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ENGINEERING OF PHENOTYPICALLY DISTINCT HUMAN CARTILAGE MICROTISSUES FOR THE BIOFABRICATION OF ZONALLY DEFINED ARTICULAR CARTILAGE GRAFTS

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of the requirements for the degree of Doctor of Philosophy.

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Nadia Rodriguez

Dublin, 31 March 2025

Summary

Articular cartilage (AC) is integral to normal synovial joint function. AC damage is very common and, as the tissue does not self-heal, often leads to further degeneration of the entire joint and the development of osteoarthritis. Current treatments, including cell-based approaches, fail to promote complete tissue regeneration, which is often attributed to the complexity of AC, whose unique function is linked to its spatial biochemical composition and architecture. Tissue engineering (TE), which aims at regenerating functional tissues, has emerged as a promising therapeutic alternative. The limited regeneration resulting from traditional TE approaches (combining cells, growth factors and scaffolds) has motivated the development of novel approaches.

Developmental engineering seeks to regenerate a specific tissue or organ by mimicking key steps of normal development. Stem/progenitor cells can self-organise into biomimetic microtissues or organoids when provided with appropriate environmental conditions, which can then be used as biological building blocks for the biofabrication of larger grafts. The choice of cell type has been shown to greatly influence tissue regeneration. While mesenchymal stem/stromal cells (MSCs) have successfully been used to generate cartilage, their inherent tendency to progress along an endochondral pathway and generate bone represents a major limitation. This has motivated increased research into the development of novel strategies to engineer phenotypically stable AC using MSCs. For example, hypoxia has been shown to support chondrogenesis and suppress hypertrophy, but the effect of distinct physiological levels of oxygen on chondrogenesis of MSCs remains unclear. In addition, gene silencing therapies have recently emerged as a novel approach to finely regulate the chondrogenic differentiation of MSCs. This thesis aims to leverage these findings in an attempt to engineer phenotypically distinct human cartilage microtissues for the biofabrication of zonally defined articular cartilage grafts.

Firstly, the potential of human articular cartilage progenitors (hACPs) to engineer stable AC was explored. It has shown that hACP-derived microtissues differentiated in the presence of transforming growth factor (TGF)- β 3 supported the development of fibrocartilage, but cocultures of hACPs and human MSCs (hMSCs) were successfully differentiated into cartilage tissue. Specific coculture conditions were found to support significantly more glycosaminoglycan (GAG) deposition than hMSC-only controls and increased the production of type II collagen. Furthermore, minimal

type X collagen was also observed in this coculture. To conclude, hACPs showed some potential for AC TE, but further studies are required to consolidate their potential.

Secondly, the effect of oxygen on the chondrogenic differentiation of hMSCs was explored. Microtissues were cultured at altered oxygen levels, similar to that found in the different zones of AC. Lower oxygen levels significantly increased GAG production and supported a collagen/GAG synthesis ratio more mimetic of the deep region of the native tissue, while type II collagen deposition was not negatively impacted by culture at these lower oxygen levels. In addition, a short priming at lower oxygen levels was found to enhance longer-term GAG production. Therefore, differentially oxygen-primed microtissues could potentially be used as biological building blocks for the fabrication of the different zones of AC.

Large cartilage grafts were next fabricated by fusing hMSC-derived microtissues onto a melt electrowritten (MEW) mesh inside a custom-made seeding well. This novel modular assembly approach allowed the macroscopic geometry of the engineered tissue to be controlled. In addition, hanging this construct upside down in culture was found to modulate the organisation of the collagen network, resulting in the development of a more biomimetic tissue. Next, oxygen primed microtissues with distinct phenotypes were sequentially seeded onto the mesh to recapitulate the biochemical gradient of native tissue. While it was not possible to generate a biochemical gradient, this novel modular assembly approach could be used to engineer large cartilage graft while minimising the presence of foreign material.

Finally, a gene silencing approach was explored to engineering hyaline cartilage by delivering the pro-chondrogenic factor, anti-miR-221. Human MSCs were successfully transfected, showing high viability, metabolic activity and effective silencing of miR-221. Transfection supported type II collagen deposition and suppressed the production of type X collagen. In addition, such microtissues were fused into larger grafts, again showing no signs of early hypertrophy. To conclude, highly hyaline cartilage microtissues were engineered and could be used as *in vitro* models of AC or as cartilage biological building blocks.

To conclude, this thesis explored three different approaches to enhance the development of hyaline cartilage microtissues. By exploring the effect of cell type, physiological oxygen and gene silencing therapy, it was possible to engineer phenotypically distinct cartilage microtissues. In addition, a novel modular assembly approach was developed to engineer scaled-up grafts which holds potential to improve the quality and complexity of such regenerative constructs.

“...from Hippocrates down to the present age, we shall find, that an ulcerated cartilage is universally allowed to be a troublesome disease... and that, when destroyed, it is never recovered.”

Hunter, W.

Of the structure and diseases of articulating cartilages
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And as José says: 'The cell is the best engineer, but I [tried my best]!'

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1. Introduction

1.1. The Clinical Problem

Hyaline articular cartilage (AC) is found at the surface of long bones within synovial joints [1]. This connective tissue is only 2–4 mm thick yet withstands challenging mechanical loads and facilitates near frictionless joint articulation [2]. This functionality is linked to the unique structure and composition of AC [3]. Focal lesions to AC from traumatic injury or “wear and tear” do not self-repair and lead to the onset of osteoarthritis (OA), an inflammatory and degenerative injury of the entire joint [4]. OA is highly painful and debilitating to such an extent that it is the world-leading cause of loss in quality of life with high socio-economic costs [5]. The prevalence of knee OA is estimated to 365 million people worldwide and is expected to continue to increase due to changing demographics and increasing rates of obesity [6].

Current treatments for AC defects (e.g. debridement, shaving, microfracture) do not promote tissue regeneration, and after three decades of tissue engineering research, cartilage true regeneration remains elusive. This can be linked to the complexity of the tissue and challenging environment in which it operates [7-9]. While surgical interventions like autografting/allografting of osteochondral plugs have shown some promise, drawbacks such as formation of biomechanically inferior fibrocartilage, limited tissue availability and donor site morbidity have impeded its widespread clinical use [4]. Total knee arthroplasty, performed in patients presenting advanced stages of OA, has a first revision rate of 2.8% through 5 years and a second revision rate of 18% after 5 years [10]. In addition, patients less than 65-year-old are projected to represent more than half of the patients and more than half of the revision patients [11].

Cell-based approaches, such as implantation of autologous chondrocytes (ACI) seeded within a supporting matrix (third generation ACI), are promising but still present several drawbacks such as graft hypertrophy, pain and variable long-term results (>2 years after implantation) [12]. Furthermore, such cell-based approaches are not recommended in advanced OA patients [12]. Currently, only one product, Spherrox® (implantation of chondrocyte spheroids), is available in the European Union (approximately 10 products available in the worldwide market) [12, 13]. A couple of

early-stage products include allogenic stem cells or redifferentiated chondrocytes but to date, no product has reported complete and long-term cartilage regeneration and functional restoration [7, 12]. Therefore, there is an urgent need for novel treatments and innovative tissue engineering solutions.

1.2. Current Trends in Articular Cartilage Tissue Engineering

Tissue engineering (TE) strategies offer a promising alternative to surgical approaches, potentially providing stable and functional replacement of tissues on demand. Such TE approaches combine innovative biomaterials, bioactive external cues and cells [14]. Central to many TE strategies is the identification of an abundant cell population capable of generating phenotypically stable hyaline cartilage grafts.

Selection of the appropriate cell for TE typically requires careful consideration of two key factors, ease of access to the source tissue and the performance of the resulting cells [15]. Autologous chondrocytes have been widely used for cartilage TE, but *in vitro* expansion, to increase the initially low cell number, leads to rapid dedifferentiation and loss of the native phenotype [15]. Mesenchymal stem/stromal cells (MSCs) represent a promising alternative cell source due to their multipotent differentiation potential, anti-inflammatory and immunomodulatory properties [16]. MSCs have been extensively used in the engineering of AC. However, despite successful differentiation into hyaline cartilaginous-like grafts, such tissues have an inherent tendency to undergo endochondral ossification and form bone, potentially limiting their use in the regeneration of synovial joints [17]. Hence, new strategies are required to engineer phenotypically stable hyaline cartilage.

Whilst autologous transplantation presents reduced risk of cross-contamination, cell performance and cell quantity are highly inconsistent with age and health status of the patients [7]. Human cells are a clinically relevant source of cells. To consolidate the clinical outcome of cell-based TE strategies, a robust and stable differentiation protocol is needed to improve the regenerative performances of human MSCs (hMSCs).

Soluble factors, such as growth factors (GFs), as well as environmental cues, such as altered levels of oxygen and mechanical signals, are factors commonly used to induce and enhance cartilage formation [18, 19]. Oxygen levels in AC are low and decrease with distance from the surface as oxygen diffuses through the extracellular matrix (ECM), from around 5% O₂ at the surface to 1% O₂ in the deeper regions of the tissue [20]. Chondrocytes metabolism is adapted to such hypoxic conditions and

orientated towards anaerobic glycolysis to produce cellular energy [21]. Thus, chondrocyte consumption of oxygen also contributes to the establishment of the oxygen gradient [22]. In addition, during cartilage injury and OA, cells experience non-physiological oxygen levels, highlighting the importance of a controlled oxygen environment [23]. Many studies have shown the positive role of hypoxia on the chondrogenic differentiation of hMSCs [24, 25]. However, most of these studies have compared hypoxia to environmental oxygen (21% O₂). Here a balance needs to be struck, as very low oxygen levels can also be detrimental to cell survival [26]. Hence, a better understanding of the effect of physiological oxygen levels on chondrogenesis could benefit the field of AC TE.

More recently, cell delivery of genetic material, plasmid deoxyribonucleic acid (pDNA) or ribonucleic acid (RNA), has been used to engineer cells by modifying the expression profile of existing genes to enhance cartilage regeneration [19, 27]. To date, one OA treatment, Invossa™, currently under the assessment of the Food and Drug Administration (FDA), consists in injections of gene-delivered transforming growth factor (TGF)- β 1 overexpressing chondrocytes [28]. In recent years, there has been increased interest in the therapeutic use of micro-RNAs (miRs), which are short noncoding nucleotides (20–24 nucleotides) that bind and modulate the translation of messenger ribonucleic acid (mRNA) [29]. Unlike pDNA, miRs can modulate the expression of several genes/signalling pathways as they target several mRNAs at the same time [30]. This valuable approach could also circumvent limitations associated with culture supplementation with exogenous GFs: absence of post-transcriptional modifications, off-target side effects or overexposure [31, 32]. Besides, the role of the TGF- β is still unclear as hypertrophy is often observed in TGF- β differentiated MSCs and it was demonstrated that TGF- β also plays a role during the development and progression of OA [33]. Several miRs are known to regulate cartilage homeostasis and MSC chondrogenic differentiation [34]. MiR-221 is of particular interest as it was studied in the context of both cartilage homeostasis and MSC chondrogenesis, but so far little is known about its potential applications in the MSC-based regeneration of hyaline cartilage [35, 36].

Finally, in the context of AC TE, scaffolds have been designed to provide a biomimetic and inductive environment for cell differentiation and tissue regeneration [37]. Examples include hydrogel-based scaffolds, whose high-water content is similar to the AC environment [38] and can be engineered to ensure sustained and physiological release of exogenous cues [39], ECM-based scaffolds, which retain tissue-specific biochemical cues [40], or synthetic polymer-derived scaffolds, which provide

mechanical stability in a highly reproducible manner [41]. Despite promising results, these approaches have been unable to support the development of truly biomimetic AC grafts [42]. This has motivated increased interest in developmentally inspired cartilage TE strategies, which often employ scaffold-free approaches that leverage the ability of cells to generate their own biomimetic ECM [15]. Cellular aggregates, microtissues and organoids can be engineered by leveraging the capacity of cells to self-organise, and can be used as transplantation therapeutics, *in vitro* tissue/organ models and biological building blocks for the engineering of larger three-dimensional (3D) tissues [43]. However, biofabrication using such microtissues can be challenging due to the difficulties in controlling their size, phenotype or their overall handleability [44]. In line with the 3Rs (Replacement, Reduction and Refinement) principles of *in vivo* animal research issued by the European Medical Agency (EMA) [45] and the United States FDA's new legislation which places less emphasis on animal drug testing prior to human trials [46], microtissue models with high biomimicry are of the highest interest. In addition, biofabrication, combining cellular aggregates, microtissues and/or organoids with 3D printing or other technologies, potentially enables the development of TE grafts with increased complexity, but still remains technically challenging in many aspects [47].

1.3. Thesis Objectives

The objective of this thesis is to use human stem/progenitor cells to engineer phenotypically stable hyaline cartilage microtissues, which can potentially be used as *in vitro* AC models or in the modular assembly of larger AC grafts. Specifically, this thesis will explore the use of human articular cartilage progenitors (hACPs), altered levels of oxygen, and the delivery of pro-chondrogenic anti-miR-221 to MSCs as strategies to engineer human hyaline cartilage microtissues. Such microtissues will then be used in a new biofabrication strategy with the goal of engineering large articular cartilage grafts with biomimetic composition and spatial organisation.

1.3.1. Aim 1—An Articular Cartilage Progenitor Cell Approach: Can Hyaline Cartilage Aggregates Be Engineered Using Human Articular Cartilage Progenitors?

This thesis will first explore the use of hACPs to engineer hyaline cartilage aggregates. First, the effect of cell number on cartilage-specific ECM production will be

assessed. This thesis will also explore if different cocultures of hACPs and hMSCs can be used to enhance cartilage tissue development.

1.3.2. Aim 2—An Oxygen Approach: Mimicking *in Situ* Oxygen Levels to Engineer Microtissues with Distinct Phenotypes

The second aim of this thesis is to explore the effect of altered oxygen levels, representative of the physiological levels found in native AC, on chondrogenesis of human bone marrow MSC-derived microtissues. Resulting phenotypes will be assessed and compared to the native tissue to evaluate their relevance for the engineering of zonally defined AC grafts. Finally, short exposure to altered oxygen levels will also be explored to assess the potential synergic effects of such cues on the chondrogenesis of hMSCs.

1.3.3. Aim 3—Modular Assembly of Zonally Defined Articular Cartilage Graft

Based on the results from Aim 2, oxygen-primed microtissues with distinct phenotypes will be used as biological building blocks for engineering the superficial and deep zones of AC using a modular assembly approach. A novel biofabrication method using melt electrowritten meshes will be explored to provide some structural support to the fusing microtissues whilst minimising the presence of foreign material within the engineered graft. Finally, the role of the orientation of growth (upwards or downwards) in directing collagen network organisation in such engineered grafts will be assessed.

1.3.4. Aim 4—A Silencing Approach: Transfection of Human Mesenchymal Stem Cells with Anti-miR-221 Supports the Development of Hyaline Cartilage Microtissues

Finally, delivery of anti-miR-221, a novel pro-chondrogenic therapeutic, using a polymeric vector, will be assessed in an attempt to engineer more hyaline cartilage-like microtissues using hMSCs. A dosage study will be performed to identify the effect of transfection dosage and regimen on cell viability, cell metabolism and microtissue differentiation. Large numbers of such ‘silenced’ microtissues will also be fused together in an attempt to engineer large-scale hyaline cartilage grafts.

2. Literature Review

2.1. Articular Cartilage

2.1.1. Composition and Organisation

The extracellular matrix (ECM) of articular cartilage (AC) is mainly composed of water (70–80% w/w), collagen (15% w/w), proteoglycans and hyaluronic acid, which all are organised into a complex three-dimensional (3D) network [3]. Traditionally, only one cell type, the chondrocyte, was described within the ECM and their slow metabolism contributes to the limited regeneration capacity of the tissue [48]. Type II collagen represents 90–95% of total collagen proteins. It is a 300 nm long homotrimer coil, stabilised by intramolecular and intermolecular bonds that participate to its tensile strength and stiffness as well as its resistance to enzymatic degradation by collagenase [3, 49]. Other minor subtypes of collagen like type I, IV, V, VI, IX and XI help to stabilise type II collagen fibres [3]. For example, type VI and type IX collagens, present in the pericellular region, stabilise type II collagen via non-covalent and covalent interactions, respectively [2]. Globular type X collagen, which is typically synthesised by hypertrophic chondrocytes, makes up 18% of collagen in the deep region of AC (see paragraph below describing the different layers within AC) [50].

Proteoglycans are a family of proteins which is highly decorated with covalently bound glycosaminoglycans (GAGs) lateral chains, mainly chondroitin sulphate and keratan sulphate (aggrecan) [51]. In addition, another type of GAGs, hyaluronic acid (HA), covalently binds aggrecan [51]. Repulsion between the negative charges of GAGs contributes to the tissue resistance to compression and its elastic deformation, while the collagen fibre network limits tissue swelling [52].

The spatial composition and organisation of the ECM, as well as changes in cell morphology, define the different layers of AC (**Figure 2.1**). In the top layer (~10–20% of the total AC thickness), the chondrocytes are flattened and synthesise lubricin (proteoglycan 4) onto the AC surface which protects it and limits friction, whilst collagen fibres (mainly type II collagen) are aligned parallel to the articular surface. In the middle layer (~40–60% of the total AC thickness), chondrocytes are rounder and collagen fibres are thicker and organised into the characteristic Benninghoff's arcade

pattern [38, 53]. Finally, the deep layer (~30% of the total AC thickness) is rich in proteoglycans (highest content of the AC; mainly aggrecan and HA) [2, 3]. Chondrocytes are enlarged and form columns that are perpendicular to the surface, while collagen fibres are also aligned perpendicular to the joint surface [38]. In healthy tissue, cell density varies from approximately 24,000 cells/mm² (in the top layer) to 8,000 cells/mm² [54]. The tidemark separates the deep layer from the calcified region that is attached to the subchondral bone. The latter participates in load transmission to the bone and contains hypertrophic chondrocytes and type X collagen which contributes to its calcification [2].

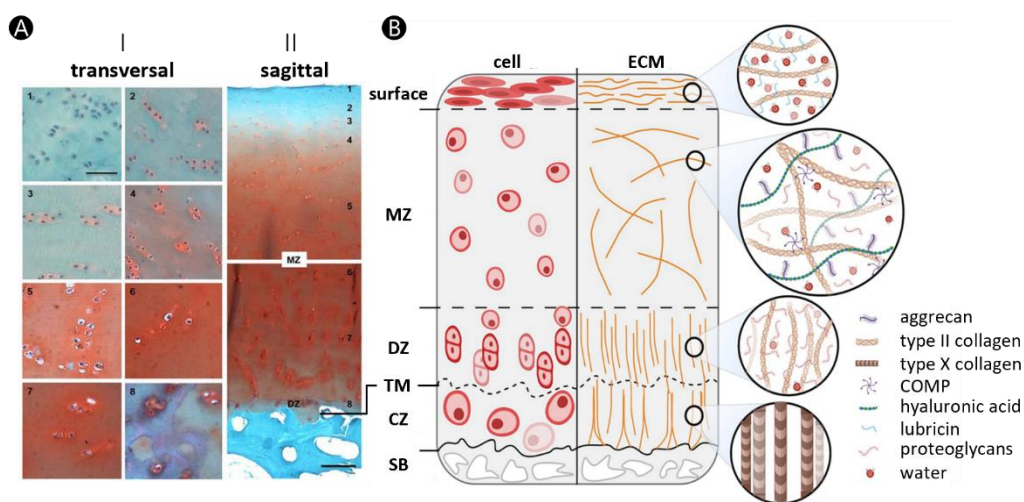


FIGURE 2.1 Articular cartilage cell morphology and extracellular matrix zonal organisation.

Gradient distribution of proteoglycans (red staining) from the surface to the tidemark in healthy tissue (A-II). Numbering of transversal images (A-I) corresponds to depth as indicated on the sagittal section. Sections 1–4 were taken 20 μ m apart, sections 5–8 were taken 400 μ m apart. MZ: middle zone; DZ: deep zone. Adapted from [55]. Schematic representation of the composition and structure of AC (B). TM: tidemark; CZ: calcified zone; SB: subchondral bone. Scale bar = 200 μ m (sagittal images)/50 μ m (transversal images). COMP: Cartilage Oligomeric Matrix Protein (glycoprotein). Adapted from [56].

2.1.2. The Origin of Articular Cartilage

At birth, hyaline cartilage is found in both the joint and the growth plate. While the formation and regulation of the growth plate are well understood, much less is known about AC development [57]. Growth plate is transient as it undergoes endochondral ossification into bone at puberty. In contrast, AC remains stable through the entire life.

The embryonic development of AC begins with the proliferation and condensation of mesenchymal cells, shortly followed by their differentiation into chondrocytes forming a continuous cartilage anlage [58, 59]. At the location of the future joint, the interzone forms where cells elongate and lose their *COL2A1* and *ACAN* expression. This suggests a direct lineage between these interzone cells with anlage chondrocytes (dedifferentiation), while growth and differentiation factor-5 (*GDF-5*) is believed to play an important role in this process [59]. It has also been shown that an additional GDF-5 positive cell population originates from outside the anlage and is recruited into the developing joint [59]. Later on, GDF-5 expressing cells were found to give rise to many joint tissues, including AC [60].

Joint cavitation at the interzone occurs at the same time as hypertrophic chondrocytes are identifiable in the surrounding cartilage anlage and is potentially explained by two different hypotheses: apoptosis of central cells of the interzone or filling of hyaluronan-rich fluid [61]. Non-apoptotic interzone cells may contribute to the formation of the superficial layer of AC as well as other joint structures such as the ligaments, meniscus and synovial membrane [58].

The newly opposing cartilage anlage bodies become the template for endochondral ossification of cartilage into bone tissue, with an initial ossification centre at the anlage diaphysis and a secondary ossification centre developing at the cartilaginous anlage epiphysis, both characterised by hypertrophic and proliferating chondrocytes, and vessel invasion [62]. Flanked between both ossification centres, the growth plate will undergo the same endochondral ossification mechanism during growth, resulting in the elongation of the developing bones [61]. One hypothesis of the origin of AC suggests that it originates from the remnant cartilage template that simply failed to be replaced by bone [60].

Two AC growth mechanisms have been proposed in the literature: interstitial and appositional growth. The 'interstitial growth' hypothesis suggests that as cells distinguish themselves into the three different zones, found in mature AC, they each thicken by chondrocyte enlargement and ECM accumulation [63]. The 'appositional growth' hypothesis suggests that cells from the superficial layer subsequently mature into the other regions of AC [57, 64]. In support of this hypothesis, the labelling of proteoglycan (PRG)-4 expressing cells from the surface layer of developing embryonic joints were found in all regions of AC in 12-month-old mice (**Figure 2.2A**), while the progeny of PRG-4 expressing cells from the surface layer of 1-month-old mice were only found in the top two thirds of AC in 18-month-old mice (**Figure 2.2B**). These results indicate that the deep region develops first, followed by the middle region [57]. Hence,

the ‘appositional growth’ hypothesis suggests that the surface layer is responsible for the differential synthesis of ECM and the expression of growth factors (GFs) driving the development of the entire tissue [65, 66].

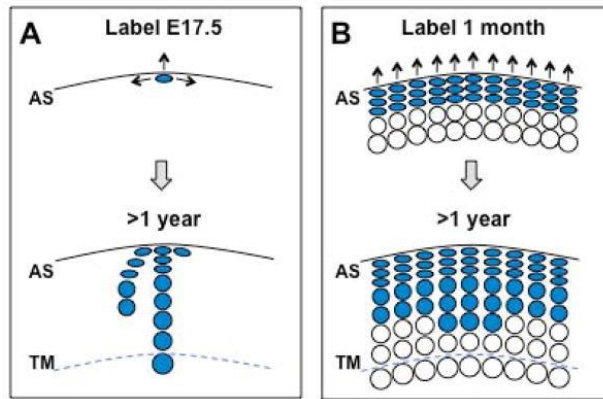


FIGURE 2.2 Schematic of the sequential birth of AC layers by appositional growth. (A) PRG-4 positive cells (blue) from E17.5 extended as columnar clones throughout the tissue depth including possibly under the tidemark in 12-month-old mice giving birth to the deep layer of AC first. **(B)** Surface PRG-4 positive cells from 1-month-old mice extended only to two thirds of the AC depth in 18-month-old mice, giving rise to the middle region of AC. E17.5: embryonic day 17.5; AS: articular surface; TM: tidemark. Adapted from [57].

2.2. Articular Cartilage Progenitor Cells

2.2.1. Identification of the Articular Progenitor Cell Population

While the origin of AC chondrocytes is not clearly determined yet, a new subpopulation of progenitor-like cells, different from resident chondrocytes, was isolated (based on differential adhesion to fibronectin) from the superficial layer of bovine AC (~10% of the total cell population were found to adhere) by Dowthwaite *et al.* [64]. Since then, similar populations of cells have been identified in other species, including humans, horses and mice [57, 67].

These newly isolated cells present stem-like properties: self-renewal (clonality), multilineage differentiation, and expression of stem cell-related surface markers [67]. In a standard colony-forming assay, 1–2% of isolated cells possessed high colony forming efficiency (>32 cells/colony) [64]. Clonality was maintained upon passaging, and *in vitro* proliferation was confirmed during *in vitro* expansion of healthy and osteoarthritic progenitor cells, which were able to undergo 18 and 13 population doublings, respectively [68].

Tripotential differentiation showed that progenitor cells were able to undergo chondrogenic differentiation, with limited signs of hypertrophy compared to mesenchymal stem/stromal cells (MSCs), and limited capacity to undergo osteogenic and adipogenic differentiations (**Figure 2.3-A**) [69]. However, other studies have shown successful osteogenesis, but to a lesser extent than MSCs, and even successful adipogenic differentiation [70, 71]. Under low oxygen conditions (5% O₂), human articular cartilage progenitors (hACPs) produced a hyaline cartilage ECM containing a dense type II collagen network and low levels of type I collagen. Furthermore, unlike human MSCs (hMSCs) which produced type X collagen (a marker of early hypertrophy), hACPs were able to maintain a hyaline cartilage phenotype as observed by the absence (or weak staining) of type X collagen (**Figure 2.3-B**) [69]. At the gene level, *ACAN* and *COL2A1* expression levels were lower in ACPs compared to chondrocytes after 28 days of chondrogenic differentiation, pointing to some subtle differences between the differentiation of both cell types [68]. In general, these results highlight the potential of articular cartilage progenitors (ACPs) to generate mature and stable hyaline AC.

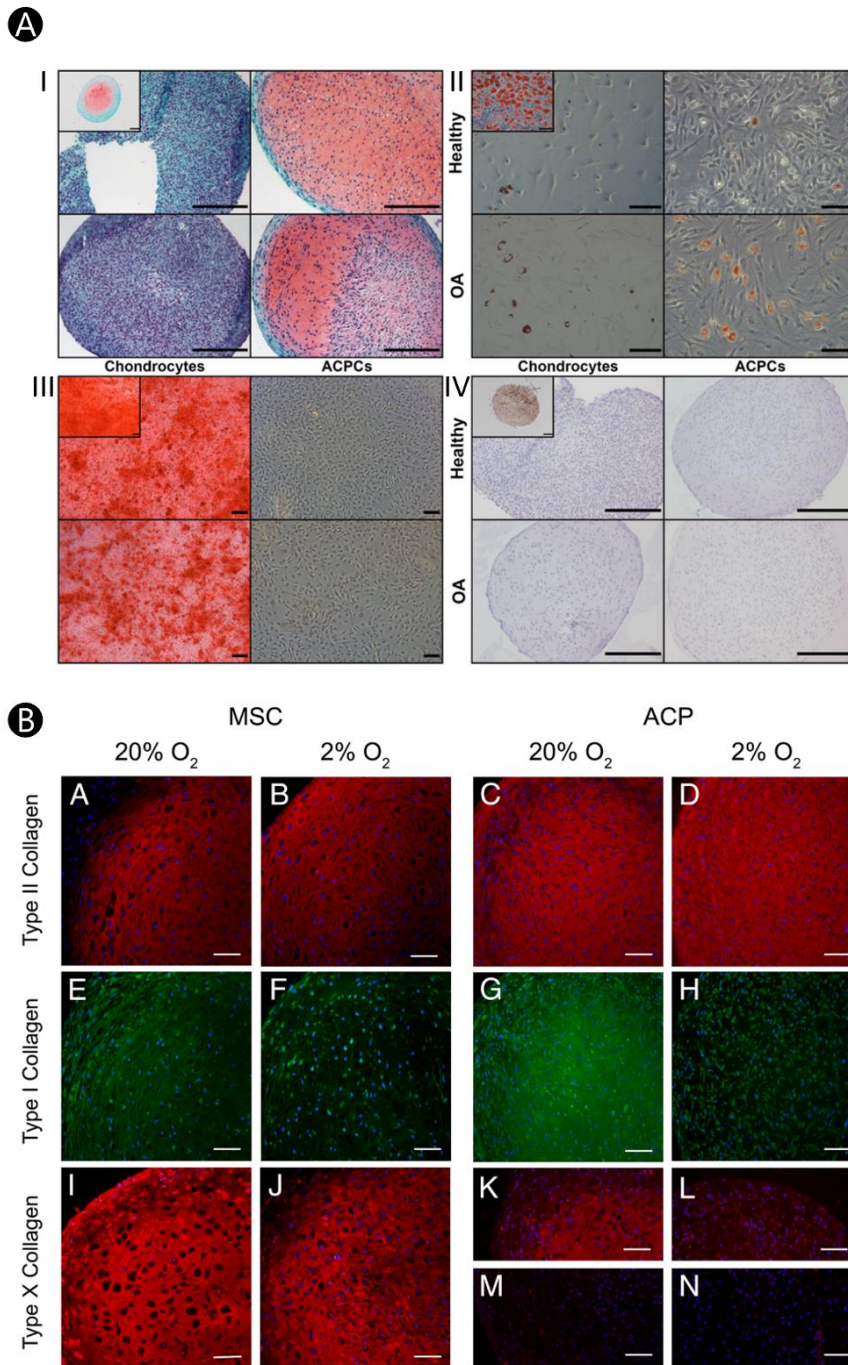


FIGURE 2.3 Tripotentiality of hACPs. Staining of proteoglycans (A-I), lipid droplets (A-II), calcium accumulation (A-III) and type X collagen (A-IV), after chondrogenic differentiation (A-I, A-IV), osteogenic differentiation (A-III) or adipogenic differentiation (A-II) of healthy and osteoarthritic (OA) chondrocytes and articular cartilage progenitor cells (ACPCs). Inserts represent results from corresponding differentiation in bone marrow-derived MSCs. Scale bar = 200 μ m. Adapted from [68]. (B) Immunofluorescence staining of collagen subtypes in mesenchymal stem cells (MSCs) and articular cartilage progenitors (ACPs) pellets after 14 days of chondrogenic differentiation under normoxia (20% O_2) or physioxia (2% O_2): type II collagen (A, B, C, D), type I collagen (E, F, G, H) and type X collagen (I, J, K, L, M, N). Donor variability of type X collagen staining was observed (K-L versus M-N). Scale bar = 100 μ m. Adapted from [69].

Attempts have been made to characterise ACPs using MSC-like cluster of differentiation (CD) surface markers like CD105 (transforming growth factor- β receptor III), CD73, CD90, CD106 and CD44 (hyaluronan receptor), or using markers of putative chondro-progenitor cells like CD29 (integrin β 1), CD49e (integrin α 5), CD151, CD146, CD166 [72, 73]. Despite their extensive proliferation capacity and chondrogenic potential, identification of clear specific markers of ACPs would help to purify the isolated population from chondrocytes and harmonise characterisation results of this new population of cells [74].

Finally, it has been suggested that ACPs are able to migrate to the site of injury which highlights their potential in novel tissue engineering (TE) approaches, especially those based on the recruitment of endogenous cells. In a blunt impact model, ACPs migrated to the injury site after 7 to 14 days in response to cellular lysate [75]. Migration of ACPs was also stimulated by exposure to platelet-derived growth factor-BB, insulin-like growth factor 1 or supernatant of *in vitro* traumatised cartilage, but was inhibited by the addition of inflammatory cytokine interleukin-1 beta or tumour necrosis factor alpha [76].

2.2.2. Articular Cartilage Progenitors and Tissue Engineering of Articular Cartilage

Human ACPs are increasingly being used in the engineering of hyaline cartilage tissue. For example, when seeded onto nanofibrous polycaprolactone/poly-L-lactic acid scaffolds, *COL2A1* and *COL1A1* expression was higher in hACPs than in adipose tissue-derived MSCs [77]. Similarly, ACPs from osteoarthritic hip AC were isolated by tissue outgrowth, seeded on a polyglycolic acid-hyaluronic acid scaffold and implanted ectopically in nude mice for 28 days [78]. Resulting tissues contained type II collagen but little type X collagen (a slight positive signal was observed at the surface of the scaffold), suggesting that tissues generated using ACPs undergo minimal hypertrophic differentiation *in vivo* (**Figure 2.4-A**) [78]. In another study, hACPs were seeded in a fibrin hydrogel polyurethane composite scaffold and were mechanically stimulated or/and adenoviral-mediated transduced with the bone-morphogenetic protein (BMP)-2 [79]. Interestingly, as early as day 7 of culture in growth-factor free medium, mechanical stimulation alone upregulated *COL1A1* compared to the unloaded group but had no effect on *COL10A1*. The overexpression of BMP-2 enhanced the expression of *COL10A1* and *ALP* suggesting that ACP capacity to resist hypertrophy decreases with long-term exposure to BMP-2 [79]. Platelet-rich plasma (PRP) hydrogels, containing transforming growth factor (TGF)- β and platelet-derived growth factor, were used to

support the differentiation of ACPs and showed positive staining of GAGs in the presence or the absence of external GFs, highlighting the bioactive potential of PRP-derived biomaterials (**Figure 2.4-B**) [80].

One pilot clinical study was reported in humans and demonstrated the capacity of hACPs, seeded in fibrin hydrogels, to generate a tissue rich in proteoglycans and type II collagen, in the absence of type I and X collagens [81].

Anderson *et al.* seeded hACPs on fibronectin-coated surfaces to fabricate large constructs [69]. After 28 days of culture in hypoxia (5% O₂), the equilibrium modulus of these scaffold-free tissues was within the same range as native adult AC. Tissues were richer in type II collagen than type I collagen, they did not contain type X collagen and they contained less pericellular type IV collagen compared to chondrocytes (**Figure 2.4-C**) [69]. Like MSCs, oxygen conditions supported a reduction of type I and type X collagens expression in ACPs [82].

Finally, hACPs isolated from osteoarthritic patients showed decreased chondrogenic capacity compared to cells isolated from healthy donors. This capacity was also reduced with disease progression (OA grades 1–2 versus OA grades 3–4) [83, 84]. Interestingly, the addition of BMP-9 during TGF- β 1-driven chondrogenic differentiation of passage 1 osteoarthritic hACPs supported higher GAG accumulation and higher *COL2A1* and *COL1A1* expression without affecting *COL10A1* expression [85].

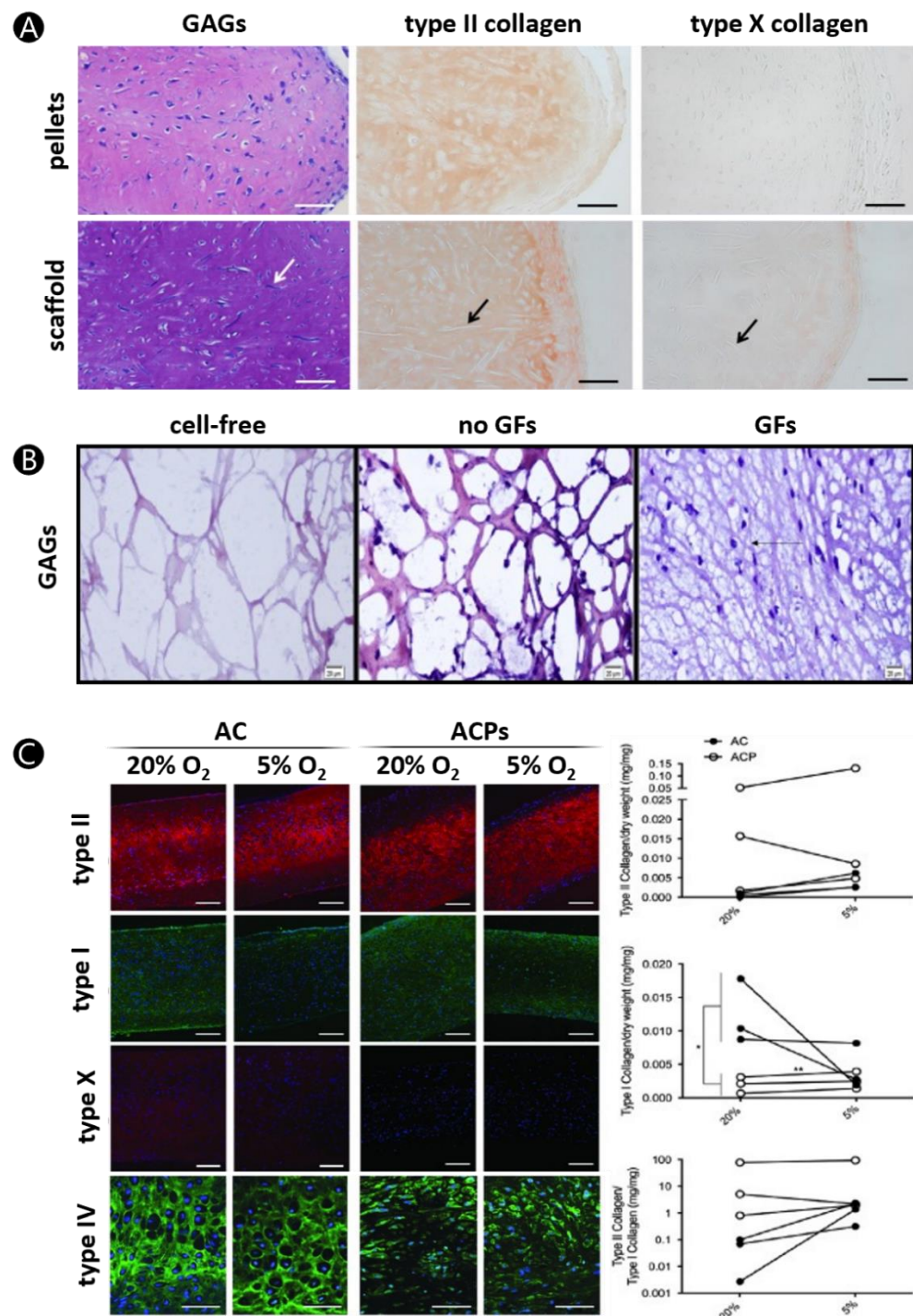


FIGURE 2.4 Current TE studies including hACPs. (A) Staining of hACP-derived pellets and TE constructs following subcutaneous implantation. Arrows indicate remnants of scaffold fibres. Scale bar = 50 μ m (pellet)/100 μ m (scaffold). Adapted from [78]. (B) Alcian blue staining of GAGs secreted by hACPs in platelet-rich plasma scaffolds. Arrows indicate matrix surrounding cells that were counterstained with Nuclear Fast Red. Representative images. 40X. GFs: growth factors. Adapted from [80]. (C) Immunofluorescence staining of collagen subtypes and ELISA quantification of type II and type I collagens of scaffold-free hACP-derived constructs. Scale bar = 100 μ m (type II, type I, type X)/20 μ m (type IV). AC: articular chondrocytes; type II: type II collagen; type I: type I collagen; type X: type X collagen; type IV: type IV collagen. Adapted from [69]. GAGs: glycosaminoglycans.

2.3. Cartilage Tissue Engineering Using Microtissues

TE aims at providing long-term solutions to repair or restore damaged and degenerated tissues or organs whose function has been impaired [86, 87]. Traditional TE approaches involve the combined use of cells, soluble growth factors and biodegradable scaffolds [88]. However, these approaches are still challenging. Timely scaffold degradation, phenotype alteration or obstruction of biophysical and biomechanical cues are commonly reported [89]. Developmentally inspired approaches have emerged as a promising alternative enabling the regeneration of tissues, including AC, by mimicking normal developmental steps [90]. These key steps include cell condensation, proliferation and differentiation, matrix deposition and tissue maturation [87]. Hence, such approaches solely rely on the ability of cells to produce their own ECM [15]. *In vitro*, cell condensation can be achieved on nonadherent substrates, using spinner flasks or by the hanging drop technic, resulting in tissues with various dimensions [91, 92]. While condensation supports abundant cell-cell and cell-matrix interactions, cellular aggregates have been shown to enhance cell viability, cell differentiation and matrix production [43]. For example, bone marrow-derived MSCs, self-assembled into a large disc and presented with appropriate GFs, were successfully differentiated into a cartilage-like tissue enriched in GAGs and type II collagen [93].

Large constructs are often associated with diffusional gradients resulting in inhomogeneous matrix deposition and the appearance of a necrotic core [87]. Hence, in the field of cartilage engineering, studies have demonstrated that lower initial cell numbers promote chondrogenesis. Microtissues of only hundreds of dedifferentiated articular chondrocytes supported cell redifferentiation and cartilaginous ECM deposition [94]. In another study, the expression of *COL2A1* and *ACAN* in chondrocyte-derived aggregates was significantly upregulated with the decrease of initial cell number [95]. In addition, comparison between these scaled-down aggregates with single cells (both seeded into a hydrogel) improved the *COL2A1/COL1A1* expression ratio [95]. Finally, the differentiation of MSC-derived aggregates with lower initial cell number has shown to enhance ECM deposition and suppress the formation of a necrotic core compared to larger aggregates [96].

Such small diameter microtissues are used as tissue models in a variety of *in vitro* assays or injected *in vivo* as therapeutic solutions [43]. However, their application in TE usually requires the fusion of multiple individual microtissues into a continuous graft with clinically relevant size [92]. Microtissues have been used as biological

building blocks to engineer AC [97]. The fusion of a few cartilaginous microtissues was demonstrated onto nonadherent dishes and supported stronger proteoglycan and type II collagen deposition than individual microtissues [98]. In another study, external constraints were applied onto numerous microtissues to ensure proper contact area and to guide their fusion [99]. While this simple method demonstrated the engineering of large tissues with homogeneous matrix deposition and native-like biomechanical properties, and the integration with surrounding tissues (bone matrix plug and *ex vivo* cartilage defect model), it also highlighted that the fusion ability of such individual microtissues decreased with their maturation stage.

Recapitulating the complex organisation of AC, defined by spatial gradients in tissue composition and architecture, is central to the regeneration of functional engineered grafts [100]. Therefore, different biofabrication strategies have emerged and aim at guiding the fusion of microtissues into organised constructs. In one study, magnetic particles were internalised within stem cell-derived spheroids and were used to guide the fusion of these 'magnetic spheroids' into a cartilage sheet [101]. Several bioprinting technologies have enabled the biofabrication of scaled-up AC graft with more complex geometry and organisation [102]. Aspiration assisted 3D bioprinting (ABB) allows the aspiration of individual microtissues and their precise positioning into compact and complex 3D geometries prior to fusion [102]. As a proof of concept, pyramids and rings of microtissues were successfully printed (into a hydrogel supporting bath), fused and differentiated into cartilage [103]. Besides, ABB of chondrogenic and osteogenic microtissue layers supported the biofabrication of the osteochondral interface [104]. In the Kenzan method, microtissues are skewered in a needle array which maintains them in a predefined compact geometry [105]. MSC-derived grafts fabricated with this method supported *in vivo* cartilage repair. To date, encapsulated bioprinting has shown potential to precisely deposit chondrogenic spheroids containing bioinks, but the low spheroid density prevented their fusion [106].

These scaffold-free approaches have enabled the engineering of large cartilage grafts, but their mechanical stability is still poor. The modular assembly of microtissue building blocks and 3D printed scaffolds is sought to mitigate these limitations [102]. Thereby, polymeric macroporous scaffolds, printed by fused deposition modelling, were seeded with phenotypically distinct microtissues to engineer the osteochondral unit [107]. The printed scaffold guided the fusion of microtissues and the organisation of the collagen network which was similar to the native tissue, and the implantation of this hybrid graft into an osteochondral defect supported joint resurfacing. Melt

electrowriting (MEW), which enables the printing in presence of an electric field of scaffolds with controlled pore geometry and micron sized fibres, was also used to modulate the differentiation of hMSCs [108]. By printing a MEW scaffold with a gradient in pore size, an increase in chondrogenic marker was observed along the gradient [109]. MEW meshes have also been combined with cellular aggregates to generate regenerative patches in the context of bone repair [110]. A couple of studies have also shown that collagen fibre alignment can be achieved in microtissue-forming MEW scaffolds [111, 112]. While these hybrid approaches supported the engineering of organised cartilage graft with biomechanical properties comparable to the native tissue, the fabrication of hybrid cartilage graft with clinically relevant dimensions is still challenging.

2.4. Gene Silencing

2.4.1. Biogenesis of Ribonucleic Acid Interference Molecules

The mechanisms of gene silencing, also known as gene interference, were described for the first time in *Caenorhabditis elegans* by injection of double-stranded ribonucleic acid (RNA) which enabled the regulation of muscle-related gene expression [113]. Further research identified the presence of well-conserved small (21–23 nucleotides) noncoding single-stranded RNAs, called microRNAs (miRNAs or miRs), which possess sequence complementarity with messenger RNA (mRNA) and prevent their translation into proteins [114]. A half of miRs comes from intragenic deoxyribonucleic acid (DNA) sequences, mostly intronic, whose transcription is carried by RNA polymerase II and III, while the other half is intergenic and is transcribed independently of genes, both resulting in double-stranded oligonucleotides [115]. There are two maturation pathways, canonical and non-canonical, depending on the structure of the double-stranded RNA precursor (short hairpin RNA (shRNA), mirtrons, pri-miRNA, m⁷G-pre-miRNA), the subsequential enzymatic cleavages (Dorsha/DGCR8 or Dorsha/DGCR8-independent or Spliceosome), the cytoplasm exporting complex (Exportin 5 or Exportin 1) and the unpairing mechanism (Dicer or AGO2) [115]. Both pathways result in cytoplasmic mature single-stranded RNAs. These RNAs bind and silence mRNA via the miRNA-induced silencing complex (RISC) with full complementarity (small interfering RNA (siRNA)) or partial complementarity (miRNA) upon a short sequence (2 to 8 nucleotides) at the 5' end (called the 'seed region') of the miRNA and the conserved 3' untranslated region (3'UTR) of the mRNA (**Figure 2.5**) [116]. Finally, the mRNA degradation mechanisms are not fully understood but could

result from preventing ribosome recruitment or ribosomal elongation, or from indirect degradation of the newly synthesised protein [117].

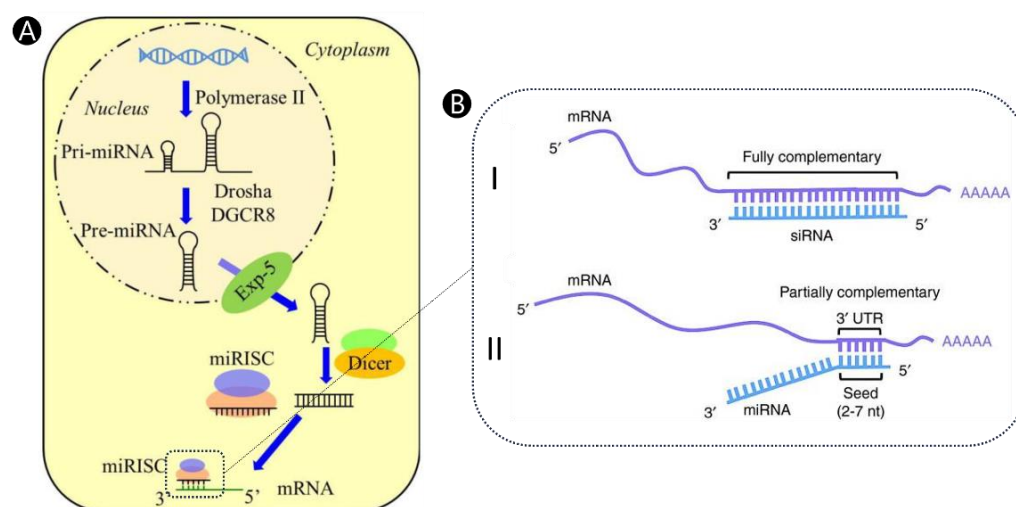


FIGURE 2.5 Biosynthesis, maturation and targeting of RNA interference molecules. (A) Example of the canonical pathway biogenesis of miRNA. Pri-miRNA: primary microRNA; Pre-miRNA: precursor microRNA; Exp: exportin; miRISC: microRNA-induced silencing complex. Adapted from [118]. **(B)** mRNA target binding by (I) full complementarity for siRNA or (II) partial complementarity for miRNA. siRNA: small interfering RNA; UTR: untranslated region; miRNA: microRNA. Adapted from [116]. mRNA: messenger RNA.

2.4.2. Gene Silencing Potential for Tissue Engineering

2.4.2.1. Different Types of Gene Silencing Therapeutics

GFs have been largely used in the regeneration of musculoskeletal tissues as illustrated by the commercialisation of INFUSE® from Medtronic, a bone graft substitute delivering recombinant BMP2 to promote bone formation [119]. GF-based regeneration presents several limitations and risks such as short *in vivo* half-life, ectopic bone formation, cytotoxicity and carcinogenicity [27, 120]. Regulation of differentiation pathways via GF-mediated cell surface receptors may involve many proteins to specify a lineage, while regulation of pivotal genes can potentially be achieved by selective miR-mediated silencing [121]. Understanding gene silencing mechanisms has provided tissue engineers with a powerful new tool for directing tissue and organ regeneration.

Current therapeutic strategies seek either to mimic miR sequences, thereby enhancing, replacing or restoring the endogenous effect of a miR of interest, or to be complementary to miR sequences, thereby antagonising a specific miR effect [122]. MiR mimics include synthetic double-stranded RNAs or short hairpin RNAs. Both are included into the cell endogenous maturation mechanisms, therefore, they can only be

lightly chemically modified and can induce the saturation of the maturation machinery [122]. On the other side, miR antagonists include single-stranded antisense anti-miRs (or antagomiRs), miRNA sponges that contain several miRNA complementary binding sites, uncharged polymeric peptide nucleic acid mimics or miR masks that specifically prevent miRs from binding to mRNA. They are usually chemically modified to improve stability and affinity (e.g. 2'-O-methyl-modified ribose sugar, terminal phosphorothioate linkage, cholesterol group at the 3' end, 2'-O-methoxyethyl, 2'-fluoro, locked nucleic acid) [117, 118]. Finally, the miR biogenesis steps, previously described, are all potential targets for small pharmacological molecules, for example by antagonising Dicer or Drosha enzymatic sites [123].

2.4.2.2. Delivery of Gene Silencing Therapies

Effective delivery of nucleic acid is essential to ensure optimal therapeutic effect. Naked miRs are negatively charged with limited cell membrane attachment and inability to escape the endo-lysosomal degradation [27]. Delivery vectors that can enhance cellular uptake are divided into two types: viral and nonviral (physical and chemical methods) vectors.

Non-pathogenic viral vectors benefit from the high delivery efficiency inherent to virus infection mechanisms, their ability to specifically target cells and integrate the cellular replicative machinery. For this reason, they represent around 70% of gene therapy based clinical trials, including retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses [124]. However, use of viral vectors carries several limitations associated to manufacturing, inherent immunogenicity and most importantly mutagenic potential by insertion into host DNA sequences or modification of gene expression [27, 125].

Physical methods of transfection enhance cell membrane permeability by applying physical stimuli such as electric pulses (electroporation), temperature (thermal assisted gene transfer), kinetic energy (biolistics), pressure (microinjections, hydrodynamic gene transfer), laser radiation (opto-injection, laser-influenced stress waves, photochemical internalisation, light-absorbing particles), ultrasound (ultrasound-assisted gene transfer), magnetic field (magneto-transfection) or mechanical force (mechanical massage) [126]. The high reliability of many of these methods is counterbalanced by the low cellular viability as the integrity of the cell membrane is often compromised [126].

Nonviral chemical vectors directly complex nucleic acids and support their entry into the cell. Lipids are the most widely used chemical vectors and there are several commercially available products which differ in their lipidic composition and targeted cell type: Lipofectamine, siPORT™ *Lipid*, SilentFect™, DharmaFECT, Gene Eraser™, RiboJuice™ [127, 128]. Cationic lipids interact with RNAs by electrostatic interactions to form a so-called lipoplex, which increases the encapsulation efficiency compared to uncharged lipids. They are also able to bind the cell membrane and the endosomes which promote intracellular liberation of the cargo [129]. However, lipids have low transfection efficiency and low cell viability due to their membrane disruptive entry mechanism [130]. Synthetic polymer vectors are easy to synthesise and functionalise, they have a high encapsulation efficiency (e.g. polyethyleneimine) and are easily hydrolysed and metabolised in the Krebs cycle (e.g. poly lactic-co-glycolic acid) [130]. Natural polymers, usually polysaccharides, bear ionisable anionic groups which highly interact with the negative charges of nucleic acids [128]. Finally, inorganic materials like gold, mesoporous silica nanoparticle or iron oxide nanoparticles have also been studied, and their efficiency depends on particle size, solubility and surface chemistry/topography [129].

2.4.2.3. Poly-Beta Amino Esters as Delivery Vectors

The potential of gene delivery strongly relies on the development of efficient, safe, and robust systems. Poly-beta amino esters (pBAEs), synthesised by the addition of diacrylates and amine monomers, are positively charged polymers whose structure, including biodegradation products, has demonstrated low cytotoxicity by outperforming polyethyleneimine by 3 to 10 times [131]. Indeed, the ester backbone is highly sensitive to hydrolysis, with a fast biodegradation rate in aqueous solutions [132].

pBAEs have been used to condensate via electrostatic interactions DNA and RNA into stable polyplex nanoparticles [133, 134]. The overall positive charge of pBAEs is also favourable to its retention within cartilage which contains negatively charged GAGs [135]. In their study, Perni *et al.* manufactured a mono-directional diffusion chamber and found that AC uptakes and retains pBAEs for more than two hours in healthy and diseased tissues [135].

Transfection efficiency of end-modified pBAEs was validated in hMSCs, demonstrating up to fourfold increase compared to Lipofectamine [136]. Upon cell entry, acidic endosomal degradation limits intracellular availability of the cargo, but

cationic polymers, including pBAEs, can escape degradation by virtue of the proton sponge effect [27, 137]. Protonation of amine groups ($pK_a \sim 4.5$ equivalents to the endosomal pH) from the continuous influx of hydrogen ions, to lower the pH of the endosome, results in swelling and rupturing of the endosome liberating the genetic cargo to the cytoplasm [27].

The versatility of pBAE polymerisation allows the inclusion of several monomer moieties such as alcohol, ethers or tertiary amines, resulting in a vast library of functionalised molecules [138]. Terminal modification of pBAEs with naturally inspired oligopeptides has improved transfection efficiency and cell viability, highlighting the potential of pBAE vectors [139, 140]. pBAE-mediated gene delivery for the treatment of cancer was already assessed *in vitro* and *in vivo* [133]. However, to date, few studies report the biocompatibility and efficiency of pBAEs and miRNA polyplexes for the TE of musculoskeletal tissues.

2.4.3. Role of Micro-RNAs During Chondrogenesis

Chondrogenesis is a complex process that is spatially and temporally regulated by specific transcription factors and growth factors. The SRY-Box Transcription factor 9 (SOX9), described as the master regulator of chondrogenesis, is highly expressed in chondrocyte precursors where it directly upregulates the expression of *COL2A1*, *COL9A1*, *COMP* and *ACAN* and downregulates the expression of the osteogenic master regulator Runt-related transcription factor 2 (*RUNX2*) [141, 142]. The transcription factor hypoxia-included factor 1 α and GFs, including TGF- β , BMP, fibroblast growth factors or Indian hedgehog-parathyroid hormone-related peptide positively regulate *SOX9* while Wnt, Notch or the retinoic acid pathways negatively regulates the expression of *SOX9* [142]. During endochondral ossification, levels of *SOX9* are lowered in hypertrophic chondrocytes while *RUNX2* drives osteogenic differentiation [142, 143].

In addition to protein-mediated regulation of chondrogenesis, several modulatory miRs have recently been identified to regulate the fate of hMSCs. The miR-30a sequence is fully complementary to the *SOX9* 3' UTR sequence and the delivery of miR-30a mimic resulted in the inhibition of chondrogenesis in hMSCs, even in the presence of TGF- β 3, by sequestration of *SOX9* [144]. Similarly, another anti-chondrogenic miR, miR-495, has been shown to possess *SOX9* sequence complementary. It has been identified in hMSC pellets differentiated in the presence

of TGF- β 3, and transfection with anti-miR-495 has shown to increase the deposition of GAGs and collagen [145].

Other miRs indirectly regulate the expression of *SOX9*. After identifying by miR profiling that miR-29a was involved in the regulation of chondrogenesis, the delivery of pre-miR-29a (miR-29a precursor) and antagomiR-29a directly targeted Forkhead box O3 (*FOXO3A*), which targets *SOX9*. Thus, transfection of hMSCs with the anti-chondrogenic miR-29a mimic maintains cells in an undifferentiated state [17, 146]. Karlsen *et al.* analysed the miR profile of hMSCs undergoing chondrogenesis in alginate hydrogels and identified an upregulation of miR-140-5p and a downregulation of miR-221, while opposite results were observed in dedifferentiated chondrocytes [147]. In addition, transfection of anti-miR-140-5p decreased protein levels of *SOX9*, type II collagen and GAGs. The authors hypothesised that miR-140-5p targets *SOX9* via the Ras-related protein Ral-A (*RALA*) [147]. Another pro-chondrogenic miR, miR-520d-5p, has been shown to target histone deacetylase 1 (*HDAC1*), which compacts DNA and participates in the modulation of gene transcription [148, 149]. Chondrogenic differentiation of hMSC monolayers resulted in a significant decrease of *HDAC1* levels. Similar results were observed after transfection with a miR-520d-5p mimic in hMSC monolayers and in primary or osteoarthritic chondrocytes, which was associated with downregulation of an ECM catabolic enzyme, matrix metalloproteinase 13 [149]. Finally, miR-574-3p is upregulated during early chondrogenesis, while transfection of pre-miR-574-3p (miR precursor) resulted in the upregulation of *COL2A1* and *COL10A1*, likely via the silencing of the retinoid X receptor (*RXR*) [142, 150].

Previously described miR-mediated regulation of chondrogenesis was carried out in presence of TGF- β . Transfection of miR-23b induced the chondrogenesis of hMSCs in absence of exogenous growth factors, and resulted in the upregulation and synthesis of aggrecan, which was mediated via the protein kinase A pathway [151]. Similarly, silencing of miR-221 via the delivery of anti-miR-221, a miR-221 inhibitor, supported the deposition of type II collagen by hMSCs in absence of TGF- β 3 [33]. Unlike other miRs, specifically the highly characterised miR-140 [146], which promote stem cell differentiation into chondrocytes but also chondrocyte hypertrophy and endochondral ossification, to date, miR-221 has been identify to specifically regulate the differentiation of MSCs into chondrocytes only [152]. In addition, hydrogel-mediated delivery of anti-miR-221 effectively guided endogenous cartilage repair *in vivo*, highlighting the potential of miR-221 targetting for cartilage tissue engineering [153]. A summary of pro-chondrogenic and anti-chondrogenic miR and their confirmed or potential targets are described in **Figure 2.6**.

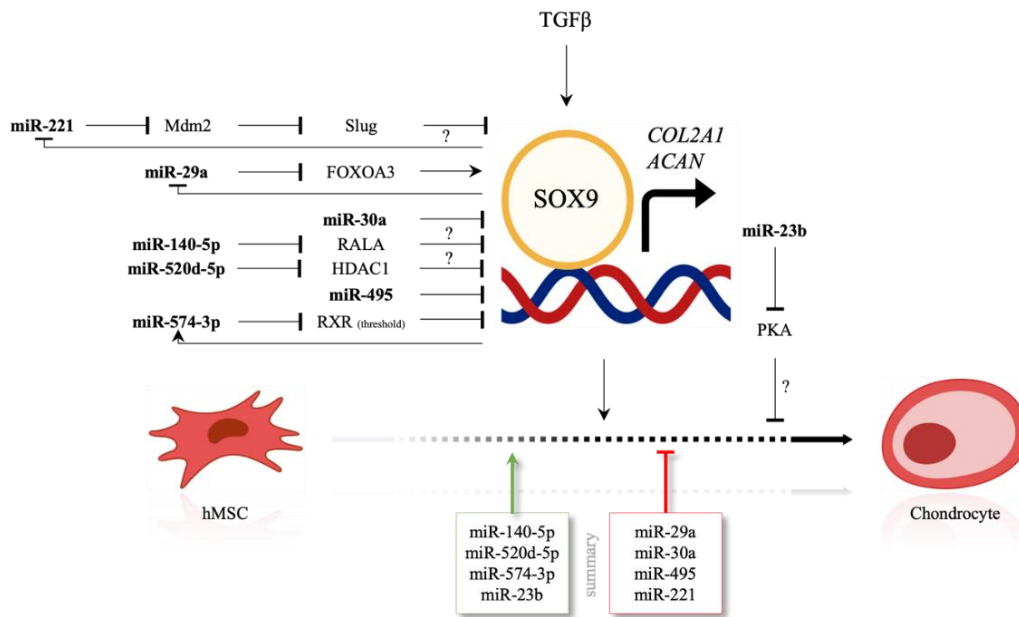


FIGURE 2.6 Schematic representation of miRNA potential targets and potential effect during the chondrogenesis of hMSCs. miR: micro-RNA; RALA: the Ras-related protein Ral-A; HDAC1: histone deacetylase 1; RXR: retinoid X receptor; TGF: transforming growth factor; SOX9: SRY-box transcription factor 9; PKA: proteinase K; hMSC: human mesenchymal stem/stromal cells.

3. An Articular Cartilage Progenitor Cell Approach: Can Hyaline Cartilage Aggregates be Engineered Using Human Articular Cartilage Progenitors?

3.1. Introduction

Articular cartilage (AC) defects are very common with prevalence as high as 65% observed during exploratory knee arthroscopic surgery [154]. As AC has limited regenerative properties, tissue defects do not self-heal, leading to further joint degeneration and the onset of osteoarthritis (OA) [155]. To date, repair of hyaline cartilage remains an ongoing challenge and OA has become the leading cause of movement disability worldwide [5, 156]. Current treatments include the implantation of *in vitro* expanded autologous chondrocytes (ACI) harvested from nonbearing regions of the AC [157]. As chondrocytes are cartilage resident cells, they can be isolated from biopsies of non-load bearing cartilage [158]. However, extensive culture induces a loss of chondrocyte phenotype which limits their usefulness for the repair of large defects [159]. While the long-term clinical outcomes of ACI are generally positive [160], the resulting tissue is often fibrocartilaginous in nature and mechanically inferior to the native tissue [161], increasing the risk of long-term failure. This has motivated increased interest in alternative cell sources for cartilage tissue engineering (TE) that can overcome the drawbacks associated with chondrocyte-based approaches [71].

Multipotent mesenchymal stem/stromal cells (MSCs) have emerged as a promising alternative for the regeneration of AC and they have already been used in several clinical trials [162, 163]. MSCs can be harvested from diverse sources, including bone marrow, adipose tissue, umbilical cord tissue or synovial membrane [164]. MSCs can be expanded to provide a high number of cells while maintaining their chondrogenic differentiation potentiality [165]. However, several studies have demonstrated the phenotypic instability of the resulting tissue, with chondrogenically primed MSCs typically undergoing hypertrophy and producing type X collagen [166, 167]. Such tissues have the inherent tendency to progress towards endochondral ossification, potentially limiting their use in the regeneration of hyaline AC [17].

A new population of cartilage resident cells with multilineage potency was recently identified in the surface of AC [64]. These cells, referred to as cartilage stem cells or articular cartilage progenitors (ACPs), can potentially be used for the TE of stable AC [68]. Their high level of integrin- $\alpha 5$ expression at the cell membrane has allowed their isolation from the chondrocyte population via fibronectin adhesion, which has been confirmed in several species, including mouse [168], rat [169], human [72] and equine model [170]. In addition, ACPs have been successfully isolated from healthy and OA human patients [68]. While they represent only 0.1-1% of the cartilage cell population in adult humans, they have a higher population doubling than chondrocytes while still maintaining their differential potential, in particular their chondrogenicity [68, 71]. Unlike MSCs, ACPs did not show signs of hypertrophy upon chondrogenic differentiation [170].

When seeded onto fibrous scaffolds or into hydrogels, or used in scaffold-free approaches, ACPs have been shown to generate a tissue rich in glycosaminoglycans (GAG) and type II collagen, with little evidence of type X collagen deposition [69, 77, 78, 80]. Besides, ACPs have been used in autologous cell therapy in an equine model [171] and in one human pilot study [81]. These studies motivate the use of ACPs to generate hyaline cartilage microtissues for use as biological building blocks in the engineering of larger cartilaginous grafts.

The aim of this study is to engineer hyaline AC using human ACPs (hACPs). First, the effect of cell density on the chondrogenic potential of cellular aggregates will be assessed to explore the potential of downsized cellular aggregates as biological building blocks for the modular assembly of hyaline AC. Then, the effect of hACP and human MSC (hMSC) cocultures on the quality and phenotype of the engineered cartilage will be evaluated as an alternative to hMSC-only based approaches.

3.2. Materials and Methods

3.2.1. Cell Isolation and Cell Expansion

3.2.1.1. Human ACPs

Cartilage shaves from one patient undergoing partial to total arthroplasty were kindly provided by and isolated with the help of Dr. Tom Hodgkinson, Anatomy and Regenerative Medicine, Royal College of Surgeons in Ireland (RCSI), Dublin, Ireland. Tissue was collected from healthy-looking regions and hACPs were isolated following the protocol from Johnstone *et al.* [69]. Briefly, cartilage shaves were minced and digested in sequential pronase (70 U/mL for 20 min at 37 °C) and type I collagenase (3,000 U/mL at 37 °C overnight) solutions. Then, hACPs were isolated through differential adhesion to fibronectin for 20 min at 37 °C. Nonadherent cells were removed, and ACP expansion media (ACP-XPAN) was added. ACP-XPAN consisted of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Cytiva) (containing L-Glutamine (2.5 mM) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (15 mM)), 10% v/v foetal bovine serum (Gibco), 50 U/mL penicillin, 50 µg/mL streptomycin (Gibco), 1 ng/mL transforming growth factor (TGF)-β 1 (Peprotech), 5 ng/mL fibroblast growth factor (FGF)-2 (Prospect Bio) and 0.1 nM L-ascorbic acid-2-phosphate (Sigma). Half of the media was changed every 3 to 4 days and cells were expanded until subconfluency at 5% O₂ and 5% CO₂ at 37 °C in humidified atmosphere. Upon passaging, cells were seeded at 10,000 cells/cm² and they were used at passage 3.

3.2.1.2. Human MSCs

Human MSCs were isolated from one bone marrow donors (Lonza) as previously described [172]. Following isolation, cells were expanded until passage 3 at 5% O₂ and 5% CO₂ at 37 °C in humidified atmosphere. They were seeded from an initial cell density of 3,000 cells/cm². Expansion media (XPAN) contained high glucose Dulbecco's Modified Eagle Medium GlutaMAX, 10% v/v foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin (all Gibco) and 5 ng/mL FGF-2 (Prospect Bio). Media was changed three times per week.

3.2.2. Chondrogenic Differentiation

Cells were trypsinised (Sigma), counted and resuspended to appropriate number in ACP-XPAN (hACPs) or XPAN (hMSCs) prior to aggregate formation.

Chondrogenic differentiation media (CDM) contained high glucose Dulbecco's Modified Eagle Medium GlutaMAX, 100 U/mL penicillin, 100 µg/mL streptomycin (both Gibco), 1.5 mg/mL bovine serum albumin, 40 µg/mL L-proline, 100 µg/mL sodium pyruvate, 1 × insulin-transferrin-selenium, 100 nM dexamethasone, 50 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid (all from Sigma) and 10 ng/mL human TGF-β3 (Peprotech). CDM was replaced by fresh media every 2–3 days for the entire culture period. Aggregates were maintained at 5% O₂ and 5% CO₂ for 21 days at 37 °C in humidified atmosphere.

3.2.2.1. Cell Density Study

Human ACP aggregates were fabricated using different cell numbers: 4,000 cells, 10,000 cells and 100,000 cells. The 4,000-cell and 10,000-cell groups were fabricated using an agarose microwell array fabricated as previously described [173]. Microwells were 1.5 mm in depth and 1 mm in diameter. Cell suspensions containing 1,604,000 cells/3 mL or 4,010,000 cells/3 mL were seeded into the microwell array, settled for 15 min and spun at 300 × g for 5 min, resulting in microtissues containing 4,000 cells or 10,000 cells, respectively. After 24 h, ACP-XPAN was discarded and replaced by 3 mL CDM. Human ACP-derived aggregates containing 100,000 cells were fabricated using V-bottomed polypropylene 96-well plates (Fischer). A cell suspension of 500,000 cells/mL was prepared, 200 µL was pipetted inside each well and spun at 300 × g for 5 min, resulting in aggregates containing 100,000 cells. After 24 h, ACP-XPAN was discarded and replaced by CDM. Volume of CDM was adapted to match the volume/cell of the 4,000-cell group. At the end of the differentiation culture, single microtissues were dislodged from their microwell and the number of harvested microtissues was recorded.

3.2.2.2. Coculture Study

Human MSC and hACP cocultures were fabricated at different ratios: 1:0, 2:1, 1:2 or 0:1. A cell suspension containing a total of 500,000 cells/mL were placed into polypropylene tubes and spun at 300 × g for 5 min, resulting in individual pellets containing 250,000 cells each. Tubes were pierced to ensure adequate gas exchange. After 24 h, XPAN was replaced by freshly prepared CDM. Volume of CDM was adapted to match the volume/cell of the 4,000-cell group.

3.2.3. Biochemical Analysis

At the end of the culture period, samples were retrieved from their culture media, washed with phosphate buffer saline (PBS) and digested in presence of 3.88 U/mL papain enzyme in papain buffer extract (PBE – containing 100 mM sodium phosphate buffer and 5 mM ethylenediaminetetraacetic acid disodium salt solution) containing 10 mM L-cysteine (pH 6.5) overnight at 60 °C. Deoxyribonucleic acid (DNA) content was quantified by bisBenzimide Hoechst fluorescent assay as described by the manufacturer's instructions. Total GAGs were quantified using a 0.002% w/v 1,9-dimethyl-methylene blue dye solution containing 0.25% w/v sodium chloride and 0.3% w/v glycine (pH 1.5). Diluted series of shark chondroitin sulphate in PBE were used as standards. Extinction coefficients (ϵ) at 530 nm and 590 nm wavelengths were measured using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc) and the $\epsilon_{530}/\epsilon_{590}$ ratio was used to extrapolate the concentration of GAGs in samples [174-176]. Finally, measurements of the total hydroxyproline content were used to determine the total collagen content of samples [177, 178]. Samples were hydrolysed in 12 M hydrochloric acid overnight at 110 °C. They were dried out and the solid material was resuspended in ultrapure deionised water. Then, a 2.82% w/v oxidising chloramine T solution in citrate buffer (pH 6.1) was added for 20 min at room temperature (RT) in the dark, followed by the addition of 50% w/v 4-(dimethylamino)benzaldehyde for 20 min at 60 °C. Serial dilutions of trans-4-hydroxy-L-proline (Fluka) were used as standards. The absorbance of blank, standards and samples was measured at the 570 nm wavelength using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc). Finally, the collagen content was calculated assuming a hydroxyproline-to-collagen ratio of 1:7.69 [178]. All reagents were from Merck if not mentioned otherwise.

3.2.4. Histology

At the end of the culture period, samples were retrieved from their culture media, washed with PBS and fixed using a 4% w/v paraformaldehyde solution overnight at 4 °C. Samples were embedded in 2% w/v agarose prior to dehydration in a graded series of ethanol solutions (10% - 70%). Final dehydration steps were made using an automatic tissue processor (Leica TP 1020). Samples were embedded in paraffin wax and sectioned into 7 μ m thick sections using a rotary microtome (Leica RM2235). Samples were stained with Haematoxylin and Eosin. GAGs were stained with 1% w/v Alcian Blue 8GX in 0.1 M hydrochloric acid (pH 1) and cells were counterstained with

0.1% w/v Nuclear Fast Red. Alternatively, GAGs were stained with 0.2% w/v Safranin O counterstained with 0.04% w/v aqueous Fast Green and cells were stained with Weigert's Iron Haematoxylin. Finally, 0.1% w/v Picrosirius Red staining was used to visualise collagen production and 1% w/v Alizarin Red (pH 4.1) was used to determine calcium deposition (all from Merck). Stained sections were imaged using a slide scanner (Aperio ScanScope CS and Olympus SLIDEVIEW VS200).

3.2.5. Immunofluorescence Staining of Collagen Subtypes

Sample sections were rehydrated in graded series. Antigen retrieval was carried out on an initial treatment with 35 U/mL, for type I and II collagens, or 3.5 U/mL, for type X collagen, pronase for 25 min at 37 °C in a humidity chamber (HC) followed by 4,000 U/mL hyaluronidase for 45 min at 37 °C in a HC. Non-specific antigen sites were blocked using 10% v/v donkey serum (abcam) and 1% w/v bovine serum albumin in 1X PBS solution (blocking buffer) for 1 h at RT in a HC. Primary antibodies were diluted in blocking buffer prior to incubation overnight at 4 °C in a HC. Dilutions were as follows: 1:100 for anti-type II collagen (sc-52658, Santa Cruz Biotechnology), 1:500 for anti-type I collagen (ab138492, abcam) and 1:50 for anti-type X collagen (14-9771-82, Invitrogen). Fluorescently conjugated secondary antibodies for type II collagen (Alexa Fluor 594; dilution 1:500; A21203, Invitrogen), type I collagen (Alexa Fluor 647; dilution 1:500; ab150075, abcam) and type X collagen (Alexa Fluor 594; dilution 1:200; A21203, Invitrogen) were diluted in blocking buffer and incubated for 1 h at RT in the dark in a HC. Finally, slides were mounted in Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI). All reagents were from Merck if not mentioned otherwise. Sample images taken with a scanning confocal microscope (LeicaTCS SP8) are superimposed z-stacks of 10 images. Porcine native tissues were used as positive (+) and negative (-) controls: articular cartilage (+) and ligament (-) for type II collagen, ligament (+) and articular cartilage (-) for type I collagen and hypertrophic articular cartilage incubated in the presence (+) and in the absence (-) of primary antibody for type X collagen.

3.2.6. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, CA, United States). Analysis of Gaussian distribution and statistical differences are described on a case-by-case basis in figure captions. Significance was accepted at a level of $p < 0.05$.

3.3. Results

3.3.1. Human Articular Cartilage Progenitor-Derived Aggregates Failed to Support Robust Chondrogenesis Independent of Cell Density

Cellular aggregates were fabricated using increasing numbers of cells (4,000, 10,000 and 100,000 cells per aggregate) to analyse the effect of cell density on chondrogenesis of hACPs. As expected, DNA levels increased with initial cell numbers in the aggregates (**Figure 3.1-I**). After 21 days of chondrogenic differentiation, GAGs/DNA levels were not significantly different between the groups (**Figure 3.1-II**), with a trend towards increased GAG production observed in the higher cell number aggregates (p-value (4,000 versus 100,000 cells) = 0.0655 and p-value (10,000 versus 100,000 cells) = 0.0778). Overall, GAG production remained relatively low, independent of the cell density (means <1.5 $\mu\text{g}/\mu\text{g}$). Collagen synthesis was also not significantly different across the experimental groups (**Figure 3.1-III**). Similar to GAGs, collagen production was relatively low with less than 4.5 $\mu\text{g}/\mu\text{g}$ deposited after 21 days of culture.

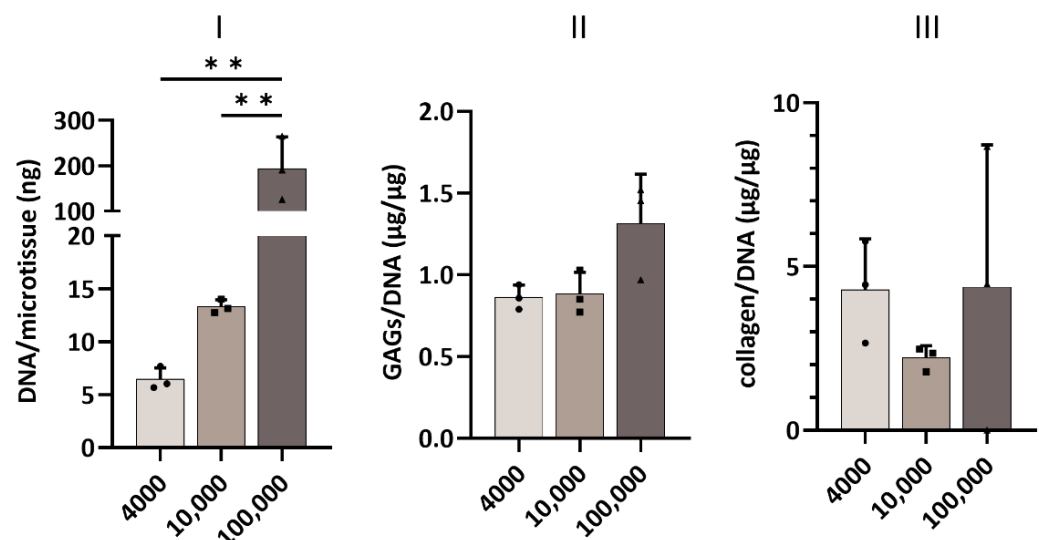


FIGURE 3.1 Biochemical comparison of hACP aggregates fabricated using different initial cell numbers. Quantification of DNA (I), GAGs (II) and collagen (III) in aggregates containing 4,000, 10,000 or 100,000 cells. All data is represented as individual data points and mean $\pm$ standard deviation. Statistical differences were determined using a one-way ANOVA test with Tukey's multiple comparison test on Gaussian distributed data (according to Shapiro-Wilk's normality test). N = 3. ** denotes p < 0.01. DNA: deoxyribonucleic acid; GAGs: glycosaminoglycans.

Further analysis of the quality of the hACP-derived extracellular matrix (ECM) was performed by histology staining and immunofluorescence staining of different

collagen subtypes (**Figure 3.2**). Aggregates containing 4,000 cells did not stain positive for GAGs (Safranin O) deposition, but stained positive for collagens (Picrosirius red), with immunofluorescent staining indicating that only type I collagen was present. In addition, cell morphology was elongated and did not look chondrocyte-like in Eosin-stained sections. In aggregates containing 10,000 cells and especially aggregates containing 100,000 cells, the presence of peripheral chondrocyte-like cells and a necrotic core were observed. While both higher density groups stained positive for GAGs (with a more intense staining in the 100,000-cell group) and type I collagen, only the 100,000-cell group stained positive for type II collagen at the periphery, with no signs of type X collagen or calcium deposition.

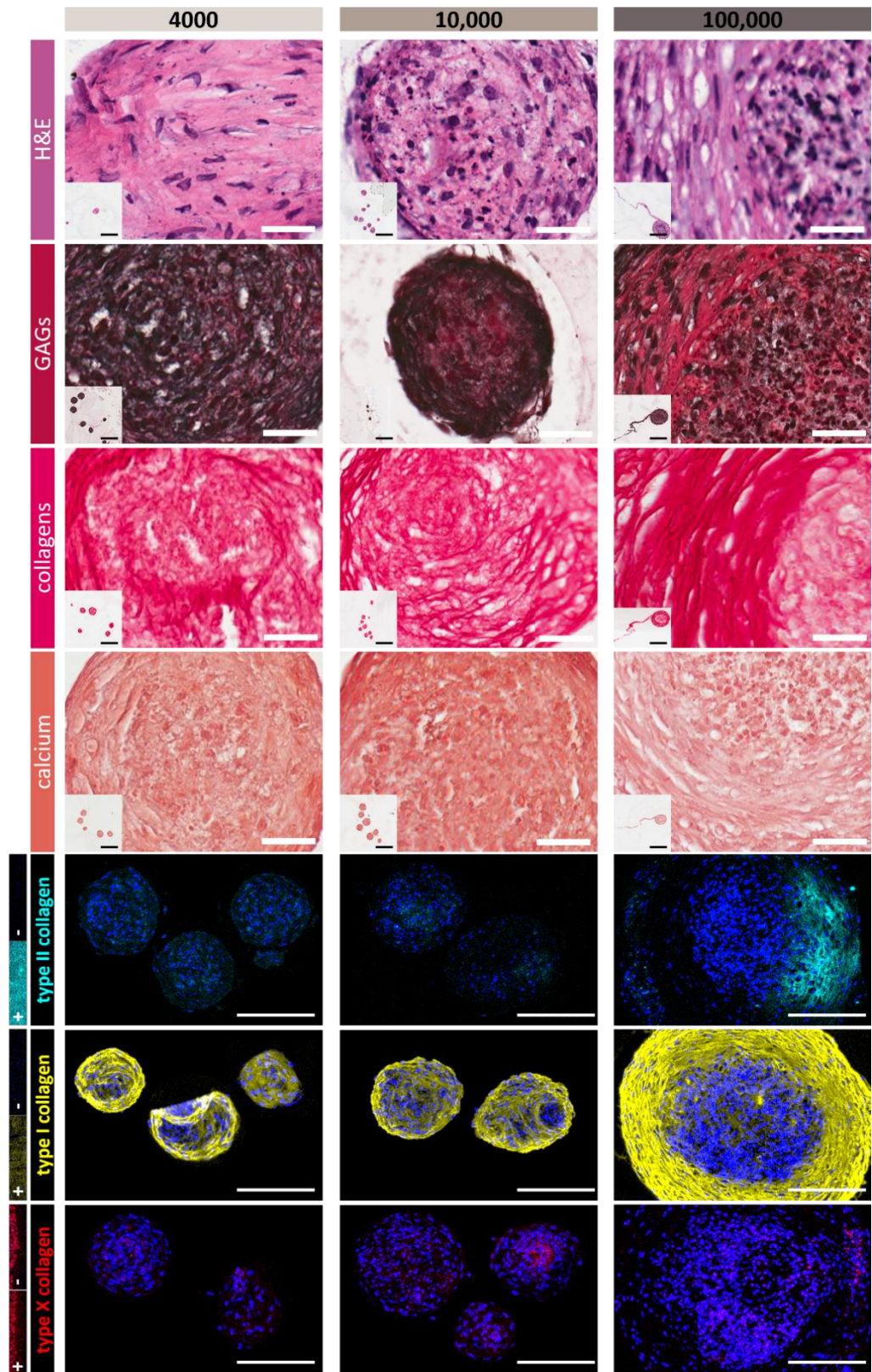


FIGURE 3.2 Histology panel of hACP aggregates fabricated using different initial cell numbers.

Staining of extracellular matrix key cartilaginous components with H&E, Safranin O to visualise GAGs, Picrosirius Red for collagens, Alizarin Red to stain calcium, and immunofluorescence staining of type II, type I and type X collagens (nuclei were stained with DAPI and are shown in dark blue). Scale bar = 25 μm (histology staining)/400 μm (inserts)/100 μm

(immunofluorescence staining). H&E: Haematoxylin and Eosin; GAGs: glycosaminoglycans; +: positive control; -: negative control.

3.3.2. Chondrogenesis Within Cocultures of Human Articular Cartilage Progenitors and Human Mesenchymal Stem/Stromal Cells

Based on relatively low cartilage-specific ECM production by hACP-derived aggregates, cocultures of hMSCs and hACPs (hMSC:hACP) at different ratios (1:0, 2:1, 1:2 and 0:1) were next fabricated in pellet culture systems. Compared to the hMSC-only (1:0) control group, which contained $7.1 \text{ pg} \pm 1.2 \text{ pg}$ DNA per cell, the 2:1 and 1:2 groups did not contain significantly different amounts of DNA (**Figure 3.3**). However, DNA levels in the hACP-only (0:1) group was significantly lower than all the other groups. In addition, the latter group did not respond to the chondrogenic differentiation media, as observed by the low levels of GAGs and collagen. Cocultures of hMSCs and hACPs supported comparable levels of GAG and collagen synthesis to the hMSC-only (1:0) control group. The 1:2 hMSC:hACP coculture contained the highest levels of GAGs/DNA.

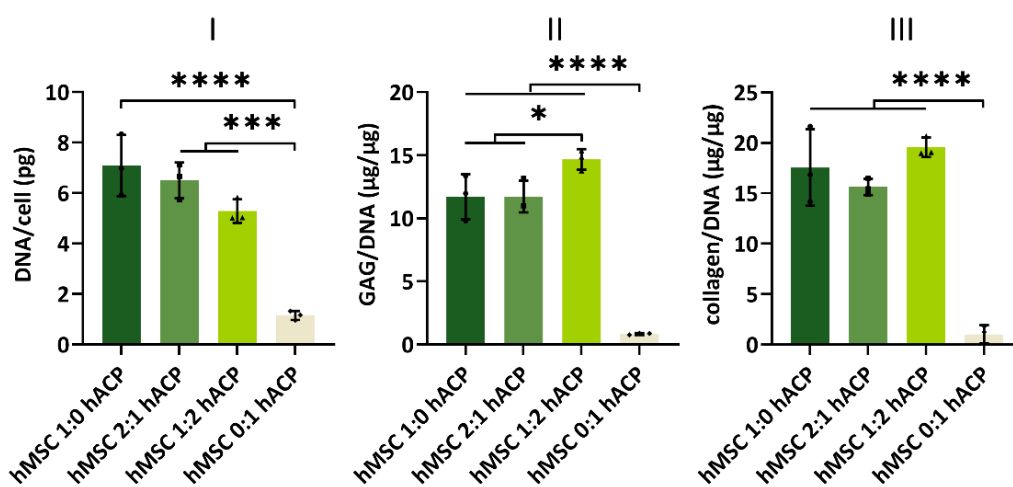


FIGURE 3.3 Biochemical composition of pellets from hMSC and hACP-derived cocultures. Quantification of DNA (I), GAGs (II) and collagen (III) in hMSC:hACP pellet cocultures. All data is represented as individual data points and mean $\pm$ standard deviation. Statistical differences were determined using a one-way ANOVA test with Holm-Šidák's multiple comparison test. N = 3. * denotes p < 0.05, *** denotes p < 0.001, **** denotes p < 0.0001. DNA: deoxyribonucleic acid; GAGs: glycosaminoglycans; hMSC: human mesenchymal stem/stromal cell; hACP: human articular cartilage progenitor.

The poor chondrogenesis within the hACP-only (0:1) pellets was confirmed by histology, with weak GAG and collagen staining and the absence of chondrocyte-like cells (**Figure 3.4**). Despite the same initial cell number, hACP-only (0:1) pellets were relatively small, while the hMSC-only (1:0) group was the largest. Similar to the hMSC-only (1:0) pellet control, both coculture groups stained positive for GAGs and collagens. These tissues contained round chondrogenic-like cells embedded in a GAG-rich ECM. The coculture of hACPs and hMSCs did not calcify, as observed by negative calcium staining. Unlike the hACP-derived pellets, cocultures contained type II collagen and type I collagen (**Figure 3.5**). Staining was homogeneous, with minimal type X collagen immunofluorescent staining observed in the centre of the 1:2 hMSC:hACP group. Finally, coculture containing one part of hMSCs for two parts of hACPs (1:2) appeared to enhance type II collagen production with a stronger staining intensity compared to pellets containing a higher ratio of hMSCs (hMSC-only and 2:1 hMSC:hACP).

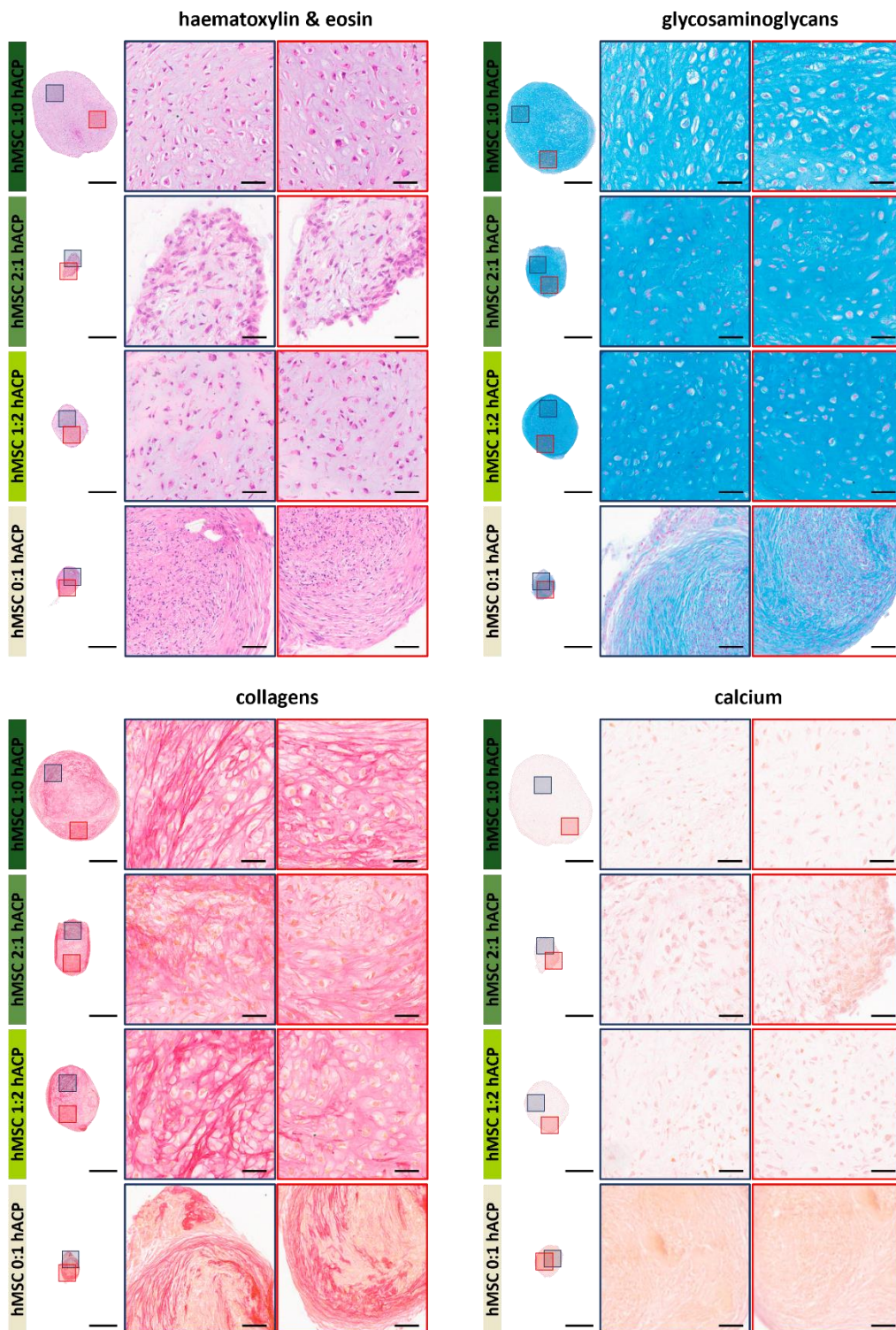


FIGURE 3.4 Histology panel of pellets from hMSCs and hACP-derived cocultures. Extracellular matrix key cartilaginous components of hMSC:hACP pellet cocultures were stained with H&E, Alcian Blue to visualise GAGs, Picrosirius Red for collagens, Alizarin Red to stain calcium. Scale bar = 500 μ m/50 μ m (magnifications). hMSC: human mesenchymal stem/stromal cell; hACP: human articular cartilage progenitor.

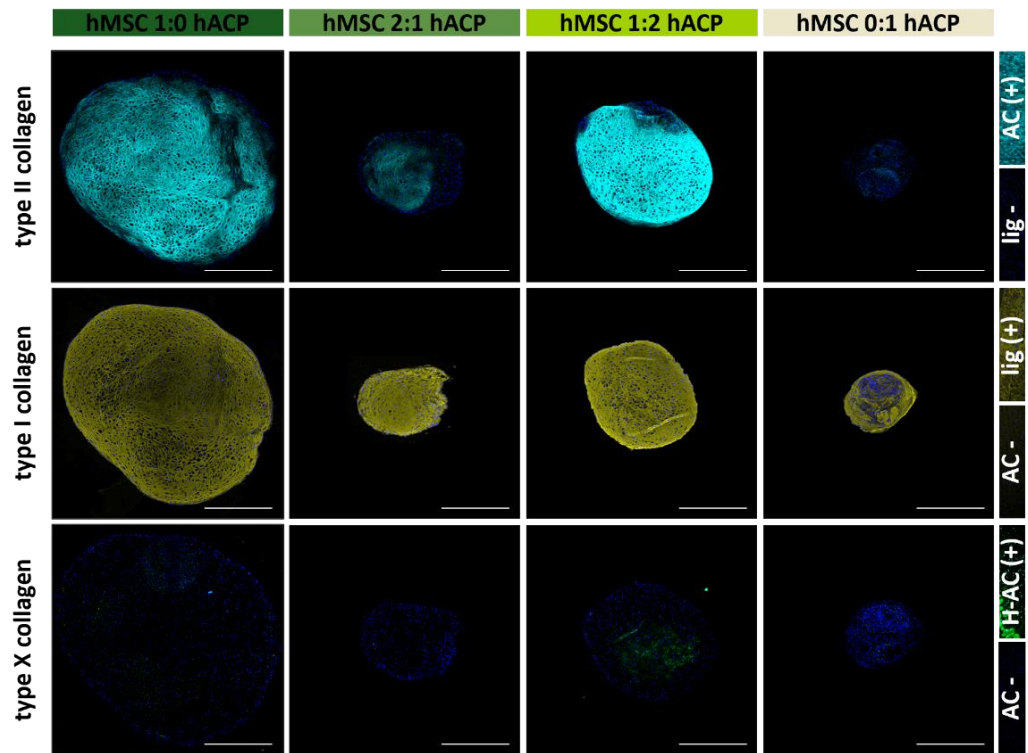


FIGURE 3.5 Staining of collagen subtypes in hMSCs and hACP-derived cocultures. Immunofluorescence staining of type II, type I and type X collagens (nuclei were stained with DAPI and are shown in dark blue). Scale bar = 500 μ m. hMSC: human mesenchymal stem/stromal cell; hACP: human articular cartilage progenitor; AC: articular cartilage; lig: ligament; H-AC: hypertrophic articular cartilage; (+): positive control; -: negative control.

3.4. Discussion

The goal of this study was to engineer hACP-derived cellular aggregates and assess their chondrogenic potential. The initial cell number within hACP-derived aggregates had little effect on the overall levels of GAG and collagen synthesis. However, increased cell numbers enhanced the secretion of type II collagen, a marker for hyaline cartilage, while no signs of the hypertrophic marker type X collagen were observed. A coculture of hACPs and hMSCs appeared to support superior chondrogenesis compared to hACP-only (0:1) pellets, with a 1:2 hMSC:hACP coculture supporting enhanced GAG synthesis and type II collagen deposition, indicating a synergic effect on chondrogenesis.

The choice of cell source is crucial to the success of cell-based TE approaches to regenerate hyaline cartilage. ACPs represent a promising alternative to chondrocytes as they can be extensively expanded *in vitro* without losing their phenotype [179], and appear to better resist cartilage hypertrophy and endochondral ossification compared to MSCs following chondrogenic differentiation [170]. In addition, ACPs preferentially support type II collagen synthesis over type I collagen production [180], with previous studies reporting a higher type II-to-type I ratio [69]. Here, minimal type X collagen synthesis was observed in all hACP containing cultures. However, type I collagen staining was very intense in hACP-derived tissues, while type II collagen was absent in cellular aggregates with low initial cell numbers (4,000 and 10,000). Only larger aggregates containing 100,000 cells supported type II collagen deposition (but also type I collagen), suggesting that hACP-derived aggregates were most likely fibrocartilaginous in nature. In addition, production of GAGs ($<1.5 \mu\text{g}/\mu\text{g}$ normalised by DNA) and collagen ($<4.5 \mu\text{g}/\mu\text{g}$ normalised by DNA) was very low, which was also reflected by the small sizes of the aggregates ($\sim 100 \mu\text{m}$ and $\sim 250 \mu\text{m}$). Human ACPs used in this study were harvested from healthy-looking regions of AC from patients undergoing partial to total arthroplasties. Human ACPs have been found in both healthy and diseased patients [68], and it was shown that a pool of ACPs from osteoarthritic cartilage could still retain their regenerative potential [181]. However, it is likely that this potential varies with OA severity and could explain the limited chondrogenic potential observed in this study [83, 84].

The initial stem/progenitor cell number within an aggregate has previously been shown to regulate subsequent chondrogenesis. Superior chondrogenesis has been observed in MSC-derived aggregates with lower initial cell numbers [96], which contrasts with the results observed here using hACPs. Maturation of MSC-derived

three-dimensional aggregates is regulated by biochemical and biomechanical signals resulting from intensive cell-cell and cell-matrix interactions [182], conditions which likely change based on the size and cellularity of the construct. For example, it may be that the lower oxygen conditions generated in larger aggregates are more supportive of hACP chondrogenesis [69]. Alternatively, the larger aggregates may support higher levels of paracrine signalling necessary for chondrogenesis of hACPs. While ACPs are often suggested to resemble MSCs, due to their common set of surface markers and multilineage potential, this result supports the argument that ACP characterisation should not be based on MSCs [74].

Human ACPs were then cocultured with hMSCs at different ratios to assess their capacity to support chondrogenesis. MSCs cocultured with chondrocytes have previously been shown to enhance ECM production with reduced hypertrophy [183, 184]. In an attempt to mimic the zonal organisation of AC, ACPs and MSCs have also been used to fabricate bilayered constructs where different cell populations could communicate through the secretion of soluble factors. This was shown to enhance GAG and type II collagen production by the MSC layer compared to single layers of ACPs [71, 185]. Here it was found that a 1:2 coculture of hMSCs and hACPs significantly enhanced production of GAGs compared to hACP-only and hMSC-only and supported superior type II collagen production. These results suggest a potential synergy between both cell types that could result from paracrine signalling, including MSC-secreted growth factors [186]. While further studies are necessary to reveal the potential of this new population of chondro-progenitors, it is worth mentioning that some recent works have also highlighted the importance of defining optimal and more ACP-specific differentiation conditions, including chondrogenic media [85, 187, 188].

3.5. Conclusion

The results of this study highlighted some potential strengths and weaknesses of hACPs as a cell source for TE hyaline articular cartilage. Increasing the initial cell number within an aggregate tends to improve the deposition of GAGs and type II collagen. However, this study failed to provide strong support for the use of diseased hACPs for the engineering of hyaline cartilage microtissues. Microtissues containing only hACPs failed to produce high levels of ECM and were at best fibrocartilaginous in nature. Coculture pellets of hMSCs and hACPs significantly improved ECM production. In addition, specific cocultures (one part of hMSCs and 2 parts of hACPs) significantly increased GAG production compared to the use of hACPs or hMSCs in isolation, with a more intense and homogeneous type II collagen staining. While these results support the use of coculture of hMSCs and hACPs for AC TE, further work is required to justify their continued use. Therefore, in the next chapter, a novel approach to engineer hyaline cartilage microtissues with region-specific phenotypes only bone marrow-derived hMSCs will be explored.

4. An Oxygen Approach: Mimicking *In Situ* Oxygen Levels to Engineer Microtissues with Distinct Phenotypes

4.1. Introduction

Articular cartilage (AC) has a spatially defined organisation with a zonal gradient of glycosaminoglycans (GAGs) from the superficial to the deep region of the tissue where the highest concentrations of GAGs are found [189]. Due to its avascular nature, oxygen diffuses through the depth of the tissue and its availability varies with distance from the surface [20]. Hence, oxygen levels are relatively low and range from 5–7% at the surface to 0.5–1% in the deeper regions of the tissue [190-193]. AC has poor self-healing ability [194] and untreated damaged tissue typically degenerates, increasing the risk of post-traumatic osteoarthritis (OA) progression, with a 50% chance of OA onset after 20 years [195]. Shortcomings of current treatments for focal cartilage damage have motivated increased interest in innovative tissue engineering (TE) alternatives [196]. However, the engineering of regenerative grafts that mimic the intricate zonal nature of AC remains a challenge [156].

Local oxygen levels are key regulatory cues in tissue development, tissue homeostasis and tissue repair [197]. Oxygen demand depends on cell type and metabolism [198], cell cycle phase [191] and the number of cells [199]. During limb development, hypoxia plays a key role in normal cartilage formation via the regulation of the hypoxia-inducible factor (HIF)-1 α transcription factor [200]. Cells used in the regeneration of AC have been shown to be highly sensitive to low oxygen environments, with hypoxic conditions typically promoting a chondrogenic phenotype and extracellular matrix (ECM) production [24, 25]. Hypoxia-induced chondrogenesis has been observed in different cell types (chondrocytes [201], mesenchymal stem cells (MSCs) [202-207], articular cartilage progenitors (ACPs) [82]), including stem/progenitor cells isolated from different tissue sources (bone marrow [205, 206], infrapatellar fat pad [204, 207, 208], embryonic [202], synovium-derived [203], cartilage [82]), in healthy [82, 202] or diseased tissues [201, 203, 205]. Besides,

different tissue engineering approaches (cocultures [201], self-assembly [202-205], cell-seeded hydrogels [206, 208] or scaffolds [207]) have also highlighted the importance of low oxygen conditions during chondrogenesis. However, in all these studies, hypoxia, commonly defined in such studies as 2–5% O₂, is usually compared to non-physiological normoxic levels (20–21% O₂). In the context of AC repair, inherent gradients are found in large engineered grafts due to diffusion and cell consumption [209]. However, these gradients are poorly defined, and their intensity varies with time in culture [210]. Engineered cartilage grafts are often inhomogeneous with cells producing high quality cartilage in some regions and inferior quality tissue in other regions of the same construct [20]. To improve the quality and organisation of zonal grafts, the role of oxygen variations, within the physiological range, on cartilage tissue development needs to be better defined.

Developmentally inspired engineering strategies commonly rely on the ability of cells to self-organise into high cell-density aggregates and to secrete ECM components in response to external cues [211]. The resulting cellular environment enables differentiation and maturation into engineered units resembling the native tissue [212]. Differentiated cellular aggregates, called microtissues, can vary in size depending on the initial cell number [107]. It was previously demonstrated that reducing the size of such microtissues accelerates chondrogenesis and improves the quality of deposited tissue [96]. Chondrocyte-derived microtissues with a diameter of less than 280 µm displayed no evidence of a necrotic core, which was linked to sufficient and homogeneous oxygen diffusion [213]. As limited oxygen gradients will develop in such microtissues, they represent an ideal system for exploring how human MSCs (hMSCs) undergoing chondrogenesis will respond to altered levels of oxygen. Furthermore, cartilage microtissues engineered under such altered oxygen conditions could potentially be used in the modular assembly of zonally defined AC grafts.

The aim of the study was to engineer cartilage microtissues with distinct phenotypes using hMSCs differentiated under oxygen levels representative of the surface (5%) and deep (2%) region of native AC. In addition, with the later goal of incorporating such microtissues into layered engineered grafts, the effect of short-term exposure to different oxygen levels on chondrogenesis was assessed. For this study, microtissues were cultured for 7 days at 2% O₂ before increasing it to 5% O₂ until the end of the differentiation period. Chondrogenic phenotypes were then assessed by biochemical quantification, histology and immunofluorescence staining.

4.2. Materials and Methods

4.2.1. Tissue Isolation and Processing

Human tissue was kindly provided by Dr. Tom Hodgkinson, Anatomy and Regenerative Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland. Cartilage was collected from surgical waste of patients undergoing partial or total knee replacement. Chondral plugs were cut using biopsy punches from healthy-looking regions until the subchondral bone. Tissues were washed with phosphate buffer saline (PBS) and frozen until processing. The plugs were weighted and were cut into two regions: the surface region corresponding to approximately the top one third and the deep region corresponding to approximately the bottom two thirds. The weight of the surface and deep regions was recorded. Tissues were then prepared for biochemical analysis.

4.2.2. Culture of Human Mesenchymal Stem Cells

4.2.2.1. Cell Expansion

MSCs from three different human bone marrow donors were isolated as previously described [172]. Cells were expanded from an initial cell density of 3,000 cells/cm² at 5% O₂ and 5% CO₂ at 37 °C in humidified atmosphere until subconfluency was reached. Expansion media (XPAN) contained high glucose Dulbecco's Modified Eagle Medium GlutaMAX, 10% v/v foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin (all Gibco) and 5 ng/mL fibroblast growth factor 2 (Prospect Bio). All cells were expanded until passage 3 prior to microtissue formation.

4.2.2.2. Single Microtissue Formation and Chondrogenic Differentiation

Agarose (4% w/v in PBS) microwells (1.5 mm depth × 1 mm diameter) were moulded under sterile conditions following the protocol of Nulty *et al.* and were soaked in XPAN for 24 h prior to cell seeding [173]. Soaking media was removed and a cell suspension of 1,604,000 cells/3 mL of XPAN was pipetted into the microwells, settled for 15 min, spun at 800 × g for 5 min, resulting in microtissues containing 4,000 cells each. After 24 h, XPAN was discarded and chondrogenic differentiation media (CDM) was added. CDM contained high glucose Dulbecco's Modified Eagle Medium GlutaMAX, 100 U/mL penicillin, 100 µg/mL streptomycin (both Gibco), 1.5 mg/mL bovine serum albumin, 40 µg/mL L-proline, 100 µg/mL sodium pyruvate, 1 × insulin-transferrin-selenium, 100 nM dexamethasone, 50 µg/mL L-ascorbic acid-2-phosphate,

4.7 µg/mL linoleic acid (all from Sigma) and 10 ng/mL human transforming growth factor (TGF)-β3 (Peprotech). CDM was replaced by freshly prepared media every 2–3 days for the entire culture period. Single microtissues were maintained at desired oxygen conditions (2% or 5% O₂ and 5% CO₂ at 37 °C in humidified atmosphere) for the entire differentiation period (2 days, 7 days or 21 days), except for the priming group (2–5%) where microtissues were maintained at 2% O₂ (5% CO₂ at 37 °C in humidified atmosphere) for 7 days and then placed at 5% O₂ (5% CO₂ at 37 °C in humidified atmosphere) for 14 days (for a total study time of 21 days). At the end of the culture period, single microtissues were dislodged from their microwell and the number of harvested microtissues was recorded.

4.2.3. Biochemistry Quantification of Extracellular Matrix Key Components

At determined timepoints, samples were washed with PBS and digested in presence of 3.88 U/mL papain enzyme in papain buffer extract (PBE – containing 100 mM sodium phosphate buffer and 5 mM ethylenediaminetetraacetic acid disodium salt solution) containing 10 mM L-cysteine (pH 6.5) overnight at 60 °C. Total deoxyribonucleic acid (DNA) content of digested samples was quantified by bisBenzimide Hoechst fluorescent assay as described by the manufacturer's instructions. Total GAGs were quantified using a 0.002% w/v 1,9-dimethyl-methylene blue dye solution containing 0.25% w/v sodium chloride and 0.3% w/v glycine (pH 1.5). Diluted series of shark chondroitin sulphate in PBE were used as standards. The extinction coefficient (ϵ) of blank, standards and samples was determined at 530 nm and 590 nm wavelengths using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc). The $\epsilon_{530}/\epsilon_{590}$ ratio was used to extrapolate the concentration of GAGs in samples [174-176]. Total collagen content was indirectly calculated from the measurement of the hydroxyproline content as previously described [177, 178]. Briefly, proline residues were hydrolysed in 12 M hydrochloric acid overnight at 110 °C. After drying, the solid material was resuspended in ultrapure deionised water. A 2.82% w/v oxidising chloramine T solution in citrate buffer (pH 6.1) was added for 20 min at room temperature (RT) in the dark, followed by addition of 50% w/v 4-(dimethylamino)benzaldehyde (reacting with pyrrole residues) for 20 min at 60 °C. Serial dilutions of trans-4-hydroxy-L-proline (Fluka) were used as standards. The absorbance of blank, standards and samples was determined at the 570 nm wavelength using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc). Finally, the collagen content was calculated assuming a hydroxyproline-to-

collagen ratio of 1:7.69 [178]. All reagents were from Merck if not mentioned otherwise.

4.2.4. Tissue Evaluation

4.2.4.1. Histology

At determined timepoints, samples were retrieved from their culture media, washed with PBS and fixed using 4% w/v paraformaldehyde overnight at 4 °C. Following embedding in 2% w/v agarose, samples were dehydrated in a graded series of ethanol solutions (10–70%) and final dehydration steps were done using an automatic tissue processor (Leica TP 1020). All samples were embedded in paraffin wax and sectioned into 7 µm thick sections using a rotary microtome (Leica RM2235). Sections were stained with Haematoxylin and Eosin, 0.1% w/v Picrosirius Red to visualise collagen production and 1% w/v Alizarin Red (pH 4.1) to determine calcium deposition. GAGs were stained using 1% w/v Alcian Blue 8GX in 0.1 M hydrochloric acid (pH 1) and cells were counterstained using 0.1% w/v Nuclear Fast Red. All reagents were from Merck. Stained sections were imaged using a slide scanner (Aperio ScanScope CS for 21-day-old microtissues and Olympus SLIDEVIEW VS200 for 7-day-old microtissues).

4.2.4.2. Immunofluorescence Staining

Sample sections were rehydrated in graded series. Antigen retrieval was carried out on an initial treatment of 35 U/mL, for type II and type I collagens, or 3.5 U/mL, for type X collagen, pronase (Merck) for 25 min at 37 °C in a humidity chamber (HC), followed by 4,000 U/mL hyaluronidase (Merck) for 45 min at 37 °C in a HC. Non-specific antigen sites were blocked using 10% v/v donkey serum (abcam) and 1% w/v bovine serum albumin (Merck) in 1X PBS solution (blocking buffer) for 1 h at RT in a HC. Anti-type II collagen (dilution 1:100; sc-52658, Santa Cruz Biotechnology), anti-type I collagen (dilution 1:500; ab138492, abcam) and anti-type X collagen (dilution 1:50; 14-9771-82, Invitrogen) primary antibodies diluted in blocking buffer were incubated overnight at 4 °C in a HC. Fluorescently conjugated secondary antibodies for type II collagen (Alexa Fluor 594, dilution 1:500, A21203, Invitrogen), type I collagen (Alexa Fluor 647, dilution 1:500, ab150075, abcam) and type X collagen (Alexa Fluor 594, dilution 1:200, A21203, Invitrogen) diluted in blocking buffer containing DAPI (2 µg/mL, Merck) were incubated for 1 h in the dark at RT in a HC. Slides were coverslipped using ProLong™ Gold Antifade Mountant (Invitrogen). Porcine native tissues were used as

positive (+) and negative (-) controls: articular cartilage (+) and ligament (-) for type II collagen, ligament (+) and articular cartilage (-) for type I collagen and hypertrophic articular cartilage incubated in the presence (+) and in the absence (-) of primary antibody for type X collagen. The different collagen subtypes were visualised using a scanning confocal microscope (LeicaTCS SP8) and images were taken as z-stacks of 10 images.

4.2.4.3. Quantification of Staining Intensity

Histology—Quantification of histology staining intensity was performed on multicoloured brightfield images using the Fiji Colour Histogram tool. The mean values of red, green, and blue colours from a selected region of interest (ROI) were averaged (RGB). Microtissues' ROI corresponded to the entire microtissue area (RGB μ T). Four different microtissues were used per experimental condition. RGB values from three background areas surrounding the sample were averaged (RGBbackground). The staining intensity was graphed using the following formula: $(1 - (RGB\mu T / RGBbackground))$, so the value of 0 corresponds to RGB μ T equals to RGBbackground.

Immunofluorescence staining—Quantification of fluorescence staining intensity was performed on 8-bit images using Fiji. The Mean Grey Value (MGV) of a selected ROI was measured. Microtissues' ROI corresponded to the entire microtissue area (MGV μ T). At least three different microtissues were used per experimental condition. MGV from three background areas surrounding the samples were averaged (MGVbackground). The staining intensity was graphed using the following formula: $(MGV\mu T - MGVbackground)$, so the value of 0 corresponds to a MGV μ T equals to MGVbackground.

4.2.5. Microtissue Diameter

The diameter of microtissues was measured using the Fiji length measurement tool on brightfield microscopy images. The diameter of each microtissue was averaged from three measurements.

4.2.6. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, CA, United States). Analysis of Gaussian distribution and statistical

differences are described on a case-by-case basis in figure captions. Significance was accepted at a level of $p < 0.05$.

4.3. Results

4.3.1. Altering Oxygen Levels Yields Chondrogenic Microtissues with Extracellular Matrix Compositions Resembling Different Regions of the Native Tissue

The composition of native AC was spatially analysed to determine levels of key cartilaginous ECM components. In chondral plugs, harvested from nonbearing regions in human AC, GAGs represented $3.3\% \pm 0.9\%$ and total collagen represented $19.6\% \pm 4.6\%$ of the total tissue wet weight (**Figure 4.1A-II**), with both values falling into the range reported in the literature [3]. The collagen to GAGs ratio was 6.2:1 (**Figure 4.1A-V**). The surface layer of the chondral plugs, corresponding to the top one third of the total tissue height (and 33.2% of the total tissue wet weight) (**Figure 4.1A-I**), contained $4.7\% \pm 2.3\%$ GAGs (**Figure 4.1A-III**) and $50.4\% \pm 30.5\%$ collagen (**Figure 4.1A-IV**), while the deep layer, defined as the bottom two thirds of the total tissue height (and 66.8% of the total tissue wet weight), contained $3.8\% \pm 1.2\%$ GAGs and $16.0\% \pm 7.4\%$ collagen. For hMSC-derived microtissues engineered under altered oxygen tensions (2% or 5% O₂), oxygen availability did not significantly affect DNA levels per microtissue at all experimental timepoints (**Figure 4.1B-I**). DNA levels after 21 days of chondrogenic differentiation were significantly lower than levels observed on day 2. Levels of GAGs/DNA and collagen/DNA significantly increased with time in culture for both oxygen tensions (**Figure 4.1B-II** and **Figure 4.1B-III**). GAGs/DNA was significantly higher at 2% O₂ than at 5% O₂, reaching $11.3 \mu\text{g}/\mu\text{g} \pm 2.2 \mu\text{g}/\mu\text{g}$ on day 21 at 2% O₂ (**Figure B-IV**). Total collagen synthesis was not significantly affected by oxygen conditions 21 days of culture (**Figure B-V**). The ratio of collagen to GAGs synthesis was calculated and compared to the ratio calculated for the native tissue (**Figure 4.1B-VI**). After 21 days of differentiation at 2% O₂, the collagen to GAG ratio within the engineered tissues was $2.7 \mu\text{g}/\mu\text{g} \pm 0.4 \mu\text{g}/\mu\text{g}$, within the range measured for the deep region of native AC ($2.5\text{--}6.4 \mu\text{g}/\mu\text{g}$). The ratio of $19.8 \mu\text{g}/\mu\text{g} \pm 15.0 \mu\text{g}/\mu\text{g}$ calculated for the 5% O₂ group was within the range measured for the surface region of human native AC ($1\text{--}29.4 \mu\text{g}/\mu\text{g}$). These results indicated that the composition of hMSC-derived microtissues is altered by the oxygen environment during chondrogenic differentiation and that zonally defined cartilage microtissues can potentially be engineered by careful control of local oxygen levels.

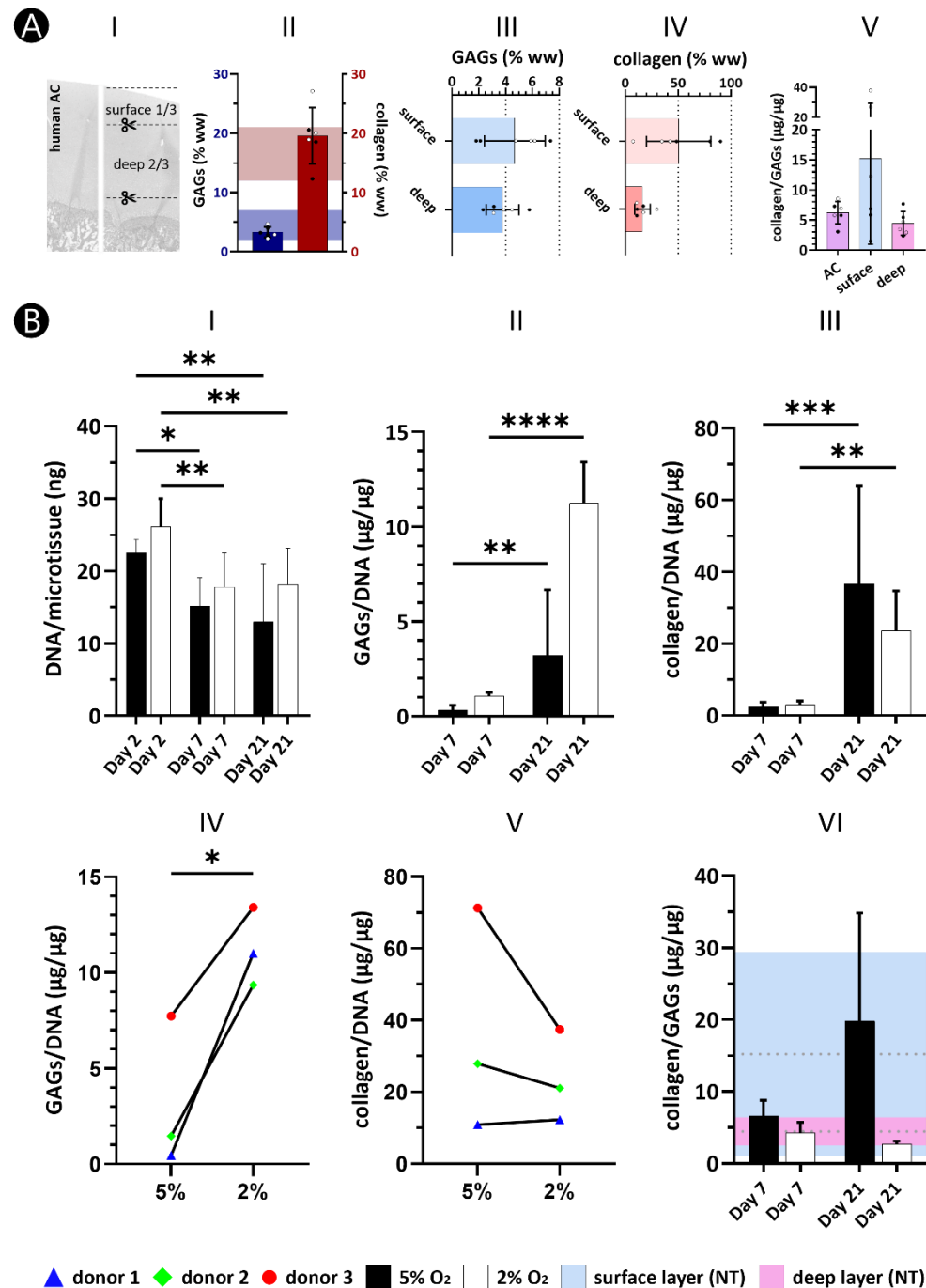


FIGURE 4.1 Biochemical comparison of native human articular cartilage and engineered human mesenchymal stem cell-derived chondrogenic microtissues. Quantification of GAGs normalised by wet weight and collagen normalised by wet weight of the full-thickness (**A-II**) and the superficial and the deep (**A-III** and **A-IV**) regions of human native AC cut into approximately 1:2 (**A-I**). The collagen to GAGs ratio (**A-V**) of the full-tissue thickness, the superficial and the deep regions. All data is represented as individual data points and mean $\pm$ standard deviation. The light red and light blue boxes in **A-II** correspond to values found in the literature [3]. $\bullet$, $\circ$ represent two different human donors. AC: articular cartilage; GAGs: glycosaminoglycans; ww: wet weight. Biochemical assay of DNA per microtissue (**B-I**), GAGs normalised by DNA (**B-II**) and inter-donor variability at day 21 (**B-IV**), collagen normalised by DNA (**B-III**) and inter-donor variability at day 21 (**B-V**). The collagen to GAGs ratio (**B-VI**) of microtissues differentiated under

2% or 5% O₂ for 21 days. All data is represented as mean ± standard deviation or individual data points (**B-IV** and **B-V**). Statistical differences were determined using a one-way ANOVA with Holm-Šidák's multiple comparison test and a paired t-test (**B-IV** and **B-V**). Normality was determined using D'Agostino & Pearson's test and Shapiro-Wilk's test (**B-IV** and **B-V**). N = 9 and 3 (**B-IV** and **B-V**). * denotes p < 0.05, ** denotes p < 0.01, *** denotes p < 0.001, **** denotes p < 0.0001. DNA: deoxyribonucleic acid; NT: native tissue.

4.3.2. 2% and 5% Oxygen Support Type II Collagen Deposition with no Type X Collagen Deposition

After 21 days of culture microtissues stained positive for GAGs (Alcian Blue) and collagens (Picrosirius Red) but were negative for calcium accumulation (Alizarin Red) (**Figure 4.2A**). GAG staining was generally homogeneous throughout the microtissue but more intense for the 2% O₂ group than the 5% O₂. A semi-quantitative evaluation of staining intensity confirmed these observations (**Figure 4.2B**). Collagen staining was generally homogeneous at 5% O₂, but at 2% O₂ was generally stronger at the periphery of the microtissues and less intense in the centre. Cells in both the centre and periphery appeared chondrocyte-like, with a round shape and the presence of a lacuna. Immunofluorescence staining of type I, II and X collagens was then carried out. Under both oxygen environments, except for one donor, microtissues stained positive for type II collagen, with no statistical differences in staining intensity observed. Microtissues were also positive for type I collagen. Type X collagen staining was negative for both oxygen groups but slightly visible for one donor.

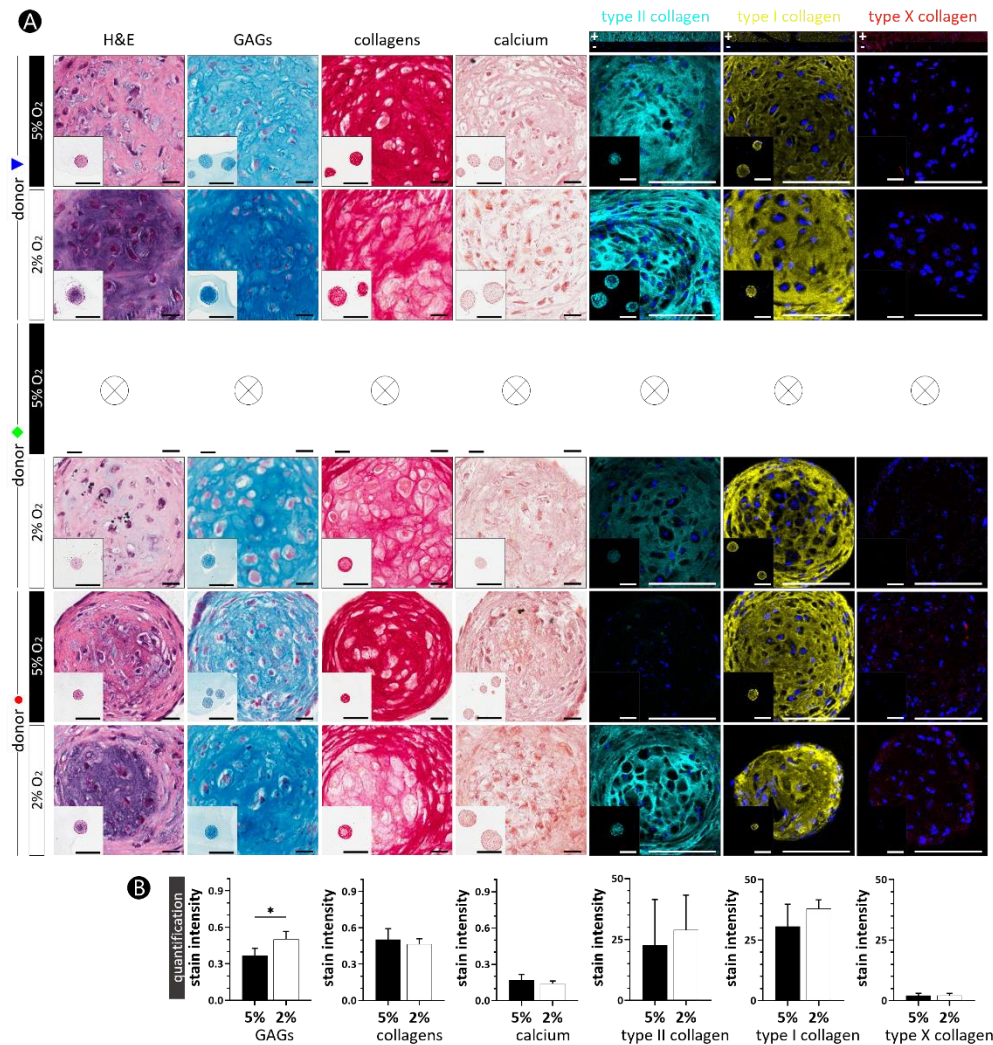


Figure 4.2 Histology and immunofluorescence staining panel after chondrogenic differentiation at 2% and 5% O₂. (A) Staining of microtissue-synthesised extracellular matrix with H&E, Alcian Blue to visualise GAGs, Picrosirius Red for collagens, Alizarin Red to stain calcium, and immunostaining of type II, type I and type X collagens (nuclei were stained with DAPI, shown in dark blue), and quantification (B) of the staining intensity at the 21-day timepoint. Scale bar = 100 μm/400 μm (inserts). ⊗: not available; H&E: haematoxylin and eosin; GAGs: glycosaminoglycans. All data is represented as mean ± standard deviation. Statistical differences were determined using a Student t-test for histology staining (N = 4, normal distribution according to Shapiro-Wilk's test) and a nonparametric Mann-Whitney test for immunofluorescence staining (N ≥ 3). ▼, ◆ and ● represent three different human donors. *p < 0.05.

4.3.3. After 7 Days of Low Oxygen Conditions, Microtissues Are Primed Towards High Glycosaminoglycan Production

With the aim at fabricating scaled-up constructs with zonal organisation, microtissues differentiated under varying oxygen conditions can be used as building blocks of the different regions of AC. Fusion of these biological building blocks needs

to be done after a short-term culture to ensure cohesion of the resulting graft. Therefore, phenotypic changes emerging in microtissues in response to shorter (≤ 7 days) exposure to altered oxygen levels were assessed. Biochemical analysis confirmed the effect of oxygen on the chondrogenic microtissue phenotype, as early as 7 days after the introduction of TGF- β 3 in culture. Oxygen-driven differences between such 7-day-old microtissues were also apparent after staining for cartilage ECM components. Chondrocyte-like cells and initial deposition of GAGs were detected (**Figure 4.3**). In addition, GAG staining was more intense when microtissues were differentiated at 2% O_2 compared to differentiation at 5% O_2 .

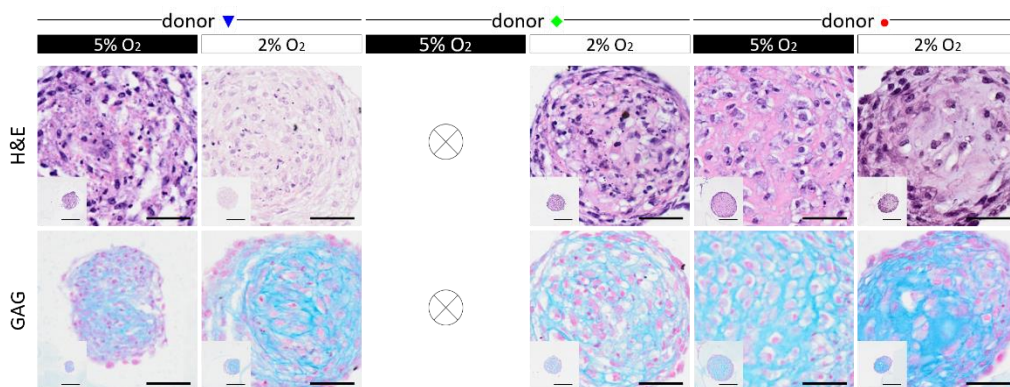


Figure 4.3 Histology staining of 7-day-old microtissues. Microtissues were stained with H&E and Alcian Blue for GAGs. $\blacktriangledown$, $\blacklozenge$ and $\bullet$ represent three different human donors. Scale bar = 50 μ m/200 μ m (inserts). $\otimes$: not available; H&E: haematoxylin and eosin; GAGs: glycosaminoglycans.

4.3.4. Microtissues Primed for One Week at 2% Oxygen Secrete Higher Levels of Glycosaminoglycans in Long-term Culture

Having confirmed that 7 days of differentiation under distinct oxygen environments was long enough to visualise differences of oxygen-driven GAG production, the effect of a 7-day-long oxygen priming on ECM composition was further analysed. Microtissues were either differentiated at 5% or 2% O_2 for 21 days, or first primed at 2% O_2 for 7 days and then moved to 5% O_2 for 14 days (a total culture period of 21 days) to assess if phenotypic changes associated with a relatively short-term exposure to 2% O_2 will persist when such microtissues are then exposed to higher oxygen levels (5% O_2) (**Figure 4.4A**). As an early indicator of microtissue growth, the diameter of the microtissues was measured over this 21-day period (**Figure 4.4B**). Compared to the 5% O_2 group used as control, the diameter of the 2% O_2 (two donors) and the 2–5% O_2 (all donors) microtissues was significantly higher at the final

timepoint. These statistical differences were also observed through the second or third week of differentiation. Macroscopic images of microtissues show a contraction of microtissues after one week of differentiation before an increase in diameter with differentiation time in most groups. Oxygen had no effect on DNA levels (**Figure 4.4C-I**), including when microtissues were exposed to altering oxygen conditions (2–5% O_2). GAG levels were significantly higher in microtissues exposed to altering oxygen conditions (2–5% O_2) than those continuously exposed to 5% O_2 or 2% O_2 (**Figure 4.4C-II**). These results suggest that oxygen-primed microtissues can be used to engineer the zonal organisation of AC ECM in innovative engineering strategies.

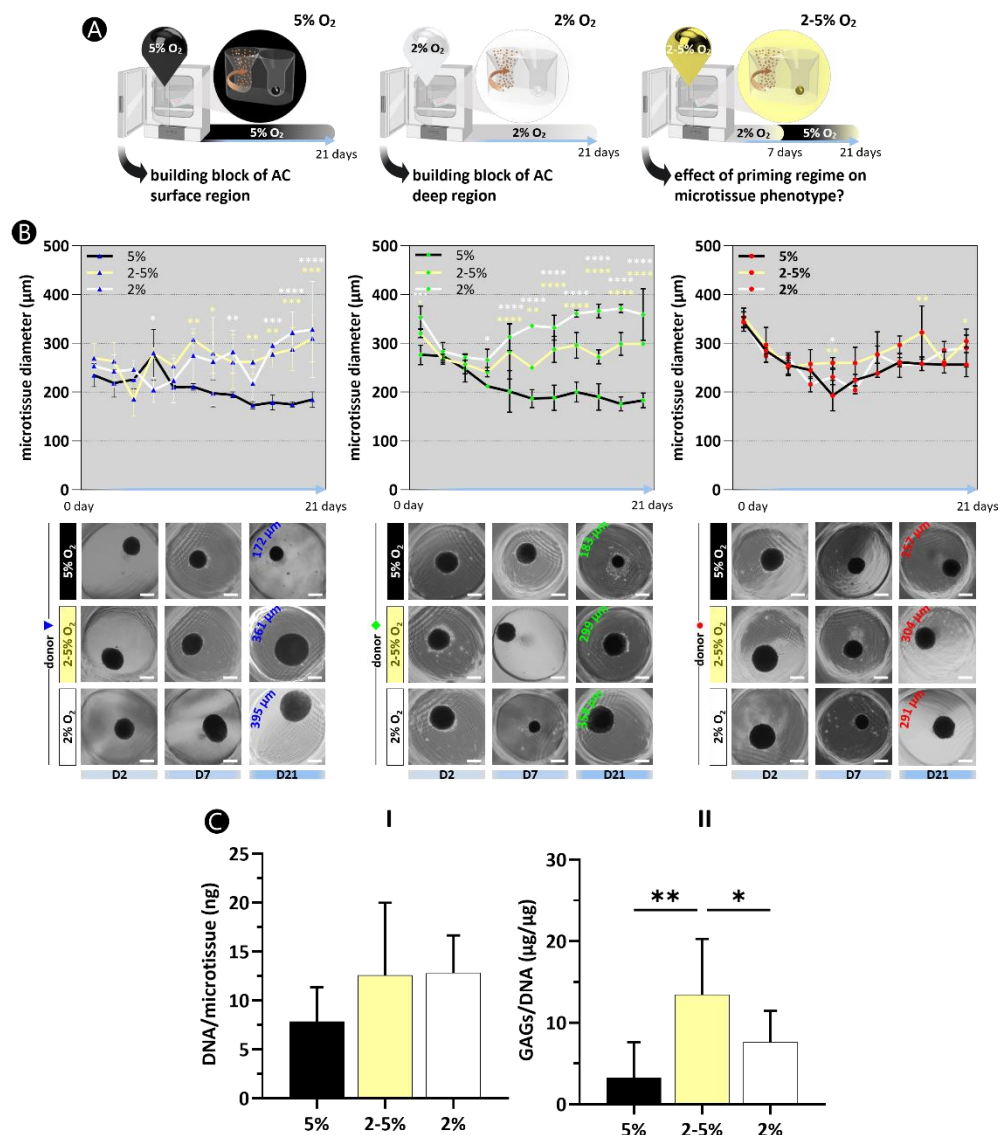


Figure 4.4 Biochemical comparison of three different oxygen conditions used during chondrogenesis of human mesenchymal stem cell-derived microtissues. (A) Microtissues were cultured at 5% or 2% O_2 for 21, or for 7 days at 2% followed by 14 days at 5% O_2 (2–5%P). **(B)** Graphical representation of microtissue diameter, accompanied with representative images,

during chondrogenic differentiation. DNA (C-I) and GAGs (C-II) content at the 21-day timepoint. Scale bar = 300 μ m. All data is represented as mean $\pm$ standard deviation. Statistical differences were analysed by a two-way ANOVA test with Holm-Šídák's multiple comparison test for microtissue diameter and a one-way ANOVA test with Holm-Šídák's multiple comparison test for biochemical results where all groups followed a Gaussian distribution according to D'Agostino and Pearson's or Kolmogorov-Smirnov's normality tests. N = 3 for microtissue diameter and N = 9 for biochemical analysis. ▼, ◆, ● represent three different human donors. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. White * denotes differences between the 2% O₂ and the 5% O₂ groups and yellow * denotes differences between the 2–5% O₂ group and the 5% O₂ group. GAGs: glycosaminoglycans, DNA: deoxyribonucleic acid; D: day.

4.4. Discussion

This study demonstrated that hMSC-derived microtissues with distinct cartilaginous phenotypes can be engineered by altering environmental oxygen levels. Lower oxygen levels (2% O₂) promoted the production of GAGs while maintaining type II collagen synthesis. The collagen to GAG ratio of such microtissues was closer to that of the deep region of native AC compared to microtissues differentiated at 5% O₂. In addition, only one week of 2% oxygen priming was necessary to generate microtissues with this phenotype, pointing to their use as biological building blocks for the biofabrication of spatially heterogeneous tissues.

Although overall levels of ECM accumulation were still lower than those of native AC, 2% O₂ significantly increased GAG production, but had no significant effect on total collagen production. These results confirm the beneficial role of hypoxia on chondrogenesis of hMSCs. Although oxygen-mediated mechanisms are still not fully understood, the transcription factor HIF-1 α has been identified as a key mediator of chondrogenesis in low oxygen environments [214]. In the presence of oxygen, HIF-1 α is hydroxylated, by prolyl-hydroxylases using oxygen as a reaction cofactor, and is then presented to the proteasome for degradation. Alternatively, under hypoxia, HIF-1 α is stabilised and enters the nucleus where it binds the HIF responsive element (HRE) gene promoters [190]. Hence, via gene expression regulation, HIF-1 α is known to regulate chondrocyte differentiation, proliferation and survival, and to increase ECM deposition [215, 216].

Having demonstrated a more potent chondrogenic effect of 2% O₂ compared to 5% O₂, the relevance of this approach for engineering zonally defined cartilage microtissues was assessed by comparing the collagen-to-GAG ratios to that found in the native tissue. The composition of microtissues differentiated at 2% O₂ was more similar to the deep region of native AC, where low levels of oxygen are found [192]. Tissue donors were undergoing partial to total knee surgery, even though tissues were harvested from healthy looking regions. However, values of GAGs and collagen measured in these native tissues were reasonably in agreement with published data and provided a benchmark for engineered tissues [3]. An analysis of donor-donor variability suggests that lower oxygen conditions are particularly beneficial for MSCs with a lower inherent chondrogenic potential (compared to that observed at 5%) and could be a robust inducer of chondrogenesis (**Figure 4.1B-V** and **Figure 4.1B-VI**). In line with this hypothesis, a similar trend was observed with the differentiation of hMSCs and human ACPs where clones with inherent low-GAG production levels were

significantly more responsive to low oxygen conditions with a significant increase of the GAG production [82]. While age is one of the major sources of donor variability in hMSCs [217], lower oxygen could support more robust differentiation conditions.

Characterisation of collagen subtypes by immunofluorescence staining confirmed the generation of cartilage with a strong type II and type I collagen staining even under low oxygen conditions. In addition, no signs of early hypertrophy, characterised by the presence of type X collagen, were visible which is consistent with previous findings in hypoxic conditions [218]. These results were obtained from three different donors. One donor displayed relatively poor chondrogenesis at 5% O₂, with little or no production of type II collagen, which was overcome at 2% O₂. Weak staining for type X collagen was observed at both oxygen levels. Interestingly, this donor also produced more total collagen than other donors at 5% O₂. While lower oxygen does not improve total collagen production, it could be beneficial for generating more hyaline-like cartilage tissue.

The spatial distribution of collagens also appeared to depend on the environmental oxygen conditions. While homogeneous in microtissues differentiated at 5% O₂, staining for collagens of the 2% group was heterogeneous with an intense staining at the periphery and a weaker staining at the centre (without showing signs of necrosis). Previous studies have reported that oxygen levels below 5%, in combination with glucose depletion due to increased cellular consumption in hypoxia, could be detrimental to collagen deposition [191, 219]. As microtissues significantly swell more at 2% O₂ compared to 5% O₂ (**Supplementary Figure 4.1**), resulting from the superior GAG production, a larger size could lead to the presence of a diffusion gradient affecting collagen deposition at the centre.

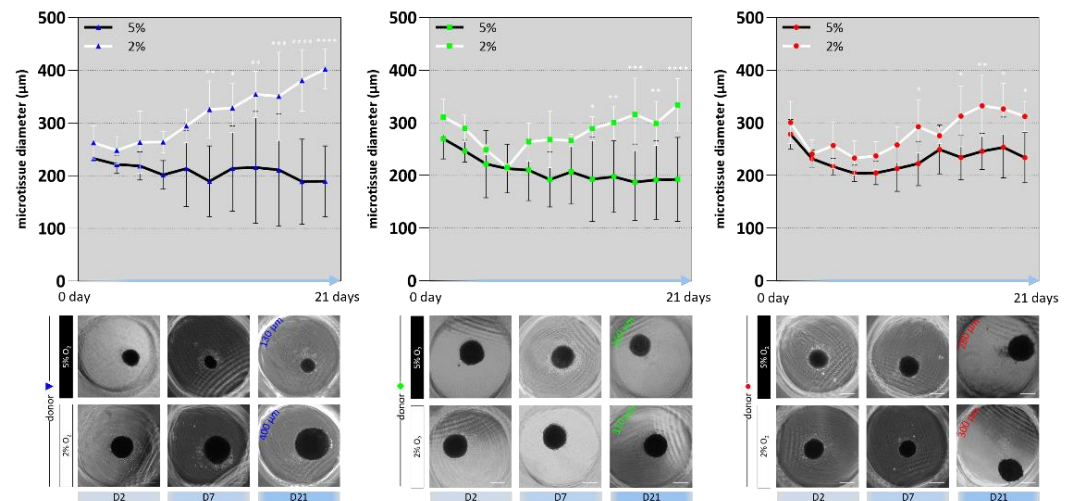
From a tissue engineering point of view, such phenotypically distinct microtissues can be used as biological building blocks for the engineering of zonally organised tissue, in particular to recapitulate the gradient in biochemical composition of AC. The presence of type II collagen with high levels of GAGs is characteristic of the deep region of AC. Hence, microtissues differentiated at 2% O₂ can be used as building blocks for the deep region of AC, while microtissues differentiated at 5% O₂ which contain lower levels of GAGs, could potentially be used as building blocks for the superficial region of AC. While different oxygen levels can easily be set for the culture of individual microtissues, spatial variation of oxygen during culture of larger grafts would require more complex settings. Hence, microtissues were exposed to temporal variations of oxygen from 2% O₂ for the first 7 days and then switched to 5% O₂ for 14 days (termed '2–5%'), to assess whether the effect of short-term exposure to low

oxygen can be maintained once subsequently exposed to higher oxygen levels. In terms of GAG production, microtissues from the 2–5% group outperformed the 5% group but was not significantly higher than the 2% group. During cartilage development and tissue maturation, levels of oxygen vary with the developmental stage (hypoxia promotes cell proliferation and oxygenation promotes organogenesis) and the tissue demand (mechanical loading, circadian rhythm, aging) [197, 220, 221]. In another study, short oxygen priming did not promote the chondrogenesis of cell-laden microsphere hydrogels. However, cell-cell and cell-matrix interactions, which drive chondrogenesis, are different in such scaffolds [22]. It was previously demonstrated that bone marrow MSCs increase their oxygen consumption to levels similar to chondrocytes within a maximum of 14 days, suggesting that the priming period could have been too short to fully capitalise on its beneficial effect [191]. On the other hand, very short temporal exposure (1 day) to growth factors like TGF- β was shown to be equivalent to 21 days exposure [222]. Hence, the effect of external cues, such as growth factors and oxygen, are complex and interconnected [208], and should be taken into account when trying to elucidate their spatiotemporal role during chondrogenesis and AC homeostasis.

4.5. Conclusion

The results of this study confirm the beneficial role of hypoxia on chondrogenesis of hMSCs. At both levels of oxygen, 5% O₂ and 2% O₂, representative to levels found at the surface and deep zones of the tissue, microtissues were successfully generated and were rich in type II collagen. The GAG production was significantly enhanced at 2% O₂ compared to 5% O₂. Besides, the collagen-to-GAGs ratio of microtissues at 2% O₂ were more similar to the deep region of native AC, making them suitable biological building blocks for the fabrication of the deep zone of AC. In addition, a short exposure to low oxygen was enough to maintain and even to enhance the beneficial effect of 2% O₂ on the GAG production compared to 5% O₂. This result stresses the importance of controlling oxygen levels during the chondrogenesis of hMSCs to support higher tissue quality. In the next chapter, such microtissues will be used as biological building blocks in an attempt to generate a hierarchical construct with a biomimetic GAG gradient.

4.6. Supplementary Figures



SUPPLEMENTARY FIGURE 4.1 Evolution of microtissue diameter. Graphical representation of microtissue diameter accompanied with representative images during chondrogenic differentiation at 5% O₂ (black) and 2% O₂ (white). Scale bar = 300 μm. All data is represented as mean ± standard deviation. Statistical differences were analysed by a two-way ANOVA test with Šidák's multiple comparison test where all groups followed a Gaussian distribution (according to Kolmogorov-Smirnov's normality test). N ≥ 3. ▼, ◆, ● represent three different human donors. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. D: day.

5. Modular Assembly of Zonally Defined Articular Cartilage Graft

5.1. Introduction

Inspired by normal developmental processes, in recent years there has been an increased interest in the use of cellular aggregates, microtissues and organoids as building blocks to engineer large cartilage grafts [223]. Such microtissues are typically generated by the self-organisation of cells within environments supportive of cellular aggregation [87, 224]. For example, seeding mesenchymal stem/stromal cells (MSCs) into nonadhesive micro-moulds has been shown to promote cell-cell interactions and the development of hyaline cartilage microtissues [94, 225, 226]. Self-assembly of such spheroids or microtissues into larger tissues presents several challenges, including maintenance of tissue geometry, handleability, and ensuring sufficient nutrient and oxygen transport into the scaled-up graft [227, 228]. Three-dimensional (3D) printing of microtissues, potentially using hydrogel inks or long-term support baths, allows the fabrication and stabilisation of complex geometries [229-232]. However, mechanical deformations during printing, exposure to post-printing cross-linking agents or the negative effects associated with the surrounding printing bath can compromise the success of such approaches. In addition, these approaches have typically only enabled the 3D printing of one population of microtissues, thereby limiting the fabrication of more spatially heterogeneous tissues [102].

To increase the structural stability of engineered tissues, macroporous scaffolds have been used in convergence with cellular spheroids or microtissues. This modular assembly approach consists in the stepwise biofabrication of large constructs by integrating defined tissue units or cellular building blocks with structural scaffolds [223]. One concern with this approach is that large volumes of scaffold material might negatively impact microtissue (re)modelling and/or promote a foreign body response once implanted into the body. For example, the fibres of scaffolds printed by fused deposition modelling (FDM) are usually thick ($>100\text{ }\mu\text{m}$) and could account for more than 30% of the construct [110]. Similar to FDM, melt electrowriting extrudes molten polymers through a nozzle but in the presence of a high electric field, resulting in the

production of extremely thin fibres, down to 2 μm , and the generation of highly porous scaffolds (typically from 80–98% pore volume) with lower polymer contents [233-235].

The geometry of such melt electrowritten (MEW) scaffolds can be precisely modulated to help direct MSC differentiation [108] and guide collagen fibre organisation [236, 237]. Cellular spheroids generated within MEW scaffolds using inkjet bioprinting were previously shown to generate a large sheet-like tissue with a stratified collagen orientation, demonstrating that such hybrid engineered constructs can spatially organise collagen networks with a native-like organisation [111]. MEW meshes have also been directly used in combination with preformed spheroids to fabricate biosheets. A MEW membrane designed with box-like pores was designed to accommodate individual spheroids, using a catching fibre at the bottom of the pore to maintain microtissues in place [234, 238]. In another study, bone-forming biosheets were fabricated using human periosteum-derived cells [110]. Spheroids were directly seeded onto a gelatine-coated MEW mesh ($116\ \mu\text{m} \times 116\ \mu\text{m} \times 10$ layers) used to maintain the shape of the engineered tissue that was then successfully used as a patch to treat critically sized tibial defects *in vivo*.

Successfully engineering complex tissues such as articular cartilage (AC) or the osteochondral unit will also require the integration of phenotypically distinct microtissues into spatially organised grafts. Cartilaginous tissues with a native-like gradient in their composition have been engineered by spatially patterning chondrogenic and hypertrophic cartilage self-assembled discs on either side of a supporting membrane [239], although it remains unclear if such strategies support the development of tissues with a biomimetic collagen organisation. Using an alternative modular assembly approach, spheroids at three different stages of maturation (7-day-old, 14-day-old, and 21-day-old spheroids) were employed as the hyaline cartilage, hypertrophic cartilage and subchondral bone layers of an osteochondral graft, which upon *in vivo* implantation could only generate a fibrocartilage-like tissue [143]. This suggests that further work is required to spatially control the phenotype of grafts produced using MSC-derived microtissues.

Hypoxia and associated cellular signalling pathways play a key role in controlling MSC chondrogenic differentiation [17, 240]. In **Chapter 4** of this thesis, it was demonstrated that 2% O_2 supports the development of more glycosaminoglycan (GAG)-rich microtissues compared to 5% O_2 . However, appropriate control of oxygenation in larger engineered tissues remains a major challenge in current AC tissue engineering strategies [241]. Recently, a discrete gradient MEW scaffold was printed with different decreasing pore dimensions, from top to bottom, resulting in an increase

of chondrogenic expression markers, which was attributed at least partially to a pore size associated gradient in oxygen tension [109].

The objective of this study is to assemble cartilaginous microtissues, primed at different oxygen tensions, into a geometrically defined graft with a biomimetic collagen network organisation. To achieve this, microtissues were integrated with a thin MEW mesh with custom pore sizes which functioned to collect and nest individual microtissues in a highly organised manner. The MEW mesh was designed with minimal material volume so as to not impede microtissue fusion whilst also potentially guiding the organisation of the secreted collagen fibres. This approach also minimises the fraction of synthetic material within the construct and increases biocompatibility. It is hypothesised that the collagen fibres deposited by microtissues adjacent to the MEW mesh will align parallel to the guiding structure. Furthermore, how direction of growth will influence the development of such engineered tissues was investigated by hanging the microtissues from the MEW mesh. Finally, a layered construct was created by using microtissues primed under altered oxygen conditions in an attempt to recapitulate the GAG gradient observed through the depth of native AC. As previously demonstrated, microtissues differentiated at 5% O₂ produced less GAGs than microtissues differentiated at 2% O₂ and will be used as modular building blocks to engineer the surface layer and deep layer of AC, respectively.

5.2. Materials and Methods

5.2.1. Human MSC Chondrogenic Differentiation

5.2.1.1. Expansion of hMSCs

Human MSCs (hMSCs) were either expanded to passage 3 from a commercial source (RoosterBio) or isolated and expanded from one human bone marrow donor (Lonza) for the single-layered engineered tissue study or the bilayered engineered tissue study, respectively. Seeding cell density was 3,000 cells/cm². Cells were kept at 5% O₂ and 5% CO₂ at 37 °C in humidified atmosphere until 80–90% confluency. Expansion media (XPAN) was changed twice per week. XPAN contained high glucose Dulbecco's Modified Eagle Medium GlutaMAX, 10% v/v foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin (all Gibco) and 5 ng/mL fibroblast growth factor 2 (Prospect Bio).

5.2.1.2. Single Microtissue Culture

Microtissues were fabricated as described elsewhere [173]. Briefly, an array of microwells (1.5 mm depth × 1 mm diameter) was moulded in sterile liquid 4% w/v agarose dissolved in phosphate buffer saline (PBS) using 3D printed acrylic positive moulds. After gelation of the agarose, positive moulds were removed and microwells were soaked in XPAN. Twenty-four hours later, a cell suspension (1,604,000 cells/3 mL of XPAN) was added, settled for 15 min and spun at 800 × g for 5 min, resulting in microtissues containing 4,000 cells each. After 24 h of incubation at respective oxygen conditions, XPAN was then replaced by chondrogenic media (CDM) containing: high glucose Dulbecco's Modified Eagle Medium GlutaMAX, 100 U/mL penicillin, 100 µg/mL streptomycin (both Gibco), 1.5 mg/mL bovine serum albumin, 40 µg/mL L-proline, 100 µg/mL sodium pyruvate, 1 × insulin-transferrin-selenium, 100 nM dexamethasone, 50 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid (all from Sigma) and 10 ng/mL human transforming growth factor (TGF)-β 3 (Peprotech). CDM was changed every 2–3 days. Single microtissues were primed for a total of 7 days at 2% O₂ or 5% O₂ (5% CO₂ at 37 °C in a humidified atmosphere). After 7 days of culture, microtissues were harvested from their microwells and pooled into a larger agarose well (described below) to undergo fusion.

5.2.2. Graft Modular Assembly

5.2.2.1. Fabrication of a MEW Mesh-Bottomed Well

Polydimethylsiloxane (PDMS) insert—PDMS was prepared according to the manufacturer's instruction (SYLGARD™ 184 Silicone Elastomer Kit, Dow). Inserts with the following dimensions were punched using biopsy punches: ① 5 mm outer diameter, 3 mm inner diameter and 5 mm in height, or ② 4 mm in diameter and 5 mm in height. Inserts were washed in PBS followed by ethanol for 30 min and finally were rinsed with PBS before use.

MEW mesh—MEW meshes were printed using a custom-built MEW printer [242]. The Capa 6500D polycaprolactone (50,000 MW, Perstorp) was melted at 80 °C and extruded at 5 mm from the collector through a 23G needle heated up to 85 °C under 0.6 bar. An initial voltage of 6 kV was applied with an increase of 0.025 kV per layer. Fibre diameter was 10 µm and fibres were deposited following predefined geometries. Dimensions of the printed pores were: 200 µm × 200 µm, 210 µm × 210 µm, 220 µm × 220 µm, 230 µm × 230 µm, 240 µm × 240 µm or 250 µm × 250 µm. Pore wall was only six fibres in height (six fibres stacked in the x-direction and six fibres stacked in the y-direction—corresponding to theoretical value between ~60 µm and ~120 µm in height) to minimise the presence of biomaterials without compromising the pore wall stability. After selecting a fitting pore size, a 1 cm × 1 cm mesh was cut out and treated with 5 M sodium hydroxide for 30 min, washed in PBS and ethanol for 30 min. The mesh was air-dried overnight and ultraviolet (UV) sterilised for 30 min. Finally, the mesh was coated with 10 µg/mL of fibronectin (Merck) in PBS for 24 h.

Fabrication of a MEW mesh-bottomed well—MEW mesh-bottomed wells were fabricated using 6-well plates. A fibronectin-coated MEW mesh was maintained between PDMS inserts (see ① in **Section 5.2.2.1.** for insert dimensions). Sterile 2% w/v agarose in PBS was molten at 60 °C, to avoid melting the polycaprolactone (PCL) fibres, and poured around the inserts to allow the embedding of the MEW mesh. After gelling of the agarose, the inserts were pulled out, creating a 5 mm × 5 mm well which bottom part consisted of a MEW mesh. Finally, 6 mL of XPAN was added for 24 h prior to microtissue seeding.

5.2.2.2. Fusion of Individual Microtissues into a Scaled-Up Graft

5.2.2.2.1. Single-Layered Hybrid Tissue

Microtissues were primed for 7 days at 2% or 5% O₂. The priming period was selected based on previous results to maximise the effect of oxygen on the resulting

cartilaginous phenotype (**Figure 4.4**) without compromising microtissue fusion [212]. After 7 days, microtissues were liberated from their microwells and a theoretical number of 1,200 microtissues were suspended in 100 to 200 μ L of CDM. The microtissue suspension was seeded into the MEW mesh-bottomed well with selected pore size based on microtissue diameter to collect and compact them at the bottom of the well. Each well was topped up with 6 mL of freshly prepared CDM (media was changed every 2 days for the rest of the culture period) and cultures were returned to their initial oxygen environment. After 2 days of fusion, microtissues initiated attachment to the mesh. To investigate the effect of direction of growth on the organisation of the collagen network, at both oxygen levels, constructs were flipped upside down so the fusing graft was hanging and growing downwards from the MEW mesh. These groups are called 'hanging'. At both oxygen levels, other constructs were kept as control with tissue developing upwards from the MEW mesh. These groups are called 'sitting'. Culture conditions are summarised in **Table 5.1** Description of different culture conditions used during the modular assembly of a single-layered hybrid tissue. Fusion lasted 13 days for a total of 21 days of differentiation culture. At the end of the differentiation period, the scaled-up tissues were cut in half. One half was digested prior to biochemical assays and the other half was used for histology and immunofluorescence staining.

Table 5.1 Description of different culture conditions used during the modular assembly of a single-layered hybrid tissue.

Groups	Single Microtissues—O ₂ (%)	Fused Microtissues	
		O ₂ (%)	configuration
5H	5	5	hanging
5S	5	5	sitting
2H	2	2	hanging
2S	2	2	sitting

5.2.2.2.2. Bilayered Hybrid Tissue

Microtissues, that will be used to fabricate the first layer of construct, were cultured for 7 days at 5% O₂. Microtissues, that will be used to fabricate the second layer of the construct, were cultured for 7 days at 2% O₂. Similarly to the modular assembly of the single-layered hybrid tissue, the first layer (of 7-day-old microtissues primed at 5% O₂) was added onto the MEW mesh-bottomed wells (pore size selected based on microtissue diameter) and was topped with CDM for 2 days until the second

layer was added. To do this, 7-day-old microtissues primed at 2% (theoretical number of 1,200) were suspended in 50 to 100 μL of CDM and added over the first layer. The engineered graft was added with CDM and returned at 5% O_2 (5% CO_2 at 37 °C in a humidified atmosphere) for the rest of the culture period. To facilitate the fusion of the second layer and the integration between layers, the constructs were left in the sitting configuration for 2 days. Then, the system was hung upside down. The total culture period was 42-day long and CDM was changed three times per week. Timeline and culture conditions are summarised in **Table 5.2**.

Table 5.2 Timelines and culture conditions used during the modular assembly of a bilayered hybrid tissue. FD: fusion day.

Day	O_2 (%)	Step	FD
0	5	priming single microtissues	-
2	2	priming single microtissues	-
7	5	seeding of 5% O_2 primed microtissue on MEW mesh (1 st layer)	0
9	5	seeding of 2% O_2 primed microtissue (2 nd layer)	2
11	5	hanging of the bilayered engineered tissue	4
42	5	histology and immunofluorescence analysis	35

5.2.3. Microtissue Fusion in a Nonadherent Agarose Well

To confirm the guiding effect of the MEW mesh on the collagen network, a 4 mm diameter nonadherent well (5 mm in height) was fabricated using 2% w/v agarose in PBS and a PDMS insert (see ② in **Section 5.2.2.1**. for insert dimensions). After 7 days of culture at 5% O_2 and 5% CO_2 and 37 °C in humidified atmosphere, a theoretical number of 1,200 microtissues were seeded in this nonadherent well for fusion in CDM. Tissues were maintained at 5% O_2 and 5% CO_2 for 21 days at 37 °C in humidified atmosphere. CDM was changed three times per week. At the final timepoint, scaled-up grafts were collected and prepared for further histology analysis.

5.2.4. Microtissue and MEW Mesh Characterisation

5.2.4.1. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was carried on gold/palladium coated MEW mesh samples previously mounted on carbon adhesive covered pin stubs. Coating was carried for 60 s at 40 mA current using the Cressington 208HRD sputter

coater (Cressington Scientific Instrument Ltd.). Images were taken using the Zeiss ULTRA plus SEM (Zeiss).

5.2.4.2. Immunofluorescence Imaging of the Fibronectin Coating

Meshes coated with 10 µg/mL fibronectin were incubated in 10% v/v goat serum and 1% w/v bovine serum albumin (both from Merck) in 1X PBS for 1 h at room temperature (RT) in a humidity chamber (HC) to block unspecific antigen sites. Anti-fibronectin (dilution 1:200; ab23751, abcam) primary antibody diluted in blocking buffer was incubated overnight at 4 °C in a HC. A goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (dilution 1:250; A11037, Invitrogen) diluted in blocking buffer was incubated for 1 h at RT in the dark in a HC. Slides were coverslipped using Prolong™ Gold Antifade Mountant (Invitrogen). A fibronectin-coated mesh incubated in blocking buffer without primary antibody was used as negative control. Staining was visualised using a scanning confocal microscope (LeicaTCS SP8) and images were taken as z-stacks of 10 images.

5.2.4.3. Microtissue and Pore Size

The average diameter was calculated from the microtissue area (area of a circle) measured using the Fiji area tool on 8-bit brightfield microscopy images. Images were manually threshold. More than 450 measurements from two different donors were taken for each oxygen condition.

The size of the MEW mesh pores was measured using the Fiji length measurement tool on brightfield microscopy images. Three hundred measurements of fibre interspacing were taken from three different regions of printed meshes.

The MEW mesh height (h) relative to the microtissue height was calculated using the following equation: $h = \frac{D}{2} - 0.5 * \sqrt{D^2 - P^2} - 60$, where D represents the diameter of microtissues, P represents the size of the mesh pores, and 60 represents the height of the MEW mesh walls (theoretical value of 60 µm).

5.2.5. Tissue Staining

Single-layered, bilayered engineered constructs and control were retrieved from the culture media, washed with PBS and fixed using a 4% w/v paraformaldehyde solution overnight at 4 °C. Samples were then embedded in 2% w/v agarose, dehydrated in a graded series of ethanol solutions (10%–70%) and processed using an

automatic tissue processor (Leica TP 1020). Finally, all dehydrated samples were embedded in paraffin wax prior to rotary sectioning (Leica RM2235) into 7 µm thick tissue sections. Single-layered samples were entirely sliced and two different cuts corresponding to the central and peripheral regions of the engineered tissue were stained. Best, medium and worst replicates were determined based on the macroscopic evaluation of tissue homogeneity and shape.

5.2.5.1. Histology

Sections were stained with Haematoxylin and Eosin, with 0.1% w/v Picrosirius Red to visualise collagens and with 1% w/v Alizarin Red (pH 4.1) to determine calcium deposition. GAGs were either stained with 1% w/v Alcian Blue 8GX in 0.1 M hydrochloric acid (pH 1) and 0.1% w/v Nuclear Fast Red (to counterstain cells) or with 0.2% w/v Safranin O, 0.04% w/v aqueous Fast Green counterstaining and Weigert's Iron Haematoxylin (to counterstain cells). All stains were from Merck. Stained sections were imaged using a slide scanner (Aperio ScanScope CS).

5.2.5.2. Immunofluorescence Staining

Sample sections were rehydrated in graded series prior to antigen retrieval using 35 U/mL of pronase for type I and II collagens or 3.5 U/mL of pronase (Merck) for type X collagen for 25 min at 37 °C in a HC and followed by 4,000 U/mL hyaluronidase (Merck) for 45 min at 37 °C in a HC. Blocking of non-specific antigen sites was performed using 10% v/v donkey serum (abcam) and 1% w/v bovine serum albumin (Merck) in 1X PBS solution (blocking buffer) for 1 h at RT in a HC. Primary antibodies and fluorescently labelled secondary antibodies were diluted in blocking buffer (summarised in **Table 5.3**). Primary antibodies were incubated overnight at 4 °C in a HC and secondary antibodies were incubated for 1 h at RT in the dark in a HC.

Table 5.3 Antibody types and dilutions. I^{ary}: primary antibody; II^{ary}: secondary antibody.

Target	Catalogue	Supplier	Dilution
I ^{ary} : anti-type I collagen	ab138492	abcam	1:500
II ^{ary} : donkey anti-rabbit	ab150075	abcam	1:500
I ^{ary} : anti-type II collagen	sc-52658	Santa Cruz Biotechnology	1:100
II ^{ary} : donkey anti-mouse	A21203	Invitrogen	1:500
I ^{ary} : anti-type X collagen	14-9771-82	Invitrogen	1:50
II ^{ary} : donkey anti-mouse	A21203	Invitrogen	1:200

Prior to imaging, slides were mounted in Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI) and scanned (z-stacks of 10 images) with a scanning confocal microscope (LeicaTCS SP8). Positive and negative controls used to set the microscope gain and offset are summarised in **Table 5.4**.

Table 5.4 Immunofluorescent staining controls. AC: articular cartilage.

Stain	Specie	Positive	Negative
type II collagen	pig	AC	ligament
type I collagen	pig	ligament	AC
type X collagen	pig	hypertrophic AC	AC or hypertrophic AC without primary

5.2.6. Biochemistry Quantification

At the end of the *in vitro* culture, constructs were collected from the culture media and washed with PBS. Digestion with 3.88 U/mL papain enzyme in papain buffer extract (100 mM sodium phosphate buffer and 5 mM ethylenediaminetetraacetic acid disodium salt solution) containing 10 mM L-cysteine (pH 6.5) was left overnight at 60 °C. Digests were analysed for their deoxyribonucleic acid (DNA), GAG and collagen contents. Total DNA was quantified by bisBenzimide Hoechst fluorescent assay as described by the manufacturer's instructions. Total GAGs were quantified using a 0.002% w/v 1,9-dimethyl-methylene blue dye solution containing 0.25% w/v sodium chloride and 0.3% w/v glycine (pH 1.5). Shark chondroitin sulphate dilutions were used as standards. The ratio of the extinction coefficient at 530 nm and 590 nm wavelengths was measured using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc) and used to extrapolate the concentration of GAGs in samples [174-176]. Total collagen was quantified by the hydroxyproline assay as previously described [177, 178]. After hydrolysis of proline residues using 12 M hydrochloric acid overnight at 110 °C, solid material was dried out and resuspended in ultrapure deionised water. An oxidising solution of 2.82% w/v chloramine T in citrate buffer (pH 6.1) was added for 20 min at RT in the dark. Then, 50% w/v 4-(dimethylamino)benzaldehyde, reacting with pyrrole residues, was added for 20 min at 60 °C. trans-4-Hydroxy-L-proline (Fluka) dilutions were used as standards. The absorbance of blank, samples and standards at the 570 nm wavelength was measured using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc). Finally, the collagen content was calculated assuming a hydroxyproline-to-collagen ratio of 1:7.69 [178]. All reagents were from Merck if not mentioned otherwise.

5.2.7. Polarised Light Microscopy

Polarised light microscopy (PLM) was used to visualise collagen fibres in tissue sections stained with Picrosirius Red. Colour maps of the collagen network orientation were generated using 8-bit PLM images and the OrientationJ Analysis plugin in Fiji.

Mapping of the orientation distribution of collagen fibres—Maps of the orientation distribution were generated using the Directionality feature in Fiji. Sections from the 5S, 5H, 2S and 2H groups, as well as from the nonadherent control and from AC native tissue were divided into 10 zones of equal height, from the surface to depth. Within each zone, the orientation distribution of three regions of interest (ROIs) were analysed. Two sections, from the periphery and the centre of the construct, were analysed per replicate. Two replicates were analysed per experimental group. Construction of the map of orientation distribution is detailed in **Supplementary Figure 5.1**. In addition, the regional (surface (top 10%), middle (intermediate 80%) and deep regions (bottom 10%)) organisation of the engineered tissues was analysed separately.

Dominant fibre orientation and fibre coherency—Quantification of the dominant fibre orientation and fibre coherency of the collagen fibres were measured using the OrientationJ Dominant Direction plugin (Fiji Software). To analyse the effect of the MEW mesh on the collagen organisation, ROIs were taken from the zone containing the MEW mesh. Multiple ROIs from two replicates were analysed per experimental group. To quantify the dominant orientation and coherency of collagen fibres outside the MEW pores, ROIs were taken outside the zone containing the MEW mesh. Two sections, from the periphery and the centre of the construct, were analysed per replicate. Two replicates were analysed per experimental group.

5.2.8. Statistical Analysis

The GraphPad Prism software (GraphPad Software, CA, United States) was used to analyse statistical differences. Analysis of Gaussian distribution and statistical tests are described on a case-by-case basis in figure captions. Significance was accepted at a level of $p < 0.05$.

5.3. Results

5.3.1. Fusion of Microtissues in a Nonadherent Agarose Well Supports the Development of a Cartilage Graft with Isotropic Collagen Network Organisation

Large cartilage constructs were generated by fusing 7-day-old microtissues in a cylindrical nonadherent agarose well (4 mm diameter × 5 mm height). The seeded microtissues covered the bottom surface of the agarose well. After 3 days of fusion, single microtissues were still visible but had started to self-organise, generating a ~3 mm large construct (**Figure 5.1-A**). After 21 days of chondrogenic differentiation, a compact spherically shaped tissue was formed, which contracted to ~2 mm in diameter. Histological analysis of the resulting tissue with Alcian Blue and Picrosirius Red confirmed the presence of GAGs and collagens in the engineered tissue (**Figure 5.1-B**).

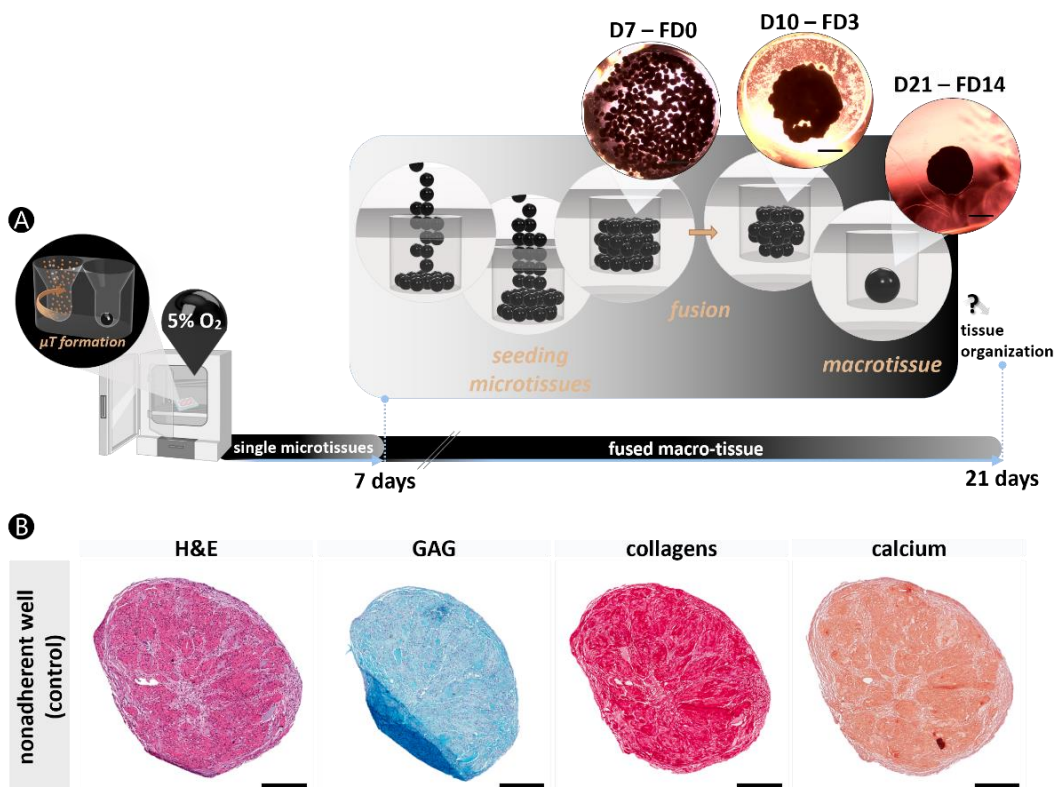


FIGURE 5.1 Microtissue fusion in a nonadherent agarose well. **(A)** Schematic of the fabrication steps of a scaled-up construct using a nonadherent agarose well. Scale bar = 1 mm. μT: microtissue; D: day; FD: fusion day. **(B)** Analysis of graft extracellular matrix composition. Scale bar = 300 μm. H&E: haematoxylin and eosin; GAG: glycosaminoglycans.

5.3.2. Fabrication of a MEW Mesh to Support Microtissue Fusion

MEW scaffolds have previously been used to guide collagen orientation within engineered tissues. In an attempt to guide the orientation of collagen fibres deposited by hMSC-derived microtissues, a novel modular assembly strategy was developed where a MEW mesh maintained inside an agarose well was leveraged to structurally support the fusion of microtissues, see **Figure 5.2**. To this end, a MEW mesh sheet (1 cm × 1 cm) was placed between two PDMS cylindrical (5 mm × 3 mm × 5 mm) inserts (①-④). The inserts were maintained in place with a custom-made 3D printed handle cover (not represented). Molten 2% w/v agarose was added around the inserts to entrap the MEW mesh (⑤). After gelation, the top insert could easily be removed using tweezers (⑥). The agarose allowed an easy handling of the MEW mesh. Then, the system was flipped upside down to remove the other insert (⑦, ⑧). As seen in the top and side views, the positive inserts left behind an empty cylindrical hole with the MEW mesh remaining in its centre creating a MEW mesh-bottomed well. The system was soaked in XPAN until microtissue seeding (⑨).

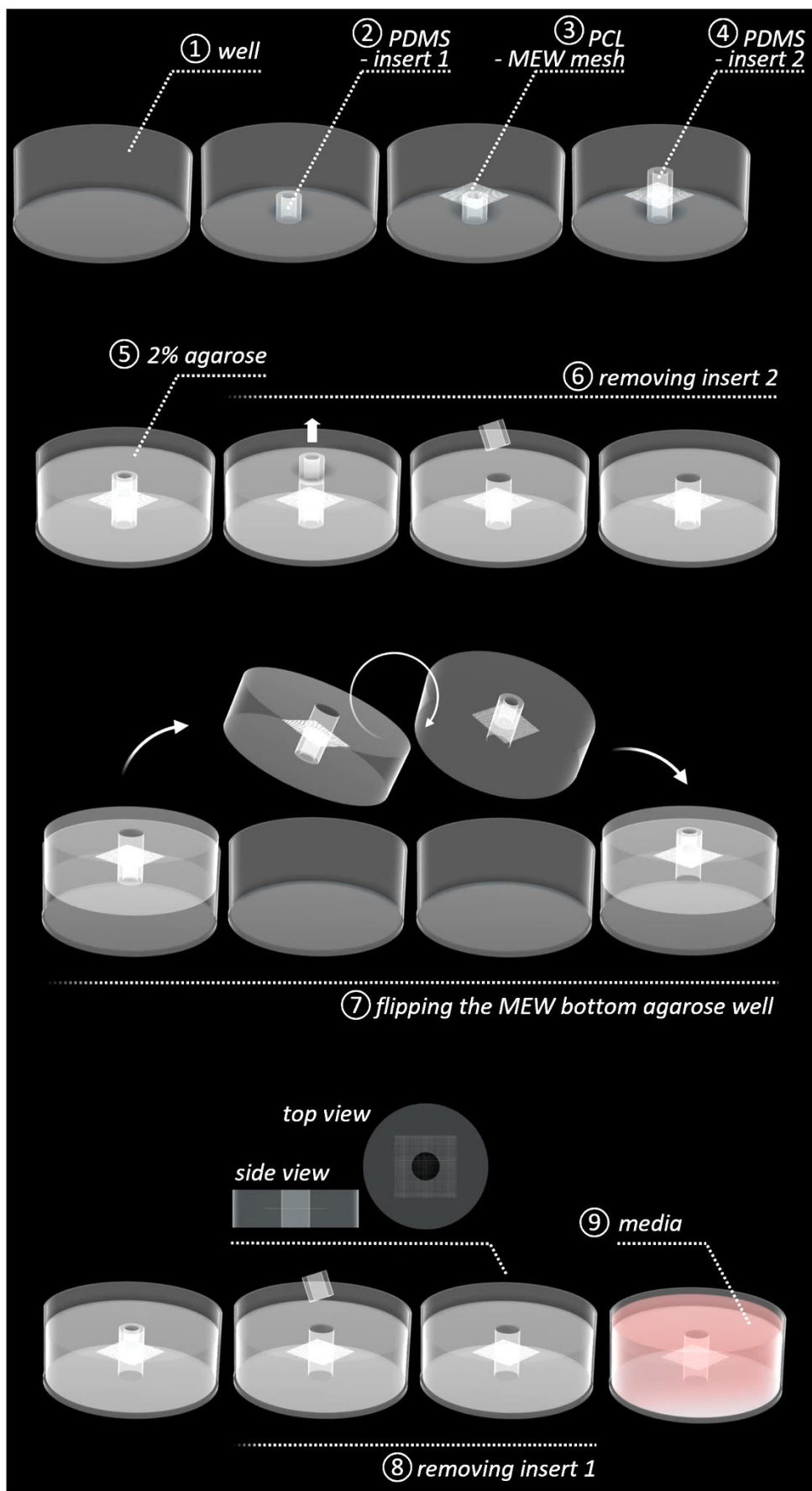


FIGURE 5.2 Fabrication of a MEW mesh-bottomed well. PDMS: polydimethylsiloxane; PCL: polycaprolactone; MEW: melt electrowritten.

To guide the orientation of collagen fibres, the MEW mesh was designed to support a single individual microtissue inside each of its pores. The pore size was adapted to the size of microtissues to collect them in each pore without letting them pass through (**Figure 5.3-A**). This configuration should promote the fusion and direct the growth of microtissues into a millimetre scale construct. In addition, the presence of a highly porous MEW mesh at the bottom of the well should allow nutrients and oxygen to reach the bottom surface of the construct, supporting reasonably consistent nutrient supply to both surfaces of the construct. Before their seeding onto the MEW mesh, the diameter of the microtissues after 7 days of chondrogenic differentiation measured on average $227\ \mu\text{m} \pm 32\ \mu\text{m}$ at 5% O_2 and $260\ \mu\text{m} \pm 45\ \mu\text{m}$ at 2% O_2 , with a range from $46\ \mu\text{m}$ to $452\ \mu\text{m}$ (both measured in the 2% O_2 group) and with most of the microtissues measuring between $200\ \mu\text{m}$ to $300\ \mu\text{m}$ (**Figure 5.3-B**). More specifically, more than half of the microtissues measured $221\ \mu\text{m}$ to $261\ \mu\text{m}$ (median values of both groups).

MEW meshes were successfully printed demonstrating good fibre stacking and no collapsing of the pore wall (**Figure 5.3-CI**). To enhance cell adhesion and promote maintenance of tissue geometry, the MEW fibres were treated with sodium hydroxide and successfully coated with fibronectin prior to the fabrication of the MEW mesh-bottomed well (**Figure 5.3-CII**) [69]. Finally, meshes, with different theoretical pore dimensions designed to support microtissue dimensions previously measured ($200\ \mu\text{m} \times 200\ \mu\text{m}$, $210\ \mu\text{m} \times 210\ \mu\text{m}$, $220\ \mu\text{m} \times 220\ \mu\text{m}$, $230\ \mu\text{m} \times 230\ \mu\text{m}$, $240\ \mu\text{m} \times 240\ \mu\text{m}$, $250\ \mu\text{m} \times 250\ \mu\text{m}$) were printed. Measurements of the pore size showed that pore size fidelity increased with printed pore size (**Figure 5.3-CIII**). The fitting map in **Figure 5.3D** was calculated to anticipate the MEW pore size to be printed according to the size of growing microtissues. The goal was to print a mesh with a pore size that allowed the collection of microtissues without having them sitting too high, which could interfere with microtissue fusion, while simultaneously preventing them from passing through the bottom of the MEW pores.

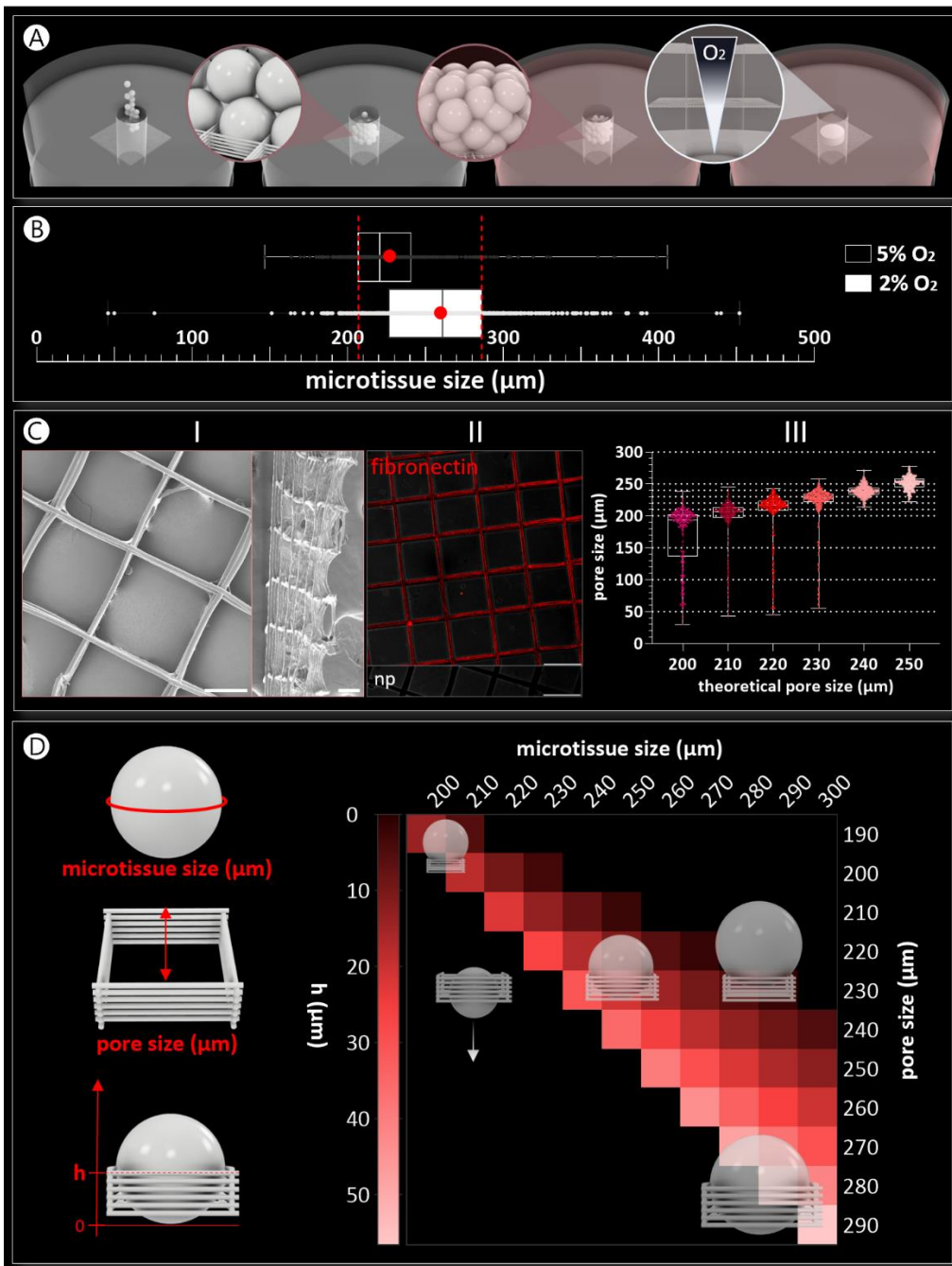


FIGURE 5.3 Seeding microtissues on a MEW mesh: matching microtissue size and MEW mesh pore size. (A) Schematic of microtissue seeding onto a MEW mesh using a MEW mesh-bottomed agarose well. (B) Distribution of the microtissue size depending on oxygen levels. Data is represented as individual data points and min to max box whisker plot. Red dotted lines indicate the lowest of 1st quartiles (Q1) and the highest of 3rd quartiles (Q3) from either group. The red dot indicates the mean value of each distribution. $N \geq 450$. (C) SEM images (scale bar = 100 μm), fibronectin coating confocal images (scale bar = 200 μm) and distribution of measured MEW pore size for each theoretical printing dimension. A MEW mesh incubated with no primary (np) antibody was used as negative control. (D) Fitting map to match the MEW mesh pore size with the microtissue size to allow optimal fitting of microtissues within the MEW mesh pores. h : height position of the MEW mesh relative to the bottom part of the microtissue.

5.3.3. Single Microtissues Fuse into a Large Construct with Controlled Macroscopic Geometry in the Presence of a MEW Mesh

The new fabrication platform was tested by seeding microtissues (theoretical number of 1,200) in the MEW mesh-bottomed well and maintaining the construct in TGF- β 3 for 21 days. To investigate their fusion capacity, microtissues generated at 2% O₂ and 5% O₂ were seeded onto the MEW mesh. In addition, the role of gravitational forces on fusion and tissue development within this system was also explored. To this end, in one group (called 'sitting') microtissues stayed on top of the MEW mesh, while in a separate group (called 'hanging') the construct was flipped upside down shortly after microtissue seeding to investigate the effect of growing direction on the resulting collagen network organisation (**Figure 5.4**). The selected pore size was 230 μ m $\times$ 230 μ m based on the measured microtissue diameter (mean value: 241 μ m $\pm$ 48 μ m for the 5% O₂ group, 236 μ m $\pm$ 55 μ m for the 2% O₂ group). As seen in brightfield images of all groups, microtissues were successfully collected, resulting in a layer depth of 2–3 microtissues being deposited onto the surface of the MEW mesh. After the initial fusion, no significant contraction of the newly formed tissue was observed with an average of 29% $\pm$ 15% of contraction across all experimental groups (as had been observed in the absence of the guiding mesh with 53% $\pm$ 3% of contraction; see **Figure 5.1**). Most importantly, the hanging group stayed anchored to the mesh after flipping the system upside down. The resulting constructs were approximately 2.5 mm in diameter and 1.5 mm in thickness.

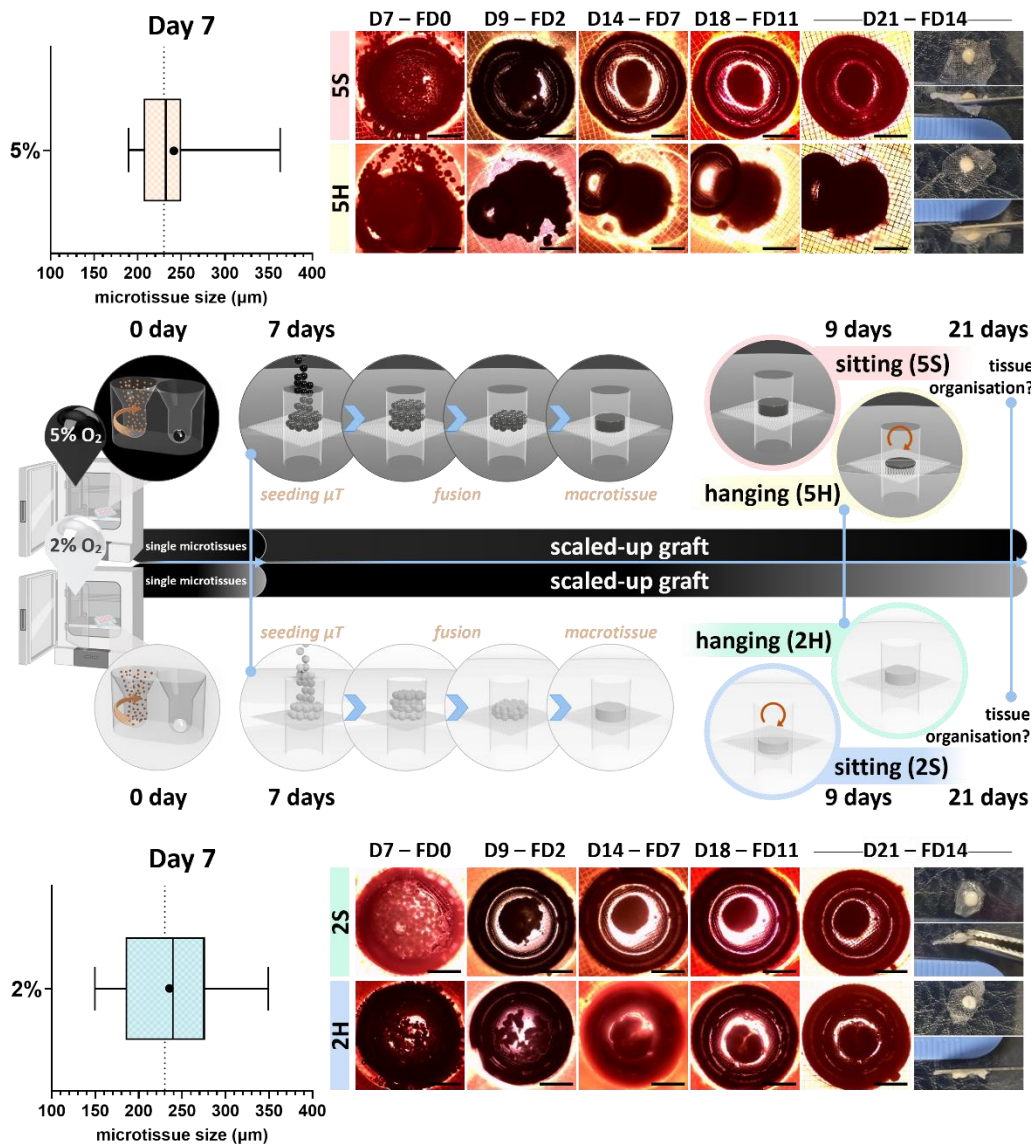


FIGURE 5.4 Single-layered engineered tissue: microtissue fusion in a MEW mesh-bottomed well. Schematic representing the four experimental conditions used during the single-layered fusion study: differentiation at 5% O₂ and hanging tissue (5H), differentiation at 5% O₂ and sitting tissue (5S), differentiation at 2% O₂ and hanging tissue (2H), differentiation at 2% O₂ and sitting tissue (2S); accompanied with macroscopic images monitoring the fusion process. Size of 7-day-old microtissues is represented as min to max box whiskers plot. Black circle represents mean value, and dashed line represents the selected MEW pore size. N = 12. Scale bar = 2 mm. μT : microtissue; D: day; FD: fusion day.

The weight of resulting constructs was not statistically different between the different groups. Hanging tissues from the MEW mesh did not negatively affect the levels of DNA compared to the sitting configuration. Additionally, total GAGs and collagen production were not statistically different across the groups (**Figure 5.5-A**). In histology staining, the attachment points of the neo-tissue to the MEW mesh were visible at the edge of the constructs (at the bottom of the tissues for the sitting groups

and at the top of the tissues for the hanging groups (**Figure 5.5-B**). All constructs formed a relatively flat and elongated tissue, especially visible in the cuts from the centre of the construct (cut #1), unlike those generated in the absence of the supporting MEW mesh. Anchoring one layer of microtissues to the MEW mesh was enough to direct tissue geometry. Even though single microtissues were still visible, the tissue remained compact even in the hanging configuration. All engineered tissues stained positive for GAGs and collagens. Calcium deposition was observed in some regions of the tissues. Immunofluorescent staining for type II collagen was observed primarily at the periphery of the construct, with the highest levels of staining was observed in the 2H group. All groups stained positive for type I collagen. Type X collagen was only detectable in one of the biological replicates of the 5H group after 21 days of chondrogenic differentiation. These results suggest the positive role of the hanging configuration and the 2% O₂ environment to promote type II collagen production. Histology and immunofluorescent staining of biological replicates are found in **Supplementary Figure 5.2** and **Supplementary Figure 5.3**.

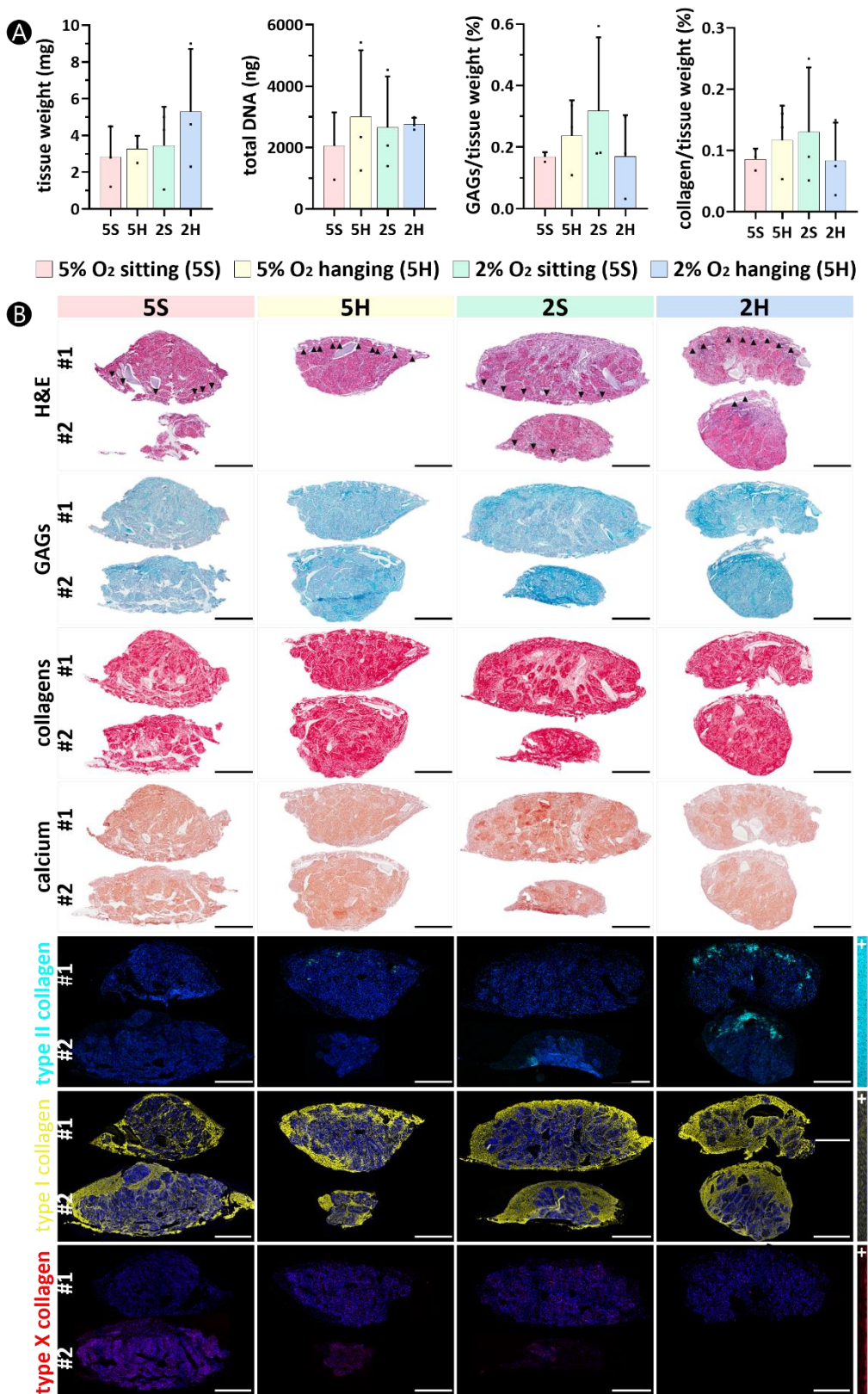


FIGURE 5.5 Single-layered engineered tissue: extracellular matrix characterisation. (A) Biochemical quantification of constructs weight and DNA, GAGs, and collagen normalised by weight after culture at 5% or 2% O₂ both in hanging or sitting configurations. All data is represented as individual data points and mean $\pm$ standard deviation and tested for differences with a one-way ANOVA test with Holm-Šídák's multiple comparison test. N = 3. DNA: deoxyribonucleic acid. **(B)** Histology panel of engineered tissues after fusion in a MEW mesh-

bottomed well. Two sections, from the centre and the periphery of the construct, are shown. Black arrows point at the MEW fibre anchoring points (only represented in the H&E staining). Nuclei were stained with DAPI, shown in dark blue. Scale bar = 500 μ m. H&E: haematoxylin and eosin; GAGs: glycosaminoglycans; #: cut number; (+): positive control; -: negative control.

5.3.4. The MEW Mesh in Hanging Tissues Directs the Orientation of Collagen Fibres with Improved Coherency

Next, whether the MEW mesh and the hanging configuration direct the orientation of the collagen fibre network was assessed using PLM. Unlike the middle region of AC, in all the groups, the collagen orientation distribution in the middle zone was not orientated perpendicular (90°) to the tissue surface (**Supplementary Figure 5.4**). To see if the spatial organisation (through depth) of the engineered tissues was similar to native AC, the constructs (including controls) were divided into 10 zones from the surface to the bottom of the tissue, and the collagen orientation distribution was assessed for each zone (see **Supplementary Figure 5.4** for details on the construct of orientation distribution maps). In native AC, a characteristic collagen network with fibres parallel to the top surface (0°) and fibres orientated perpendicular to the surface (90°) through the majority of the tissue depth was observed (**Figure 5.6**). As a result of its spherical geometry, the control fused in a nonadherent agarose well showed a radial organisation, with fibres aligned circumferentially around the very outside, and then radially out from the centre through much of the thickness. In the presence of a supporting MEW mesh, strong birefringence was observed in all four culture conditions (**Supplementary Figure 5.5**). All groups showed $\sim 0^\circ$ alignment of the collagen fibres at the top surface of the constructs, which closely resembled the surface zone of native AC. Moving down through the section, fibres in the 2H group more coherently orientated in the same direction. Within the central region of the tissue (40% to 80% depth through tissue), fibres aligned at 45° to the surface. In contrast, all the other groups became less organised with depth, showing fibres going towards all different directions within each zone, pointing to a greater level of stratification within the 2H group.

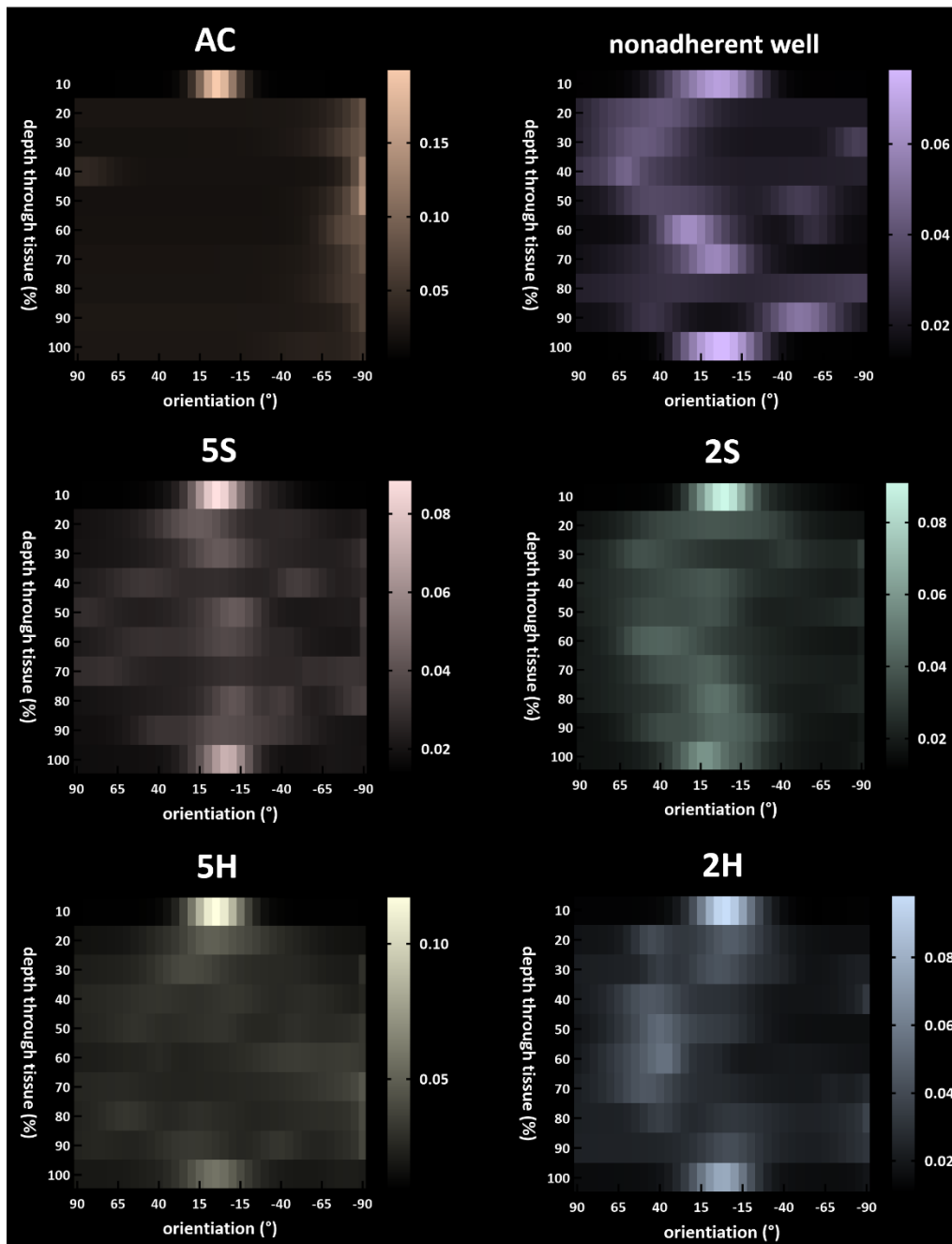


FIGURE 5.6 Single-layered engineered tissue: map of the orientation distribution of the collagen network. Orientation distribution maps were generated for microtissues fused in nonadherent well, native articular cartilage (AC), microtissues fused at 5% O₂ in the sitting configuration (5S), 5% O₂ in the hanging configuration (5H), 2% O₂ in the sitting configuration (2S) and 2% O₂ in the hanging configuration (2H). N = 12 for experimental groups and N = 3 for controls. Data is represented as mean.

Further quantification of the collagen network within the pores of the MEW mesh showed that the dominant fibre orientation was not statistically different from that observed in the surface zone of native AC (**Figure 5.7-A**). Interestingly, the fibre coherency, which reflects the variance of the dominant fibre orientation, of the

hanging groups were significantly increased compared to the sitting groups and were the closer to native values. In addition, the quantification of the dominant fibre orientation of the collagen fibres outside the pores of the MEW mesh in the 2H group was significantly higher than other groups (**Figure 5.7-B**). Hence, PLM quantification highlighted that our new fabrication approach, consisting of anchoring a MEW mesh into one layer of microtissues during the fusion process and hanging the tissue from the mesh, yields an engineered tissue with a collagen network orientation displaying greater similarities with the native tissue. The hanging condition was then selected in the bilayered engineered tissue study.

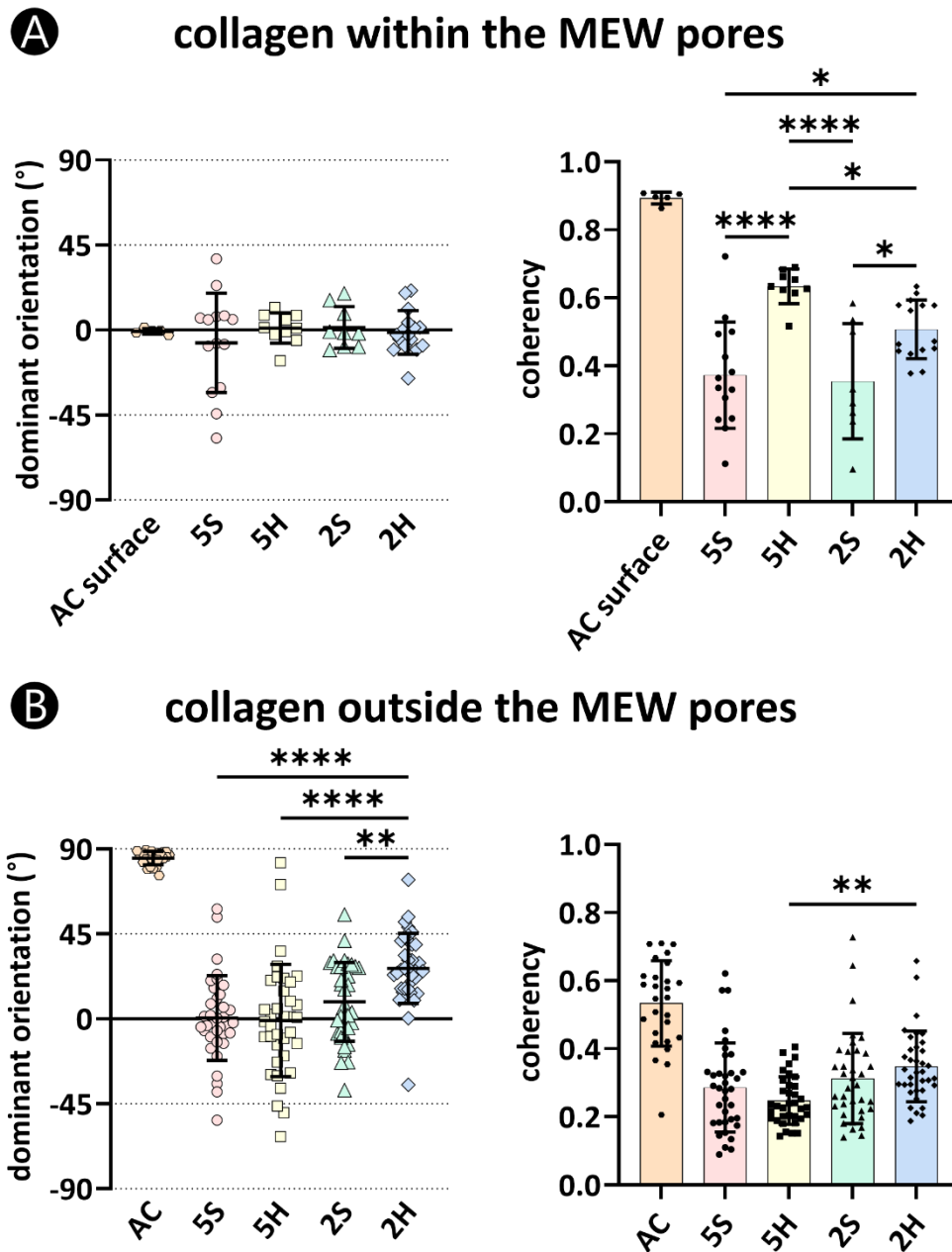


FIGURE 5.7 Single-layered engineered tissue: dominant orientation and coherency of the collagen network. Dominant orientation and coherency of collagen fibres within in the MEW pores (**A**) and outside the MEW pores (**B**). All data is represented as individual data points and mean $\pm$ standard deviation. Normality was verified by Kolmogorov-Smirnov's test. Statistical differences were determined using a one-way ANOVA test followed by Holm-Šidák's multiple comparisons test. $N \geq 5$ in (**A**) and $N \geq 27$ in (**B**). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. MEW: melt electrowritten; ACS: articular cartilage surface; AC: articular cartilage; 5S: 5% O₂ sitting; 5H: 5% O₂ hanging; 2S: 2% O₂ sitting, 2H: 2% O₂ hanging.

5.3.5. Modular Assembly of a Bilayered Hybrid Cartilaginous Tissue

As already demonstrated, chondrogenic differentiation under altered oxygen conditions yielded microtissues with distinct cartilaginous phenotypes. Microtissues differentiated at 2% O₂ produced GAG-rich extracellular matrix (ECM) also containing type II collagen, while microtissues differentiated at 5% O₂ produced an ECM with a higher collagen to GAGs ratio. These different microtissues were used as the building blocks of the different layers of AC to mimic the GAG gradient found in native tissue: 5% O₂ differentiated microtissues to engineer the top region of AC and 2% O₂ differentiated microtissues to engineer the bottom region of AC. With an interval of 2 days, single microtissues were primed at both 5% O₂ or 2% O₂, both for 7 days. At the 7-day timepoint, 5% O₂ microtissues were seeded onto the MEW mesh followed, 2 days later, by 2% O₂ microtissues added on top of the first layer. As the mean microtissue diameter was 212 $\mu\text{m} \pm 20 \mu\text{m}$, the pore size of the mesh was selected to 200 $\mu\text{m} \times 200 \mu\text{m}$. Two days after seeding the second layer of microtissues, the system was flipped upside down to hang the engineered tissues. Finally, the constructs were kept in TGF- β 3 for 42 days (**Figure 5.8**).

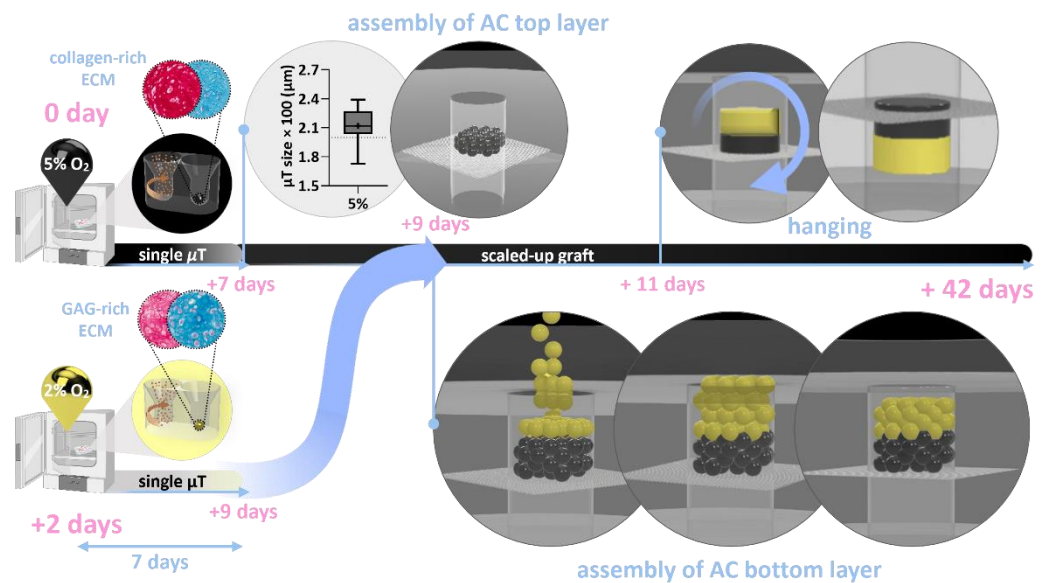


FIGURE 5.8 Bilayered engineered tissue: modular assembly timeline. Data is represented as min to max box whiskers. Cross represents mean value, and dashed line represents size of MEW mesh pore size. N = 8. μT : microtissue; GAG: glycosaminoglycan.

At day 11, prior to hanging the tissues, histology staining revealed that the two populations of microtissues (altered oxygen priming regimes) were spatially patterned in a two-layer manner (**Figure 5.9A**). The top layer, corresponding to microtissues

primed at 5% O₂, contained more GAGs than the bottom layer, corresponding to microtissues primed at 2% O₂. Histological analysis after 42 days of ECM maturation revealed a strong GAG staining with no obvious gradient despite the pre-priming of the single microtissues (**Figure 5.9B**). Collagen staining was also strong and revealed partial fusion of the microtissues. In general, the regions between the unfused microtissues appeared homogeneous and positive for collagens and contained chondrocyte-like cells embedded in ECM (**Figure 5.9 C**). Some weak staining of calcium deposition was observed at unfused microtissues.

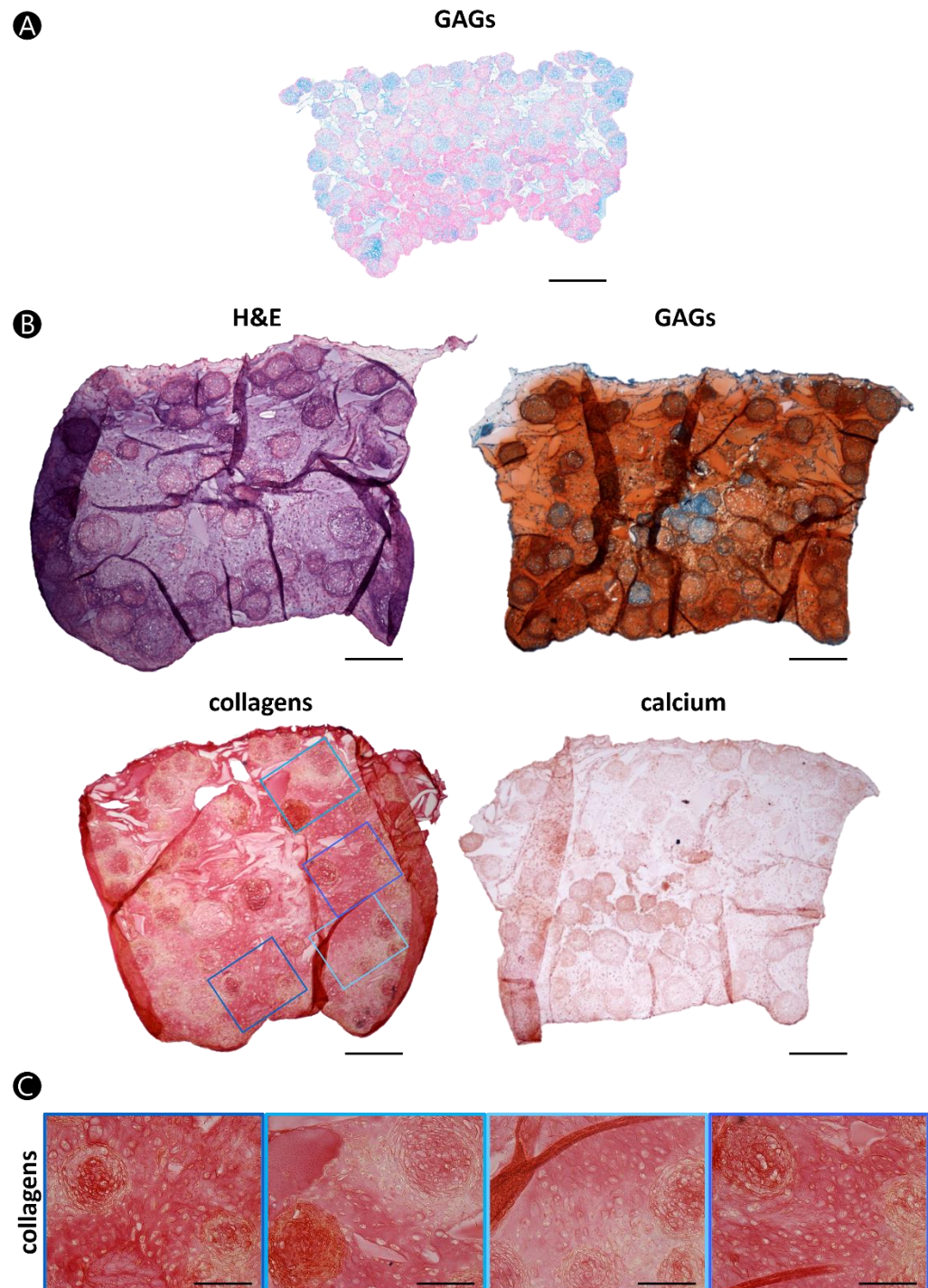


FIGURE 5.9 Bilayered engineered tissue: histology panel. Histology staining of the bilayered tissue at (A) day 11 (two days after the fusion of the second layer) and (B) day 42 of culture. (C) Magnifications of the inter-microtissue space at day 42. H&E: Haematoxylin & Eosin; GAGs: glycosaminoglycans. Scale bar = 500 μm /200 μm (magnification).

The analysis of the different collagen subtypes by immunofluorescent staining demonstrated the presence of type II collagen, type I collagen and type X collagen (Figure 5.10). While the type I collagen was homogeneously distributed over the engineered tissue, type II collagen was positive at the periphery of the tissue and

absent at the core of the tissue. In contrast, type X collagen was mainly observed at the centre of the tissue after 42 days of chondrogenic differentiation. In addition, no clear biomimetic organisation was observed in these larger tissues by PLM (**Supplementary Figure 5.6**).

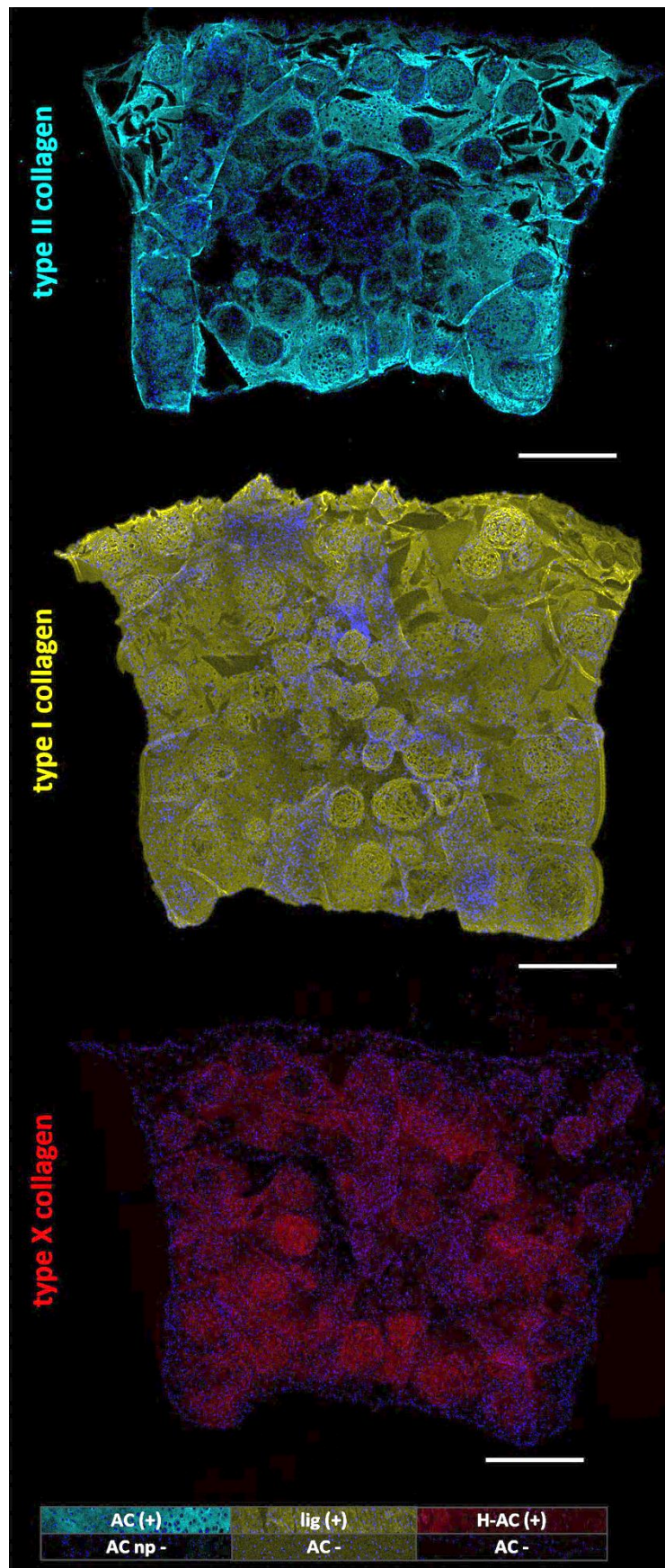


FIGURE 5.10 Bilayered engineered tissue: immunofluorescent staining panel. Nuclei were stained with DAPI and are shown in dark blue. AC: articular cartilage; np: no primary; lig: ligament; H-AC: hypertrophic articular cartilage; (+): positive control; -: negative control. Scale bar = 500 μm .

5.4. Discussion

As a means of engineering an AC graft with both a predefined geometry and zonal organisation, a novel modular assembly method that integrates cartilaginous microtissues and a MEW mesh was developed. Using this approach, it was possible to spatially pattern phenotypically distinct cartilage microtissues into a supporting MEW mesh, enabling the generation of a scaled-up graft. Such approaches hold great potential for fabricating complex tissues with biomimetic composition and collagen fibre organisation.

An in-house modular assembly system was fabricated using a MEW mesh and agarose. The agarose, gelled in presence of positive PDMS inserts, formed a seeding well with easily adaptable diameter, removing the need to use commercially available inserts to seed the microtissues onto the MEW mesh [238]. The MEW mesh served as the bottom of the agarose well to collect and integrate with seeded microtissues. Success of this modular assembly strategy relies in part on the ability to generate meshes with highly accurate dimensions that can retain seeded microtissues. Hence, the use of melt electrowriting allowed printing of meshes with well-defined box-like pore dimensions. For all printed pore dimensions, the measured median size corresponded to the theoretical printing dimensions with an averaged 1.5 μm standard deviation. In addition, the printing precision increased with pore dimensions, as residual charges around the fibres post-printing likely disturb the stability of the printed mesh with smaller dimensions [243]. Previous studies on the modular assembly of biosheets have found that the resulting construct contains ~2% polymeric material, with MEW scaffolds porosity of 80–98% [110, 235]. In this study, the MEW mesh was six-layer thick, while the resulting single-layered grafts were approximately 1.5 μm thick. Thus, this new modular approach is believed to yield a hybrid cartilage graft with a minimal volume of biomaterial, especially when compared with FDM approaches [244]. Minimisation of the presence of foreign material in combination with PCL, which has already been used in Food and Drug Administration-approved therapeutics, is anticipated to favour clinical translation and biosafety [245].

Integrating cartilage microtissues into a MEW mesh was found to prevent cell mediated contraction of the final construct whilst still supporting robust chondrogenesis. After 21 days of chondrogenic differentiation, limited contraction of the fused graft was observed (approximately 2.5 mm $\times$ 1.5 mm) even though only the bottom part of the construct was maintained by the MEW mesh [110, 227, 238]. Chondrogenesis was not suppressed despite limited contraction as seen by the positive

GAG and collagen staining. In a previous study, adipose-derived stem cells were seeded in PCL buckyballs used to fabricate hybrid spheroids with consistent diameter [246]. While tissue contraction was attenuated by the incompressible volume of the buckyball “cage”, chondrogenesis and fusion of these hybrid tissues were not hampered as it was reported that low cell density could favour nutrient and oxygen diffusion and that low ECM density could favour rapid fusion kinetics [247]. Additionally, the MEW mesh-bottomed agarose well allowed both sides of the tissue, top and bottom, to be exposed to media. Diffusion of TGF- β from the media-exposed surface is beneficial over short distances (<0.5 mm) [248]. As the construct was not placed on culture plastic, diffusion of nutrients from both surfaces could favour more homogeneous chondrogenesis. Finally, the MEW mesh was entrapped within the secreted ECM but cells did not migrate beyond the mesh as previously observed with microtissues seeded on a nylon mesh, which contributed to the thickening of the tissue by 40% [227]. The fibronectin coating of the MEW fibres could contribute to enhancing cell attachment to the MEW surface [69].

Allowing the microtissues to hang from the MEW mesh was found to support type II collagen deposition and the development of a more biomimetic collagen architecture throughout the depth of the engineered tissue. As previously demonstrated, the MEW fibres were used to guide the orientation of *in situ* newly synthesised collagen fibres [236, 237]. To guide the organisation of the tissue present outside of the MEW mesh, the effect of orientation on the growth and the organisation of the collagen network was investigated by hanging the tissue upside down, as opposite to the sitting configuration where the tissue grew upwards from the MEW mesh. Mapping of the collagen orientation distribution by depth of the tissue revealed that the 2H group (hanging configuration fabricated with microtissues differentiated at 2% O₂) showed some level of biomimetic arch-like collagen network organisation as fibres started to orientate 45° to the surface moving into the tissue depth. The dominant orientation of collagen fibres outside of the MEW mesh in the 2H group was also closer to native tissue. Collagen fibres in contact with the MEW fibres were orientated parallel to the surface in both configurations, similar to the orientation of collagen fibre found at the surface of native AC. However, in the hanging configuration, orientation coherency of the collagen fibres within the mesh was more similar to the native tissue. Tailored mechanical properties of embedded scaffolds can support the chondrogenic differentiation potential of MSCs [249]. Here, the combination of the MEW mesh mechanical properties and the weight of the scaffold itself yields a more biomimetic fibre coherency. Further experiments should aim at identifying mechanical

forces exerted by cells depending on the configuration, with for example the staining of the yes-associated protein (YAP) mechano-sensor [250].

Fusion of 7-day-old single microtissues was partial in both single-layered and bilayered tissues, which may be linked to the production of tenascin at the microtissue boundaries during the priming period [99]. However, as seen in the bilayered construct, cells, most likely from the microtissue surface [251], were able to migrate outside of the microtissues and filled the inter-microtissue space with ECM containing GAGs and type I, II and X collagens. After 42 days of culture, no signs of tissue necrosis were observed despite the construct being several millimetres in thickness. In a similar study, a 1.6 mm thick biosheet, resulting from the assembly of microtissues between two nylon meshes, underwent core necrosis [227]. However, the biosheet was cultured under normoxic conditions (21% O₂ and 5% CO₂). Therefore, oxygen priming of microtissues under altered low oxygen conditions for 7 days could be enough to maintain high tissue viability. On the other hand, 7 days of oxygen priming were not enough to maintain a hyaline cartilage phenotype, as after 42 days of culture type X collagen was detected, suggesting progression towards endochondral ossification of the cartilage graft [252].

It was possible to spatially pattern microtissues primed at 2% O₂ onto microtissues primed at 5% O₂ to generate a bilayered cartilaginous construct. Hypoxia promotes the chondrogenic differentiation of MSCs [253] and as demonstrated in **Chapter 4**, the variation of oxygen tension from 5% to 2% O₂ significantly increases the production of GAGs in hMSCs. To mimic the GAG gradient found in native AC, microtissues differentiated at 5% O₂ and 2% O₂ were then used to engineer the surface region and the deep region, respectively. By modular assembly, different cell populations were spatially positioned by sequentially seeding a first layer of microtissues onto the MEW mesh, and then seeding a second layer of microtissues onto the first one. Layers were seeded 2 days apart to strike a balance between fusion of the first layer with its boundary integration, yet not mixing, with the second layer. Spatial patterning of the 5% O₂ and 2% O₂ primed microtissue populations was observed 96 h after the beginning of the modular assembly. At this point, the difference in staining intensities between layers is likely due to the different maturation stage of the two microtissue populations (2 days apart) rather than their different priming regimes. At the final timepoint, layer integration was successful, despite the hanging configuration, but the initial layering of distinct microtissue populations was not visible anymore. This loss of spatial organisation might be due to the mixing of both microtissues populations or a loss of the beneficial effect of oxygen priming in long-

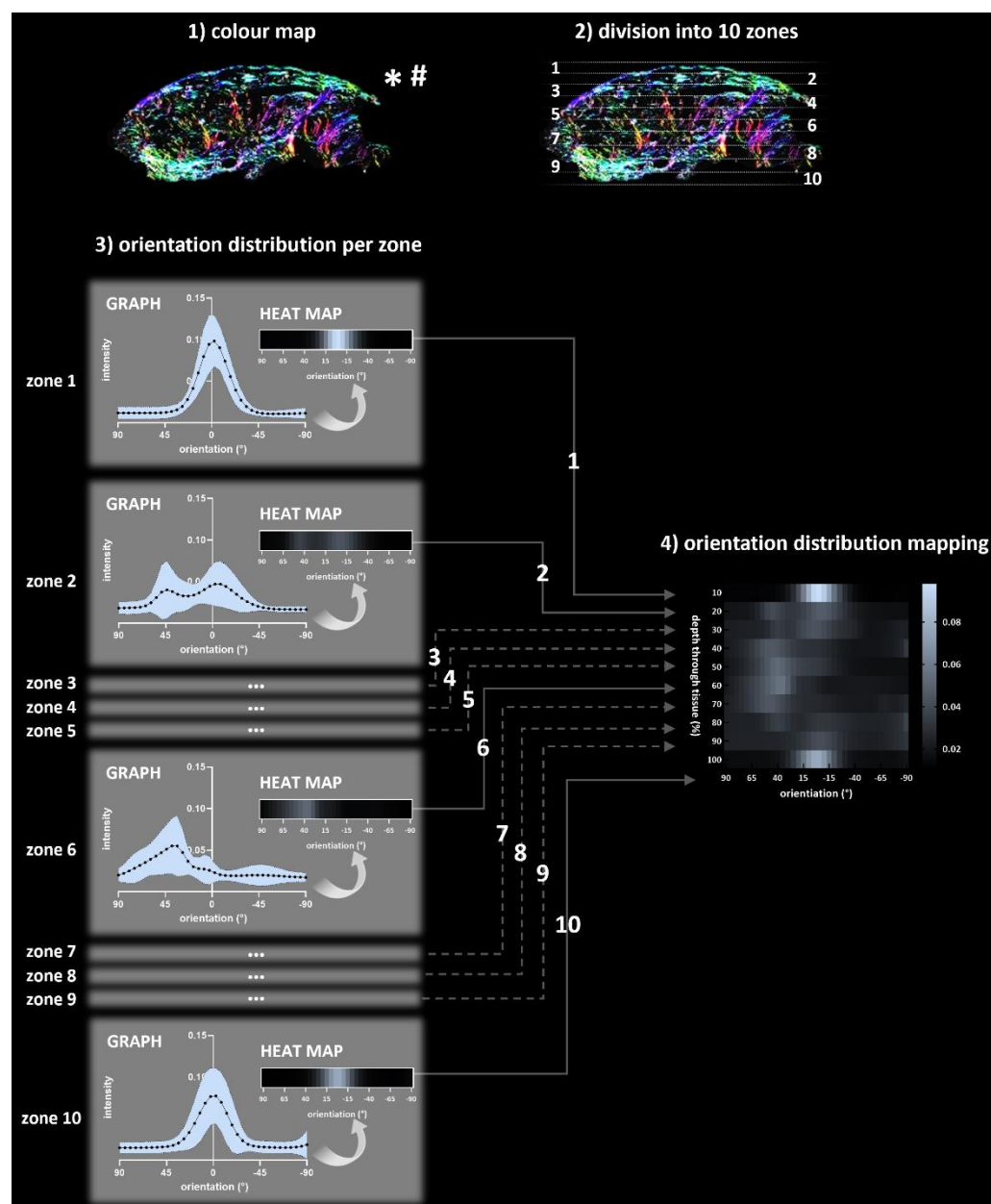
term culture. In a scaffold-free approach, chondrogenic and osteogenic microtissue populations, sequentially assembled in a PDMS mould, were able to maintain their layered organisation, but 14-day-old microtissues differentiated in osteogenic media were used as the second layer and the assembled construct was only cultured for an additional 8 days, which might be too short to observe the remodelling of these slow-fusing microtissue building units [254]. Alternatively to oxygen priming, the incorporation of oxygen-delivering building units, like the haemoglobin-heparin gelatine microspheres developed by Norberg *et al.*, could be added to maintain distinct levels of oxygenation within large constructs [13]. Finally, the tissue geometry was maintained but, unlike the single-layered tissue, it is most likely due to steric confinement, as a large volume of microtissues was used to assemble the bilayered construct, as seen elsewhere [100].

It is worth mentioning that this novel modular assembly method holds the potential to engineer spatially complex and heterogeneous tissues with minimal use of foreign biomaterials. However, complexity, heterogeneity and quality of the resulting tissue could benefit from other 3D technologies. Microtissues were pipetted in the MEW mesh-bottomed well and arranged inside each pore as media was drained through the mesh. Aspiration-assisted bioprinting, even in combination with array fabrication of microtissues [212], could be used to more precisely deposit microtissues in each mesh pores prior to fusion [104]. Fluidic-based singularisation and injection modules, integrated to a commercial 3D printer, demonstrated precise positioning of individual microtissues in a 3D printed scaffold [88]. The Kenzan method of skewering individual microtissues prior to fusion is also another method that helps to maintain the organisation of tissues engineered using scaffold-free approaches [255]. While the MEW mesh improves the handleability of the graft [256], other specifications like mechanical integrity, surface lubrication, graft fixation and integration with surrounding tissues should also be considered [223].

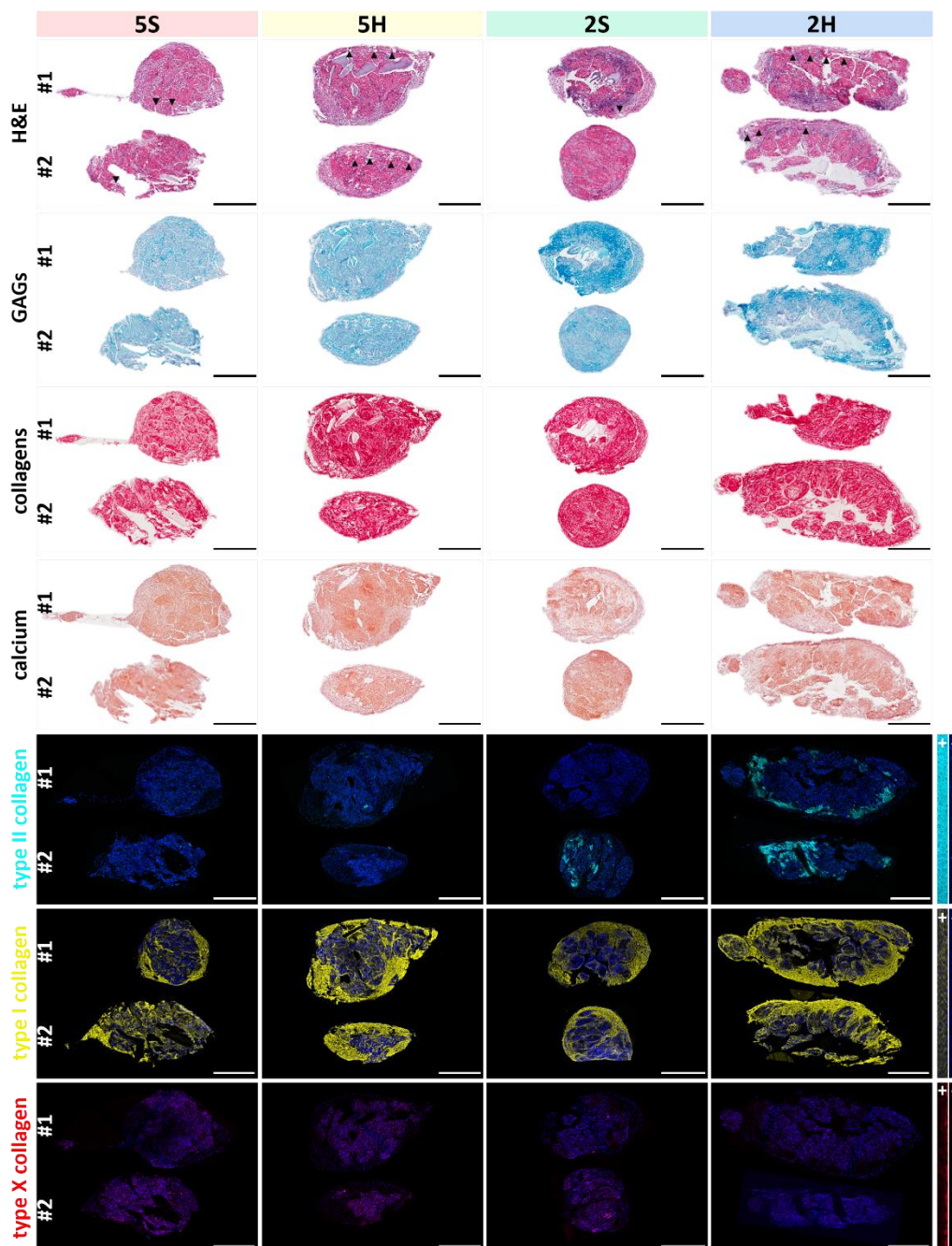
5.5. Conclusion

A new modular assembly method was developed based on the fabrication of an in-house agarose MEW mesh-bottomed well. A thin MEW mesh (6 layers of printed PCL) was printed to minimise the presence of polymeric material, incorporating a pore size that was adapted to the size of microtissues to collect them in individual pores and support several layers of microtissues on top. After chondrogenic differentiation under altered oxygen tensions and the use of either hanging or sitting culture configurations, the 2H group showed stronger type II collagen staining and some degree of collagen network organisation biomimicry. In addition, the hanging configuration enhanced the coherency of collagen fibres synthesised inside the MEW mesh, more similar to native AC than the sitting configuration. Finally, modular assembly of distinct microtissue populations onto a MEW mesh led to the development of a bilayered construct that at least initially contained a gradient in GAG content, although further work is required to support the longer-term development of these engineered tissues.

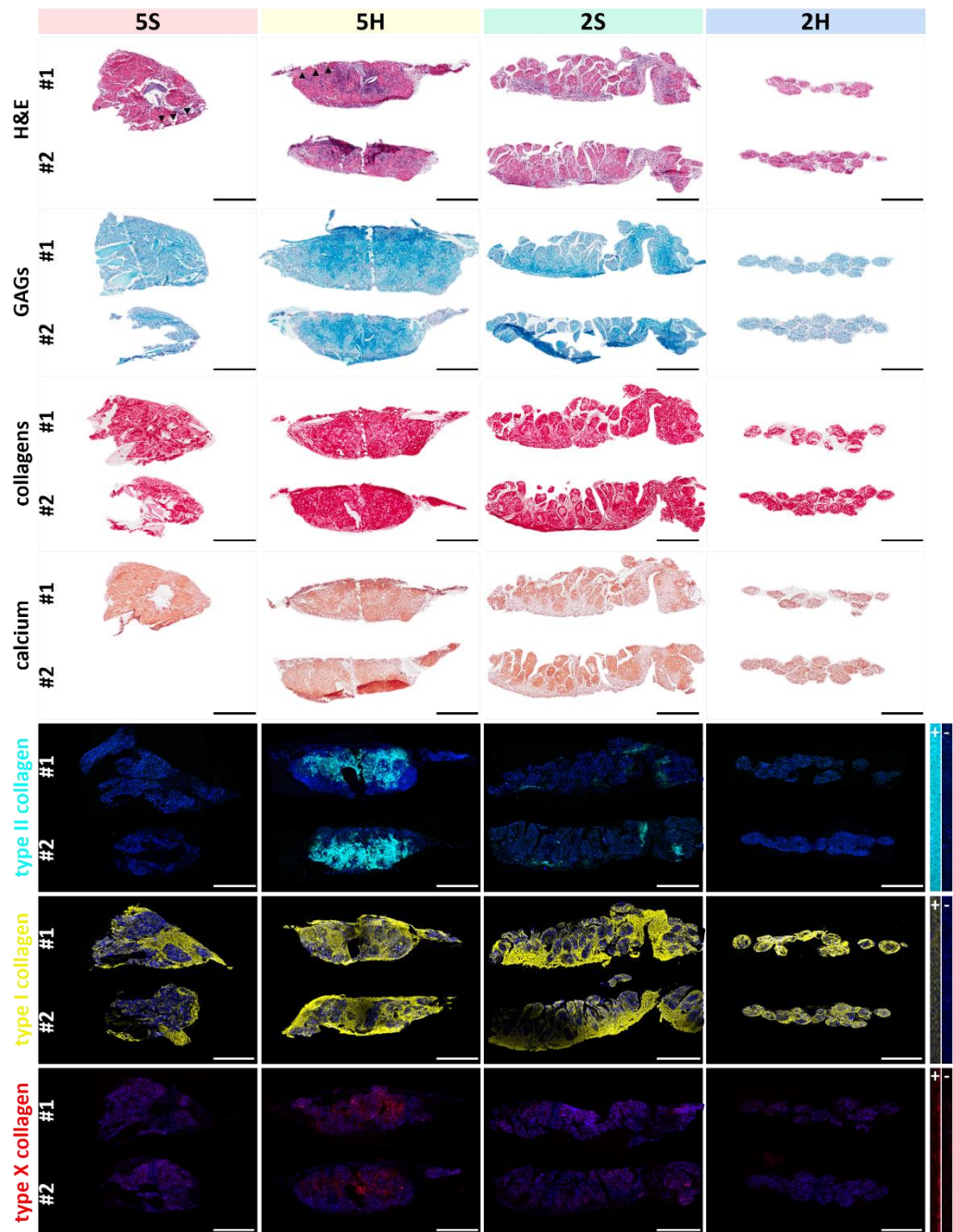
5.6. Supplementary Figures



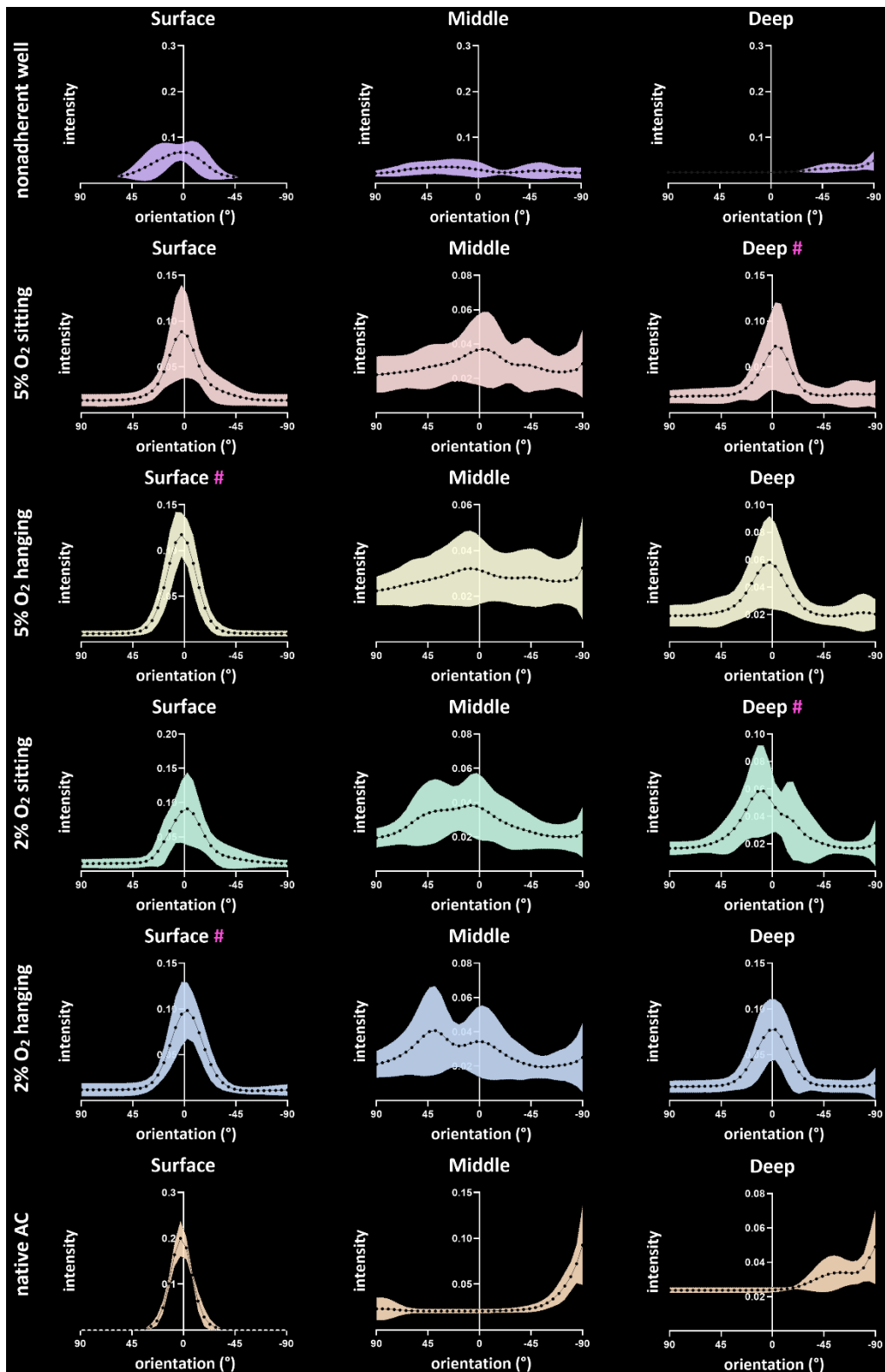
SUPPLEMENTARY FIGURE 5.1 Construction of the orientation distribution maps: example of the 2H group. The colour map (only one section represented) was divided into 10 zones from the surface to the bottom of the constructs and the orientation distribution of each zone was quantified. The resulting distribution graph was transformed into a heat map that represents the orientation distribution of each zone. * indicates the top surface of the tissue; # indicates the position of the melt electrowritten mesh.



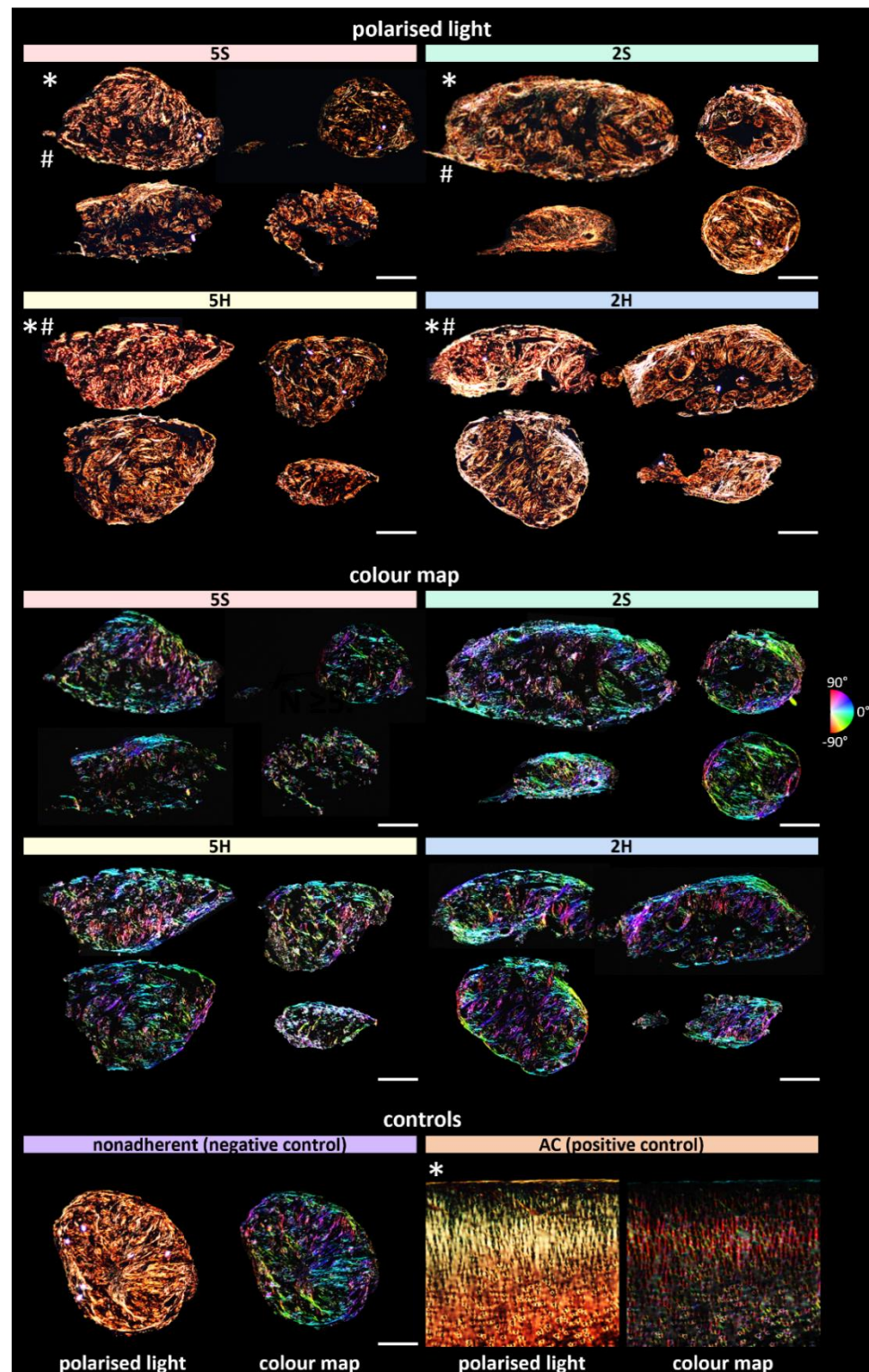
SUPPLEMENTARY FIGURE 5.2 Single-layered engineered tissue: histology and immunofluorescence panel of the medium replicate. Two sections from the centre and the periphery of the construct are shown. Black arrows point at the MEW fibres (only represented in the H&E staining). Nuclei were stained with DAPI, shown in dark blue. Scale bar = 500 μm . H&E: haematoxylin and eosin; GAGs: glycosaminoglycans; #: cut number; (+): positive control; -: negative control.



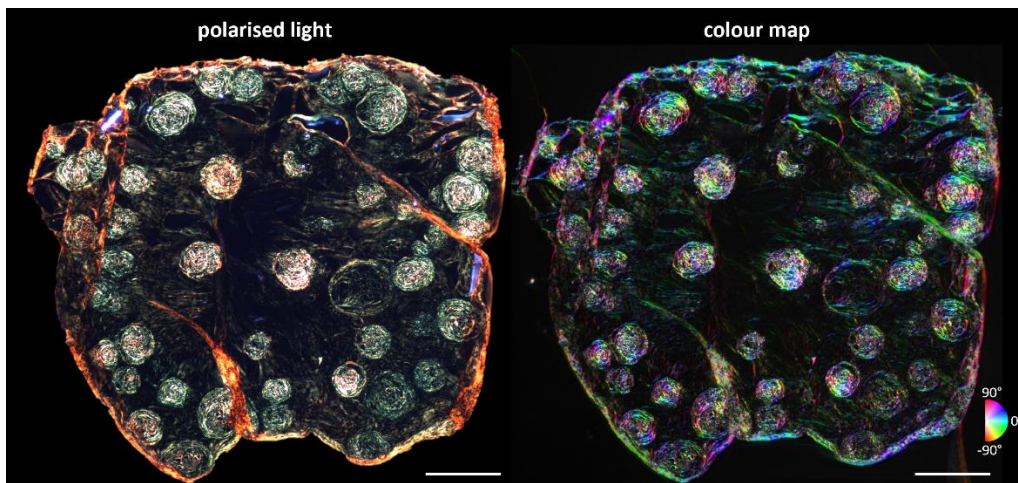
SUPPLEMENTARY FIGURE 5.3 Single-layered engineered tissue: histology and immunofluorescence panel of the worst replicate. Two sections from the centre and the periphery of the construct are shown. Black arrows point at the MEW fibres (only represented in the H&E staining). Nuclei were stained with DAPI, shown in dark blue. Scale bar = 500 μ m. H&E: haematoxylin and eosin; GAGs: glycosaminoglycans; #: cut number; (+): positive control; -: negative control.



SUPPLEMENTARY FIGURE 5.4 Single-layered engineered tissue: orientation distribution of the surface, middle and deep regions. All data is represented as mean $\pm$ standard deviation (coloured area). $N \geq 3$. 5S: 5% O₂ sitting; 5H: 5% O₂ hanging; 2S: 2% O₂ sitting, 2H: 2% O₂ hanging; AC: articular cartilage. # indicates the presence of the MEW mesh.



SUPPLEMENTARY FIGURE 5.5 Single-layered engineered tissue: polarised light microscopy and colour map panel. Colour maps generated from PLM images. Colour hue indicates fibre orientation with red denoting $-90^{\circ}/90^{\circ}$ and cyan denoting 0° . Two replicates and two sections per replicate from the centre and the periphery of the construct used to build the maps of orientation distribution, and the fibre mean orientation, fibre coherency and regional orientation distribution graphs are shown. One cut is shown for controls. Scale bar = 500 μm . * denotes the surface of the construct; # denotes the position of the MEW mesh. AC: articular cartilage; 2: 2% O_2 ; 5: 5% O_2 ; H: hanging; S: sitting.



SUPPLEMENTARY FIGURE 5.6 Bilayered engineered tissue: polarised light microscopy and colour map. Bilayered engineered graft were cultured for 42 days. Scale bar = 500 μm .

6. A Silencing Approach: Transfection of Human Mesenchymal Stem Cells with Anti-miR-221 Supports the Development of Hyaline Cartilage Microtissues

6.1. Introduction

Developmentally inspired tissue engineering (TE) strategies leverage the intrinsic ability of stem/progenitor cells to self-organise in response to environmental cues and produce an organised, functional tissue, at least in part by mimicking key aspects of normal tissue development [257]. Processes such as cellular self-assembly and self-organisation can be used to generate scaffold-free tissues, thereby overcoming several limitations associated with conventional TE and biofabrication strategies [7]. These processes typically exclude toxic chemicals [258], polymerising ultraviolet (UV) light [259] or high shear stress [260] which compromises cell viability. The absence of exogenous materials and associated degradation products reduces the risk of a foreign body response post-implantation [89], and should lead to improved integration with surrounding tissues [261].

In the context of cartilage TE, scaffold-free TE strategies support the cell-cell and cell-matrix interactions which are known to promote chondrogenesis [262] and functional cartilage tissue development [263]. Such approaches have enabled the engineering of cartilage grafts of clinically relevant dimensions for the treatment of cartilage defects [264], as well as the development of constructs with mechanical properties comparable to the native tissue [265]. Cellular aggregates and microtissues have been used as *in vitro* tissue or disease models, *in vitro* platforms for drug screening or even as transplanted cell therapy to deliver biomimetic signals to diseased tissue [43, 87]. They have also been used as biological building blocks as they can spontaneously assemble with each other to generate macroscale tissues for use in the repair of critically sized defects [43, 266]. Tissue complexity can be recreated by using phenotypically distinct microtissue populations [143, 267] which can be processed into

complex structures using three-dimensional (3D) bioprinting and other emerging biofabrication technologies [268]. Multiple mesenchymal stem/stromal cell (MSC)-derived cartilage microtissues have been combined to generate scaled-up grafts with biomimetic composition and organisation [100]. However, one major limitation of MSCs is their inability to generate phenotypically stable hyaline cartilage that is resistant to hypertrophy and progression along an endochondral pathway [17]. Thus, the generation of stable articular cartilage (AC) microtissues using MSCs is still an ongoing challenge.

The discovery and characterisation of micro ribonucleic acids (miRNAs or miRs) have highlighted their potent silencing of gene expression at the post-transcriptional level, playing key roles in development, regeneration and disease [117]. In mammalian cells, miRNAs are short (~22 nucleotides), single-stranded, noncoding ribonucleic acids (RNAs) with a binding complementary sequence to messenger RNAs (mRNAs), preventing their translation and silencing the expression of encoded genes [115]. Numerous miRNAs have been shown to play an important role in cartilage homeostasis and osteoarthritis [34]. Chondrogenic differentiation of MSCs is also finely regulated by miRNAs, positively or negatively, targeting cell proliferation, cell differentiation or hypertrophy [269, 270]. One example of miRNAs that was studied in both cartilage homeostasis and chondrogenesis is miR-221. Microarray analysis of articular cartilage weight-bearing regions identified the upregulation of miR-221, suggesting its implication in the regulation of chondrocyte mechano-responsiveness and tissue homeostasis [271]. MiR-221 has been shown to be upregulated during chondrocyte dedifferentiation in monolayer expansion, while redifferentiation in 3D culture suppressed expression levels, suggesting an anti-chondrogenic role for miR-221 [272]. Jun N-terminal kinase (JNK)-inhibitor mediated upregulation of miR-221, in chick wings buds chondroprogenitor cells, reduced cell proliferation prior to precartilage condensation, an essential step in cartilage formation. miR-221 was identified to directly target Mouse double-minute 2 homolog (MDM2) and to silence its positive effect on progenitor proliferation confirming its anti-chondrogenic action [152].

Besides understanding biological processes, miRNAs hold great potential as biomarkers for disease but also represent a new class of innovative therapeutic [142]. Micro RNA inhibitors (anti-miRNAs or anti-miRs) are synthetic small RNAs with sequence complementarity to miRNAs. By preventing miRNAs binding to their mRNA targets, they reduce or suppress the endogenous silencing effect of miRNAs [273-275]. Therapeutics aims to increase the activity of disease suppressors or to reduce the activity of disease inducers [273]. One major strength of miRNA targeting therapies is

the ability of miRNAs to target hundreds of complementary mRNAs, due to their short sequence, therefore potentially silencing hundreds of genes [273].

Anti-miRNAs, and in particular anti-miR-221, have also been shown to regulate chondrogenesis of stem/progenitor cells [276]. Transfection of Wharton's Jelly human MSCs (hMSCs) with anti-miR-221, in the absence of other exogenous cues, was found to silence miR-221 expression [277]. Silencing of miR-221 in bone marrow-derived MSCs was found to support type II collagen deposition, whose levels were comparable to controls stimulated with transforming growth factor (TGF)- β 3, with no evidence of type X collagen deposition, pointing to the potential of anti-miR-221 for cartilage TE [33]. Anti-miR-221 transfected MSCs encapsulated in hydrogels showed upregulation of the chondrogenic genes *COL2A1* and *ACAN*, as well as increased glycosaminoglycan (GAG) deposition [278]. A hydrogel functionalised with anti-miR-221 (complexed to a supporting vector) has been shown to silence miR-221 in target cells in an *in vivo* ectopic osteochondral defect model, leading to enhanced cartilage repair [153, 279]. In another recent study, a miR-221 inhibitor activated scaffold seeded with hMSCs has been shown to silence miR-221 and downregulate the expression of *RUNX-2* compared to the delivery of TGF- β 3 alone [280]. Taken together, these studies support the strong pro-chondrogenic potential of anti-miR-221 and its application to cartilage regeneration. Integrating such cues in cellular aggregates or cartilage microtissues could potentially enable the engineering of phenotypically stable hyaline cartilage microtissues.

In this study, it was hypothesised that the inhibition of miR-221 in hMSCs could be mediated using a poly- β -amino-ester (pBAE) vector and that the incorporation of such miR-221 silenced cells into microtissues will enhance type II collagen deposition whilst simultaneously suppressing hypertrophy and progression along an endochondral pathway. The pro-chondrogenic anti-miR-221 was delivered using pBAE polymers, specifically the thiol terminated arginine peptide (Cysteine-Arginine-Arginine-Arginine) [281], abbreviated as C6-CR3, to efficiently transfect hMSCs with an optimal dosage in order to maximise hMSC responsiveness to miR-221 silencing. These silenced cells were then used to fabricate microtissues for the biofabrication of scaled-up cartilage grafts. This was done using different anti-miR-221 transfection doses and regimens (either transfection in 2D monolayer culture alone, or 2D transfection followed by additional 3D transfection following microtissue generation). Finally, fusion of these silenced building blocks will be demonstrated as proof of concept of the fabrication of silenced scaffold-free cartilage grafts.

6.2. Materials and Methods

6.2.1. Anti-miR-221:C6-CR3 Polyplex Formation

Polyplex nanoparticles were formed as previously described [281]. Briefly, a C6-CR3 polymer stock solution (100 mg/mL in dimethyl sulfoxide, kindly provided by Dr. Pere Dosta Pons, University of Texas Southwestern, Medical Center, Dallas, United States) was diluted in 12.5 mM sterile filtered sodium acetate (AcONa) solution (Merck), pH 5.5, at different concentrations to obtain desired anti-miR-221:C6-CR3 complexation ratios (for example, a 0.625 µg/µL AcONa solution is used to complex the anti-miR-221:C6-CR3 1:12 w/w ratio). The miRIDIAN microRNA Human has-miR-221-3p hairpin inhibitor (anti-miR-221) and the miRIDAN microRNA Hairpin Inhibitor Negative Control #2 (scramble) were resuspended to 20 µM stock solution in 1X siRNA buffer according to the manufacturer's instructions (Horizon Discovery), from which 0.05 µg/µL (in AcONa) hairpin inhibitor working solutions were prepared. Finally, to equal volumes, the C6-CR3 solution, with desired concentration, was added over the hairpin inhibitor working solution, incubated for 10 min at room temperature (RT), and precipitated in two volumes of sterile-filtered 1X phosphate buffer saline (PBS) for 10 min at RT.

6.2.2. Polyplex Characterisation: Agarose Retardation Assay and Dynamic Light Scattering

Anti-miR-221:C6-CR3 polyplex solutions with different complexation ratios, ranging from 1:12 to 1:250 w/w (1:0 used as uncomplexed anti-miR-221 control), were freshly prepared. Retardation of anti-miR-221 was evaluated by gel electrophoresis to determine the optimal complexation ratio. A 2% w/v agarose gel in 1X Tris-Acetate-ethylenediaminetetraacetic acid (EDTA) buffer containing 0.1 µL/mL SYBR Safe deoxyribonucleic acid (DNA) Gel Stain (Invitrogen) was loaded with polyplex solutions (including the GeneRuler 1 kb DNA Ladder, Thermo Scientific), run for 15 min at 80 V, and visualised under UV light (ChemiDoc MP Imaging System, BioRad). Absence of a miRNA visible band at the same level as the control group (1:0 uncomplexed anti-miR-221) indicates complete complexation. Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter, the polydispersity index, and the zeta potential of the polyplex solutions. Nanoparticle solution of selected complexation ratio was diluted 1:100 in filtered 1X PBS prior to measurements (at 0 h and 24 h after complexation) at RT with the Zetasizer Nano ZS (Malvern) using a 633 nm wavelength

laser. The loading capacity was calculated by dividing the mass of anti-miR-221 (μg) by the mass of C6-CR3 (mg) used at the selected complexation ratio.

6.2.3. Transfection of Cell Monolayer

6.2.3.1. Human MSC Expansion

MSCs were isolated from the bone marrow (Lonza) of one human donor as previously described [172], and then expanded from an initial cell density of 3,000 cells/ cm^2 at 5% O_2 and 5% CO_2 at 37 °C in humidified atmosphere until subconfluency. Expansion media (XPAN) contained high glucose Dulbecco's Modified Eagle Medium GlutaMAX, 10% v/v foetal bovine serum, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin (all Gibco), and 5 ng/mL fibroblast growth factor 2 (Prospect Bio). Cells were fed twice per week. All cells were expanded to passage 2 prior to two-dimensional (2D) cell transfection.

6.2.3.2. Polyplex Dose Screening—Transfection of Cell Monolayer

Passage 2 hMSCs were expanded in XPAN as previously described. After 3 days of expansion, 2D cell monolayers were incubated with transfection media (TM) containing anti-miR-221:C6-CR3 polyplex in XPAN for 4 h at 5% O_2 and 5% CO_2 at 37 °C in humidified atmosphere. The TM volume was adjusted to the growing area (23 $\mu\text{L}/\text{cm}^2$). The anti-miR-221:C6-CR3 was prepared at a 1:12 w/w ratio. To optimise the anti-miR-221 dose, TM was prepared with different concentrations of anti-miR-221 ($\text{MM} = 18458.1 \text{ g/mol}$): 0 nM, 10 nM, 20 nM, 50 nM, or 100 nM. After 4 h, TM was removed and replaced by XPAN. At day 6 of expansion, a second transfection was performed following the same protocol. Cells were expanded for 7 days to compare population doubling (PD) with the 0 nM control group. The PD was calculated following the next equation: $PD = 3.322 \times (\log(\text{final cell density}) - \log(\text{initial cell density}))$. The transfection regime of the scramble nucleic acid was adjusted for confocal imaging and polymerase chain reaction (PCR). See respective Sections 6.2.6. and 6.2.10. for more details. This protocol is illustrated in **Figure 6.2A**.

6.2.4. Cell Viability and Transfection Yield

Live/Dead staining—Human MSCs were expanded and transfected with a range of anti-miR-221 concentrations as previously described. Here a C6-CR3 polymer fluorescently labelled with the Alexa Fluor 647 was used to assess the transfection yield. After 7 days of 2D culture, cells were detached, counted by trypan blue exclusion,

resuspended ($\geq 300,000$ cells/mL), and stained with the eBioscience Fixable Viability Dye eFluor 506 (Invitrogen) to assess particle cytotoxicity. Briefly, 1X PBS washed cells were stained with 1:1,000 in 1X PBS diluted eFluor506 for 15 min at RT in the dark. Cells were then washed in 1X PBS, spun at $350 \times g$ for 5 min, prior to fixation in 2% paraformaldehyde (PFA) for 15 min at RT in the dark, and then left in 1X PBS at 4 °C overnight before flow cytometry acquisition. The positive control for cell viability consisted of an equal mix of live cells and cells killed for 10 min at 60 °C (both taken from the 0 nM group), and then stained with eFluor 506. The viability negative control group consisted of live cells only (from the 0 nM group). The transfection positive control consisted of an equal mix of untransfected cells (0 nM group) and cells transfected with 50 nM, and the transfection negative control consisted of untransfected cells only (0 nM group).

Annexin V/PI co-staining—Cells were expanded for 7 days from passage 2 to passage 3, and transfected in 2D with 0 nM, 10 nM, 20 nM, 50 nM, or 100 nM of anti-miR-221 as previously described before Annexin V/PI co-staining using the APC Annexin V and Propidium Iodide (PI) Solution (BioLegend) following the manufacturer's protocol. Briefly, cells were washed twice with BioLegend Cell Staining Buffer (BioLegend) and resuspended at a concentration of 1×10^6 cells/mL. Then, 100 μ L of cell suspension was mixed with 5 μ L of APC fluorescently labelled Annexin V (APC-Annexin V) and 10 μ L of PI, incubated for 15 min at RT in the dark before adding 400 μ L of Annexin V Binding Buffer and proceeding with flow cytometry measurements. The APC-Annexin V and PI double positive control consisted of cells killed for 10 min at 60 °C (from the 0 nM group) and co-stained. The APC-Annexin negative control consisted of live cells (0 nM group) stained for APC-Annexin V only, and the PI negative control consisted of live cells (0 nM group) stained for PI only. Both negative controls were compared to an unstained live cell control (from 0 nM group) to gate out background fluorescence. The co-staining results correspond to: APC-/PI- live cells (Q4, bottom left quadrant), APC+/PI- early apoptotic cells (Q1, top left quadrant), APC+/PI+ late apoptotic cells (Q2, top right quadrant), and APC-/PI+ dead cells (Q3, bottom right quadrant).

Cell staining was detected with the BD FACS Canto II (BD Biosciences), and data was analysed with the FlowJo v.10 Software (BD). All gating strategies can be found in **Supplementary Figure 6.1**.

6.2.5. Cell Metabolic Activity

6.2.5.1. Alamar Blue Reduction

The cell metabolic response to particle transfection was assessed by reduction of the alamarBlue Cell Viability Reagent (AB, Invitrogen). As described above, 2D cell monolayers were incubated twice for 4 h in increasing concentrations of anti-miR-221:C6-CR3. Culture media was removed, and the AB working solution (1:10 dilution in XPAN) was added to cells ($40 \mu\text{L}/\text{cm}^2$) for 2 h 30 min in the dark under 5% O_2 and 5% CO_2 at 37 °C in humidified atmosphere. A sample of the AB working solution, prior to cell incubation, was kept as blank control (blank was subtracted from experimental readings). After incubation, AB solutions were collected, and cells were washed in 1X PBS and added XPAN. Then, AB solution absorbance was measured at the 570 nm wavelength using the Synergy HT Multi-Mode Microplate Reader (Biotek Instruments). The evolution of the metabolic activity was measured at different timepoints: (A) before the first transfection at day 3, AB was replaced by TM, (B) after the first transfection at day 3, AB was replaced by fresh XPAN, (C) 24 h after the first transfection at day 4 while the culture XPAN was collected and kept at 37 °C to then replace AB, (D) before the second transfection at day 6, AB was replaced by TM, (E) after the second transfection at day 6, AB was replaced by fresh XPAN, (F) 24 h after the second transfection at day 7 before cells were detached.

6.2.5.2. DNA Quantification

To assess DNA levels, samples were digested in 3.88 U/mL of papain enzyme buffer extract (PBE – containing 100 mM sodium phosphate buffer and 5 mM EDTA disodium salt solution) containing 10 mM L-cysteine (pH 6.5) overnight at 60 °C under rotation. Total DNA was quantified using the bisbenzimidazole Hoechst 33258 solution (0.2 $\mu\text{g}/\text{mL}$ in 1X Fluorescent Assay Buffer, 5 min incubation at RT in the dark) and diluted series of calf thymus DNA (0–1.4 $\mu\text{g}/\text{mL}$ in PBE) as standards. All reagents were from Merck. Fluorescence was measured at the 360 nm excitation and the 460 nm emission wavelengths using the Synergy HT Multi-Mode Microplate Reader (Biotek Instruments). DNA levels were used to normalise the cellular metabolic activity and to calculate the cell metabolism relative to the cell metabolism of the 0 nM group.

6.2.6. Silencing Effect of Anti-miR-221 at the Gene Expression Level

To validate the silencing effect of anti-miR-221, quantitative real-time PCR (qRT-PCR) of 2D transfected hMSCs using 0 nM, 10 nM, 20 nM of anti-miR-221:C6-CR3

or 10 nM of scramble:C6-CR3 1:12 w/w ratio ($MM_{\text{scramble}} = 18394 \text{ g/mol}$) was conducted. Cells were seeded at 3,000 cells/cm² and transfected during expansion from passage 2 to passage 3 on day 3 and day 6. To confirm the beneficial effect of two rounds of transfection, cells transfected twice were compared to cells transfected once at day 6 only. At day 7, total RNA was isolated using the miRNeasy Mini kit (Qiagen), and reverse transcribed to complementary DNA (cDNA) using the TaqMan Advanced miRNA Assays (Applied Biosystems). Then, 1.4 to 3.5 ng cDNA was used for the detection of miR-221 silencing (miR-221 primers from Applied Biosystems) and gene expression levels of *COL1A1*, *COL2A1*, *ACAN* and *SOX9* (all primers from Biosciences) using validated TaqMan Advanced miRNA primers (summarised in **Table Supplementary 6.1**). RT-qPCR was detected with the 7500 real-time PCR System (Applied Biosystems). The relative expression of RNAs was obtained by the $2^{-\Delta\Delta C_t}$ method using the 18 S ribosomal RNA (primer from Biosciences) as housekeeping gene.

6.2.7. Transfection of hMSC-Derived Microtissue

6.2.7.1. Biofabrication and Differentiation of Single Microtissues

A microwell array (1.5 mm depth × 1 mm diameter wells) was fabricated under sterile conditions as previously described [173] and soaked in XPAN for 24 h prior to cell seeding. A suspension of transfected cells (0 nM, 10 nM and 20 nM) of 1,604,000 cells/3 mL XPAN was added to the microwells, settled for 15 min and spun at 800 × g for 5 min, resulting in individual microtissues containing 4,000 cells each. After 24 h, XPAN was replaced by chondrogenic media (CDM+) containing high glucose Dulbecco's Modified Eagle Medium GlutaMAX, 100 U/mL penicillin, 100 µg/mL streptomycin (both Gibco), 1.5 mg/mL bovine serum albumin, 40 µg/mL L-proline, 100 µg/mL sodium pyruvate, 1 × insulin-transferrin-selenium, 100 nm dexamethasone, 50 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid (all from Sigma) and 10 ng/mL human TGF-β3 (Peprotech). Experimental groups were called: 0 nM, 2D-10 nM and 2D-20 nM. To decouple the chondrogenic effect of anti-miR-221 from TGF-β3, microtissues were also cultured using CDM not supplemented with TGF-β3 (CDM-). Experimental groups were called: 2D-10 nM-CDM- and 2D-20 nM-CDM-. Finally, to evaluate the potential positive effect of miR-221 silencing during early chondrogenesis, 2-day-old microtissues were also transfected in three dimensions (3D) with 10 nM or 20 nM, matching doses used during 2D transfection. Experimental groups were called: 2D+3D-10 nM and 2D+3D-20 nM (summarised in **Table 6.1**). Fresh media was added three

times per week. Microtissues were maintained for 21 days at 5% O₂ and 5% CO₂ at 37 °C in humidified atmosphere. At the final timepoint, single microtissues were harvested and counted. This protocol is illustrated in **Figure 6.5A**.

6.2.7.2. Fusion of Silenced Microtissues into a Large-Scale Cartilaginous Graft

Nonadherent 2% w/v agarose fusion wells (4 mm diameter × 5 mm height) were fabricated using polydimethylsiloxane (SYLGARD™ 184 Silicone Elastomer Kit, Dow) positive inserts. To support the biofabrication of large-scale functional cartilaginous grafts using silenced microtissues, single microtissues, from the different experimental conditions previously described, were harvested from their microwell array, and pipetted into fusion wells (theoretical number of 400 microtissues/fusion well). As microtissue capacity to fuse and remodel decreases with microtissue maturity, they were harvested after 3 days of culture [212]. Fusion was macroscopically monitored for 21 days. Fused tissues were maintained at 5% O₂ and 5% CO₂ at 37 °C in humidified atmosphere in respective differentiation media (CDM+ or CDM-), which was changed three times per week. At the final timepoint, engineered tissues were harvested and prepared for further downstream analysis.

Table 6.1 Experimental outline of hMSC transfection and scaffold-free biofabrication. Y/N: yes/no; CDM+: chondrogenic media; CDM-: chondrogenic media not supplemented with TGF-β3, 2D: two dimensions; 3D: three dimensions.

Group	2D Transfection		3D Transfection		Media	Biofabrication
	Y/N	nM	Y/N	nM		
0 nM	No	0	No	0	CDM+	microtissue, fused
2D-10 nM	Yes	10	No	0	CDM+	microtissue, fused
2D-20 nM	Yes	20	No	0	CDM+	microtissue
2D-10 nM (CDM-)	Yes	10	No	0	CDM-	microtissue
2D-20 nM (CDM-)	Yes	20	No	0	CDM-	microtissue
2D+3D-10 nM	Yes	10	Yes	10	CDM+	microtissue, fused
2D+3D-20 nM	Yes	20	Yes	20	CDM+	microtissue

6.2.8. Biochemical Quantification of Engineered Cartilage

Total GAG content was quantified using 0.002% w/v 1,9-dimethyl-methylene blue dye, 0.25% w/v sodium chloride and 0.3% w/v glycine solutions (pH 1.5). Diluted series of shark chondroitin sulphate in PBE were used as standards. The ratio of

extinction coefficients (ϵ) at 530 nm and 590 nm wavelengths was measured in standards and samples using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc) and was used to extrapolate the concentration of GAGs in samples [174-176]. Total collagen was indirectly calculated from the detection of hydroxyproline as previously described [177, 178]. Briefly, the hydrolysis of proline residues was done in 12 M hydrochloric acid overnight at 100 °C. The solid material was dried at 50 °C and then resuspended in ultrapure deionised water. An oxidising 2.82% w/v chloramine T solution in citrate buffer (pH 6.1) was added for 20 min at RT in the dark, followed by 50% w/v 4-(dimethylamino)benzaldehyde for 20 min at 60 °C to react with pyrrole residues. Serial dilutions of trans-4-hydroxy-L-proline (Fluka) were prepared as standards. The absorbance was measured at the 570 nm wavelength using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc). Collagen content was calculated from the hydroxyproline-to-collagen ratio of 1:7.69 [178]. All reagents were from Merck if not mentioned otherwise.

6.2.9. Histology

At the end of the differentiation period, samples were harvested, washed with PBS, fixed in 4% w/v PFA for 20 min at RT, washed again with PBS and then embedded in 2% w/v agarose. First, samples were dehydrated in a graded series of ethanol (10–70%) before final dehydration steps were done using an automatic tissue processor (Leica TP 1020). Finally, samples were embedded in paraffin wax prior to sectioning using a rotary microtome (Leica RM2235). Tissue sections of 7 μ m in thickness were stained with Haematoxylin and Eosin (H&E), collagens were stained using 0.1% Picrosirius Red, and calcium deposition was visualised using 1% w/v Alizarin Red (pH 4.1). GAGs were stained using 0.2% w/v Safranin O counterstained with 0.04% w/v aqueous Fast Green and cells were counterstained using Weigert's Iron Haematoxylin; alternatively, GAGs were stained with 1% w/v Alcian Blue 8GX in 0.1 M hydrochloric acid (pH 1) and cells were counterstained using 0.1% w/v Nuclear Fast Red. All reagents were from Merck. Finally, stained sections were imaged with a slide scanner (Aperio Scanscope CS).

6.2.10. Confocal Imaging

Cellular uptake—To validate transfection efficiency, polyplex imaging using the Alexa Fluor 647 conjugated C6-CR3 was performed. The anti-miR-221 and the scramble were complexed at 1:12 w/w ratio as previously described. To validate 2D transfection,

hMSCs were transfected during expansion at day 6 only. To validate 3D transfection, transfection was done on 2-day-old microtissues only with no transfection done during 2D expansion. A concentration of 100 nM of anti-miR-221 or scramble was used. Twenty-four hours post-transfection, cells and microtissues were washed in 1X PBS and stained. Monolayers were stained with Rhodamine Phalloidin (Invitrogen) following the manufacturer's instructions. Briefly, cells were fixed in 4% PFA for 10 min, permeabilised with 0.5% Triton for 10 min, stained with 1:40 Rhodamine Phalloidin for 1 h and 1:500 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 10 min in 2.5% w/w bovine serum albumin, and left in 1X PBS prior to imaging. Microtissues were stained with 2 μ M Calcein AM (Biotium) for 30 min, 100 μ g/mL Hoechst 33258 (ThermoFischer) solution for 30 min, and then left in 1X PBS prior to imaging.

Immunofluorescent staining of type I, II and X collagens—Different subtypes of collagen were identified by immunofluorescent staining. After rehydrating tissue sections in graded series, antigen retrieval was done using 35 U/mL (type I and type II collagens) or 3.5 U/mL (type X collagen) pronase for 25 min followed by 4,000 U/mL hyaluronidase for 45 min, both at 37 °C in a humidity chamber (HC). Blocking, using 10% v/v donkey serum (abcam) and 1% w/v bovine serum albumin (blocking buffer (BB)) for 1 h at RT in a HC, was followed by primary antibody incubation overnight at 4 °C in a HC. Fluorescently labelled secondary antibodies were incubated for 1 h at RT in the dark in a HC. Antibodies were diluted in BB as follows: 1:500 anti-type I collagen (ab138492, abcam), 1:100 anti-type II collagen (sc-52658, Santa Cruz Biotechnology), 1:50 anti-type X collagen (14-9771-82, Invitrogen), 1:500 donkey anti-rabbit IgG Alexa Fluor 647 (ab150075, abcam) for type I collagen, 1:500 donkey anti-mouse IgG Alexa Fluor 594 (A21203, Invitrogen) for type II collagen and 1:200 donkey anti-mouse IgG Alexa Fluor 594 (A21203, Invitrogen) for type X collagen. Slides were mounted in Fluoroshield with DAPI. Staining was imaged using a scanning confocal microscope (LeicaTCS SP8) as z-stacks of 10 images. All reagents were from Merck if not mentioned otherwise. Porcine native tissues were used as positive (+) and negative (-) controls: ligament (+) and articular cartilage (-) for type I collagen, articular cartilage (+) and ligament (-) for type II collagen, and hypertrophic articular cartilage (+) and cartilage (-) for type X collagen.

Quantification of fluorescence intensity was performed using the Fiji software using 8-bit images.

- Single Microtissues: the mean grey value (MGV) of each microtissue and the MGV of the background (averaged from four areas) were measured and subtracted. A

total of three to six distinct microtissues were analysed for each experimental group.

- Fused Microtissues: the MGv of each section (averaged from three distinct regions per replicate) and the MGv of the background (averaged from four areas) were measured and subtracted. Three replicates were measured for each experimental group.

Quantification of the type II collagen positive area was calculated using the Fiji software and represents the ratio of the positive staining area by the entire area of the scaled-up tissue. Three replicates were measured for each experimental group.

6.2.11. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software. Analysis of Gaussian distribution and statistical differences are described in each figure caption. Significance was accepted at a level of $p < 0.05$.

6.3. Results

6.3.1. Generation of Anti-miR-221:C6-CR3 Polyplexes with Positive Zeta-Potential for Particle-Mediated MiRNA Cell Delivery

The C6-CR3 polymer has previously been shown to form a stable polyplex with various miRNAs based on electrostatic interactions in AcONa buffer and polyester hydrophobic aggregation in PBS [281-284]. The complexation of C6-CR3 with anti-miR-221, into polyplexes with a range of anti-miR-221:C6-CR3 from 1:12 to 1:250 w/w ratio (**Figure 6.1A**) was evaluated using a gel retardation assay. Retardation, reflected by electrophoresis mobility of free anti-miR-221 (1:0 control), was observed at all tested ratios indicating complete complexation (**Figure 6.1B**). At 0 h, biophysical characterisation by DLS of the anti-miR-221:C6-CR3 1:12 w/w ratio indicated an average size of $228 \text{ nm} \pm 5.42 \text{ nm}$ with a polydispersity index of 0.084 ± 0.007 (<0.1 for monodisperse solutions) and a zeta potential of $16.9 \text{ mV} \pm 1.97 \text{ mV}$ (**Figure 6.1C**). The slightly positive zeta potential, which was not stable over 24 h, was suitable for polymeric nanoparticle mediated cell transfection [285, 286] and the 1:12 w/w ratio was selected for further experiments.

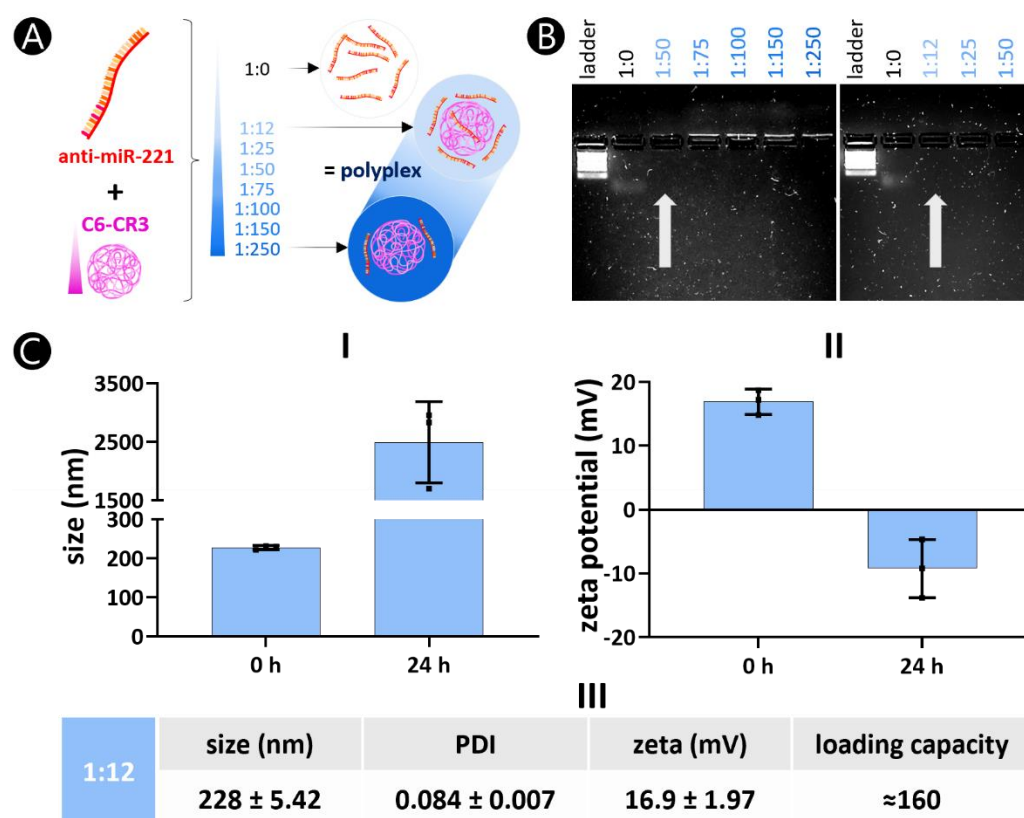


FIGURE 6.1 Retardation and biophysical characterisation of the anti-miR-221:C6-CR3 polyplex nanoparticles. (A) Schematic of the anti-miR-221:C6-CR3 complexation prepared by incubating

the anti-miR-221 solution (0.05 µg/µL) with increasing concentrations of C6-CR3 solution resulting in anti-miR-221:C6-CR3 1:12 to 1:250 w/w ratio range (1:0 is the uncomplexed control). **(B)** Polyplex retardation assay evaluated by gel electrophoresis. Biophysical characterisation of anti-miR-221:C6-CR3 by DLS at 0 and 24 h post-complexation: **(C-I)** size, **(C-II)** surface charge, and **(C-III)** polydispersity index (PDI) of the anti-miR-221:C6-CR3 1:12 w/w ratio. Results are represented as mean ± standard deviation. N =3.

6.3.2. Human MSC Viability Following Transfection with a Wide Range of Anti-miR-221:C6-CR3 Polyplex Concentrations

To validate the capacity of the new polyplex to cross the cell membrane, anti-miR-221 was complexed with the Alexa Fluor 647 conjugated C6CR3 at a 1:12 w/w ratio, transfected at day 6 and assessed by confocal imaging at day 7. The presence of particles in the cell cytoplasm was observed, confirming cellular uptake by hMSCs (**Figure 6.2B**). Cellular uptake of scramble (100 nM) using the fluorescent C6-CR3 vector was also observed. Flow cytometry quantification of live/dead staining and apoptotic cell death showed high cell viability (>99%) across all concentrations, with an overall average of $1.3\% \pm 1.5\%$ of cells undergoing apoptosis (**Figure 6.2 C**), confirming the low cytotoxicity of the pBAE polymer vectors. The transfection yield significantly increased with concentration with more than 80% of the cell population transfected in all groups, exceeding 99% for concentrations of 50 nM and above (**Figure 6.2D**). In addition, polyplex fluorescent intensity, graphed as mean fluorescent intensity (MFI), increased as the concentration increased from 0 nM to 100 nM. These results indicated that an increasing number of cells was transfected and that more transfection events per cell were taking place as the concentration of polyplex increased.

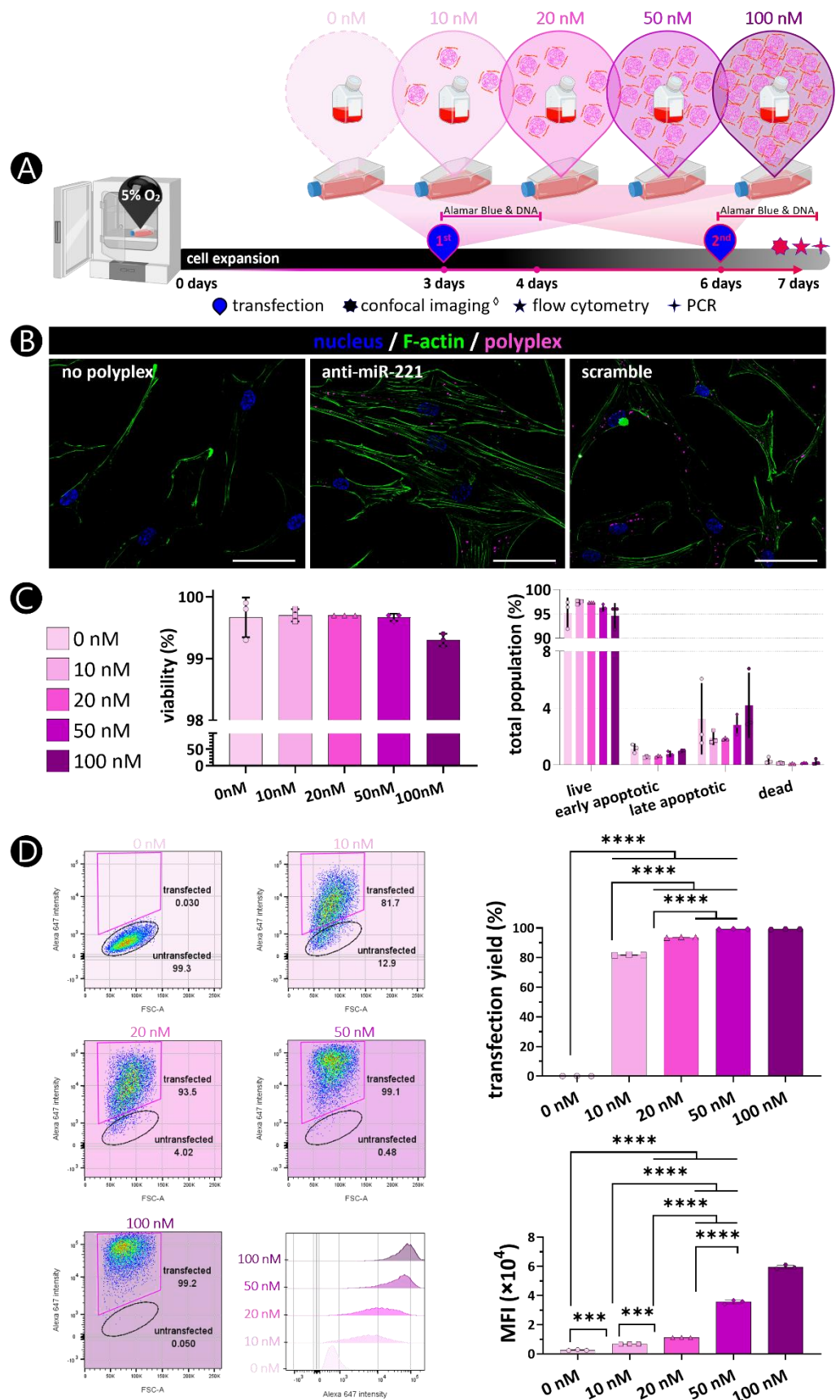


FIGURE 6.2 Cytotoxicity and transfection efficiency of anti-miR-221:C6-CR3 2D transfected hMSCs. (A) Schematic of the 2D transfection study to assess cell behaviour and gene expression levels. Human MSCs were incubated during 2D monolayer expansion at day 3 (1st) and day 6 (2nd) for 4 h in presence of increasing concentrations of polyplex solutions from 0 to 100 nM.

transfection, the DNA levels in the 0 nM group were statistically higher than the levels of the 100 nM group. At day 7, DNA levels in the 0 nM and 10 nM groups were significantly higher than the 50 nM and 100 nM groups. In addition, DNA levels of the 0 nM group, but not the 10 nM group, remained statistically higher than the DNA levels of the 20 nM group. No statistical differences between the 20 nM, 50 nM and 100 nM groups were observed at this final timepoint.

Metabolic activity was assessed by normalising AB reduction levels (**Supplementary Figure 6.2**) to DNA levels (**Figure 6.3 C**). Metabolic activity increased with time for all polyplex doses, except for the 100 nM higher dose where metabolic activity was suppressed (fold changes in **Supplementary Figure 6.3**). In addition, 24 h after the first transfection and before the second transfection, metabolic activity was suppressed in all polyplex transfection groups compared to the 0 nM control (**Figure 6.3D**). This recovered with time in the 10 nM and 20 nM but remained suppressed for the higher concentration groups.

Based on these results, the 50 nM and the 100 nM doses were ruled out. At these doses, cell number (**Figure 6.3A**) and cell metabolism remained low through the expansion period. The 10 nM and 20 nM groups were selected for further evaluation of their silencing effect and pro-chondrogenic potential.

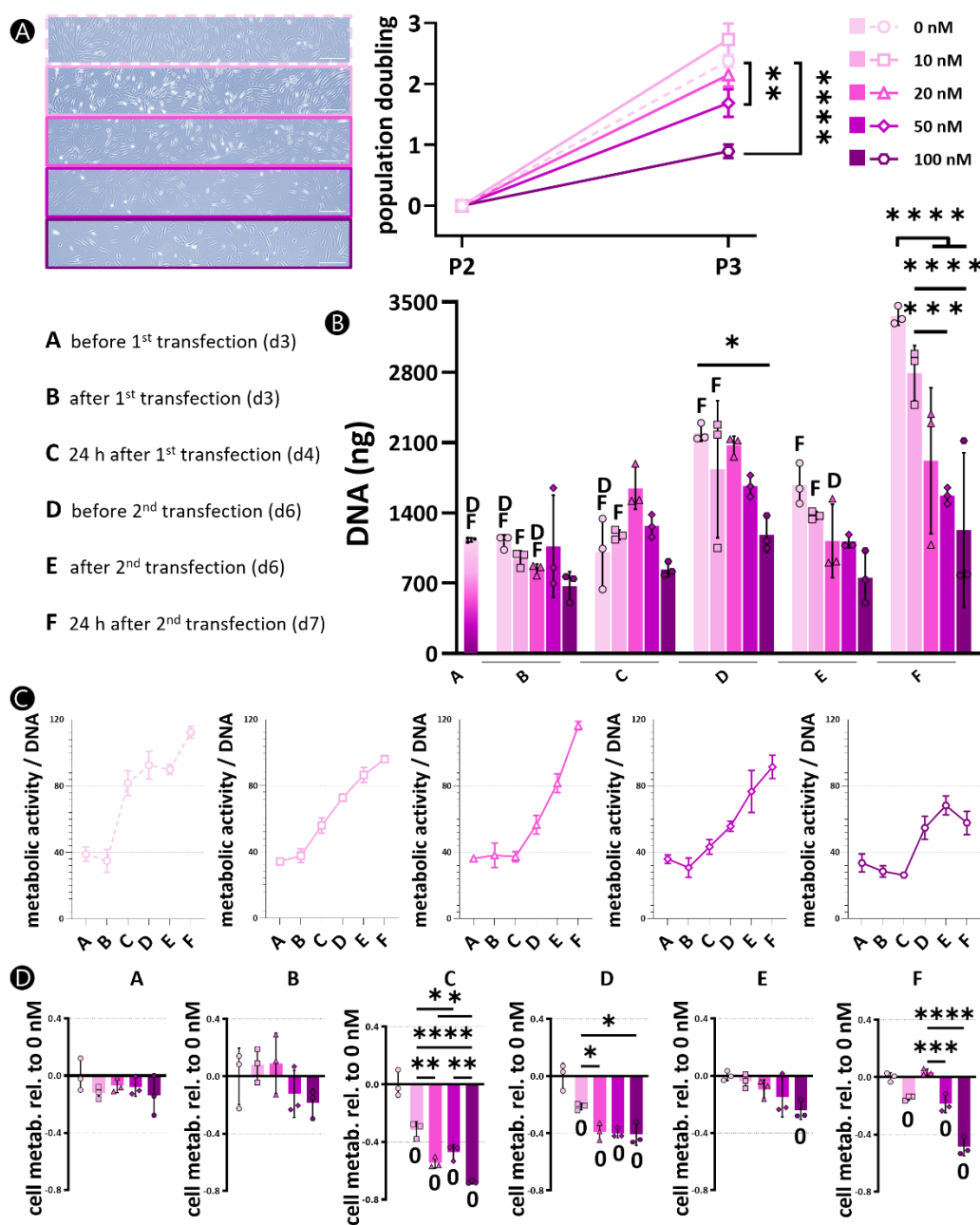


FIGURE 6.3 Evolution of cellular metabolism of hMSCs over the 2D transfection period. (A) Representative images of cell density at the end of the expansion period of 2D transfected cells and corresponding population doubling. P: passage; scale bar = 250 μ m. (B) DNA level quantification, (C) metabolic activity normalised by DNA, and (D) cell metabolism relative to the 0 nM group (cell metab. rel. to 0 nM) measured at different timepoints during the expansion of cells transfected in 2D. All data is represented as individual data points and/or mean $\pm$ standard deviation. Normality was verified by Shapiro-Wilk's test. Statistical differences between population doubling and cell metabolism relative to 0 nM were determined using a one-way ANOVA test and statistical differences between DNA levels were determined using a two-way ANOVA test. Holm-Šidák's test was used for multiple comparisons. Not all statistical differences are shown. N = 3. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. D: denotes a statistically lower value than the one from the same concentration at the D timepoint; F: denotes a statistically lower value than the one from the same concentration at the F timepoint; 0: denotes a statistically lower value than the one from the 0 nM group.

denotes a statistically lower value than the one from the 0 nM group; DNA: deoxyribonucleic acid; d: day.

6.3.4. Anti-miR-221 Delivery Mediated by C6-CR3 Efficiently Silences miR-221

The effect of anti-miR-221 on the expression of target genes in hMSCs was next assessed (**Figure 6.4**). No significant differences in expression levels of miR-221 and *COL1A1* were detected between the control (0 nM) and the scramble group for both transfection regimens. Both the 10 nM and 20 nM anti-miR-221 groups reduced miR-221 expression, with a more pronounced effect after two 10 nM transfections. The expression of chondrogenic genes *COL2A1*, *ACAN* and *SOX9* was not detectable in these monolayer cultures. Two rounds of transfection and both concentrations were used in subsequent experiments.

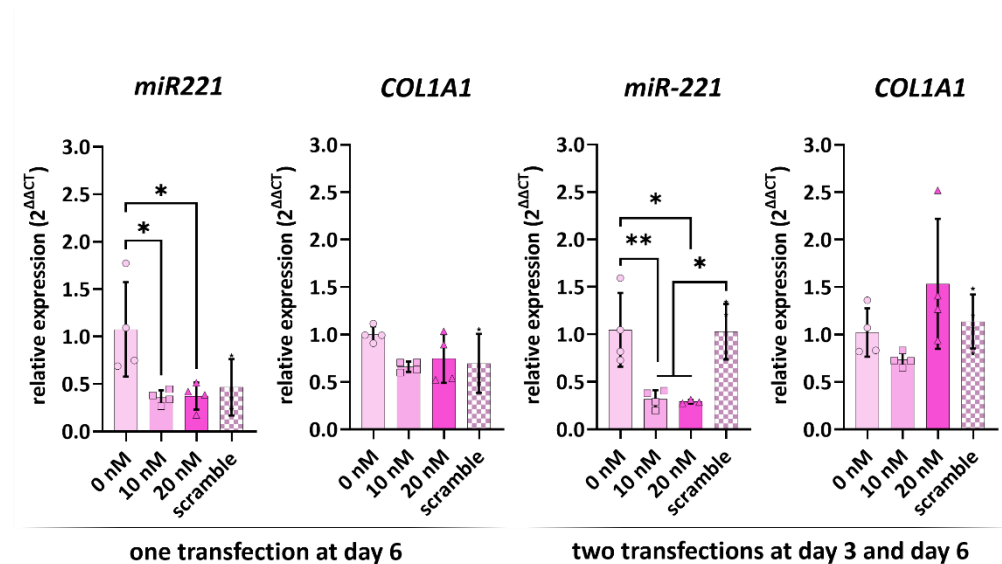


FIGURE 6.4 Evaluation of anti-miR-221 effect at the gene expression levels of 2D transfected hMSCs. RT-qPCR detection of miR-221 silencing and gene expression levels of *COL1A1* after (A) one or (B) two transfections of anti-miR-221:C6-CR3 at varying concentrations (0 nM, 10 nM and 20 nM) in hMSC monolayers. All data is represented as individual data points, mean $\pm$ standard deviation, and were following a Gaussian distribution validated by Shapiro-Wilk's test. Statistical differences between groups were determined using a one-way ANOVA test followed by Tukey's multiple comparisons test. Not all statistical differences are shown. $N \geq 3$. * denotes $p < 0.05$, ** denotes $p < 0.01$.

6.3.5. Microtissues Fabricated with Pro-Chondrogenic Silenced hMSCs Promotes Collagen-Rich Extracellular Matrix Deposition

Chondrogenic differentiation within microtissues generated using hMSCs transfected with anti-miR-221 was next evaluated. Cells were transfected twice during

2D expansion (days 3 and 6) with 0 nM, 10 nM, or 20 nM of anti-miR-221 (**Figure 6.5A**). At day 7, cells were lifted and successfully aggregated into microtissues using an agarose microwell array (**Supplementary Figure 6.4**). The influence of anti-miR-221 delivery on the chondrogenesis of these microtissues after 21 days of culture in the presence or absence of TGF- β 3 was then assessed. Cell aggregation was maintained even in the absence of TGF- β 3, but the size of these microtissues decreased over time. An additional 3D transfection, confirmed by confocal microscopy (**Figure 6.5B**), was also performed 2 days following microtissue generation to assess any beneficial effect on chondrogenesis and potential therapeutic applications. Microtissue integrity was maintained after 3D transfection and their diameter increased over time. The microtissues formed using transfected hMSCs contained significantly lower levels of DNA compared to the control, especially in the presence of TGF- β 3, but the additional 3D transfection did not lead to a further reduction in DNA levels compared to 2D transfection only (**Figure 6.5 C**). Silenced cells synthesised significantly less GAGs compared to non-transfected cells. This suppression of GAG synthesis was lower for lower anti-miR-221 concentrations. Anti-miR-221 transfection mediated via C6-CR3 did not prevent the deposition of collagen during 3D chondrogenic differentiation, as no statistical differences were observed between experimental and control groups (except for groups differentiated in the absence of TGF- β 3). After the extra 3D transfection, collagen synthesis significantly increased for both dosages compared to 2D transfection alone. These results suggest that anti-miR-221 delivery, in the absence of additional TGF- β 3 stimulation, does not promote significant cartilage specific extracellular matrix (ECM) deposition.

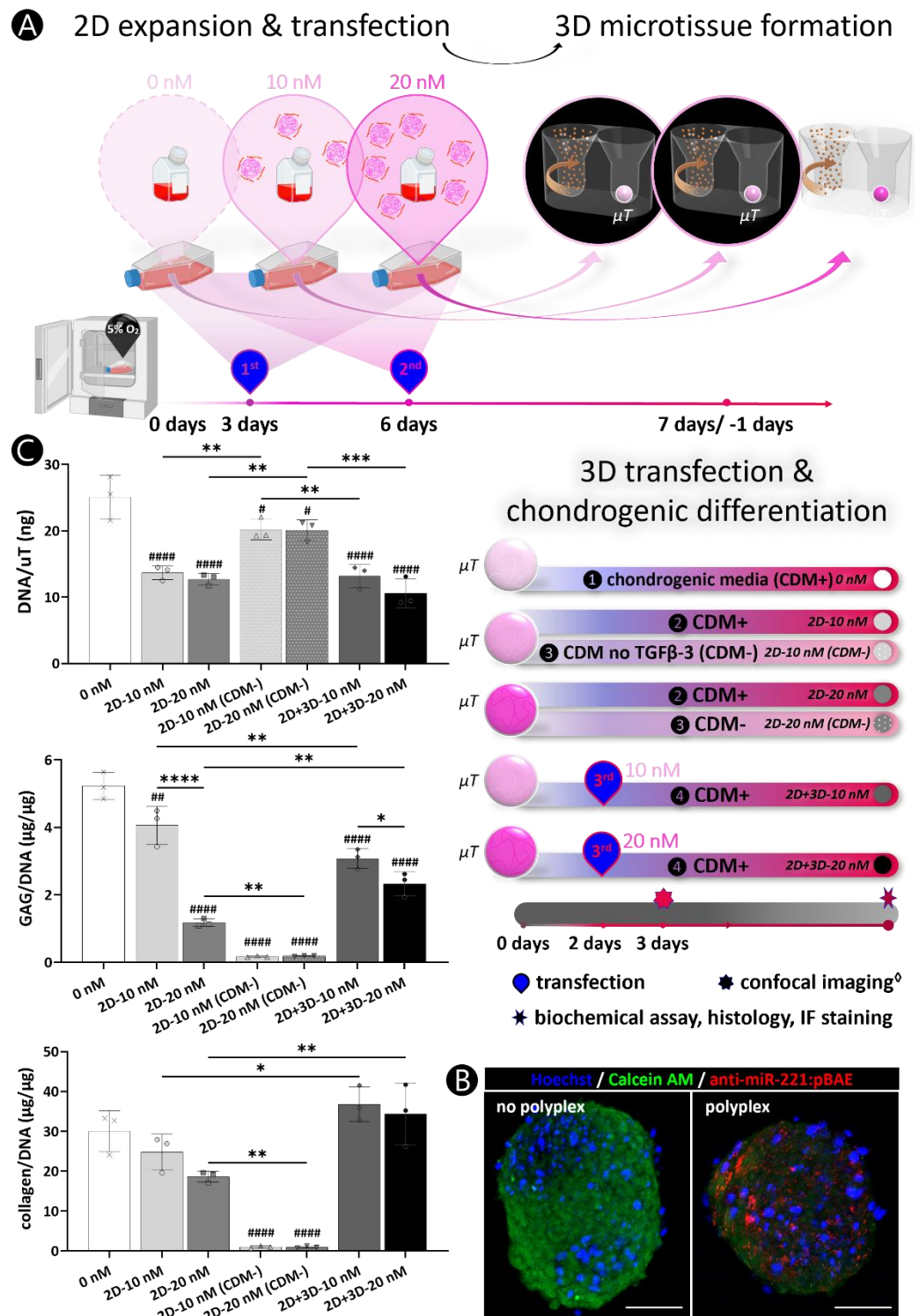


FIGURE 6.5 Assessment of tissue deposition in miR-221 silenced microtissues. (A) Schematic of the fabrication process of microtissues using miR-221 silenced hMSCs. After transfecting cells in 2D (0 nM, 10 nM and 20 nM) as described in **Figure 6.2**, microtissues were fabricated in low adherence agarose microwells and differentiated for 21 days in chondrogenic media containing (groups: 0 nM, 2D-10 nM and 2D-20 nM) or not containing (groups: 2D-10 nM-CDM- and 2D-20 nM-CDM-) TGF- β 3. In addition, microtissues differentiated in presence of TGF- β 3 were transfected a third time in 3D on day 2 with the same concentration as used during 2D transfections (groups: 2D+3D-10 nM and 2D+3D-20 nM). μ T: microtissue; CDM+: chondrogenic media; CDM-: chondrogenic media not supplemented with TGF- β 3, IF: immunofluorescence.

(B) Confocal imaging of 2-day-old hMSC-derived microtissues transfected with 100 nM of fluorescently labelled anti-miR-221:C6-CR3 or not transfected. Nuclei were stained with Hoechst stain and are shown in blue. To assess 3D transfection efficiency, cells were transfected day 2 after microtissue formation only with no transfection performed during 2D expansion. Scale bar = 100 μ m. (C) Biochemical evaluation of DNA content and key cartilaginous ECM components (GAGs and collagen). All data is represented as individual data points and mean $\pm$ standard deviation. Statistical differences were determined using a one-way ANOVA test with Holm-Šídák's multiple comparison test. Selected statistical differences are displayed. N = 3. The # symbol denotes differences with the control group (0 nM). #, * denote p <0.05, ##, ** denote p <0.01, *** denotes p <0.001, ####, **** denote p <0.0001. CDM-: chondrogenic differentiation media not supplemented with TGF- β 3; DNA: deoxyribonucleic acid; GAGs: glycosaminoglycans.

Histologically, all groups of microtissues differentiated in the presence of TGF- β 3 stained positively for GAGs and collagens (**Figure 6.6**). Safranin O staining was visibly more intense for the 0 nM and 2D-10 nM groups, which correlates with the biochemical results. GAG deposition in these groups was also homogeneous. All groups stained equally positive for collagens with no signs of calcium deposition. The cell morphology in the Picrosirius Red stained sections was chondrocyte-like with round/elliptical shaped lacunae.

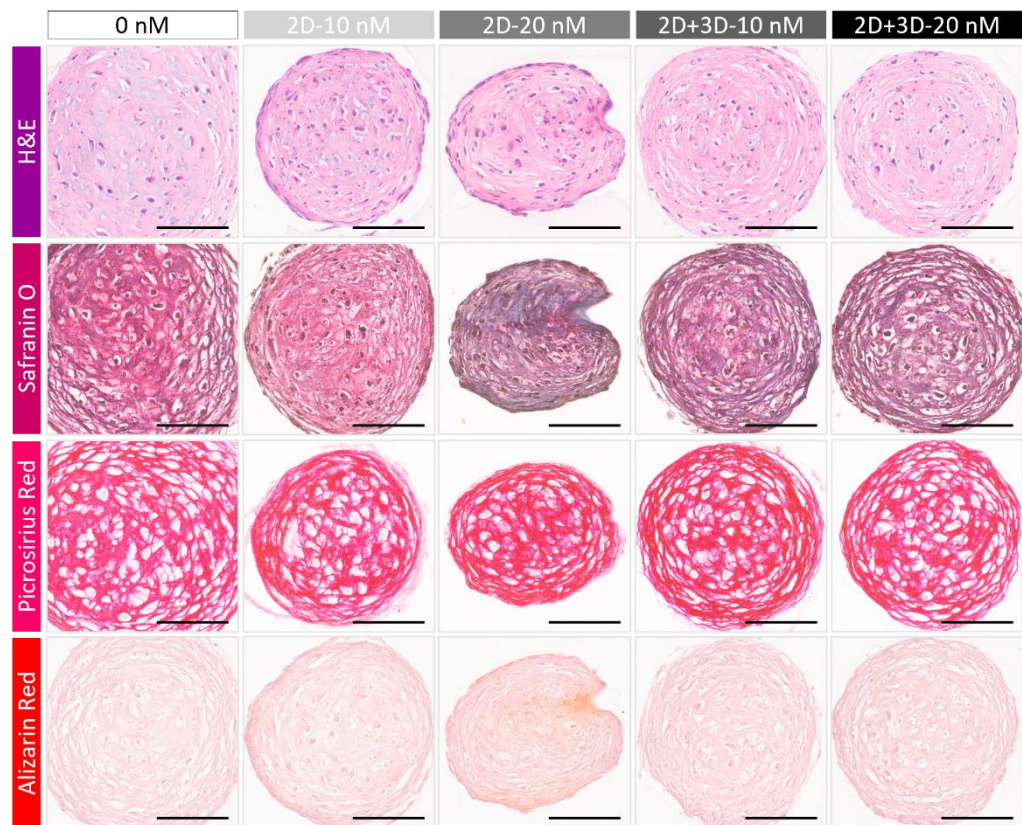


FIGURE 6.6 Histology panel of single microtissues after 21 days of chondrogenesis driven by different differentiation conditions. Representative staining images for Haematoxylin and eosin (H&E), GAGs (Safranin O), collagens (Picrosirius Red) and calcium (Alizarin Red) staining of silenced microtissues transfected with 10 nM or 20 nM in 2D only (2D-10 nM and 2D-20 nM) or in 2D and 3D (2D+3D-10 nM and 2D+3D-20 nM). Cells were not transfected in the 0 nM control group. Scale bar = 100 μ m.

6.3.6. Transfection with 10 nM Anti-miR-221 Promotes the Development of More Hyaline-Like Cartilaginous Microtissues

Further characterisation of silenced hMSC-secreted ECM collagen subtypes was undertaken to determine the tissue phenotype. In the control 0 nM group, positive staining for type I, II and X collagens was observed, indicating hypertrophy and progression along an endochondral pathway (**Figure 6.7**). For groups transfected with 10 nM in 2D, and even more so when transfected in 2D and in 3D, a very intense and relatively homogeneous staining of type II collagen was apparent, with less type X collagen deposition observed. All groups stained positive for type I collagen. Quantification of staining intensity revealed significantly more intense type II collagen staining and lower type X collagen staining after three rounds of transfection with 10 nM of anti-miR-221 (**Figure 6.8**). Surprisingly, the higher anti-miR-221 dose of 20 nM almost completely suppressed type II and type X collagen deposition but maintained

(or even increased) type I collagen deposition indicative of a fibrotic phenotype. To conclude, varying doses of anti-miR-221 yield distinct microtissue phenotypes: hyaline, hypertrophic and fibrotic. As the 10 nM dose enhanced type II collagen production in single microtissues, this concentration was selected in the final fusion study.

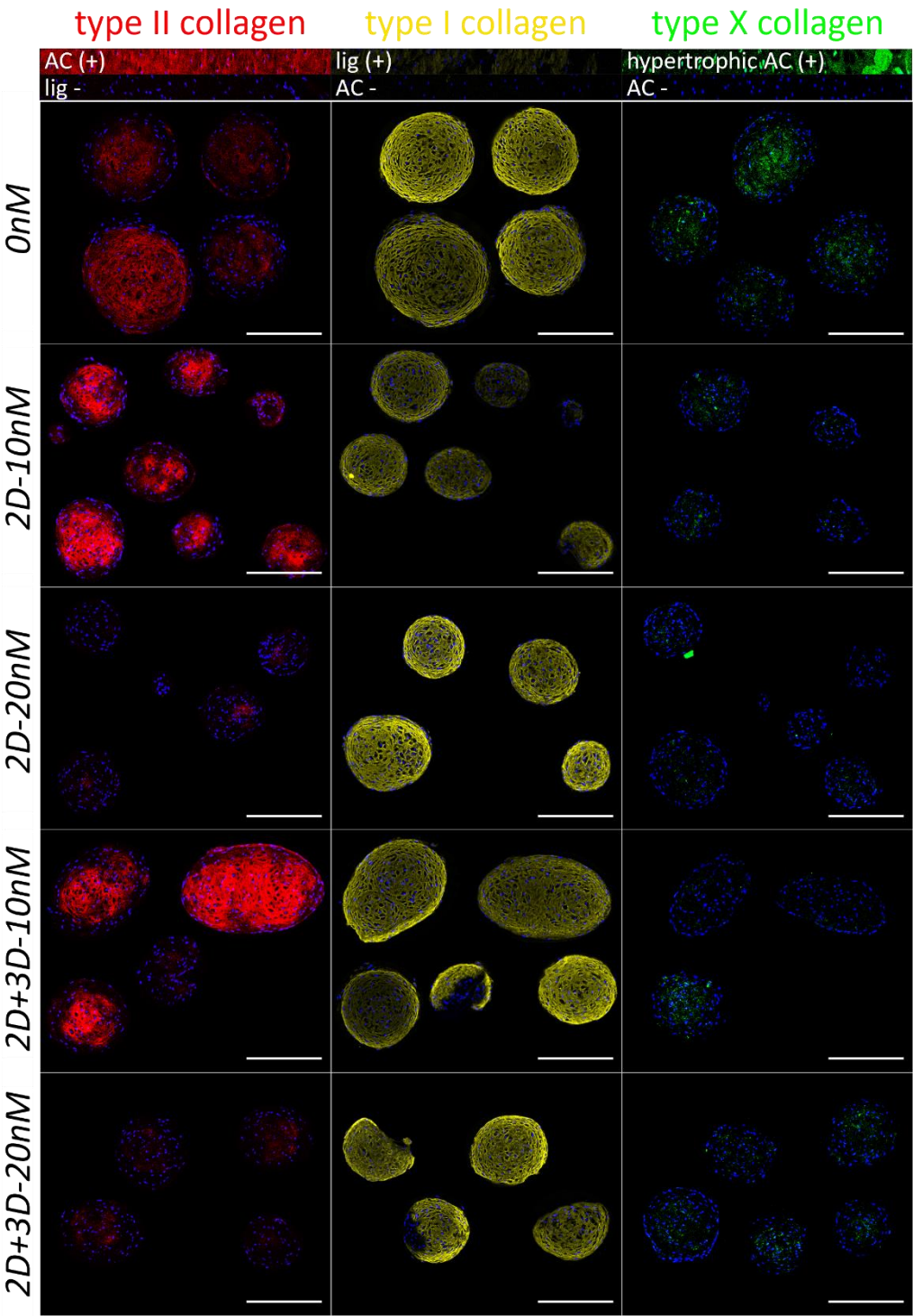


FIGURE 6.7 Phenotype characterisation of silenced cartilaginous microtissues. Immunofluorescent staining of type II collagen (red), type I collagen (yellow), type X collagen (green) and nuclei (blue, DAPI). Microtissues were transfected with 10 nM or 20 nM in 2D only (2D-10 nM and 2D-20 nM) or in 2D and 3D (2D+3D-10 nM and 2D+3D-20 nM). Cells were not

transfected in the 0 nM control group. Scale bar = 250 μ m. AC: articular cartilage; lig: ligament; (+): positive control; -: negative control.

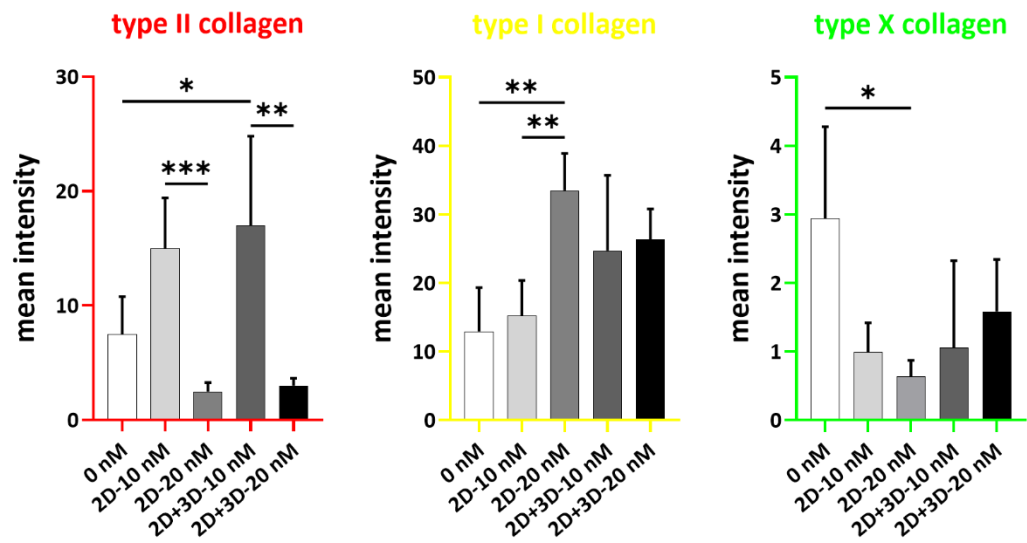


FIGURE 6.8 Quantification of fluorescence staining intensity of collagen subtypes (II, I, X). Microtissues were fabricated using cells transfected with 10 nM or 20 nM of anti-miR-221 in 2D alone (2D-10 nM and 2D-20 nM) or in 2D and 3D (2D+3D-10 nM and 2D+3D-20 nM). Cells were not transfected in the 0 nM control group. All data is represented as mean $\pm$ standard deviation. Statistical differences were determined using a one-way ANOVA test with Tukey's multiple comparison test. $N \geq 3$. * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

6.3.7. Silenced Microtissues Can Be Fused into a Larger Graft with Hyaline Phenotype

Silenced microtissues were next used as building units to generate a larger cartilage graft. After 3 days of culture in a microwell array system, 400 microtissues were seeded in a 4 mm $\times$ 5 mm nonadherent agarose well (**Supplementary Figure 6.4**). After 24 h, single microtissues fused with each other and began to generate a cohesive scaled-up graft. At day 21, ECM production was visualised by histology and immunofluorescence staining. Picosirius Red staining of collagen fibres confirmed fusion, as single microtissues were not identifiable in all groups suggesting that cell and microtissue transfection did not hinder the fusion capacity of single microtissues into a larger construct (**Figure 6.9**). Collagen deposition was generally inhomogeneous, with higher deposition at the periphery of the constructs. In transfected groups, GAG staining was also heterogenous. Chondrocyte-like cells were present in GAG-rich regions of the engineered grafts. The cell morphology in unstained area suggested the presence of a necrotic core, which was absent in the control group. Finally, there was no sign of calcium deposition.

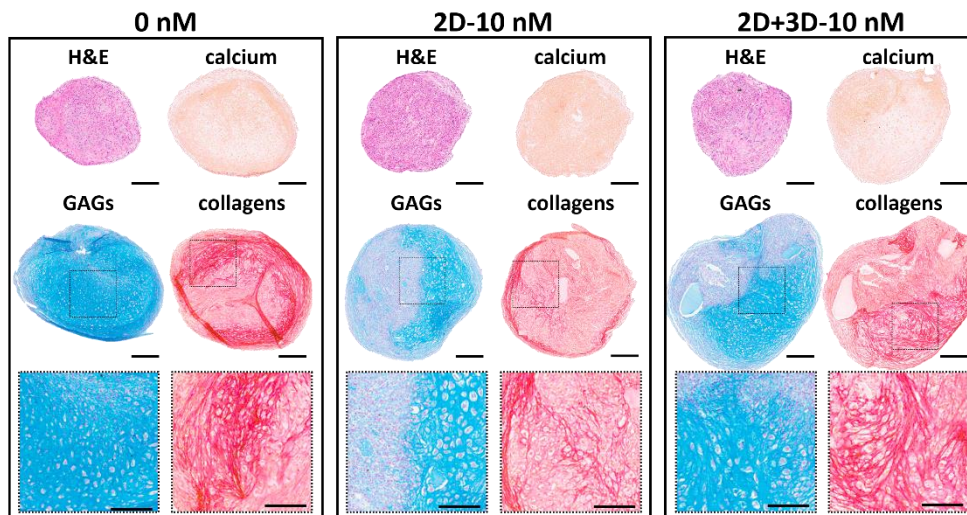


FIGURE 6.9 Histology panel of silenced and fused grafts. Representative staining images.

0 nM: not transfected control; 2D-10 nM: cell transfected with 10 nM in 2D; 2D+3D-10 nM: cell transfected with 10 nM in 2D and 3D; H&E: Haematoxylin and Eosin; GAGs: glycosaminoglycans. Scale bar = 200 μ m/100 μ m (zoom).

Finally, immunofluorescent staining of collagen subtypes was analysed to further characterise the phenotype of these larger constructs (**Figure 6.10**). Type II collagen staining was apparent in the control group, covering $58.5\% \pm 5.8\%$ of the surface of the tissue (**Figure 6.11**). Unlike that observed in individual microtissues, staining of type II collagen in the 2D+3D-10 nM group and even more in the 2D-10 nM groups was not homogeneous, covering less than half of the tissue surface, and weaker (or similar in the 2D+3D-10 nM group) than the control group. All groups stained positive for type I collagen. The 0 nM control group showed some positivity for type X collagen, while the transfected groups were negative for type X collagen. Hence, the 2D+3D-10 nM group appeared to best support the engineering of scaled-up hyaline-like constructs resistant to hypertrophy.

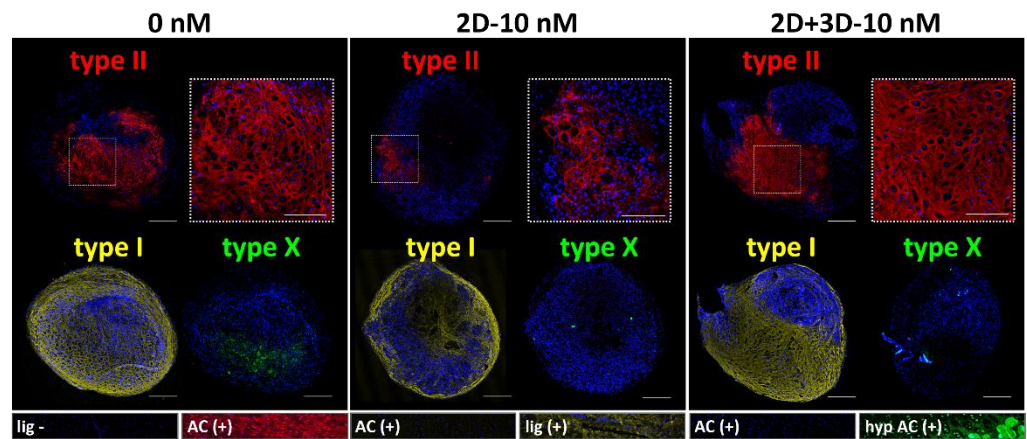


FIGURE 6.10 Immunofluorescent staining of collagen subtypes in fused grafts after chondrogenic differentiation under different conditions. Immunofluorescent staining of type II collagen (red), type I collagen (yellow), type X collagen (green) and nuclei (blue, DAPI). Representative images. Scale bar = 200 μ m/100 μ m (zoom). 0 nM: not transfected control; 2D-10 nM: cell transfected with 10 nM in 2D; 2D+3D-10 nM: cell transfected with 10 nM in 2D and 3D; lig: ligament; AC: articular cartilage; hyp AC: hypertrophic AC; (+): positive control; -: negative control.

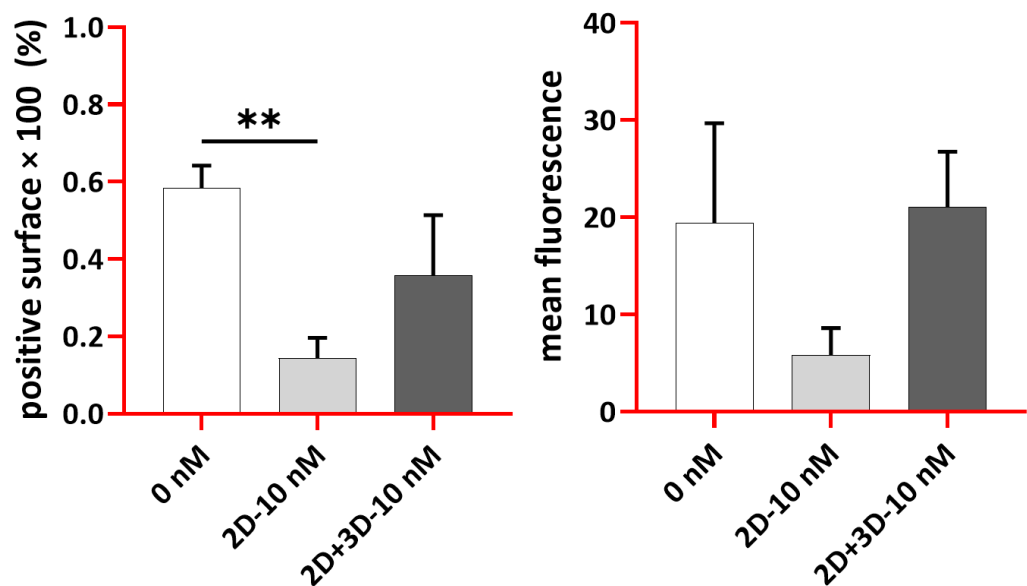


FIGURE 6.11 Quantification of positive area and type II collagen staining intensity in scaled-up cartilage grafts. All data is represented as mean $\pm$ standard deviation. Gaussian distribution was verified by Shapiro-Wilk's normality test. Statistical differences were determined using a one-way ANOVA test with Holm-Šidák's multiple comparison test. N = 3. ** denotes $p < 0.01$. 0 nM: not transfected control; 2D-10 nM: cell transfected with 10 nM in 2D; 2D+3D-10 nM: cell transfected with 10 nM in 2D and 3D.

6.4. Discussion

The overall goal of this study was to establish a novel protocol for TE hMSC-derived hyaline cartilage microtissues via the delivery of the pro-chondrogenic anti-miR-221. The delivery of a wide range of anti-miR-221 concentrations, from 0 nM to 100 nM, using the pBAE polymeric vector on 2D monolayers was confirmed via confocal imaging, with subsequent cell viability and potential apoptosis assessed using flow cytometry. A preferred 2D transfection concentration was determined based on an analysis of cellular metabolic activity, DNA quantification and qRT-PCR for miR-221. Microtissues generated using cells transfected with anti-miR-221, either in 2D only or in combination with transfection in 3D (10 nM dose), supported the development of type II collagen-rich cartilage microtissues containing lower levels of type X collagen. Additionally, anti-miR-221 treated microtissues supported the fabrication of scaled-up AC grafts with lower levels of type X collagen production. Taken together, these results indicate that optimised pBAE-mediated delivery of anti-miR-221 efficiently silences its miR-221 target, enhancing chondrogenesis of hMSC-derived microtissues whilst suppressing markers of hypertrophy.

In-house synthesised C6-CR3 polymer was used as an efficient transfection vector for anti-miR-221, with transfection rates of ~80% and minimal cell death/apoptosis. The pBAE polymer has successfully been used as a transfection vector for plasmids, nucleic acids and small interfering RNAs (siRNAs) in the context of cancer treatment and immunotherapy, including severe acute respiratory syndrome coronavirus 2 (COVID-19) infections due to its versatility and high degree of functionalisation [287-289]. The high transfection success rate can be explained by the cationic nature of pBAE, which enables higher interaction and condensation of negatively charged genetic cargos by electrostatic interactions than other polymeric nanoparticles or even lipid-based nanoparticles whose condensation is limited by the liposome/micelle size [139, 281]. Positively charged polyplexes, as observed in this study (polyplex zeta potential of $16.9 \text{ mV} \pm 1.97 \text{ mV}$), interact with the cell membrane and escape endolysosomal degradation, subsequently increasing the intracellular presence of the polyplex [133]. Via the proton-sponge hypothesis, pBAE amines buffer the acidification mechanism of internalised endolysosomes which prevents degradation and releases the polyplex in the cytosol [290]. In addition, pBAE has been shown to be less cytotoxic than polyethylenimine, which are commercially available and often considered a gold standard for polymeric vectors, due to its excellent biocompatibility and high biodegradability of its hydrolysable ester backbone [285,

291, 292]. The relatively low anti-miR-221:C6-CR3 1:12 w/w ratio is characteristic of arginine-terminated polymers due to the presence of end-cap readily protonated primary and secondary amines, which increases the oligonucleotide condensation capacity of pBAE [282]. The diameter of the selected anti-miR-221:C6-CR3 polyplex was $228 \text{ nm} \pm 5.42 \text{ nm}$. The value is slightly higher than the suggested recommendations of $\sim 200 \text{ nm}$ predictive for high transfection efficiency [136]. However, larger nanoparticles (300 nm) have also successfully been used as a transfection vector [293]. Indeed, nanoparticle compaction has been observed in foetal bovine serum, as contained in the XPAN used in this study, which could result from the electrostatic interactions of polyplexes with serum proteins.

Flow cytometry showed that the transfection yield and number of transfection events per cell increased with increasing anti-miR-221 concentrations. This was associated with a reduction in DNA levels and slowdown in cell division, particularly for higher polyplex concentrations. Increasing the dose of transfecting agent is typically associated with a decrease of cell viability due to particle toxicity, however here cells remained alive, again highlighting the low cytotoxicity of C6-CR3. It should be noted, however, that higher concentrations did negatively impact the cellular metabolic activity. Cellular uptake uses cell endocytosis machinery, resulting in the internalisation of membrane-derived vesicles and the recycling and degradation by the endosome and lysosome [27, 294]. However, the lysosome has an important role in the homeostasis of cell metabolism by selectively retaining or releasing its nutrient reservoir in response to external and internal cues [295]. Further studies are needed to confirm the potential role of the lysosome in cell metabolism following transfection with such complexes.

After demonstrating a superior inhibition of miR-221 after two rounds of 2D transfection (in both the 10 nM and 20 nM groups), these silenced cells were used to fabricate microtissues which were maintained in culture for 21 days in the presence or absence of TGF- β 3. TGF- β 3 plays complex yet diverse roles in cartilage development and homeostasis [296]. It has previously been demonstrated that silencing of miR-221 supports chondrogenesis of MSCs and type II collagen deposition in the absence of additional TGF- β stimulation [33, 153, 280]. Here, anti-miR-221 treatment failed to support chondrogenesis within the microtissues in the absence of additional TGF- β 3 stimulation. Difference in the size of microtissues compared to traditional pellet culture systems, and associated changes in oxygen and nutrient transport [297], could potentially contribute to these differences in findings. In addition, differences in transfection agents or silencing efficiency could also explain these discrepancies. In the presence of TGF- β 3, treatment with anti-miR-221 was found to generally suppress GAG

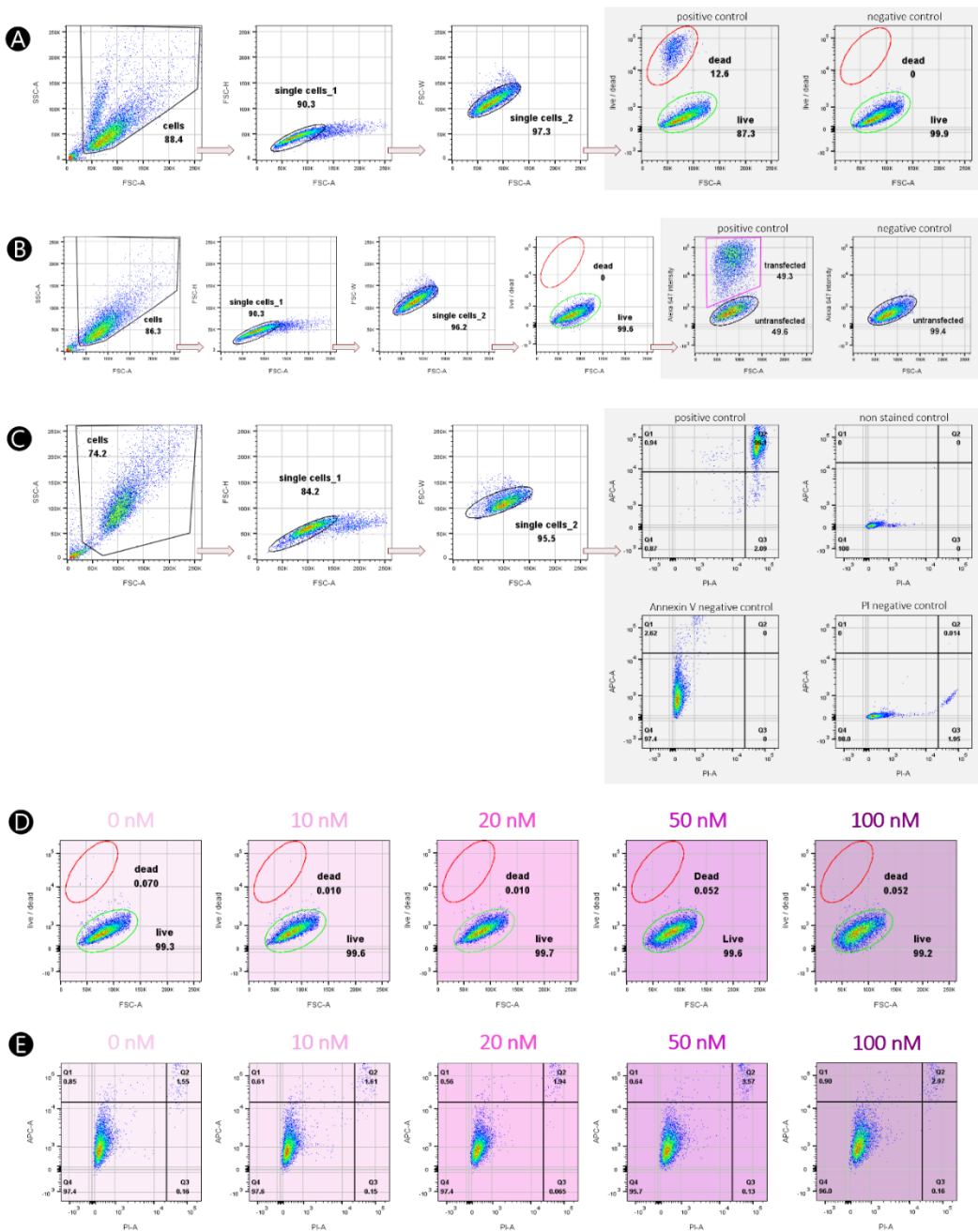
synthesis compared to untreated controls, but at the appropriate dosing level (10 nM), was found to increase type II collagen deposition. This was accompanied by suppression of type X collagen accumulation, a well-established marker of chondrocyte hypertrophy. Type II collagen represents 90% to 95% of the total collagen content of cartilage ECM [3]. Engineering cartilage with sufficiently high levels of type II collagen is a well-established challenge in the field, with traditional approaches often resulting in the development of GAG-rich, but collagen-poor tissues. Furthermore, cartilage grafts engineered using bone marrow-derived MSCs typically become hypertrophic and generate bone *in vivo* [298]. This inherent tendency to progress along an endochondral pathway would appear to be suppressed by treatment with anti-miR-221. Unexpectedly, the 20 nM group did not perform as well. Type II collagen staining intensity was significantly lower compared to the 10 nM group, highlighting the importance of identifying the appropriate dose when using such anti-miR therapies, as has previously been demonstrated in the context of bone regeneration [299].

Finally, silenced microtissues were harvested from their individual agarose well and fused to form larger cartilage grafts. Microtissues were harvested after 3 days to leverage their fusion capacity [212]. Transfection of hMSCs did not appear to hinder the capacity of microtissues to fuse and generate such larger constructs. However, unlike the untransfected control, GAG and collagen deposition within the scaled-up transfected grafts appeared rather heterogeneous. The mechanism of miR-221 is still poorly understood. Only MDM2, a negative regulator of Slug, and trocho-rhino-phalangeal syndrome type I factor (TRPS1), a negative regulator of Runt-related transcription factor 1 (Runx2), have been experimentally validated as miR-221 targets [33, 277]. Potentially, as a result of MDM2 and TRPS1 inhibitions, Slug negatively regulates SRY-Box transcription factor 9 (SOX9), the master regulator of chondrogenesis, and Runx2 positively regulates gene associated with cartilage hypertrophy [33]. Additionally, high-throughput screening has identified *SOX9* as a potential target of miR-221 [300]. Deeper understanding of the mechanism of action of miR-221 could help to understand and improve current discrepancies and should be addressed in future studies.

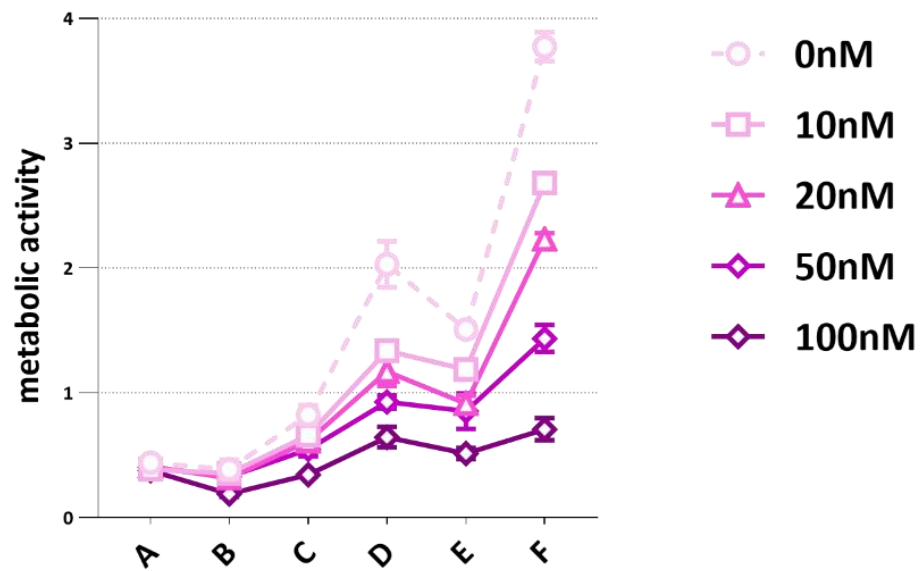
6.5. Conclusion

In conclusion, the anti-miR-221:C6-CR3 polyplex was used to efficiently transfect hMSCs in 2D monolayers as well as in 3D microtissue aggregates leading to the generation of AC mimetic microtissues displaying a hyaline cartilage phenotype with less evidence of premature hypertrophy. The dose and regimen of polyplex transfection were optimised to maximise the pro-chondrogenic effect of anti-miR-221 without compromising downstream biofabrication strategies. The 10 nM dose transfected twice during monolayer organisation plus an extra transfection in 3D appeared to best support chondrogenesis in single microtissues. Fusion of silenced microtissues was not compromised by transfection, but dose and regimen optimisation challenges likely remain and should be addressed in future studies. Taken together, these studies describe a novel approach for generating more biomimetic AC microtissues, which have diverse potential uses from drug testing and disease modelling to the engineering of biological building blocks for the biofabrication of large AC tissue grafts.

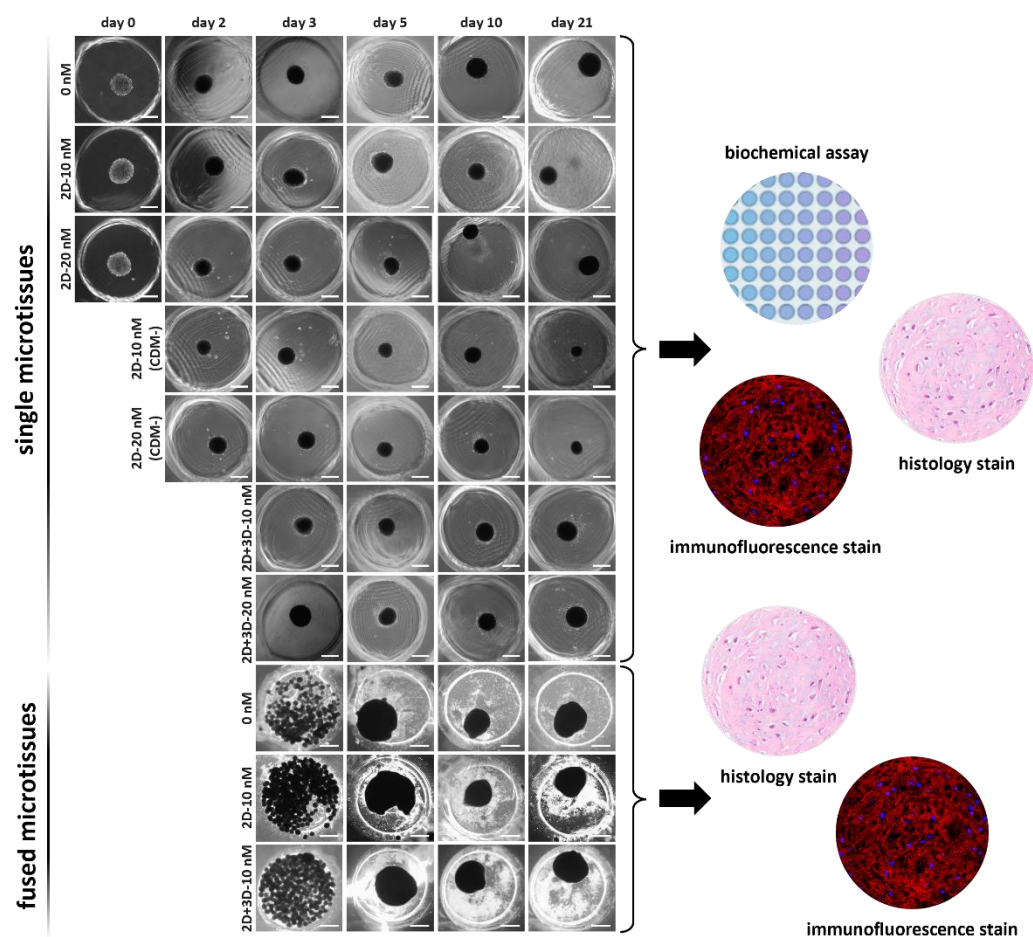
6.6. Supplementary Figures



SUPPLEMENTARY FIGURE 6.1 Gating strategy and representative plots of the flow cytometry analysis. (A&D) Live/dead (eFluor 506) staining, (B) Alexa 647 C6-CR3, and (C&E) Apoptosis (Annexin V/PI) staining. SSC: side scatter; FSC: forward scatter; A: area; H: height; W: width; PI: propidium iodide.



SUPPLEMENTARY FIGURE 6.2 Evolution of the metabolic activity of hMSC transfected in 2D with increasing concentrations of anti-miR-221 polyplexes. All data is represented as mean $\pm$ standard deviation. N= 3. Timepoints: A: before first transfection, B: after first transfection, C: 24 h after first transfection, D: before second transfection, E: after second transfection, F: 24 h after second transfection.



SUPPLEMENTARY FIGURE 6.4 Evolution of the macroscopical aspect of silenced single or fused microtissues during chondrogenic differentiation. Brightfield microscopy images of microtissues. Scale bar = 200 μ m (single)/1 mm (fused). CDM-: chondrogenic media not supplemented with TGF- β 3.

TABLE SUPPLEMENTARY 6.1 List of TaqMan advanced miRNA primers used to analyse gene expression levels following anti-miR-221 transfection.

Target Gene	Target Gene Reference	Catalogue Code
has-miR-221-3p	477981_mir	A25576
collagen type II alpha 1 chain (<i>COL2A1</i>)	Hs00264051_m1	4331182
collagen type I alpha 1 (<i>COL1A1</i>)	Hs00164004_m1	4331182
aggrecan (<i>ACAN</i>)	Hs00153936_m1	4331182
SRY-box 9 (<i>SOX9</i>)	Hs00165814_m1	4331182
eukaryotic 18S rRNA	Hs99999901_s1	4448485

7. Discussion

7.1. Summary

The objective of this thesis was to explore different tissue engineering (TE) approaches to support the use of human-derived cells in developmentally inspired strategies to regenerate biomimetic articular cartilage (AC). The success of engineering AC requires careful consideration of the cell type, environmental cues and soluble factors. In addition, recapitulating the organisation of the tissue, particularly the glycosaminoglycan (GAG) gradient and the collagen network arcade-like structure, is central to the success of such engineered tissues. Bone marrow-derived mesenchymal stem/stromal cells (MSCs) have been extensively used in the field of TE, but their application for hyaline cartilage regeneration is still challenging. As a potential alternative to human MSCs (hMSCs), human articular cartilage progenitors (hACPs) were first used to fabricate cartilage aggregates in isolation or in coculture with hMSCs. While it was possible to identify culture conditions favourable to the differentiation of hACPs, their capacity to generate hyaline cartilage tissue was somewhat limited. As an alternative to engineer phenotypically stable hyaline cartilage microtissues, this thesis next explored the effect of oxygen levels on chondrogenesis of hMSC aggregates. By altering oxygen levels to values more representative of the *in situ* tissue environment, the quality of synthesised extracellular matrix (ECM) was significantly enhanced and mimicked the specific composition of the different regions of AC. Additionally, when used in combination with a guiding melt electrowritten (MEW) mesh, such microtissues were capable of generating a macroscale tissue with a more biomimetic collagen network. Notably, this novel modular assembly approach minimised the presence of foreign material while maintaining the geometry of the grafts. Finally, another promising approach to direct the differentiation of hMSCs is through the delivery of pro-chondrogenic microRNA (miRNAs or miRs). Through the selection of adequate dose and regimen, the production of type II collagen was significantly enhanced with early signs of phenotype stability. This thesis established novel approaches for engineering more biomimetic hMSC-derived microtissues, which could advance *in vitro* models and the biofabrication of AC.

7.1.1. Alternatives to hMSCs to Engineer Hyaline AC

Numerous hMSC-based therapies to regenerate AC are currently under investigation, as not only are the cells relatively easy to harvest, but they overcome limitations associated with primary chondrocytes following extensive *in vitro* culture, including maintenance of their chondrogenic potential [15]. However, the use of these cells comes with their own limitations. Differentiation of MSCs in cellular aggregates [165] or in scaffold-based approaches [301] has shown histological evidence of key cartilaginous ECM components, especially type II collagen, but maintenance of the hyaline phenotype remains a challenge as MSCs have the inherent tendency to undergo hypertrophy [222]. In a first study, hACPs, which are AC resident cells with stem-like characteristics, were explored as a novel cell type to engineer phenotypically stable hyaline cartilage microtissues. While hACPs have been shown to increase type II collagen and decrease type I and X collagen synthesis [82], in this study hACP-derived aggregates mostly failed to support robust chondrogenesis. Notably, hACPs were isolated from diseased patients undergoing knee arthroplasty, albeit from healthy-looking regions. The potential of articular cartilage progenitors (ACPs) has been shown to decrease with osteoarthritis progression and could explain the relatively poor chondrogenesis observed [83, 84]. In addition, the differentiation of these hACP-derived aggregates was carried out using the transforming growth factor (TGF)- β 3, a differentiation protocol established for MSCs [165]. The optimal differentiation conditions for ACPs are currently being refined and could improve the differentiation of hACP-derived microtissues [188]. Finally, isolation protocols, cell characterisation or culture expansion protocols are different between studies, which likely results in heterogeneous cell populations, making them difficult to compare [74]. The lack of a specific ACP surface marker raises the question whether APCs are a homogeneous or heterogeneous population and questions their continuous use as an alternative cell source, especially when isolated from diseased patients.

7.1.2. Improving the Chondrogenic Potential of hMSCs

Having ruled out hACPs as a cell source, this thesis next investigated the effect of environmental cues or the potential of gene silencing on chondrogenesis of hMSCs in a microtissue format. Human cells represent a more clinically relevant choice as results from animal studies, including gene-transfection efficiency, do not always translate into humans [302-304].

The effects of low oxygen conditions on chondrogenesis of MSCs have extensively been studied [24, 25]. In such studies, low oxygen conditions (e.g. 5% O₂) is typically compared to 'normoxic' oxygen levels (21% O₂). As part of this thesis, the influence of different oxygen levels (5% O₂ and 2% O₂), comparable to those found at different depths within the native tissue [20], were directly compared for the first time using hMSC-derived microtissue models. Lower oxygen (2% O₂) significantly improved the deposition of GAGs, while still supporting type II collagen production. Notably, lower oxygen was particularly beneficial for donors with less intrinsic chondrogenic potential (compared to differentiation at 5% O₂) and almost completely suppressed differences between donors. Therefore, these results suggest that 2% O₂ should be considered in chondrogenic differentiation protocols for hMSCs, especially those exploring the microtissue format. Notably, despite such lower oxygen conditions significantly increasing GAG production, levels are still lower than similar studies including animal MSC-derived microtissues [100]. Further improvements of hMSC differentiation conditions are still needed.

To control the chondrogenic differentiation of hMSCs, delivery of novel silencing nucleic acids was employed. Anti-miR-221 (miR-221 inhibitor) therapeutics has previously been shown to be pro-chondrogenic [153, 277, 280]. This study confirms the positive effect of anti-miR-221 on chondrogenesis of hMSCs, either when used during two-dimensional (2D) transfection or when also delivered to cellular aggregates (2D+3D transfection; 3D: three-dimensional). For the first time, the effect of anti-miR-221 (10 nM) was investigated in a developmentally inspired microtissue model where it was found to support type II collagen deposition (2D+3D-10 nM best group). Unexpectedly, despite miR-221 silencing at the gene level, higher dose of anti-miR-221 (20 nM) was detrimental and appeared to suppress type II collagen and increase type I collagen deposition. Such microtissue models highlight the importance of a well-defined dose and regimen, especially for further scaffold-mediated delivery. Indeed, one limitation of the 2D approach concerns the time-consuming process of pre-treating the cells and their limited translation into readily available off-the-shelf scaffold products [305].

The mechanisms behind the effect of miR-221 silencing are still poorly understood and, this study did not investigate it any further. Mechanisms of action could only be hypothesised from the current literature. Although *SOX9*, *COL2A1* and *ACAN* have all been hypothesised to be direct targets of miR-221 [33], in hMSC monolayers not otherwise exposed to chondrogenic factors, these genes were not detectable despite significant silencing of miR-221. Elucidation of the mechanisms

underpinning the action of anti-miR-221 would contribute to a better understanding of its potential therapeutic use.

In both studies, altering oxygen levels or anti-miR-221 transfection (**Chapter 4** and **Chapter 6**), no signs of type X collagen or calcium accumulation were observed. As MSCs have the inherent tendency to undergo hypertrophy [222], long-term studies are needed to confirm whether this tendency is delayed or at best suppressed.

7.1.3. Recapitulating AC Organisation

While single microtissues have shown improved chondrogenesis under altered oxygen environment, achieving cartilage zonal features in terms of biochemical gradients and structural organisation is critical to restore the function of AC [90]. Phenotypically distinct individual microtissues primed under altered oxygen environments were used as biological units to engineer the surface and deep regions of AC. The self-assembly process of individual microtissues typically supports the engineering of scaled-up grafts lacking controlled geometry and hierarchical organisation [225]. To address these issues, a new modular assembly method was successfully developed. Microtissues from different populations were (alone (single-layered) or sequentially (bilayered)) seeded onto a guiding MEW mesh, maintained in place in a custom-made agarose well. The presence of the MEW mesh contributed to controlling the geometry of the hybrid graft, whilst the very high specific surface area of the scaffold minimised the presence of polymeric material. Within the MEW mesh, collagen fibres aligned parallel to the surface similar to the surface organisation of native tissue, whilst hanging the tissue from the MEW mesh enhanced the coherency of fibre orientation. In addition, hanging the scaffold from the MEW mesh improved the organisation of the collagen fibres through the depth of the engineered construct, showing some level of biomimicry. Increased spatial organisation was then observed, but measurements of the mechanical forces deployed inside such developing scaled-up tissues, by the presence of the MEW mesh and/or from hanging the tissue, was lacking in the study. Mechanical cues have been shown to regulate chondrogenesis of MSCs [306], and understanding its extent in this novel hybrid system is highly valuable for engineering functional AC. In addition, mechanical stability of such biomimetic graft is yet to be confirmed.

Despite initial spatial patterning (in a bilayered manner), the organisation of sequentially seeded distinct microtissue population was lost during the differentiation period. While, microtissues seeded onto the mesh are believed to stay anchored and

not to mix with others [143], future studies using fluorescent cell labelling or similar could be used to evaluate the long-term spatial positioning of the different microtissue populations within the scaled-up graft. A short oxygen priming in altered levels was shown to enhance the GAG production in single hMSC microtissues. However, GAG staining in bilayered grafts was homogeneous, highlighting a major limitation of the short oxygen priming approach. Despite large scaffolds developing an oxygen gradient, oxygen levels are poorly estimated and controlled [307]. As an alternative to oxygen priming, novel oxygen delivery systems, and/or hypoxia mimicking factors [197], could be incorporated into such constructs to generate an environment more favourable to stable chondrogenic differentiation.

Results from this thesis have demonstrated enhancement of the chondrogenic potential of human-derived microtissues to promote their usefulness in engineering AC and *in vitro* tissue models. In addition, the fabrication of scaled-up grafts using a MEW mesh-bottomed well developed in this thesis sets the basis of novel and promising modular assembly methods.

7.2. Conclusive Remarks

- A coculture of hACPs and hMSCs can support cartilage tissue development with increased GAG and type II collagen deposition compared to hACPs alone. However, ACP-only aggregates failed to support robust chondrogenesis, which challenges their usefulness as a potential alternative cell source to MSCs.
- Altering oxygen to levels representative of the oxygen gradient of native AC supports differential chondrogenesis in hMSCs, with the resulting microtissues displaying key features of different regions of hyaline cartilage. In such microtissue models, short-term exposure to lower oxygen levels improves GAG deposition compared to continuous exposure to lower oxygen levels.
- A novel modular assembly method using a guiding MEW mesh supports the fusion of single hMSC-derived microtissues while controlling the geometry of large scaled-up grafts. In addition, the spatial organisation of the collagen network of these hybrid grafts shows some level of biomimicry. Sequential incorporation of different

populations of oxygen-primed microtissues into such MEW scaffolds supports an initial layered organisation within the engineered graft.

- Poly-beta amino esters mediated delivery of anti-miR-221 is highly efficient and minimally cytotoxic to hMSCs. Such polyplexes successfully enhance chondrogenesis of hMSC-derived microtissues, specifically the deposition of type II collagen, a key component of hyaline AC. The resulting differentiation is particularly dependent on the careful selection of the transfected dose. Silenced microtissues are also capable of fusing into scaled-up grafts with no signs of early cartilage hypertrophy.

8. Future Outlook

This thesis presents several tissue engineering approaches to leverage the potential of human mesenchymal stem/stromal cells (hMSCs)-derived cartilage microtissues as biological building blocks for the biofabrication of larger articular cartilage (AC) grafts. MiR-221 silencing successfully enhanced the production of type II collagen within such microtissues, while relatively low levels of glycosaminoglycans (GAG) synthesis is still a concern. The positive effect of lower oxygen levels on cartilage-specific extracellular matrix (ECM) production, particularly on GAG synthesis, in anti-miR-221 transfected microtissues, could be studied to overcome this limitation. Besides, one question remains on the ability of these approaches to truly sustain a stable hyaline phenotype and address the challenging intrinsic tendency of hMSCs to undergo hypertrophy and support endochondral bone formation. Thus, long-term *in vivo* studies are required to validate such approaches. Particularly, this could be achieved by sequentially seeding transfected microtissues, to engineer hyaline cartilage, and non-transfected microtissues, to engineer hypertrophic cartilage, into melt electrowritten (MEW) scaffolding structures. Preliminary work on the MEW mesh-mediated modular assembly showed potential to guide the organisation of scaled-up AC grafts. Superior efficiency and reliability could result from the implementation of bioprinting and/or robotic bioassembly approaches [308, 309]. Finally, spatially controlled oxygen delivery and/or hypoxia mimicking systems could be implemented to leverage the potential of altered oxygen levels to recapitulate the biochemical gradient of native AC into scaled-up engineered grafts.

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