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Cartilage extracellular matrix-derived scaffolds for joint regeneration

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Trinity College Dublin, January, 2016

A thesis submitted to the University of Dublin in partial fulfilment of the requirements for the degree of

Doctor in Philosophy

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Declaration

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Henrique Martiniano Vazão de Almeida

Dublin, January, 2016

Summary

The goal of this thesis was to address key limitations associated with autologous chondrocyte implantation (ACI) for articular cartilage regeneration, specifically the need for two hospital stays, cell culture and high cost. As an alternative, this thesis explored the combination of freshly isolated stromal cells and a novel chondroinductive scaffold as a putative alternative to ACI. Consequently, the first objective of this thesis was to develop and optimize a chondro-permissive device able to deliver stem cells and other chondrogenic factors. Extracellular matrix (ECM)-derived materials have previously been used to enhance cartilaginous tissue formation and regeneration. Hence, the first step was to develop a scaffold derived from articular cartilage ECM that could be used as a growth factor delivery system to promote chondrogenesis. Porous scaffolds were fabricated using devitalized cartilage, which were then seeded with human infrapatellar fat pad-derived stem cells (FPSCs). It was found that these scaffolds promoted chondrogenesis, especially when stimulated with transforming growth factor (TGF)-β3. The superior chondrogenesis in the presence of exogenously supplied TGF-β3, led to explore whether this scaffold could be used as a growth factor delivery system. When these scaffolds were loaded with TGF-β3, comparable chondrogenesis to continuous adding TGF-β3 to the media was observed.

The next step of this thesis was to optimize the scaffold itself and demonstrate that this scaffold could promote chondrogenesis of freshly isolated stromal cells *in vivo*. By freeze-drying cryomilled cartilage ECM of differing concentrations, it was possible to produce scaffolds with different architectures. Migration, proliferation and differentiation of FPSCs depended on the scaffold concentration/porosity, with greater sGAG accumulation observed with increases in pore size. Next, it was sought to demonstrate that fresh stromal cells, when seeded onto a TGF-β3 eluting ECM-derived scaffold, could promote chondrogenesis *in vivo*. While a more cartilage-like tissue could be generated using culture expanded FPSCs compared to non-enriched freshly isolated cells, fresh $CD44^+$ stromal cells were capable of producing a tissue *in vivo* that stained strongly for sGAGs and type II collagen. These findings open up new possibilities for in-theatre cell based therapies for joint regeneration.

Therefore, once it was demonstrated that it was possible to deliver growth factor and chondro-potent cells in an optimized ECM-derived scaffold *in vitro* and *in vivo*, the next step was to assess the effect of different doses of exogenously supplied TGF-β3 in different FPSCs donors (healthy and diseased). After comparing the different donors in escalating TGF-β3 conditions it was possible to conclude that the high dose enabled higher matrix formation consistently for all donors. No disparity was observed between healthy and diseased donors.

ECM-based biomaterials are commonly xenogeneic, which may elicit an adverse immune response. Native human ECM can be used as an alternative to xenogeneic tissue; however, its supply is limited leading to the need for more readily available source of material. Hence, scaffolds were produced using ECM from xenogeneic articular cartilage, and sheets of engineered cartilage using stem cells. Engineered ECM presented some of the features of

native cartilage, although it contained lower levels of type II collagen. Scaffolds produced using both engineered and native ECM possessed similar properties. However, engineered ECM-derived scaffolds supported inferior chondrogenesis when seeded with FPSCs. TGF-β3 eluted in engineered ECM-derived scaffolds enhanced their capacity to support chondrogenesis, to levels comparable to the native ECM-derived constructs.

Cartilage ECM was then used to further functionalize well known biomaterials, specifically a fibrin hydrogel and an alginate scaffold. This thesis first explored functionalizing an injectable fibrin hydrogel with cartilage ECM particles and TGF-β3 for cartilage regeneration. Even in the presence of such levels of ECM, chondrogenesis of FPSCs within these fibrin constructs was enhanced when additionally stimulated with TGF-β3. ECM particles could also be used to control the delivery of TGF-β3 to FPSC within fibrin hydrogels *in vitro*, and furthermore, led to higher levels of sGAG and collagen accumulation compared to control constructs loaded with gelatin microspheres. *In vivo*, freshly isolated stromal cells generated a more cartilage-like tissue within fibrin hydrogels functionalized with cartilage ECM particles. These tissues stained strongly for type II collagen and contained higher levels of sGAGs.

Finally, the overall goal of the last part of the thesis was to develop a mechanically stable anisotropic alginate scaffold featuring shape-memory and biomimetic properties to be used in cartilage regeneration. For this end an architectural and an additional collagen functionalization were performed. The architectural change was created using a directional freezing technique. This enabled the creation of an aligned structure, which improved the mechanical properties. The functionalization with type II collagen improved cell recruitment and consequent tissue formation throughout the construct. Incorporating such collagen into the alginate scaffold did not negatively influence the shape-memory properties of the structure. Coating with type II collagen enabled superior chondrogenesis when seeded with human FPSCs. Compared with coating with type I collagen, type II collagen improved cell proliferation, higher sGAG and collagen accumulation, and the development of a stiffer tissue. These findings open up the possibility of using cartilage ECM-derived type II collagen to functionalize anisotropic shape-memory alginate scaffolds in order to enhance their capability to regenerate cartilage.

Both the ECM-derived scaffold and other biomaterials (fibrin and alginate) functionalized with ECM, should be considered for cartilage tissue regeneration in man. These devices in combination with stromal cells and growth factors carried by the ECM-derived scaffold or ECM functionalized devices have shown significant promise as therapeutics for driving articular cartilage repair, overcoming current cell-based limitations observed for example in ACI.

Acknowledgements

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Nomenclature

AB – Alcian blue
AC – Articular cartilage
ACI – Autologous chondrocyte implantation
ACL – Anterior cruciate ligament
ASC – Adipose-derived mesenchymal stem cell
AF – Aldehyde fuchsin
Align – Alginate scaffold with alignment
ASC – Adipose-derived stem cells
BM-MSC – Bone marrow-derived mesenchymal stem cell
BMP – Bone morphogenic protein
BSA – Bovine serum albumin
C1B – Alginate scaffold with alignment and type I collagen blended
C1C – Alginate scaffold with alignment and type I collagen coated
C2B – Alginate scaffold with alignment and type II collagen blended
C2C – Alginate scaffold with alignment and type II collagen coated
CDM – Chemically defined chondrogenic medium
Coll II – Type two collagen
dH_2O – Deionised water
DHT – Dehydrothermal
DMEM – Dulbecco's Modified Eagle Medium
ECM – Extracellular matrix
EDAC – 1-Ethyl-3-3dimethyl aminopropyl carbodiimide
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme-linked immunosorbent assay
Eng – Engineered cartilage
Eng-MS – cartilage engineered in the presence of TGF-β loaded microspheres
FD – Freeze-drying
FGF – Fibroblast growth factor
FPSC – Infrapatellar fat pad-derived stem cell
GAG – Glycosaminoglycans
GMP – Good manufacturing practice
H&E – Haematoxylin and Eosin
HA – Hyaluronic acid

HCL – Hydrochloric acid

HIM – Helium ion microscopy

IFP – Infrapatellar fat pad

IGF – Insulin-like growth factor

MACI – Matrix-induced autologous chondrocyte implantation

MACS – Magnetic-activated cell sorting

MSC – Mesenchymal stem cell

Non-Al – Alginate scaffold without alignment

OA – Osteoarthritis

PBS – Phosphate buffered saline

PCM – Pericellular matrix

PFA – Paraformaldehyde

PLGA – Poly(lactic-co-glycolic) acid

PLM – Polarized light microscopy

PR – Picro-sirius red

PRP – Platelet-rich plasma

RT – Room temperature

SDS – Sodium dodecyl sulfate

SEM – Scanning electron microscopy

sGAG – Sulphated glycosaminoglycans

TE – Tissue engineering

TGF – Transforming growth factor

UV – Ultraviolet

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Patents

Two patents were submitted during the current PhD. One related with ECM-derived scaffold, and other concerning the use of scaffolds and freshly isolated stromal cells for single-stage therapies for cartilage repair.

Chapter 1

Introduction

1. Introduction

1.1. Cartilage and clinical need

Tissues in the body can undergo self repair, but in many cases a therapeutic intervention is required to facilitate regeneration. Autografts are commonly used to promote repair, however harvesting healthy tissue from within the body is constrained by limited supply and donor site morbidity. Many clinical attempts have been made to induce healing of lesions within articular cartilage (AC), with the aim of re-establishing the functionality of the injured joint. Usually, AC lesions only partially heal and are frequently related with disability and joint pain, leading to osteoarthritis (OA) [1-3]. More than 35 million people in USA suffer from some form of arthritis [4], and nearly 10% of the population worldwide is affected by this disease [5]. Such lesions are generated during the course of many joint diseases or due to trauma. However, unlike bone, cartilage lacks the intrinsic ability to naturally regenerate, due to its avascularity and lack of mobility of the chondrocytes [1, 2].

Unfortunately, a successful and universally accepted approach for cartilage tissue treatment still does not exist [1, 2]. In 1723, the anatomist William Hunter stated “an ulcerated cartilage is a troublesome problem and once destroyed, it never repairs” [6]. Currently, treatment strategies are limited to surgical procedures that seek to encourage the intrinsic capacity of cartilage and subchondral bone to self-heal by facilitating contact with the underlying marrow, or to fill the defect with grafts or cells capable of chondrogenesis. Current surgical techniques typically involve drilling holes into the subchondral bone, thereby allowing blood to invade the damaged area, in theory allowing regeneration of the tissue [1]. These procedures, including abrasion arthroplasty [1, 7, 8], drilling [1, 9-11] and microfracture [1, 12, 13], showed large variability in outcomes with unpredictable quality of the new tissue ranging from no cartilage, to fibrocartilage or occasionally hyaline cartilage [1, 2]. Tissue grafting is

limited by the fact that it requires inflicting damage to healthy tissue [1, 14, 15]. Autologous chondrocyte implantation (ACI) is a common method used in some countries, also requiring the excision of tissue from an undamaged region of the joint with the goal of isolating chondrocytes, expand these cells over many weeks of *in vitro* culture, and finally implanting the cells or the tissue engineered graft into the defect [16, 17]. This approach is limited by high costs (€30-40k) and requires two surgical procedures: one to isolate the cells, and a second to re-implant the expanded cells or engineered tissue.

Tissue engineering (TE) is an exciting field, which uses a combination of biomaterials, cells and bioactive agents to facilitate the regeneration of a damaged or diseased tissue. As another example, we have Matrix-induced autologous chondrocyte implantation (MACI), a variant of ACI where a scaffold is used to support the implanted cells [18]. Alternatively, or in addition, such scaffolds can be loaded with bioactive factors and implanted at the defect site where host cells are recruited and promote tissue repair. Regardless of the specific TE strategy, scaffolds provide the foundation to conduct and sustain regeneration. This tissue healing happens due to the delivery capability of cells and bioactive factors from the loaded constructs [1, 2]. Hence, to develop clinical relevant biomaterial-based scaffolds presents distinct challenges, and requires interdisciplinary work, mainly because of the need for a deep understanding of material science in combination with clinical challenges. Moreover, cell biology, tissue properties and controlled release of bioactive agents (e.g. growth factors) are crucial for the success in this field [2].

In conclusion, although current approaches are reasonably effective in achieving symptomatic relief and improved joint function, they have not been universally successful in preventing the long-term degeneration of the articular joint. While clinical results with tissue engineering strategies such as ACI have improved patient outcomes, such strategies have had limited clinical uptake, mostly due to the high cost, complexity and significant regulatory challenges associated with such

approaches. Hence, novel single-stage strategies for joint regeneration are urgently required [19-22].

1.2. Scaffolds, cells and growth factors for cartilage tissue engineering

1.2.1. Criteria

Scaffolds play an indispensible role in TE, functioning as a three-dimensional carrier of cells, growth factors and other bioactive agents [23]. Scaffolds are not only able to transport biological cues, but can also be engineered to deliver cells into the injured tissue [2, 24]. Scaffolds can also be used as reservoirs of cellular regulators (e.g. growth factors) to initiate host cell recruitment or directly influence differentiation (or maintenance of phenotype) of transplanted cells [2, 23, 24].

To ensure success, scaffolds should be porous to support and allow homogeneous infiltration of seeded cells, and/or migration of host cells and consequent tissue ingrowth. Diffusion of waste products and nutrients is another reason why this type of structures should present adequate porosity [2]. Integration with the surrounding tissue is also crucial for success. To achieve this goal, the scaffold should degrade at a rate that matches that of new tissue formation [2, 25]. To get the ideal degradation rate it is necessary to produce a scaffold with a suitable biomaterial [23]. This material can be from natural or synthetic origin, should be biocompatible and ideally not cytotoxic, to minimize adverse reactions after implantation [2, 23, 26, 27].

1.2.2. Materials

Structurally and biochemically, scaffolds should mimic aspects of the native environment of the tissue to be regenerated, with the aim of providing morphological, chemical and biomechanical cues until the newly produced cell-matrix takes over [28]. Examples of naturally-derived biomaterials [27] used in cartilage tissue engineering are collagen [29], chitosan [30], alginate [31], fibrin [32], silk [33], gelatine [34], hyaluronic

acid (HA) [35] and ECM cartilage-derived materials [36]. Collagen and HA are two of the most commonly used natural-derived biomaterials used clinically in regenerative medicine and TE [24, 35]. Some of the advantages of using these materials are: biodegradability, biocompatibility and the possibility of being engineered to a specific application. In addition, mechanical properties and performance can be adjusted just by altering the crosslinking technique or its percentage. Naturally-derived native extracellular matrix (ECM) has been also used as a scaffold material for cartilage TE [36-39].

On the other hand, synthetic polymeric biomaterials are an alternative to naturally derived materials. Using this type of materials brings some advantages, such as mechanical strength, controlled degradation kinetics, as well as the multitude of methods available to engineer and recreate the biofunctionality of native ECM [24]. Polyglycolic acid (PGA) and polylactic acid (PLA) are examples of synthetic biomaterials used in tissue engineering [40]. Nevertheless, synthetic biomaterials present a number of limitations when compared to natural-derived materials, including integration with the host tissue and problematic degradation products [38].

1.2.3. Cell sources

Success in cartilage TE can maybe best achieved through the optimal combination of cells, materials and biochemical cues. Cells play a critical role on the regeneration of damaged tissue. Chondrocytes or mesenchymal stem cells (MSCs) are the typical cell types used for cartilage TE [24]. Stem cells can be isolated from a range of tissues such as bone marrow [41-47], subcutaneous fat [34, 48-50], infrapatellar fat pad (IFP) [20, 46, 51-60] and synovium [49, 61-65].

This thesis will focus on the use of IFP-derived stem cells (Figure 10), a particularly attractive source of chondro-progenitor cells for cartilage TE, mainly due to two characteristics of this type of cells: (1) they are easily accessible, and (2) possess a strong potential to generate cartilaginous tissue [20, 51, 58]. Furthermore, the yield

of such cells from the IFP is high, opening up the potential of using freshly isolated IFP-derived stromal cells as part of a single-stage therapy for cartilage regeneration [19-22]. Furthermore, it is also pertinent to explore the isolation of specific chondro-potent stromal cells sub-populations (e.g. $CD44^{+}$) [66, 67]. Such single-stage approaches could potentially overcome the need to expand cells *in vitro* for cartilage regeneration therapies, as is currently the case with ACI. To recognise the optimal combination of material and biochemical factors that generate robust chondrogenesis will be crucial for any new stem cell therapy for articular cartilage repair.

1.2.4. Growth Factors

Chondrocyte or stem cell matrix formation is regulated by a synergistic effect between physical and chemical stimulation [68]. These chemical factors include growth factors, which play a key role in cartilaginous tissue maintenance and regeneration. Growth factors known to be chondro-inductive include transforming growth factor-β (TGF-β), insulin-like growth factors (IGFs) and fibroblast growth factor (FGF) [68, 69]. Members of the TGF-β family of growth factors, which play a key role in driving chondrogenesis of stem cells [47, 48, 52, 70-73], have a natural affinity for ECM components such as proteoglycans [74, 75]. This family includes TGF-β1 [76], TGF-β3 [77], bone morphogenic protein-2 (BMP-2) [78], BMP-4 [79] and BMP-7 [80] among others, which have been proven as efficient agents promoting cartilage ECM production [24]. TGF-β family of growth factors can be delivered *in situ* by a panoply of biomaterials such as collagen [81] and fibrin [32].

1.3. Objective

The global objective of this thesis was to develop a novel cartilage ECM-derived scaffold capable of promoting robust chondrogenesis of stem cells. Furthermore, with the ultimate goal of developing a single-stage or in-theatre strategy for joint regeneration, it was aimed to assess whether combining such an ECM-derived scaffold with freshly isolated IFP-derived stromal cells could be used to generate cartilage tissue *in vitro* and *in vivo*. Therefore, the objectives of this thesis are:

- To develop a porous native cartilage ECM-derived scaffold capable of delivering chondrogenic growth factors while efficiently enhancing stem cell chondrogenesis.
- To optimize the ECM-derived scaffold by altering parameters such as porosity to enhance its efficiency in promoting robust chondrogenesis of stem cells.
- Once the scaffold is optimized, the goal is to combine it with freshly isolated stromal cells to be used in off-the-shelf, single-stage therapies for cartilage repair.
- It is recognized that growth factors are critical driving chondrogenesis; however there is still a lack of understanding of the optimal dosing. Hence, an objective of this thesis is to assess the effects of different doses of TGF-β3 in regulating chondrogenesis of human IFP-derived stem cells isolated from diseased and healthy donors.
- Xenogeneic ECM-derived materials maybe are an optimal source of ECM; however it carries a number of limitations. Thus, a further goal of this thesis is to investigate the performance of allogeneic engineered cartilage ECM, and to compare it with native xenogeneic ECM, as a biomaterial to produce porous scaffolds for cartilage TE.
- This thesis will assess if functionalizing a fibrin hydrogel with cartilage ECM will enhance the chondro-inductivity of this biomaterial.

- Cartilage ECM-derived scaffolds may not possess sufficient mechanical properties for the highly demanding joint environment. Consequently, a final objective of this thesis is to functionalize a more mechanically robust anisotropic alginate scaffold with cartilage ECM components to induce chondrogenesis of stem cells.

Addressing these questions is crucial to the clinical translation of single-stage cartilage therapies for joint regeneration.

Chapter 2

Literature Review

2. Literature Review

2.1. Articular cartilage and extracellular matrix

2.1.1. Introduction

The extracellular matrix (ECM) of cartilage has an intricate architecture that creates a complex environment consisting of different types of collagens and proteoglycans in which growth factors, integrins, and functional peptides are integrated (Figure 1). As previously mentioned, this level of complexity will probably never be achieved by highly sophisticated new biomaterials. Moreover, it has been argued that oversimplified biomaterials for cartilage repair are still being used and these limitations are driving tissue engineers to use biomaterials or scaffolds based on processed natural ECM [38, 82].

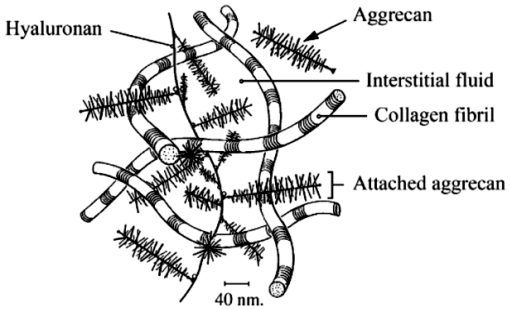


Figure 1 – Representation of collagen matrix interacting with the proteoglycan network, forming a porous fibre reinforced solid ECM in articular cartilage [83].

To regenerate articular cartilage (AC) is a key challenge in the field of TE, and will be the main subject of the present thesis. AC is an avascular, specialized and complex connective tissue, with a unique composition and structure (Figure 2), that it is usually present at the end surfaces of articulating bones [1, 2, 4].

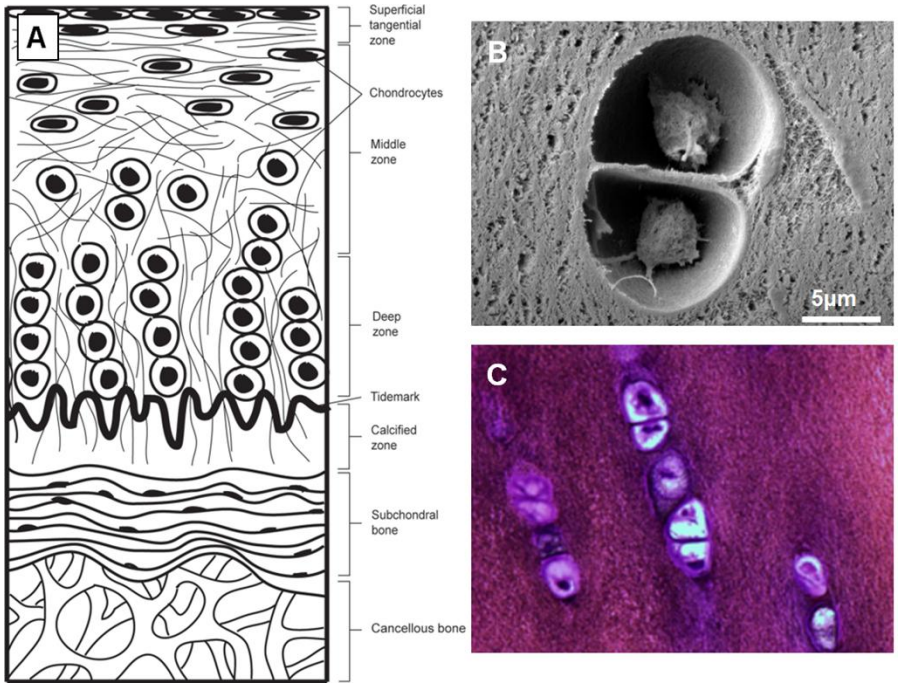


Figure 2 - Structure of articular cartilage (A) [2], chondrocyte scanning electron microscopy (SEM) micrograph (B), and chondrocytes in articular cartilage stained with H&E [84].

Hyaline cartilage is there to provide a low friction surface and facilitate a load transfer between bones and joint [85]. This type of tissue exhibits a high matrix to cell ratio as it is composed predominantly of ECM. Cartilage ECM is produced and maintained by chondrocytes (the cell type within cartilage-Figure 2B and C), and consists on a reinforced network of collagen fibrils that constrains a hydrated proteoglycan gel-like structure [2]. The tissue is able to resist high compressive forces, due in part to the osmotic pressure created by the negative charge within the matrix [2, 4, 86]. Chondrocytes, which maintain the ECM in a normal joint, represent approximately 1% of the total volume of hyaline cartilage [4], and are a product of stem cell differentiation (Figure 3) during embryogenesis.

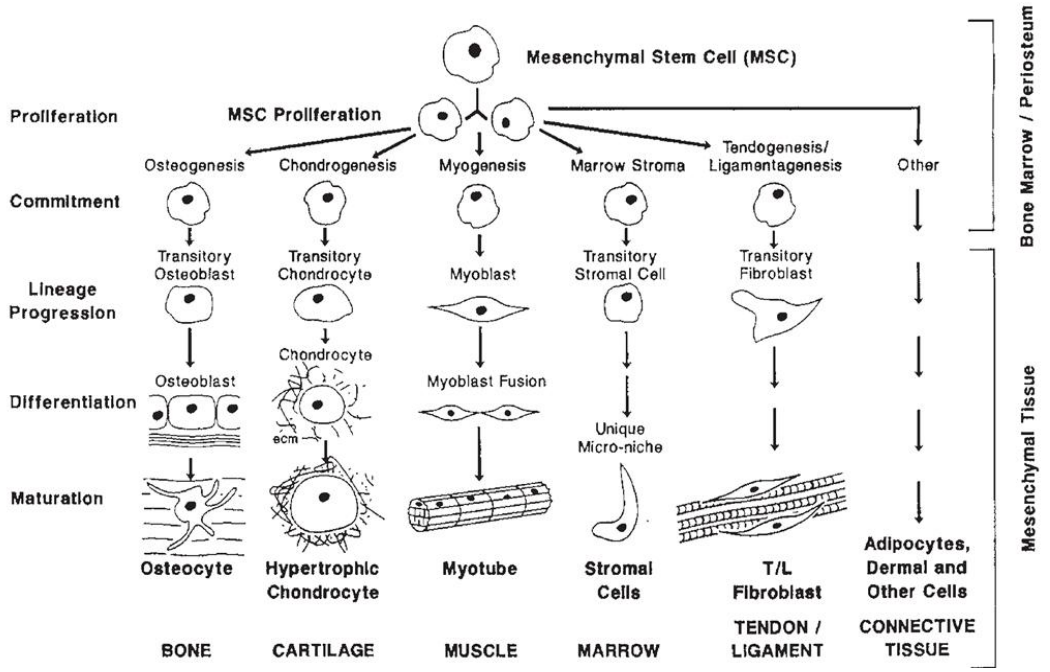


Figure 3 – MSCs have the capacity to differentiate in different type of tissues such as cartilage [87].

The cells pass through several lineages, located in what soon will be bone, until they reach the chondrogenic state, and continue to the final stage where chondrocytes become hypertrophic. When chondrocytes undergo hypertrophy they produce proteins necessary for calcification and to the process of endochondral bone formation (Figure 4). In the outside edge of the newly formed bone, chondrocytes secrete collagen and glycosaminoglycans (GAGs) to produce hyaline cartilage [4].

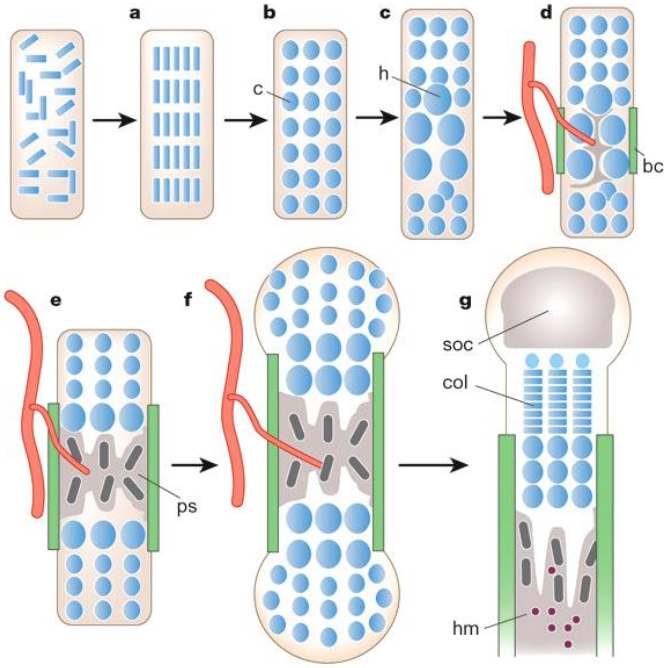


Figure 4 – Representation of the process of endochondral bone formation: stem cells start to condense (a); cells become chondrocytes-c (b); chondrocytes become hypertrophic-h (c); perichondrial cells adjacent to hypertrophic chondrocytes become osteoblasts forming bone collar-bc (d); hypertrophic chondrocytes direct the formation of mineralised matrix, attract blood vessels, and undergo apoptosis; osteoblasts of primary spongiosa accompany vascular invasion, forming the primary spongiosa-ps (e); chondrocytes continue to proliferate to increase the bone (f); osteoblasts of primary spongiosa are precursors of eventual trabecular bone; osteoblasts of bone collar become cortical bone (g). At the end of the bone, the secondary ossification centre (soc) forms through cycles of chondrocyte hypertrophy, vascular invasion and osteoblasts activity. The growth plate below the secondary centre of ossification forms orderly columns of proliferating chondrocytes-col. Haematopoietic marrow-hm expands in marrow space along with stromal cells [88].

The ECM consists of type II collagen (90-95% of the collagen present), VI, IX, and XI, which makes up 10 to 20% of total cartilage mass, proteoglycans (10 to 15% of total cartilage mass), non-collagenous proteins, and tissue fluids (up to 80% of the wet weight) [4]. Important GAGs present are hyaluronic acid and chondroitin sulphate among others [4]. Architecturally, cartilage is not homogeneous and it is composed of distinct layers such as the superficial, middle, deep and calcified zones (Figure 2A) [2, 85]. Each one of these zones has different composition, architecture, cellular, mechanical and metabolic characteristics [2, 85]. Given its complex structure,

regenerating articular cartilage is a significant challenge for regenerative medicine techniques. Therefore, the next section will focus on this subject.

2.1.2. Injuries in cartilage and osteoarthritis

Injuries and response in articular cartilage are different from the normal healing process in the body, mainly due to both its avascular nature and the fact that the chondrocytes are entrapped in the cartilage ECM. As a result, this static nature of chondrocytes limits not only its mobility but also the migration of chondrocytes from healthy tissue to the injury site [89]. In what concerns cartilage tissue damage, it is possible to distinguish three types of defects/injuries, namely: matrix disruption, partial thickness and full thickness [4, 90]. Matrix disruption occurs from trauma, in which ECM damage can be repaired by resident cells as long as the extension of the injury is not high. On the other hand, partial thickness injuries occur when the surface of the tissue is ruptured as a result of trauma, mechanical injury or wear and tear. However, is not extended to the subchondral bone [91]. For this type of injury the cells can regenerate part of the tissue briefly after the injury, however stopping this process before the defect is healed. In the case of full thickness defects, the cartilage tissue is damaged to the subchondral bone, and by reaching this area it is possible to observe a formation of a clot mainly composed by fibrinogen. During this process, cells from the marrow travel to the newly formed tissue and initiate the production of a hyaline-like tissue and/or fibrocartilage [4]. Such tissue does not possess hyaline cartilage mechanical characteristics and degenerates with time. Additionally, the healing of the cartilage defect can be influenced by the size of it and the age of the patient, and in both cases less is better. It was previously reported that a small defect inferior to 3 mm can completely heal, when compared with wider cartilage defects [92].

Osteoarthritis (OA) (Figure 5), the most common joint condition, can occur if severe cartilage defects are not treated [93] (Figure 5A). Specifically, this disorder causes joint pain, degeneration and dysfunction, which influences negatively the

patients' wellbeing and lifestyle. Furthermore, OA results from the breakdown and wear of the cartilage, which causes structural anomalies, followed by attempted and failed repair, as well as remodelling and sclerosis of the subchondral bone. This medical condition is sometimes followed by abnormal formation of osteophytes [94]. More importantly, this disease has a considerably high prevalence rate worldwide. Approximately 0.8 million people, just in Ireland (from Arthritis Ireland), and 35 million in the United States of America [4], are known to be suffering from some form of OA. The significant number of individuals affected by OA highlights the clinical need to develop more efficient techniques that may overcome the limitations associated with this medical condition.

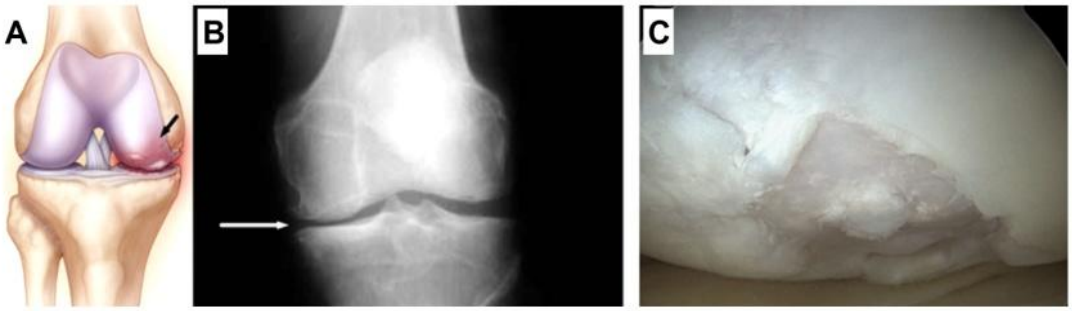


Figure 5 – Osteoarthritis (arrow) of the medial side of the knee (A) and radiograph showing osteoarthritis (B), with narrowing of the medial joint space [3]; cartilage defect (C) [95].

There are no current treatments to prevent or cure OA. The procedures to tackle the disease are surgical replacements with metallic implants that despite providing pain relief, as well as functional recovery, do not restore cartilage nor subchondral bone. Also, this surgical procedure has a finite lifespan and can degenerate over time [96-98], which is not advisable for a younger population suffering from OA [96, 99]. A more advisable alternative to younger patients is the osteotomy technique, which can decrease OA symptoms by surgically modifying the mechanical load from the injured site to healthy cartilage surface [90]. Such procedure can provide temporary pain relief, with acceptable results lasting from 3 to 12 years [89].

Nevertheless, the abovementioned approaches are still insufficient and limited as solutions to OA. However, new developments in the treatment of OA have revealed several techniques that can be used to promote cartilage healing, as discussed in the next sections.

2.2. Articular cartilage repair techniques

In articular cartilage regeneration traditional surgeries, there are usually key approaches used to restore its structure and functionality. One of the most common is a technique that uses marrow stimulation, which enhances the capacity of the cartilage and subchondral bone to restore the natural state of the native tissue. Conversely, another method uses a transplanted chondrogenic tissue graft to regenerate the cartilage defect. Simple interventions that merely reduce clinical symptoms of the damaged joint are also occasionally used (e.g., lavage, debridement and shaving) to provide temporary pain relief [1]. Nevertheless, in what concerns the treatment of critical focal cartilage defects, the most commonly used methods include bone marrow stimulation techniques, autologous/allogeneic tissue transplantation (e.g. mosaicplasty), or cell-based therapies [100]. The following section will focus on these cartilage regeneration approaches.

2.2.1. Bone marrow stimulation techniques

Perforation of the subchondral bone is one of the oldest techniques to stimulate cartilage regeneration, as well as one of the most commonly used [90]. This method, appropriate for full thickness chondral defects with subchondral bone exposed, works by penetrating the subchondral bone plate and consequently disrupting blood vessels. This process enables the formation of a fibrin clot [90], a neo-tissue invaded by progenitor cells that will differentiate into chondrocytes [90]. Techniques comprising marrow exposure rely on vascular elements such as fibrin clot, blood and marrow cells, cytokines, growth factors and vascularisation [89].

Moreover, bone marrow stimulating techniques include drilling, microfracture, spongialization and abrasion arthroplasty [1, 89]. More specifically, drilling subchondral bone with the Pridie method consists in creating holes in cartilage defects, which exposes the bone marrow and enables the formation of fibrocartilage [1]. Microfracture, on the other hand, is similar to the Pridie method, although it creates smaller diameter punctures [1] and can be performed through a minimally invasive arthroscopic approach [1]. Spongialization is usually applied in the patella, and involves complete removal of the subchondral bone plate at the lesion site [1]. Abrasion arthroplasty technique is used as a first line treatment for articular cartilage defects as it is easy to perform and can be used in combination with other intervention, however, it is considered palliative not curative [101].

2.2.2. Autologous/Allogeneic tissue transplantation

An alternative to marrow stimulating techniques is to transplant cartilaginous tissue from healthy areas to a defect site. Perichondrial and periosteal grafts are examples used in this approach. The first involves transplantation of tissue from the rib to the defect site, while the periosteal approach, based on the same principle, uses periosteal tissue adjacent to the defect site [4]. In previously reported studies comparing both techniques, superior chondrogenesis was observed in the periosteal approach [102]. Such procedures have proven to be relatively effective; however, both result in donor site morbidity and additional surgical problems [4].

Mosaicplasty or autograph osteochondral transplantation appears as an alternative to the previous methods, and involves the transplantation of one or more osteochondral plugs from a non weight bearing region (e.g. patellar groove) into the defect (Figure 6A and B) [1, 102]. Such technique has the disadvantage of using healthy tissue from an undamaged non weight bearing area of the joint to a high load region, which is going to lead to its degeneration as a result of overloading [1].

Additionally, it is also associated with much potential collateral damage to the cartilage of the articular joint [1].

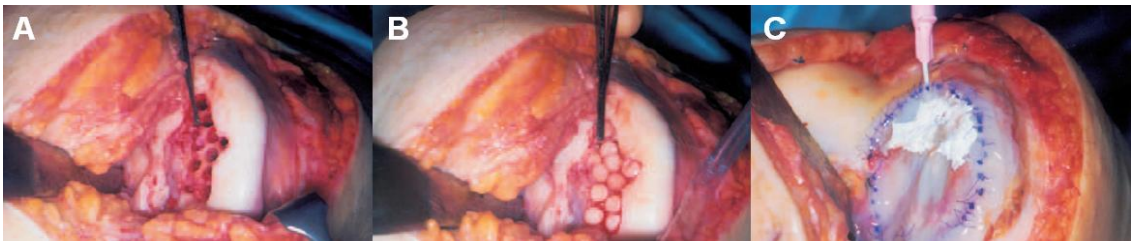


Figure 6 – Mosaicplasty osteochondral autograft transplantation procedure (A, B) and autologous chondrocyte implantation (ACI, C) for the repair of a defect of the medial femoral condyle [103].

Cadaveric allogeneic grafts can also be used while performing this approach in cartilage repair. However, issues with immunological response, tissue availability, problems with handling and storage, as well as risk of disease, are restrictions usually associated with allogeneic transplants that limit the general use of this approach [1]. A possible solution to overcome these issues may be the cryopreservation of the tissue. However, freezing is responsible for chondrocyte death deteriorating mechanical performance, structural properties, and lifespan when compared with fresh tissue [1, 104, 105]. Hence, the previously approached techniques provide a moderately successful outcome to cartilage regeneration. Table 1 highlights limitations associated with current techniques for cartilage repair, including donor morbidity, tissue availability, immune response and disease transmission [106].

Table 1 – Limitations of current cartilage repair techniques [106].

	Hindrance to technique				
	Donor-site morbidity	*Multiple surgical sites*	*Tissue scarcity*	*Immune response*	*Disease transmission*
Autogenic					
Osteochondral plug transplantation	+++	++	+++	–	–
Mosaicplasty	++	+++	++	–	–
Chondrocyte implantation	++	++	++	–	–
Chondrocyte-seeded scaffolds	++	++	++	–	–
Microfracture	–	–	–	–	–
Mesenchymal stem cell implantation	+	+	+	–	–
Allogenic					
Allograft transplantation	–	–	++	–	+
Chondrocyte implantation	–	–	++	+++	+
Tissue-engineered constructs	–	–	++	+	+
Xenogenic					
Chondrocyte implantation	–	–	–	++	++
Chondrocyte-seeded scaffolds	–	–	–	++	++

Hindrance level: –, none; +, minor; ++, moderate; +++, major.

Limitations such as poor biocompability, donor site morbidity, poor fixation, wear and pathogen transmission, have motivated the interest in regeneration cartilage using cell-based approaches to engineer tissue [100, 107]. Such topic is going to be the subject of the following section.

2.3. Cell-based therapies

2.3.1. Tissue engineering

Tissue engineering (TE), constitutes a promising approach for cartilage regeneration in which the main objective is to regenerate damaged tissue with the help of biological substitutes that are able to restore, maintain, and improve tissue function and performance [23, 108]. The scaffolds in TE are going to be seeded with cells, growth factors or be subjected to mechanical stimuli, to create a 3D environment which enhances tissue development mainly created by cell ECM production [23]. This symbiotic interaction between cells, scaffold and environmental factors represents the TE triad (Figure 7).

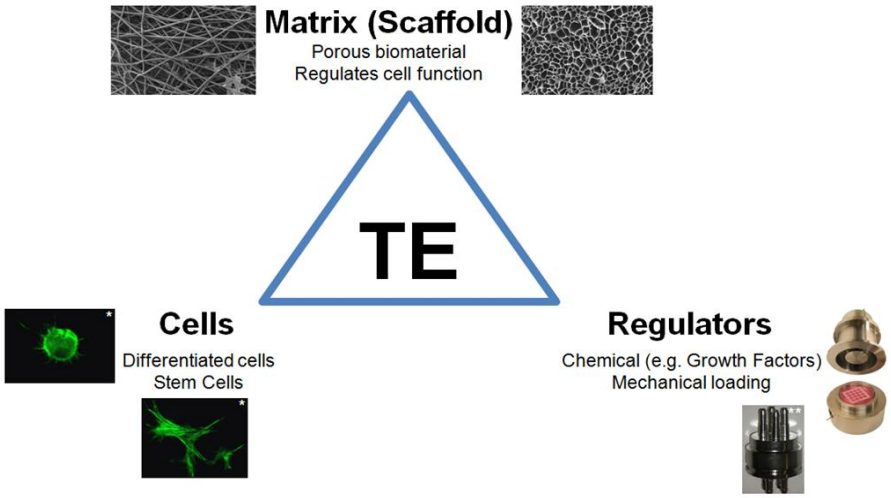


Figure 7 – Tissue engineering (TE) triad, which includes biomaterial-based scaffold, chemical and physical regulators** [109] and cells*[110] (adapted from [23]).

In vitro culture is usually one of the approaches used in cell-based therapies for cartilage TE to generate tissue, which can be implanted in the defect after culture. As an alternative, these scaffolds can be directly implanted, without *in vitro* culture, into

the degenerated joint in which the tissue is produced *in vivo* [23]. Although the *in vitro* culture approach is a highly controllable environment, it is overly simplistic when compared with the native tissue complexity and the unknown ideal conditions for full tissue regeneration [108]. On the other hand, the *in vivo* approach does not have this limitation, given that the construct with cells and biological chondrogenic cues will be included in a "natural bioreactor" with the native articular environment, perfect for cartilage regeneration [108, 111]. In order for this type of approach to succeed, it is important to implant the adequate number of chondrogenic cells, and that these can produce the ECM necessary to restore the damaged tissue *in situ*. Additionally, the cells should not leach out of the construct instantaneously and it is crucial that the newly formed tissue is properly integrated in the surrounding tissue while it is being created [23].

2.3.2. ACI and MACI

Autologous chondrocyte implantation (ACI) is another method commonly used, which utilizes both culture strategies, *in vitro* and *in vivo* (Figure 8). This technique can be considered the first clinical example of cartilage TE, mainly due to the regeneration and treatment of focal articular cartilage defects [100]. It was introduced in 1987 by Brittberg [112], and published following FDA approval for clinical studies [100].

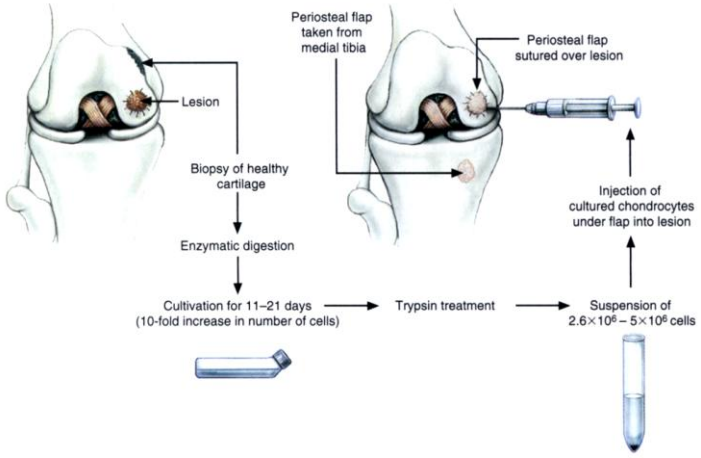


Figure 8 – Representation of autologous chondrocyte implantation (ACI) [112].

The ideal patient for the ACI procedure suffers from a full thickness cartilage defect with healthy cartilage in the periphery, which is not often the case of osteoarthritic patients. The first step for the ACI method requires removal/harvest of tissue from healthy cartilage, a procedure that is generally a full thickness biopsy to the subchondral bone. This harvest usually happens in the edge of the femoral condyle with the goal of isolating chondrocytes, expand these cells over many weeks of *in vitro* culture, and finally implant them (or the tissue engineered graft) into the defect [16, 17]. In this process, the damaged joint is exposed by the surgeon, and a flap of periosteom is sutured over the defect. The expanded cells are injected into the defect with a typical amount of 2 million/cm^2 in the defect [113]. This approach implies high costs and requires two surgical procedures, one to isolate the cells and a second one to re-implant the expanded cells or engineered tissue. The ACI technique changed over time to a second generation, in which the periosteom-derived membrane was replaced by a collagen scaffold [114, 115]. Although this procedure using a collagen membrane to cover the defect reduced patient recovery morbidity, it still requires an invasive surgical intervention [116]. This fact led to the development of the next generation of this type of approach: matrix-induced autologous chondrocyte implantation (MACI). MACI is an alternative to ACI where a scaffold is used a scaffold to culture the cells, followed by implantation in the damaged joint, similar to the ACI procedure [18, 114] (Figure 9). This MACI technique is an example of a next generation ACI [114].

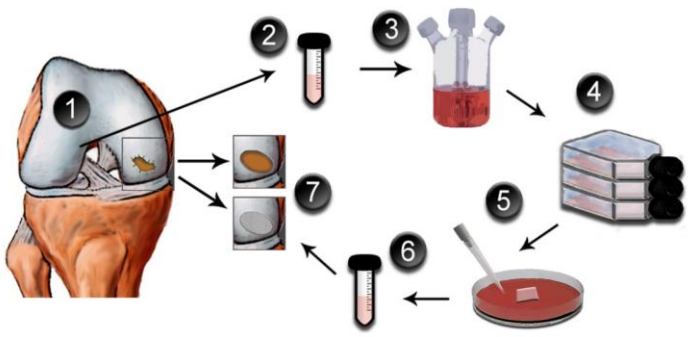


Figure 9 – Representation of the MACI procedure: initial evaluation of the injury and cartilage harvest (1); biopsy sent to the culture lab (2); tissue is digested (3); chondrocytes are culture expanded in monolayer (4); cells are seeded into the scaffold before implantation (5); the

construct is sent to the surgical room (6); final surgery with debridement of the injured cartilage followed by implantation with fibrin glue (7) [114].

MACI involves seeding of expanded chondrocytes into a type I/III collagen membrane before implantation into the cartilage defect [117]. This technique also used other types of scaffolds such as a hyaluronic acid scaffold (Hyalograft-C®), in which it was observed an adequate support for cell interaction, cluster formation and consequent ECM production [118]. Advantages of MACI over ACI include the minimally invasive surgery to implant the construct, as well as reduced time for surgery, given that it does not need membrane cover, suturing or harvest of periosteal flap [116]. For the last twenty years, a lot of effort has been applied in reducing culture period time, to use alternative cells and to produce tissue grafts easier for the surgeon to work with [114]. Unfortunately, a reduced number of these possible improvements reached the clinical context [114]. Moreover, although these ACI-type approaches have had reasonable success in the clinical context with small sized cartilage defects, these are not suitable for larger defects typical from an osteoarthritic joint. Additional limitations include a reduced amount of viable cartilage to harvest to be used in the procedure, and donor site morbidity. Hence, such strategies have had limited clinical uptake due to the high cost, complexity and significant regulatory challenge. Consequently, novel strategies to joint regeneration are required, such as single-stage therapies in which some of the mentioned limitations are not existent in such protocol [19-22].

2.3.3. Cell sources

Success in cartilage TE can only be achieved through the optimal combination of cells, materials and biochemical cues. Cells play a critical role on the regeneration of damaged tissue. A variety of cells types can be used to regenerate cartilage. However, such cells should be immuno compatible, ideally from the own patient with additional reduction of disease transmission risk. Accessibility and expansion characteristic are

also critical factors for the cells being used for TE. Chondrocytes or mesenchymal stem cells (MSCs) are the typical cell types used for cartilage TE [24].

Chondrocytes were the firsts cells used in cartilage TE, mainly due to their key role in ACI procedures. Such a choice happened because chondrocytes are the cell responsible for ECM maintenance and production in AC. However, the use of this cell in TE is limited due to tissue availability, donor site morbidity related with tissue harvest, and when expanded in monolayer, these cells differentiate towards a fibroblastic phenotype. The chondrocytes start to produce the undesired type I collagen when they start to express a fibroblastic phenotype [4, 119, 120]. Once *in vivo*, these differentiated cells lose their capability of hyaline cartilage production, similar to what happens with the previously mentioned autologous chondrocyte technique [121]. Consequently, using chondrocytes has obvious limitations in cartilage TE, and therefore a lot of attention has been given to stem cells in the field of regenerative medicine.

Stem cells are undifferentiated cells capable of self renewal and differentiation (Figure 3) [122]. With the adequate environment, cell can generate different tissue specific cell types [87]. For TE goals, MSCs represent the ideal cell type, mainly because they can differentiate into specific cell lines and proliferate, increasing the number of cells and allowing the enhancement of tissue regeneration [123]. Moreover, these cells can be expanded several times without losing their multipotency [124]. Ideally, stem cells should be found in high quantities, be obtained with minimally invasive procedures, differentiate consistently along multiple cell lineages, should also be transplanted to either an autologous or allogeneic host effectively, and finally be handled in accordance with GMP protocols [122].

Adult MSCs can be isolated from a range of tissues such as bone marrow [41-47], subcutaneous fat [34, 48-50], synovium [49, 61-65] and infrapatellar fat pad (IFP) [20, 46, 51-60]. Bone marrow MSCs were identified firstly when it was observed, and documented for the first time, their characteristic behaviour of adhering to plastic.

These were considered a rare cell population that easily formed colony forming units, which were fibroblastic and consistently expressed a set of cell surface markers [124-127]. Many studies have been performed using bone marrow MSCs [41-47], such as one where MSCs-derived from bone marrow had comparable performance to chondrocytes in ACI [128]. Nevertheless, using bone marrow MSCs has some drawbacks, particularly the natural tendency of chondrocytes originated from bone marrow MSCs to undergo hypertrophy and mineralize *in vivo*, limiting its use for chondral repair [129-131]. In addition, from a patient's perspective, it has also been claimed that the procedure used to obtain this type of cells is painful and has significant risks associated [121]. This fact has motivated researchers to develop studies using alternative sources of stem cells with vast potential in regenerative medicine [132], namely adipose, synovium, and IFP tissues.

Adipose tissue represents an abundant, accessible source of adult stem cells with the capability to differentiate into several lineages, usually presenting a high cell yield [122]. These cells are named adipose-derived stem cells (ASCs) which identify the isolated, plastic adherent, multipotent cell group [122]. In terms of proliferation rate and lineage capability, ASCs present behaviour comparable to bone marrow MSCs [133, 134]. ASCs cells are also known to be able to produce cartilage-specific matrix both *in vitro* and *in vivo* [134]. In addition, stem cells can also be found in the synovium [49, 61-65, 135]. Previous research has demonstrated that synovium-derived cells are highly proliferative and chondrogenic, showing that by suspending these cells *in vivo*, in a cartilage defect, tissue repair was promoted in rabbit and pig models [135]. Therefore, this type of cells constitutes another promising alternative for cartilage tissue regeneration [135]. Stem cells can also be found in IFP, and like synovium-derived stem cells, have been shown to be more phenotypically similar to chondrocytes than other sources [136]. The IFP (Figure 10) is a shock absorber structure located in the knee (with its proximal end connected to the patella, and its

distal end linked to the menisci) composed by adipocytes and connective tissue (containing collagen and GAG) [137].

The present thesis will focus on the use of IFP-derived stem cells (FPSCs), a particularly attractive source of chondro-progenitor cells for cartilage TE, mainly because these cells are easily accessible and possess a strong potential to generate cartilaginous tissue [20, 51, 58]. Previously presented studies argued that FPSCs can proliferate and undergo robust chondrogenesis [20, 52-55, 60, 136], and additionally it was suggested as a viable source of autologous stem cells for OA treatments [136]. This type of cell has also been shown to generate higher chondrogenic-specific matrix when compared with BM-MSCs [46], making this cell source one of the most promising for cartilage TE.

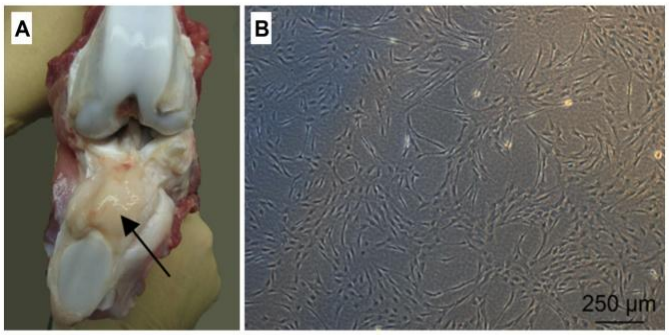


Figure 10 – Articular joint with exposed infrapatellar fat pad (IFP) (arrow) and expanded IFP-derived stem cells.

Furthermore, the yield of such cells from the IFP is high, opening up the potential of using freshly isolated (not culture expanded) IFP-derived stromal cells as part of a single-stage therapy for cartilage regeneration [19-22]. Moreover, it is also pertinent to explore the isolation of specific chondro-potent stromal cells sub-populations (e.g. $CD44^{+}$) [66, 67]. Such single-stage approaches could potentially overcome the need to expand cells *in vitro* for cartilage regeneration therapies. Single-stage therapies for cartilage repair are going to be the subject of section *2.3.6*.

2.3.4. Biomaterial-based scaffolds for cartilage tissue engineering

At this stage it is well established that cells are encapsulated, and that they proliferate and differentiate within a three-dimensional (3D) environment while inside the body [69]. Once they are isolated, chondrocytes lose their phenotype in monolayer culture [138]. Conversely, if the culture is performed in 3D they regain their phenotype [119, 139]. This shows the important role of the 3D environment in the maintenance of the phenotype of chondrocytes. Hence, 3D scaffolds are used to promote chondrogenic differentiation of stem cells while engineering cartilage [69]. In this sense, O'Brien [23] underscored that when choosing or designing a scaffold for cartilage regeneration you should have the following considerations:

- The scaffold should be biocompatible, and cells must be able to adhere and function normally.
- After implantation it must not provoke a strong immune reaction.
- The device should be biodegradable to allow host cells and implanted cells to produce ECM, and replace biomaterial.
- Products of the degradation should not be toxic and should be easily leached out.
- Scaffolds should possess from the moment they are implanted, appropriate mechanical properties, which is a challenging task in the joint environment.
- Architecture is also crucial for scaffold design, and devices should have interconnected pores, adequate porosity and pore size, to ensure cellular infiltration and nutrient diffusion to cells and newly formed tissue. Leaching of waste is also depending on pore interconnectivity.
- Finally, manufacturing technology is crucial, mainly because the scaffold must be clinically translatable and commercially viable [23].

These 3D structures can be produced with several biomaterials, which can be classified as natural-derived, which are further distinguished as protein-based (e.g.

collagen and fibrin) and polysaccharide-based (e.g. chitosan and hyaluronic acid), and into synthetic biomaterials (e.g. PLGA) [69]. Examples of naturally-derived biomaterials [27] used in cartilage TE are collagen [29], chitosan [30], alginate [31], fibrin [32], silk [33], gelatin [34], hyaluronic acid (HA) [35] and ECM cartilage-derived materials [36]. Collagen (Figure 11) is one example of versatility in natural-derived biomaterials for scaffold fabrication [24, 27, 35, 81]. Among the protein-based biomaterials, collagen is one of the most important mainly because type I and II collagens membranes are clinically available for ACI and MACI [69, 140].

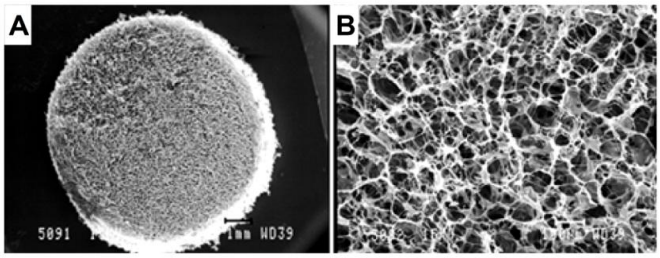


Figure 11 - Scanning electron microscopy (SEM) micrographs (low-A and high-B magnification) of a porous collagen based scaffold for cartilage tissue engineering [35].

Commercially available examples are MACI® (Verigen, Germany), Maix® (Matricel, Germany) and Chondro-gide® (Geistlich Biomaterials, Switzerland) [69]. Another commercially available collagen application is the product Atelocollagen® (Koken Co. Ltd, Japan), a gel made of type I collagen that enables 3D culture and *in vivo* implantation of human autologous chondrocytes [140] and bone marrow MSCs [69, 141]. The previously mentioned Hyalograft-C®, polysaccharide-based biomaterial which is a tissue engineered graft consisting of autologous chondrocytes in a hyaluronic acid matrix called HYAFF-11® (Fidia Advanced Biopolymers, Italy), proved to be beneficial for cartilage repair in humans [142]. Among the synthetic biomaterials, Bio-Seed®-C (BioTissue Technologies, Germany) is a porous scaffold fabricated with polyglycolic acid, polylactic acid and polydioxanone, which has been tested with autologous chondrocytes embedded in a fibrin gel (Figure 12) [143]. This approach has been reported as able to induce formation of hyaline cartilage [69, 143].

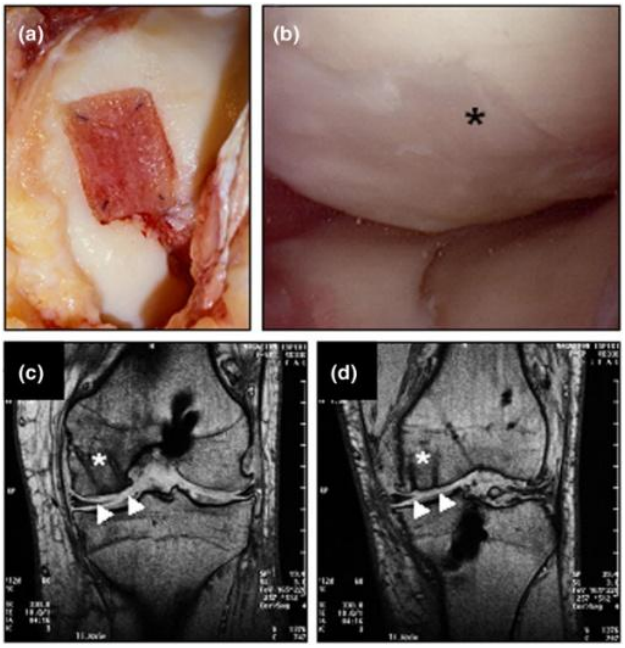


Figure 12 – Arthroscopic and magnetic resonance imaging evaluation of cartilage defects treated with autologous chondrocyte grafts (BioSeed®-C). Situation of a cartilage defect situated at the femoral condyle covered with fixed scaffold (a). At 9 months after surgery, second-look arthroscopy showed the formation of a cartilage repair tissue of a tough condition (asterisk) (b). Magnetic resonance imaging (MRI) at 6 months (c) and 12 months (d) after implantation shows. The repair tissue covers the defect (white triangles) and gives a slightly altered MRI signal [143].

Although the currently available approaches abovementioned have demonstrated promising results in terms of clinical outcomes, they all require an invasive surgery to access the joint. Hence, alternative approaches to regenerate cartilage defects (e.g. minimally invasive injectable hydrogels) are receiving more attention lately [69]. Hydrogels represent a class of biomaterials that can be injected into the cartilage defect in a minimally invasive procedure. These devices are composed of a synthetic or natural-derived polymer, which is crosslinked physically, ionically or covalently [69, 144]. More importantly, these materials have high water content, similar to the one found in native tissue, therefore mimicking cartilage environment ideal for cells [145]. Chondrogenesis of stem cells has been observed and demonstrated with different hydrogels such as fibrin [146] or alginate [34].

Fibrin hydrogel is a degradable biopolymer derived from fibrinogen. This gel mimics the last step of coagulation in blood and results in a clot of fibrin. The

mechanism of clothing is primarily possible by the thrombin-mediated removal of the fibrinopeptides from the fibrinogen structure [147]. This structural alteration and the exposure of polymerization sites enable fibrin monomers to assemble creating a stable, insoluble hydrogel [148]. Fibrin presents a combination of excellent biocompability, controllable degradation rate, adhesive properties and it can enhance healing *in situ* [149]. Such characteristics make this biomaterial a viable option for TE. This biomaterial has been widely used as adhesive for haemostasis [150], wound suture [151] and as a sealant [149, 152]. In addition, this hydrogel presents minimal inflammation and foreign body reaction after implantation, and is quickly absorbed in the body [149]. Fibrin can self-assemble creating a scaffold by mimicking the last step of the clotting process in blood enabling cell migration, proliferation, differentiation and consequent tissue regeneration [149]. Additionally, fibrin can also be used as cell carrier to keep cells viable during the delivery process. Better mechanical properties and overall performance can be achieved in fibrin just by biochemical modification and additional functionalization (e.g. ECM-derived material) [149]. Fibrin can be obtained from pooled human plasma [153]. When applied in TE, fibrin can function as both 2D and 3D cell culture scaffold (Figure 13) [154]. The traditional 2D approach seeds cells after fibrin gelation, and provides some understanding about cell-fibrin interaction. However, it cannot mimic natural physiological environment of cell *in vivo* [155]. 3D fibrin scaffolds have been used several times previously due to their ability to mimic *in vivo* environment. With the goal of developing functional engineered tissue, the construct in the 3D approach is fabricated with the cells encapsulated in the fibrin, and once the fibrin-cells system is solidified, constructs can be cultured or implanted for tissue regeneration. One of the advantages of this approach is the possibility of injecting the fibrin with cells, and potentially other chondrogenic factors, in a minimally invasive approach for tissue repair [149].

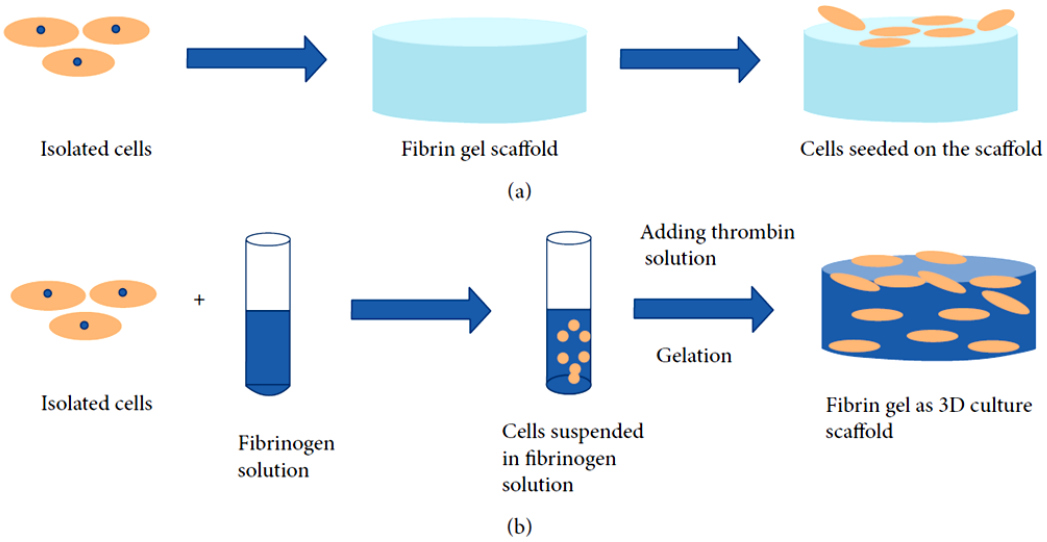


Figure 13 – Schematics of the fabrication of 2D and 3D cell culture with fibrin. The conventional 2D approach is fabricated in advance of cell seeding and the cells are seeded on the surface of the scaffold (a). In the 3D approach fibrin gels with the cells encapsulated, then the mixture can be delivered into a mould or injected into the defect (b) [149].

Fibrin has been used for cartilage TE in the past. Chondrocytes were transplanted with fibrin, achieving promising results [156]. In this procedure, cells and fibrin were injected in rabbits with formation of neocartilage after 8 weeks [156]. In another rabbit study, synovium-derived stem cells were encapsulated in fibrin, combined with collagen and hyaluronic acid, and applied *in vivo* on a model to regenerate osteochondral defects in the knee [157]. This study demonstrated that through histological analysis it was possible to detect GAGs and type II collagen, with a hyaline cartilage full structural organization after 24 weeks *in vivo* [157]. Such a biomaterial can and was used in the past, as a delivery device for cells and for growth factors [52, 158]. In this specific case [52], fibrin delivered efficiently growth factor to FPSCs in different doses, with promising chondrogenic results. However, there is evidence to suggest that fibrin is not as chondro-permissive as other well established hydrogels [110], with bone marrow and adipose-derived stem cells showing a diminished chondrogenic potential when encapsulated in fibrin [110, 159, 160]. Therefore, there is a clear need for further functionalization (e.g. with ECM) of this

versatile injectable hydrogel system to increase its chondrogenic potential for cartilage repair therapies. This subject is going to be addressed in Chapter 8.

Alginate is a natural-derived anionic biopolymer typically obtained from brown seaweed (*Phaeophyceae*) [1, 161]. This biomaterial is a carbohydrate-based polysaccharide (Figure 14), composed of sugar-rings building blocks, which is one of the front runners in cartilage TE [161]. In cartilage, matrix cells are anchored into the network which the hydrogel or scaffold design aims to mimic [161]. Once the cells are embedded in alginate, they start to renew and specialize maintaining the round shape characteristic of the cartilage-specific phenotype [161, 162]. Alginate continues to be the most widely used hydrogel for *in vitro* studies mainly because it is easy to produce, effective and not expensive [161]. More importantly, it presents appropriate biocompability, low toxicity and mild gelation by addiction of divalent cations such as Ca^{2+} [163]. Alginate is used in wound healing [164], delivery of bioactive substances [165] and cell transplantation in TE [161]. Alginate hydrogels are 3D crosslinked networks composed by hydrophilic polymeric chains, which are biocompatible, mainly because this network is structurally similar to the components present in native tissue [166].

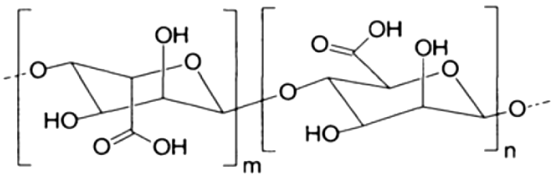


Figure 14 – Alginate polysaccharide chemical structure [161].

The most commonly used method of alginate hydrogel production is the ionic crosslinking, which involves combining alginate solution with ionic crosslinking agents such as Ca^{2+} (from $CaCl_2$) [164]. The mechanism of crosslinking is explained by calcium cations bond to the guluronate blocks of the alginate chains, and consequently, these blocks start to chemically interact with the block of the adjacent polymeric chain resulting in a gel structure [167]. One drawback of this type of crosslinking is the limited long term stability of the alginate gel in physiological

conditions [164]. An alternative approach to ionic crosslinking is to covalently crosslink the alginate, obtaining a permanent, chemical crosslink, as well as a mechanically stable structure for a long period of time [168-171]. In order to obtain this chemical crosslink, carbodiimide can be used to covalently crosslink the alginate [169]. The disadvantage of the covalent chemical crosslinking is the lack of binding sites for cell direct attachment [170].

Furthermore, there are particular situations where the alginate instability is desirable, namely *in vivo*. This degradation rate can be manipulated by altering molecular weight and composition [172]. Alginate seeded with stem cells was successfully used in the past for cartilage TE *in vitro* and *in vivo* [161, 164, 173]. However, there are still some limitations when using this biomaterial. Alginate is a relatively inert polysaccharide that usually requires some form of functionalization for TE applications [169, 174, 175]. One of the major limitations concerning alginate is its poor cell attachment properties [175]. This happens due to the hydrophilic nature of the alginate, which reduces cell seeding efficiency [169, 175]. Therefore, to overcome this limitation it is possible to functionalize the alginate with different ECM components, such as fribronectin [175] or collagen [170]. In addition, there were also reports that highlighted the benefits of collagen coatings in chondrogenesis [176-179]. This ECM functionalization of alginate scaffolds is going to be approached in Chapter 9.

Strategies for TE using ECM are already being tested [39], and represent a promising alternative to the aforementioned biomaterials for cartilage regeneration. Presently, ECM-derived materials are being highly assessed in TE, with the aim of clinical translation for a range of different tissues. These include heart valves [180], trachea [181], muscle [182], tendon [183], bone [184], and cartilage [36, 38, 39] which is the focus of the current thesis. ECM-derived biomaterials are promising and versatile for TE and regenerative medicine, and can be applied in diverse designs and applications (Figure 15).

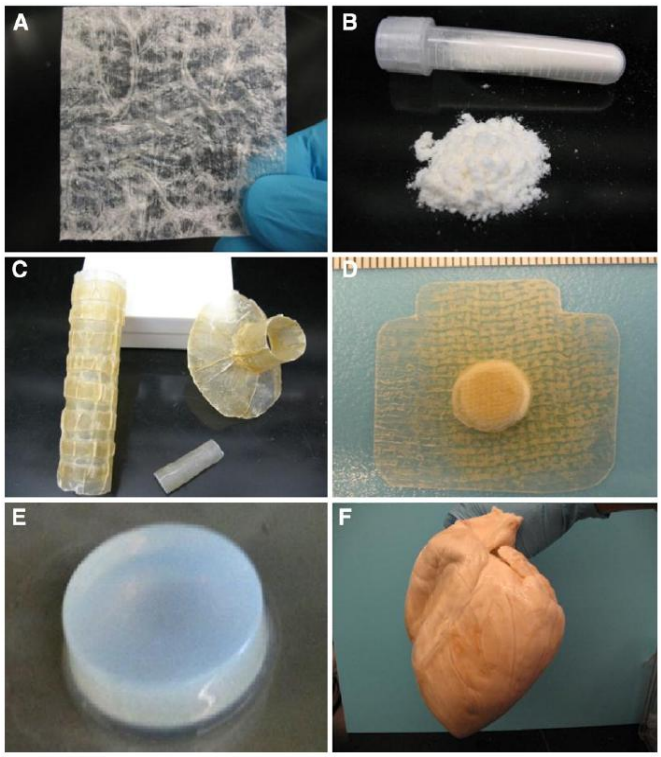


Figure 15 – Examples of ECM-derived scaffold materials: thin film (A), powder (B), tube (C), powder devices (D), hydrogel (E) and whole organs (F) [185].

One of the main reasons why ECM is being used as a biomaterial is because it can support and enhance tissue specific synthesis *in situ* (Figure 16), instead of inferior and less functional repair tissue. Badylak termed this process “constructive remodelling” [186]. However, success is dependant of several factors such as growth factor availability, architecture, immune response and mechanical *stimuli* at a cellular level [187]. These questions are going to be addressed in the section 2.4.

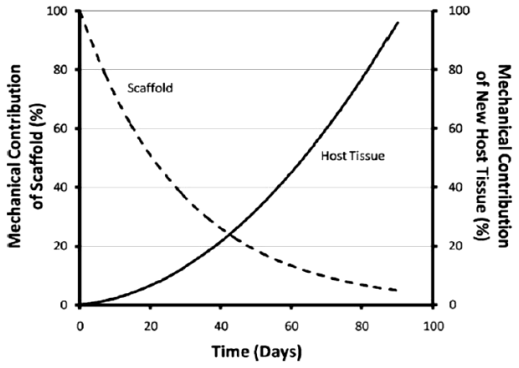


Figure 16 – Mechanical contribution of an ECM-derived scaffold over time as it degrades, and the mechanical contribution of the new host tissue as it forms during ECM remodelling in the presence of loading [37].

2.3.5. Growth factor delivery

ECM formation is regulated by a synergistic effect between physical and chemical stimulation [68]. These chemical factors include growth factors, which play a key role in cartilaginous tissue maintenance and regeneration. Growth factors that are known to be chondro-inductive include transforming growth factor-β (TGF-β) and insulin-like growth factors (IGFs) [68, 69]. Members of TGF-β family of growth factors, which play a key role in driving chondrogenesis of stem cells [47, 48, 52, 70-73], have a natural affinity for ECM components such as proteoglycans [74, 75], and some forms of collagen [188, 189]. This family include TGF-β1 [76], TGF-β3 [77], bone morphogenic protein-2 (BMP-2) [78], BMP-4 [79] and BMP-7 [80] among others, which have been proven to be efficient agents promoting cartilage ECM production [24]. TGF-β family of growth factors can be delivered *in situ* by a panoply of biomaterials [190] (Figure 17), such as collagen [81] and fibrin [32].

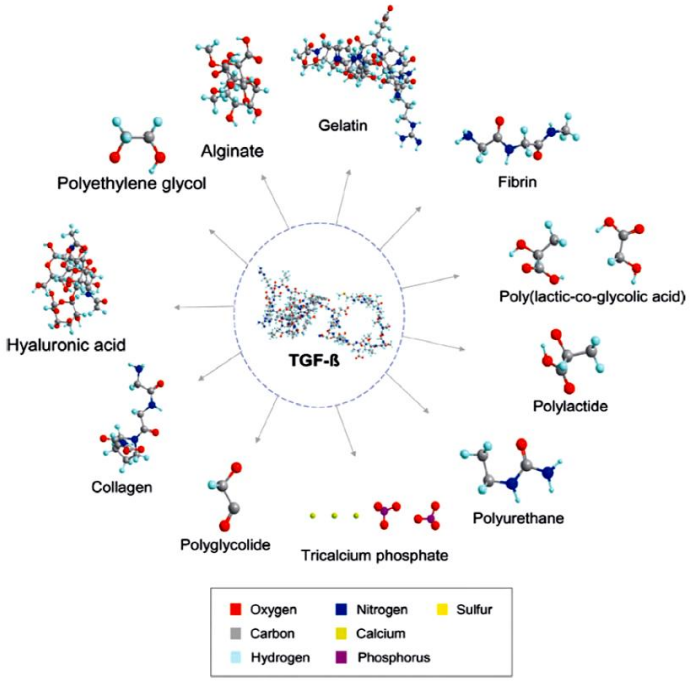


Figure 17 – Diagram showing the multitude of materials that can be used to deliver TGF-β [191].

TGF-β is a polypeptide [192] with three isoforms with a similar structure and around 60-70% of amino acid identity [190]. TGF-β is secreted in a latent form that can

further covalently bound to the binding protein that serves to hold TGF-β in the ECM until it is activated. A rapid extraction of TGF-β happens *in vivo* (in a few minutes) [193]. TGF-β signalling is mediated through membrane binding to TGF-β type II receptors causing type I receptor phosphorylation, which activates intracellular processes involving TGF-β-specific Smads also via phosphorylation [190]. This process enables translocation of the activated Smads to the nucleus modulating cartilage gene expression as part of differentiation, proliferation and ECM production in chondrogenesis [190, 194-197]. Smad-mediated transcription is not the only signalling cascade pathway activated by TGF-β [198].

There are several general requirements, in terms of dose and timing, for TGF-β delivery systems. This growth factor has been used to enhance MSC chondrogenesis in a multitude of culture systems, where the TGF-β was supplemented in dissimilar ways [199, 200]. However, there still is an overall critical limitation of the TGF-β use *in vivo*, its short viability (less then 30min) [201, 202]. Hence, it is important to maintain a threshold concentration of TGF-β for a particular period for chondrogenic culture [190]. The ideal dose for TGF-β growth factor *in vivo* is still unknown, however *in vitro* the commonly accepted dose is 10 ng/ml for MSCs culture for cartilage TE [203-205]. It is known that it is critical for MSC chondrogenesis, the continuous exposure to TGF-β in the first week of culture [206, 207]. It was previously shown that only 4 days of culture with 10 ng/ml it was enough to enhance ten times sGAG accumulation when compared with TGF-β-free control [76]. However, while the ideal dose is still unclear when enhancing chondrogenesis, it is known that high and continuous doses of TGF-β are not beneficial [190]. It is also known that continuous stimulation with growth factor (TGF-β1) can induce the accumulation of aggrecan cleavage products during chondrogenesis [76, 208], and undesired fibrosis and hypertrophic scars after implantation [202, 209]. There are also concerns when high doses of growth factor are delivered intra-articularly. Namely, this may lead to pathological changes such as osteophyte occurrence [210-216]. Therefore, due to the potency of TGF-β growth

factors it is necessary to develop and optimize devices or ways to control deliver TGF-β [217]. These biomaterial-based systems should possess characteristics typical for TE devices [23]. Ideally, this delivery system will provide the controlled release of the adequate amount of growth factor to induce and enhance chondrogenesis [190]. Such chondrogenic growth factors can be incorporated into the carrier biomaterial by direct loading, encapsulation, covalent bonding or reverse bonding (Figure 18) [218].

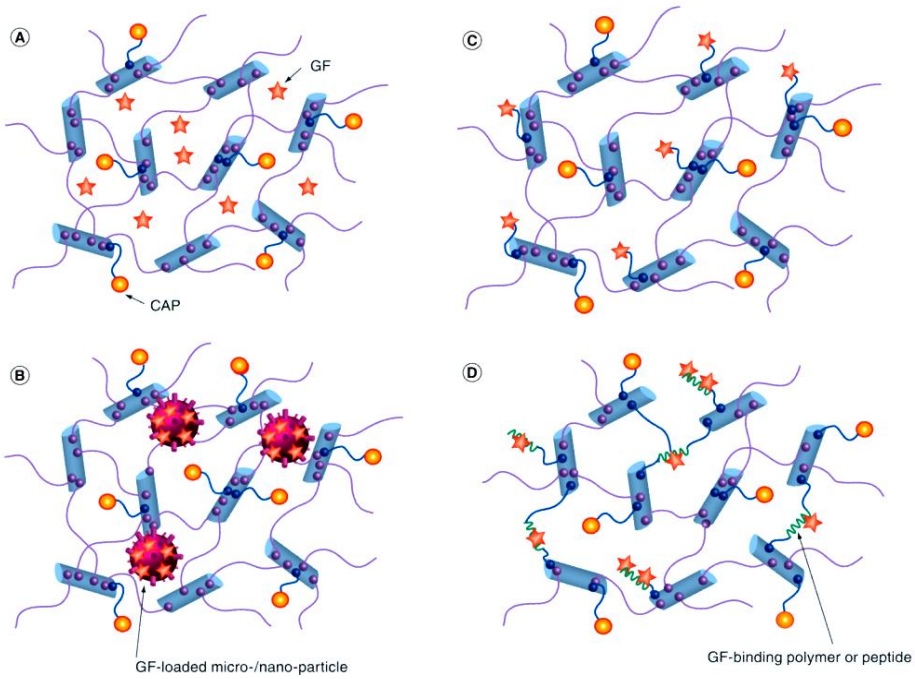


Figure 18 – Schematics of growth factor delivery systems. Direct loading were the growth factor is encapsulated into the biomaterial directly during its preparation (A); Carrier system where a particle is used to encapsulate growth factor first, which are included in the device during preparation (B); In the covalent bonding method growth factor is covalently attached to the polymeric network (C); For the reverse binding method, growth factor polymers are incorporated in to the biomaterial network by a reaction such as radical copolymerization or chemical conjugation. CAP: cell-adhesive peptide; GF: growth factor [218].

Biomaterial-based TGF-β delivery systems are natural-derived or synthetic, and can be fabricated in three different forms: hydrogels, solid scaffolds or hybrid scaffolds [190]. Hyaluronic acid (HA) hydrogels have been explored with TGF-β to induce chondrogenesis with promising results [219]. HA hydrogels have been shown to allow cell expression of specific chondrogenic markers compared with PEG, while the TGF-β was encapsulated in the HA [220]. Alginate is also an important natural-derived,

anionic polymer for growth factor delivery. Growth factors encapsulated in this polysaccharide structures are released by pore-mediated matrix diffusion, and by degradation of the polymeric network [163]. TGF-β release from alginate strongly depends of its molecular ionic nature [221], molecular weight [222] and composition [223]. Previously, alginate scaffolds have been engineered with a sulphate functionalization to sustain release of TGF-β1 by affinity interaction [224, 225]. The alginate/sulphate scaffold presented superior growth factor loading efficiency and a slower initial release when compared with alginate only scaffold, while influencing positively chondrogenesis of MSCs in a prolonged manner. There is also the possibility of combining HA and alginate biomaterials for efficient growth factor delivery [206]. This HA hydrogel combined with alginate-TGF-β3-loaded microspheres, promoted superior chondrogenesis of MSCs when compared with constructs with TGF-β3 media supplementation. Additional biomaterials such as fibrin have been also used to deliver growth factors [52, 158, 226-230]. TGF-β release from fibrin devices is related to the amount of growth factor initially loaded, and that TGF-β1 as fibrin affinity [32]. It is also known that fibrin concentration [231] and the presence of cells [232] increases retention of TGF-β1. It is also known that TGF-β loaded fibrin enhances chondrogenesis of MSCs [32], and that it can also carry gelatin microspheres loaded with TGF-β1. Gelatin microspheres are known to be capable of delivering growth factor in a controlled manner while enhancing chondrogenesis of stem cells [47, 52, 233].

Scaffolds can be fabricated with different types of biomaterials from natural or synthetic origins for cartilage defect repair [23]. These devices can be mechanically supportive and provide cell binding sites; however, is limited for growth factor delivery due to its poor absorption capabilities [234]. This limitation can be overcome by the use of growth factor loaded microspheres incorporated into the scaffold, in which the release rates can be tailored by spheres and/or scaffold material degradation [190, 235]. An example of scaffold-based delivery systems is the gelatin/chondroitin/HA scaffold containing gelatin microspheres loaded with TGF-β, which showed a release

profile with two different rates, an initial fast of 37%, and 80% released after 18 days [236, 237]. The same approach was used with sequential release with IGF-I and TGF-β1 with promising results for cartilage TE [238]. Hydrogels and scaffolds can be combined to create a hybrid growth factor release system [190]. A hybrid system build with type I/III collagen and TGF-β1-loaded fibrin enhanced chondrogenesis of MSCs [239], while a heparin/fibrin/PCL system led to *in situ* chondrogenesis of ASCs [240]. Moreover, an interesting approach is to try and fabricate a multilayer composite scaffold to recreate zonal characteristics of cartilage and to regenerate osteochondral defects [241, 242].

Growth factor delivery hydrogel systems have also been assessed *in vivo*, and one of their most attractive features is their injectability and minimally invasive nature [190, 243]. Acellular fibrin loaded with TGF-β (1, 2 or 3) was injected in mini-pig articular cartilage partial thickness defects with the objective of recruiting cells from the synovium [244, 245]. TGF-β1 was the one that promoted the highest degree of chondrogenesis. However, for high dose (>900 ng/ml) synovitis and cartilage erosion was observed, among other detrimental side effects [244]. Additionally, in another *in vivo* study (rabbit osteochondral model) a TGF-β1 high dose of 2000 ng/ml was used, and also led to undesired reactions, such as osteophytes [214]. However, the osteochondral repair outcome was satisfactory for both low (20 ng/ml) and high dose for up to 3 months [214]. Finally, in a rabbit osteochondral model where two layers of acellular alginate were loaded with TGF-β1 and BMP-4, repair was observed after 28 day *in vivo* [79]. When encapsulated with MSCs, different hydrogels demonstrated high potential for delivering TGF-β and regenerating rabbit osteochondral defects [236, 246]. These *in vivo* assessments and consequent cartilage regeneration are promising, which led to the next step of assessment in bigger animal models. In one of the pioneer experiments with autologous MSCs *in vivo*, cells were encapsulated in chitosan and fibrin, and assessed in a partial-thickness cartilage defect in an ovine model with promising results [247]. For a scaffold only approach the release of the

growth factor happened in a high delivery rate, which it is desirable for chondrogenesis [190]. It was reported that a scaffolds (PLGA) combined with microspheres from the same material loaded with TGF-β1 (50ng) and BMP-2 (5000ng), improved short term cartilage repair in rabbit osteochondral defects [248]. Additional acellular scaffold studies were performed, with TGF-β family growth factors in osteochondral rabbit defects [80, 249]. These types of scaffold systems were also assessed *in vivo* with MSCs using different biomaterials. A scaffolds combining PLGA/gelatin/chondroitin/HA was seeded with MSCs and implanted in rabbits osteochondral defects [250]. After 28 days, *in vivo* better cartilage was observed with cells implanted. However, no TGF-β was present. In addition, the same scaffolds were assessed in the same model and with MSCs, but in this experiment the best performing group was the one were the cells were preconditioned in TGF-β3 [251]. To conclude this section, it is agreed that much needs to be done in terms of *in vivo* assessment and also about the devices used to deliver TGF-β. Ideally the scaffolds and hydrogels used would be chondrogenic and also be able to deliver only the adequate amount of growth factor to induce cartilage repair. ECM-derived material, which is the subject of section 2.4, is a viable candidate to this challenging task.

2.3.6. Single-stage therapies for cartilage repair

Current cartilage therapies have been aiming to tackle symptoms. These ones include microfracture, autologous chondrocyte therapies and replacement with metallic implants [20]. However, these therapies are limited due to donor site and articular joint morbidity, the presence of alloplastic material, limited long-term performance and structural failure [18, 20]. As an alternative are available TE techniques, which are promising for cartilage repair [69]. The combination of cells, chondrogenic factors and bioactive scaffolds would be ideal to fully restore articular cartilage function and structure [69]. Currently used cell-based therapies using commercially available scaffolds (e.g. Chondrogide®) such as MACI, may lead to reasonable success in

cartilage regeneration and in improving patients' life [20]. However, the prohibitive costs of such interventions along with the two hospitals stays have been limiting the widespread adoption of such approach in the clinical context [16, 113]. The use of differentiated cells (chondrocytes) presents additional drawbacks. These include cartilage harvesting, which creates additional damage, low availability, only adequate for small defects, and when in culture, chondrocytes can present dedifferentiation [20, 113, 252]. Limitations for differentiated cells application can be overcome by using MSCs [20], which are available from different tissues (section *2.3.3*). An example of a viable source is adipose tissue, which is easy to access and possess high competent stem cell yield [122, 253]. The stromal vascular fraction is where the adipose-derived stem cells (ASCs) are present [20]. Consequently, the cell yield and the multipotent nature of such ASCs opens the door for the use of these cells for single-stage procedures for cartilage repair [20]. Single-stage procedures are based on the concept of isolating multipotent cells and implant them into the patient in one surgical procedure. This will avoid the need of costly and time consuming cell culture expansion, and the need of two hospital stays [254]. Therefore, as a result of single-stage techniques promising results, this type of approach has been getting a lot of attention lately [20-22, 255-257].

The choice of infrapatellar fat pad (IFP) as the source of stromal cells for single-stage therapies would be advantageous to the patient, considering that with a single surgery the multipotent cells would be harvested and seeded in a chondrogenic scaffold, as well as implanted in the cartilage defect [20]. Hence, to be able to develop an efficient single-stage procedure for cartilage repair the following key points must be addressed [20]:

- Rapid and efficient viable multipotent cell isolation.
- High cell yield for regenerative therapy.
- Cells with stem cell features [258].

- Chondrogenic differentiation capacity of stromal cells when seeded into a chondrogenic biomaterial-based scaffold.

This single-stage approach for cartilage regeneration was assessed in previously reported studies [20-22, 255-257]. This approach was tested chondrogenically in PLA/PCL scaffolds, where 0.5 million expanded cells and 2 million ASCs freshly isolated where independently seeded [20]. 25% of the freshly isolated cell fraction adhered to the scaffold and performed similarly to the expanded cells. These stromal cells proved to be chondrogenic and suitable for this type of single-stage approach. However, due to the variable amount of tissues harvested and cell yields, the IFP stromal fraction may only be applicable in small focal cartilage defects [20]. Hence, for larger osteoarthritic defects, subcutaneous fat could more suitable due to high amounts of tissue available [20]. In an additional single-stage therapy published study, the source for obtaining ASCs was subcutaneous adipose tissue [255]. The stromal fraction provided again a high cell yield, and such cells were seeded (2 million/scaffold) in a PLA/PCL and type I/III collagen scaffolds, both commercially available. Cells attached rapidly, and chondrogenic matrix was deposited in both scaffolds with satisfactory promising results for single-stage therapies for cartilage repair [255]. In an additional study where IFP-derived cells where encapsulated in a TGF-β3 releasing agarose hydrogels (0.3 million cells), it was observed a promising chondrogenic response [256]. In this study, freshly isolated stromal cells kept to adhere to plastic (30min), and used in the agarose gels. Results for the selective adhesion were not very promising in terms of cell yield; however, the system agarose/growth-factor/stromal cells (no selective adhesion) presented promising results for cartilage single-stage therapies [256].

Several *in vivo* studies were performed in the past with promising outcomes for single-stage therapies [21, 22, 257]. Considering that ACI has some limiting drawbacks, an alternative *in vivo* was assessed in a subcutaneous and in a goat

articular lesion model [22]. In this study, it was hypothesised that the combination of chondrons [259] and mesenchymal stromal (bone marrow) cells would improve ACI, by performing the procedure in one single-stage. Cells were delivered using fibrin as vehicle in nude mice and in the goat cartilage defect. The combination of both cells increased cartilage-specific matrix and it was efficiently delivered in a cartilage defect outperforming microfracture [22]. The same research group presented an additional *in vivo* study (goat cartilage defect), where a focal defect in cartilage was treated with freshly isolated bone marrow stromal cells and chondrocytes [21]. Fibrin was the biomaterial used, with this combination resulting in cartilage regeneration when compared with microfracture (Figure 19). Finally, a single-stage *in vivo* study in a caprine articular cartilage defect model was performed [257]. Adipose-derived stromal cells were freshly isolated and compared with expanded ASCs when seeded in type I/III collagen to regenerate cartilage defects in goats. Freshly isolated stromal cells group outperformed the others in all parameters. Hence, these *in vivo* evidences motivate the focus on such therapies. However, there is still a need to perform additional studies to optimize such single-stage procedures for cartilage repair, and enable widespread use of this approach.

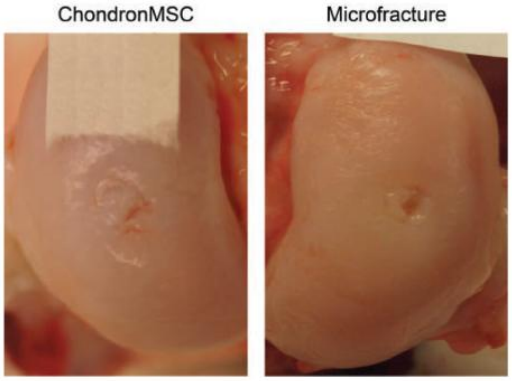


Figure 19 – Macroscopic defect repair for both chondron/MSCs and microfracture treatment [22].

To develop a single-stage therapy for cartilage repair, it is imperative to freshly isolate natural chondrogenic multipotent cells. As an example we have stromal cells that express specific surface markers, such as CD44 or CD90. Stromal cells

expressing such surface markers are promising candidates to form cartilage-like tissue. CD44 is the principal cell surface receptor for hyaluronate [260], a key component of articular cartilage. The IFP is a source of cells with a high expression of CD44 [261]. It has been previously reported that a microenvironment enriched with HA initiates and promotes chondrogenesis *via* CD44 in human ASCs [66]. In addition, previous studies have demonstrated that CD44 antibody-beads can be used for stem cell isolation and delivery, and that such complexes can effectively generate chondrogenic matrix in monolayer and 3D culture [262, 263]. The technique for stromal cell isolation will be of crucial importance for the development of single-stage procedures for cartilage regeneration [255]. Hence, to select the ideal stromal cell fraction, scaffolds and chondrogenic factors will be the main challenge to the optimization of such technique in cartilage TE. ECM-derived scaffolds have been shown as a viable chondrogenic scaffold material and promising to be used in single-stage therapies for cartilage repair, which is going to be the focus of the following section.

2.4. ECM-derived scaffolds for cartilage repair

2.4.1. Introduction

Current clinical strategies to repair cartilage defects use material harvested either from a non-load bearing joint region or from a donor. Techniques such as mosaicplasty are being utilized in the clinical context, where osteochondral plugs are used to fill defects with the aim of reconstructing damaged cartilage in the human joint [264]. As already discussed, this approach leads to significant donor site morbidity, motivating the development of novel cartilage TE strategies. The use of ECM-derived scaffolds is an innovative and emerging approach in cartilage regeneration, which has been reinforced by promising results [38, 184]. There are numerous advantages of using ECM as a raw material for scaffold fabrication, and one of the most relevant ones is the capability to retain beneficial pro-chondrogenic growth factors such as

TGF-β, FGF and IGF within the matrix [69, 265, 266]. The lack of growth factor and nutrient supply due to the absence of vascularisation in articular cartilage may be one of the main reasons why cartilage fails to spontaneously regenerate. Hence, ECM-derived scaffolds rich in chondrogenic cues may help in the challenging task of regenerating hyaline cartilage [38]. The ECM for cartilage regeneration applications can be from different sources and divided in different categories that are going to be the theme of the following paragraphs.

2.4.2. ECM-derived biomaterials and current techniques

The ECM material for cartilage regeneration can be from different sources. Consequently, due to different features of the dissimilar ECM, a few research questions need to be addressed before definitive clinical translation. Several examples of successful applications have been published recently, namely non-decellularized cartilage particles combined with a commercial available degradable polymer (fibrin), method that proved to be as effective as microfracture [267]. Chen *et al*. demonstrated that particulated devitalized ECM from osteoarthritic cartilage can be use with the same fibrin glue for a similar application [268]. Another viable alternative to use these micro-particles mixed with cell suspensions, which may enhance efficiency in ACI or MACI [269, 270]. Furthermore, cartilage can be harvested from allogeneic [271, 272] or xenogeneic [273] donors and then used in scaffold fabrication [38], with allogeneic meaning that we have tissue from a donor from the same species, and xenogeneic from a different species (Figure 20) [106].

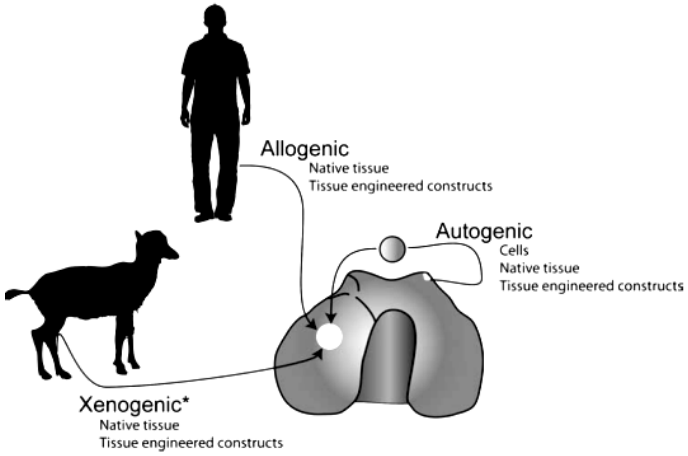


Figure 20 – Autogenic, allogeneic and xenogeneic sources for cartilage treatment. (*) not currently used clinically [106].

Currently in the biomedical market it is possible to find ECM-derived products that proved to be promising for cartilage regeneration. Examples include BioCartilage® (Anthrex, USA), that basically consists of dehydrated, micronized allogeneic devitalized cartilage. These ECM particles are implanted in conjunction with platelet-rich plasma (PRP) into a microfactured defect (Figure 21A and B) [95]. Furthermore, another single-stage technique for cartilage regeneration (allograft) uses viable juvenile particulated cartilage (DeNovo® NT, Zimmer, USA) (Figure 21C and D) [274]. The rationale for the use of juvenile cartilage is that it has been proven that human allogeneic juvenile chondrocytes have greater growth potential than adult chondrocytes [274, 275].

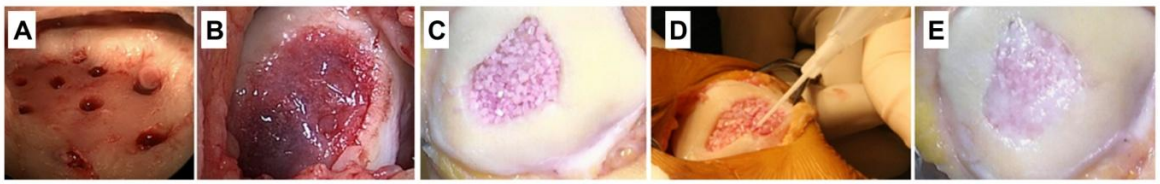


Figure 21 – Microfracture (A) and fibrin glue + micronized allogeneic cartilage (*BioCartilage*) applied into the cartilage defect (B) [95]; Particulated allogeneic juvenile cartilage (*DeNovo NT*) in defect (C), fibrin glue + cartilage particles (D) and defect filled with particles and solidified fibrin (E) [274].

Autogenic or autologous tissues refer to tissue that originates from the same individual/patient (e.g. osteochondral plugs) [106]. Autogenic approaches have shown

reasonable success by using isolated chondrocytes (e.g. ACI), native tissue autografts (e.g. mosaicplasty), and tissue constructs (e.g. MACI). Revell and Athanasiou stated in 2009 that in clinical context, allogeneic cell sources have been successful when surrounded by ECM in native tissue grafts and tissue engineered constructs [106]. Furthermore, animal studies indicate the possibility to successfully repair cartilage using tissue grafts or tissue engineered constructs [106]. However, there are several drawbacks of using native ECM-derived material such as its possible immune response. This sensible subject is going to be the focus of the following section.

2.4.3. Immune response to ECM-derived material

Numerous decellularized products are currently available in the market (Table 2) for different applications [37, 38, 276].These types of products present a wide range of remaining cellular material after decellularization, mainly because there are not strict regulatory guidelines for the degree of decellularization [276, 277]. One possible reason for this is that cellular debris in devitalized tissue may not suppress the process of regeneration of the native tissue [269, 278].

Table 2 – ECM based commercial products available in the market [276].

Product	Company	Material	Form
AlloDerm®	LifeCell™	Human skin	Natural
Axis™ dermis	Mentor	Human dermis	Natural
Bard Dermal Allograft	C R Bard	Cadaveric human dermis	Natural
CuffPatch™	Biomet Sports Medicine	Porcine small intestinal submucosa (SIS)	Cross-linked
DuraADAPT™	Pegasus Biologicals	Horse pericardium	Cross-linked
Dura-Guard®	Synovis Surgical	Bovine pericardium	Cross-linked
Durasis®	Cook® Medical	Porcine small intestinal submucosa (SIS)	Natural
Durepair®	TEI Biosciences/Medtronic	Fetal bovine skin	Natural
FasLata®	C R Bard	Cadaveric fascia lata	Natural
Graft Jacket®	Wright Medical Tech	Human skin	Natural
Oasis®	Cook® Biotech/Healthpoint	Porcine small intestinal submucosa (SIS)	Natural
OrthADAPT™	Pegasus Biologicals	Horse pericardium	Cross-linked
Pelvicol™	C R Bard	Porcine dermis	Cross-linked
Peri-Guard®	Synovis® Surgical Innovations	Bovine pericardium	Cross-linked
Permacol™	Covidien	Porcine skin	Cross-linked
PriMatrix™	TEI Biosciences	Fetal bovine skin	Natural
Restore®	DePuy	Porcine small intestinal submucosa (SIS)	Natural
SurgiMend®	TEI Biosciences	Fetal bovine skin	Natural
Surgisis®	Cook® Medical	Porcine small intestinal submucosa (SIS)	Natural
Suspend™	Mentor	Human fascia lata	Natural
TissueMend®	TEI Biosciences	Fetal bovine skin	Natural
Veritas®	Synovis® Surgical Innovations	Bovine pericardium	Cross-linked
Xenform®	TEI Biosciences/Boston Scientific	Fetal bovine skin	Natural

Gilbert *et al*. compared the amount of DNA in ECM products being used for clinical applications (Figure 22), demonstrating the existence and variability in cellular material present in current products [277].

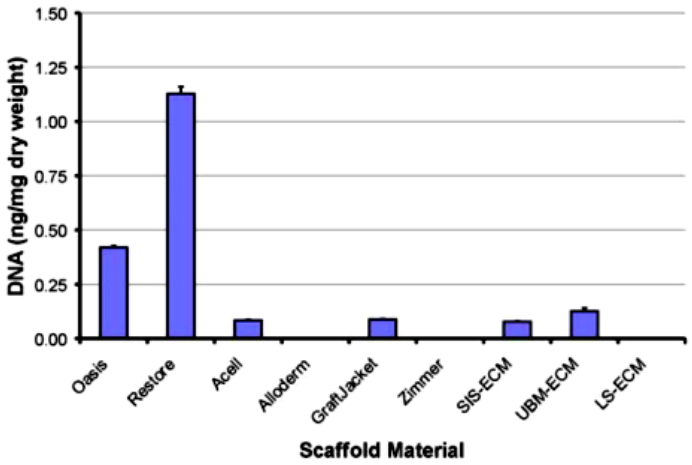


Figure 22 – DNA content determined (PicoGreen Assay) for commercial and lab products produced with ECM [277].

The necessary immune mechanism response, after foreign cellular and/or material implantation, is partially mediated by macrophages [276]. These cells take part on the event of scaffold degradation, secreting soluble factors important for new tissue formation and remodelling. As described before, decellularization can influence which factor macrophages will secrete after implantation [276]. Another important factor that needs to be taken into account is the avascular nature of cartilage, which can be detrimental regarding nutrient and growth factor supply. However, avascularity can also be beneficial because such tissues are often considered to be immunopriviliged, enabling the use of additional ECM sources (e.g. allogeneic and xenogeneic) without rejection limitations [106]. In addition, the dense matrix in cartilage ECM may diminish the immunogenic response, given that it protects chondrocytes from T and NK cells that are released during rejection [106].

In the specific case of cartilage ECM immunogenicity, a response can be initiated with surface markers in cells, matrix epidopes, and DNA [39]. However, there is a lack of understanding concerning immune response in chondral xenogeneic devices/implants [39]. Some authors claim that chondral defects are fairly immunoprivileged, when compared with osteochondral defects, mainly due to subchondral bone exposure [39]. However, recently published work claimed that the

cartilage present in the articular joint is not an entirely immunoprivileged location, in which xenogeneic implantation generated high levels of rejection [279]. Conversely, the same did not happen for allogeneic chondrocyte implantation, and additionally, this immune response depended on the location in the articular joint [279]. It was reported that this detrimental immune response in the articular joint may be related with synovium proximity [279]. Hence, additional research work is necessary to understand how immuno-privileged cartilage is, and what needs to be done to implants before implantation and further clinical translation [39]. Decellularization of ECM-derived biomaterials is a subject of interesting discussion nowadays, and it is going to be the subject of the following section.

2.4.4. Decellularization of native ECM

The preparation of tissues to extract ECM from is usually made by using a devitalization and/or decellularization step [38]. These can be accomplished by using several methods combined [38]. Methods including thermal abrupt differences, freeze-thaw cycles, and fragmentation are used to promote cell lysis and tissue rupture [38]. This will enhance effectiveness of biochemical treatment usually used [280]. Furthermore, treatments including chemicals are frequently used [38]. These include Triton-X and SDS, which are used to destroy cellular and nuclear membranes [281]. These chemicals have to be efficiently washed from ECM after treatment [281]. There are additional enzymatic treatments that can be performed, which depend on the tissue type [38]. These include trypsin and nuclease treatments which are often used to remove peptides, RNA and DNA [281]. Several previously performed studies combined physical end chemicals methods to decellularize cartilage-derived ECM [271, 272, 282-288]. These harsh treatments should ideally remove cellular elements without removing or destroying bioactive beneficial cues present in the ECM, such as growth factors and sGAG [289]. Both single tissues and entire organs can be decellularized and used as biologically accurate scaffolds, to enhance nutrient supply,

and to further re-cellularization and tissue regeneration [280]. In the particular case of tissue decellularization the process can be harsher when compared with organ decellularization treatment [38]. Criteria have been discussed for efficient decellularization (or denuclearization) [280]:

- Absence of nuclei;
- DNA below 50 ng/mg of dry tissue;
- DNA fragments below 200 bp [280].

However, these points were not obtained and standardized using cartilage, therefore it may not apply to a tissue as dense as articular cartilage [38]. These harsh decellularization treatments promote ECM changes in structural integrity, and leaching of ECM components. If total decellularization is necessary for cartilage, is still under discussion [38], mainly due to previously reported studies where not fully decellularized ECM induced comparable host remodeling to the one induced by fully decellularised ECM [276]. Hence, additional *in vitro* and *in vivo* studies need to address this issue and determine the effects of devitalization and decellularization on the ECM used to fabricate and functionalize devices for cartilage repair. ECM derived from native articular cartilage has been used to produce chondro-permissive scaffolds and assessed *in vitro* and *in vivo*. Therefore, this subject is going to be the focus of the following section.

2.4.5. Scaffolds derived from cartilage ECM

Synthetic biomaterials can provide biomimetic properties to the scaffold; however, these generally possess a limited capacity to interact with cells alone [290]. Due to the this limitation, native cartilage ECM appears as an important material for scaffold fabrication mainly because it provides the ideal physical and chemical cues that regulate proliferation, differentiation and matrix synthesis of the seeded cells [36, 186].

ECM-derived scaffolds for cartilage TE can be fabricated using treated and/or processed cartilage ECM [36, 39, 291-293]. Previous studies claim the benefits of devitalized tissue over implantation of living cartilage [294]. There are different techniques that can be used to devitalize and to give shape to processed ECM, such as the so-called lyophilisation (or freeze-drying), that proved to be effective creating porous constructs while enhancing cartilage formation [36]. Physical (e.g. UV) and chemical (e.g. carbodiimide) crosslinking techniques can also be used to mechanically stabilize the scaffold [36, 273].

Yang *et al*. developed an acellular ECM-derived scaffold from human cartilage, where freeze-drying was used to create the needed porosity [285]. Cartilage was obtained from human cadaveric joints, pulverized and resuspended in PBS before being freeze-dried. After freeze-drying the scaffold was physically and chemically crosslinked. After 4 weeks in culture the bone marrow-derived stem cells (BM-MSCs) created a cartilage-like tissue with positive staining for type II collagen [285]. Another example in literature is an acellular ECM scaffold derived from bovine articular cartilage, that was freeze-dried, crosslinked with UV, and seeded with rabbit BM-MSCs. The results *in vitro* and *in vivo* denoted a significant improvement when compared with the controls after 6 and 12 weeks [286].

Devitalized cartilage ECM has been included in several studies throughout the years [36, 273, 291, 292, 295]. Xenogeneic ECM material (i.e. ECM from another species) can be the solution for the lack of ECM availability and also a cost-effective way of dealing with this limitation [38]. Porcine articular cartilage has been used to fabricate ECM-derived scaffolds, where cartilage was homogenized into slurry, freeze-dried and seeded with ASCs from liposuction waste, and cultured *in vitro* without exogenous supplementation of growth factors [292]. The results were positive, with high type II collagen and other cartilage specific components after 4 and 6 weeks. After 6 weeks, the morphology of the construct was close to native cartilage with typical round-shaped cells in a GAG rich matrix. This ECM-derived scaffold result suggests

the induction of chondrogenesis of ASCs without exogenously supplied growth factor. This result indicates that soluble cues were entrapped in the ECM matrix and enhanced chondrogenesis [292]. Following the aforementioned study, Cheng *et al*. assessed similar ECM-derived scaffolds, with human and porcine chondrocytes seeded [295]. These ECM-derived scaffold supported proliferation of both human and porcine chondrocytes with high percentage of cartilage-specific macromolecule deposition. Human chondrocytes migrated throughout the construct, with homogeneous distribution of cells and newly formed matrix, while porcine cells tended to form a GAG rich layer on the outside of the construct. Human chondrocytes presented lower modulus at the end of the culture period. Again it was shown that this type of scaffold, in the absence of exogenous growth factors, can support neocartilage formation [295]. This study also indicated that properties of the construct are dependent of factors such as concentration (Figure 23), porosity and species of cells [295]. One of the crucial conclusions of these last two studies is that the constructs undergo cell mediated contraction throughout the culture period.

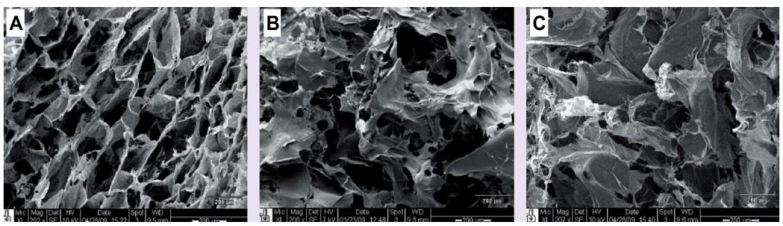


Figure 23 – SEM micrographs showed different structures of ECM-derived scaffolds made with different slurry concentrations: (A) 0.2 g/ml; (B) 0.1 g/ml; (C) 0.05 g/ml [295].

To address the challenge of cell mediated contraction of cartilage ECM-derived scaffolds, further studies have been undertaken where a crosslinking technique was used to strengthen the biomaterial-based scaffold [36, 273]. Previously described porcine ECM-derived scaffolds [292, 295] were crosslinked with genipin at 0.005%, 0.05% and 0.5%, seeded with human ASCs and cultured for 4 weeks to evaluate the effects of this process on scaffold contraction and chondrogenesis [273]. At the highest degree of crosslinking, most cells failed to attach and this resulted in poor deposition of

new ECM. The lowest crosslinking group performed similarly to the non-crosslinked group. The group that was subjected to 0.05% genipin, which corresponded to 50% crosslinking, exhibited no significant contraction. Expression of cartilage-specific genes, synthesis, deposition of collagen type II and GAG and mechanical properties were comparable to the non-crosslinked ECM-derived scaffold. The findings support the use of moderate crosslinking as a means to limit scaffold contraction [273].

Rowland *et al*. studied the effects of different crosslinking techniques in chondrogenesis of MSCs seeded in porcine ECM-derived scaffolds [36]. In this study, ECM-derived scaffolds (Figure 24) were seeded with human MSCs derived from iliac crest. It was assessed one group without crosslinking, another with dehydrothermal (DHT) physical crosslinking (120˚C, 24h), other group with UV light physical crosslinking, and finally one group with carbodiimide chemical crosslinking. After the culture period, all crosslinked groups retained the original dimensions. Physically crosslinked scaffolds facilitated significantly higher GAG and collagen deposition than carbodiimide crosslinked scaffolds and non-crosslinked scaffolds. The treatments influenced the newly formed matrix and DHT group was the one that best matched native cartilage composition. Cell adhesion was inhibited by carbodiimide treatment. The effects seem to have been mediated by alterations to cell-biomaterial communication between MSCs and the ECM scaffold [36].

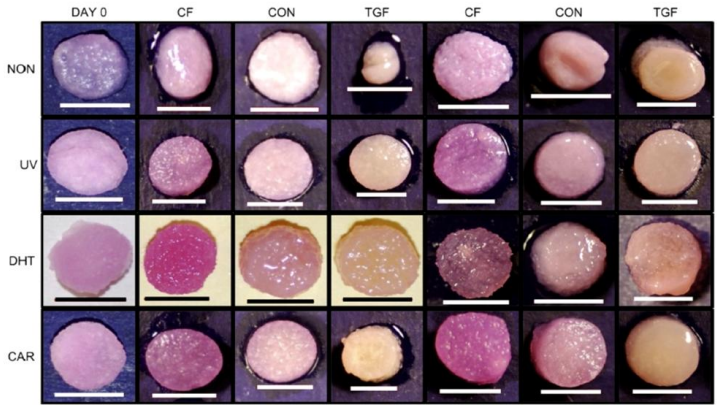


Figure 24 – Macroscopic images of all the ECM-derived scaffold groups in the crosslinking effect study at day 0 and at the end of the culture period; (CF) means cell-free; (CON) control media; (CAR) carbodiimide crosslinking [36].

Diekman *et al*. used a similar ECM-derived scaffold as abovementioned [292], and seeded ASCs or BM-MSCs, and compared those chondrogenically with alginate beads. This study involved growth factor stimulation (TGF-β3 and BMP-6). Chondrogenic growth factor induced chondrogenesis in both cell types, in alginate and in ECM-derived constructs. BM-MSCs enhanced type II collagen gene expression and hypertrophic phenotype. ASCs had higher aggrecan expression in response to BMP-6, while BM-MSCs responded more favourably to TGF-β3. This study proved the different behaviour of ASCs and BM-MSCs in response to chondrogenic growth factors, and that chondrogenesis is affected by composition of the scaffold and presence of serum [291].

Finally, ECM necessary to build this type of scaffold can be obtained from cell culture, as an alternative for the aforementioned ECM sources [39, 271, 282, 296, 297]. This cell-derived engineered ECM overcomes problems with exogenous pathogens and allows the use of ECM-derived from patient own cells [38]. The use of autologous scaffolds may also diminish inflammatory and immune responses, however there are limitations regarding availability. Lu *et al*. presented a study where an autologous ECM scaffold was fabricated by combining culture of autologous cells in a 3D template, followed by a decellularization and finalized with template extraction (Figure 25). Furthermore, the scaffold was implanted showing high biocompatibility [271]. The main limitation of the use of cell culture derived ECM is the amount of tissue available, and the main challenge is to upscale the process in a way that could be applied for human cartilage regeneration [38, 39]. Alternatives have been proposed where sheets of expanded tissue would be stacked to create a multilayered construct [272, 298].

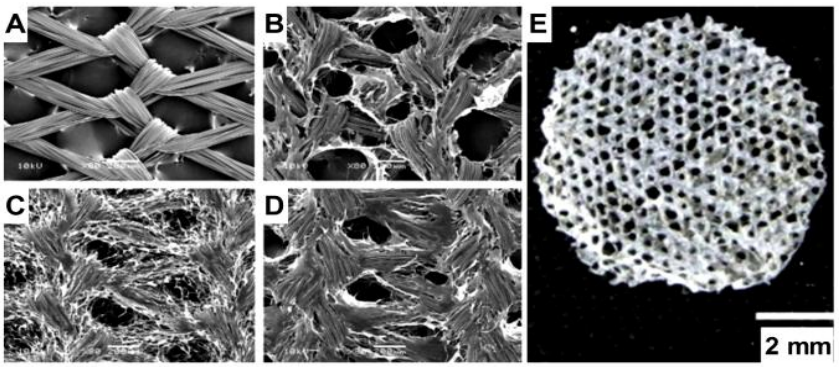


Figure 25 – SEM micrographs after 5-6 days in culture of biomaterial (PLGA) based template (a), stem cells + ECM + PLGA (b), chondrocytes + ECM + PLGA (c) and dermal fibroblasts + ECM + PLGA (d); (E) represents the autologous ECM-derived scaffold after the template removal [271].

2.4.6. In vivo assessment of ECM-derived scaffolds

An important part of the process of moving into a clinical context depends on the outcome of *in vivo* experiments. However, the success of the TE strategy is going to be dependent on the scaffold used for structural support, mainly due to its morphological features mimicking the native tissue microenvironment [299]. As already mentioned, pore size and shape [300, 301], and cell mediated contraction *in vivo* will dictate TE construct fate [273]. Several studies have supported the idea of that ECM-derived material can induce chondrogenesis and/or the formation of cartilage-like tissue *in vivo* [285, 286, 294, 302-304]. In the *in vivo* assessment of the developed scaffolds different animal models can be used, such as subcutaneous mouse [285, 294, 304] and rat [305], rabbit cartilage defect [286, 302] and canine cartilage defect [303] models, among others [21].

Peretti *et al*. assessed subcutaneously *in vivo* the performance of devitalized and living discs of porcine cartilage [294]. Cells and fibrin were sandwiched between discs and implanted for 2, 5 and 8 weeks, indicating that devitalized ECM group formed superior matrix. Conversely, further studies described subcutaneous *in vivo* assessment of freeze-dried ECM-derived scaffolds fabricated with decellularized pulverized human ECM [285, 304]. These scaffolds promoted, after 4 weeks *in vivo*,

cartilage-like tissue formation from BM-MSCs. In addition, another study was performed where human ECM was used to induce cartilage tissue formation subcutaneously in mice [304]. The results also supported the idea of the beneficial presence of biochemical cues in the ECM-derived scaffold [304].

Nevertheless, further *in vivo* studies with different animal models are necessary. The cartilage defect model in rabbits has been used to assess ECM constructs previously. Yang *et al*. used bovine cartilage to fabricate scaffolds that were freeze-dried, UV crosslinked, seeded with rabbit BM-MSCs, and further implanted in rabbit cartilage defects [286]. Constructs improved defect healing when compared with controls [286]. Similar results were obtained in the same *in vivo* context from Kang *et al*., with the difference of human native ECM and ASCs being used [302]. The positive outcome from these previously described studies points towards the use and success of cartilage ECM-derived constructs for cartilage defects repair. Further *in vivo* studies and clinical trials are necessary to clinically translate such promising approach.

2.5. Conclusion

This literature review demonstrated the existence of numerous approaches to regenerate damaged cartilage. However, some of these are only palliative and with reduced long-term effectiveness [1]. Current cell based techniques such as autologous chondrocyte implantations (ACI) have been reasonably successful clinically, however, ACI requires two hospitals stays and the cost of the procedure is prohibitive. Consequently, a new door is open for single-stage cell-based therapies, which do not require two hospital stays and the limiting cell expansion [20-22, 59, 267]. To perform single-stage approaches several requirements need to be fulfilled namely a biomaterial-based scaffold, or hydrogel, capable of supporting chondrogenesis of chondro-progenitor cells. These cells should be obtained from an adequate viable source of multipotent stromal cells, such as infrapatellar fat pad [306] or subcutaneous fat [122].

The challenge of designing and selecting the adequate scaffold can be overcome by using natural-derived materials. Cartilage ECM-derived biomaterials have been shown as highly chondroinductive *in vitro* [36, 292] and *in vivo* [285, 302, 303]. The possibility of using natural-derived material is also appealing when compared with the usage of synthetic ones available in the market, which try to mimic the native ECM. The use of native-derived ECM will lead to a more favourable response from the cells and also from the host tissue after implantation. Therefore, cartilage ECM is going to be the focus as raw-material for scaffold fabrication and functionalization in this study.

Scaffolds for cartilage tissue engineering should be able to facilitate cell infiltration, cell retention, growth factor entrapment and its efficient controlled release. To this end an adequate optimization is required, which includes obtaining a suitable porosity, mean pore size, and morphology [23]. This improvement can be achieved by changing concentration of the ECM used in the scaffold fabrication [295], enabling cell homogeneous infiltration and consequent tissue ingrowth. The process of optimizing

such ECM-derived scaffold for cartilage TE also includes the selection of the adequate crosslinking technique, to avoid undesired cell-mediated construct contraction usually observed in ECM-derived scaffolds [36].

Chondrogenic growth factors (e.g. TGF-β) have a crucial function in the process of cartilage regeneration, due to its influence in driving chondrogenesis [190]. Hence, scaffolds and hydrogels to be used in cartilage TE should be able to retain and release growth factors in an optimal window for chondrogenesis. Cartilage native tissue-derived devices emerge as a viable alternative to transport and supply these growth factors, mainly due to TGF-β strong affinity to ECM [201, 266], while enhancing chondrogenesis. One of the goals of this thesis is to assess this material as a potent chondro-inductor and also as an agent to release bio-chemical factors in an optimal time window for promoting chondrogenesis. By using the ECM as a delivery system for growth factors, it will not be necessary to use other delivery systems such as gelatin microspheres [52].

The chondro-progenitor cells to be used in cartilage cell-based therapies can be obtained from diverse tissues. However, for the current study, infrapatellar fat pad-derived stem cells (FPSCs) will be used. FPSCs are a viable choice for cartilage tissue cell-based therapies, mainly because they are accessible and possess a strong chondrogenic potential [20, 46, 51-55, 58, 60, 136]. The high cell yield from this tissue opens up the potential of using freshly isolated (not culture expanded) IFP-derived stromal cells as part of a single-stage therapy for cartilage regeneration [19-22]. Due to the importance of the cell yield and the chondrogenic potential of the cells used, it is also pertinent to explore the isolation of specific chondro-potent stromal cells sub-populations (e.g. $CD44^{+}$) [66, 67]. Single-stage approaches could potentially overcome the need to expand cells *in vitro* for cartilage regeneration therapies.

Growth factors are vital for cartilage TE, and to understand its effects on the stem cells and chondrogenesis will enable the creation of more effective cartilage TE therapies. Numerous different growth factor delivery systems have been developed for

various TE applications. However, relatively little is known about how the dose of a specific protein will influence tissue regeneration, or how different patients will respond to altered levels of growth factor delivery. Hence, it was sought to assess stem cell chondrogenesis in cartilage ECM-derived scaffolds loaded with escalating levels of exogenous TGF-β3.

ECM-derived scaffolds have been developed from devitalized native cartilage and successfully used for cartilage tissue engineering [38]. They are commonly derived from animal (xenogeneic) tissues, which may elicit an adverse immune response. Native human ECM can be used as an alternative to xenogeneic tissue; however, its supply is limited, leading to the need for a more readily available tissue source. Hence, it was sought to compare native and tissue engineered cartilaginous ECM as chondroinductive scaffold material for cartilage TE.

Native ECM-derived scaffolds have been proven as viable choices while enhancing chondrogenesis. However, strategies relying on these highly chondro-permissive structures possess limitations, such as the necessity of an invasive surgery, poor fixation in complex-shaped cartilage defects, and limited mechanical properties. Hence, to address the accessibility and fixation issues of the scaffolds, it was sought to ECM-functionalize a fibrin hydrogel to enhance chondrogenesis. Ideally, the assessment of such a system for cartilage TE should be performed *in vitro* and *in vivo*.

Due to the mechanical limitations of ECM-derived scaffolds, which ultimately will have to be implanted into the mechanical demanding joint environment, it may be necessary to explore alternatives to purely ECM-derived scaffolds. Covalently crosslinked shape-memory alginate has been shown to be suitable for TE, with adequate mechanical properties [169, 170, 307]. However, such covalently crosslinked biomaterials present some limitations that include poor cell seeding efficiency. Such limitation can be addressed by functionalizing the alginate scaffold with specific ECM components to allow superior cellular interactions. Additional morphological changes

can also be undertaken in these alginate scaffolds with the goal of maintaining or improve mechanical properties, and facilitating homogeneous tissue deposition. Such architectural changes in scaffold may also enable the formation of a more cartilage-like anisotropic tissue to be produced by seeded FPSCs.

The following chapters will describe the steps of optimization of a cartilage ECM-derived growth factor-releasing scaffold and ECM-functionalized devices to be used in conjunction with FPSCs, and also with freshly isolated stromal cells for single-stage cartilage repair therapies.

Chapter 3

General Methods

3. General methods

The following chapters focus on material that is going to be included (or already included) in publications during the current PhD timeline. As a consequence of that several methods steps are repeated during such chapters. Hence, to avoid repetition, the present general methods section was created with the aim of compiling the repeated protocols conducted during the current thesis. In the respective chapters, references to the general methods section will be made, and consequently the protocols will be condensed and easier to follow without the ineffective repetition. Therefore, each following chapter, which represents a publication, will only include relevant materials and methods used.

3.1. Porcine articular cartilage harvest

Native porcine cartilage tissue was harvested from femoral condyles, patella groove and tibial plateau using a biopsy punch (diameter 8 mm) of a female pig (3 month). The porcine breed from maternal side (50%) was half Landrace, a quarter Duroc and a quarter Large White, and the terminal side (50%) was: PIC Line 337. The native tissue was harvested in aseptic conditions. Cartilage was washed and kept in phosphate buffered saline (PBS; Sigma-Aldrich) supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml; GIBCO, Biosciences).

3.2. Freeze-drying

The cartilage slurry was transferred to custom made moulds (containing wells 5 mm in diameter and 3 mm in height) and freeze-dried (FreeZone Triad, Labconco, KC, USA) to produce porous scaffolds. The slurry was frozen to -30˚C (1˚C/min) and kept at that temperature for one hour. The temperature was then increased to -10˚C (1˚C/min) under vacuum (0.2 mbar), followed by a hold of 24 hours and then finally increased to room temperature (0.5˚C/min).

3.3. Crosslinking

The scaffolds underwent either dehydrothermal (DHT) crosslinking or both DHT and 1-Ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC; Sigma-Aldrich, Germany) crosslinking as previously described in literature [308]. The DHT process was performed in a vacuum oven (VD23, Binder, Germany), at 115˚C, in 2 mbar for 24 hours. The EDAC crosslinking consisted of chemical exposure for 2 hours at a concentration of 6 mM in the presence of N-Hydroxysuccinimide (NHS; Sigma-Aldrich, Germany), a catalyst that is commonly used with EDAC. A molar ratio of 2.5 M EDAC/M N-Hydroxysuccinimide was used [308, 309]. After EDAC crosslinking the scaffolds were washed twice for 30 minutes in sterile PBS.

3.4. Cell culture

Ethical approval for the isolation of human infrapatellar fat pad (IFP) stem cells was obtained from the institutional review board of the Mater Misericordiae University Hospital Dublin. Cells were isolated from the IFP of patients undergoing total joint arthroplasty. The IFPs were harvested from the knee joint capsule, weighed and washed thoroughly in PBS. Subsequently, the IFPs were diced in sterile conditions and followed by incubation under constant rotation at 37˚C in high-glucose Dulbecco's Modified Eagle Medium (hgDMEM, GlutaMAX^{TM}; GIBCO, Biosciences, Ireland) containing collagenase type II (750 U/ml, Worthington Biochemical, LaganBach Services, Ireland) and 1% penicillin (100 U/ml)-streptomycin (100 µg/ml) for 4 hours. A ratio of 4 ml of collagenase (750 U/ml) per gram of tissue was found to be optimal based on previous work [53-56]. After tissue digestion, cells were washed, filtered (40 µm nylon cell strainer) and centrifuged at 650 g for 5min. The supernatant (which include the majority of adipocytes) was then removed. The remaining cells were re-suspended, counted and finally plated ($5x10^3$ cells/cm^2) in T-175 flasks (Sarstedt, Wexford, Ireland). Cells were cultured in a standard media formulation, which consisted of hgDMEM containing 10% foetal bovine serum and 1% penicillin (100

U/ml)-streptomycin (100 mg/ml; GIBCO, Biosciences, Ireland) with the addition of fibroblast-growth factor-2 (FGF-2, 5 ng/ml; ProSpec-Tany TechnoGene Ltd, Israel). Cells were expanded to passage 2 (P2), with an initial seeding density of $5x10^3$ cells/cm^2 at each passage. Media changes were performed twice a week.

3.5. Construct culture

Scaffolds were seeded with human infrapatellar fat pad-derived cells. Constructs were maintained in chemically defined chondrogenic medium (CDM), as previously described, for the appropriate culture period (at 5% O_2 and 37˚C) [53]. CDM consisted of DMEM GlutaMAX™ supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml; both Gibco, Biosciences, Ireland), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1x insulin-transferrin-selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human growth factor-β3 (TGF-β3; ProSpec-Tany TechnoGene Ltd, Israel). In certain experiments, TGF-β3 was soak loaded into the scaffold. In such cases, TGF-β3 was not directly added to the culture media. The scaffolds were kept in 12 well plates and each scaffold was placed within cylindrical agarose moulds (slightly bigger than the scaffold) to prevent cell migration into the culture wells. After seeding, the scaffolds with the cells plus 40 µL of CDM were left in the incubator for two hours to allow cell attachment. After two hours, 2.5 ml of supplemented CDM were added to each well. Media changes were performed twice a week. The media was stored at -85˚C for further analysis.

3.6. Biochemical analysis

Biochemical analysis was performed at different time points, depending on the experiment, for sulphated glycosaminoglycan (sGAG), collagen, and/or DNA content. The scaffolds were enzymatically digested by incubating the constructs in papain (125 µg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma-Aldrich, Ireland) at 60˚C under rotation (10 rpm) for 18 h. The proteoglycan

content was estimated by quantifying the sGAG in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor, Northern Ireland), using bovine chondroitin sulphate as a standard. Collagen content was determined by measuring hydroxyproline content, after acidic hydrolysis of the samples at 110°C for 18 h in concentrated HCL (38%). Samples were assayed using a chloramine-T assay assuming a hydroxyproline/collagen ratio of 1:7.69 [310]. For some experiments, sGAG release from the scaffolds into the media was also determined. To this end, the culture medium was sampled for each construct at each media exchange.

3.7. Histology

Samples for histological analysis were fixed overnight at 4˚C in a 4% solution of paraformaldehyde (Sigma-Aldrich, Ireland). After being washed in PBS, samples were cut in half longitudinally, dehydrated and wax embedded. Wax embedded constructs were sectioned in 6 µm thick slices and mounted in microscope slides. Sections were stained with 1% alcian blue 8GX (Sigma-Aldrich) in HCl (0.1 M) for sGAG and with picro-sirius red for collagen. Cell nuclei were also stained with 0.1% nuclear fast red solution (Sigma-Aldrich). For some of the experiments, alizarin calcium staining was performed to assess calcium accumulation [160].

3.8. Immunohistochemistry

Immunohistochemical analysis was performed on 6 µm sections using monoclonal antibodies to type I and II collagen (Abcam, UK), depending on the experiment, as previously described [53]. Samples were washed in PBS and subjected to peroxidase activity (20 min). Slides were then incubated (1 hour, 37˚C in a moist environment) with chondroitinase ABC (Sigma, 0.25 U/ml) with the aim of enhancing the permeability of the ECM by removing the chondroitin sulphate. Slides were rinsed with PBS and blocked with 10% goat serum (30 minutes) and incubated with mouse monoclonal anti-collagen depending on the collagen to stain. A secondary antibody for the desired type of collagen (Anti-Mouse IgG Biotin antibody produced in goat;

concentration 1 g/l) binding was then applied (1 hour). By using Vectastain ABC reagent (Vectastain ABC kit, Vector, UK) for 5 minutes in peroxidase DAB substrate kit (Vector, UK) it was possible to observe a colour alteration. Samples were dehydrated with graded ethanol and xylene and mounted with Vectamount medium (Vector, UK).

3.9. Helium ion microscopy (HIM)

Freeze-dried acellular ECM-derived scaffolds were imaged using Helium ion microscopy (HIM; Zeiss Orion Plus, Germany). Image resolution of the microscope is manufacture specified at 0.35 nm, working distance was 10 mm and a 10 µm aperture was used. The beam current was 0.8 pA with a tilt angle of 15 degrees. Charge compensation was enabled using an electron beam flood gun and no additional conductive coating of the specimens was employed.

3.10. Scanning electron microscopy (SEM)

Acellular ECM-derived scaffolds were imaged using scanning electron microscopy (SEM). Structures were fixed in 4% paraformaldehyde solution (PFA) overnight. Furthermore, scaffolds were dehydrated through successive graded ethanol baths (10-100%), fixed onto aluminium stubs, coated with gold and examined under a field emission scanning electron microscope (Tescan Mira FEG-SEM XMU, Libušina, Czech Republic).

3.11. Transforming growth factor (TGF)-β3 quantification

The amount of TGF-β3 release from the growth factor loaded cartilage ECM-derived scaffolds or hydrogels was determined via ELISA. 96 well plates were coated with capture antibody, with the concentration of 360 µg/ml of mouse anti-human TGF-β3 (R&D Systems, UK). The samples (different time points depending of experiment) and TGF-β3 standards (ProSpec-Tany TechnoGene Ltd, Israel) were incubated for 2 hours. After washing and drying, detection antibody (18 µg/ml of biotinylated goat anti-human TGF-β3) was added to the plate and incubated (2 hours). The next step was to

wash, dry and incubate the plate in streptavidin-HRP (horseradish-peroxidase; R&D Systems, UK) for 20 minutes in the dark. Substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine; R&D Systems, UK) was added to each well, followed by incubation (20 min) avoiding direct light. Stop solution (2 N H_2SO_4; Sigma-Aldrich, Germany) was added and the optical density was determined immediately with a plate reader set to 450 nm.

3.12. *In vivo* subcutaneous mouse implantation

Six constructs were implanted into the back of nude mice (n=9; Balb/c; Harlan). Mice were weighed and anesthetized with an intraperitoneal injection of 10 mg/kg xylazine (Chanazine 2%; Chanelle) and 100 mg/kg ketamine (Narketan; Vetoquinol) [131]. Two skin incisions were made along the central line of the spine. Three constructs were inserted in each subcutaneous pocket, and sutured using 4-0 Vicryl plus (Ethicon, Johnson & Johnson) and tissue glue (Vetloc xcel). Euthanasia was performed 4 weeks after the surgery by CO_2 inhalation, and the constructs were analyzed histologically, immunohistochemically, and biochemically. The protocol was reviewed and approved by Trinity College Dublin ethics committee.

3.13. Statistical analysis

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

Chapter 4

Controlled Release of TGF-β3 from Cartilage Extracellular Matrix-derived Scaffolds to Promote Chondrogenesis of Human Joint Tissue-derived Stem Cells

4. Controlled release of TGF-β3 from cartilage extra cellular matrix-derived scaffolds to promote chondrogenesis of human joint tissue-derived stem cells

Abstract

The objective of this study was to develop a scaffold derived from cartilaginous extracellular matrix (ECM) that could be used as a growth factor delivery system to promote chondrogenesis of stem cells. Dehydrothermal crosslinked scaffolds were fabricated using a slurry of homogenized porcine articular cartilage, which were then seeded with human infrapatellar fat pad-derived stem cells (FPSCs). It was found that these ECM-derived scaffolds promoted superior chondrogenesis of FPSCs when the constructs were additionally stimulated with transforming growth factor (TGF)-β3. Cell mediated contraction of the scaffold was observed, which could be limited by the additional use of 1-Ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC) crosslinking without suppressing cartilage specific matrix accumulation within the construct. To further validate the utility of the ECM-derived scaffold, it was next sought to compare its chondro-permissive properties to a biomimetic collagen-hyaluronic acid (HA) optimized for cartilage tissue engineering (TE) applications. The cartilage ECM-derived scaffold supported at least comparable chondrogenesis to the collagen-HA scaffold, underwent less contraction and retained a greater proportion of synthesised sulphated glycosaminoglycans (sGAGs). Having developed a promising scaffold for TE, with superior chondrogenesis observed in the presence of endogenously supplied TGF-β3, the final phase of the study explored whether this scaffold could be used as a TGF-β3 delivery system to promote chondrogenesis of FPSCs. It was found that the majority of TGF-β3 that was loaded onto the scaffold was released in a controlled manner over 12

days of culture, with comparable long-term chondrogenesis observed in these TGF-β3 loaded constructs compared to scaffolds where the TGF-β3 was continuously added to the media. The results of this study support the use of cartilage ECM-derived scaffolds as a growth factor delivery system for use in articular cartilage regeneration.

Keywords: Articular cartilage, Extracellular matrix, Tissue Engineering, Stem Cells, Crosslinking.

4.1. Introduction

Scaffolds fabricated using devitalized extracellular matrix (ECM) have shown great promise for the regeneration of damaged tissues [186]. This approach has been used to develop different tissue-specific (e.g. heart valves, blood vessels, skin and cartilage [25, 37, 38, 48, 268, 272, 273, 292, 295, 303, 311-318]) scaffolds. In the case of articular cartilage, numerous studies have demonstrated that scaffolds derived from devitalized cartilage are chondroinductive and show great promise for regenerating damaged joints [25, 38, 48, 72, 268, 272, 273, 281, 292, 295, 303, 311-319]. For example, it has been demonstrated that ASCs secrete a matrix rich in glycosaminoglycans (GAGs) and type II collagen when seeded onto cartilage ECM-derived scaffolds, resulting in the development of a cartilaginous tissue with mechanical properties approaching that of the native tissue after 42 days in culture [273]. One of the factors that may limit ECM-derived scaffolds for cartilage tissue engineering applications is cell-mediated contraction of the construct either during *in vitro* culture or following implantation into a defect [273]. To overcome such problems different crosslinking techniques can be used to minimise or prevent contraction such as dehydrothermal (DHT) [25, 29, 35, 37, 308, 320-324], UV light [25, 322] and chemical crosslinking [25, 273, 308, 325]. Chemical methods include the use of glutaraldehyde [308, 326-328], 1-Ethyl-3-3dimethyl aminopropyl carbodiimide (EDAC) [25, 308, 309, 321, 329] and genipin [273]. DHT treatment consists of the removal of water present in the scaffold polymeric chains under vacuum and with temperature. By

removing the water from the collagen molecules, a condensation reaction occurs, leading to intermolecular crosslinking [308]. However it is unclear how these different crosslinking methods will influence the chondroinductive properties of cartilage ECM-derived scaffolds, with recent studies demonstrating that high levels of crosslinking do not support new cartilaginous ECM accumulation [273].

While cartilage ECM-derived scaffolds most likely retain endogenous growth factors that contribute to their chondroinductive properties, it may be that the combination of such a scaffold with additional exogenous growth factors will lead to more robust chondrogenesis of cells that are seeded onto or migrate into the construct. It has been shown that ECM acts as a reservoir for growth factors [72, 75, 186, 313, 330], opening up the possibility of using such materials as growth factor delivery systems. Members of the transforming growth factor-β (TGF-β) family of growth factors, which play a key role in driving chondrogenesis of MSCs [47, 48, 52, 70-73], have a natural affinity for ECM components such as proteoglycans [74, 75]. This suggests that cartilage ECM-derived scaffolds could be used as natural growth factor delivery systems, overcoming the need to introduce additional components into a scaffold such as microspheres to control the release of such soluble cues [47, 52, 74, 232, 331, 332].

Building on previous work in this field [25, 273], the global aim of this study was to develop a cartilage ECM-derived scaffold capable of promoting robust chondrogenesis of human IFP-derived stem cells (FPSCs). To this end, it was first sought to compare chondrogenesis of FPSCs within a cartilage ECM-derived scaffold in the presence or absence of exogenously supplied TGF-β3. Next, it was sought to determine whether EDAC crosslinking could be used to prevent scaffold contraction without impacting the chondroinductive properties of the construct. Having fabricated a suitable cartilage ECM-derived scaffold, then it was compared the chondro-permissiveness of this scaffold to a biomimetic scaffold produced from two key

components of cartilage tissue, specifically collagen and hyaluronic acid (HA) [35]. Finally, having demonstrated that a cartilage ECM-derived scaffold promotes robust chondrogenesis of human FPSCs in the presence of exogenously supplied TGF-β3, it was sought to determine whether the scaffold itself could be used as a delivery system to control the release of this growth factor and hence facilitate chondrogenesis of progenitor cells that are seeded into such a construct.

4.2. Materials and Methods

4.2.1. Scaffold Preparation

Porcine cartilage used in the fabrication of ECM-derived scaffolds was harvested as previously presented (section 3.1). The cartilage was first broken up into small pieces (approximately $1mm^3$) using a scalpel. These small pieces of cartilage where then minced in distilled water (dH_2O) using a homogenizer (IKAT10, IKA Works Inc, NC, USA) to create a cartilage slurry. The homogenized tissue was centrifuged and the supernatant was removed. The remaining material was re-suspended in dH_2O at a concentration of either 500 or 1000 mg/ml. The slurry was freeze-dried (section 3.2) and scaffolds underwent either DHT crosslinking or both DHT and EDAC crosslinking (section 3.3). Freeze-dryer ECM-derived scaffolds were imaged using Helium ion microscopy (section 3.9).

Collagen-HA scaffolds were fabricated using a freeze-drying method and were DHT crosslinked, as previously described [35]. Briefly, collagen-HA scaffolds were composed of collagen type I derived from bovine Achilles tendon (Collagen Matrix, USA) and hyaluronic acid sodium salt derived from *streptococcus equi* (Sigma-Aldrich, Arklow, Ireland). The final concentrations of the suspensions were composed of 0.5% (w/v) collagen and 0.05% (w/v) hyaluronic acid (HA).

4.2.2. Area determination

After 28 days in culture constructs were removed from culture and imaged next to a ruler. Pictures were analyzed with Image J to quantify area. Using a previously described approach [333], mean pore size was determined using Image J. The diameter of 15 pores present in cross-section of each scaffold was measured and averaged (n=3). Porosity was determined using liquid displacement method (n=10) [334].

4.2.3. Cell and construct culture

Stem cells used were harvested and cultured with the general method previously mentioned (sections 3.4 and 3.5). Scaffolds were seeded as previously described (sections 3.5). Each experiment used stem cells from different donors and construct culture was performed following the general protocol (sections 3.5). In certain experiments, TGF-β3 (3.2 ng in 40 µl of media) was soak-loaded into the scaffold. In such cases, TGF-β3 was not directly added to the culture media.

Histological (section 3.7), immunohistochemical (section 3.8), biochemical (section 3.6) analysis were performed at day 0 and after 28 days in culture as described in general methods section. TGF-β3 quantification analysis was performed in media samples collected during culture period, as described in general methods section (section 3.11).

4.3. Results

4.3.1. Cartilage ECM-derived scaffolds promote more robust chondrogenesis of human infrapatellar fat pad-derived stem cell in the presence of exogenously supplied TGF-β3

The architecture of scaffolds derived from freeze-dried slurries of cartilaginous ECM was found to depend on the initial concentration (either 500 mg/ml or 1000

mg/ml) of cartilage matrix within the slurry. More homogenous and spherical pores were observed in the 1000 mg/ml scaffolds, whilst less spherical pores and a wider distribution of pore sizes was observed in the 500 mg/ml scaffolds (Figure 26).

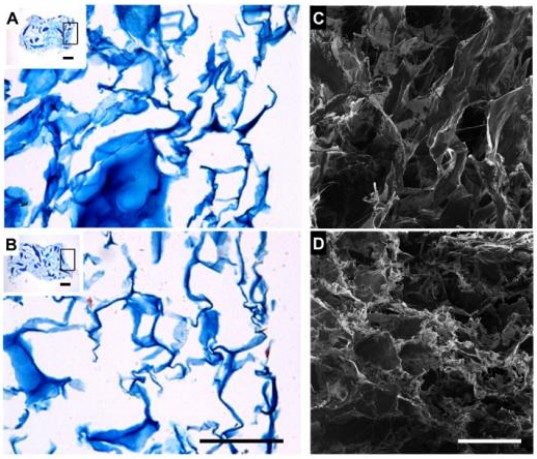


Figure 26 - Porous scaffolds at day 0. Alcian blue staining of freeze-dried extracellular matrix (ECM)-derived scaffolds (A) 500 mg/ml and (B) 1000 mg/ml at day 0. Helium ion microscopy (HIM) micrographs of porous freeze-dried ECM-derived scaffold (C) 500 mg/ml and (D) 1000 mg/ml at day 0 (scale bar: 100µm).

It was next sought to explore if the ECM-derived scaffolds, in the absence of exogenously supplied chondrogenic growth factors, could promote chondrogenesis of human FPSCs. The 1000 mg/ml scaffolds were chosen for these experiments based on the results of preliminary studies which demonstrated better handleability and less cell mediated contraction when using the higher concentration scaffolds. Tissues engineered using these ECM-derived scaffolds, in the absence of exogenously supplied TGF-β3, stained less intensely for sGAG and collagen deposition after 28 days in culture (Figure 27A) compared with constructs stimulated with this growth factor (Figure 27B). Immunohistochemistry revealed that only fragments of articular cartilage used to produce the scaffolds stained strongly for type II collagen when constructs were not additionally stimulated with TGF-β3 (Figure 27). Conversely, more diffuse deposition of type II collagen was observed in constructs where the media was additionally supplemented with TGF-β3. sGAG deposition was also significantly greater within scaffolds additionally stimulated with TGF-β3 (Figure 27C). In all cases,

a certain amount of cell mediated scaffold contraction was observed, with the diameter of the construct reducing by 16.0±4.0% over 28 days in culture (Table 3).

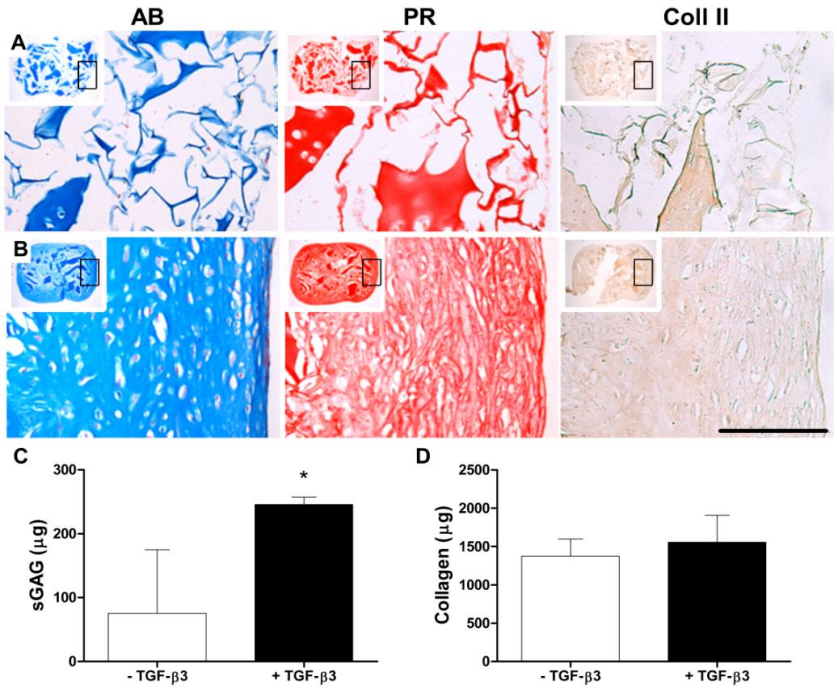


Figure 27 - Robust chondrogenesis with exogenously supplied TGF-β3. Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM-derived scaffold histological sections, after 28 days of culture. (A) No TGF-β3 supplementation; (B) With TGF-β3 supplementation. Higher sulphated glycosaminoglycan (sGAG) and collagen accumulation in the supplemented group (scale bar: 100µm). Biochemical assays results for ECM-derived scaffold with no TGF-β3 supplementation and with TGF-β3 supplementation seeded with human infrapatellar fat pad-derived stem cells (FPSC). (C) sGAG and (D) Collagen content (n=4, *p<0.05).

Table 3 – ECM-derived scaffold 500 and 1000 mg/ml (DHT crosslinked) parameters before culture. Note that there is batch-to-batch variability in these parameters. Values presented are mean ± standard deviation.

	ECM 500	ECM 1000
GAG day 0 (µg)	83±12	253±23
GAG day 28 (µg)	-	246±12
Collagen day 0 (µg)	804±162	1174±87
Collagen day 28 (µg)	-	1556±355
Porosity (%)	94.3±1.5	87.0±1.2
Pore size (µm)	104.2±49.2	98.9±37.2
Contraction (%)	-	16.0±4.0

4.3.2. EDAC crosslinking of ECM-derived scaffolds limits cell mediated contraction without suppressing chondrogenesis

In an attempt to minimise cell mediated contraction, scaffolds were additionally crosslinked chemically with EDAC as well as undergoing physical crosslinking with DHT. Collagen and sGAG accumulation within FPSC seeded constructs was unaffected by EDAC crosslinking (Figure 28).

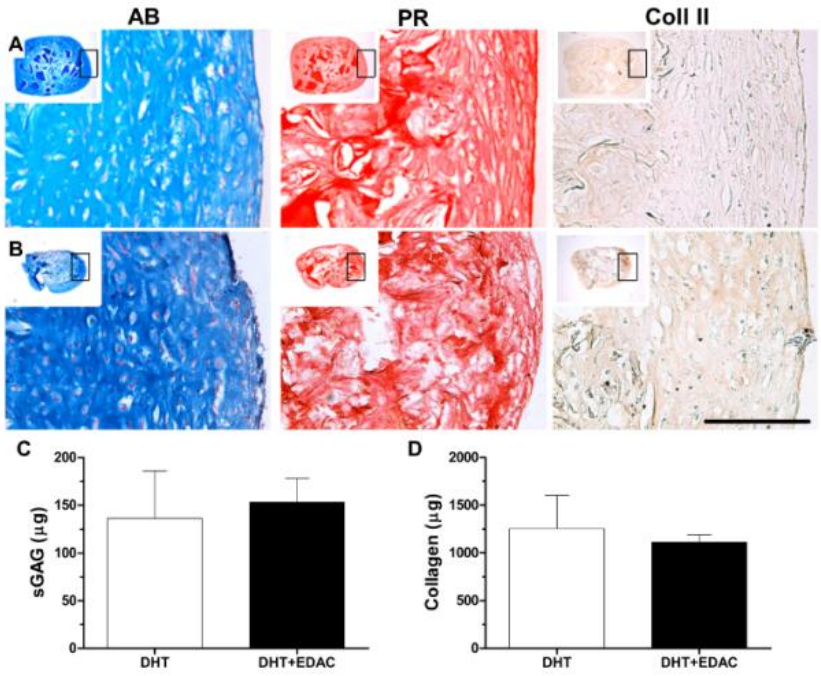


Figure 28 - Chondrogenesis was not affected by EDAC crosslinking. Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM-derived scaffolds after 28 days of culture. (A) Dehydrothermal (DHT) crosslinking; (B) DHT + 1-Ethyl-3-3dimethyl aminopropyl carbodiimide (EDAC) crosslinking. Similar sGAG and collagen accumulation in both groups (scale bar: 100µm). Biochemical assays results for ECM-derived scaffold DHT and DHT + EDAC seeded with human FPSC. (C) sGAG content and (D) Collagen content (n=4, *p<0.05). Day 0 values for EDAC group of sGAG is 83±15 µg and 1117±140 µg for collagen.

Significantly less contraction over 28 days in culture was observed in scaffolds that underwent both EDAC and DHT crosslinking (Figure 29).

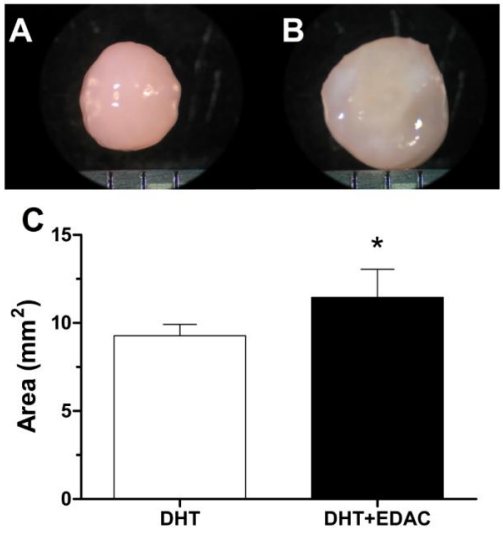


Figure 29 - EDAC crosslinking limits contraction. Area for ECM-derived scaffolds with DHT crosslinking: with and without EDAC after 28 days in culture. (A) DHT only; (B) DHT + EDAC crosslinking; (C) Scaffolds area in mm^2 (n=6, *p<0.05).

4.3.3. Cartilage ECM-derived scaffolds promote at least comparable chondrogenesis to biomimetic collagen-HA scaffolds

It was next sought to compare the chondro-permissiveness of the cartilage ECM-derived scaffold (DHT crosslinking only) to a biomimetic scaffold produced from two key components of cartilage tissue, specifically collagen and hyaluronic acid [35]. Greater cell mediated contraction was observed in the collagen-HA scaffold compared with the ECM-derived scaffold, where the construct diameter reduced by 54.0±6.0% over 28 days in culture (Table 4).

Table 4 – ECM-derived scaffold 1000 mg/ml and collagen-hyaluronic acid (DHT crosslinked) parameters before culture. Note that there is batch-to-batch variability in these parameters. Values presented are mean ± standard deviation. FPSCs from different donors were used in each experiment.

	ECM 1000	Collagen-HA
GAG day 0 (µg)	253±23	6±9
GAG day 28 (µg)	473.±35	29±6
Collagen day 0 (µg)	1174±87	469±99
Collagen day 28 (µg)	1639±145	543±135
Porosity (%)	87.0±1.2	99±0.017
Pore size (µm)	98.9±37.2	304.6±3.9
Contraction (%)	14.3±2.5	54.0±6.0

Both scaffolds stained intensely for alcian blue, indicating significant sGAGs deposition, while certain regions of the collagen-HA appeared to stain more intensely for picro-sirius red, suggesting greater levels of collagen accumulation in these scaffolds (Figure 30A and B). Quantitative biochemical assays were undertaken to quantify the composition of the scaffolds at the onset of the experiment (Table 4), the levels of sGAG released into the media over the course of the 28 day culture period and the composition of the scaffold after 28 days of culture (Figure 30). The total level of sGAG synthesised was assumed to be equal to the sum of the scaffold composition at day 28 and that released to the media, less the scaffold sGAG content at day 0. Greater levels of sGAG accumulation were observed within the cartilage ECM-derived scaffolds, but no significant difference in total collagen accumulation was observed between the two scaffold types (Figure 30C, D and E). Interestingly, this was not due to FPSCs synthesising less sGAGs within the collagen-HA scaffold, but rather due to superior retention of proteoglycans within the ECM-derived scaffold (Figure 30C). The overall levels sGAG synthesized during the 28 days of culture were similar for both scaffolds (Figure 30C).

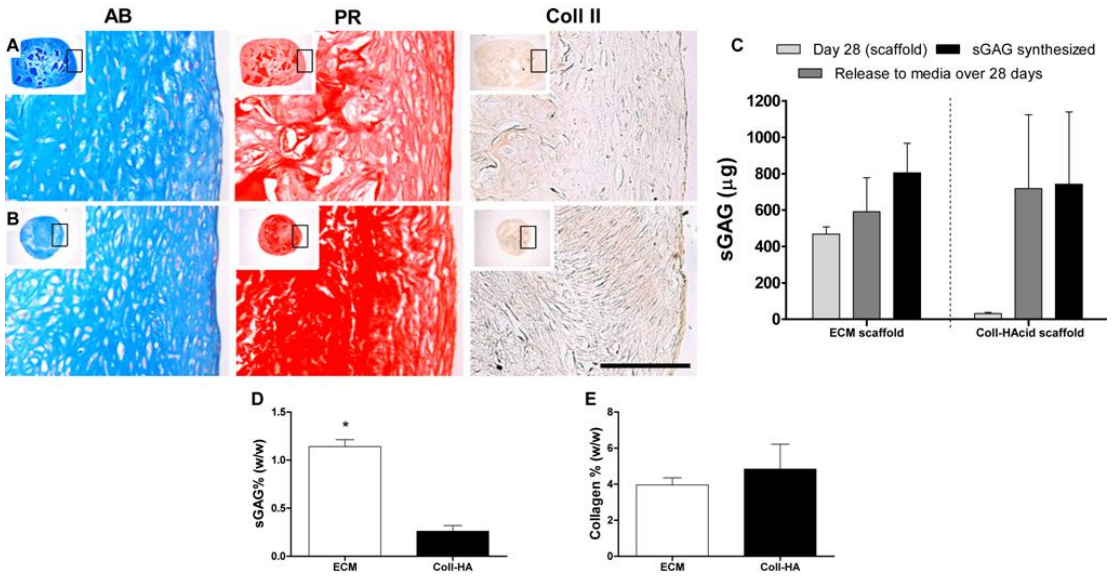


Figure 30 - Comparable chondrogenesis with a collagen-hyaluronic acid (coll-HA) scaffold. Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM-derived scaffold and coll-HA histological sections, after 28 days of culture. (A) ECM-derived scaffold; (B) coll-HA scaffold (scale bar: 100µm). Biochemical assays results for ECM-derived scaffold and coll–HA scaffold seeded with human infrapatellar fat pad-derived stem cells (FPSC). (C) sGAG for ECM-derived scaffold and coll-HA scaffold. sGAG values for day 28 in both constructs, in the media and total sGAG synthesized, by subtracting day 0 values (n=6). Coll-HA scaffold lost to the media the majority of the sGAG synthesized sGAG synthesized was calculated by subtracting day 0 value to total. (D) sGAG content per wet weight; (E) Collagen content per wet weight. Significantly higher sGAG accumulation for the ECM-derived scaffold and similar collagen content when compared with coll-HA scaffold (n=4, *p<0.05).

4.3.4. An ECM-derived scaffold can be used as a delivery system for TGF-β3 to induce chondrogenesis of diseased human infrapatellar fat pad-derived stem cells

Having previously demonstrated that superior chondrogenesis was observed in the presence of exogenously supplied TGF-β3, the final phase of the study explored whether an ECM-derived scaffold could be used as a growth factor delivery system to promote robust chondrogenesis of FPSCs. It was found that the majority of TGF-β3 that was loaded into the construct was released in a relatively controlled manner over the first 8-10 days of culture, with a burst release (of approximately 25% of the total released) occurring within the first day of culture (Figure 31).

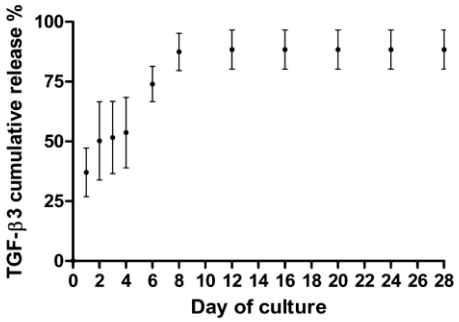


Figure 31 - TGF-β3 release profile. ELISA results for TGF-β3 release into the media from the TGF-β3 loaded ECM-derived scaffold (n=3). Cumulative release values are presented as a percentage of the initial amount of TGF- β3 loaded into the scaffold.

Chondrogenesis, as measured histologically (Figure 32A and B) and using biochemical assays for total sGAG and collagen accumulation (Figure 32C and D), appeared comparable in TGF-β3 loaded scaffolds and in scaffolds where the growth factor was added to the media.

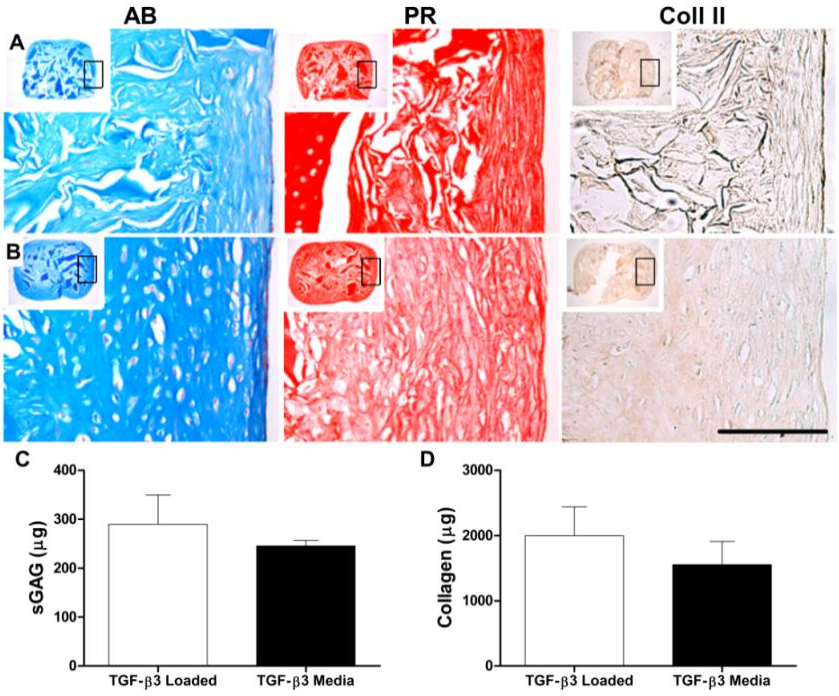


Figure 32 - ECM-derived scaffold loaded with TGF-β3 can induce robust chondrogenesis. Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM-derived scaffold loaded with TGF-β3 and TGF-β3 in media, after 28 days of culture (scale bar: 100µm). (A) TGF-β3 loaded; (B) TGF-β3 in media. Similar sGAG and collagen accumulation for both groups (n=4).

4.4. Discussion

ECM-derived scaffolds have shown great promise for cartilage tissue engineering and regenerative medicine applications. This has been attributed to a number of factors, including providing appropriate structural and mechanical cues to resident cells, as well as the retention of bioactive molecules present in the native ECM. It was hypothesised that an ECM-derived scaffold, and specifically a scaffold derived from a cartilaginous ECM, could additionally be used as a growth factor delivery system to improve chondrogenesis of stem cells that are either seeded onto, or potentially recruited into, such a scaffold. It was observed that a cartilage ECM-derived scaffold could indeed retain and slowly release TGF-β3, and in doing so drive chondrogenesis of human FPSCs. These findings open up the possibility of using such a construct as an off-the-shelf scaffold for articular cartilage repair, where stem or progenitor cells are either chemotactically recruited into the TGF-β loaded construct [335, 336], or where freshly isolated autologous stromal cells [20, 59, 255] or allogeneic MSCs from a stem cell bank are seeded into the scaffold prior to implantation.

Unlike previous studies exploring the chondroinductive nature of cartilage ECM-derived scaffolds for ASCs [273, 292], it was found that additional supplementation with TGF-β3 was required to induce robust chondrogenesis of IFP-derived stem cells. There are a number of reasons that may explain this discrepancy in findings. Firstly, it was used MSCs isolated from diseased (osteoarthritic) human IFP tissue as opposed to ASCs from presumably non-osteoarthritic donors. It is still unclear how diseases like osteoarthritis will influence resident MSCs within the joint space. While it was found that FPSCs isolated from osteoarthritic joints possess a comparable chondrogenic capacity to those derived from healthy donors in a pellet culture system [337], there may still be changes with disease that leave such stem cells less responsible to the cues provided by an ECM-derived scaffold. In addition, the scaffold

crosslinking methods used in previous studies were different to that utilized in this study [273, 292]. The use of different crosslinking methods and concentrations can influence the degradation kinetics of the scaffold and may alter release rates of growth factors [38], which in turn could explain the limited chondrogenesis observed in ECM-derived scaffolds not additionally supplemented with TGF-β3. Furthermore, the use of DHT treatment could have degraded/denatured some of the cytokines present in the ECM matrix. It should be noted, however, that a recent comparison of different crosslinking techniques demonstrated that although they all influenced the composition of newly synthesized matrix within the scaffold, that DHT treatment lead to the development of constructs that best matched the composition of native cartilage [25]. Finally, crosslinking will also stiffen the scaffold. Stiffer scaffolds have been shown to suppress chondrogenesis of stem cells [35].

In an attempt to prevent cell-mediated contraction of the ECM-derived scaffold, it was incorporated an additional EDAC crosslinking step into the fabrication procedure. It has been reported previously that different crosslinking methods (e.g. EDAC) can prevent cell-mediated contraction [25]. EDAC crosslinking had the effect of minimizing contraction, without suppressing cartilaginous ECM accumulation within the cell seeded construct. Such chemical crosslinking may be necessary to prevent scaffold contraction *in vitro* and *in vivo* and to ensure mechanical stability of the construct [25]. Reduction of contraction associated with matrix formation may be necessary in for the regeneration of cartilage defects as it should support integration of the ECM-derived scaffold with the surrounding tissue. Further *in vivo* studies are also necessary to examine other untested impacts of EDAC crosslinking, including immunological response [338].

An alternative to the use of decellularized ECM as a scaffold for cartilage repair are biomimetic scaffolds fabricated using specific ECM components. Several natural biomaterials derived from components of ECM have been used as scaffolds for tissue

regeneration including collagen, chondroitin sulphate and hyaluronic acid [27, 35]. Collagen-based scaffolds incorporating chondroitin sulphate and hyaluronic acid, key sGAGs in cartilage ECM, have previously been shown to promote the proliferation and chondrogenic differentiation of MSCs [35]. It was found that sGAG accumulation was higher in FPSC seeded cartilage ECM-derived scaffolds compared to collagen-HA acid scaffold, primarily due to greater retention rather than superior synthesis of sGAGs. Structural differences between the two scaffold types, such as porosity, or the greater levels of cell mediated scaffold contraction in the collagen-HA scaffolds, may explain these differences.

The final phase of the study sought to manipulate a key property of ECM, namely that certain matrix components such as collagen and proteoglycans can act as growth factor reservoirs [74, 75], to deliver TGF-β3 from such scaffolds in a controlled manner. Proteoglycans present in the pericellular matrix (PCM) and interstitial ECM have been shown to bind and modulate TGF-β3 supply and consequently control their availability [75]. The majority of the growth factor was released from the scaffolds within the first 10 days of culture. A certain amount of scaffold degradation may have occurred in that timeframe, although it is unlikely that this is the only mechanism by which TGF-β3 is released. Rather it is suggested that the majority of TGF-β3 is not permanently bound to the ECM, allowing the growth factor to be released within what appears to be an optimal dosing window [339]. Previous work has demonstrated that two weeks of *in vitro* culture in the presence of TGF-β3 is sufficient to promote robust expression of collagen type II, aggrecan and sox 9 in MSCs [220]. Furthermore, proteoglycan deposition in poly(ethylene glycol) diacrylate hydrogels seeded with human MSCs was shown to be enhanced by the temporal withdrawal of TGF- β3 from the media [340]. Therefore the timeframe over which this growth factor is released by the ECM-derived scaffold may be near optimal for promoting robust chondrogenesis of MSCs. Further experiments are required to verify this hypothesis.

4.5. Conclusion

In conclusion, it was found that the combination of a porcine cartilage ECM-derived scaffold and stimulation with TGF-β3 can induce robust chondrogenesis of human diseased infrapatellar fat pat-derived stem cells. When compared with a well-established chondro-inductive collagen-HA scaffold, it was found that this ECM-derived scaffold was at least as effective in promoting chondrogenesis of FPSCs. The finding that such an ECM-derived scaffold can be used as delivery system for TGF-β3 to induce chondrogenesis of MSCs opens the possibility of using such a construct as an off-the-shelf product for cartilage tissue regeneration.

The next phase of the ECM-derived scaffold development is to optimize parameters, such as porosity, and enhance its efficiency in promoting chondrogenesis of progenitor cells (Chapter 5). An additional goal for the following study of this thesis (Chapter 5) is to try to develop and assess a single-stage therapy for cartilage repair combining an optimised cartilage ECM-derived scaffold and freshly isolated infrapatellar fat pad-derived stromal cells.

Chapter 5

Coupling Freshly Isolated CD44^{+} Infrapatellar Fat Pad-derived Stromal Cells with a TGF-β3 Eluting Cartilage ECM-derived Scaffold as a Single-Stage Therapy for Joint Regeneration

5. Coupling freshly isolated CD44$^+$ infrapatellar fat pad-derived stromal cells with a TGF-β3 eluting cartilage ECM-derived scaffold as a single-stage therapy for joint regeneration

Abstract

An alternative strategy to the use of *in vitro* expanded cells in regenerative medicine is the use of freshly isolated stromal cells, where a bioactive scaffold is used to provide an environment conductive to proliferation and tissue-specific differentiation *in vivo.* The objective of this study was to develop a cartilage extracellular matrix (ECM)-derived scaffold that could facilitate the rapid proliferation and chondrogenic differentiation of freshly isolated stromal cells. By freeze-drying cryomilled cartilage ECM of differing concentrations, it was possible to produce scaffolds with a range of pore sizes. The migration, proliferation and chondrogenic differentiation of infrapatellar fat pad-derived stem cells (FPSCs) depended on the concentration/porosity of these scaffolds, with greater sGAG accumulation observed with increase in pore size. We then sought to determine if freshly isolated stromal cells, seeded onto a TGF-β3 eluting ECM-derived scaffold, could promote chondrogenesis *in vivo*. While a more cartilage-like tissue could be generated using culture expanded FPSCs compared to non-enriched freshly isolated cells, fresh CD44$^+$ stromal cells were capable of producing a tissue *in vivo* that stained strongly for sGAGs and type II collagen. These findings open up new possibilities for in-theatre cell based therapies for joint regeneration.

Keywords: Articular cartilage, Extracellular matrix, Growth factor release, Single-stage therapy, Clinical translation.

5.1. Introduction

Cartilage regeneration is still a major challenge in orthopaedic medicine. The outcomes of cartilage repair procedures are inconsistent and further joint degeneration commonly occurs [20, 273]. Cell based therapies such as autologous chondrocyte implantation (ACI) may lead to improved clinical outcomes for patients, however the high cost and need for two hospital stays have limited the widespread adoption of this technique into the clinic [20]. This has motivated increased interest in single-stage or off-the-shelf therapies for cartilage regeneration, possibly involving the use of freshly isolated cells that can potentially be harvested in-theatre and delivered back into the patient during the same procedure [20-22, 255]. Successful realization of such a concept therefore requires the identification of both a suitable cell source and the development of bioactive scaffolds capable of promoting the rapid proliferation and chondrogenic differentiation of the limited number of cells that can potentially be isolated from a patient in-theatre.

Extracellular matrix (ECM)-derived from native tissue has been proposed as a promising biological scaffold material, providing cues that enhance cell proliferation, differentiation and matrix formation [25, 186]. The ECM of articular cartilage is organized into a complex three-dimensional network, consisting primarily of type II collagen and proteoglycans in which growth factors and other cues are incorporated [38, 341]. Devitalized and decellularized ECM-derived from articular cartilage has been used to produce bioactive scaffolds for cartilage tissue engineering applications [25, 273, 291, 292, 295, 313, 341, 342]. These scaffolds have been shown to be chondro inductive *in vitro* [292], although their capacity to promote the development of functional hyaline cartilage is enhanced when additionally stimulated with exogenous growth factors such as transforming growth factor (TGF)-β3 [191, 341]. We have

previously demonstrated that cartilage ECM-derived scaffolds can also be used to control the delivery and release of TGF-β3 to stem cells [341], opening up the potential of using such biomaterials as part of an off-the-shelf strategy for joint regeneration. While cartilage ECM is clearly a promising material for the development of scaffolds for cartilage tissue engineering applications, it is still unclear what the optimal composition and architecture (e.g. pore size) of such scaffolds should be to promote robust chondrogenesis. Given that such factors have been shown to play a key role in regulating cell fate in other scaffolding systems [35, 343], it would seem highly likely that scaffold composition and pore size would need to be tailored to promote the rapid proliferation and chondrogenic differentiation of any progenitor cell population intended to be used as part of a single-stage therapy for cartilage regeneration.

The overall goal of this study was to develop a single-stage strategy for promoting chondrogenesis *in vivo* that combines an optimised cartilage ECM-derived scaffold and freshly isolated infrapatellar fat pad derived stromal cells. The infrapatellar fat pad was chosen as a source of stromal cells as it is easily accessible to a clinician during joint repair procedures. Furthermore, infrapatellar fat pad-derived stem cells (FPSCs) have been shown to have a strong chondrogenic potential [51, 306, 337] and can be used to engineer functional cartilaginous grafts [55, 344]. The objective of the first phase of the study was to develop cartilage ECM-derived scaffolds with controllable and consistent pore size and shape, and to then explore how altering their porosity influences the migration, proliferation and chondrogenic differentiation of human FPSCs when seeded onto such constructs. We then explored the potential of such scaffolds to act as growth factor delivery systems to facilitate chondrogenesis of culture expanded FPSCs *in vitro* and *in vivo*. Finally, we sought to determine if such a scaffold could be coupled with enriched freshly isolated stromal cells as a one-step or single-stage strategy for promoting chondrogenesis *in vivo*.

5.2. Material and methods

5.2.1. Scaffold preparation

Cartilage used in the fabrication of ECM-derived scaffolds was harvested as previously mentioned (section 3.1). Cartilage pieces were then fragmented using two different methods, to produce either coarse or fine scaffolds. Coarse scaffolds were produced using a previously described protocol [341], where cartilage is blended in deionised water (dH_2O) using an homogeniser (IKAT10, IKA Works Inc, NC, USA) to create a cartilage slurry. The material was re-suspended in dH_2O at a concentration of 500 mg/ml. Fine scaffolds were fabricated by first pulverising cartilage pieces within a cryogenic mill (6770 Freezer/Mill, SPEX, UK). These particles of cartilage where then blended in dH_2O using a homogenizer to create a fine cartilage slurry. Three distinct scaffolds were fabricated using different slurry concentrations: 250 mg/ml, 500 mg/ml and 1000 mg/ml. The slurry for both coarse and fine groups was freeze-dried as described in general method chapter (section 3.2). The scaffolds underwent DHT and EDAC crosslinking (section 3.3). Scaffolds were imaged using HIM (section 3.9).

5.2.2. Diameter, particle size and pore size determination

After the 4 weeks culture period, constructs were removed from culture wells and imaged. Macroscopic images were analyzed with Image J to quantify changes in construct diameter (n=4) and particle size. Pore size determination for the scaffolds was obtained by measuring the diameter of 40 pores (with Image J) in HIM micrographs of dry scaffolds (n=3) before cell seeding, based on a previously described method [333].

5.2.3. Cell and construct culture

Stem cells used were harvested and cultured with the general method previously mentioned (section 3.4). For *in vitro* chondrogenic studies, the before mentioned general protocol was used (section 3.5). Groups termed “loaded with TGF-

β3" were not supplemented with TGF-β3 in chondrogenic media during the culture period. Instead, TGF-β3 (approximately 5 ng in 40 µl of media) was soak loaded into the scaffold and was not directly added to the culture media.

Histological (section 3.7), immunohistochemical (section 3.8), biochemical (section 3.6) analysis were performed after 28 days in culture as described in general methods section. TGF-β3 quantification analysis was performed in media samples collected during culture period as described previously in general methods section (section 3.11).

5.2.4. Cell viability and distribution

Cell survival and distribution were assessed using live/dead staining for all slurry concentrations. Calcein was used to stain live cells and images were taken using confocal microscopy after 2 hours of culture, as previously described [333]. Briefly, at day 1 and 28, cell viability was assessed by LIVE/DEAD® kit (Invitrogen, Bio-science, Ireland). Constructs were washed in PBS, sectioned in half, incubated in calcein 2 µM (live/green) and 4 µM ethidium homodimer-1 (dead/red) (Cambridge Biosciences, UK). Constructs were washed again and imaged in confocal microscope 10x Olympus FV-1000 Point-Scanning Microscope (Southend-on-Sea, UK) at 515 and 615 nm channels and analysed using FV10-ASW 2.0 Viewer.

5.2.5. In vivo and cell population enrichment

ECM-derived (DHT+EDAC crosslinked) scaffolds loaded with 5 ng of TGF-β3 were seeded as follows: 1. No cells; 2. monolayer expanded (Passage 2) FPSCs; 3. Freshly isolated stromal IFP-derived cells; 4. $CD44^+$ freshly isolated stromal IFP-derived cells. All cells were isolated from porcine (female, 3 month old) infrapatellar fat pad and each scaffold was seeded with $0.5x10^6$ cells, with the exception of $CD44^+$ where the number was $0.1x10^6$ (approximately 10% of the freshly isolated fraction). $CD44^+$ cells were isolated using magnetic-activated cell sorting (MACS®,Miltenyi

Biotec, Germany). Briefly, $CD44^+$ cells were labelled with micro-beads specific to CD44 according to the manufacturer's instructions. This cell suspension was then passed through a MACS column in a magnetic field. Magnetically labelled cells $CD44^+$ cells can be separated after removing the magnet. Within 24 hours of cell isolation, seeded constructs were subcutaneously implanted into nude mice following an established protocol (section 3.12).

5.3. Results

5.3.1. Chondro-permissive scaffolds with a consistent structure and pore size can be produced using cryomilled cartilage extracellular matrix (ECM)

Scaffold pore size has been shown to regulate stem cell proliferation and differentiation [320, 343]. Hence, we first sought to develop a method to produce scaffolds from cartilaginous ECM with controllable and consistent pore size and shape. Porous scaffolds were produced using slurries of either "coarse" (ECM blended using a homogenizer) or "fine" (cryomilled ECM) porcine cartilage ECM (Figure 33A). The coarse ECM slurry contained particles with a mean size of 322±195 µm, while the fine slurry contained particles with a mean pore size of 97±26 µm. Large cartilage particles were still present in the freeze-dried scaffold fabricated using the coarse slurry (Figure 33A), while a scaffold with a more homogenous pore size and morphology was produced using the fine particulated cartilage ECM slurry. This was confirmed using helium ion microscopy, where more homogenous and spherical pores (mean diameter - 65±20 µm) were observed in the fine ECM scaffolds (Figure 33C), whilst less spherical pores and a wider distribution of pore sizes (mean diameter - 104±49 µm) was observed in the coarse ECM scaffolds (Figure 33B).

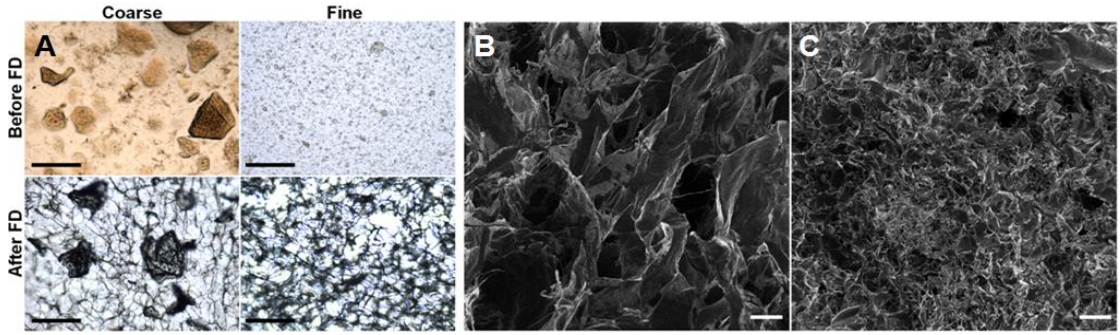


Figure 33 – (A) Light micrographs of cartilage slurries (coarse and fine) before and after freeze-drying (FD) (scale bar: 500 μm). Helium ion micrographs of cartilage ECM-derived scaffolds produced using either a coarse (B) and fine (C) slurry (scale bar: 100 μm).

We next sought to compare the capacity of both coarse and fine cartilage ECM-derived scaffolds to promote chondrogenesis of human FPSCs. Tissues engineered using both coarse and fine ECM-derived scaffolds stained similarly for sGAG and collagen deposition after 28 days in culture (Figure 34A and B). There was no significant difference in the sGAG and collagen content of tissues engineered using the two different scaffold types (data not shown).

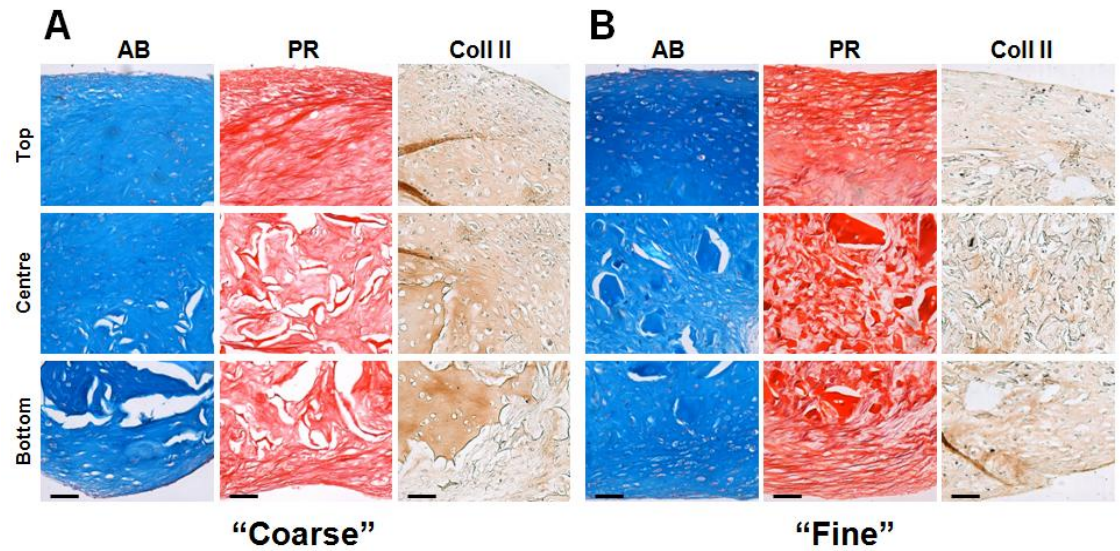


Figure 34 –Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM-derived scaffold produced with coarse (A) and fine (B) method, after 28 days of culture (scale bar: 50μm).

5.3.2. The porosity of cartilage ECM-derived scaffolds can be tailored by varying the concentration of the slurry

As the fine (or cryomilled) cartilage ECM particles could be used to produce scaffolds with a consistent pore size and shape, we next sought to determine how the concentration of such slurries would influence the pore size of the resulting scaffolds and their capacity to facilitate FPSC migration, proliferation and to promote subsequent chondrogenesis. HIM demonstrated that varying the ECM slurry concentration (250, 500 and 1000 mg/ml) lead to the development of scaffolds with different pore sizes (Figure 35). Specifically, lowering the concentration of ECM lead to the development of scaffolds with a higher mean pore size (from 32±12 µm to 65±20 µm; see Figure 35D).

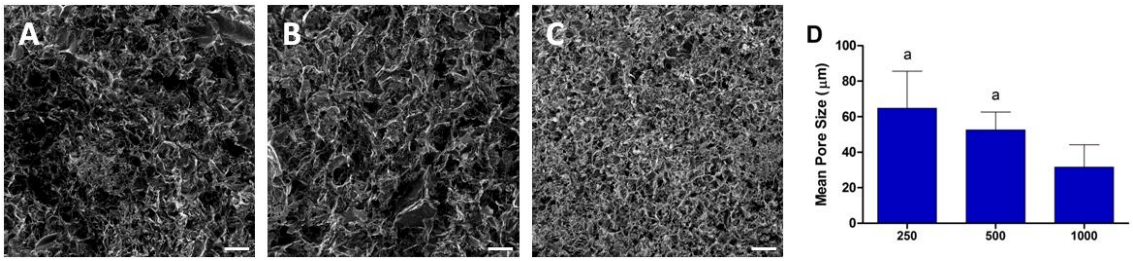


Figure 35 – (A-C) Helium ion microscopy (HIM) micrographs of scaffolds with altered cartilage ECM slurry concentrations: (A) 250 mg/ml; (B) 500 mg/ml; (C) 1000 mg/ml scaffolds (scale bar: 100 µm). (D) Mean scaffold pore size ($^{a}p<0.05$; groups with *a* are significantly different from group 1000 mg/ml).

5.3.3. Stem cell migration, proliferation and chondrogenic differentiation depends on the porosity of ECM-derived scaffolds

FPSCs were seeded onto scaffolds fabricated using a range of slurry concentrations/pore sizes. Confocal microscopy revealed that FPSCs were evenly distributed throughout the 250 mg/ml scaffolds after 1 day of culture (Figure 36A), while cells were only observed around the periphery of the lower pore size 500 and 1000 mg/ml scaffolds (Figure 36B and C). After 28 days of culture, FPSCs were observed throughout the majority of the 250 and 500 mg/ml scaffolds, except for a

region in the very centre of the 500 mg/ml scaffold, while viable cells were only observed around the periphery only of 1000 mg/ml scaffolds. Greater FPSC proliferation was also observed within the 250 mg/ml scaffolds, as evidenced by a significantly higher net increase in DNA content (164%) within the scaffold over 28 days in culture, whilst no significant change in DNA content was observed in the 500 and 1000 mg/ml scaffolds (data not shown).

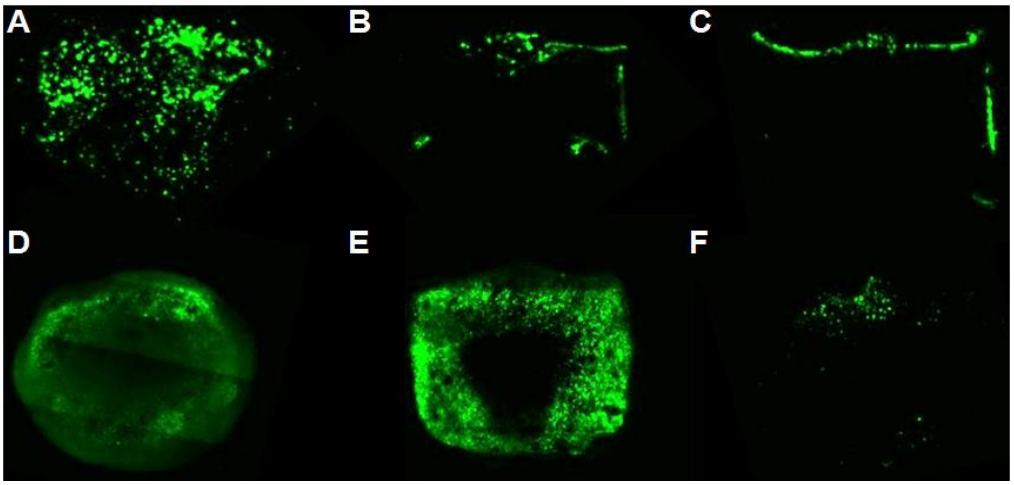


Figure 36 – (A-C) Confocal microscopy at day 1 of human infrapatellar fat pad-derived stem cells seeded in ECM-derived scaffolds; calcein was used to stain live cells: (A) 250 mg/ml, (B) 500 mg/ml and (C) 1000 mg/ml. (D-F) Scaffolds at day 28: (D) 250 mg/ml, (E) 500 mg/ml and (F) 1000 mg/ml scaffolds. Images represent a cross-section through ECM-derived constructs.

Histological analysis (nuclear fast red *nuclei* staining) on day 0, 7, 14 and 28 of culture confirmed that FPSCs were homogenously distributed throughout the 250 mg/ml scaffold (Figure 37A). Furthermore, robust proteoglycan deposition was observed throughout this construct by day 28 (Figure 37A and D). In contrast, the lower pore sized 1000 mg/ml scaffolds stained less intensely and more inhomogeneously for Alcian Blue compared to other constructs. This was confirmed by biochemical analysis of the sGAG content of the engineered tissues, which demonstrated greater levels of ECM accumulation within the 250 mg/ml scaffolds (Figure 37). Based on these results, the 250 mg/ml fine scaffold was selected for further development in the remainder of the study.

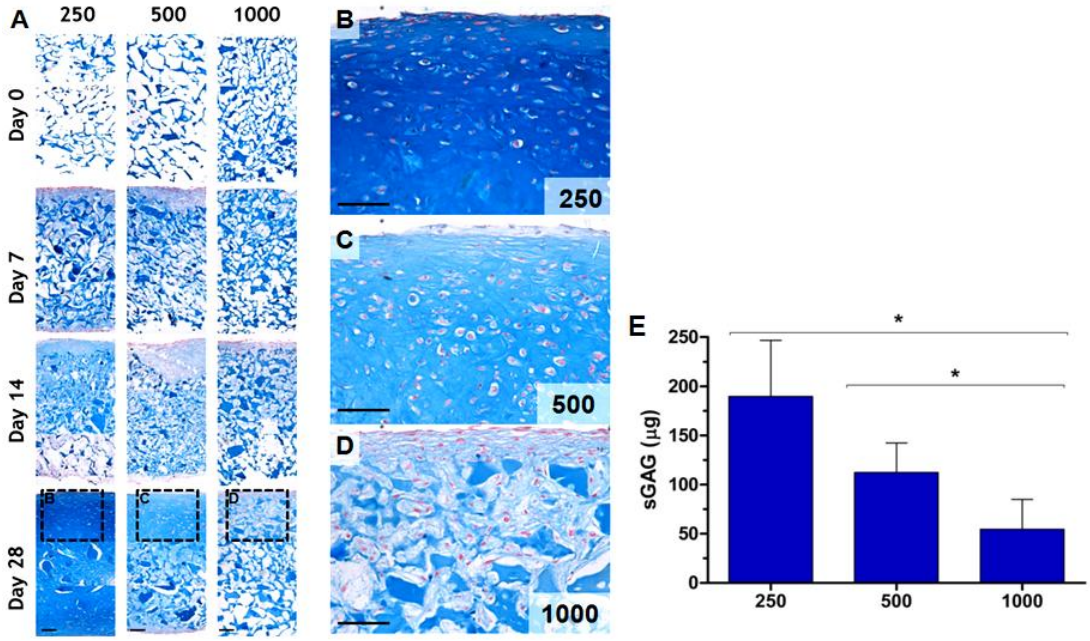


Figure 37 – Histological sections staining for glycosaminoglycans (sGAG) (alcian blue) and cell *nuclei* (nuclear fast red) in 250, 500 and 1000 mg/ml ECM-derived scaffolds (seeded with FPSCs) at day 0, 7, 14 and 28 of culture (A). (B-D) High magnification images demonstrating more robust sGAG deposition within the 250 mg/ml scaffolds (B) compared to the 500 (C) 1000 mg/ml (D) scaffolds (scale bar: 50 µm). (E) sGAG accumulation within the 250, 500 and 1000 mg/ml scaffolds (n=4, **p*<0.05).

5.3.4. EDAC crosslinking of ECM-derived scaffolds prevented cell mediated contraction with no loss in chondroinductive capacity

A potential limitation of the lower concentration cartilage ECM-derived scaffolds was that they underwent greater levels of contraction during culture compared to higher concentration scaffolds (data not shown). Previous studies have demonstrated that EDAC crosslinking is an efficient way to minimize cell mediated contraction in scaffolds for cartilage tissue engineering applications [25, 341]. Therefore, 250 mg/ml scaffolds were physically crosslinked using DHT and chemically with EDAC. Significantly less contraction was observed in scaffolds that underwent both EDAC and DHT crosslinking (Figure 38A and B). The diameter of the EDAC crosslinked scaffolds did not change over 28 days in culture, and was significantly higher than the DHT only crosslinked scaffolds.

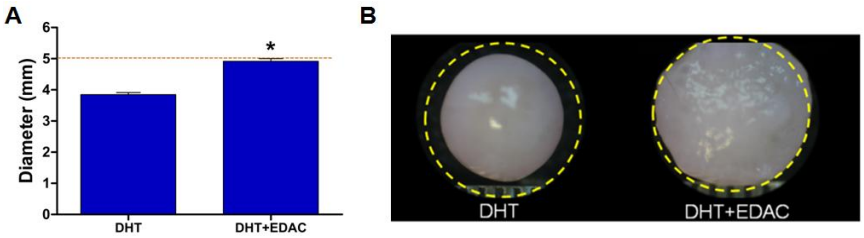


Figure 38 – (A) Diameter of ECM-derived scaffolds that had been crosslinked with DHT or DHT and EDAC after 28 days in culture (n=4; *p<0.05). (B) Macroscopic images of scaffolds (yellow represents initial diameter: 5 mm).

We next sought to confirm that the chondro inductivity of the scaffolds was not affected by the use of EDAC crosslinking. Histological analysis revealed that robust levels of cartilage ECM deposition occurred within both DHT only (Figure 39A) and EDAC + DHT (Figure 39B) crosslinked scaffolds. Furthermore, the sGAG content of DHT+EDAC scaffolds was significantly higher than that of DHT only constructs (Figure 39C), confirming that EDAC crosslinking does not suppress chondrogenesis within cartilage ECM-derived scaffolds.

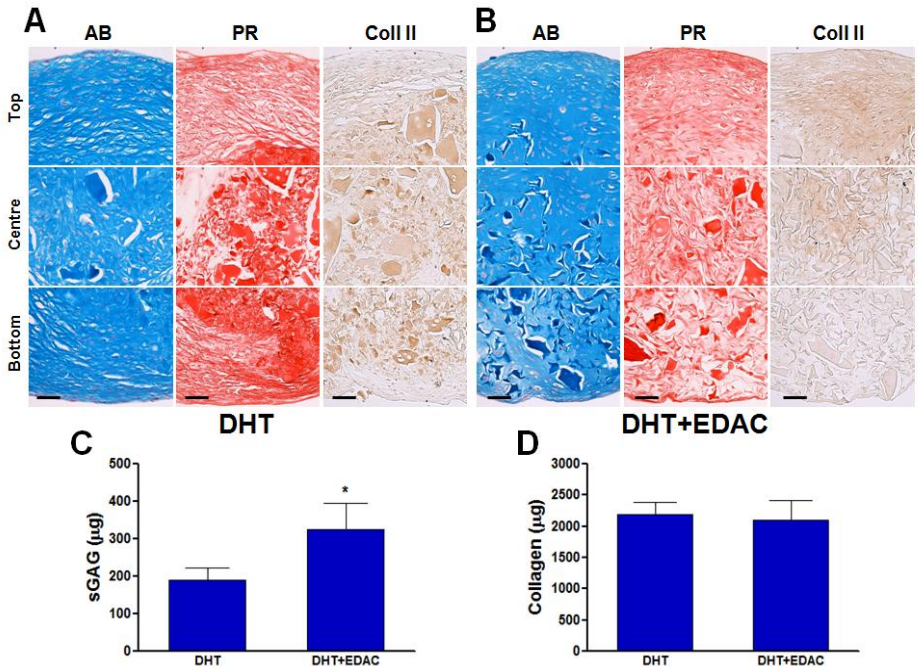


Figure 39 – Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM-derived scaffolds after 28 days of culture. (A) Dehydrothermal (DHT) crosslinking; (B) DHT + 1-Ethyl-3-3dimethyl aminopropyl carbodiimide (EDAC) crosslinking (scale bar: 50 µm). (C) sGAG and (D) collagen accumulation within DHT and DHT+EDAC crosslinked ECM-derived scaffolds seeded with human FPSCs (n=4, *p<0.05).

5.3.5. EDAC crosslinking delays the burst release of TGF-β3 from cartilage ECM-derived scaffolds

We have previously demonstrated that cartilage ECM-derived scaffolds (derived from coarse ECM particles) can be used as platforms to control the release of soak loaded growth factor and consequently induce robust chondrogenesis of FPSCs [341]. To confirm that cartilage ECM-derived scaffolds derived from a slurry of fine particles can also be used to control the release of exogenously supplied TGF-β3, and to evaluate the impact of EDAC crosslinking on growth factor release, an ELISA analysis was performed to determine the release of TGF-β3 into the media (Figure 40B). After 4 days of culture, the media of the EDAC+DHT crosslinked scaffolds contained significantly lower levels TGF-β3 compared to the DHT-only crosslinked scaffold. Both scaffolds released almost all of the TGF-β3 loaded onto the scaffold within the first 10 days of the culture period.

With the aim of confirming that loading of TGF-β3 onto the scaffold could induce comparable chondrogenesis to directly supplementing the culture media with this growth factor. Both the DHT and DHT+EDAC scaffolds that were pre-loaded with TGF-β3 were capable of inducing robust chondrogenesis of FPSCs, as evidenced by alcian blue, picro-sirius red and type II collagen immunohistochemical staining (Figure 40A and B). The sGAG content of constructs after 28 days of culture was higher for DHT crosslinked scaffolds when pre-loaded with TGF-β3 (Figure 40A) compared to scaffolds where the media was directly supplemented with TGF-β3. A similar trend was observed for DHT+EDAC crosslinked scaffolds (Figure 40A).

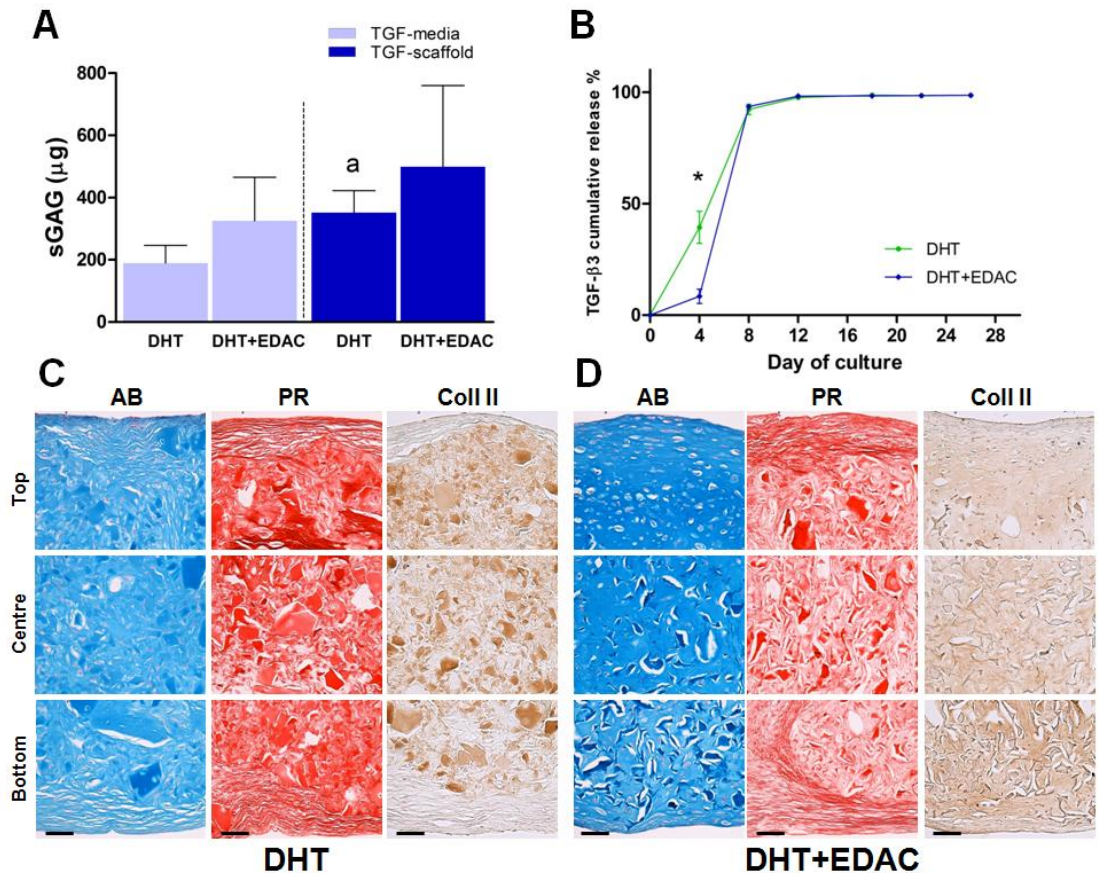


Figure 40 – sGAG accumulation values for 28 days in culture for TGF-β3 loaded (TGF-scaffold) and TGF-media groups, both with and without EDAC crosslinking (n=4; $^{a}p<0.05$; group with *a* is significantly different from group DHT only and TGF-media). ELISA results (B) for TGF-β3 release into the media from TGF-β3 loaded ECM-derived scaffold with and without EDAC crosslinking (n=6, $*p<0.05$). Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM-derived scaffold loaded with TGF-β3 with DHT (C) and DHT+EDAC (D), after 28 days of culture (scale bar: 50µm).

5.3.6. Coupling freshly isolated CD44$^+$ infrapatellar fat pad-derived stromal cells with a TGF-β3 eluting cartilage ECM-derived scaffold promotes chondrogenesis in vivo

The next step was to evaluate whether a TGF-β3 eluting cartilage ECM-derived scaffold, optimized *in vitro* to promote stem cell proliferation and chondrogenic differentiation, could be used to promote chondrogenesis of culture expanded FPSCs *in vivo*. To this end, cell-free and FPSC-loaded constructs were implanted subcutaneously into nude mice. After 28 days *in vivo*, cell free constructs had been infiltrated with host cells that synthesised a fibrous or fibrocartilaginous tissue within the scaffold. The pores of ECM-derived scaffolds seeded with culture expanded FPSCs stained more intensely for glycosaminoglycan (Alcian Blue) and type II collagen deposition compared to cell-free constructs (Figure 41A and B). With the ultimate objective of developing a single-stage therapy for articular cartilage regeneration, we next sought to determine if freshly isolated (i.e. not culture expanded) infrapatellar fat pad-derived stromal cells, seeded into the same TGF-β3 eluting cartilage ECM-derived scaffold, could be used as an alternative to culture expanded FPSCs to promote chondrogenesis *in vivo*. The tissue formed in constructs seeded with freshly isolated FPSCs was comparable to that within cell-free constructs, suggesting that this cell population was not capable of undergoing robust chondrogenesis within the scaffolds *in vivo*. Given that the infrapatellar fat pad contains a heterogeneous cell population, we finally sought to determine if freshly isolated CD44$^+$ (a putative marker of chondro-progenitors) fat pad-derived stromal cells would promote more robust chondrogenesis *in vivo*. Cartilage-like matrix deposition was observed throughout CD44$^+$ cell seeded constructs, with localized regions staining intensely for sGAG and type II collagen deposition (Figure 41G and H).

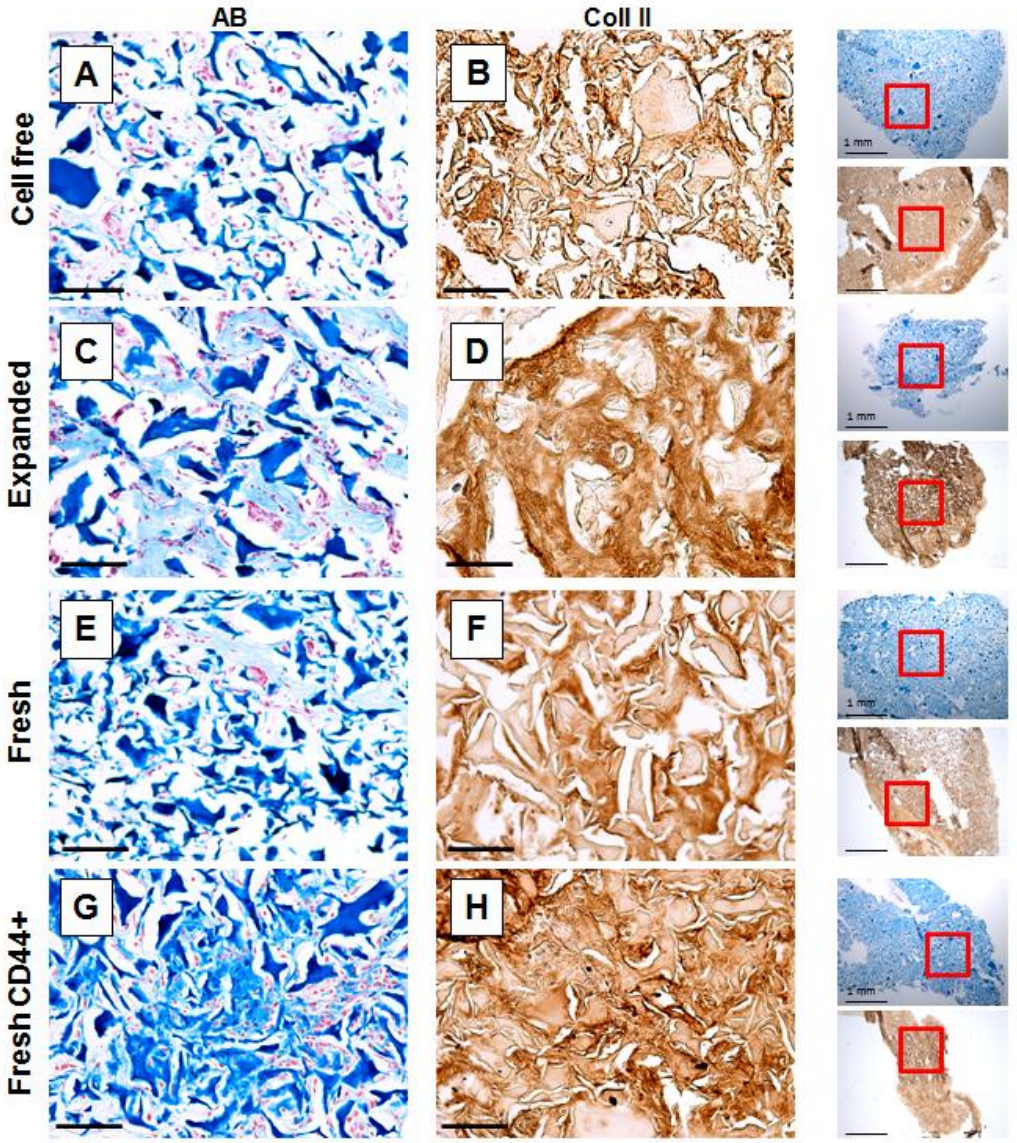


Figure 41 – Alcian blue (AB) and collagen type two (Coll II) histological staining for implanted cell free scaffolds (A and B respectively), expanded cell seeded constructs (C and D), freshly isolated cell seeded constructs (E and F) and finally the $CD44^{+}$ freshly isolated cell seeded constructs (G and H). All groups were implanted *in vivo* for four weeks (scale bar: 50µm).

5.4. Discussion

The overall goal of this study was to develop a single-stage therapy for cartilage repair combining an optimised cartilage ECM-derived scaffold and freshly isolated infrapatellar fat pad-derived stromal cells. By freeze-drying slurries of cryomilled cartilage ECM of differing concentrations, it was possible to produce scaffolds with a range of pore sizes. The migration, proliferation and chondrogenic differentiation of FPSCs depended on the concentration/porosity of the ECM-derived scaffolds, with greater sGAG accumulation observed within the scaffolds with a larger pore size. A limitation of these more porous scaffolds was that they underwent greater cell-mediated contraction; however this could be prevented with the use of combined dehydrothermal (DHT) and 1-Ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC) crosslinking, with no loss in scaffold chondroinductive capacity. Such crosslinking also functioned to retard the initial release of exogenously loaded TGF-β3 from stem cell seeded scaffolds. Finally, the optimized scaffold was seeded either with culture expanded FPSCs or freshly isolated infrapatellar fat pad-derived stromal cells and implanted *in vivo* in a subcutaneous model. The results indicate that this ECM-derived scaffold loaded with TGF-β3 supported cartilage-like tissue formation, specifically for culture expanded FPSCs or freshly isolated $CD44^+$ stromal cells, opening up the possibility of using the latter cell source as part of a single-stage therapy for joint regeneration.

Freeze-drying slurries of fine (cryomilled) cartilage ECM particles was found to result in the development of scaffolds with a consistent pore size and morphology. Similar strategies have been employed in the literature to particulate ECM [338]. From a translational perspective, the identification of robust and consistent strategies for scaffold fabrication will be important; for example, by leading to the development of production methods that minimise batch-to-batch variability. In addition, modifying ECM particles size may also influence the efficacy of the resulting scaffold to facilitate

tissue regeneration. For example, previous studies have demonstrated that the particle size of powdered ECM material can influence new tissue forming fate [345], despite the fact that similar proteins were found in both biomaterials. In spite of this, it was found that the chondro-inductive properties of cartilage ECM-derived scaffold was independent of particle size, i.e. it was similar for both coarse and fine cartilage ECM-derived scaffolds.

By altering the concentration of ECM within a slurry prior to freeze drying, it was possible to produce cartilage ECM-derived scaffolds with a range of pore sizes. Reducing the ECM concentration led to the development of scaffolds with a larger pore size, which in turn enhanced cellular infiltration, proliferation and chondrogenic differentiation. Previous studies have also reported that the porosity of ECM-derived scaffolds depend on slurry ECM concentration [295]. It is still unclear as to what the ideal scaffold pore size is for facilitating cell attachment, proliferation and migration in tissue engineering, with a wide range (5 to 500 μm) utilized in the literature depending on the cell type [346, 347]. In the context of stem cell differentiation, it has been demonstrated that chondrogenesis is enhanced in PCL scaffolds with a larger pore size [348]. Within osteochondral defects, it has been demonstrated that cell-seeded PLGA scaffolds with 100-200 μm pores in the chondral layer and 300-450 μm pores in the osseous layer best supported joint regeneration [349]. In addition to pore size, scaffold stiffness has also been shown to regulate stem cell differentiation, with softer scaffolds shown to support chondrogenesis, and stiffer scaffolds shown to support osteogenesis [350]. Therefore the enhanced chondrogenesis observed in the higher porosity/lower concentration ECM-derived scaffolds observed in this study may be due, at least in part, to alterations in scaffold stiffness as the concentration of the scaffold is reduced. Additional mechanical testing on individual scaffold struts is required to further test this hypothesis. It was also proven that composition can determine scaffold degradation kinetics and may alter release rates of biochemical

cues [38], which could explain the altered levels of chondrogenesis observed in different concentration/porosity ECM-derived scaffolds.

In agreement with previous studies, we found that EDAC crosslinking prevented contraction of cartilage ECM-derived scaffolds [341]. It was also found that sGAG accumulation was greater within EDAC+DHT crosslinked scaffolds compared with the DHT only scaffolds. This was likely due to superior sGAG retention within the scaffold and not due to superior matrix synthesis [341]. EDAC crosslinking of the scaffold is necessary, because it reduces contraction and may enhance integration with the surrounding tissue when implanted into cartilage defects. Further *in vivo* studies are also necessary to examine other impacts of EDAC, including the immunological response [338].

A further impact of EDAC crosslinking was the delay in the initial release of TGF-β3 from the scaffold. Previous studies have demonstrated that altering the degree of crosslinking of collagen-like microspheres affects the release profile of growth factors such as TGF-β [47]. Chemical crosslinking has also been shown to modulate growth factor release in protease containing media, with low crosslinking leading to rapid release compared to highly crosslinked microspheres [47]. Irrespective of the degree of scaffold crosslinking, the majority of the growth factor was released from the ECM-derived scaffolds within the first 10 days of culture. Superior chondrogenesis was generally observed in scaffold loaded with TGF-β3 compared to constructs where the growth factor was directly added to the media. This may be explained by the temporal release of TGF-β3 from the scaffold, as a number of studies have demonstrated that short-term exposure to growth factors enhances chondrogenesis [339, 341]. These results provide further support for the concept that ECM-derived scaffolds can release growth factor within an optimal dosing window to effectively promote chondrogenesis of stem cells [220, 339].

In vivo cartilage ECM-derived scaffolds supported chondrogenesis of culture expanded infrapatellar fat pad-derived stem cells. Cell free scaffolds were infiltrated by host cells, however when compared with cell seeded groups, a more fibrous or fibrocartilaginous tissue was generated. This is in agreement with previous studies that demonstrated that stem cell seeded constructs promoted superior matrix formation in a similar *in vivo* model [84]. In the context of developing a single-stage therapy for cartilage repair, freshly isolated $CD44^+$ stromal cells were also found to generate a cartilage-like tissue *in vivo*. CD44 is the principal cell surface receptor for hyaluronate [260], a key component of articular cartilage. The infrapatellar fat pad is a source of cells with a high expression of CD44 [261]. It has been previously reported that a microenvironment enriched with hyaluronan initiates and promotes chondrogenesis *via* CD44 in human ASC [66]. In addition, previous studies have demonstrated that CD44 antibody-beads can be used for stem cell isolation and delivery, and that such complexes can effectively generate chondrogenic matrix in monolayer and 3D culture [262, 263]. The technique for stromal cell isolation will be of crucial importance for the development of single-stage procedures for cartilage regeneration [255].

5.5. Conclusion

In conclusion, this study describes a robust method to control the composition and porosity of cartilage ECM-derived scaffolds, a biomaterial with potent pro-chondrogenic properties. By seeding such a scaffold with either culture expanded infrapatellar fat pad-derived stem cells, or freshly isolated $CD44^+$ stromal cells, it was possible to promote the development of a cartilage-like tissue *in vivo*. This latter finding supports the concept that enriched populations of freshly isolated stromal cells, when combined with a chondro-inductive scaffold, can induce cartilage formation and can potentially be used in ‘one-step’ or ‘single-stage’ procedures for cartilage repair. The clinical realisation of such a strategy would overcome many of the limitations believed

to be hampering the widespread clinical adoption of current cell-based approaches such as autologous chondrocyte implantation.

The following chapter is going to focus on the assessment of the effects of growth factor dosage on chondrogenesis of IFP-derived progenitor cells for different donors (diseased and healthy). Relatively little is known about how the dose of a specific growth factor will influence regeneration, or how different patient cells will respond to altered levels of delivery. Hence, the objective of the studies described in Chapter 6 is to assess the capacity of cartilage ECM-derived scaffolds to release deferent levels of TGF-β3 and its influence in chondrogenesis of stem cells. Additionally, it is going to be investigated if adult stem cells display a donor dependent response to the binding and release of different doses of TGF-β3 from such ECM-derived scaffolds.

Chapter 6

Stem Cell Display a Donor Dependent Response to Escalating Levels of Growth Factor Release from Extracellular Matrix-derived Scaffolds

6. Stem cells display a donor dependant response to escalating levels of growth factor release from extracellular matrix-derived scaffolds

Abstract

Numerous different growth factor delivery systems have been developed for various tissue engineering applications. In spite of this, relatively little is known about how the dose of a specific protein will influence tissue regeneration, or how different patients will respond to altered levels of growth factor delivery. The objective of this study was to assess stem cell chondrogenesis within cartilage extracellular (ECM)-derived scaffolds loaded with escalating levels of exogenous TGF-β3. Furthermore, we also sought to determine if adult stem cells display a donor-dependent response to different doses of TGF-β3 (low-5ng to high-200ng) released from such scaffolds. It was found that ECM-derived scaffolds possessed a remarkable capacity to bind and release increasing amounts of TGF-β3. Irrespective of the dose of TGF-β3 loaded onto the scaffold, they released between 60-75% of this growth factor into the media over 12 days of culture. After seeding these scaffolds with human infrapatellar fat pad-derived stem cells (FPSCs), it was found that cartilage specific extracellular matrix accumulation was greatest for the higher levels of growth factor loading. Importantly, soak-loading cartilage ECM-derived scaffolds with high levels of TGF-β3 always resulted in at least comparable levels of chondrogenesis as continuously supplementing the media with this growth factor. Similar results were observed for FPSCs from all donors, although the absolute level of secreted matrix did vary from donor-to-donor. This suggests that no single growth factor release profile will be

optimal for all patients, pointing to the need for personalized tissue engineering strategies.

Keywords: Extracellular matrix, Stem cells, TGF-β, Donor variability, Chondrogenesis, Growth factor dosage**.**

6.1. Introduction

Articular cartilage regeneration still is a challenge in orthopaedic medicine. While cell based therapies have been reasonably successful clinically [1, 100], they are expensive and do not consistently result in hyaline cartilage regeneration. Tissue repair can potentially be augmented by the use of porous scaffolds to provide an environment more conductive to regeneration [23, 69]. Extracellular matrix (ECM)-derived scaffolds have shown particular promise in this regard, having been used in the regeneration of different tissues including cartilage [36, 38, 39, 273, 291, 292, 295, 341, 351, 352].

Chondrogenesis is enhanced in the presence of members of the transforming growth factor (TGF)-β family of growth factors, motivating the delivery of such proteins to articular cartilage defects to enhance regeneration [190]. This multifunctional cytokine not only modulates stem cell differentiation, but has also been shown to regulate chondrocyte proliferation and matrix turnover in articular cartilage [266, 353]. Various scaffolds and hydrogels can be used to deliver cells and growth factors such as TGF-β, thereby opening up the possibility of using such functionalized biomaterials to enhance chondrogenesis during articular cartilage repair [38, 341, 351, 352]. However in the context of TGF-β mediated articular cartilage regeneration, the optimal growth factor dose or release profile is still unclear [190, 354]. Furthermore, it remains unclear how different patients will respond to altered levels of growth factor delivery.

A number of different strategies have been developed to control the release of growth factors to accelerate tissue repair [190]. TGF-β is widely used to promote chondrogenesis *in vitro* [190, 354]; however its short half-life *in vivo* can limit its

availability following delivery [190, 201, 202]. Therefore in the context of articular cartilage regeneration, it is crucial to develop scaffolds to temporally and spatially control the release of TGF-β to direct the differentiation of stem cells down a chondrogenic pathway [190, 354]. Established *in vitro* culture protocols that have been optimised to drive chondrogenesis can provide clues as to the optimal TGF-β dosing and release rates required to drive chondrogenesis using growth factor delivery systems. Specifically, temporal exposure (2 weeks) to specific concentrations (10 ng/ml) of TGF-β has been shown to promote robust chondrogenesis *in vitro* [203, 204, 220]. Furthermore, it is known that continuous stimulation with TGF-β can promote undesirable pathologies such as fibrosis and osteophyte formation [76, 190, 202, 208-215]. This motivates the development of delivery systems capable of supplying growth factor within such a dosing window [206, 207]. Different approaches have been used for exogenous chondrogenic growth factor delivery for cartilage tissue engineering, including from scaffolds [341, 351, 355] and/or from micro-sphere delivery systems [47, 52, 233, 356]. Delivery of TGF-β from cartilage ECM-derived scaffolds is particularly promising in this regard, as the biomaterial itself is inherently chondrogenic, and furthermore, this growth factor is known to bind strongly to ECM [201].

Hence, the overall objective of this study was to assess the effect of delivering different doses of TGF-β3 from cartilage ECM-derived scaffolds on chondrogenesis of human infrapatellar fat pad-derived stem cells (FPSCs). Furthermore, given the well documented donor-to-donor variability that exists in stem cell populations [357, 358], we also sought to determine the effects of delivering different doses of TGF-β3 from ECM-derived scaffolds on FPSCs isolated from a range of healthy and diseased (osteoarthritic) donors.

6.2. Material and methods

6.2.1. Scaffold preparation

The scaffolds were fabricated as previously described [351] (section 5.2.1) and imaged with SEM (section 3.10) before culture.

6.2.2. Cell and construct culture

Cells were obtained from the IFP of patients undergoing total joint arthroplasty (diseased) or ACL surgery (healthy), and were isolated and in culture, as previously described [351] (sections 3.4 and 3.5). Groups that are mentioned as “loaded with TGF-β3” were not supplemented with TGF-β3 in chondrogenic media during culture period. Instead, TGF-β3 was soak-loaded into the scaffold and was not directly added to the culture media. For the first part of the study, where the dosing effects were assessed in only one donor, the experimental groups were: Low dose (3 ng), Medium (30 ng), High (300 ng) and Media supplemented (10 ng/ml). For the second part of this study six different cell donors were assessed with similar low (5 ng), medium (50 ng), high (200 ng) and media supplementation (10 ng/ml) growth factor dosage.

Histological (section 3.7), immunohistochemical (section 3.8), biochemical (section 3.6) analysis were performed after 28 days in culture as described in general methods section. TGF-β3 quantification analysis was performed in media samples collected during culture period as described in general methods section (section 3.11).

6.3. Results

6.3.1. Cartilage ECM-derived scaffolds bind and release TGF-β3 independently of the dose added

Three different doses (low-3 ng, medium-30 ng and high-300ng) of TGF-β3 were loaded onto cartilage ECM-derived scaffolds, which were then seeded with human FPSCs and maintained *in vitro* for 4 weeks. Cartilage ECM-derived scaffolds

possessed a consistent capacity to bind and release increasing amounts of TGF-β3 (Figure 42). Irrespective of the concentration of TGF-β3 applied, the scaffolds released between 60-75% of the loaded growth factor into the media over 10 days of culture. Over the first 12 days of culture, the low group released a total of 2.4±0.2 ng of TGF-β3 (Figure 42A), the medium group released 21±2 ng (Figure 42B) and high dose group released 198±25 ng (Figure 42C). In terms of the total percentage of growth factor released over 12 days, this corresponded to 74±6% of loaded TGF-β3 released from the low group (Figure 42D), 67±7% from the medium group (Figure 42E) and finally 61±7% from the high group (Figure 42F).

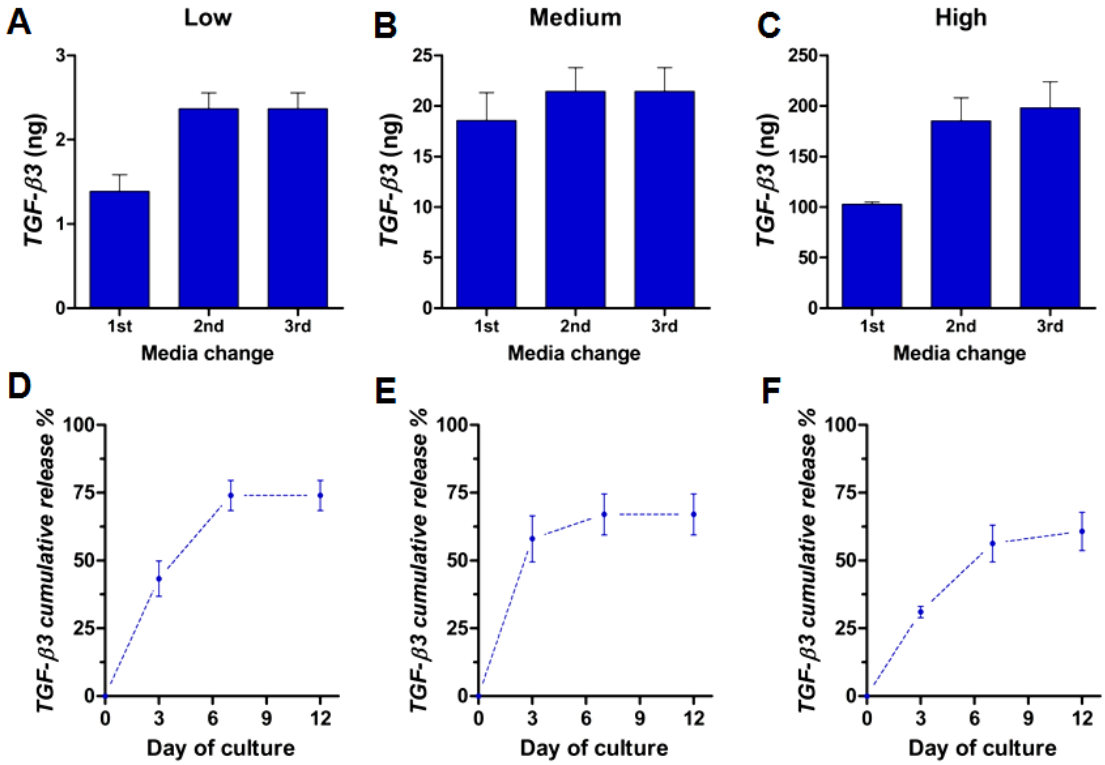


Figure 42 – Total TGF-β3 content (ELISA) of the culture media 1st, 2nd and 3rd media changes for low (A), medium (B) and high (C) growth factor loaded groups (n=3). Release profile into de media for the first 12 days of culture for low (D), medium (E) and high (F) TGF-β3 loaded groups (n=3).

6.3.2. Chondrogenesis within ECM-derived scaffolds seeded with human stem cells strongly depends on the dose of TGF-β3 loaded into the construct

After 4 weeks in culture, only scaffolds loaded with either medium or high doses of TGF-β3 resembled cartilage macroscopically (Figure 43I and M). These engineered tissues also appeared macroscopically similar to constructs where TGF-β3 was continuously added to the culture media (Figure 43Q; Note: a total of 200 ng of TGF-β3 was added to the media of this group over the 28 day culture period). Histologically, very little cartilage matrix deposition was observed in scaffolds that were not exogenously stimulated with TGF-β3 (Figure 43B-D), appearing similar to day zero scaffolds (data not shown). Alcian Blue staining suggested little sGAG accumulation within scaffolds loaded with low and medium doses of TGF-β3 (Figure 43F and J). Conversely, robust sGAG deposition was observed in scaffolds loaded with high levels of TGF-β3, or where the growth factor was directly supplemented in the culture media (Figure 43N and R). Collagen matrix staining was strong for medium dose (Figure 43K), high dose (Figure 43O) and media supplemented (Figure 43S) scaffolds. Staining for type II collagen was most intense within scaffolds loaded with high doses of TGF-β3 (Figure 43P).

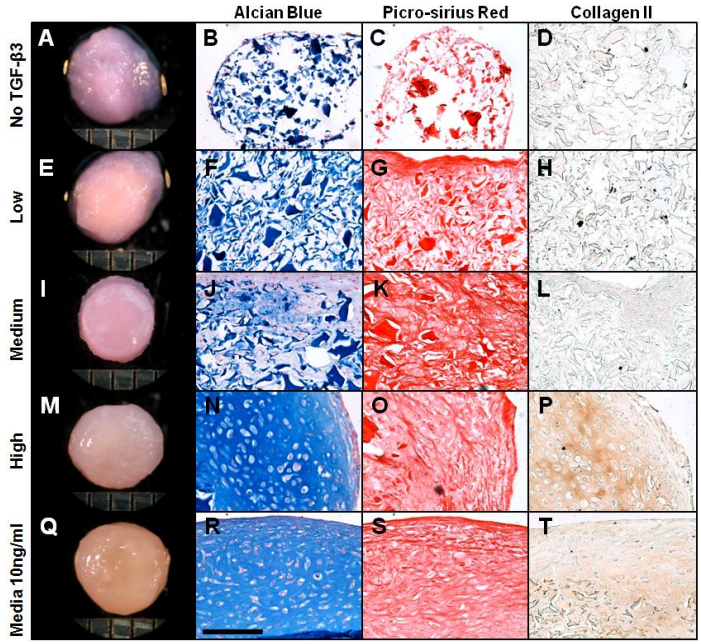


Figure 43 – Macroscopic images of ECM-derived scaffolds seeded with infrapatellar fat pad-derived stem cells after 4 weeks in culture for no TGF-β3 (A), low (E), medium (I), high (M) and direct media TGF-β3 supplementation (Q). Alcian blue, picro-sirius red and type II collagen staining for no TGF-β3 (B-D), low (F-H), medium (J-L), high (N-P) and direct media TGF-β3 supplementation (R-T). Scale bar: 50 µm.

Total sGAG content after 4 weeks of culture period was highest in scaffolds loaded with a high dose of TGF-β3, being at least comparable to constructs where this growth factor was continuously supplemented to the media (Figure 44A). Collagen content was significantly lower for the low dose group when compared with the other scaffolds (Figure 44B).

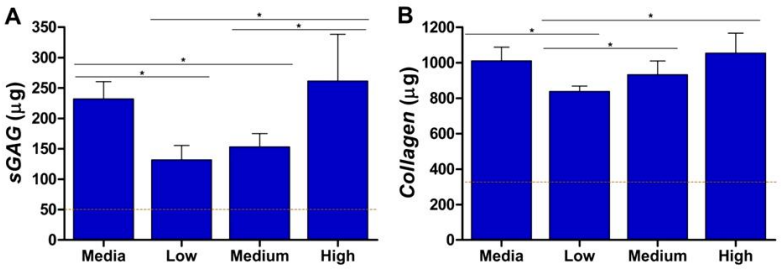


Figure 44 – (A) sGAG and (B) collagen accumulation within ECM-derived constructs seeded with infrapatellar fat pad-derived stem cells after 4 weeks of culture for media, low, medium and high TGF-β3 supplementation (n=5, *p<0.05). Red line represents day 0 values.

6.3.3. Stem cells display a donor dependent response to TGF-β3 delivery from ECM-derived scaffolds

Having demonstrated that cartilage ECM-derived scaffolds can be used to control the delivery of different doses of TGF-β3, and that chondrogenesis within these scaffolds was dependent on the amount of growth factor loaded onto the scaffold, we next sought to determine if FPSCs display a donor dependant response to the release of different doses of TGF-β3 from these constructs. To this end, FPSCs from a range of healthy and diseased donors were used. After 4 weeks in culture, sGAG staining (alcian blue) was week for all six donors (A-F) when a low dose (5 ng per scaffold) of TGF-β3 was loaded onto the scaffolds (Figure 45). For a medium dose (50 ng) of growth factor, reasonable levels of chondrogenesis (as evidenced by Alcian Blue staining for sGAG deposition) were observed for 2 of the 6 donors (donor C and F). With the exception of donor A, robust chondrogenesis was observed in all cases when high doses of TGF-β3 were loaded onto the scaffolds (Figure 45). Robust collagen deposition (picro-sirius red staining) was observed for all donors in all conditions, with the exception of donor A when stimulated with a low dose of growth factor (Figure 45). In agreement with previous studies using a pellet culture system [60], the disease state of the donors (either healthy or osteoarthritic) did not appear to impact chondrogenesis of FPSCs (Figure 45).

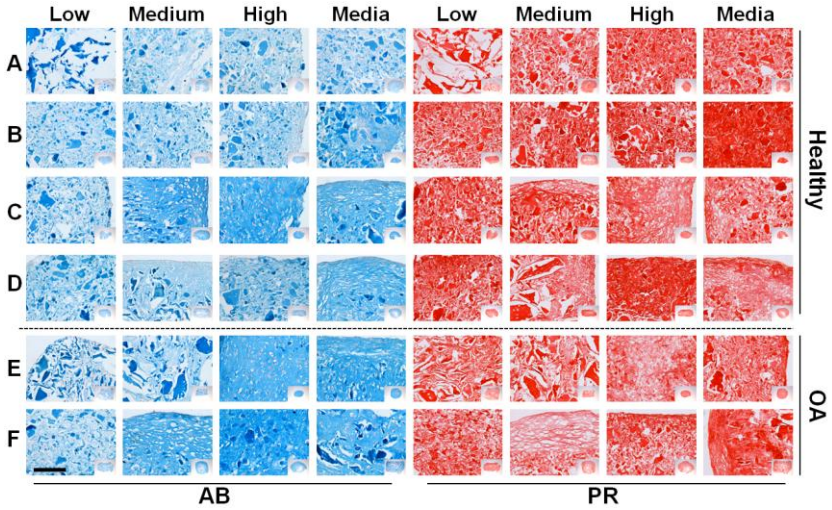


Figure 45 – Alcian blue (AB), picro-sirius red (PR) for low, medium, high and media TGF-β3 supplementation for six different donors (A-F), Healthy and diseased (osteoarthritic - OA). All micrographs are for 4 weeks in culture with human infrapatellar fat pad-derived stem cells. Scale bar: 50 µm.

Quantification of the total sGAG content after 28 days of culture within constructs initially loaded with increasing doses of TGF-β3 generally agreed with the histological analysis of the engineered tissues (Figure 46). For all donors, the highest levels of sGAG accumulation were observed in scaffolds loaded with high doses of TGF-β3. For this high dose, sGAG accumulation was highest for donor B, reaching close to 470 µg, in comparison to near 260 µg for donor A. Again, there was no evidence of diminished sGAG deposition using FPSCs isolated from diseased (OA) donors (Figure 46).

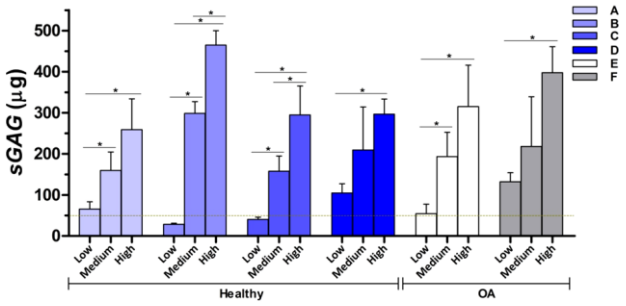


Figure 46 – sGAG accumulation within ECM-derived constructs after 4 weeks culture period with infrapatellar fat pad-derived stem cells for media, low, medium and high TGF-β3 supplementation for six different donors (A-F) (n=5, *p<0.05). Red line represents day 0 value.

6.4. Discussion

The overall goal of this study was to assess the capacity of cartilage ECM-derived scaffolds to bind and release different amounts of soak loaded TGF-β3. Furthermore, we also sought to determine if adult stem cells display a donor dependent response to the binding and release of different doses of TGF-β3 from such ECM-derived scaffolds. These scaffolds possessed a remarkable capacity to bind and deliver increasing amounts of TGF-β3. They released between 60-75% of the loaded TGF-β3 into the media over 12 days of culture, irrespective of the concentration of growth factor added to the scaffold, with the remainder sequestered within the ECM. After a 28 day culture period, only constructs loaded with either medium or high doses of TGF-β3 resembled cartilage macroscopically. Histologically, very little cartilage matrix deposition was observed in scaffolds that were not stimulated with TGF-β3. Robust sGAG and collagen deposition was observed in scaffolds loaded with high levels of growth factor, or where the TGF-β3 was regularly supplemented into the media. It was also observed that staining for type II collagen, the main collagen type in articular cartilage, was most intense within scaffolds loaded with high doses of TGF-β3. Having demonstrated that cartilage ECM-derived scaffolds can be used to control the delivery of different doses of TGF-β3, and that chondrogenesis within these scaffolds was dependent on the amount of growth factor loaded onto the scaffold, we next sought to determine if FPSCs display a donor dependant response to the release of different doses of TGF-β3 from these constructs. To this end, FPSCs from a range of healthy and diseased donors were seeded into cartilage ECM-derived scaffolds. Chondrogenesis was observed in most of the cases when high doses of growth factor were used. In agreement with previous studies [60], the donor's disease state (healthy or osteoarthritic) did not impact chondrogenesis.

The release profile of TGF-β3 from cartilage ECM-derived scaffolds was relatively insensitive to the dose of growth factor loaded into the stem cell laden

construct, with between 60 and 75% of the growth factor released over the first 12 days of culture. The growth factor interaction with the scaffold is possible due to known non-specific binding sites present in native cartilage ECM [266]. Binding sites for TGF-β include proteoglycans, glycosaminoglycans, collagens and glycoproteins, and these interactions are known to influence growth factor availability [266]. Previous studies have used gelatin (denatured collagen) microspheres to control release TGF-β [52]. Positively charged growth factors can be absorbed by these lyophilized microspheres by creation of a polyion complex with gelatin [52]. TGF-β is released when this complex is compromised, or via material degradation due to cell mediated proteolysis [52]. Additionally, the rate of release of such growth factor is dependent on the polymer degree of crosslinking [52, 359]. With these gelatin microspheres, approximately 10% of loaded TGF-β1 is released over 15 days. Chondroitin/hyaluronic-acid scaffolds containing gelatin microspheres have also been used as TGF-β3 delivery systems, which showed an initial burst release of 37% of loaded growth factor, with 80% released after 18 days [236, 237]. Other examples include a hybrid system consisting of type I/II collagen and TGF-β1-loaded fibrin [239], and a heparin/fibrin/PCL system which supported *in situ* chondrogenesis of adipose-derived stem cells while releasing approximately 65% of the initial TGF-β1 loaded into the construct during the first week of culture [240]. In the particular case of this cartilage ECM-derived scaffold it is likely that only a percentage of growth factor binds to the ECM, and the remainder is internalized by FPSCs. This cellular internalization is likely the same for all TGF-β3 doses. Release of bound growth factor from scaffolds may also depend on the rate of new tissue development within the scaffold, which may act as a new link for TGF-β3 as it is released from the scaffold. Higher levels of new tissue is deposited within scaffolds loaded with the high doses of exogenous TGF-β3, which may explain why a higher percentage of growth factor appears sequestered within these construct after 12 days of culture.

In agreement with previous studies [341, 351], additional growth factor (TGF-β3) stimulation was necessary to induce chondrogenesis of FPSCs, even in the presence of native cartilage ECM. Cartilage specific matrix accumulation within these FPSCs seeded scaffolds was greatest for the higher levels of growth factor loading. It has previously been shown that TGF-β enhances chondrogenesis of stem cells in a dose dependent manner [360]. The gold standard for promoting chondrogenesis of mesenchymal stem cells *in vitro* is to supplement the media with 10 ng/ml of TGF-β3; however the dosage required to drive chondrogenesis *in vivo* from a growth factor delivery scaffold has yet to be elucidated. Previously reported TGF-β dosing studies have demonstrated that a brief exposure to high concentrations of TGF-β (100 ng/ml for 30 min) was beneficial to chondrogenesis [361]. This suggests that the TGF-β burst release from the ECM-derived scaffold observed in the first three days of the current study is advantageous for driving robust chondrogenesis of stem cells seeded in the scaffold. There are, however, potential drawbacks associated with excessive TGF-β release from a scaffold into the joint space that need to be considered when developing any growth factor delivery system. For example, it has previously been reported that 21 days after a single joint injection of 200 ng of TGF-β1, sGAG synthesis was significantly increased in a murine knee joint model [362]. However, repeated growth factor injections lead to TGF-β1-induced osteophyte formation [362]. Therefore, there is a clear need to perform additional studies to understand which dose of growth factor will be acceptable when cartilage regeneration is concerned.

Human stem cells displayed a donor dependant response to escalating levels of exogenous growth factor stimulation within ECM-derived scaffolds, although importantly the greatest levels of chondrogenesis were consistently observed within constructs loaded with the higher levels of TGF-β3. It is known that bone marrow-derived mesenchymal stem cells from different donors may have dissimilar responses to environmental *stimuli* [233, 357, 358, 363]. In the current study we observed that if

the dose of TGF-β3 loaded onto cartilage ECM-derived scaffolds is increased (specifically to the 200 ng dose), it is possible to observe a similar level of chondrogenesis using infrapatellar fat pad-derived stem cells from a range of healthy and diseased donors. This is an important finding, as other studies that have not used such cartilage ECM-derived scaffolds to drive chondrogenesis of stem cells have observed notable donor dependency in their results. Such consistency in minimizing patient-to-patient variability will facilitate the development, and future implementation, of more effective treatments for human cartilage repair.

6.5. Conclusion

The overall objective of this study was to assess the effect of delivering different doses of exogenous TGF-β3 from cartilage ECM-derived scaffolds on stem cells isolated from a range of healthy and diseased donors. We observed that by delivering a high dose of growth factor from these scaffolds that it was possible to minimize donor-to-donor variability in chondrogenesis of human infrapatellar fat pad-derived stem cells. Furthermore, no major difference was observed between healthy and diseased donors. The combination of an ECM-derived scaffold, human infrapatellar fat pad-derived stem cells, and a sufficient dose of growth factor consistently results in robust chondrogenesis. Such consistency will facilitate future clinical translation of such treatments for human cartilage repair using growth factors.

The next stage of this thesis is going to assess alternative sources of ECM, mainly due to xenogeneic material limitations. As an alternative we have allogeneic engineered cartilage, which can possibly be used to fabricate the already-known chondro-permissive native ECM-derived scaffolds.

Chapter 7

A Comparison of Engineered and Native Cartilage Extracellular Matrix as a Scaffold for Cartilage Tissue Engineering

7. A comparison of engineered and native cartilage extracellular matrix as a scaffold for cartilage tissue engineering

Abstract

ECM-derived scaffolds have been developed from devitalized native cartilage and successfully used for cartilage tissue engineering. They are commonly derived from animal tissues, which may elicit an adverse immune response. Native human ECM can be used as an alternative to xenogeneic tissue. The goal of this study was to compare native and tissue engineered cartilaginous ECM as chondroinductive scaffolds for tissue engineering. To this end, porous scaffolds were produced using ECM derived from porcine articular cartilage and sheets of cartilaginous tissues engineered using human bone marrow derived mesenchymal stem cells (MSCs). Their capacity to support chondrogenesis was then assessed. Engineered cartilage ECM presented some of the features of native articular cartilage, although it contained lower levels of type II collagen. Scaffolds produced using both engineered and native ECM possessed similar mechanical properties, pore size and GAG content. In spite of this, engineered ECM derived scaffolds supported less robust matrix deposition when seeded with human infrapatellar fat pad-derived stem cells. However, soak-loading these engineered ECM-derived scaffolds with TGF-β3 enhanced their capacity to support chondrogenesis, to levels comparable to the native ECM. The results of this study demonstrate that engineered ECM can be used to fabricate scaffolds for cartilage tissue engineering, overcoming stock limitations and other barriers associated with allogeneic and xenogeneic tissues. These findings open the door for the clinical translation of such off-the-shelf chondroinductive scaffolds for cell based therapies in human cartilage repair.

Keywords: Tissue engineering, cartilage, ECM, allogeneic, scaffold, stem cell, TGF-β.

7.1. Introduction

Articular cartilage is a complex avascular tissue with limited capacity for self repair [3, 100]. Injuries to cartilage can be treated using different strategies, including cell-based therapies [100]. Tissue engineering is an encouraging approach to regenerate tissues and organs through the use of cells, scaffolds and biochemical and biophysical cues [364]. Scaffolds are designed to support cell adhesion, proliferation and differentiation, often by mimicking aspects of the biochemical and biophysical characteristics of the native extracellular-matrix (ECM) [26, 188, 365, 366]. In particular, decellularized or devitalized ECM-derived scaffolds have been developed from numerous different tissues and used in tissue engineering and regenerative medicine [37, 38]. As with any biomaterial, the success of ECM-derived scaffolds is dependent on the host response following implantation [367]. The immune response to such scaffolds is dependent on several factors, including fabrication methods and the origin of the ECM [367].

Cartilage ECM-derived scaffolds have been shown to be chondro-permissive and to facilitate articular cartilage repair [36, 38, 286, 302, 341, 351]. In general, however, such approaches rely on the use of animal-derived tissues (xenogeneic), with the associated risk of an adverse immune response and disease transmission after implantation into man [82, 368]. As an alternative to the use of xenogeneic tissues, native human cartilage ECM (either autologous or allogeneic) has also been used in scaffolds for joint regeneration [82, 106]. However, the supply of autologous native tissues is limited, motivating the use of *in vitro* engineered cartilage ECM from human chondrocytes or mesenchymal stem cells (MSCs) [47, 189, 233, 271, 369]. In addition to lowering the risk of disease transmission and an inappropriate immune response, engineered ECM-derived scaffolds could be considered to be more

developmentally immature and hence provide a milieu of features more conducive to chondrogenesis [189]. Engineered cartilage ECM will be compositionally different to native articular cartilage, which will impact how cells and growth factors will interact with scaffolds generated using such biomaterials [188]. The collagen present in the immature ECM engineered cartilage scaffolds will be critical in the interaction with cells, and particularly with TGF-β, where are expected to exist specific binding sites for this growth factor family [188, 189]. Conversely, these chordin-cysteine binding sites, which are not present in mature tissue, are available in early developed type II collagen and will bind to TGF-β family growth factors [188]. This interaction between collagen and TGF-β is expected to enhance chondrogenesis of stem cells [177, 179, 189]. In addition, higher density of glucosamine present in glycosaminoglycans in mature cartilage inhibits chondrogenesis of mesenchymal stem cells [370, 371].

The goal of this study was to compare devitalized native and engineered cartilage ECM when used to produce chondroinductive scaffolds for cartilage tissue engineering. Engineered cartilaginous ECM was produced using a self-assembly technique using a high density of human bone marrow derived MSCs [47]. This self-assembling approach was performed with or without transforming growth factor (TGF)-β1 releasing gelatin microspheres [47]. These microspheres, when included in high density sheets of MSCs, can uniformly deliver chondrogenic growth factor efficiently over a sustained period, enabling homogenous differentiation and robust cartilage tissue formation compared to simply supplementing the media with growth factor [47]. A further objective of the study was therefore to assess if scaffolds produced using ECM engineered in the presence of such TGF-β3 loaded microspheres would be more chondroinductive. To access the potential of these different scaffolds to support chondrogenesis, human Infrapatellar fat pad derived stem cells (FPSCs) were seeded onto the ECM-derived scaffolds and *in vitro* chondrogenesis was assessed over a 4 week culture period. A further goal of this study was to assess feasibility of these

different ECM-derived scaffolds to act as growth factor delivery platforms to enhance chondrogenesis of FPSCs.

7.2. Material and methods

7.2.1. Engineering of Human Cartilaginous ECM using Bone Marrow-Derived Mesenchymal Stem Cells

Human bone marrow mesenchymal stem cells (hMSCs) from bone marrow aspirates were obtained from Case Comprehensive Cancer Center Hematopoietic Biorepository and Cellular Therapy Core under University Hospitals of Cleveland Institutional Review Board approval, as previously described [356]. Firstly, bone marrow aspirates were washed with culture media (DMEM-LG; Sigma-Aldrich, St. Louis, MO) with 10% bovine serum (Gibco Qualified FBS; Life Technologies, Carlsbad, CA). Mononuclear cells were separated using a Percoll gradient (Sigma-Aldrich), plated in expansion media and cultured in incubator (37˚C; 5% CO_2). Cells were supplemented with expansion media with 10 ng ml^{-1} fibroblast growth factor-2 (FGF-2; R&D Systems, Minneapolis, MN) twice a week. hMSCs were cultured to passage 3, and used in this study to engineer human cartilage sheets [47]. Transwell inserts (12 mm; Corning) were incubated with 0.75 ml expansion media in well plate (2 hours; 37˚C) after which an additional 0.75 ml of basal pellet media (BPM) comprised of DMEM-HG (Sigma-Aldrich), 1% ITS+ Premix (Corning Inc, Corning, NY), 10^{-7} M dexamethasone (MP Biomedicals, Solon, OH), 1 mM sodium pyruvate (HyClone Laboratories), 100 mM non-essential amino acids (Lonza Group, Switzerland), 37.5 mg/ml ascorbic acid-2-phosphate (Wako Chemicals USA) and 100 U/ml penicillin-streptomycin (Corning) was added to the plate well. Furthermore, 2 million hMSCs were resuspended in 500 µl of BPM and allowed to settle onto the membranes of transwell inserts in incubator (48h; 37°C; 5% CO_2). Additionally, TGF-β1 (10 ng/ml; Peprotech, Rocky Hill, NJ) was supplemented to the media every media change.

After 1 day, the media was replaced with 1.5 ml BPM. A complete media change (2 ml) occurred after 48 hours and regularly twice a week [47].

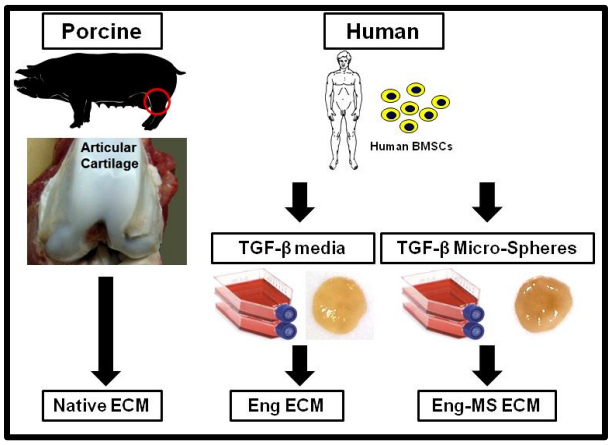
Gelatin microspheres (11.1 w/v% Type A; Sigma-Aldrich) were produced in a water-in-oil emulsion, as previously described [47, 356]. Microspheres were crosslinked with genipin (2 hours; 1 w/v%; Wako Chemicals, USA), washed with deionised water, lyophilized and rehydrated with PBS (HyClone Laboratories, Logan, UT) containing TGF-β1 (400 ng per mg of microspheres). Light microscopy micrographs were obtained using a TMS microscope (Nikon, Japan) with a Coolpix 995 camera (Nikon). Microspheres were sterilized (UV) followed by loading with 400 ng TGF-β1 per mg of microspheres (2 hours; 37˚C), as previously described [47]. The assemblage and media formulation was the same as the normal human engineered sheets (no TGF-β1 loading), as mentioned earlier in the text. Moreover, microspheres (1.5 mg) and hMSCs (2 million) were suspended in 500 µl of BPM and allowed to attach to membranes of previously mentioned transwell inserts. Culture conditions were the same as the human engineered sheets, however with no TGF-β1 supplementation.

7.2.2. Preparation of Cartilage ECM-Derived Scaffolds

Cartilage (native and engineered) was maintained aseptic before and after being sectioned into small pieces using a scalpel, each group separately. The cartilage was harvested as described in general methods section (section 3.1). Furthermore, the scaffolds were fabricated as previously described [351] (section 5.2.1) and imaged with SEM (section 3.10) before culture. Briefly, scaffolds were fabricated by pulverising cartilage pieces within a cryogenic mill (6770 Freezer/Mill, SPEX, UK), as before mentioned [351]. Pulverized cartilage was then blended in UPW using a homogenizer (IKAT10, IKA Works, USA) to create a fine cartilage slurry. Scaffolds were produced by using a previously used known method, where a native cartilage slurry was initially created with 250 mg/ml as concentration [351]. Slurry was freeze-dried, DHT and

EDAC crosslinked as previously described (sections 3.2 and 3.3). Identical process of fabrication of the ECM-derived scaffolds was performed for the three different types of ECM: derived from porcine ECM (Native), from human engineered sheets (Eng) and from human engineered sheets with microspheres (Eng-MS) (Table 5).

Table 5 – Schematics of cartilage ECM origin and production.



7.2.3. Mechanical Testing

Scaffolds (acellular; 5 mm diameter; 3 mm height) were mechanically tested dry with a standard materials testing device with a 5 N load cell (Zwick Z005, Roell, Germany) [55]. Moreover, preload (0.03 N) was applied to ensure direct contact between the scaffold and the loading platens. A ramp compressive strain (10%; 0.001 mm/s) was applied to samples, from which the Young's modulus was determined from the slope of the stress-strain curve. Engineered tissues at day 28 were mechanically tested with a similar protocol (compressive strain). Constructs were hydrated and maintained in a bath of PBS (RT; 10%; 0.001 mm/s), until equilibrium was reached, as previously described [55].

7.2.4. Cell Isolation and Culture

Cells were isolated and in culture, as previously described [351] (sections 3.4 and 3.5). Groups termed "TGF-β3 in scaffold" did not have additional TGF-β3 added to the medium during the culture period. Instead, TGF-β3 (200 ng) was soak-loaded into

the ECM-derived scaffold for 15 minutes, and was not added to media. Each scaffold was placed within cylindrical agarose moulds to increase cell-seeding efficiency. After cell seeding scaffold with cells were left in the incubator for two hours. After two hours, 2.5 ml of supplemented CDM was added, and media changes were performed twice a week.

Histological (section 3.7), immunohistochemical (section 3.8), biochemical (section 3.6) analysis were performed after 28 days in culture as described in general methods section.

7.2.5. Histology and immunohistochemistry

Constructs were fixed overnight (4˚C) in paraformaldehyde (4%) (Sigma-Aldrich), followed by washing steps and wax embedded, as previously described [341, 351] (section 3.7 and 3.8). With the aim of imaging the orientation/organization of the collagen fibril, picro-sirius red stained sections were imaged using a polarised light microscope (Nikon Eclipse E400 POL), as previously described [85]. Alizarin calcium staining was performed to assess calcium accumulation [160].

7.2.6. Measurement of TGF-β3 release from ECM scaffolds

The TGF-β3 present in the ECM-derived scaffolds was extracted using a previously reported protocol [266]. Briefly, the scaffolds were treated with a solution of 4 M guanidine hydrochloride (Pierce) at 4°C for 2 days to extract their TGF-β content. TGF-β3 content was determined via ELISA, as previously described [266, 341, 372] (section 3.11).

7.3. Results

7.3.1. Engineered and native cartilage ECM is compositionally distinct

Porous scaffolds were fabricated using three distinct cartilage-derived ECM materials: (1) native porcine articular cartilage; (2) engineered cartilage (Eng); and (3)

cartilage engineered in the presence of TGF-β1 loaded microspheres (Eng-MS). Each of these three tissues was structurally and compositionally different (Figure 47). Both the Eng and Eng-MS tissues stained strongly for glycosaminoglycans (Figure 47G and L), which was comparable to native articular cartilage (Figure 47B). Native tissues stained more intensely for collagen compared to engineered tissues (Figure 47C, H, and M). Polarized light microscopy (PLM) revealed that engineered tissues did not possess the same degree of cartilage fibril organization as native articular cartilage (Figure 54D). All tissues stained negatively for calcium accumulation (Figure 54E, J and O).

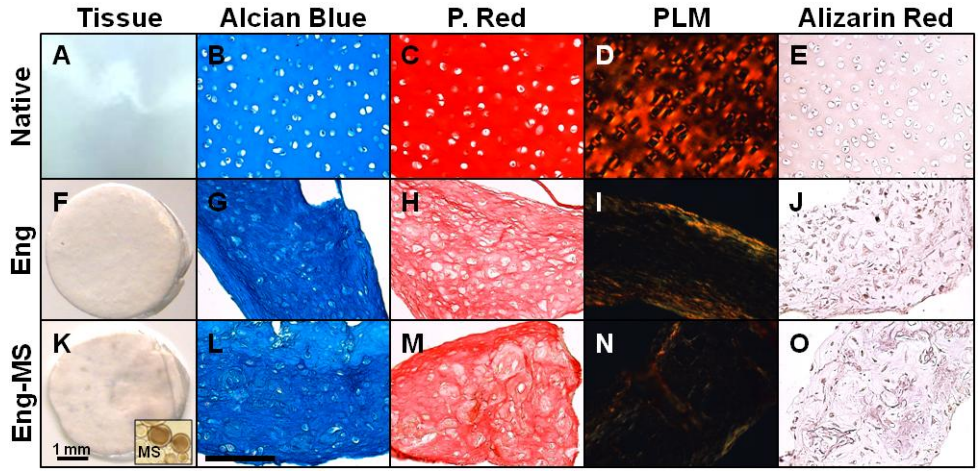


Figure 47 – Macroscopic appearance of native (A), engineered (Eng - F) and engineered with microspheres (Eng MS - K) cartilage. Histological staining for alcian blue (AB) and picro-sirius red (PR) for native (B, C), Eng (G, H) and Eng-MS (L, M) cartilage groups. Polarized light microscopy (PLM) micrographs of the collagen fibrils architecture for native (D), Eng (I) and Eng-MS (N) cartilage. Alizarin red calcium staining for native (E), Eng (J) and Eng-MS (O) cartilaginous tissues. Scale bar: 50 µm.

Immunohistochemistry was used to determine the specific types of collagen within the different ECMs. The native, Eng and Eng-MS tissues all stained weakly for type I collagen deposition (Figure 48A, D, and G). As expected, native cartilage stained strongly for type II collagen (Figure 48B), but less intensely in engineered ECM. Eng-MS group stained slightly for type II collagen (Figure 48H). Some weak staining for type X collagen staining was observed in native and Eng (Figure 48C and

F) tissues. However, the type X collagen staining was superior for Eng-MS group (Figure 48I).

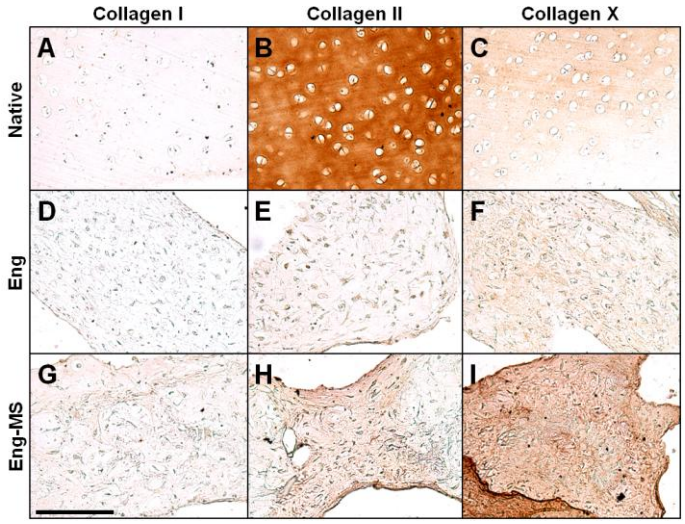


Figure 48 – Immunohistochemical analysis for type I, II and X collagen for native (A-C), Eng (D-F), and Eng-MS (G-I). Scale bar: 50 µm.

7.3.2. Native cartilage ECM-derived scaffolds support greater levels of chondrogenesis than engineered ECM-derived scaffolds

Porous scaffolds were first fabricated using a freeze drying protocol [351] using devitalized native and engineered ECM. Scanning electron microscopy (SEM) was used to characterize the porosity of both native and Eng scaffolds (Figure 49A-D). The architecture (Figure 49A-D), porosity, Young's modulus (Figure 49E) and mean pore size was comparable for both scaffold types (Figure 49F). Both native and Eng scaffolds stained positive for GAG (Figure 49G, I) and collagen (Figure 49H, J). The GAG content was similar for both groups, while the collagen content of the native ECM-derived scaffold was higher than the engineered group (data not shown).

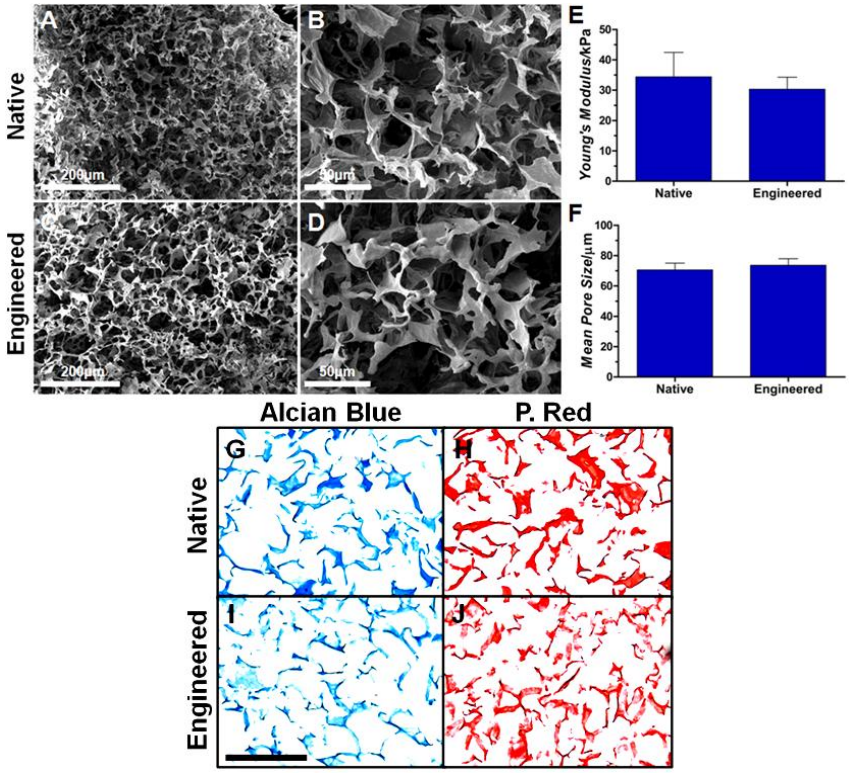


Figure 49 – Scanning electron microscopy (SEM) micrographs for native (A, B) and Eng (C, D) ECM-derived scaffolds. Young's modulus (E) and mean pore size (F) for both native and engineered groups (n=4). Acellular ECM-derived scaffolds histological staining for Alcian blue (AB) and picro-sirius red (PR) for native (G, H) and Eng (I, J) groups. All micrographs are for the dry scaffolds before culture period. Scale bar: 50 µm.

To assess the capacity of the two scaffolds to support chondrogenesis, they were seeded with FPSCs and maintained for 4 weeks in media supplemented with TGF-β3. Pilot studies revealed that neither scaffold was able to induce robust chondrogenesis in the absence of exogenously supplied TGF-β3 (data not shown). In the presence of endogenous TGF-β3, the native and engineered scaffolds resembled cartilage macroscopically (Figure 50A and E). Histological analysis suggested that GAG and collagen deposition was higher within native ECM scaffolds compared to the engineered ECM scaffolds (Figure 50B and C). In addition, staining for type II collagen deposition was more intense in native cartilage ECM scaffolds (Figure 50D). After 4 weeks of culture, the DNA, GAG and collagen content was higher in the native cartilage ECM-derived scaffolds compared to the engineered groups (*p<0.05; Figure

50I-K), demonstrating that the native ECM-derived scaffolds support greater levels of FPSC proliferation and cartilage-matrix specific matrix accumulation. Finally, the equilibrium *moduli* of the two different constructs were not statistically different, however native cartilage ECM-derived group denoted a slightly more mechanically robust construct (Figure 50L).

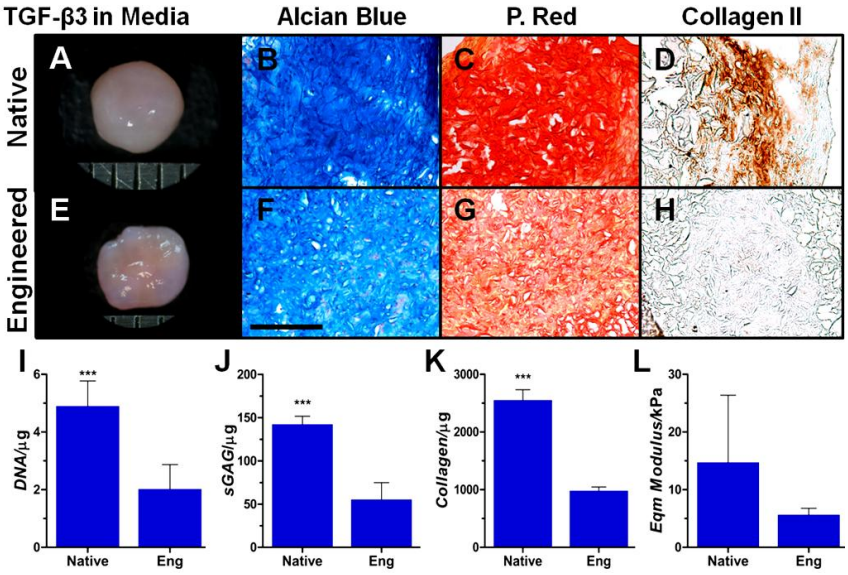


Figure 50 – Macroscopic appearance of native (A) and Eng (E) ECM-derived constructs after 28 days in culture (TGF-β3 in media). Histological staining for Alcian blue (AB), picro-sirius red (PR) and type II collagen for native (B-D) and Eng (F-H) groups. All micrographs are for 28 days culture period. Scale bar: 50 μm. DNA (I) (day 0 values subtracted to total DNA content), GAG (J), Collagen (K) and Equilibrium Modulus (L) for native and Eng groups (n=5; ***p<0.001).

7.3.3. Loading engineered cartilage ECM-derived scaffolds with TGF-β3 enhances chondrogenesis

We have previously shown that native cartilage ECM derived scaffolds can be used to control the release of TGF-β3, inducing robust chondrogenesis of FPSCs [341, 351]. To access if engineered cartilage ECM derived scaffolds could be used in a similar manner, they were soak loaded with TGF-β3, seeded with FPSCs and maintained in culture for 28 days. At the end of this period, both the native and engineered constructs resembled cartilage macroscopically (Figure 51A and E). GAG

and collagen histological staining was similar for both the Native and Eng groups (Figure 51B, C, F, and G).

Immunohistochemical staining for type II collagen was locally more intense in constructs fabricated using native cartilage (Figure 51D). The higher DNA content in the native construct again indicated that this scaffold supported higher levels of proliferation (Figure 51I). While the collagen content was higher in native ECM scaffolds soak loaded with TGF-β3 (Figure 51K), which is due (at least in part) to the higher collagen content in these scaffolds at day 0, the GAG content and equilibrium modulus was not significantly different to that of engineered ECM-derived constructs (Figure 51J and L).

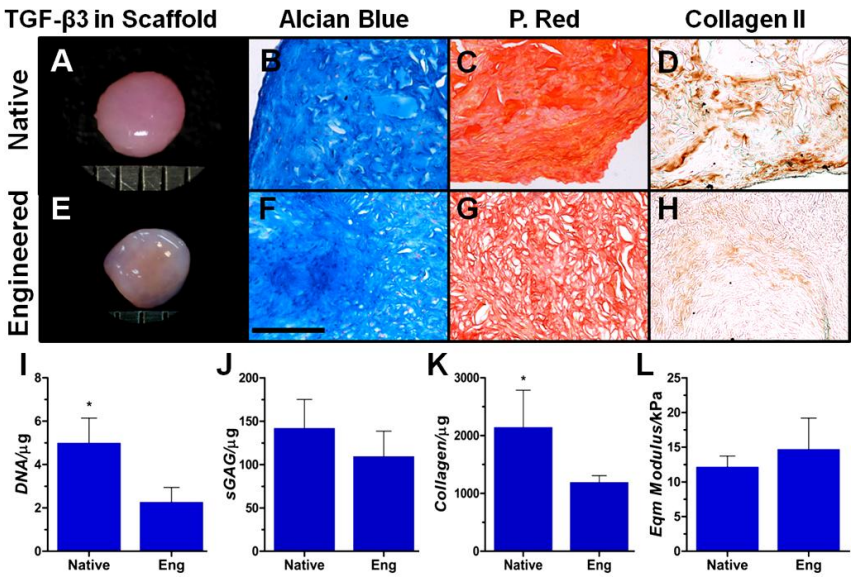


Figure 51 – Macroscopic appearance of native (A) and Eng (E) ECM-derived constructs after 28 days in culture (TGF-β3 in scaffold). Histological staining for Alcian blue (AB), picro-sirius red (PR) and type II collagen for native (B-D) and Eng (F-H) groups. All micrographs are for 28 days culture period. Scale bar: 50 μm. DNA (I), GAG (J), Collagen (K) and Equilibrium Modulus (L) for native and Eng groups (n=5; *p<0.05).

7.3.4. Cartilaginous ECM engineered in the presence of TGF-β loaded microspheres retain high levels of growth factor which enhances its capacity to promote chondrogenesis

The final phase of the study sought to determine if the mechanism by which cartilage ECM is engineered would influence the chondro-inductivity of the resulting scaffold. Cartilaginous tissues that were engineered using TGF-β loaded microspheres (Eng-MS) where compositionally different to both native and Eng tissues (Figure 47). It was again possible to produce porous scaffolds using Eng-MS ECM using an identical freeze-drying technique to the previously prepared scaffolds. The GAG and collagen content of the Eng-MS was slightly higher than the Eng group (data not shown). Prior to seeding with FPSCs, an ELISA was used to quantify the amount of TGF-β present in each of the scaffold groups. Higher levels of TGF-β3 were present in scaffolds engineered using Eng-MS ECM compared with the other groups, suggesting that TGF-β3 was retained in the microspheres and ECM after engineering the tissues and processing them into scaffolds (Figure 52C).

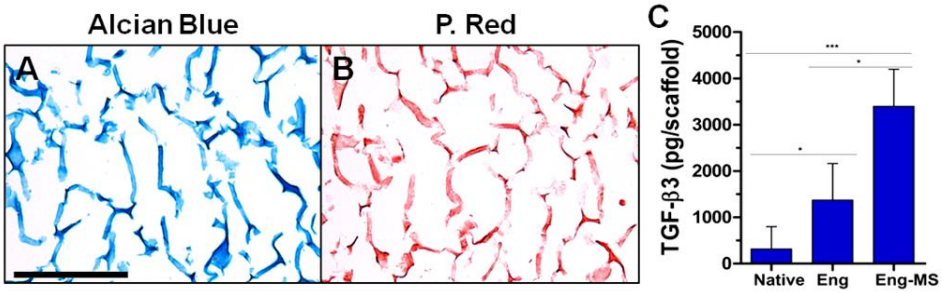


Figure 52 – Acellular ECM-derived scaffold histological staining for Alcian blue (AB) and picro-sirius red (PR) for Eng-MS (A, B) group. All micrographs are for the dry scaffold before culture period. TGF-β3 content (ELISA) (C) of the native, engineered (Eng) and engineered plus microspheres (Eng-MS) ECM-derived scaffolds before culture period (n=4; *p<0.05; ***p<0.001). Scale bar: 50 µm.

Scaffolds fabricated with Eng and Eng-MS ECM were seeded with FPSCs, with scaffolds either directly loaded with TGF-β3, or where this growth factor was added to the culture media. After 28 days in culture, all constructs resembled cartilage

macroscopically (Figure 53A, E, I and M). Histologically, all constructs stained positive for GAG and collagen deposition. Type II collagen immuno-staining appeared slightly more intense in the Eng-MS ECM derived scaffolds (Figure 53D and L). When TGF-β3 was added to the media, the Eng-MS ECM derived constructs accumulated higher levels of GAG (Figure 53Q). No significant difference in GAG accumulation was found when TGF-β3 was loaded into the scaffold before cell culture (Figure 53Q). Finally, alizarin red staining revealed no evidence of calcification in any of the engineered cartilaginous constructs (data not shown).

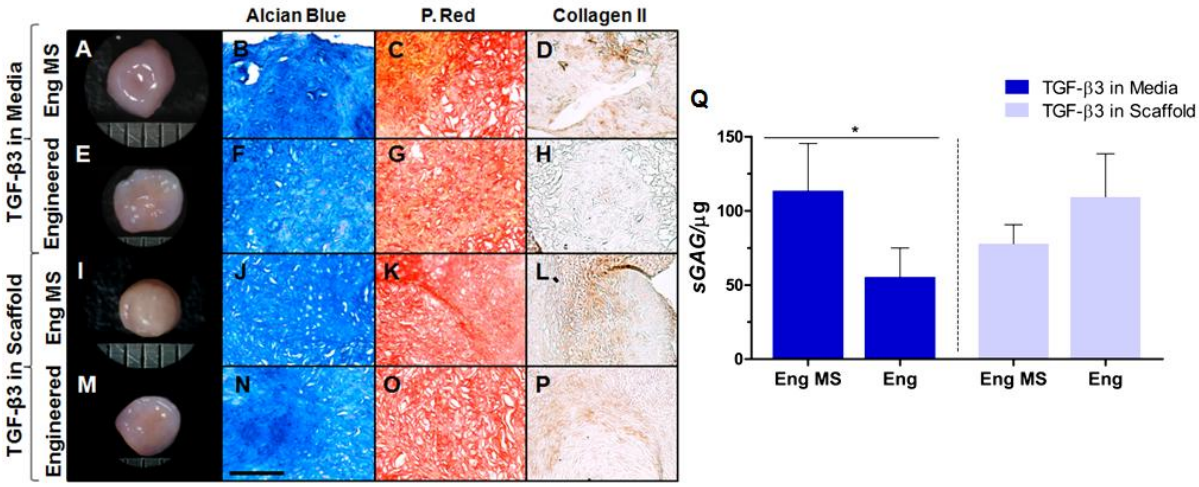


Figure 53 – Macroscopic appearance of Eng-MS (A) and Eng (E) groups (TGF-β3 in media culture), and Eng-MS (I) and Eng (M) groups (TGF-β3 in scaffold). Histological staining for Alcian blue (AB), picro-sirius red (PR) and type II collagen for Eng-MS media (B-D) or scaffold supplemented (J-L), and Eng media (F-H) or engineered scaffold (N-P) TGF-β3 supplemented groups. All micrographs are for 28 days culture period. Scale bar: 50 μm. GAG (Q) content for Eng-MS and Eng groups with media or scaffold TGF-β3 supplementation (n=5; *$p<0.05$).

7.4. Discussion

The purpose of the current study was to assess the potential of using devitalized engineered cartilaginous ECM as a biomaterial to fabricate scaffolds for cartilage tissue engineering. We have previously shown that devitalized native porcine articular cartilage ECM-derived scaffolds are chondro-permissive when seeded with human infrapatellar fat pad stem cells and maintained in the presence of TGF-β3 [341,

351]. Previous studies have reported that devitalized human engineered cartilage can also be used as a base material to fabricate porous scaffold to facilitate chondrogenesis of stem cells [189, 271], although we have shown that such biomaterials can become osteoinductive if the engineered cartilage becomes hypertrophic and begins to mineralise prior to scaffold production [369]. The goal of this study was to directly compare the capacity of native and engineered cartilage ECM-derived scaffolds to support chondrogenesis of human FPSCs [341, 351]. The porosity, architecture and mechanical properties of scaffolds derived from devitalized engineered and native ECM was similar, although differences in scaffold composition were observed. After seeding with human FPSCs, the native ECM-derived scaffolds facilitated the development of a more cartilage like tissue than the engineered ECM. However when TGF-β was directly loaded into the engineered ECM derived scaffolds, they supported the development of cartilaginous tissues with a comparable GAG content and stiffness to that of native ECM-derived scaffolds. Furthermore, the composition of the devitalized engineered cartilaginous ECM used to fabricate the scaffolds could be modulated by incorporating TGF-β eluting microspheres into the cell sheets during culture. Altering the composition of the engineered ECM derived scaffolds in turn influenced the development of the cartilaginous tissues that formed within these constructs. These engineered human ECM derived scaffolds are promising scaffolds for cartilage repair, overcoming limitations associated with the availability and xenogeneic immune reaction to native ECM derived scaffolds [367].

When compared with native ECM, engineered ECM derived scaffolds supported less robust cartilage specific matrix deposition when TGF-β3 was directly added to the media. Both scaffolds had a pore size of approximately 75 μm, and a porosity of >90%, which has previously been shown to support chondrogenesis [341, 351]. Furthermore, the initial mechanical properties were similar for both types of scaffolds. While the porosity and mechanical properties of these scaffolds were similar,

their composition was different. The native cartilage ECM was richer in type II collagen compared to the engineered tissue. Previous studies have shown that hydrogels and scaffolds functionalized with type II collagen support more robust levels of chondrogenesis [176, 178, 179]. Another potential difference between native and engineered ECM scaffolds is the level of remnant growth factors. While engineered ECM derived scaffolds contained higher levels of TGF-β (which is to be expected given that this growth factor was used to engineer the cartilaginous ECM), native cartilage is known to contain numerous different growth factors [38] which may subsequently support chondrogenesis within native ECM derived scaffolds.

Soak-loading engineered ECM derived scaffolds with TGF-β enhanced their capacity to support chondrogenesis of FPSCs, to levels comparable to the native ECM derived scaffolds. This suggests that early, local and homogenous growth factor stimulation is beneficial to supporting chondrogenesis within engineered ECM derived scaffolds. As TGF-β is not permanently bound to the ECM, it allows the growth factor to be released within what may be an optimal dosing window for supporting chondrogenesis [220, 339, 341, 351]. A number of previous studies have also shown that the temporal presentation and then withdrawal of TGF-β is beneficial to stem cell chondrogenesis [340].

As expected, scaffolds derived from devitalized cartilaginous ECM which was engineered in the presence of TGF-β loaded microspheres contained higher levels of remnant growth factor than all other scaffolds. This may explain the superior chondrogenesis observed in these Eng-MS scaffolds when compared with Eng groups, although the higher levels of type II collagen in the devitalized ECM used to fabricate these scaffolds may also be playing a role. When TGF-β is media supplemented, growth factor transport limitations may occur due to slow diffusion through the construct, as well as preferential TGF-β uptake by cells in the scaffold periphery [41, 47]. When TGF-β was directly loaded onto the scaffold prior to cell seeding, the Eng

and Eng-MS ECM derived scaffolds supported similar levels of cartilage specific matrix deposition, which may be due to more uniform local growth factor availability within these scaffolds. Together these results suggest that the specific growth factor stimulation technique will influence the development of cartilaginous constructs within these porous ECM-derived scaffolds.

Further studies need to be performed with the aim of understanding the immune/inflammatory response, and the role of devitalization/decellularization on the capacity of ECM-derived scaffolds to promote chondrogenic differentiation *in vitro* and cartilage regeneration *in vivo*. The engineered human ECM-derived scaffold developed in this study was able to support robust chondrogenesis of FPSCs, especially when loaded with growth factor (TGF-β). Engineered tissue maturity needs to be taken into account, mainly because the degree of development of the ECM present will dictate TGF-β entrapment and availability to the cells [188, 189]. It is relevant to plan future studies where ECM and scaffold composition is assessed, mainly due to the fact that collagen and GAG ratios will influence directly chondrogenesis of mesenchymal stem cells in this context [189, 370, 371]. These findings open the door for the clinical translation of such allogeneic “off-the-shelf” chondroinductive scaffolds for cell based therapies in human cartilage repair.

7.5. Conclusion

In conclusion, this study describes a viable method to engineer human cartilage and use the ECM extracted from this tissue as a biomaterial to fabricate chondro-permissive scaffolds for cartilage regeneration. By seeding these scaffolds with infrapatellar fat pad stem cells and loading the construct with TGF-β3, it was possible to generate robust cartilage like grafts *in vitro*. The use of such engineered ECM-derived scaffolds could overcome the limitations associated with allogeneic and xenogeneic grafts and scaffolds. These engineered ECM-derived scaffolds could

potentially be used as off-the-shelf chondroinductive scaffolds to support cell-based therapies for cartilage regeneration.

The ECM-derived scaffolds proved to be chondro-permissive while releasing TGF-β3, and can potentially be fabricated with a more controllable and viable source of ECM. However, scaffolds may have limitations when implanted in cartilage defects. These include poor fixation in complex defects, and surgical procedures are not entirely minimally invasive. Therefore, as an alternative can be used a hydrogel systems which can overcome such scaffolds limitations. The hydrogel can be injected in a minimally invasive procedure, and also can adapt to the complex-shaped cartilage defects. Hence, the goal of the study presented in Chapter 8, is to develop an injectable ECM-functionalized hydrogel, to be used in a single-stage approach to promote chondrogenesis *in vivo*.

Chapter 8

Fibrin Hydrogel Functionalized with Particulated Cartilage Extracellular Matrix and Incorporating Freshly Isolated Stromal Cells as an Injectable for Cartilage Regeneration

8. Fibrin hydrogels functionalized with particulated cartilage extracellular matrix and incorporating freshly isolated stromal cells as an injectable for cartilage regeneration

Abstract

Freshly isolated stromal cells can potentially be used as an alternative to *in vitro* expanded cells in regenerative medicine. Their use requires the development of bioactive hydrogels or scaffolds which provide an environment to enhance their proliferation and tissue-specific differentiation *in vivo*. The goal of the current study was to develop an injectable fibrin hydrogel functionalized with cartilage ECM micro-particles and transforming growth factor (TGF)-β3 as a putative therapeutic for articular cartilage regeneration. ECM micro-particles were produced by cryomilling and freeze-drying porcine articular cartilage. Up to 2% w/v ECM could be incorporated into fibrin without detrimentally affecting its capacity to form stable hydrogels. Even in the presence of such levels of ECM, chondrogenesis of infrapatellar fat pad (IFP)-derived stem cells within these fibrin constructs was enhanced when additionally stimulated with exogenous TGF-β3. To further access the chondroinductivity of cartilage ECM, we then compared chondrogenesis of IFP-derived stem cells in fibrin hydrogels functionalized with either particulated ECM or control gelatin microspheres. Cartilage ECM particles could be used to control the delivery of TGF-β3 to IFP-derived stem cells within fibrin hydrogels *in vitro*, and furthermore, led to higher levels of sulphated glycosaminoglycan (sGAG) and collagen accumulation compared to control constructs loaded with gelatin microspheres. *In vivo*, freshly isolated stromal cells generated a more cartilage-like tissue within fibrin hydrogels functionalized with cartilage ECM

particles compared to the control gelatin loaded constructs. These tissues stained strongly for type II collagen and contained higher levels of sGAGs. Hence, these results allow new possibilities for single-stage, cell-based therapies for in-theatre cartilage joint regeneration.

Keywords: Articular Cartilage, Single-Stage Therapy, Fibrin Hydrogel, Extracellular Matrix, Growth Factor.

8.1. Introduction

Articular cartilage is an avascular tissue with a complex structure that has a limited capacity for self repair [100]. Regenerating articular cartilage is still is a challenge in the field of tissue engineering [3, 100]. Chondral and osteochondral lesions often result in pain and swelling, followed by further joint degeneration and osteoarthritis [3, 100]. Injuries to cartilage can be treated with a range of approaches, including marrow stimulating techniques, mosaicplasty and cell-based therapies [100]. Autologous chondrocyte implantation (ACI) and matrix-induced ACI (MACI) are examples of currently used techniques in articular cartilage regeneration, which proved to be reasonably successful clinically [100, 113]. However, these procedures require two surgeries and are significantly more expensive than traditional approaches to articular cartilage repair [373].

A number of different single-stage, cell-based procedures have been proposed that would theoretically overcome the need for two surgical procedures and autologous cell expansion [20, 373, 374]. Freshly isolated cells can potentially be obtained from harvested tissue in the surgical room and efficiently implanted in one single procedure [20-22]. Freshly isolated stromal cells from the infrapatellar fat pad (IFP), which we have previously shown to be a viable source of chondro-progenitor cells [337, 341, 344, 351], are a particularly promising cell type for single-stage procedures. In addition to identifying a suitable cell type, the successful realization of such in-theatre procedures also requires the development of a bioactive scaffold or hydrogel able to

promote the proliferation and chondrogenic differentiation of a limited number of multipotent cells, which can be obtained from a donor in one surgical intervention.

Fibrin is a commonly used biomaterial for tissue engineering and is in widespread clinical use, typically as a hemostatic and/or a sealant agent [158]. It has also been investigated as a cell vehicle and as a therapeutic drug delivery system for different tissue engineering applications [52, 158, 160]. In the context of articular cartilage tissue engineering, there is evidence to suggest that fibrin is not as chondro-permissive as other well established hydrogels [110], with bone marrow and adipose-derived stem cells showing a diminished chondrogenic potential when encapsulated in fibrin [110, 159, 160]. There is therefore a clear need for further functionalization of this versatile injectable hydrogel system to optimise its utility for cartilage repair therapies.

Articular cartilage extracellular matrix (ECM)-derived materials have been previously used to engineer cartilage grafts with promising results [36, 38, 341, 351]. In addition, such ECM-derived biomaterials have been used to bind and release chondrogenic factors such as transforming growth factor (TGF)-β3 [341, 351]. Furthermore, ECM particles have also been used to functionalize other biomaterials in an attempt to enhance chondrogenesis [375-377]. For example, fibrin has been combined with ECM particles to develop implants for focal cartilage defect repair [95]. The use of such injectable hydrogels can also overcome limitations associated with pre-formed scaffolds, including challenges associated with fixation to complex cartilage defects and poor retention of newly synthesized ECM [378]. Therefore, such ECM functionalized fibrin hydrogels could potentially be used as an injectable carrier for freshly isolated stromal cells, with such a construct forming the basis of a single-stage therapy for articular cartilage regeneration. Hence, the objective of this study was to functionalize fibrin hydrogels with particulated cartilage ECM, and to assess the capacity of this construct to promote chondrogenesis of freshly isolated stromal cells *in vivo*. As promoting robust chondrogenesis *in vivo* may also necessitate exogenous

growth factor presentation, this study also assessed the capacity of particulated cartilage ECM components to act as a controlled delivery system for TGF-β3 within a fibrin hydrogel. The chondro-inductivity of these cartilage ECM components was then compared to gelatin microspheres. These specific strategies were evaluated both *in vitro* and *in vivo* with the aim of assessing their potential as putative single-stage therapies for cartilage repair.

8.2. Material and methods

8.2.1. Preparation of particulated cartilage ECM

Articular cartilage for the fabrication of ECM particles was obtained from the articular joint of pigs (female, 3 months old), as presented in section 3.1. Briefly, cartilage was fragmented, and further fine ECM micro-particles were fabricated by pulverizing/devitalizing the cartilage ECM in pieces with a cryogenic mill (6770 Freezer-Mill, SPEX). The ECM-derived particles were freeze-dried (FreeZone-Triad, Labconco, USA). Temperature was decreased to -30˚C (1˚C per minute), followed by a drying phase to -10˚C under vacuum (0.2 mbar, 24 hours), and finally to 20˚C. Micro-particles were physically crosslinked and sterilized using dehydrothermal treatment at 110°C under vacuum (1 day). ECM-derived particles were imaged using SEM (section 3.10). Images were analyzed with Image J to quantify particle size.

8.2.2. Fabrication of Fibrin/ECM hydrogels

Fibrin hydrogels were produced using a method previously described [52, 160]. Briefly, fibrin hydrogel constructs were fabricated by dissolving 100 mg/mL bovine fibrinogen (Sigma-Aldrich) in 10,000 KIU/mL aprotinin (Nordic Pharma, Sweden) containing 19 mg/mL sodium chloride (NaCl). Solution of Thrombin (5 U/mL) was made in 40 mM calcium chloride and adjusted to pH 7.0. Moreover, the optimal percentage loading of ECM particles in fibrin constructs was assessed based on literature [376, 379] and additional experimental work. ECM was mixed with fibrinogen

(fibrin) solution in 2% (w/v) and 10% (w/v) based on similar approaches [376, 379]. An additional fibrin only was also prepared to serve as a control group. Fibrin/ECM or fibrin only solutions were mixed at a ratio of 1:1 with a 5 U/mL thrombin in 40 mM $CaCl_2$ solution and allowed to gel at 37°C for 30 minutes yielding a final concentration of 50 mg/mL fibrin, 2.5 U/mL thrombin, 5,000 KIU/mL aprotinin, 17 mg/mL NaCl and 20 mM $CaCl_2$ [52, 160]. The final acellular hydrogels were 60 µl and were produced by using cylindrical agarose moulds (3% w/v; Sigma-Aldrich, Ireland), 5 mm in diameter. Furthermore, these fibrin/ECM hydrogels were scaled up for assessing the scalability of the method. The fibrin only, fibrin/ECM 2% and 10% (w/v) acellular hydrogels were prepared by using a 1.5 ml eppendorf tube as a mould.

8.2.3. Fabrication of Gelatin Microspheres and Fibrin/Gelatin hydrogels

Fibrin/gelatin hydrogels were produced using a previously described protocol [52], using a method similar to fibrin/ECM. However, in this particular case, gelatin microspheres [52, 232, 332] where used and incorporated into the fibrin hydrogel as previously described [52]. Briefly, microspheres were produced by a water-in-oil emulsion method. Gelatin was dissolved in deionised water and added drop-by-drop to 100 ml of olive oil heated to 45°C while being continuously stirred. Gelatin concentration of 11% (w/v) was used in this study. After 10 minutes, the solution was cooled with additional stirring for 30 minutes, after which 40 ml of acetone was added and left for 1 hour. Formed gelatin microspheres were collected through sieving (50 µm) and repeated washings in acetone. Microspheres were next crosslinked in 100 mL of glutaraldehyde solution (0.1% w/v; Sigma-Aldrich, Ireland) with 100 µl Tween 80 (Sigma-Aldrich, Ireland) for 18 hours while being stirred. Then they were removed from the glutaraldehyde solution and stirred in 100 ml of glycine solution (25 mM, Sigma-Aldrich, Ireland) solution for 1 hour. Microspheres were sieved to a controlled range (50–70 µm), which was used for previously reported release studies [52]. Finally,

microspheres were freeze-dried overnight, weighed and sterilized using dehydrothermal treatment.

8.2.4. Cell isolation and culture

Cells were isolated from the infrapatellar fat pad of a patient (male, age 26) undergoing anterior cruciate ligament (ACL) surgery. Cells were isolated and in culture as previously described (section 3.4 and 3.5). For in vitro studies, $1x10^6$ culture expanded human infrapatellar fat pad-derived stem cells were encapsulated to form hydrogel constructs (5 mm diameter and 3mm height). Groups termed “loaded with TGF-β3” were not supplemented with TGF-β3 in chondrogenic media during the culture period. Instead, TGF-β3 (200 ng) was soak-loaded into the ECM and gelatin microspheres (15 minutes), and was not directly added to the culture media.

Histological (section 3.7), immunohistochemical (section 3.8), biochemical (section 3.6) analysis were performed after 28 days in culture as described in general methods section. TGF-β3 quantification analysis was performed in media samples collected during culture period as described in general methods section (section 3.11).

8.2.5. In vivo subcutaneous implantation

For the first *in vivo* assessment groups with fibrin/ECM, with and without TGF-β3, seeded with freshly isolated infrapatellar fat pad-derived human stromal cells were implanted subcutaneously in nude mice to assess effect of growth factor loading into constructs. Furthermore, after TGF-β3 loading viability assessment, additional fibrin hydrogels were loaded with TGF-β3 (200 ng) and the following groups were implanted: 1. Acellular Fibrin/Gelatin; 2. Stromal cells in Fibrin/Gelatin; 3. Acellular Fibrin/ECM; 4. Stromal cells in Fibrin/ECM. Stromal cells were freshly isolated (not culture expanded) from the infrapatellar fat pad of patients undergoing total joint arthroplasty. Within 24 hours of cell isolation, constructs were implanted subcutaneously into the back of nude mice (Female, 6 weeks old, Balb/c; Harlan), with n=9 per group, as previously

mentioned (section 3.12). Histological (section 3.7), immunohistochemical (section 3.8), biochemical (section 3.6) analysis were performed after 28 days *in vivo* as described in general methods section.

8.3. Results

8.3.1. Development of stable ECM functionalized fibrin hydrogels

Scanning electron microscopy (SEM) was used to characterize the morphology and size distribution of the particulated ECM (Figure 54 A-C), with the mean particle size after cryomilling measured as 97±26 µm. Fibrin was then mixed with particulated ECM material at two different concentrations. To assess if the incorporation of different concentrations of ECM would impact the capacity of fibrin to form a stable gel, the components were added to a cylindrical mould (Figure 54 D-F) and an eppendorf tube (Figure 54 G, H) and their capacity to maintain their shape was determined. The ECM free fibrin hydrogel appeared semi-transparent (Figure 54 D) and with the addition of ECM became more opaque (Figure 54 E, F). The capacity of the hydrogels to maintain a fixed shape also diminished with the incorporation of higher concentrations of ECM, as it was possible to observe a stable structure for fibrin only (data not shown) and fibrin/ECM 2% w/v (Figure 54 G), however when the ECM concentration was increased (10% w/v) the structure failed to maintain its initial shape (Figure 54 H). This motivated the use of 2% w/v ECM embedded in fibrin hydrogel for further assessment.

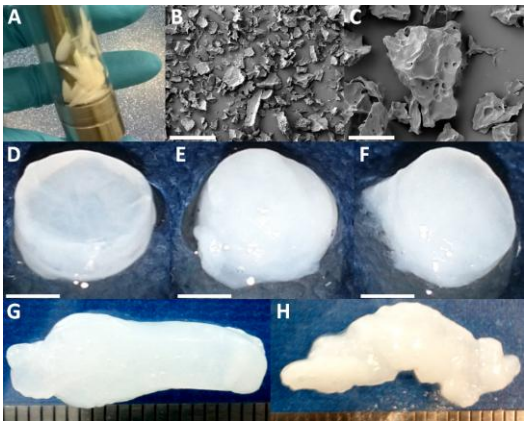


Figure 54 – Cartilage before cryomilling (A). SEM micrograph of the pulverized ECM (B) and high magnification for cartilage particles (C) (scale bar: 500 µm (B) and 100 µm (C)). Cylindrical-shaped hydrogel macroscopic outline: fibrin only (D), fibrin/ECM 2% w/v (E) and fibrin/ ECM 10% w/v (F) (scale bar: 2 mm). Alternative scaled-up design for fibrin/ECM 2% w/v (G) and fibrin/ECM 10% w/v. (H).

8.3.2. Fibrin hydrogels functionalized with particulated cartilage ECM support robust chondrogenesis when stimulated with endogenous TGF- β3

We next sought to determine the capacity of fibrin hydrogels functionalized with cartilage ECM to support chondrogenesis of infrapatellar fat pad-derived stem cells *in vitro* in the presence and absence of endogenous TGF-β3. After 28 days of *in vitro* culture, fibrin-ECM composites supplemented with TGF-β3 resembled cartilage macroscopically (Figure 55E). When compared with growth factor free constructs (Figure 55A-D), the TGF-β3 supplemented hydrogels (Figure 55E-H) contained higher levels of cartilage specific matrix components. Histological staining for sGAG (Figure 55F), collagen (Figure 55G) and type II collagen (Figure 55H) was more intense for TGF-β3 supplemented constructs. The DNA, sGAG and collagen content of TGF-β3 supplemented hydrogels was significantly higher than non-supplemented constructs (Figure 55I-K).

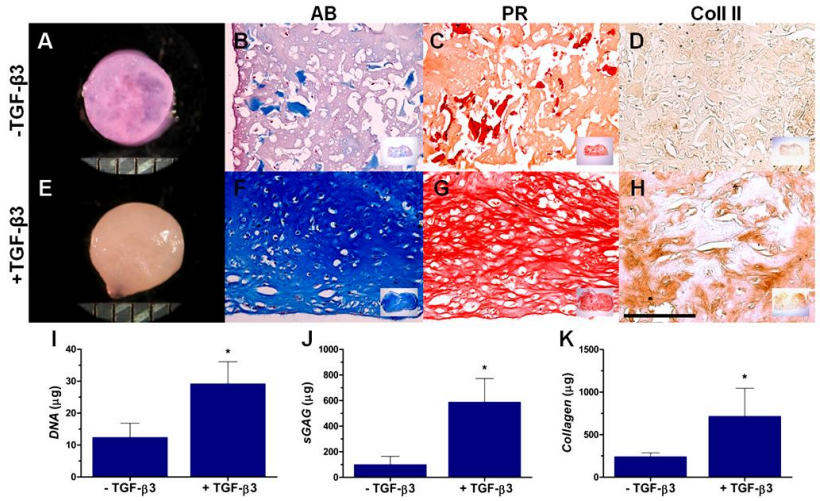


Figure 55 – Macroscopic view of fibrin/ ECM (A) and fibrin/ECM with TGF-β3 (E) hydrogel. Alcian blue (AB), picro-sirius red (PR) and type II collagen (Coll II) staining for fibrin/ ECM (B-D) and fibrin/ECM with TGF-β3 hydrogel (F-H). DNA (I), sGAG (J) and collagen (K) accumulation within fibrin/ECM (-TGF-β3) and fibrin/ECM with TGF-β3 (+TGF-β3) hydrogel seeded with human fat pad-derived stem cells (n=4, *p<0.05). All the data corresponds to 28 days in vitro culture. Scale bar: 50 µm.

8.3.3. ECM-derived particles can be used to deliver TGF-β3 and enhance chondrogenesis of human infrapatellar fat pad-derived stem cells

Having demonstrated superior chondrogenesis in the presence of TGF-β3, and with a view towards developing an off-the-shelf therapeutic for cartilage regeneration, we next sought to assess the potential of particulated cartilage ECM to bind and release TGF-β3 within a stem cell laden hydrogel, and to subsequently support chondrogenesis over 28 days of *in vitro* culture. Furthermore, we sought to determine if the incorporation of ECM into fibrin would enhance chondrogenesis compared to that within control fibrin hydrogels containing gelatin microspheres. After loading the ECM-derived particles with TGF-β3 it was possible to observe a progressive release of the growth factor into the surrounding culture (Figure 56), comparable to that obtained using gelatin microspheres embedded in the same stem cell-laden fibrin hydrogel (Figure 56).

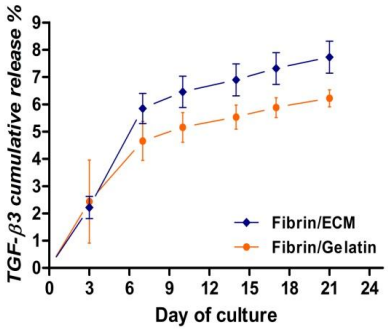


Figure 56 –TGF-β3 release into the media from the fibrin/ECM with TGF-β3 and fibrin/Gelatin with TGF-β3 hydrogels, as measured by ELISA (n=3). Cumulative release values are presented as a percentage of the initial amount of TGF- β3 loaded into the scaffold.

At the end of the 28 day *in vitro* culture period, both types of constructs resembled cartilage macroscopically (Figure 57A and E). Histologically, the tissue generated with the ECM functionalized hydrogels was richer in sGAGs and type II collagen (Figure 57B-D). In addition, significantly higher levels of sGAG (Figure 57J) and collagen (Figure 57K) accumulation were observed with ECM functionalized constructs. No significant difference in DNA content was observed between the two groups (Figure 57 I).

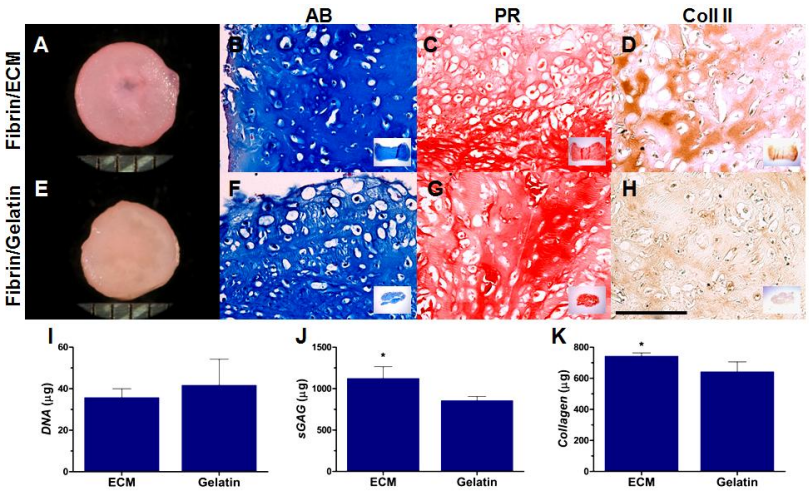


Figure 57 – Macroscopic view of fibrin/ECM with TGF-β3 (A) and fibrin/Gelatin with TGF-β3 (E) hydrogel. Alcian blue (AB), picro-sirius red (PR) and type II collagen (Coll II) staining for fibrin/ECM with TGF-β3 (B-D) and fibrin/Gelatin with TGF-β3 hydrogel (F-H). DNA (I), sGAG (J) and collagen (K) accumulation within fibrin/ECM with TGF-β3 and fibrin/Gelatin with TGF-β3 hydrogel seeded with human fat pad-derived stem cells (n=4, *p<0.05). All the data corresponds to 28 days in vitro culture. Scale bar: 50 µm.

8.3.4. Cartilage ECM enhances chondrogenesis in vivo in the presence of TGF-β3

It was pertinent to determine the potential of fibrin hydrogels functionalized with particulated cartilage ECM hydrogels to promote chondrogenesis *in vivo* in a subcutaneous nude mouse model when seeded with freshly isolated infrapatellar fat pad-derived stromal cells. The particulated ECM was either loaded with TGF-β3 (+TGF-β3) or left empty (-TGF-β3) prior to implantation. After 4 weeks *in vivo* the Fibrin-ECM constructs loaded with TGF-β3 generated more robust chondrogenesis of human infrapatellar fat pad stromal cells, with superior sGAG (Figure 58F), collagen (Figure 58G) and type II collagen (Figure 58H) deposition compared to the non-supplemented group (Figure 58B-D).

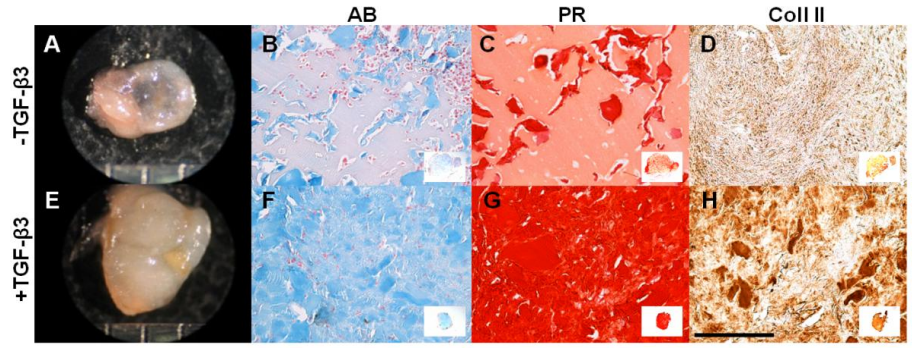


Figure 58 – Macroscopic view of fibrin/ECM, with (E) and without (A) TGF-β3. Alcian blue (AB), picro-sirius red (PR) and type II collagen (Coll II) staining for fibrin/ECM, with (F-H) and without (B-D) TGF-β3. All data corresponds to fibrin based hydrogels implanted for 28 days *in vivo*. Scale bar: 50 µm.

The final goal of the study was to determine whether particulated cartilage ECM is chondroinductive *in vivo*. To this end, the capacity of fibrin hydrogels functionalized with either gelatin microspheres or particulated cartilage ECM to promote ectopic cartilage formation was compared. Both the gelatin and cartilage ECM particles were loaded with TGF-β3 and the constructs were either implanted cell free or with freshly isolated stromal cells. Little matrix appeared to accumulate within acellular constructs after 4 weeks *in vivo* (Figure 59). Macroscopically, ECM constructs seeded with freshly

isolated stromal cells appeared cartilage-like in appearance (Figure 59M), unlike the other fibrin based groups. More intense staining for sGAG (Figure 59N), collagen (Figure 59O) and type II collagen (Figure 59P) deposition was observed in fibrin-ECM constructs when compared with the fibrin-gelatin constructs. sGAG synthesis in the fibrin-ECM implants was significantly higher than in the fibrin-gelatin constructs (Figure 59Q).

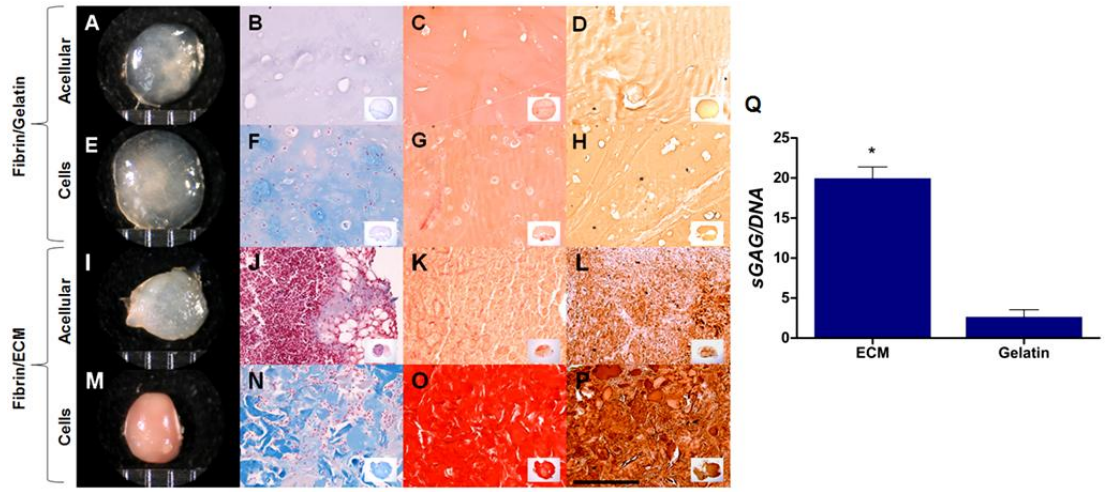


Figure 59 – Macroscopic view of acellular fibrin/gelatin (A), acellular fibrin/ECM (I), fibrin/gelatin seeded with freshly isolated stromal cells (E) and fibrin/ECM seeded with freshly isolated stromal cells (M). All constructs were loaded with TGF-β3 prior to implantation. Alcian blue (AB), picro-sirius red (PR) and type II collagen (Coll II) staining for acellular (B-D) and cell-laden (F-H) fibrin/gelatin constructs and acellular (J-L) and cell-laden (N-P) fibrin/ECM constructs. sGAG/DNA accumulation within cell-laden fibrin/ECM constructs compared to fibrin/gelatin constructs (Q) (n=6, *p<0.05). All data corresponds to constructs implanted for 28 days in vivo. Scale bar: 50 µm.

8.4. Discussion

The goal for this study was to develop an injectable, single-stage approach to promote chondrogenesis *in vivo*. The method consists of functionalizing a well-established biomaterial (fibrin) with particulated cartilage ECM loaded with TGF-β3, and using this construct to promote chondrogenesis of freshly IFP-derived stromal cells. By cryomilling and freeze-drying the ECM it was possible to produce micro-particles that were easily incorporated into fibrin hydrogels at a known concentration.

In spite of the fact that these particles are potentially chondroinductive on their own, due to the sGAGs, collagen and innate chondrogenic cues within the ECM, additional TGF-β3 stimulation was necessary to induce robust chondrogenesis both *in vitro* and *in vivo*. *In vitro*, ECM-derived particles were able to retain and prolong the release of TGF-β3, proving to be as effective in this regard as the well established gelatin microsphere delivery system [52]. Fibrin hydrogels functionalized with particulated cartilage ECM promoted superior chondrogenesis *in vitro* and *in vivo* when compared with fibrin-gelatin constructs. These findings open up the possibility of using ECM functionalized fibrin hydrogels, in combination with freshly isolated stromal cells, to regenerate articular cartilage defects.

Cryomilling and freeze-drying of cartilage ECM was found to result in the development of particulated tissue with a consistent size and morphology, with comparable strategies being employed in the literature [380]. While we demonstrated that the specific size of ECM particles used in this study enhanced chondrogenesis *in vitro* and *in vivo*, further studies need to be performed to understand the role of ECM particle size in promoting chondrogenesis using this approach. Previous studies have demonstrated that the particle size of powdered ECM material can influence new tissue formation, regardless of initial biomaterial composition [345]. Furthermore, particle size will also determine the effectiveness of devitalization and decellularization treatments that will likely influence the immune response [39]. Additional studies need to be undertaken to better understand the immunogenicity of cartilage ECM-derived materials, and consequently understand and optimise production and decellularization methods to enable clinical translation of such powerful naturally-derived biomaterials [39].

In agreement with previous studies [341, 351], additional growth factor (TGF-β3) stimulation was necessary to induce robust chondrogenesis of IFP-derived stem cells, even in the presence of particulated cartilage ECM. This motivated us to explore

the use of cartilage ECM particles as a growth factor delivery system. These ECM particles were comparable to traditional gelatin microspheres in their capacity to bind and release TGF-β3. It is well known that ECM components can act as reservoirs for efficient growth factor release [74, 75, 341, 351]. Proteoglycans (negatively charged) present in the pericellular and extracellular matrix have been shown to bind and modulate TGF-β3 (positively charged) supply and consequently control its availability [75].

While the amount and rate of TGF-β3 release from cartilage ECM was similar to that from gelatin microspheres, superior chondrogenesis was observed in the ECM-functionalized hydrogels. This difference indicates that the ECM-derived particles are able to provide additional chondroinductive cues to the IFP-derived stem cells that are not present in the fibrin/gelatin hydrogels, strongly suggesting that these bioactive ECM micro-particles are more than simple growth factor carriers. The presence of growth factors in cartilage ECM, particularly from the TGF-β family, may explain the superior chondrogenesis observed in cartilage ECM micro-particles group [38, 39, 266, 292, 352]. Moreover, these growth factors can bind and be stored in a panoply of molecules present in native cartilage ECM [266]. Additionally, the presence of type II collagen in cartilage ECM is known to be advantageous for chondrogenesis [176-179], which may explain the superior results observed in the cartilage ECM-functionalized biomaterials.

Growth factor release from the hydrogel to the media was less than 10% of the initial amount loaded into the micro-particles. Fibrin by itself has been used previously as a growth factor delivery system [52, 158], and hence it is likely helping to retain the TGF-β3 within the construct. Results from ELISA indicate that the release of growth factor from particles occurs at a relatively slow rate throughout the culture period, except for a more rapid release profile during the first 8 days. It is not clear what percentage of growth factor that remains within the construct is available to seeded

cells. Regardless, this specific release profile is sufficient to initiate the chondrogenic pathway. Temporal growth factor exposure is known to be beneficial in chondrogenesis [220, 339-341, 351]. Further studies are necessary to identify the optimal amount of TGF-β3 that needs to be added to the delivery device in order to promote robust chondrogenesis of embedded cells. Previous studies highlight that low levels of growth factor may need to be added to ECM-based hydrogels or scaffolds to enhance cartilage formation [39, 292, 295, 341, 351, 381].

ECM-derived materials have been used previously to deliver growth factors such as TGF-β3 [341, 351]. In the present study, the same principle was assessed in a subcutaneous nude mouse model, where the functionalized fibrin was the vehicle to deliver freshly isolated IFP-derived stromal cells and growth factor. The devitalized cartilage ECM-derived particles facilitated robust chondrogenesis of the encapsulated stromal cells. These stromal cells were critical to generate cartilage ectopically, as acellular fibrin/ECM complexes did not induce chondrogenesis. When compared with the well established gelatin microspheres system, the ECM particles induced superior cartilage matrix formation, emphasizing the important role and potential of such particles *in vivo*.

Finally, it is relevant to affirm that further studies need to be performed to assess immune response, alternative methods of devitalization and decellularization, and the capacity of the proposed therapy to regenerate cartilage defects in large animal models. From a translation perspective, the proposed strategy of freshly isolating fat pad stromal cells and embedding them in an ECM functionalized fibrin hydrogel seems promising, as it may be used as a minimally invasive approach for single-stage cartilage repair. The strategy of cryomilling and devitalizing native ECM to produce bioactive particles, and incorporating them in a well-established fibrin hydrogel with stromal cells and/or growth factors, has multiple potential applications for tissue regeneration.

8.5. Conclusion

The objective of this study was to functionalize an injectable fibrin hydrogel with a view to developing a single-stage therapy for cartilage repair. We demonstrate that ECM-derived micro-particles can deliver TGF-β3 and that this system can induce chondrogenesis of freshly isolated fat pad-derived stromal cells *in vivo*. This finding supports the concept that populations of freshly isolated stromal cells, when combined with a chondroinductive hydrogel, can induce cartilage formation and can potentially be used in single-stage, minimally invasive procedures for cartilage regeneration. The translation of such a strategy would overcome many of the current limitations associated with clinically available cell-based therapies for cartilage repair.

It is documented that ECM components, such as type II collagen, are pro-chondrogenic, however, when used to fabricate scaffolds on their own they hamper the formation of a mechanical robust construct immediately after cell seeding. Hence, these ECM components can be used in conjunction with more mechanically robust biomaterials such as covalently crosslinked alginate. Therefore, the overall goal of the study described in Chapter 9 is to develop a biomimetic, shape-memory alginate scaffold for cartilage tissue engineering applications.

Chapter 9

Anisotropic Shape-memory Alginate Scaffolds Functionalized with Type II Collagen for Cartilage Tissue Engineering

9. Anisotropic shape-memory alginate scaffolds functionalized with type II collagen for cartilage tissue engineering

Abstract

Regenerating articular cartilage is still is a challenge in orthopaedic medicine. While a range of different scaffolds have been developed for joint repair, none have facilitated the development of a tissue that mimics the complexity of normal articular cartilage. Furthermore, many of these scaffolds are not designed to function in mechanically challenging joint environments. The overall goal of this study was to develop a porous, biomimetic, shape-memory alginate scaffold for directing articular cartilage regeneration. To this end, a scaffold was designed with architectural cues to guide cellular and neo-tissue alignment, and was additionally functionalized with a range of extracellular matrix (ECM) cues to direct stem cell differentiation towards the chondrogenic lineage. Shape-memory properties were introduced by covalent crosslinking, while the architecture of the scaffold was modified using a directional freezing technique. Introducing such an aligned porous structure was found to improve the mechanical properties of the scaffold whilst maintaining its shape-memory capacity. Furthermore, this anisotropic pore architecture directed cellular alignment and lead to higher levels of sGAG and collagen deposition within the scaffold compared to scaffolds with isotropic (non-aligned) pore geometry. Functionalization with collagen improved stem cell recruitment into the scaffold and facilitated more homogenous cartilage tissue deposition throughout the construct. Incorporating type II collagen into the scaffolds lead to greater cell proliferation, higher sGAG and collagen accumulation and the development of a stiffer tissue compared to scaffolds functionalized with type I collagen. The results of this study demonstrate how both

scaffold architecture and composition can be tailored in a shape-memory alginate scaffold to direct stem cell differentiation and support the development of a complex tissue such as articular cartilage.

Keywords: Shape-memory, Articular Cartilage, Collagen, Alginate, Scaffold, Anisotropy.

9.1. Introduction

Articular cartilage it is a highly specialised anisotropic tissue that functions to support joint contact forces and to reduce friction within the joint [85, 382]. Regenerating this complex tissue is still a challenge in the field of orthopaedic medicine, which is due, in part, to its avascular nature [3, 100]. Chondral and osteochondral lesions often result in pain and swelling, followed by further joint degeneration and osteoarthritis [3, 100]. Injuries to cartilage can be treated with a range of approaches, including marrow stimulating techniques [383], mosaicplasty [264] and cell-based therapies [100, 253] such as autologous chondrocyte implantation (ACI) [384] or matrix-induced autologous chondrocyte implantation (MACI) [385]. An alternative to the use of differentiated cells (e.g. chondrocytes) are stem cells, which can be delivered *in vitro* or *in vivo* using a biomaterial-based scaffold [108]. This approach uses the scaffold as carrier, stem cells to generate the newly formed tissue, and other chondrogenic factors (e.g. growth factors) with the aim of cartilage regeneration [100]. However, novel scaffolds are required that are not only mechanically robust and compatible with the load bearing environment of a synovial joint, but which can also direct progenitor cells down chondrogenic pathway and promote the development of an organized tissue.

Porous scaffolds for cartilage tissue engineering can be fabricated using different raw-materials and manufacturing techniques [26, 27, 108, 386]. Ideally these biomaterials are instructive to cells that are seeded or recruited into the scaffold [108, 387]. Extracellular matrix (ECM) [38, 39] and its derivatives such as collagen [350,

388] and hyaluronic acid [355], as well as other natural polymers such as chitosan [178, 179] and alginate [169, 170, 174, 389], are commonly used as biomaterials in cartilage tissue engineering. Furthermore, inert biomaterials can be functionalised to promote chondrogenesis through the incorporation of cartilage specific ECM components such type II collagen [176-179]. Alginate is a relatively inert polysaccharide that is commonly used as a biomaterial in the field of tissue engineering [108, 174]. Highly porous shape-memory scaffolds can be produced by covalently crosslinking alginate using carbodiimide chemistry [169, 170, 307]. Such scaffolds can be compressible 11-fold and return to their original shape when rehydrated [307]. Cellular infiltration into such scaffolds can be improved through the incorporation of different ECM components, such as fribronectin [175] or collagen [170]. The unique mechanical properties of these shape-memory alginate scaffolds make them highly attractive for articular cartilage tissue engineering, however they will first need to be optimised for chondrogenesis.

It is known that tissue architecture plays a key role in native tissue function and in its mechanical performance [26]. Musculoskeletal tissues are highly anisotropic, with a composition and structure optimized to enable performance in high load environments [85, 382]. The structure of native tissues has inspired scaffold designs with aligned pore geometries to direct cell and neo-tissue alignment [312, 390-392]. It is possible to produce aligned scaffolds by using different methods such as ionotropic gelation [175, 393], 3D bioprinting [394], electro-spinning [395, 396] and directional freezing [312, 390]. Using a directional freezing method, pore alignment can be modulated by changing the angle of the freezing surface, which in turn determines the direction of ice crystal growth [390]. Such unidirectional technique can enable the fabrication of biomaterial-based scaffolds with an anisotropic architecture and enhanced mechanical behaviour [312, 390, 397, 398].

The overall goal of this study was to develop a porous, biomimetic, shape-memory alginate scaffold for directing articular cartilage regeneration. In order to explore how scaffold pore geometry can influence cellular recruitment and direct cellular alignment, scaffold with both isotropic (and non-aligned) and anisotropic (or aligned) pore geometries were produced using freeze-drying only and unidirectional freezing followed by freeze-drying techniques. Furthermore, these scaffolds were functionalised with the addition of either type I or type II collagen (coating or blending). The chondro-inductivity of these scaffolds was then assessed *in vitro* over a 4 weeks culture period following seeding with human infrapatellar fat pad-derived stem cells.

9.2. Material and methods

9.2.1. Fabrication of alginate scaffolds without alignment (Non-Al)

Non-aligned alginate scaffolds (Non-Al) were fabricated using a protocol that was previously reported [169, 170]. Briefly, the biopolymer used to fabricate the scaffold was sodium alginate (Pronova UP LVG; Novamatrix, Sandvika, Norway). This polysaccharide was covalently crosslinked using carbodiimide chemistry [169, 170]. In brief, the polymer was dissolved in 2-(N-Morpholino)ethanesulfonic acid buffer (MES; pH 6.0 at 0.1 M) with NaCl (0.2 M), to a final concentration of 3.3% (w/v). Solutions of N-Hydroxysuccinimide (NHS) and 1-ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC) were homogenised with alginate solution (5 minutes) with a final molar ratio of 2:1:2 for EDAC:NHS:COO^-. Furthermore, a crosslinking agent, adipic acid dihydrazide (AAD), was added to the solution (molar ratio of 45% compared to alginate n_{NH2}/n_{COOH}), quickly homogenized and transferred into a mould. The reaction was performed overnight at room temperature. Finally, the alginate hydrogel was washed in ultrapure water (UPW) for 24 hours with several water cleaning bath changes, with the objective of removing remnant chemical contaminants. After the washing step, cylinders (5mm in diameter and 3mm in height) of the hydrogel were cored out from the main hydrogel

slab using a biopsy punch (5 mm). To create a porous scaffold, a freeze-drying process was used, as previously described [169, 170, 351]. Briefly, the hydrogels were placed in a Petri dish on the cooling shelf of the freeze-dryer (Labconco Triad^{TM}, Kansas City, MO, USA) and frozen to -30°C (1°C min^{-1}) and maintained at that temperature for 1 hour. The temperature was then increased under vacuum (0.2 mBar) to -10 °C (1°C min^{-1}) and held for 24 hours, before being increased to room temperature (0.5°C min^{-1}). All chemicals used were obtained from Sigma-Aldrich.

9.2.2. Fabrication of alginate scaffolds with aligned pores (Align)

The aligned alginate scaffolds (Align) were fabricated using a modified version of a previously described unidirectional freezing technique [312, 390, 391]. The covalently crosslinked alginate hydrogel (3.3% w/v) described above was maintained in UPW after several washes. A 20 mm thick stainless steel plate was cooled in liquid nitrogen, and promptly the cylindrical alginate hydrogels were dropped vertically onto the cold flat metal surface. Moreover, without letting the ice crystals thaw, the frozen blocks were transferred quickly with tweezers into a freeze-dryer set at -30°C. The freeze-drying process was the same as that used for Non-Al scaffolds.

9.2.3. Collagen functionalization of alginate scaffolds

In the current study only the Align scaffolds were functionalized with type I and II collagen. The type I collagen was obtained from a commercial source (extracted from rat tail, BD Biosciences, Oxford, United Kingdom). Type II collagen (collagen content of 95%±4%) was isolated from bovine knees and hocks using a previously reported protocol [399-401]. Briefly, bovine joints were obtained from a local butcher and were washed thoroughly with 70% alcohol. Cartilage slices were removed from the joints using a scalpel. Subsequently, they were minced into small pieces (1-2 mm). 1 ml of sterile filtered NaOH (0.2M) was added to each 50 mg of wet tissue and rotated for 24 hours (4°C). The suspension was centrifuged (2500 g; 10 min; 4°C) and the

supernatant discarded (repeated 3 times). Pellet was then re-suspended in 1 ml of activated pepsin solution for each 50 mg of original wet tissue. With the aim of dissolving the pellet, the suspension was homogenized vigorously. The solution was then incubated on a rotating platform for 24 hours (20°C; 4 rpm). The solution was centrifuged (2800g; 1hr; 4°C) and the supernatant transferred to a tube. Sterile filtered NaCl (5M) was slowly added into the tube in order to get a final concentration of 0.9M NaCl. The solution was homogenized and allowed to equilibrate overnight (4°C). Thereafter, the mixture was centrifuged, the supernatant discarded and the pellet washed with UPW. Collagen was re-suspended in sterile filtered acetic acid (10 ml; 0.5M) and allowed to dissolve overnight (T<20°C; 4 rpm). Acidic solubilized collagen was transferred to a previously prepared dialysis membrane (MWCO; 10 kDa). The solution was dialyzed with mild rotation against 0.02 M Na2HPO4 (pH 9.4) for 24 hours at 4°C. The dialysate was removed after 12 hours and it was added to fresh dialyzing solution. The dialyzed collagen solution was freeze-dried and stored in a freezer at -20°C degrees until use. All chemicals used were obtained from Sigma-Aldrich.

Scaffolds were functionalised by either coating the scaffold with collagen, or blending collagen into the alginate prior to scaffold production. For coated scaffolds functionalized with type I (C1C) and type II (C2C) collagen, collagen solutions were prepared (0.05% w/v) in 0.5M acetic acid. After freeze-drying the alginate, 100 µl of the collagen solution was soak-loaded into the dry porous scaffolds (1 hour; RT). The coated scaffold was then freeze-dried a second time using the same protocol. For the blended scaffolds, type I (C1B) and II (C2B) collagen was mixed with the initial alginate solution at a concentration of 0.05% (w/v) of collagen in the total volume. Align scaffolds were additionally coated and blended with chondroitin sulfate as an additional control for functionalization (Appendix 1). The subsequent freeze-drying steps were the same as that for the non-functionalized Align scaffold.

9.2.4. Assessment of mechanical properties

Scaffolds were mechanically tested as previously described using a standard materials testing machine with a 5 N load cell (Zwick Z005, Roell, Germany) [55, 170, 351]. For day 0 and day 28, a preload of 0.01 N was applied to ensure direct contact between the scaffold and the loading platens. Stress relaxation tests were performed, in which a ramp and hold cycle (displacement of 1 mm/s until 10% strain) was obtained and maintained until equilibrium was reached. The compressive equilibrium modulus was determined by using the stress determined at equilibrium divided by the applied strain (10%). To assess shape-memory property of alginate scaffold, after collagen functionalization at day 0, the same compressive test with 10% strain was applied to samples. However, in this case the mechanical assessment was performed before and after fifty compressive cycles of 10% strain. All compressive tests were unconfined and maintained in PBS. Finally, for a macroscopic assessment of the mechanical properties of the alginate scaffold, tweezers was used to compress all the scaffold groups. A macrograph was taken before and after the mechanical stimulus to assess consequences in scaffold shape after compressive conditioning.

9.2.5. SEM imaging of scaffolds

Alginate scaffolds were imaged using scanning electron microscopy (SEM) as previously presented [170]. Briefly, scaffolds before SEM observation were fixed in paraformaldehyde solution (4%) overnight and followed by several phosphate buffed saline (PBS) washings. Scaffolds were dehydrated through successive graded ethanol baths (10-100%), fixed onto aluminium stubs, coated with gold and examined under a field emission SEM (Tescan Mira FEG-SEM XMU, Libušina, Czech Republic). Images were analyzed with Image J to quantify mean pore size.

9.2.6. Cell isolation and culture

For the use of human cells it was necessary an ethical approval for the isolation of human infrapatellar fat pad (IFP) stromal cells, which was obtained from the institutional review board of the Mater Misericordiae University Hospital, Dublin, Ireland. Stromal cells were obtained from an infrapatellar fat pad of a healthy (non-osteoarthritic) patient undergoing anterior cruciate ligament surgery. Stem cells used were harvested and cultured with the general method previously mentioned (section 3.4). For *in vitro* chondrogenic studies, the before mentioned general protocol was used (section 3.5).

9.2.7. Biochemical analysis

Alginate constructs were biochemically analyzed at day 28, for DNA, sulphated glycosaminoglycans (sGAG) and collagen content, as previously described in general methods section (section 3.6) after 28 days in culture.

9.2.8. Histology and immunohistochemistry

Histological (section 3.7) and immunohistochemical (section 3.8) analysis were performed after 28 days in culture as described in general methods section. Day 0 acellular Non-AL and Align sections were stained with 1% alcian blue 8GX (Sigma-Aldrich) in HCl (0.1 M) for sGAG to examine scaffold structure and material distribution. With the aim of monitoring cell colonization and the newly formed matrix, constructs were histologically analysed by staining with aldehyde fuchsin (AF) and haematoxylin and eosin (H&E) at day 0 and 28, as previously described [169]. Immunohistochemical analysis was performed on 6 µm sections using monoclonal antibody to type II collagen (Abcam, UK) as previously described (section 3.8) [53, 169, 170, 341].

9.2.9. Cell Viability and Actin/DAPI staining

Cell survival and distribution were assessed using live/dead staining. Calcein was used to stain live cells. Constructs images were taken using confocal microscopy for day 1 and 10 of culture, as previously described [170, 351]. Briefly, cell viability was assessed by LIVE/DEAD kit (Invitrogen, Bio-science, Ireland). Constructs were washed in PBS, sectioned in half, incubated in calcein ($2x10^{-6}$M) (live/green). Moreover, constructs were washed and imaged using confocal microscopy 10x Olympus FV-1000 Point-Scanning Microscope (Southend-on-Sea, UK) at 515 and 615 nm channels and analyzed using FV10-ASW 2.0 Viewer. For actin staining, as previously described [170], scaffold cross-sectional slices were in incubation with fluorescent agent rhodamine-conjugated phalloidin (dilution 1:40, Biotium, Hayward, USA) combined with Hoechst (4',6-diamidino-2-phenylindole (DAPI), dilution 1:50, VWR, Ireland) in order to identify cell morphology and observe in red F-actin filaments of the cytoskeleton, and in blue the nucleus of the cell.

9.3. Results

9.3.1. Development of a shape-memory alginate scaffold with aligned pores

Shape-memory alginate scaffolds with both a non-aligned (Non-Al) and an aligned (Align) pore structure was produced by freeze-drying covalently crosslinked alginate hydrogels. Scanning electron microscopy (SEM) was used to characterize the morphology and size distribution of the channels/pores in both the Non-Al and Align scaffolds (Figure 60). There was no obvious preferred pore alignment in the Non-Al scaffolds (Figure 60A-C). Conversely, aligned honeycomb-like pore architecture was observed in the Align scaffolds (Figure 60D-F). These differences were also observed upon histological evaluation of the scaffolds (Figure 60H). The mean pore diameter for the Non-Al scaffolds was 176±70 μm, while the mean pore/channel diameter for the

Align scaffold was 32±12 µm. Acellular Align scaffolds were stiffer than the Non-Al group (Figure 60J).

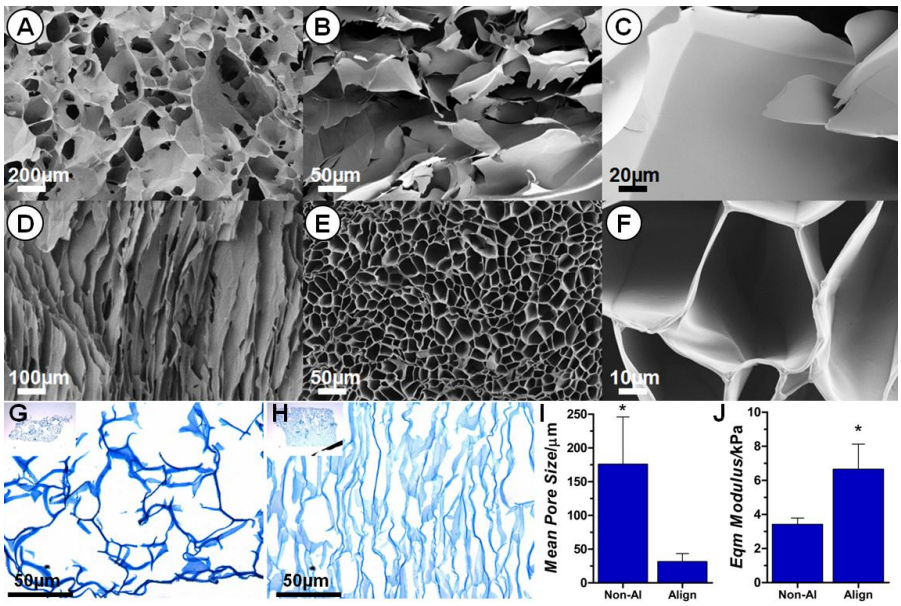


Figure 60 – Scanning electron microscopy (SEM) micrographs for alginate non-aligned (Non-Al; A-C) and alginate aligned (Align; D-F) acellular scaffolds. Alcian blue staining for Non-Al (G) and Align (H) before cell culture. Mean pore size (I) and equilibrium modulus (J) comparison between Non-Al and Align acellular scaffolds (n=3; *p<0.05).

Both covalently crosslinked scaffolds (Non-Al and Align) possessed shape-memory characteristics. When mechanically compressed, both scaffold groups retained their original shape on unloading (Figure 61). While a collagen-based ECM-derived scaffold control did not maintained its original shape after compression in the same conditions (Figure 61G-I). Furthermore, no reduction in mechanical properties was observed after fifty cycles of mechanical compression (data not shown).This assessment was performed in acellular scaffolds while hydrated.

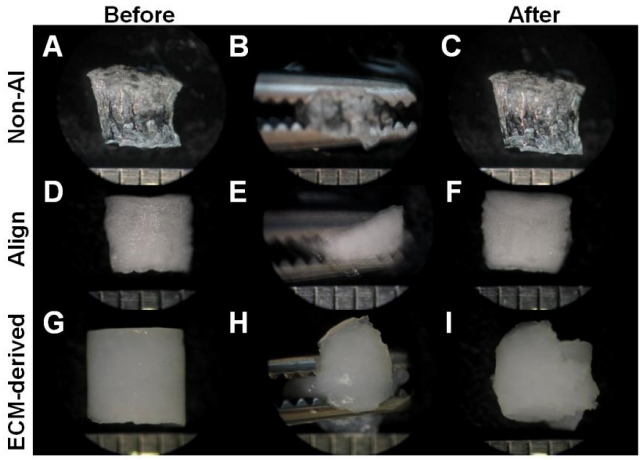


Figure 61 – Macrographs of acellular scaffold alginate groups Non-Al (A, B and C), Align (D, E and F) and a collagen-based ECM-derived scaffold control, before and after mechanical compression and culture period.

9.3.2. Scaffold pore directionality determines stem cell alignment and the extent of extracellular matrix deposition

Both the isotropic (Non-Al) and the anisotropic (Align) scaffolds were then seeded with FPSCs and maintained for up to 28 days in chondrogenic culture conditions. Actin/DAPI (Figure 62) staining was used to assess stem cell alignment in Non-Al and Align scaffolds at day 1, and after ten days of culture. Viable FPSCs were observed within both scaffolds at day 1, with clustering of cells evident in both groups (Figure 62A). By day 10 it was possible to observe new matrix being produced around these clusters of cells (Figure 62A). Within the anisotropic (Align) scaffolds, the cells and the matrix they produced were aligned parallel to the pore structure (Figure 62A). This organization was also observed in the H&E stained sections of the engineered tissues after 28 days in culture (Figure 62B). The composition of the tissues that formed with the scaffolds also depended on the underlying pore geometry, with higher DNA, sGAG and collagen content measured in the Align scaffolds compared to the Non-Al scaffolds after the 4 weeks culture period (Figure 62C). Macroscopically, scaffold anisotropy had a noticeable influence on tissue formation within FPSC seeded scaffolds, with more peripheral tissue deposition within Non-Al scaffolds (Figure 3D).

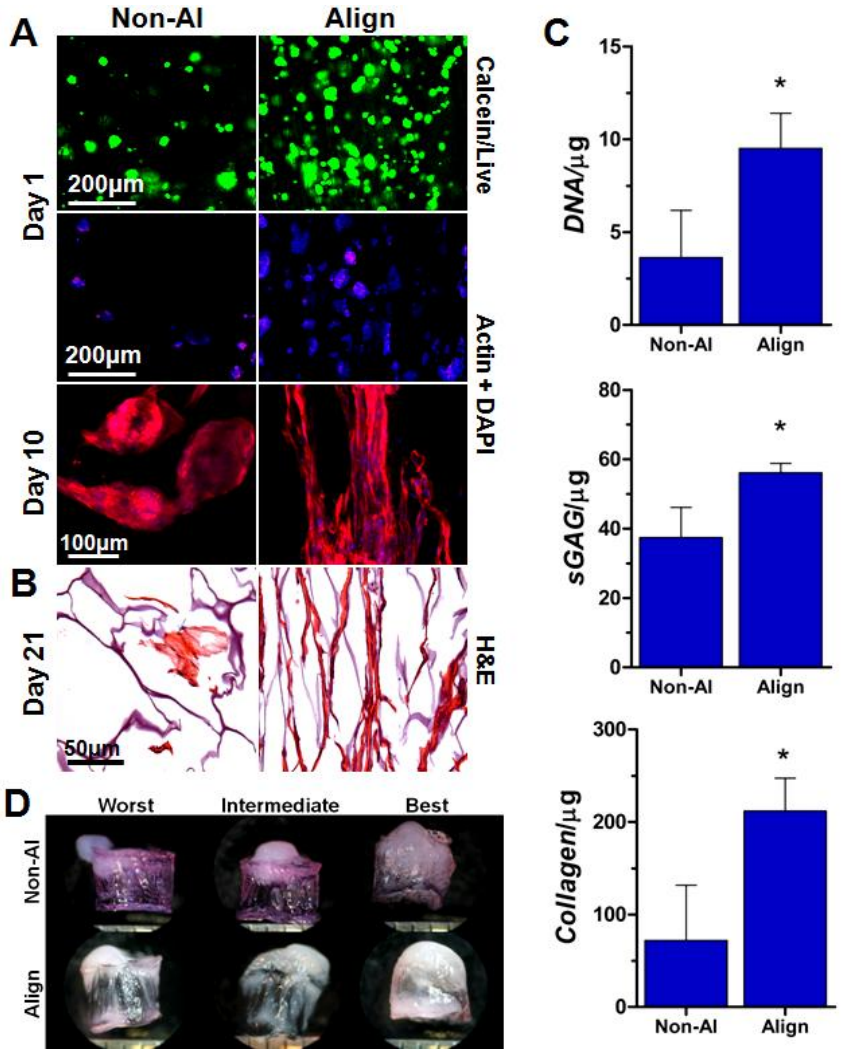


Figure 62 – Confocal calcein live cells and actin/DAPI staining micrographs for day 1 and day 10 of culture with FPSCs for alginate non-aligned (Non-Al) and alginate aligned (Align) (A). H&E staining for day 21 of culture for both groups (B). DNA, sGAG and collagen content after the 4 weeks culture period for Non-Al and Align groups (n=3; *p<0.05) (C). Macrographs (worst to best tissue deposition) of non-aligned (Non-Al) and alginate aligned (Align) scaffolds after 4weeks culture period with FPSCs.

9.3.3. Functionalization of shape-memory alginate scaffolds with either type I or type II collagen

Having demonstrated that aligned scaffolds promote more robust and homogenous tissue deposition within the scaffolds, we next sought to functionalize these anisotropic scaffolds with specific ECM components to further enhance their capacity to support chondrogenesis. Functionalization was performed by either coating

the scaffold with collagen and then freeze-drying for a second time, or blending this ECM protein into the alginate prior to freeze drying. We first sought to determine the impact of such ECM functionalization (type I and II collagen) on the mechanical properties of shape-memory alginate scaffolds. This functionalization was performed only in Align scaffolds given the fact that these were more chondrogenic and mechanically superior based on obtained results (Figure 62).

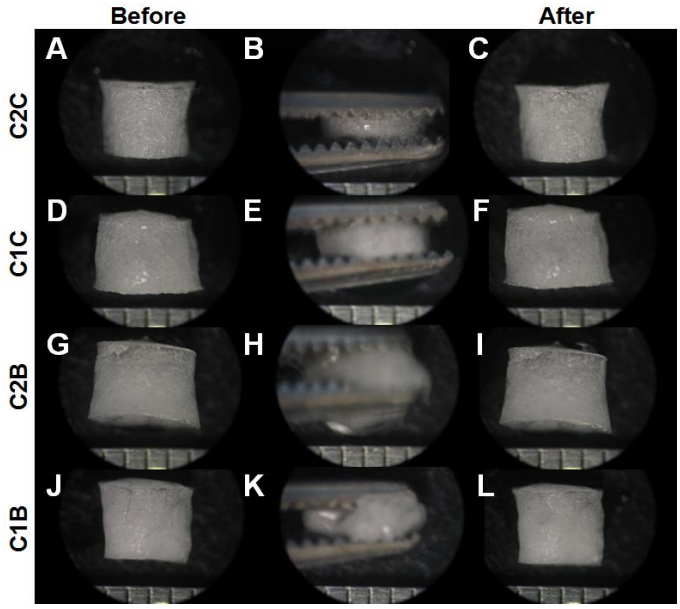


Figure 63 – Macrographs of alginate groups with type II coated (C2C), type I coated (C1C), type II blended (C2B) and type I blended (C1B) before and after mechanical compression with a tweezers before culture period.

Wet scaffolds were firstly compressed using a tweezers and again were able to return to original shape on unloading (Figure 63). To determine the stability of these ECM functionalised scaffolds, they were next subjected to continuous mechanical compression for fifty cycles. Only the collagen coated (C2C group) scaffolds demonstrated a significant decrease in mechanical properties (equilibrium modulus) after the application of cyclic loading (Figure 64). However, the stiffness of these C2C scaffolds was still similar to the non-coated (NC) scaffold after the conditioning phase.

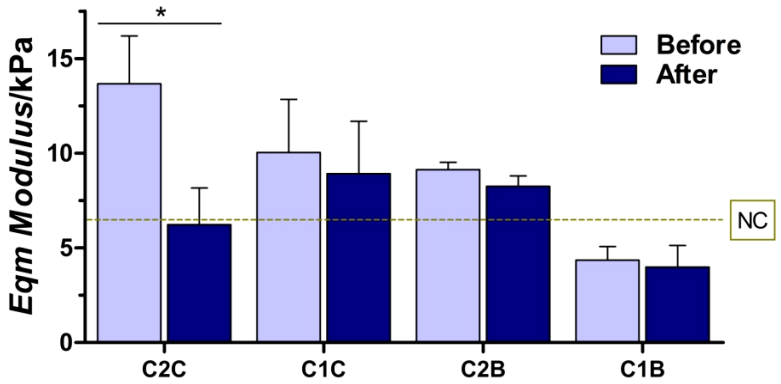


Figure 64 – Equilibrium modulus for all collagen coated (C2C and C1C) and blended (C2B and C1B) acellular scaffold groups, before and after the fifty compressive 10% strain cycles, before culture period (n=4; *p<0.05). Green line represents not coated Align (NC) scaffold equilibrium modulus after conditioning phase.

9.3.4. Functionalizing anisotropic shape-memory scaffolds with type II collagen promotes stem cell infiltration and homogenous cartilage tissue deposition

FPSCs were next seeded into the type I and II collagen functionalized Align scaffolds. Viable cells were observed in all functionalized scaffolds after ten days in culture (Figure 65). Even with ECM functionalization, FPSCs appeared to Align themselves in a direction parallel to the underlying scaffold pore structure (Figure 65).

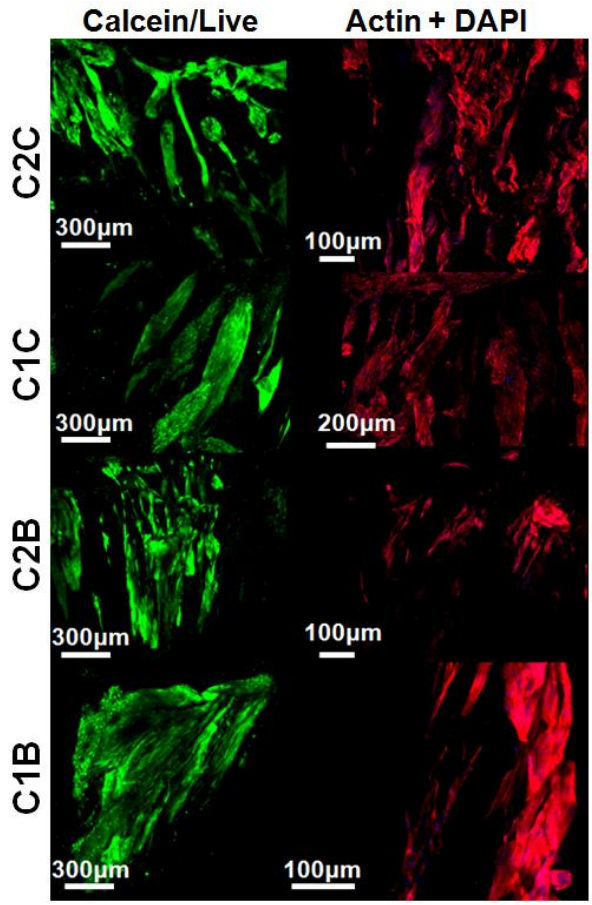


Figure 65 – Confocal calcein/live cells and actin/DAPI micrographs for day 10 of culture with FPSCs for alginate aligned scaffolds (Align) coated with type II (C2C) and type I collagen (C1C), and blended type II (C2B) and type I (C1B) collagen.

Coating with type II collagen was found to result in more homogenous tissue deposition within scaffolds seeded with FPSCs (Figure 66G-I). Similar results were observed with blended type II collagen scaffolds, although not as consistently as with coated scaffolds (Figure 66N and O). Pockets of newly formed tissue were observed in type I collated coated (C1C) scaffolds (Figure 66J-L). When blended into the alginate, type I collagen typically promoted external tissue formation, with poor tissue ingrowth inside the body of the scaffold (Figure 66P-R).

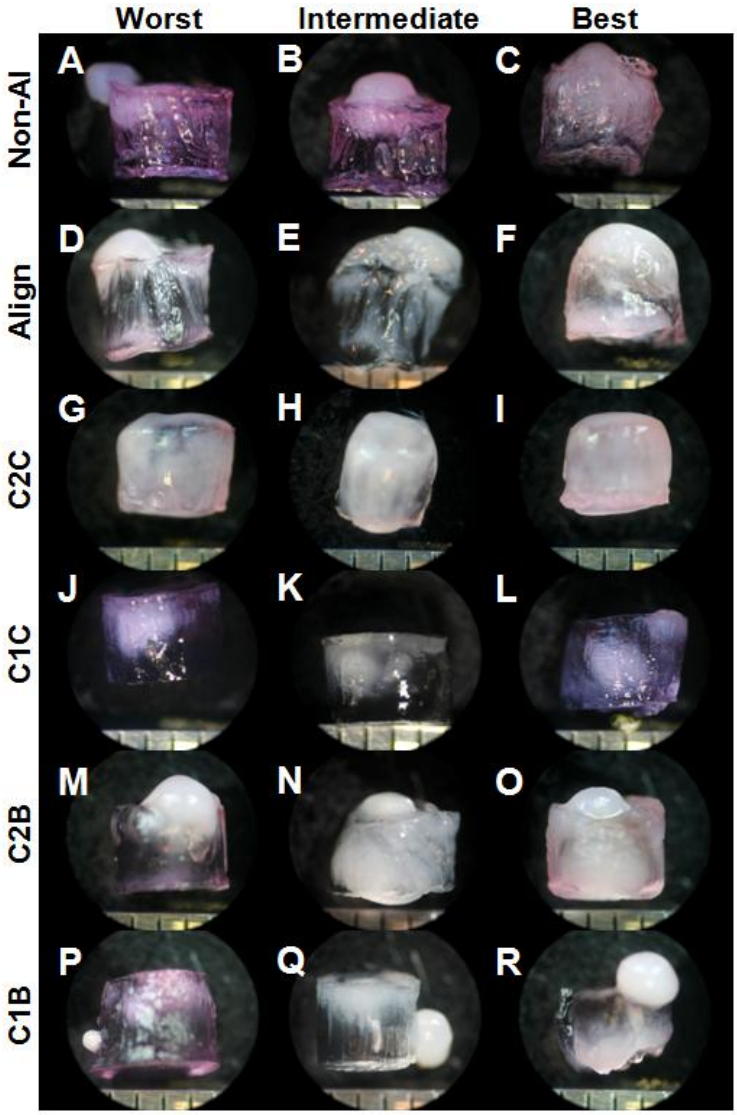


Figure 66 – Macrographs of constructs after 4 weeks in culture with FPSCs (worst to best): alginate non-aligned (Non-Al; A-C), aligned (Align; D-F), aligned coated with type II collagen (C2C; G-I), aligned coated with type I collagen (C1C; J-L), aligned blended with type II collagen (C2B; M-O) and aligned blended with type I collagen (C1B; P-R).

Aldehyde fuchsin and H&E histological staining was performed after 4 weeks in culture to assess spatial tissue deposition within the scaffolds. As noted previously, ECM was primarily deposited on the periphery of scaffolds not functionalised with collagen. Only small pockets of new tissue formation were observed inside the body of the scaffolds (Figure 67I and L). Coating the scaffolds with type II collagen (C2C) promoted the robust deposition of new matrix inside the body of the constructs (Figure

67O and R). While coating with type I collagen (C1C) also facilitated tissue deposition within the body of the scaffold, staining for sGAG deposition was generally less intense than the C2C scaffolds (Figure 67S-X). Blending of alginate with type II collagen (C2B) prior to scaffold fabrication was also found to result in more homogenous tissue deposition within the scaffold, although the intensity of staining was less that in C2C scaffolds (Figure 67Y-d). Tissue was mainly deposited on the surface of the type I collagen blended (C1B) scaffolds (Figure 67e and h; exclusively above dash line).

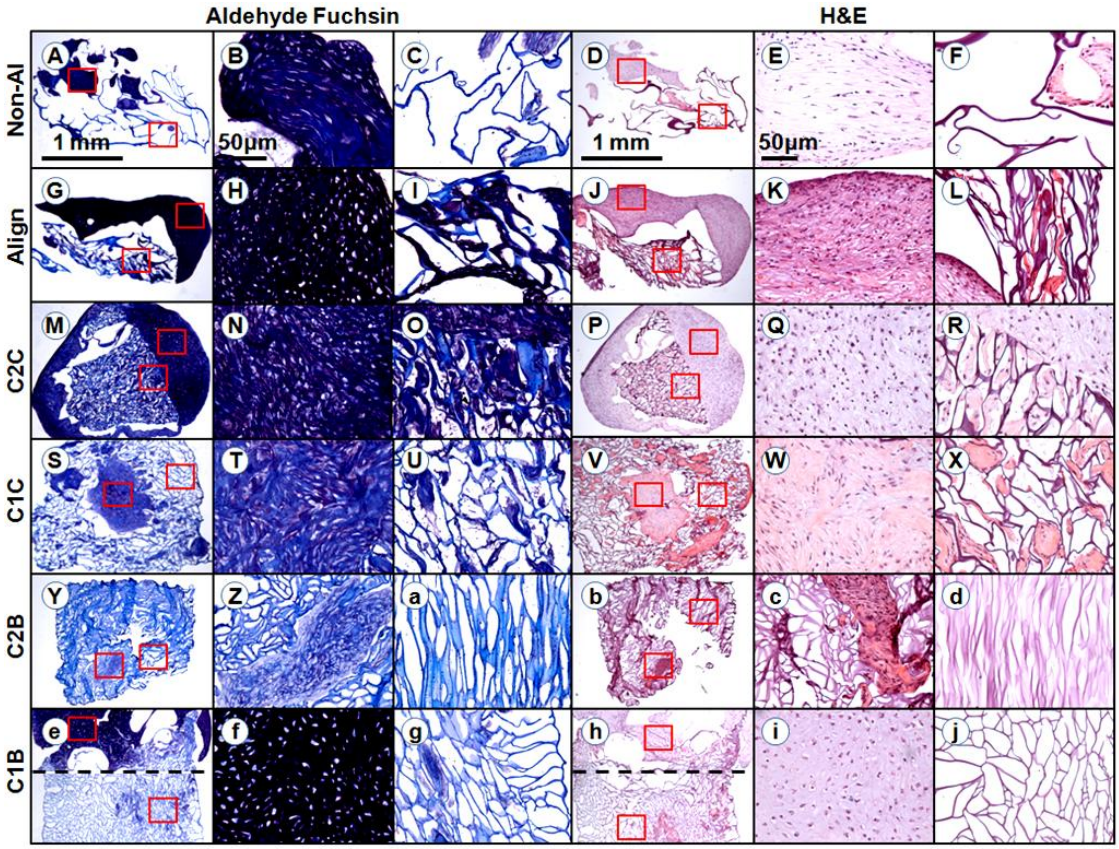


Figure 67 – Micrographs of aldehyde fuchsin and H&E staining of constructs after 4 weeks in culture with FPSCs: alginate non-aligned (Non-Al; A-F), aligned (Align; G-L), aligned coated with type II collagen (C2C; M-R), aligned coated with type I collagen (C1C; S-X), aligned blended with type II collagen (C2B; Y-d) and aligned blended with type I collagen (C1B; e-j). Red squares indicate location of the high magnification micrographs.

The specific types of collagen that filled the pores of the scaffolds also depended on the type of collagen they were functionalised with. The tissue that formed

within C2C scaffolds stained more homogenously and intensely for type II collagen than all other groups, including uncoated and C1C scaffolds. However, the newly formed tissue within these C2C scaffolds also stained positively for type I collagen (Figure 68M-O). It was also possible to observe some localized staining for type II collagen for Non-Al (Figure 68E), C1C (Figure 68V-X) and C1B (Figure 68i).

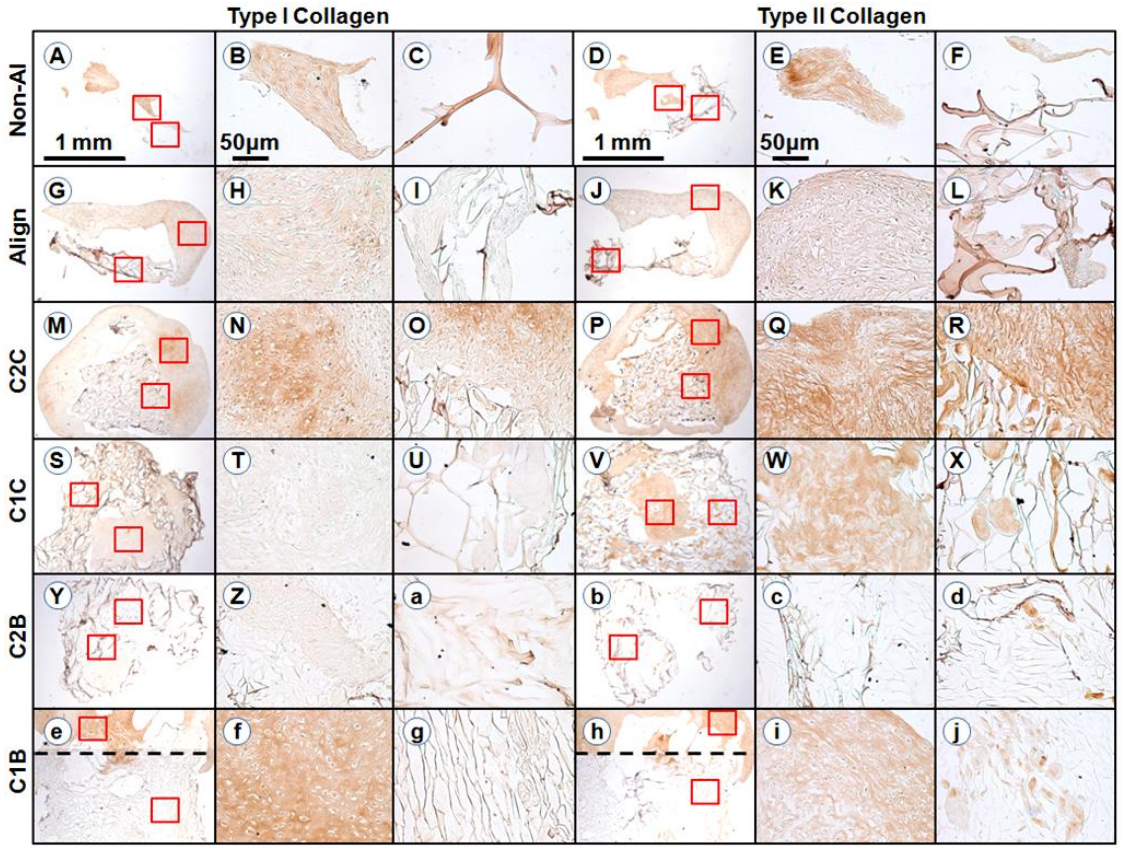


Figure 68 – Micrographs of type I collagen and type II collagen immuno staining of constructs after 4 weeks in culture with human FPSCs: alginate non-aligned (Non-Al; A-F), aligned (Align; G-L), aligned coated with type II collagen (C2C; M-R), aligned coated with type I collagen (C1C; S-X), aligned blended with type II collagen (C2B; Y-d) and aligned blended with type I collagen (C1B; e-j). Red squares indicate location of the high magnification micrographs.

FPSCs were also seeded for 4 weeks in alginate Non-Al and Align, either coated or blended with chondroitin sulphate (Appendix 1 - Figure A1). Results denoted cell viability (Appendix 1 - Figure A1A) and also newly formed tissue developing in parallel to aligned pores (Appendix 1 - Figure A1A and B), however newly formed matrix was poor in sGAG when compared with C2C group (Appendix 1 - Figure A1C).

9.3.5. Functionalizing anisotropic scaffolds with type II collagen results in the development of a more mechanically functional cartilaginous matrix

Tissues engineered with these collagen functionalized scaffolds were also analysed biochemically to determine their DNA, sGAG, and collagen content, and mechanically tested to determine their equilibrium stiffness (Figure 69). FPSC proliferation (as measured by DNA content) was greater in the collagen type II coated (C2C) scaffolds (Figure 69A). sGAG (Figure 69B) and collagen (Figure 69C) accumulation was in C2C compared to C1C scaffolds. Mechanically the C2C scaffolds were stiffer than both the control NC and C1C groups (Figure 69D).

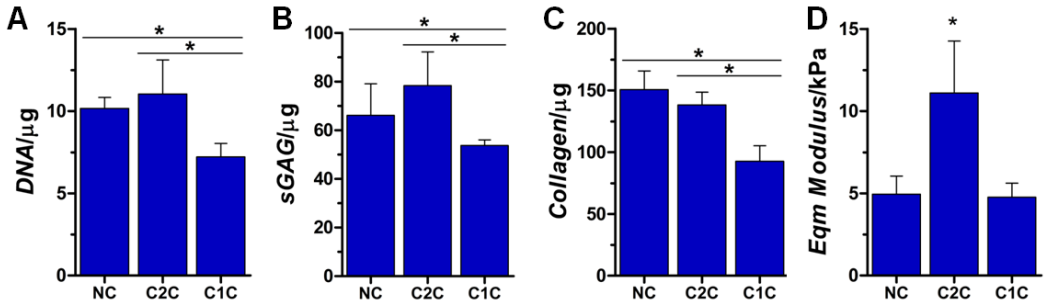


Figure 69 – DNA (A), sGAG (B), collagen (C) and equilibrium modulus (D) for alginate aligned not coated (NC), aligned scaffold coated with type I collagen (C1C) and type II collagen (C2C), after 4 weeks in culture with human FPSCs (n=4; *p<0.05).

9.4. Discussion

The overall goal of this study was to develop a biomimetic, shape-memory alginate scaffold for cartilage tissue engineering applications. Our objective was to improve the mechanical properties of covalently crosslinked alginate scaffolds whilst simultaneously enhancing its chondroinductivity. To this end the pore architecture was modified to mimic the alignment of the deep zone of articular cartilage (the largest region of the tissue), and the scaffold was additionally functionalised with type II collagen. Porosity was modified using a directional freezing technique [391], enabling the creation of aligned pores within the alginate scaffold. These anisotropic scaffolds

were stiffer than scaffolds with a more isotropic pores, and furthermore these geometrical features were found to direct stem cell alignment and facilitated the development of a more homogenous and cartilage-like tissue. Furthermore, the additional incorporation of type II collagen onto the surface of these scaffolds and facilitated the formation of a cartilage-like tissue rich in sGAG and type II collagen. The tissues that formed within these type II collagen coated scaffold were stiffer than those generated within scaffolds coated with type I collagen. These findings open up the possibility of using anisotropic shape-memory scaffolds, functionalised with type II collagen extracted from articular cartilage, and seeded with human FPSCs, to regenerate hyaline cartilage defects.

A directional freezing followed by freeze-dying strategy was used to develop covalently crosslinked alginate scaffolds with anisotropic pore geometry, which improved the mechanical properties of the scaffold without compromising its shape-memory properties. It has previously been shown that introducing vertical pore alignment in chitosan-gelatin scaffolds improves their structural mechanical properties [390]. A further advantage of modifying scaffold pore geometry is that it can provide structural cues to resident cells, which in turn can facilitate the engineering of complex tissues [175, 312, 390, 391, 402, 403]. In the context of articular cartilage regeneration, such an approach can potentially be leveraged to recapitulate the structural anisotropy of the tissue [390]. Indeed, introducing pore alignment was found to enhance stem cell proliferation and the deposition of cartilage-specific extracellular matrix within the scaffold.

In spite of the promising features of the aligned shape-memory alginate scaffold (Align), they still suffered from a number of limitations, including poor cell attachment, limited cell proliferation and inhomogeneous neo-tissue deposition throughout the construct [169, 175]. This is due the hydrophilic nature of the alginate and the absence of binding sites within the unmodified alginate to facilitate cell-scaffold

interactions, which reduces cell seeding efficiency that leads to heterogeneous tissue formation [175]. To overcome such a limitation it is possible to functionalize the alginate with biological native ECM-derived components, such as fibronectin [175] and collagen [170]. Motivated by previous studies [176], we functionalized these alginate scaffolds with either type I or type II collagen. In general, the alginate scaffolds retained their shape-memory capability following ECM functionalization, with type II collagen coating found to increase the stiffness of the scaffold relative to uncoated controls. However this C2C scaffold also demonstrated a significant reduction in mechanical properties after cyclic loading, likely due to physical damage to the collagen coating during cyclic loading. The collagen network that coats the alginate is not strongly chemically bonded with the underlying scaffold, but is rather physically attached to the structure.

Functionalizing the alginate scaffold with collagen enhanced cartilage-specific ECM accumulation, particularly when coated with type II collagen. Several previous studies have used type I and/or II collagen as cues to enhance tissue engineering strategies [176, 177, 404-406]. While functionalization with type I collagen also enhanced neo-tissue deposition within the body of the scaffold, it did not create comparable levels of cell proliferation and chondrogenesis as type II collagen coated scaffolds. This is in agreement with previous studies that have demonstrated the superiority of type II over type I collagen for promoting chondrogenesis [177-179, 388, 404, 405]. For example, superior cell differentiation and chondrogenic gene expression was previously observed when bovine stem cells were encapsulated in a type II collagen device, when compared with type I collagen [177]. Another important finding of this study was the more efficient penetration and robust proliferation of stem cells within the type II compared to the type I collagen coated scaffolds. This finding is also in agreement with a previous study were chondrocytes were seeded into collagen-sGAG scaffolds containing different types of collagen [388]. It was found that cells

were mainly located at the periphery of type I collagen scaffolds, whilst type II collagen containing matrices supported a homogeneous distribution of the cells throughout the scaffold [388]. The favourable chondrogenesis observed in such type II collagen devices may be mediated by specific integrin binding, which is crucial to maintain the chondrogenic phenotype and chondrocyte function [176, 407-410]. Type II collagen can improve chondrogenesis through integrin interactions, which modulate transforming growth factor signalling cascade critical for chondrogenesis [179, 411]. This collagen type is also known to improve cell attachment and chondrogenesis not only via integrins, but also with discoidin and annexin V receptors [179, 406, 410, 412, 413]. However there are limitations which hamper the use of type II collagen alone as scaffolding material, which include poor mechanical properties.

In spite of the poor mechanical performance of type II collagen alone, which limits its use as bulk biomaterial for cartilage tissue engineering, this limitation does not affects its beneficial effect as coating molecule [414]. Coating scaffolds with type II collagen is an efficient method to maximize stem cell adhesion in cartilage tissue engineering [178, 179]. However, type II collagen still needs to be used in conjunction with an additional biomaterial to ensure a mechanically robust scaffold. In the current study we combine attractive features of chemically crosslinked alginate with type II collagen to produce a scaffold ideally suited to articular cartilage tissue engineering.

9.5. Conclusion

The objective of this study was to develop an anisotropic, biomimetic, shape-memory, covalently crosslinked, and type II collagen functionalized alginate scaffold for cell based therapies for cartilage repair. We have demonstrated that the alignment created increases in the mechanical performance of the previously developed scaffold, and that the type II collagen functionalization enhances chondrogenic response of human infrapatellar fat pad-derived stem cells *in vitro*. These findings support the concept that anisotropy and coatings with chondro-specific molecules overcome

limitations previously seen in over-simplified scaffolds for cartilage tissue engineering. Furthermore, the use of such powerful scaffolds will enable the restoration of tissues of high complexity such as hyaline cartilage. The shape-memory feature of this scaffold will also enable the use of such a device for minimally invasive surgeries for cell based therapies in the mechanical demanding cartilage regeneration within a clinical context.

Chapter 10

Discussion

10. Discussion

10.1. Summary

It is recognized the existence of numerous approaches to regenerate damaged cartilage. However, some of these are only palliative and lacking long-term effectiveness as discussed earlier [1]. Current cell based techniques such as ACI are a promising alternative to known cartilage therapies. However, ACI and similar current cell based therapies have been reasonably successful clinically. Techniques such as ACI require two hospitals stays, cell culture, and high cost, limitations that hamper its widespread use. Therefore, there is an opportunity for new single-stage cell-based therapies, which do not require the hospital stays and the limiting cell expansion [20-22, 59, 267]. To achieve this, and perform effectively single-stage approaches, several requirements need to be fulfilled, such as the presence of a biomaterial-based scaffold, or hydrogel, capable of supporting chondrogenesis of progenitor cells. Hence, the global objective of this thesis was to develop and optimize a novel cartilage ECM-derived scaffold capable of promoting efficiently robust chondrogenesis of stem cells. It was also an objective to assess whether combining such an ECM-derived scaffold with freshly isolated IFP-derived stromal cells could be used to generate cartilage tissue *in vitro* and *in vivo*, with the ultimate goal of developing a single-stage or in-theatre strategy for joint regeneration.

With the aim of developing this single-stage approach for cartilage therapy, the first objective of this thesis was to develop and optimize a bioactive scaffold using a suitable biomaterial (Chapter 4). This scaffold would need ideally to be porous and capable of delivering cells and growth factors while enhancing cartilage repair. ECM-derived scaffolds appear as a viable choice for this application, having shown great promise for cartilage tissue engineering and regenerative medicine applications [38, 39]. For the first study of this thesis we also hypothesised that an ECM-derived

scaffold, and specifically a scaffold derived from a cartilaginous ECM, could be used as a growth factor delivery system to improve chondrogenesis of stem cells that are either seeded onto, or potentially recruited into, such a scaffold. It was observed that a cartilage ECM-derived scaffold could indeed retain and release TGF-β3, and in doing so drive chondrogenesis of human FPSCs. These findings open up the possibility of using such a construct as an off-the-shelf scaffold for articular cartilage repair, where stem or progenitor cells are either chemotactically recruited into the TGF-β loaded construct [335, 336], or where freshly isolated autologous stromal cells [20, 59, 255] are seeded into the scaffold prior to implantation.

The next phase of the ECM-derived scaffold development was to optimize parameters such as porosity and enhance its efficiency in promoting chondrogenesis of progenitor cells (Chapter 5). An additional goal for the second study of this thesis was also to try to develop and assess a single-stage therapy for cartilage repair combining an optimised cartilage ECM-derived scaffold and freshly isolated infrapatellar fat pad-derived stromal cells. By using ECM of differing concentrations building the scaffold, it was possible to produce structures with a range of pore sizes. The migration, proliferation and chondrogenic differentiation of FPSCs depended on the concentration/porosity of the ECM-derived scaffolds, with greater sGAG accumulation observed within the ones with a larger pore size. A limitation of these more porous scaffolds was that they underwent greater cell-mediated contraction. However, this could be prevented with the use of combined dehydrothermal DHT and EDAC crosslinking, with no loss in scaffold chondroinductive capacity. Such crosslinking also functioned to retard the initial release of exogenously loaded TGF-β3 from stem cell seeded scaffolds. Finally, the optimized scaffold was seeded either with culture expanded FPSCs or freshly isolated infrapatellar fat pad-derived stromal cells and implanted *in vivo* in a subcutaneous mouse model. The results indicate that this ECM-derived scaffold, with TGF-β3, supported cartilage-like tissue formation, specifically for culture expanded FPSCs or freshly isolated $CD44^+$ stromal cells,

opening up the possibility of using the latter cell source as part of a single-stage therapy for joint regeneration. The technique for stromal cell isolation will be of crucial importance for the development of single-stage procedures for cartilage regeneration [255]. At this point the main objectives of the first two chapters were addressed. The ECM-derived scaffold was developed, optimized, and able to release TGF-β3 while enhancing chondrogenesis of IFP-derived stem cells, and stromal cells *in vitro* and *in vivo*. The next stage was to assess affects of growth factor dosage on chondrogenesis of IFP-derived progenitor cells for different human donors (diseased and healthy).

Numerous growth factor delivery systems have been developed for cartilage TE. However, relatively little is known about how the dose of a specific protein will influence tissue regeneration, or how different patients will respond to altered levels of growth factor delivery. Hence, the objective of the studies described in Chapter 6 was to assess the capacity of cartilage ECM-derived scaffolds to bind and release escalating levels of TGF-β3 and its influence in stem cell chondrogenesis. Furthermore, it was also sought to determine if adult stem cells display a donor dependent response to the binding and release of different doses of TGF-β3 from such ECM-derived scaffolds. These possessed a remarkable capacity to bind and deliver increasing amounts of TGF-β3. They released between 60-75% of the loaded TGF-β3 into the media over 12 days of culture, irrespective of the concentration of growth factor added to the scaffold, with the remainder sequestered within the ECM. After a 28 day culture period, only constructs loaded with either medium or high doses of TGF-β3 resembled cartilage macroscopically. Robust sGAG and collagen (with high type II) deposition was observed in scaffolds loaded with high levels of growth factor.

Having demonstrated that cartilage ECM-derived scaffolds can be used to control the delivery of different doses of TGF-β3, and that chondrogenesis within these scaffolds was dependent on the amount of growth factor loaded onto the scaffold, we next sought to determine if FPSCs display a donor dependant response to the release of different doses of TGF-β3 from these constructs. To this end, FPSCs from a range

of healthy and diseased donors were seeded into cartilage ECM-derived scaffolds. Chondrogenesis was observed in most of the cases when high doses of growth factor were used. It was observed that if the dose of TGF-β3 loaded onto cartilage ECM-derived scaffolds is increased (specifically to the 200 ng dose) it is possible to observe a similar level of chondrogenesis using FPSCs from a range of healthy and diseased donors. This is an important finding, as other studies that have not used such cartilage ECM-derived scaffolds to drive chondrogenesis of stem cells have observed notable donor dependency in their results. Such consistency in minimizing patient-to-patient variability will facilitate the development, and future implementation, of more effective treatments for human cartilage repair. Once these questions were answered the next stage was to assess alternative sources of ECM, mainly due to xenogeneic material limitation. As an alternative we have allogeneic engineered cartilage, which can possibly be used to fabricate the already-known chondro-permissive native ECM-derived scaffolds.

The purpose of the study described in Chapter 7 was to assess the potential of using engineered cartilaginous ECM as a biomaterial to fabricate scaffolds for cartilage tissue engineering. We have previously shown that xenogeneic ECM-derived scaffolds are chondro-permissive when seeded with FPSCs and maintained in the presence of TGF-β3 [341, 351]. Previous studies have reported that devitalized human engineered cartilage can also be used as a base material to fabricate porous scaffold to facilitate chondrogenesis of stem cells [189, 271], although we have shown that such biomaterials can become osteoinductive if the engineered cartilage becomes hypertrophic and begins to mineralize prior to scaffold production [369]. The goal of this study was to directly compare the capacity of native (xenogeneic) and engineered cartilage (allogeneic) ECM-derived scaffolds to support chondrogenesis of human FPSCs. The porosity, architecture and mechanical properties of scaffolds derived from devitalized engineered and native ECM were similar, although differences in scaffold composition were observed. After seeding with human FPSCs, the native ECM-derived

scaffolds facilitated the development of a more cartilage like tissue than the engineered ECM. However when TGF-β3 was directly loaded into the engineered ECM-derived scaffolds, they supported the development of cartilaginous tissues with a comparable GAG content and stiffness to that of native ECM-derived scaffolds. Furthermore, the composition of the devitalized engineered cartilaginous ECM used to fabricate the scaffolds could be modulated by incorporating TGF-β3 eluting microspheres into the cell sheets during culture. Altering the composition of the engineered scaffolds in turn influenced the development of the cartilaginous tissues that formed within these constructs. These engineered human ECM-derived scaffolds are promising scaffolds for cartilage repair, overcoming limitations associated with the availability and xenogeneic immune reaction to native ECM-derived scaffolds [367].

The ECM-derived scaffolds proved to be chondro-permissive while releasing TGF-β3, and can potentially be fabricated with a more controllable and viable source of allogeneic ECM. However, scaffolds may have limitations when implanted in cartilage defects. These include poor fixation in complex defects, and implantation surgical procedures are not entirely minimally invasive. Therefore, as an alternative can be used a hydrogel systems which can overcome such scaffolds limitations. The hydrogel can be injected in a minimally invasive procedure, and also can adapt to the complex-shaped cartilage defects. Hence, the goal of the study presented in Chapter 8, was to develop an injectable, single-stage approach to promote chondrogenesis *in vivo*. This method consists of functionalizing a well-established biomaterial (fibrin) with particulated cartilage ECM loaded with TGF-β3, and using this construct to promote chondrogenesis of freshly isolated IFP-derived stromal cells. *In vitro*, the ECM-derived particles were able to retain and prolong the release of TGF-β3, proving to be as effective in this regard as the well established gelatin micro-sphere delivery system [52]. Fibrin hydrogels functionalized with particulated cartilage ECM promoted superior chondrogenesis *in vitro* and *in vivo* when compared with fibrin-gelatin constructs. These findings open up the possibility of using ECM-functionalized fibrin hydrogels, in

combination with freshly isolated stromal cells, to regenerate articular cartilage defects. From a translation perspective, the proposed strategy of freshly isolating IFP stromal cells and embedding them in an ECM functionalized fibrin hydrogel seems promising, as it may be used as a minimally invasive approach for single-stage cartilage repair.

It is recognized that ECM components, such as type II collagen, are beneficial for chondrogenesis, however, when used to fabricate scaffolds on their own they hamper the formation of a mechanically robust construct immediately after cell seeding. Hence, these ECM components can be used in conjunction with a potentially more mechanically robust biomaterial such as alginate. Therefore, the overall goal of the study described in Chapter 9 was to develop a biomimetic, shape-memory alginate scaffold for cartilage tissue engineering applications. Our objective was to improve the mechanical properties of covalently crosslinked alginate scaffolds whilst simultaneously enhancing its chondroinductivity. To this end, the pore architecture was modified to mimic the alignment of the deep zone of articular cartilage (the largest region of the tissue), and the scaffold was additionally functionalized with type II collagen. Porosity was modified using a directional freezing technique [391], enabling the creation of aligned pores within the alginate scaffold. These anisotropic scaffolds were stiffer than scaffolds with a more isotropic pores, and furthermore these geometrical features were found to direct stem cell alignment and facilitated the development of a more homogenous and cartilage-like tissue. Furthermore, the additional incorporation of type II collagen onto the surface of these scaffolds facilitated the formation of a cartilage-like tissue rich in sGAG and type II collagen. The tissues that formed within these type II collagen coated scaffold were stiffer than those generated within scaffolds coated with type I collagen. These findings open up the possibility of using anisotropic shape-memory scaffolds, functionalized with type II collagen extracted from articular cartilage, and seeded with human FPSCs, to regenerate hyaline cartilage defects. In the current study we combine attractive

features of chemically crosslinked alginate with type II collagen to produce a scaffold ideally suited to articular cartilage tissue engineering.

In summary this thesis demonstrated the potential of ECM-derived material to fabricate chondro-permissive devices with the characteristic of being able to transport cells and chondroinductive factors. This ECM-derived scaffold was able to induce chondrogenesis of expanded cells and freshly isolated stromal cells. Hence, it is appropriate to say that this scaffold can be used to enhance the performance and effectiveness of single-stage cartilage therapies *in vivo*. Additionally, the combination of this TGF-β3 releasing scaffold with $CD44^+$ freshly isolated cell can open the door for a next generation of single-stage therapies. Where a more chondrogenic stromal fraction will be isolated quickly and implanted into the cartilage defect, while seeded in the growth factor loaded scaffold. The new insight on the amount of growth factor to include into the scaffold, despite the cell donor origin or condition, will facilitate the clinical translation of this approach. It is known that animal-derived ECM has limitations in TE, thus it is possible to engineer human cartilage reducing risks and shortage. Therefore we can use it as raw-material to fabricate scaffold for cartilage repair, and particularly while loaded with TGF-β3, enhance chondrogenesis in single-stage therapies for human cartilage repair. It was proven that the ECM from native tissue is a powerful biomaterial to build chondro-permissive scaffolds, so the next step was to try to use such material to enhance the performance of already in use biomaterials. Firstly, it was possible to functionalize fibrin with ECM micro-particles and load these ones with TGF-β3. This system proved to be efficient *in vitro* and *in vivo* (subcutaneously) while enhancing chondrogenesis of IFP-derived stem cells. This fact will allow the use of such minimally invasive method to regenerate cartilage defect in one single procedure. Finally, it is known that alginate scaffolds have some limitation such as its seeding efficiency. To overcome such limitation coatings with collagen were performed with promising results, especially for type II collagen. The additional architectural change of the previously developed scaffold enhanced mechanical

performance. Hence, it was developed an anisotropic, with shape-memory, type II collagen coated, and chondro-permissive scaffold for cartilage TE. Such shape-memory feature will also allow the use of such scaffold in minimally invasive surgeries for single-stage cartilage therapies. A summary table comparing advantages and disadvantages for approaches used in this thesis for cartilage regeneration was performed (Table 6).

Table 6 – Summary of characteristic for approaches used in this thesis for cartilage TE. Chapter 6 is not included due to nature of the study.

Chapters	4	5	7	8	9
Approach	Coarse ECM scaffold	Fine ECM scaffold	Eng. ECM	Fibrin	Alginate
Advantages	• Easy to produce • Robust chondrogenesis • TGF release	• Robust chondrogenesis • Pore size control • Reproducibility • Cell infiltration • TGF release • *In vivo* • Single-stage • CD44+ cells	• Robust chondrogenesis • Allogeneic • Reproducibility • TGF release	• Injectable • Good Chondrogenesis • TGF release • *In vivo* • Single-stage • Easy to produce	• Shape-memory • Mechanical properties • Anisotropy • Reproducibility • Cell infiltration (coll II)
Limitations	• Low reproducibility • No morphology control • Difficult to decellularize • Xenogeneic • Poor mechanical properties • Contraction • Isotropic • DHT and EDAC Crosslinking	• Liquid nitrogen • Cryo-milling • Xenogeneic • Mechanical properties • Contraction • Isotropic • DHT and EDAC Crosslinking	• Liquid nitrogen • Cryo-milling • Mechanical properties • Contraction • Isotropic • DHT and EDAC Crosslinking	• Liquid nitrogen • Cryo-milling • Xenogeneic • Isotropic • Extra functionalization	• Cell infiltration (Coll I and CS) • Extra ECM functionalization • Xenogeneic (functionalization) • Liquid nitrogen • Difficult to produce • No growth factor release

An additional direct comparison between the developed ECM-derived scaffolds and the ECM-functionalized alginate scaffold was prepared (Figure 70). After 4 weeks culture period it was possible to observe a superior sGAG and collagen deposition for the ECM-derived scaffold, proving that such scaffold is more chondro-permissive than the alginate one (Figure 70A and B). Equilibrium modulus for the cartilaginous constructs was not superior for 10% strain, however when the percentage of strain was increased, a superior equilibrium modulus was observed (Figure 70C). While the alginate functionalized constructs maintained its equilibrium modulus for the three different strain values. This fact indicates that the ECM-derived construct enhanced the formation of a robust cartilaginous tissue with depth dependent mechanical properties, superior when compared with alginate constructs. However, the mechanical stability of the ECM-derived scaffold at day 0 is inferior when compared with the type II collagen functionalized alginate scaffold presented in Chapter 9. Hence, both scaffolds offer important features (especially ECM-derived in long-term and alginate in short-term

mechanical properties), which will enable their use separately or in conjunction as a hybrid scaffold to be used in the future for next generation cartilage TE therapies. Techniques for cartilage regeneration using ECM-derived devices may be patient-dependent, where the surgical intervention can range from simple to complex.

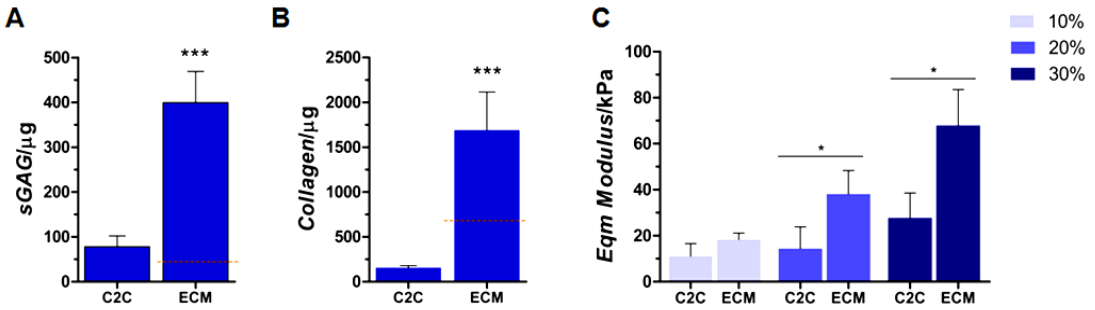


Figure 70 – sGAG (A), collagen (B) and equilibrium modulus (strain: 10%, 20% and 30%) (C) for alginate aligned type II collagen coated (C2C) compared with ECM-derived scaffold, after 4 weeks in culture with human FPSCs (n=4; *p<0.05; ***p<0.001). Red line represents day 0 values for ECM scaffold; C2C values were negligible.

10.2. Conclusions

- In conclusion in Chapter 4 it was found that the combination of a porcine cartilage ECM-derived scaffold and stimulation with TGF-β3 can induce robust chondrogenesis of human diseased infrapatellar fat pad-derived stem cells (FPSCs).
- The finding that such an ECM-derived scaffold can be used as a delivery system for TGF-β3 to induce chondrogenesis of MSCs opens the possibility of using such a construct as an off-the-shelf product for cartilage tissue regeneration (Chapter 4).
- In Chapter 5 a robust method was described to control the composition and porosity of cartilage ECM-derived scaffolds, a biomaterial with potent pro-chondrogenic properties.
- By seeding such a scaffold with either culture expanded FPSCs, or freshly isolated $CD44^{+}$ stromal cells, it was possible to promote the development of a

cartilage-like tissue *in vivo*. This latter finding supports the concept that enriched populations of freshly isolated stromal cells, when combined with a chondro-inductive scaffold, can induce cartilage formation and can potentially be used in single-stage procedures for cartilage repair (Chapter 5).

- It was observed that by delivering a high dose of growth factor from these scaffolds it was possible to minimize donor-to-donor variability in chondrogenesis of human infrapatellar fat pad-derived stem cells. Furthermore, no major difference was observed between healthy and diseased donors (Chapter 6).
- The combination of an ECM-derived scaffold, FPSCs, and a sufficient dose of growth factor consistently results in robust chondrogenesis. Such consistency will facilitate future clinical translation of such treatments for human cartilage repair using growth factors (Chapter 6).
- The study presented in Chapter 7 describes a viable method to engineer human cartilage and use ECM extracted from this tissue as a biomaterial to fabricate chondro-permissive scaffolds for cartilage regeneration.
- By seeding these scaffolds with infrapatellar fat pad stem cells and loading the construct with TGF-β3, it was possible to generate robust cartilage-like grafts *in vitro* (Chapter 7).
- The use of such allogeneic engineered tissue-derived scaffolds could overcome the limitations associated with xenogeneic scaffolds, and human cartilage grafts. These engineered ECM-derived scaffolds could potentially be used as off-the-shelf chondro-permissive devices to support cell-based therapies for cartilage regeneration (Chapter 7).
- The objective of the study mentioned in Chapter 8 was to functionalize an injectable fibrin hydrogel with a view of developing a single-stage therapy for cartilage repair. It was demonstrated that ECM-derived micro-particles can

deliver TGF-β3 and that this system can induce chondrogenesis of freshly isolated fat pad-derived stromal cells *in vivo*.

- This finding supports the concept that populations of freshly isolated stromal cells, when combined with a chondroinductive hydrogel, can induce cartilage formation and can potentially be used in single-stage, minimally invasive procedures for cartilage regeneration. The translation of such a strategy would overcome many of the current limitations associated with clinically available cell-based therapies for cartilage repair (Chapter 8).
- The objective of the final chapter was to develop an anisotropic, biomimetic, shape-memory, covalently crosslinked, and type II collagen-functionalized alginate scaffold for cell-based therapies for cartilage repair. It was demonstrated that the alignment created increases the mechanical performance of the previously developed scaffold. Additionally, type II collagen functionalization enhances chondrogenic response of human infrapatellar fat pad-derived stem cells *in vitro* (Chapter 9).
- These findings support the concept that anisotropy and coatings with chondro-specific molecules overcome limitations previously seen in over-simplified scaffolds for cartilage tissue engineering. The use of such powerful scaffolds will enable the restoration of tissues of high complexity such as hyaline cartilage (Chapter 9).
- The shape-memory feature of this scaffold will also enable the use of such a device for minimally invasive surgeries for cell based therapies in the mechanically demanding cartilage regeneration within a clinical context (Chapter 9).

10.3. Limitations and future work

The goal of this thesis was to develop and assess an ECM-derived scaffold, and ECM-functionalized devices, as delivery systems for cells and growth factors. These would be used to streamline single-stage therapies for cartilage repair. Promising results were achieved, however, some of the studies and approaches discussed in previous paragraphs have limitations or points that need to be assessed in the future. These include the method of ECM production, tissue decellularization, effects of scaffold crosslinking, characterization of ECM growth factors, IFP as a source of cells, mechanical performance and oversimplified morphology, and necessity of additional *in vivo* models. Some of these issues are already being addressed.

A major limitation of using ECM-derived biomaterials is the variability in biological material. Despite the fact that the methods being used are improving batch-to-batch variability (e.g. cryomilling), there is always a variability donor-to-donor. Additionally, one of the possible steps of the process which is hampering the standardization of this approach is the use of a freezing step during device fabrication. It has been previously shown that freezing tissues will influence negatively the outcome of the graft used for cartilage regeneration [1]. The improvement and assessment of new/optimized methods will allow the standardization of the ECM isolation processes, which will include decellularization.

One of the most important subjects which need to be addressed is the issue of decellularization. As previously mentioned, no consensus is achieved yet, with the claim that current standards were established based on work performed in other tissues other than cartilage [38]. Cartilage possesses a very intricate and dense structure, with no vascularisation, and consequently partially immuno-privileged. Hence, further additional studies should be performed to assess the immune response to such ECM-derived devices and effects of decellularization. This decellularization step will alter the ECM structure and composition. The harsh physical and chemical

treatments, commonly used in decellularization methods, will breakdown tissue orientation and topography, leach sGAGs and damage collagen fibrils. Additionally, other important factors will be removed or denatured, such as intrinsic growth factors and other unidentified chondrogenic cues. Therefore, a full ECM-derived device characterization (e.g. compositional and immunological), before and after decellularization treatment, will be pertinent to answer some of these questions. To test and understand immunologically the scaffold is an important step to assess the safety of the referred structures before clinical translation. These decellularization studies are already in progress, with parallel work being performed at the moment, to fully assess viable methods of decellularization, and its consequences in the performance of such ECM-scaffolds. The preliminary data indicated that by using decellularization methods the amount of sGAGs in scaffolds is reduced. Such difference interferes with the growth factor releasing kinetics. An additional immunological characterisation of the ECM is already being performed with promising preliminary results.

In the production of the ECM-derived scaffolds there are some steps that can be detrimental to the material. One of the examples is the crosslinking dehydrothermal treatment (DHT), commonly used to physically crosslink natural-derived scaffolds for TE. It is known that this treatment is efficient concerning crosslinking and also while sterilizing the scaffold. However, high temperatures used can irreversibly denature components of the bioactive device. These include sGAG, collagens, and also fragile growth factors present. This fact can be a limitation of our approach. It should be noted, however, that a comparison of dissimilar crosslinking methods demonstrated that although they all influenced the composition of the ECM-derived scaffold, DHT treatment lead to the development of constructs that best matched the composition of native cartilage [25]. There is also a previously reported study were the denaturation of ECM proteins exposed previously deactivated cell binding sites [415]. Hence, a detailed characterization of the ECM before and after the process of scaffold

fabrication will elucidate about its consequences. It is also known that ECM-derived from native cartilage possess intrinsic chondrogenic cues, such as growth factors [266]. Hence, studies are already underway with the objective of full growth factor characterization present in the native cartilage ECM used in the fabrication of these scaffolds. To understand which remaining chondrogenic factors are present in the ECM, will enable the deeper understanding of the tissue, and help in the selection of the doses of growth factor to use in future *in vivo* studies. These preliminary studies indicate the presence of growth factors and additional chondrogenic cues. Further gene expression assessment is going to be performed in response to ECM and growth factors present in the construct.

An additional possible limitation of the current thesis is related with the use of IFP as source of progenitor cells. This source is suitable for small cartilage defect repair, but it is lacking enough cell numbers for larger defects. The reduced amount of the tissue available will limit this source to be used in single-stage procedures [20]. Alternative sources include subcutaneous fat that proved to be a viable source of stromal cells [20]. Conversely, it would be important to continue to explore the potential of CD44 positive cells in future experiments, to further validate such chondro-potent cells in cartilage therapies. It would be also important to identify additional subpopulations with similar properties and suitable for cell-based approaches. Once all these questions are answered an important advance for clinical translation is made for this potential approach for cartilage repair. *In vivo* studies are being performed at the moment, to regenerate cartilage defects in where CD44 positive cells are being used as the implanted chondro-progenitor cells.

An additional pertinent research question is the mechanical performance of the ECM-derived scaffold. The previously developed ECM-derived structure still shows signs of poor mechanical stability in the first few hours after cell seeding. There are different approaches to achieve the desired mechanical behaviour, such as incorporating a stronger biomaterial, similar to alginate presented in Chapter 9.

Alternatively it can also be assessed a modification to the architecture of the pure ECM-derived scaffold by altering manufacturing parameters, similarly to what was done to the anisotropic alginate scaffold. It was previously proposed a new approach in which was possible to orientate collagen fibres (Figure 71) [312]. These oriented scaffolds were produced using a modified unidirectional freezing technique followed by freeze-drying, similar to the alginate study approach (Chapter 9). Moreover, this orientation of the fibres led to an increase of the Young's modulus of the scaffold [312]. Furthermore, this aligned architecture promoted rapid chondrocyte orientation throughout the construct, mainly along the fibres, with newly formed sGAG and collagen. Values for both were similar to control scaffolds with no fibre alignment [312]. Therefore, just by altering parameters of the manufacturing process it would be possible to enhance mechanical performance without compromising chondrogenesis only using native ECM.

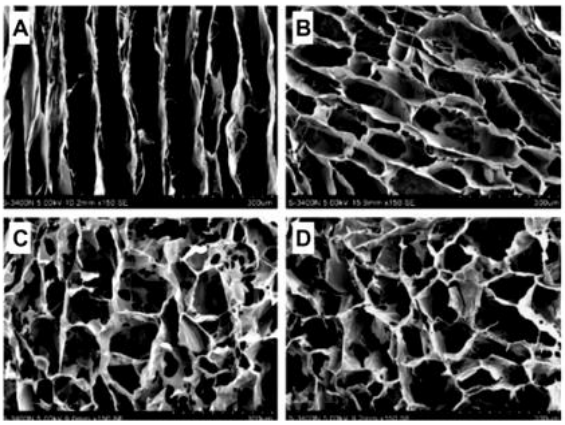


Figure 71 – SEM micrographs of the oriented scaffold in (A) vertical section and (B) cross section. (C) and (D) correspond to the non-oriented one [312].

Additionally, in future studies is necessary to further assess and characterize growth factor release, chondrogenesis, and construct mechanical performance under free-swelling and dynamic compression culture conditions. In future work, alternative methods of mechanical characterization will be used to assess and improve ECM-derived scaffolds and hydrogels performance. In further characterization is important to

take into account the morphology (e.g., particle and pore size), rheology, and moduli of the devices to assure superior performance after surgery.

Another critical issue in cartilage TE is the complexity of AC tissue. There are different zones which have dissimilar functions, mainly because its composition and chondrocyte phenotypes are different [416]. With the aim of recreating the native environment of cartilage, as previous mention [298], the staking of cartilaginous sheets looks promising, particularly if the ECM was originally produced by zone specific cell types [38]. It has been proposed an alternative approach to recreate native architecture in cartilage by using 3D bioprinted constructs, to deposit zone-specific material [417]. The method can also create tailored constructs with a mixture of ECM and synthetic materials capable of the improvement of mechanical properties [38, 418]. Synthetic materials based scaffolds could also be improved in terms of cell response with the incorporation of ECM [38], similarly to fibrin and alginate functionalization present in the previous chapters.

The current thesis presented *in vivo* subcutaneous implantations of the ECM-derived scaffolds and ECM-functionalized fibrin hydrogel. Such implantation was performed to assess feasibility of the single-stage procedures while using such delivery devices. However, the subcutaneous model of implantation in immunoprivileged mice it is limited when compared to articular cartilage defects in larger animal models, with full operational immune system. Native articular cartilage represents a different environment biomechanically and biochemically. It is also relevant to investigate the effects of the growth factor delivery and doses in the native environment once *in vivo*. Hence, it is necessary in the future work to assess such an approach in more adequate animal models where the environment is closer to the one in the human joint.

After achieving promising results *in vitro* and *in vivo* (subcutaneously) with the ECM-derived scaffolds and ECM-functionalized fibrin, it is imperative to move to larger animal models and cartilage defects. The outcome of these studies in rabbits and

goats will dictate the fate of our concepts in future clinical translation. Consequently, the objectives for future work are to assess this ECM-derived scaffold and the ECM-functionalized fibrin injectable, capable of carrying growth factor and support chondrogenesis of stromal cells, in an orthotopic defect within both a rabbit and a goat model. These studies are going to be concluded soon, where an ECM-derived scaffold was implanted in a rabbit cartilage defect, and where fibrin functionalized with ECM was implanted in a goat orthotopic model. Both surgeries used freshly isolated cells and growth factor (equivalent to high dose of TGF-β3 in Chapter 6), to assess a single-stage therapy for cartilage repair. The interpretation of the results will determine the effectiveness of the scaffold and hydrogel *in situ* and the need of improvements. These changes may permit advances in the method, and efficiency in clinical context, of single-stage procedures for cartilage repair, overcoming current ACI limitations.

Chapter 11

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Appendix 1

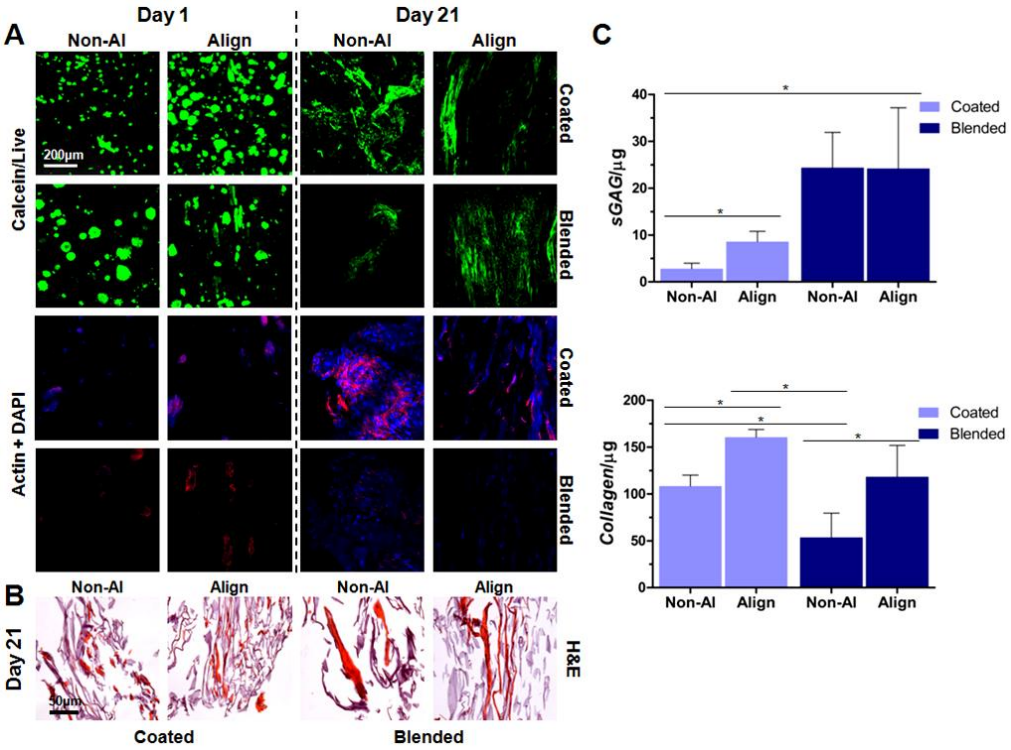

Figure A1 – Confocal calcein live cells and actin/DAPI staining micrographs for day 1 and day 21 of culture with FPSCs for alginate non-aligned (Non-Al) and alginate aligned (Align) with either coated of blended with chondroitin sulphate (A). H&E staining for day 21 of culture for both groups (B). sGAG and collagen content after the 4 weeks culture period for Non-Al and Align groups either coated of blended with chondroitin sulphate (n=3; *p<0.05) (C).