. 13056

Second measurement CW-lamp energy

Emission side ................. Above

CW-Lamp Control ............... Stabilized Energy

Excitation Aperture .......... N/A

Fig 2.5 Pico Green Assay Protocol
2.15 Results

Results Contents

2.15.1 Pico Green Assay
2.15.2 Live/Dead Assay
2.15.3 SEM Microscopy
2.15.4 Cryosectioning

2.15.1 Pico Green Assay

Each well from the seeding experiment described above was analysed in triplicate during the Pico Green Assay run. This yielded 9 results for each study group. The average of these readings was taken to give a representative figure of the remaining DNA levels in wells in each group. Analysis of the DNA standards revealed an $R^2$ value of 0.9998, indicating a high level of accuracy in the assay run (Fig 2.6). The readings show that increasing the cell seeding concentration results in increased attachment of cells onto the scaffolds, as evidenced by the decreasing amount of DNA remaining in the well (Fig 2.7), which is proportional to the amount of cells remaining in each well, that therefore did not undergo attachment. From this, we can see that the optimal treatment concentration studied is $1.2 \times 10^6$ cells per scaffold.

When comparing fibronectin pre-treatment, whilst it had no effect in the lower concentration groups, it resulted in better cell adherence in the optimal $1.2 \times 10^6$ cells concentration group, as shown by the decreased DNA quantity remaining in the wells (4210 vs 2142 ng/ml).
Following staining with the proprietary Live/Dead assay, the scaffolds were imaged to assess the viability of cells on the surface, with cells staining green being considered viable. Fluorescent images were also taken. Images showed numerous cells present on the scaffold (Fig 2.8). In addition, the majority of cells stained green, indicating that they remained viable on the scaffold (Fig 2.9)
Fig 2.8 Fluorescent Image showing cells on scaffold (4x magnification). Cells appear as bright green dots, and can be seen distributed throughout the surface of the scaffold.

Fig 2.9 Live cells on the scaffold (20x magnification), with the bright green dots representing the nucleus of each cell.

2.15.3 SEM Microscopy

Imaging was performed using SEM to look at the cell attachment, distribution and migration on the surface and cross-sectional planes of the scaffolds. This technique allowed us to look closer at the samples, looking for individual cells, their
morphology, adherence and interaction with other cells. At the 0.6 x 10⁶ cells concentration without fibronectin, sparsely distributed individual cells are visible on the surface, with little or no migration into the scaffold interior (Fig 2.10). As the concentration increases in the group untreated with fibronectin, the number of cells on both the surface and interior increases, with the cells starting to form a thin confluent layer (Figs 2.11 and 2.12). When looking at the fibronectin treated groups, again the cell numbers and migration increases as the concentration increases (Figs 2.13-2.15). When compared with their counterpart concentrations in the untreated group, increased numbers of cells, cell migration, and a thicker confluent cell layer is seen. This can be seen most markedly when comparing fig 2.12 with Fig 2.15.

Fig 2.10 0.6 x 10⁶ cells, no fibronectin (Left: Surface; Right: Cross-sectional)

SEM performed on scaffolds following fixation in 10% Formalin, dehydration in alcohol and HDMS, and coating in gold.
Fig 2.11 0.9 x 10^6 cells, no fibronectin (Left: Surface; Right: Cross-sectional)

Fig 2.12 1.2 x 10^6 cells, no fibronectin (Left: Surface; Right: Cross-sectional)

Fig 2.13 0.6 x 10^6 cells, with fibronectin (Left: Surface; Right: Cross-sectional)
Fig 2.14 $0.9 \times 10^6$ cells, with fibronectin (Left: Surface; Right: Cross-sectional)

Fig 2.15 $1.2 \times 10^6$ cells, with fibronectin (Left: Surface; Right: Cross-sectional)

2.15.4 Cryosectioning

Cryosectioning was unsuccessful due to physical destruction of the scaffold during sectioning, and no usable slides were obtained.
2.16 Discussion

In order for chondrogenesis to occur following implantation of the scaffold, a number of factors need to be present. There needs to be cells present on, and throughout, the material, and these cells need to be alive and viable. The cells need to be numerous enough, and close enough together to form interactions and connections, without the material being too overpopulated causing competition. The seeding method needs to be accurate, convenient and easily reproducible.

The seeding method used in our study is simple, quick and accurate. Cell attachment and distribution was confirmed using the images obtained with fluorescent microscopy and scanning electron microscopy. These images demonstrated that cells adhere to the surface of the scaffold, and also migrate into the internal channels of the material. The scanning electron microscopy images showed that increasing cell seeding density resulted in increased cell adherence and migration, with the maximum levels seen in the \(1.2 \times 10^6\) cells concentration, especially when pre-treatment with fibronectin is performed. This combination of parameters resulted in numerous cells migrating into the interior channels, and a complete and confluent cell layer on the surface. Pico-green assay identified that higher concentrations of cells decreased the amount of DNA left in the plate wells. From this, we can infer that less cells remain in the well, and therefore more adhere and migrate onto the scaffold. Finally, Live/Dead assay confirmed the viability of cells on the scaffold after 24 hours of incubation.
Having carried out the above experiments and correlated the results, a standardised protocol for cell seeding of the scaffold was instituted for the remainder of the study. The protocol is outlined below in its entirety.

2.17 Standardised Cell-seeding Protocol

1. Prepare a solution of cells to a concentration of 30 million cells/mL. This yields the desired $1.2 \times 10^6$ cells per scaffold in a volume of 40µL.

2. Place scaffold in well of a 48-well plate.

3. Prepare a fibronectin solution to a concentration of 10µg/mL by adding 10µg of fibronectin to 1ml of D-PBS.

4. Add 100µL of the fibronectin solution to each scaffold-containing well using a pipette.

5. After 1 hour of soaking in the fibronectin solution, remove the scaffold and rinse with D-PBS.

6. Using a P200 pipette, add 20µl of cell suspension to the scaffold, raising a droplet on the surface.

7. Incubate the scaffold at 37 °C for 30 minutes.

8. Invert the scaffold using a sterile tweezers.

9. Using a P200 pipette, add 20µl of cell suspension to the other side of the scaffold.

10. Incubate at 37 °C for 30 minutes.

11. Add 500µl of cell culture medium (Alpha MEM, 10% Foetal Bovine Serum, 1% Penicillin/Streptomycin, FGF 1ng/ml) to each well containing cell-seeded scaffold.

12. Incubate at 37 °C for 24 hours.
3.1 Introduction

Explant models allow for more information to be obtained before progression to an animal model study. An explant model involves using animal tissue in an *in vitro* experiment. Explant tissue can be taken from different animals in different constructs. For this project, the decision was taken to use bovine explant tissue. An osteochondral explant model previously described by Theodoropoulos et al\(^\text{20}\) was felt to be an appropriate and useful model to use. Other models that were considered included a chondral explant described by Hunter et al\(^\text{21}\).

3.2 Bovine Osteochondral Explant Model

3.2.1 Aim

- To adapt and optimise a model for harvesting osteochondral explant constructs

- To develop and optimise a model for creating a chondral defect in explant constructs

- To evaluate scaffold fixation methods

- To evaluate cartilage repair *in vitro* using an explant model and to compare it to spontaneous regeneration and regeneration using an autologous chondral graft
3.2.2 Groups

4 groups were studied as outlined below, with n=3 in each group:

1. Empty defect (n=3)

2. Empty scaffold (n=3)

3. MSC seeded scaffold (n=3)

4. Autologous chondral graft (n=3)

Each group will have 1 replicate fixated with each of Hyaluronic Acid (HA), Surgical Cyanoacrylate glue, and suture.

3.2.3 Endpoints

- Gross examination of scaffold retention
- Histological imaging of constructs and cartilage repair

3.2.4 Duration

7 days from insertion of scaffolds

3.2.5 Methodology

As mentioned above, Theodoropoulos et al’s osteochondral explant model was identified for use in our study. This required the harvesting of the constructs from bovine metacarpophalangeal joints (MCPJ). The joints were sourced from a local abattoir. They were received fresh and were transported in a cool box to maintain tissue integrity. The joints were obtained by cutting the metacarpal bone 10cm
proximal to the MCPJ. This ensured that the joint capsule was kept intact to maintain sterility. It also allowed the specimen to be secured with a G-clamp during explant harvest. To harvest the construct, an arthrotomy into the MCPJ was performed. The proximal articular condyles were exposed. A circular coring drill bit (Ø 15mm) was used to create a construct to a depth of 10mm, with an electric saw blade used to remove the cylinder of tissue (Fig 3.1). The explants were subjected to three 20 minute washes in PBS before being transferred into Ham’s F12 (supplemented with 1% Penicillin/Streptomycin) and incubated overnight at 37°C. The following day, a 3mm biopsy punch was used to create a full thickness chondral defect. These procedures were all performed in such a way as to maintain sterility of the samples.

Fig 3.1 Osteochondral Explant Construct

3.2.6 Pilot Study

We performed a 1 week pilot study using three constructs (one empty defect, one empty scaffold, one autologous chondral graft (Fig 3.2)). During the course of the pilot study, it was found that the above mentioned construct dimensions meant that
the constructs were too large for 6-well culture plates. 50ml Falcon tubes were used as an alternative. The samples were cultured in complete chondrogenic medium (CCM) (Fig 3.3), which was changed every 48 hours. At the first medium change, it was noted that the constructs had rotated position, dislodging both the scaffold and the chondral graft. The pilot study was ended early because of this.

Fig 3.2 Pilot Study Explant Constructs

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (to make 100ml)</th>
<th>Final Concentration</th>
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<tr>
<td>DMEM (HG)</td>
<td>95ml</td>
<td></td>
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<tr>
<td>Dexamethasone 1mM</td>
<td>10µl</td>
<td>100nM</td>
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<tr>
<td>Ascorbic acid 2-P: 5mg/ml</td>
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<td>50µg/ml</td>
</tr>
<tr>
<td>L-Proline: 4mg/ml</td>
<td>1ml</td>
<td>40µg/ml</td>
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<tr>
<td>ITS+ supplement</td>
<td>1ml</td>
<td>6.25 µg/mL bovine insulin 6.25 µg/mL transferrin</td>
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<tr>
<td></td>
<td></td>
<td>6.25 µg/mL selenous acid 5.33 µg/mL linoleic acid</td>
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<td>1ml</td>
<td>100U/mL penicillin 100µg/mL streptomycin</td>
</tr>
<tr>
<td>TGFβ-3</td>
<td>0.5µl</td>
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</tr>
</tbody>
</table>

Fig 3.3 Complete Chondrogenic Medium (CCM)
3.2.7 Limitations

This method presented limitations that precluded its use in our study. Difficulties were encountered during the harvesting of the explant constructs with the electric tools. There was a high loss of constructs due to cartilage shearing from the drill bit (Fig 3.4), and inadvertent expulsion of constructs onto non-sterile surfaces by vibration of the saw blade. It was difficult to achieve uniformity of samples due to variation in size of the articular facet, and the need to maintain sterility compromising the technique. Integrity of construct tissue was affected from bone burning from the high-speed saw/drill and also repeated manipulations. There was also a certain danger to the technicians from the high-speed equipment, frequent blade breakages, and equipment slipping off bone. Efforts were made to overcome these limitations by using an Osteochondral Autograft Transfer System (OATS®) harvest device to create the explant constructs. However, the hardness of the bone again caused difficulty, resulting in damage to the device and unsuccessful harvesting of constructs (Fig 3.5).

Fig 3.4 Cartilage Damage from saw blade and drill bit
3.2.8 Results

Due to the difficulty of the harvest technique and the high rate of loss of samples, the decision was made to change to a chondral explant model. Also, the technical difficulties raised by the pilot study precluded any further study of fixation methods.

3.3 Chondral Explant Model

3.3.1 Change of Model

Following the decision to change to a chondral explant construct, Hunter et al’s$^{21}$ model was felt to be the most viable option. This model uses cartilage discs from the tibial plateau of a bovine stifle joint.

3.3.2 Aim

- To adapt and optimise a model for harvesting chondral explant constructs
- To develop and optimise a model for creating a chondral defect in explant construct
• To assess scaffold fixation methods and retention rates

### 3.3.3 Groups

4 groups were studied as outlined below, with n=3 in each group

1. Control (n=3)

2. Hyaluronic Acid Solution (Suplasyn® Joint Injection, Mylan Healthcare) (n=3)

3. Surgical cyanoacrylate glue (Dermabond®, Ethicon US) (n=3)

4. Suturing-circumferential, crossover and subchondral (n=3)

### 3.3.4 Endpoints

- Gross examination of scaffold retention
- Histological imaging of constructs and cartilage repair

### 3.3.5 Duration

7 days from scaffold insertion

### 3.3.6 Methodology

Bovine stifle joints were sourced from a local abattoir. The joint capsule was left intact to maintain sterility of the cartilage until the harvesting procedure. Using a sterile technique, an arthrotomy was performed and the tibial plateau was exposed. A sharp blade was used to shave the articular cartilage off in one large piece (Figs 3.6 and 3.7). An 8mm trephine was then used to create the construct discs (Fig 3.8).
Following this, a 3mm biopsy punch was used to create a defect to a depth of 1mm (Fig 3.9). The discs were culture overnight in Alpha MEM (containing 10% Foetal Bovine Serum and 1% Antibiotics). Next, the defects were washed out with dPBS to prepare them for scaffold insertion. Empty scaffolds were secured into the defects using the various methods (Figs 3.10-3.14). The constructs were incubated in a 12-well plate in Alpha MEM (containing 10% Foetal Bovine Serum and 1% Antibiotics) for 7 days at 37°C. The medium was changed every 48 hours. Any dislodged scaffolds were recorded at each medium change.

Fig 3.6 Tibial plateau following removal of cartilage

Fig 3.7 Cartilage with trephine
Fig 3.8 Harvested constructs

Fig 3.9 Construct with created defect

Fig 3.10 Hyaluronic Acid Solution
Fig 3.11 Surgical Cyanoacrylate Solution

Fig 3.12 Crossover Suture Technique

Fig 3.13 Subchondral Suture Technique
3.3.7 Macroscopic Results

Scaffold retention rate was assessed at each medium change. All scaffolds secured with the HA solution failed to be retained, with loss of retention at day 2, day 4 and day 5 for the respective samples (Fig 3.15). The crossover suture technique also failed, with loss of retention at day 2 (Fig 3.16). The other suture techniques and the surgical cyanoacrylate glue method all resulted in scaffold retention in the defect to the day 7 endpoint (Figs 3.17-3.19).
Fig 3.16 Crossover Suture Failure

Fig 3.17 Subchondral Suture Technique

Fig 3.18 Circumferential Suture Technique
3.3.8 Microscopic Results

Samples were fixed in 10% Formalin for 10 days. Samples were then processed for microscopy by dehydration in increasing concentrations of ethanol (50%, 75%, 80%, 90%, 100%) for 1 hour at each concentration. Lastly, the samples were placed in Histoclear® for 1 hour. Next, they were mounted in paraffin wax. Unfortunately, despite careful sectioning, no useable slides were obtained for histological examination.
3.4 Discussion

The explant study resulted in two possible methods being considered for use in vivo for scaffold fixation, namely either surgical cyanoacrylate glue, or one of the two successful suturing techniques. Given the clinical relevance and real-life application of suture fixation, this was chosen as the preferential method. In addition, it was felt that the cyanoacrylate glue would provide a physical barrier to cell migration in and out of the scaffold, as well as potentially damaging nearby cartilage. The circumferential suture technique was chosen as the method for use in our pilot study, as it replicates the technique used to secure the periosteal flap in Autologous Chondrocyte Implantation (ACI).
In Vivo Chondral Defect Pilot Study
4.1 Introduction
Following on from our explant study, we wanted to investigate further an appropriate method for creation of a chondral defect in vivo, and also to further evaluate the fixation methods found to be successful i.e. circumferential suturing. This involved a two stage experiment. The first stage was an in vitro concept testing experiment. Two methods of chondral defect creation were to be assessed, namely a novel method, which would be compared with a method adapted from one described by Aroen et al. Following identification of the optimal defect creation method, the second stage, a pilot in vivo study to identify the best fixation method was undertaken.

4.2 In vitro Defect Creation Optimisation

4.2.1 Aims

- To compare two methods of chondral defect creation for use in a rabbit model

4.2.2 Methods

- 6 post-mortem rabbit femurs (giving 12 condyles)
- Surgical procedures as outlined below

4.2.3 Groups

2 groups were studied as outlined below, with n=6 in each group

1. Novel freehand method (n=6)

2. Adapted microscopic method (n=6)
4.2.4 Endpoints

- Histological examination of defects to assess adequacy of cartilage removal, sharpness of lateral wall of defect, adequate depth of defect (to subchondral plate), and integrity of subchondral plate

4.2.5 Chondral Defect Creation Optimisation

A novel defect creation method was compared with a method adapted and modified from a technique originally described by Aroen et al\textsuperscript{22}. The novel method consisted of defect creation using a Ø3mm biopsy punch freehand under direct visualisation. Firstly, the punch was used to defined the circumference of the defect, pushing until resistance from the subchondral plate was felt. Next, the punch was used to remove the cartilage within the defined defect. The modified technique from Aroen et al\textsuperscript{22} consisted of defect creation using a dental curette, visualised under an operating microscope. The microscope was positioned to give a good view of the medial femoral condyle. Again, a Ø3mm biopsy punch was used to define the borders of the defect, with the surgeon pushing until he felt the resistance of the subchondral plate. The dental curette was then used to remove all the cartilage within the defined defect, down to the subchondral plate (Figs 4.1 - 4.4).
Fig 4.1 Margins of defect created with biopsy punch

Fig 4.2 Dental curettage of cartilage

Fig 4.3 Dental curettage of cartilage
Fig 4.4 Finished defect

4.2.6 Sample Processing

Following defect creation, the distal femurs were fixed for 10 days in 10% Formalin. Following fixation, samples were decalcified in Surgipath® Decalcifier. The solution was changed every 3 days. Decalcification was deemed to be complete following 2 consecutive negative tests using 5% Ammonium Oxalate/5% Ammonium Hydroxide and removed Surgipath® Decalcifier solution in a 1:1:1 ratio (Fig 4.5). It was also confirmed by testing the subchondral bone with a 27G hypodermic needle. Once decalcification was confirmed, the samples were processed using an automatic tissue processor. They were then mounted in paraffin wax prior to sectioning.
4.2.7 Histological Evaluation

5μm sections were taken from the centre of the created defects. Staining with Toluidine Blue was performed. Sections were then visualised and photographed using a light microscope connected to a computer.

4.2.8 Histological Results

Following staining, the slides were visualised using light microscopy. Defects from both methods were assessed, looking for adequacy of cartilage removal, sharpness of lateral wall of defect, adequate depth of defect (to subchondral plate), and integrity of subchondral plate. The freehand method resulted in irregular removal of the cartilage, with cartilage remaining in the defect in nearly all cases (Fig 4.6). In some cases, the required depth wasn’t reached, with cartilage remaining in the base of the defect (Fig
4.6A). Integrity of the subchondral plate couldn’t be guaranteed with this method, as a number of samples showed evidence of breaching of the plate (Fig 4.6C). In comparison, the operating microscope method was superior, with uniform defects, free of cartilage, with clean lateral walls and an intact subchondral plate (Fig 4.7).

Fig 4.6 Freehand technique defects
Fig 4.7 Microscope technique

4.2.9 Discussion

The operating microscope method resulted in a reproducible pure chondral defect, which was well defined and maintained the integrity of the subchondral plate. This method was used as the optimal procedure for chondral defect creation in our pilot study.
4.3 In Vivo Chondral Defect Pilot Study

4.3.1 Aims

• To establish a full thickness chondral defect in the medial femoral condyle

• To assess ACI style sutures as a fixation method.

• To assess cellular migration from the marrow to the scaffold following defect creation

4.3.2 Number of Subjects

3 New Zealand White rabbits (6 knees)

4.3.3 Groups

3 groups were studied as outlined below, with n=2 in each group

1. Untreated defect with empty scaffold (2 knees)

2. Microfractured defect with empty scaffold (2 knees)

3. Untreated defect with MSC loaded scaffold (2 knees)

4.3.4 Timepoint

28 days from surgical procedure
4.3.5 Endpoints

- Scaffold retention in defect
- Gross appearances of cartilage surface and joint structures
- Histological appearance of defect

4.3.6 Scaffold Seeding Technique

Scaffolds were placed in wells of a 48-well plate. Fibronectin solution was made to a concentration of 10µg/mL by adding 10µg of fibronectin to 1ml of D-PBS. 9 scaffolds were placed in individual wells of a 48-well plate. 100µL of the fibronectin solution was added to each scaffold-containing well. After 1 hour of soaking in the fibronectin solution, the scaffolds were removed and rinsed with D-PBS. Using a P200 pipette, 20µl of cell suspension was added to each scaffold (made to a concentration of 30 million cells/mL; 1.2 million cells per scaffold), raising a droplet on the surface. The scaffold was incubated at 37 °C for 30 minutes, and then inverted using a sterile tweezers. Using a P200 pipette, 20µl of cell suspension was added to the other side of each scaffold. Again, this was incubated at 37 °C for 30 minutes. Following this, 500µl of cell culture medium (Alpha MEM, 10% Foetal Bovine Serum, 1% Penicillin/Streptomycin, FGF 1ng/ml) was added to each well containing a cell-seeded scaffold. The scaffold was incubated overnight at 37 °C.

4.3.7 Surgical Procedure

The rabbits’ knees were shaved prior to surgery. Subjects were anaesthetised using a weight-adjusted dose of ketamine (35mg/kg) and xylazine (10mg/kg). When
required, isoflurane was given as maintenance anaesthesia via face mask. Pulse oximetry monitoring was established by placing a probe on the subject’s non-operative hind paw. The surgeon scrubbed, then prepped and draped the subject’s leg. Access to the knee joint was achieved via an anterior midline skin incision, followed by a median para-patellar joint capsule incision. The patella was dislocated laterally to provide increased exposure of the medial femoral condyle. The operating microscope was positioned to give a good view of the medial femoral condyle. As described before, a Ø3mm biopsy punch was used to define the borders of the defect, with the surgeon pushing until he felt the resistance of the subchondral plate. A dental curette was then used to remove all the cartilage within the defined defect, down to the subchondral plate. Following this, fixation methods were undertaken. The patella was relocated and closure was in layers using Vicryl® 4/0 sutures. Antibiotic prophylaxis with Enrofloxacine (10mg/kg), and post-operative analgesia with butenorphanol (2mg/kg), were given via IM injection. The subject was recovered and allowed to mobilise freely post-operatively. Daily weights and wound inspections were undertaken for the first 7 days, and weekly thereafter.

4.3.8 Rabbit 1

The first animal was randomised to have one untreated defect with an empty scaffold, and one microfractured defect with an empty scaffold. The first chondral defect was created successfully as described above. A 10/0 Ethilon® microsurgical suture was used to fix the scaffold into the defect (Fig 4.8). However, due to the narrow thickness of the articular cartilage, it was impossible to achieve any good purchase
with the suture, despite repeated careful attempts. This resulted in the suture repeatedly slicing through the cartilage and causing excessive damage (Fig 4.9). Given the failure of the fixation method and the increasing length of surgical time, the procedure was abandoned. The wound was closed in layers and the rabbit awoken and recovered.

Fig 4.8 Suturing of scaffold

Fig 4.9 Cartilage damage from suture
4.3.9 Rabbit 2

Following the failure of the suture fixation method, the decision was made to investigate the use of surgical cyanoacrylate glue further. The second rabbit had been randomised to receive MSC-seeded scaffolds in untreated defects bilaterally. Chondral defects were created bilaterally as above. In the first defect, the scaffold was covered in glue and placed into the defect. The wound was closed in layers. In the second defect, a sterile PLCL membrane was glued over the scaffold to fix it in the defect. Again, the wound was closed in layers. However, again, this method was very technically challenging. The scaffolds were very prominent in the joint and appeared clinically to disrupt the normal anatomy of the knee joint by occupying the joint space and impeding the range of joint motion.

4.3.10 Rabbit 3

Following the difficulties outlined above, and the decisions made following the results, it was felt that it would be unethical to continue with the procedure on the final rabbit.

4.3.11 Results

The two subject rabbits were sacrificed at 28 days using intramuscular (IM) ketamine (35mg/kg) and xylazine (10mg/kg), followed by an intravenous (IV) overdose of sodium pentobarbital (100mg/kg). Final weights were taken prior to sacrifice. The samples were inspected grossly. The sample from the failed suture fixation showed evidence of some fibrocartilage repair. Both of the scaffolds fixed using glue had
become dislodged. They both promoted an inflammatory response and were found encapsulated in the soft tissues of the joint. The associated condyles both showed evidence of significant deterioration, with marginal osteophytic change (Fig 4.10 & 4.11).

Fig 4.10 Glue only subject (with inflammatory mass circled)

Fig 4.11 Glue and film subject (with inflammatory mass circled)
4.4 Discussion

Chondral defect models in rabbits have previously been used successfully in studies looking at periosteal flaps, ACI, and gel matrices. We were not aware of any studies at the time of our work that had used a chondral defect model to look at synthetic scaffolds. We had hoped to adapt the current models to be compatible with our scaffold. We demonstrated a technique that resulted in a reproducible chondral defect in rabbit medial femoral condyles. However, we were unable to develop a fixation method that was reliable and easily reproducible in vivo. The study identified a number of reasons why a chondral defect model wasn’t optimal for studying our scaffold. Firstly, the thin nature of rabbit articular cartilage precluded the use of sutures as a fixation method. Secondly, the use of surgical glue was unsuccessful in retention, and also promoted a clinically visible inflammatory response. Finally, the prominence of our scaffold after fixation in the defect required a very secure fixation method to ensure retention, which we were unable to achieve. For these reasons, a subchondral model was identified as a more viable working model, and our further work focused on this avenue of research.
Chapter 5

Pre-clinical Rabbit Study
5.1 Introduction

Planning of a pre-clinical study requires careful consideration of a number of important factors\(^\text{23}\). The aim of a pre-clinical study is proof of concept and assessment of safety before progression to clinical trials in humans. The ideal animal for use in pre-clinical studies would conform to certain characteristics that replicate the disease processes in adult humans. For cartilage repair, these characteristics include development of spontaneous cartilage lesions in cartilage of comparable thickness to humans, in a similar anatomical location. The comparable thickness should allow for easy fixation and retention of repair constructs. The animal should be large enough to allow for gait analysis and to allow ease of surgical interventions. Pain should be easily quantifiable, and should correspond to lesion severity. In reality, an ideal animal is hard to find so careful selection to maximise the above factors should be undertaken. Economical and housing factors also influence choice of animal.

Human cartilage thickness on the medial femoral condyle was measured arthrographically as 4.3mm in men and 3.6mm in women\(^\text{24}\). More recent cadaveric studies have shown thicknesses in the range of 1.65-2.65mm\(^\text{25, 26, 27}\). Animal cartilage thickness varies with species: rabbit 0.3mm, mini-pig 1.5mm, pig 3.2mm, sheep 0.5mm, goat 0.7-1.5mm\(^\text{27}\). Obviously, studies using species with a comparable cartilage thickness to humans (mini-pig, pig, goat) are desirable. However, these are expensive studies to run and are usually preceded by pilot or proof of concept studies in smaller animals. Rabbit models have long been used in cartilage repair research. Advantages of rabbit models include lower cost, smaller facilities required for
housing, ease of handling, and the ability to use large numbers of genotypically similar replicants. Drawbacks arise from the anatomy, biomechanics and inherent healing ability of the rabbit knee\textsuperscript{28}. Cartilage thickness of 0.3mm limits the size of defect created, as well as posing technical difficulties in creation of the defect, as I discovered throughout the course of my work. Increased knee flexion compared to humans results in unique loading mechanisms in the knee joint, with rabbits using their patellar groove as a partial weight-bearing surface. Human cartilage has little or no ability for spontaneous repair. In comparison, rabbit cartilage has been shown to demonstrate significant intrinsic repair capability. These factors can reduce the ability to draw definitive comparisons from results in rabbits with what occurs in humans. However, rabbit models are still an extremely useful tool, as a both a precursor to larger animal models, as well as for biocompatibility work. The International Cartilage Regeneration Society (ICRS) recommends that animals should be 8 months of age to ensure skeletal maturity\textsuperscript{23}. Weight can be used as a guide for skeletal maturity, with the range of 2.5-3.5kg used\textsuperscript{39}.

Studies looking at cartilage regeneration in rabbit models generally use one of two defects, either the previously mentioned chondral defect\textsuperscript{22,30,31,32}, or a 3-5mm deep osteochondral defect\textsuperscript{33,34,35,36,37,38,39}. Both are well validated models.

The medial femoral condyle was chosen as the site for defect creation, as there is less spontaneous repair at this location\textsuperscript{31}.

As we showed in our pilot study, the chondral defect model was unsuitable for use with our scaffold. Similarly, the osteochondral defect was felt to be too deep to be a
viable model, and no pilot study was performed. We decided that a defect to match the dimensions of our scaffold (3mm diameter x 1mm depth) was the most appropriate option. Using a defect of this depth would result in a subchondral defect model. At the time of the study, we were not aware of any previous work using a subchondral defect rabbit model. We developed a theoretical model using an electric drill (outlined below), in the hope it would be effective, and comparable to the well-established osteochondral model, insofar that it would cause enough cartilage damage to induce spontaneous repair. The second arm of this study looked at the biocompatibility of the scaffold, and also the effects on early cartilage repair in the subchondral defect.

5.2 **Aims**

- To develop a novel 1mm deep subchondral defect
- To assess the biocompatibility of the proprietary scaffold
- To assess spontaneous repair in the novel subchondral defect
- To look at the effects of the scaffold on early cartilage repair tissue

5.3 **Methods**

- 12 skeletally mature New Zealand White rabbits weighing 3.0-3.5kg were used
- Bilateral medial femoral condyle defects were created during a single surgery
5.4 Groups

4 groups were studied as outlined below, with n=6 in each group

1. 3mm untreated osteochondral defect (n=6)

2. 1mm untreated subchondral defect (n=6)

3. 1mm subchondral defect treated with empty scaffold (n=6)

4. 1mm subchondral defect treated with rabbit mesenchymal stem cell seeded (rMSC) scaffold (n=6)

5.5 Subject Randomisation

Subjects were randomised to each study group using Microsoft Excel® as below, with the four-digit number representing the rabbit’s unique identification number, and ‘L’ or ‘R’ representing the left or right femoral condyle. Each femur was treated as an individual, meaning that most rabbits had a different treatment or non-treatment on each knee. This helped give good inter- and intra-specimen variation.

| 1027L | Empty Scaffold |
| 1027R | Subchondral Defect |
| 1028L | Empty Scaffold |
| 1028R | Empty Scaffold |
| 1029L | Subchondral Defect |
| 1029R | Subchondral Defect |
| 1030L | Osteochondral Defect |
| 1030R | Osteochondral Defect |
| 1031L | rMSC Seeded Scaffold |
| 1031R | Empty Scaffold |
| 1032L | rMSC Seeded Scaffold |
| 1032R | Osteochondral Defect |
| 1033L | rMSC Seeded Scaffold |
| 1033R | Osteochondral Defect |
| 1034L | Subchondral Defect |
| 1034R | Empty Scaffold |
| 1035L | rMSC Seeded Scaffold |
| 1035R | Subchondral Defect |
| 1036L | Osteochondral Defect |
| 1036R | rMSC Seeded Scaffold |
| 1037L | Subchondral Defect |
| 1037R | rMSC Seeded Scaffold |
| 1038L | Empty Scaffold |
| 1038R | Osteochondral Defect |

Fig 5.1 Subject Randomisation
5.6 Timepoint

28 days from surgical procedure

5.7 Endpoints

- Gross appearance photo-documentation and assessment using modified ICRS guidelines\textsuperscript{37}
- Histological imaging following staining with Toluidine Blue, and staining for Types I and II Collagen

5.8 Scaffold Seeding Procedure

1 \times 10^6 P0 rabbit Mesenchymal Stem Cells (rMSCs) from each of 3 donors were thawed and plated into T175 flasks and cultured at 37°C until 5 \times 10^6 cells from each donor were cultured. Once this amount were present, they were trypsinised and counted. Using the seeding technique described previously, 3 scaffolds were seeded with 1.2 \times 10^6 rMSCs per scaffold from each donor. This yielded 9 seeded scaffolds, with 3 scaffolds from each donor. 2 scaffolds were to be used in the experiment, with 1 spare in case of unforeseen loss of the study scaffolds.

5.9 Surgical Procedure

The rabbits’ knees were shaved prior to surgery. Subjects were anaesthetised using a weight-adjusted dose of ketamine (35mg/kg) and xylazine (10mg/kg), given intramuscularly. When required, isoflurane was given as maintenance anaesthesia via face mask. Pulse oximetry monitoring was established by placing a probe on the subject’s
non-operative hind paw. The subject’s operative leg was secured in a retort stand (Fig 5.2). The surgeon scrubbed, then prepped and draped the subject’s leg. Access to the knee joint was achieved via an anterior midline skin incision, followed by a median para-patellar joint capsule incision (Fig 5.3). The patella was dislocated laterally to provide increased exposure of the medial femoral condyle. The defect was created with an electric hand drill, with a pre-sterilised 2.8mm diameter drill bit, using a depth stop to achieve the desired depth, either 1mm or 3mm (Fig 5.4, Fig 5.5). The edges of the defect were finished with a dental curette. If the subject was in a scaffold group, press fitting of the scaffold into the defect was performed (Fig 5.6). The patella was relocated and closure was in layers using Vicryl® 4/0 sutures. Antibiotic prophylaxis with Enrofloxacine (10mg/kg), and post-operative analgesia with butenorphanol (2mg/kg), were given via intra-muscular injection. The subject was recovered and allowed to mobilise freely post-operatively. Daily weights and wound inspections were undertaken for the first 7 days, and weekly thereafter.
Fig 5.2 Leg position for surgery

Fig 5.3 Medial para-patellar capsular incision
Fig 5.4 Electric drill bit with depth stop

Fig 5.5 Empty defect

Fig 5.6 Scaffold press-fitted into defect
5.10 Harvesting of Samples

Animals were sacrificed at 4 weeks using intra-muscular ketamine (35mg/kg) and xylazine (10mg/kg) for sedation, followed by an intra-venous overdose of sodium pentobarbital (100mg/kg). Final weights were taken prior to sacrifice. The distal femurs were retrieved and were photographed prior to and following clearing of soft tissue (Fig 5.7). The gross appearances were recorded on a proforma sheet, adapted from ICRS guidelines\(^\text{40}\) (Table 5.1). Samples were fixed for 10 days in 10% Formalin. Following fixation, samples were decalcified in Surgipath\(^\text{®}\) Decalcifier. The solution was changed every 3 days. Decalcification was deemed to be complete following 2 consecutive negative tests using 5% Ammonium Oxalate/5% Ammonium Hydroxide and removed Surgipath\(^\text{®}\) Decalcifier solution in a 1:1:1 ratio. It was also confirmed by testing the subchondral bone with a 27G hypodermic needle. Once decalcification was confirmed, the samples were processed using an automatic tissue processor. They were then mounted in paraffin wax prior to sectioning.
Fig 5.7 Photographs of sample in situ and following cleaning

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<td>Macroscopically visible roughening</td>
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<td>Fibrillations on articulating surface</td>
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<td>Degradation of articular surfaces</td>
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<td>Presence of osteophytes</td>
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<td>Evidence of synovial inflammation</td>
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<td>Evidence of hypertrophy</td>
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<td>Evidence of pannus</td>
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Table 5.1 Macroscopic evaluation of joint and repair tissue after necropsy (ICRS Guidelines)
5.11 Tissue Sectioning

Following processing, 5µm sections were taken at intervals of 0.75mm, 1.5mm, and 2.25mm from where the edge of the defect was identified. These measurements corresponded respectively to one quarter, one half, and three quarters of the way into the defect. Sections from the centre of the defect were preferentially stained, as they would show the most amount of detail.

5.12 Toluidine Blue Staining

Sections were stained with 1% toluidine blue for 5 min. at 60°C, following dehydration in solutions of increasing concentrations of ethanol.

5.13 Collagen I and Collagen II Staining

Two methods were undertaken to stain for each collagen subtype. Each method was carried out on separate slides. A positive staining for each collagen subtype was indicated by the presence of dark brown/black colour in the defect. The presence of Collagen I in repair tissue is indicative of fibrocartilage, with the presence of Collagen II indicating hyaline-like cartilage.

For collagen type I and II immunostaining, an endogenous hydroxide quench was performed with 0.3% hydrogen peroxide (H$_2$O$_2$) in methanol after rehydration. Thereafter, antigen retrieval was performed using pepsin (DAKO, S3002 4% in 0.2 N HCl, Agilent Technologies, Dublin) for 30 min, followed by blocking with 5% rabbit serum in trisbuffered saline (TBS 0.05 M tris, 0.15 M NaCl, pH 7.6) for collagen type I and 10% goat serum (KPL 71-00-27, Insight Biotechnology, Middlesex) in TBS for
collagen type II. Sections were incubated overnight at 4 °C with goat polyclonal anti-type I collagen antibody (1:100, S1301-01, Southern Biotech, Birmingham, AL, USA) and with mouse monoclonal anti-rabbit type II collagen antibody (1:50; AF5710, Acris, Herford, Germany). Sections were then incubated with biotinylated secondary antibodies against rabbit anti-goat (H + L) (1:1000; 305-065-003 Jackson ImmunoResearch, Newmarket) for collagen type I or goat anti-mouse (H + L) (1:1000; KPL 71-00-29, Insight Biotechnology, Middlesex) for collagen type II followed by peroxidase-conjugated streptavidin (KPL 71-00-38) at room temperature for 30 min each and stained for visualisation with 3,3¢-diaminobenzidine (DAB) (Abcam, substrate kit, ab94665, Cambridge, UK). Sections were counterstained with Harris hematoxylin for 10 s and eosinphloxine B for 1 min. After staining, all sections were dehydrated in a series of alcohols (70, 95 and 100%), cleared with histoclear and mounted for imaging.

5.14 Results

Results Contents

5.14.1 Gross Results
5.14.2 Toluidine Blue Staining
5.14.3 Collagen Subtype Staining

5.14.1 Gross Results

At necropsy, the study joints were assessed for gross signs of degradation and arthritic changes. All samples were photo-documentated in situ and following removal and cleaning. The signs of joint damage and degradation were examined using
guidelines adapted from the ICRS (Table 5.1), and were recorded on a proforma
document adapted from the same.

In the untreated subchondral defect, 5 out of 6 samples showed osteophyte formation,
4 out of 6 showed evidence of synovial involvement (inflammation, hypertrophy,
adhesions), 4 out of 6 showed roughness of the cartilage surface. When the defect fill
was assessed, 5 samples showed a moderate fill, and the remaining 1 showed a good
fill. However, only 3 out of 6 showed homogenous fill. In the osteochondral defect
group, 4 out of 6 samples showed osteophyte formation, 5 out of 6 showed evidence
of synovial involvement, 3 out of 6 showed roughness of the cartilage surface. When
the defect fill was assessed, 1 sample showed minimal fill, 4 samples showed a
moderate fill, and the remaining 1 showed a good fill. 5 out of 6 showed homogenous
fill. These results demonstrate that the novel subchondral method is sufficient to
induce an osteoarthritic model, and is comparable to the well-described osteochondral
method.

In the empty scaffold treated subchondral defect group, no samples showed
osteophyte formation, 3 out of 6 showed evidence of synovial involvement, 1 out of 6
showed roughness of the cartilage surface. When the defect fill was assessed, 3
sample showed minimal fill, and 3 samples showed a moderate fill. However, all
samples had a homogenous fill. In the rMSC seeded scaffold treated group, 1 sample
showed minimal osteophyte formation, 4 out of 6 showed evidence of synovial
involvement, no sample showed roughness of the cartilage surface. When the defect
fill was assessed, 2 sample showed minimal fill, and 3 samples showed a moderate fill, and 1 showed good fill. Again, all samples showed homogenous fill.

The gross inspection results show that the novel subchondral model is sufficient and suitable to use, as it results in an osteoarthritic reaction, and also induces spontaneous repair, as evidence by the untreated group, and when compared to the traditional osteochondral model. Cartilage damage and arthritic changes were reduced in the scaffold treated groups. In addition, the gross cartilage repair quality is improved and more consistent in the treated groups, when compared to the untreated groups.

Fig 5.8 Representative images showing gross appearances in each group
5.14.2 Toluidine Blue Staining

This technique was used to look at a number of criteria of the defects. Firstly, the bio-compatibility of the scaffold was elucidated by looking for the stigmata of material rejection and tissue inflammation, namely bone cyst formation, giant cells, monocytes and macrophages. None of these features were seen in any sample, indicating that the material is inert, doesn't promote a foreign body response, and is bio-compatible with rabbit tissue.

Next, the depth and diameter of the subchondral defects were measured. A depth of 1mm and diameter of 3mm was seen in all subchondral defects (Figs 5.9-5.11). This indicated that the novel creation method was consistent, accurate and reproducible.

Finally, the repair tissue present in each defect was visualised, looking specifically at the tissue organisation, cell distribution, and cell morphology. All empty defects, both subchondral and osteochondral, showed evidence of spontaneous cartilage repair. However, the repair tissue was of poor quality, with no lamination or organisation of the cells (Fig 5.9). These features are indicative of fibrocartilage repair. The presence of cartilage repair tissue in the subchondral defect proves that the novel model is sufficient to induce spontaneous repair, and is comparable to the traditional osteochondral model.

In the scaffold groups (Figs 5.10 & 5.11), both empty and seeded, the repair tissue was more organised, with consistent distribution of the cells in a layered fashion, with a decrease in the number of cells as you approach the articular surface, similar to native cartilage. There is good integration of the neo-tissue with the native tissue. In
addition, the repair tissue cells are small with nicely rounded nuclei, and have the appearance of ‘neo-chondrocytes’. Interestingly, the repair tissue in the empty scaffold group (Fig 5.10) appears to be of a better quality than the rMSC seeded scaffold group (Fig 5.11), with greater defect fill and tissue organisation.
Fig 5.9 Empty subchondral defect (A 10x magnification; B 20x magnification)

Fig 5.10 Empty scaffold treated subchondral defect (A 10x magnification; B 20x magnification)

Fig 5.11 rMSC scaffold treated subchondral defect (A 10x magnification; B 20x magnification)
5.14.3 Collagen Subtype Staining

Collagen subtype staining demonstrated the presence of large amounts of Collagen I in the untreated subchondral defects, with little or none present in the scaffold treated defects, both empty and rMSC seeded (Fig 5.12). Conversely, no Collagen II was visualised in the untreated subchondral defects, but with large amounts seen in the scaffold groups (Fig 5.13). The repair tissue in the empty scaffold treated group stained more intensely for Collagen II than the rMSC seeded scaffold group. These results indicate that the repair tissue in the untreated defects is fibrocartilagenous in nature, whereas the scaffold treated groups have repaired with hyaline-like cartilage.

Fig 5.12 Collagen I Staining

Fig 5.13 Collagen II Staining
5.15 Discussion

Our pre-clinical portion of the study yielded a number of positive results. In the surgical method developed, we had produced a novel subchondral model that had not been described in a rabbit before. We showed that the technique was simple, efficient, user-friendly and reproducible. This technique produces consistent and accurate defects throughout all samples. It may be applicable for other researchers to use in future studies, and is easily adaptable to any depth of defect. Most importantly, we demonstrated that the depth of the defect is sufficient to induce spontaneous cartilage repair, which allows for successful control groups.

The absence of any signs of material rejection or inflammatory response confirmed the biocompatibility of the scaffold material, and its suitability for use with *in vivo* models. This is significant, as any degree of inflammatory reaction would alter the dynamics of the cartilage repair process.

Whilst our study ended at a time-point of 28 days, in comparison to ICRS’s guideline of 84 days for optimal cartilage repair studies, we can make some reasonable assumptions in respect of the repair tissue. As evidenced by the Toluidine Blue images, the scaffold groups produced repair tissue that was more structured, and which resembled the multiphasic nature of native articular cartilage. In addition, the repair cells took on the appearance of early chondrocytes. These appearances were in stark contrast with the disorganised spontaneous repair tissue visualised in the untreated defect groups.
The presence of Collagen II in the scaffold treated groups suggests the presence of the more desirable hyaline-like cartilage in the repair tissue, which is further suggested by the relative absence of Collagen I in these groups. Indications that the spontaneous repair tissue in the untreated defect is the biomechanically inferior fibrocartilage are seen with the presence of a large amount of Collagen I visualised.

As noted previously, it was found that the quality of repair tissue differed between the scaffold treated groups, with the empty scaffold producing superior repair when compared with the rMSC-seeded scaffold. We postulate that this may be due to the host rabbit forming some sort of rejection response to the donor cells, which affected the cartilage repair process. In addition, with the empty scaffold, there was access to the defect site for the subject’s own stem cells from the marrow to migrate, and attach unimpeded. It would appear that the scaffold forms the more important component when promoting repair, with it’s unique design providing a structural framework for organised tissue formation and deposition by stem cells.

Our pre-clinical rabbit study demonstrated that we had developed a successful novel subchondral defect model; that the material in the scaffold is bio-compatible; and that treatment of subchondral defects with the scaffold promotes improved early cartilage repair.

My work was the pre-clinical part of a larger study, with other investigators conducting research into the optimal biomechanical, chemical and physical structure; cell viability on the scaffold; and in vitro chondrogenesis using the scaffold. A paper
detailing the work was published in the Annals of Biomedical Engineering, a peer-reviewed international journal (Article Pubmed ID 26438451).
Chapter 6

Discussion
This study was designed to assess the biocompatibility of a novel proprietary scaffold in a rabbit model, and to look at the effect this scaffold had on early cartilage repair. Whilst an initial methodology was devised, shortcomings discovered in the implementation of the proposed outline resulted in the need to rethink and to adapt the protocols and procedures used. In some instances, this required the development of novel approaches and models.

The cell seeding process was optimised as the first step in the process. It was shown that pre-treatment with fibronectin produced increased adherence and migration of cells, for which an optimal concentration of $1.2 \times 10^6$ cells per scaffold was identified. Cells seeded onto the scaffold migrated throughout the porous network in the interior of the material, and they remained viable after a period of 24 hours. Therefore, they were viable at time of surgical implantation. This series of in vitro work resulted in a protocol that was refined to provide the best conditions for the mesenchymal stem cells to undergo differentiation and commence chondrogenesis, and facilitate cartilage repair.

The explant phase of the study commenced with the identification of a bovine osteochondral model described by Theodoropoulos et al. Unfortunately, this model proved technically difficult to adapt to our requirements, and the decision was made to use an alternative chondral explant, as originally described by Hunter et al. This model had a number of problems also, but positive results were yielded in the form of
two potential scaffold fixation methods for use in further work, namely suturing and surgical cyanoacrylate glue.

The results of both the above experiments were translated into the pilot chondral defect study, conducted using three rabbits. It quickly became evident that the dimensions of scaffold, with the associated technical limitations in respect of fixation and interference with joint biomechanics, precluded the use of a pure chondral defect model. On ethical grounds, the study was concluded early.

Review of the literature demonstrated an apparent absence of suitable subchondral defect models described in rabbit subjects. This necessitated the development of a novel surgical approach to create a defect of appropriate dimensions and depth. Histological evaluation validated the reproducibility and accuracy of this model, with evidence of uniformity of defects throughout all the samples.

The subchondral defect is of sufficient depth to induce spontaneous repair, which is an important characteristic as it means that empty replicates can function as a control and comparison. This confirms its suitability as a novel alternative to the well established osteochondral defect used throughout the literature. The dimensions of the defect are also conducive to adaptation to the individual study requirements. The gross anatomical changes differed between the untreated groups, and the scaffold treated groups, with the former demonstrating an increased preponderance towards degenerative changes. The improved gross appearance outcome seen in the scaffold
treated groups suggests that it has a positive effect on delaying or preventing arthritic changes. This could be considered an important result in its own right, irrespective of the histological appearance, as these degenerative changes are the cause of the clinical manifestations and presentations of human arthritis, and the associated morbidity and suffering. If the scaffold prevents these sequelae, whether by biomechanical or biological means or both, then it is an important finding.

Within the pre-clinical model, one of the key questions was the biocompatibility of the PLCL material in the scaffold, and any possible adverse effects on the native tissue. The criteria of evidence of inflammatory response was used, and assessed on the histological images. The absence of monocytes, macrophages, giant cells and cyst formation proved that the PLCL material is inert, and has no effect on the host’s immune system or native tissue. Any provocation of the inflammatory system has a detrimental effect on the formation and lay down of cartilage repair tissue. The ideal repair tissue will replicate native cartilage, both in terms of physical structure, biochemical make up, and biomechanical properties. The closer any reparative attempt can be to natural cartilage, and any influences that can facilitate this, should result in improved outcomes clinically for sufferers of degenerative arthritic conditions. Our scaffold induces the repair tissue to develop in a manner that is very similar to native articular cartilage, and is superior to the repair in the untreated defect. The repair achieved with the scaffold shows organised distribution of cells, and structured struts of tissue. The cells demonstrate the appearance of neo-
chondrocytes. In addition, the repair construct has integrated with native tissue, as evidenced at the lateral walls of the treated defects.

Interestingly, the repair tissue in the empty scaffold group is superior to rMSC seeded scaffold group, with more organisation, integration, and increased numbers of neo-chondrocytes. One possibility to explain this is that the depth of the subchondral defect allows access to the subject’s bone marrow, and the associated mesenchymal stem cells. These may produce the improved repair, as there is no interplay of factors such as host-donor response, or cell degradation, which may occur when donor MSCs are used. It appears to suggest that the scaffold itself is sufficient to improve cartilage repair, possibly by providing the support structure and impetus for chondrocytic differentiation, and the framework for organised tissue formation and deposition.

The work within our study provides the groundwork for progression in this area of research. We set out to study the biocompatibility of the novel scaffold, and the effect it had on cartilage repair. We were successful in our aims, in that we showed that the scaffold was biocompatible, and that it positively influenced the quality of cartilage repair tissue. Another positive outcome that arose was the need to develop a surgical method to create a novel subchondral defect model. This model may now be utilised by other researchers looking at similar sized constructs, and it is also easily adaptable to constructs of other dimensions.
If used as a biocompatibility and proof of concept study, the results of our work would justify funding for progression to a larger animal study, with the joint and cartilage characteristics mentioned previously more replicative of human joints. This would bring the scaffold closer to a human model, and if our results were replicated could allow progression to clinical trials.

Our scaffold improves the quality of early cartilage repair. The work carried out has scope to provide the basis for further research. A study looking at the biomechanical, as opposed to histological, characteristics of the repair cartilage would be of benefit, as this information would help predict the impact on the outcome in humans. Another potential avenue of research would be the modification of the scaffold to include chondrogenic promoting factors within the design, to further improve the repair process.

Our study achieved its aims, and also produced some interesting results in terms of the factors that affected the quality of early cartilage repair. It will provide a solid basis for further study and development of the proprietary scaffold, which will hopefully result in eventual progression to clinical trials.

Since completing this study, the field of cartilage regeneration has continued to develop, and current research is now moving towards the use of natural construction materials in scaffold fabrication. Kang et al have demonstrated success with the use of human cartilage extra-cellular matrix (ECM) derived 3D scaffolds, achieving
improved repair when used in conjunction with autologous adipose derived stem cells\textsuperscript{41}. Other materials being successfully studied include material such as fibrin hydrogels\textsuperscript{42}, collagen\textsuperscript{43}, agarose\textsuperscript{44}, and cellulose\textsuperscript{45,46}.

The optimum cell type, or even whether to use cells at all, continues to be researched, with numerous studies looking at variations of combinations. The most successful repairs appear to be from a combination of stem cells with chondrocytes. Dahlin et al demonstrated that co-cultures of MSCs with chondrocytes improved cartilage repair in rats when compared to empty scaffolds, or either cell type in isolation\textsuperscript{47}. De Windt et al studied co-cultures of allogenic MSCs and recycled chondrons in 35 human patients\textsuperscript{48}. They found a statistically significant clinical improvement at 12 months in all patients. Analysis of biopsied cartilage repair in these patients demonstrated patient DNA only, and no trace of the allogenic stem cells. These studies suggest that stem cells may play a role in cartilage regeneration through trophic and paracrine effects.

The area of cartilage regeneration remains an evolving and innovative field of research, and as evidenced in the preceding paragraphs, is a rapidly progressing discipline.
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