Stem cells derived from adipose tissue and umbilical cord blood for cartilage tissue engineering in scaffold cultures

PhD thesis

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University of Aarhus

2013

Stem cells derived from adipose tissue and umbilical cord blood for cartilage tissue engineering in scaffold cultures

Experimental cell studies

PhD thesis

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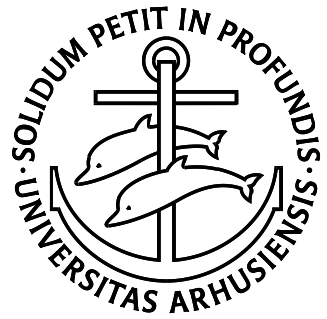

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Life can only be understood backwards, but it must be lived forwards – just like a PhD.

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Preface

This thesis is based on experimental studies performed at the Orthopaedic Research Laboratory, Department of Orthopaedics, Aarhus University Hospital, Denmark in collaboration with the Interdisciplinary Nanoscience Center (iNANO), Aarhus University; the Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Canada; and the Department of Biomedical Sciences, Guelph, Canada. I carried out the studies during my enrolment as a PhD student at the Faculty of Health Sciences, Aarhus University during 2008-2012.

My sincerest thanks go to my main supervisor, Kjeld Søballe, MD, Prof., for introducing me to the field of orthopaedic research and for providing great research facilities. I also owe special thanks to my project supervisor Thomas Gadegaard Koch, DVM, PhD. Your always timely precision, dedication, enthusiastic approach, encouragement and support have taught me many perspectives of research and in the end made this thesis possible. I would also like to thank our highly skilled laboratory technicians Anette Baatrup, Jane Pauli, Anna Bay Nielsen and Lisa Feng for their expertise and their continuous help.

Doing a PhD is not a simple task – many times you face issues you did not even know existed. That is why I am especially thankful to all those people who were not directly involved in my PhD. Thanks to all co-workers for creating a great working environment where we have generously supporting one other's struggles. Mette Sørensen, Kasra Zainali, Jørgen Baas, Casper Foldager, Muwan Chen and Ryan Figueroa, thanks for all the personal and moral support, for becoming friends and for taking care of the "new guy in town". Mikkel Albrecht Auning Hansen, Thomas Alexander Hoelgaard, Ea Voergaard, Anders Barasa, Gobind Kalsi, Jane Fjermestad, Johannes Korfits Møller, Sara Gilling, Dang Le, Henrik Saksø, Jamal Butt, Tanja Lykke Schmidt, Annemette Palm, Louise Thomsen, Stine Lisa Sandø, Ditte Pedersen and Andreas (dr. squash) Lambert, my deepest thanks go to you guys for encouraging me, coaching me in squash and pulling me out for kitesurfing (or, rather, ice swimming), acting as movers and reminding me to keep focused – basically thanks for being there for me. Finally, I would like to thank my parents Ahmed and Parveen Munir and my brother Amer Munir for your unconditional support, love and patience.

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Samir Munir

This PhD thesis is based on the following papers:

I. Hypoxia Enhances Chondrogenic Differentiation of Human Adipose Tissue-Derived Stromal Cells in Scaffold-Free and Scaffold Systems. **Munir S,** Foldager C B, Lind M, Zachar V, Soballe K, Koch T G (Accepted, Cell Tissue Research).

II. Culturing in Reduced Oxygen Enhance Chondrogenic Differentiation of Human Cord Blood Multilineage Progenitor Cells in Structurally Graded Scaffolds of Polycaprolactone. **Munir S,** Figueroa R, Le D Q S, Foldager C B, Nygaard J V, Soballe K, Koch T G (In 2nd revision, PlosOne)*.

III. Collagen-Coated Polytetrafluoroethane Membrane Inserts Enhances Chondrogenic Differentiation of Human Cord Blood Multilineage Progenitor Cells. **Munir S,** Lee W D, Soballe K, Koch T G (In 2nd revision, Tissue Engineering)**.

The papers will be referred in the text by their Roman numerals (I-III).

* Presented at the meeting for The Nordic Orthopaedic Federation, Aarhus 2010 and the annual meeting for The Canadian Connective Tissue Conference, Toronto 2010, and at the autumn meeting for "The Danish Orthopaedic Society", Copenhagen 2011.

** Presented at the autumn meeting for "The Danish Orthopaedic Society", Copenhagen 2012.

Contents

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACI	Autologous chondrocyte implantation
ANOVA	Analysis of variance
AT-MSC	Adipose tissue-derived mesenchymal stromal/stem cell
BM-MSC	Bone marrow-derived mesenchymal stromal/stem cell
BMP	Bone morphogenetic protein, a group belonging to the TGF-β superfamily
CDM	Cartilage derived matrix
CD-RAP / MIA	Cartilage-derived retinoic acid-sensitive protein / Melanoma inhibitory activity
CB-MSC	Cord blood-derived mesenchymal stromal/stem cell
CPP	Calcium polyphosphate
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified essential medium
DMEM-HG	Dulbecco's modified essential medium high glucose
DMMB	Dimethylmethylene blue
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FD	Freeze-dried
FDA	U.S. food and drug administration
FD-PCL	Freeze-dried polycaprolactone
FDM	Fused deposition modelling
GUSB	β-glucuronidase, a protein involved in vital cellular processes
h	Human
H&E	Haematoxylin and eosin
HKG	Housekeeping gene
ITS	Insulin-transferrin-selenium
MACI	Matrix-assisted autologous chondrocyte implantation
MLPC	Multilineage progenitor cell
MMA	Methylmethacrylate
MPa	Mega pascal
MSC	Mesenchymal stromal/stem cell
OA	Osteoarthritis
PBS	Phosphate-buffered saline

PCL	Polycaprolactone
PX	Cells of X^{th} passage
qPCR	Quantitative polymerase chain reaction
Runx2	Runt-related transcription factor-2, essential for osteoblast differentiation and ossification
SDS	Sodium dodecyl sulfate (soap)
SEM	Scanning electron microscopy
sGAG	Sulphated glycosaminoglycan
SGS	Structurally graded scaffold
SMA	Smooth muscle actin
SOX9	SRY (sex determining region Y) box 9
TBP	TATA-box binding protein, a general transcription factor
TE	Tissue engineering
TB	Toluidine blue
TIPS-FD	Thermally induced phase separation combined with freeze-drying
TGF-β	Transforming growth factor-β
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, a protein involved in vital cellular processes

English summary

By nature, cartilage is a complex tissue that has limited repair potential. Cartilage injuries often progress to osteoarthritis, which has a major socioeconomic impact in the form of costs to society and disability in the individual. At present, treatment options are unable to eliminate the long-term consequences of cartilage injuries. During the past decade, researchers in the field of tissue engineering have worked intensively on developing technologies to produce artificially made tissues. Tissue engineering combines cells, growth factors and biologically compatible scaffolds to stimulate and enhance the production of tissue. Stem cells are particularly appealing in tissue engineering because harvesting is primarily done non-invasively from various organs. However, tissue-specific differentiation is often needed before the harvested stem cells may be used in the clinic. This is a complex task that currently limits their clinical application.

This PhD project initially focused on the chondrogenic differentiation of stem cells with the aim to produce a cartilage implant.

Two sources of stem cells were investigated.

- Primary cultures of human adipose tissue-derived stem cells.
- Monoclonal umbilical cord blood-derived stem cells.

Stem cells were used to test three hypotheses:

1. Reduced oxygen tension increases chondrogenesis of adipose tissue-derived stem cells in collagen type I/III scaffold cultures.
2. Reduced oxygen tension increases chondrogenesis of human umbilical cord blood-derived stem cells in pellet and scaffold cultures of polycaprolactone.
3. Three-dimensional culture on semipermeable membranes and ceramic calcium polyphosphate increases chondrogenesis of human umbilical cord blood-derived stem cells.

Histology, gene expression analysis, quantification of cartilage-specific proteins and the novel marker for cartilage anabolism cartilage-derived retinoic acid-sensitive protein (CD-RAP) were assessed as outcome parameters.

The overall results demonstrate that stem cells display enhanced chondrogenesis when differentiated during reduced oxygen tension. Chondrogenesis of mesenchymal stromal cells (MSCs) is significant when differentiation is carried out in three-dimensional (3D) scaffolds. However, a zonal configuration similar to that of native cartilage was less pronounced in tissue-engineered neocartilage. I observed the most

pronounced cartilage differentiation in high-density culturing on semipermeable membranes.

Future studies should aim to optimise chondrogenic differentiation of MSCs in standard cultures, e.g., by customising the supplement of different growth factors to yield a well-differentiated stem cell prior to culturing in 3D systems.

Danish summary

Brusk er et komplekst væv med ringe helingsevne. Bruskskader progredierer ofte til slidgigt, hvilket har store socioøkonomiske konsekvenser for samfundet og helbredsmæssige konsekvenser for det enkelte individ. På nuværende tidspunkt er man behandlingsmæssigt ikke i stand til at eliminere langtidsfølgerne. Forskere inden for feltet *tissue engineering* har i det sidste årti arbejdet intensivt på at udvikle teknologier til at lave kunstigt fremstillet væv. *Tissue engineering* kombinerer celler, vækstfaktorer og biologisk kompatible materialer (skaffolder) til at stimulere udviklingen af væv under kunstige forhold. Stamceller har en særlig interesse, da de nemt og relativt harmløst kan udvindes fra forskellige organer. Udfordringen er dog, at cellerne før deres kliniske anvendelse skal stimuleres med henblik på at blive modnet (differentieret) til det ønskede væv, herunder brusk.

Initialt har dette ph.d.-projekt haft fokus på at udvikle et implantat ved at differentiere stamceller til brusk (chondrogenese). Der er forsket i to typer stamceller:

- Stamceller udvundet som primær kultur fra humant fedtvæv
- Monoklonale stamceller fra humant navlestrengsblod

Stamcellerne er anvendt til at undersøge tre hypoteser:

1. Reduceret iltspænding øger chondrogenesen af stamceller fra fedtvæv under dyrkning i collagen I/III baserede scaffolder.
2. Reduceret iltspænding øger chondrogenesen af stamceller fra navlestrengsblod ved dyrkning på et porøst polymer scaffold baseret på polycaprolactone.
3. Tredimensional dyrkning på semipermeable membraner samt keramisk calcium polyfosfat øger chondrogenesen af stamceller fra navlestrengsblod.

De målte effektparametre inkluderer histologi, genanalyser, kvantificering af bruskproteiner, samt måling af en relativt ny markør for bruskanabolisme CD-RAP.

Resultaterne viser overordnet, at de anvendte stamceller under reduceret ilt differentieres i chondrogen retning. Differentiering er mest markant, hvis cellerne dyrkes på 3-dimensionelle scaffolder. Dog har det under de anvendte forhold ikke været muligt at opnå den samme zonale lagdeling af væv som ved naturligt brusk. Den bedste bruskdifferentiering ses ved at dyrke stamceller i høj densitet på semipermeable membraner.

Ph.d.-projektets undersøgelser viser også, at fremtidige forsøg bør fokusere på at optimere bruskmodningen af stamcellerne i standardkulturbetingelserne, fx ved at

skræddersy brugen af flere forskellige vækstfaktorer, så man har en veldifferentieret stamcelle inden, der overgås til dyrkning i scaffolder.

1 Introduction

Synovial joint cartilage injuries are highly prevalent and once the cartilage has been injured, the condition is chronic. A significant amount of adults and young adults suffer from chondral defects due to trauma and sports-related injuries. This requires medical attention. Cartilage is an avascular tissue incapable of regeneration and for this reason, among others cartilage defects often progress to osteoarthritis (OA), which has socio-economic as well as health-related consequences [1, 2]. Pain, decreased mobility and decreased activity of daily living are major claims in patients suffering from OA.

Surgical treatment relies on cellular activation or transplantation of cells capable of healing chondral defects [1, 3]. As cells that occupy the cartilage matrix, chondrocytes have precisely this potential. They can be isolated from mature tissue and cultivated *in vitro* on scaffolds to produce neocartilage for implantation. However, problems associated with this approach include (a) few tissue harvest sites, (b) tissue donor site morbidity, (c) limited *in vitro* expansion potential and (d) dedifferentiation of the cartilage-specific phenotype [4, 5]. Furthermore, these established techniques are unable to produce repair tissue with the same characteristics as native hyaline cartilage and OA is therefore often the final stage [6].

Tissue engineering through the combination of cells, scaffolds and growth factors is a promising strategy for repairing articular cartilage. However, due to the limitations mentioned above, autologous chondrocytes are not an optimal cell source.

MSCs from tissues such as bone marrow, adipose tissue and umbilical cord blood have been shown to contain cells capable of chondrogenic differentiation when subjected to appropriate culturing conditions [7-10]. The problems associated with the use of mature chondrocytes may be avoided by the use of MSCs for cartilage tissue engineering. However, it remains unclear how specific signals coordinate to promote the differentiation and maturation of MSCs into articular chondrocytes. Proposed signals and conditions that stimulate chondrogenesis are numerous and include (a) high-density seeding, (b) the biomechanical properties of the scaffold, (c) mechanical stimulation, (d) supplementation of various growth factors to the culture medium, (e) the supply of nutrients, (f) the shape and morphology of the cell and (g) surface interaction between the cell and the biomaterial and (h) culturing in reduced oxygen tension.

Within this context, this PhD project focused on how to differentiate MSCs into cartilage tissue to produce a cartilage implant.

2 Aim, hypothesis and outline of the thesis

The objective of the present thesis was to produce cartilage constructs with the aid of MSCs and scaffolds. I evaluated the chondrogenic differentiation of two sources of MSCs and examined the inducing effect of culturing in reduced oxygen tension (hypoxia) within scaffolds.

This PhD thesis is based on testing the following three hypotheses:

1. Reduced oxygen tension increases the chondrogenesis of adipose-derived stem cells in collagen type I/III scaffold (Chondrogide®) cultures (I).
2. Reduced oxygen tension increases the chondrogenesis of human umbilical cord blood-derived stem cells in pellet and scaffold cultures of porous polycaprolactone (II).
3. A three-dimensional culture on semipermeable membranes and ceramic calcium polyphosphate increases the chondrogenesis of human umbilical cord blood-derived stem cells (III).

The present thesis is structured as follows: the Background Section provides a brief overview of (a) the biology of cartilage, (b) types of cartilage injures and their treatment, (c) tissue engineering strategies, (d) cells that may be used for cartilage tissue engineering, and (e) a brief review of recent literature on hypoxic culturing and mechanical stimulation of MSCs. The Material and Methods Section summarises the applied methods: detailed descriptions are presented in Papers I-III. The results are presented individually. The Discussion Section aims to relate findings in the studies to the current literature. The Conclusion Section presents a general conclusion and offers suggestions for future research. Major differences between the studies are outlined in Table 1, p. 23.

3 Background

3.1 Hyaline cartilage biology

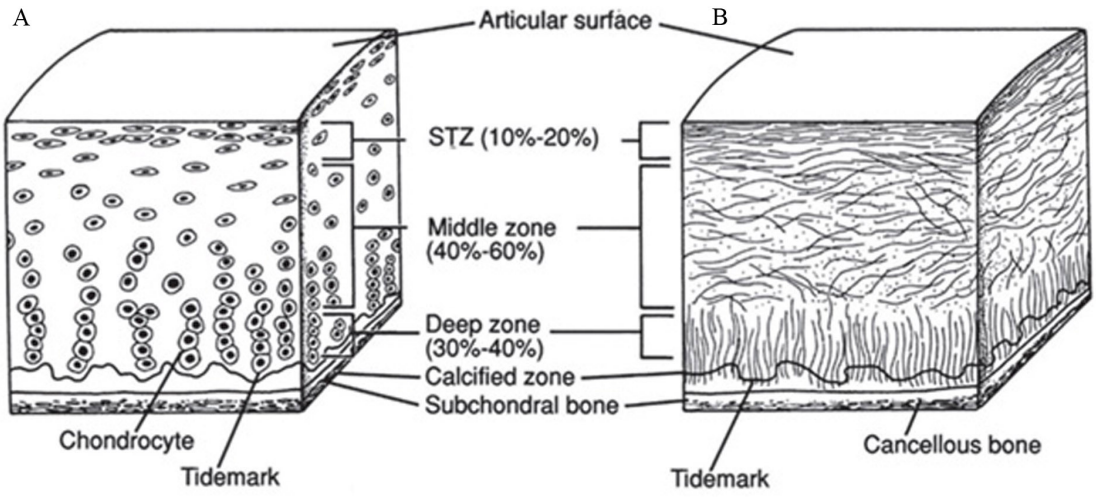

Figure 1 Schematic diagram of the zonal chondrocyte (A) and collagen fibre (B) organization in the three main zones of the hyaline articular cartilage. Adapted from Buckwalter et al, 1994 [11].

Hyaline articular cartilage is located in weight-bearing joints. Its function is to reduce friction between two opposing bones and to support and distribute loads, thereby protecting the underlying bones from any pressure that could cause damage. Synovial fluid fills the open cavity between the bones and reduces the frictional forces over the cartilage which aids the ease with which the joint is moved. The tissue consists of chondrocytes surrounded by an extracellular matrix (ECM) that consists of water, proteoglycans and collagens (primarily collagen type II) [12]. The cells are responsible for tissue turnover, whereas the ECM ensures tissue function. Cartilage is avascular and tissue maintenance and nourishment therefore depends on (a) passive diffusion of fluid through the cartilage, (b) compressive mass-transport during motion of the joint and (c) fluid-induced shear across the surface of the joint [12]. The avascularity also contributes to the limited regenerative ability of articular cartilage [13].

The complex organisation of cells and ECM components in cartilage is responsible for its mechanical properties. Cartilage is not a homogeneous tissue; rather, it can be divided into four zones: (a) the calcified cartilage zone/tidemark, (b) the deep zone, (c) the intermediate zone and (d) the superficial zone. This zonal classification is based upon differences in the size, shape, number and distribution of (a) chondrocytes, (b) proteoglycans and (c) collagens (Figure 1). Adjacent to the subchondral bone is the calcified cartilage zone, which serves as the boundary between cartilage and bone. The collagen fibres are longitudinally oriented and anchor the cartilage to the underlying

bone. The deep zone (≈30% of the total thickness) contains mostly proteoglycan with firmly packed collagen fibres orthogonally oriented to the surface, and relatively little water. In this zone, chondrocytes are aligned in columns. The intermediate zone (≈40-60% of the total thickness) is located between the surface and the deep zone and has less proteoglycan, and collagen fibres are randomly oriented. The superficial zone (≈10-20% of the total thickness) is exposed to the synovial fluid flow, has the least amount of proteoglycan and has densely packed collagen fibres that are organised into layers parallel to the articular surface which provides tissue wear-resistant protection. The cells here are oval in shape and are aligned tangentially to the articulating joint surface [1, 12, 14].

Chondrocytes are responsible for the synthesis, secretion and organisation of collagen, glycosaminoglycan (GAG), proteoglycan (primarily aggrecan) and hyaluronan composing the ECM surrounding them (Figure 2) [15]. The high density of matrix proteins protects the chondrocytes from damage due to mechanical forces when cartilage is under loading.

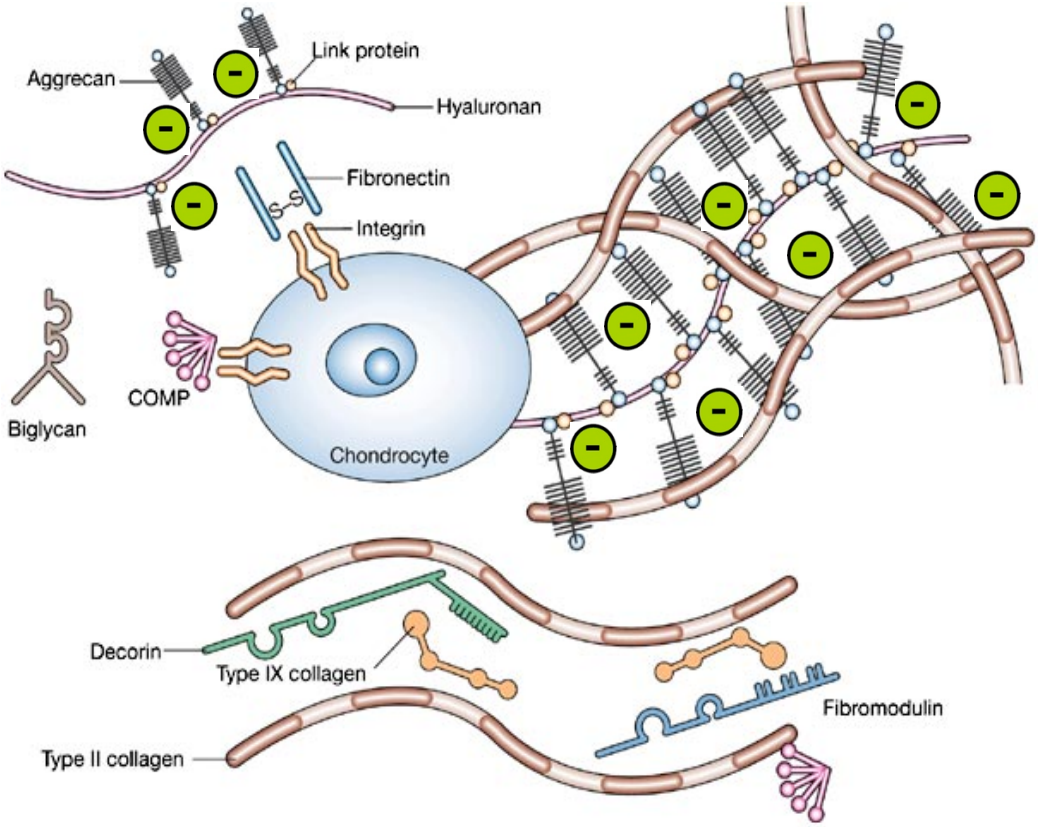

Figure 2 Schematic presentation of the ECM in articular cartilage. Three classes of proteins exist in articular cartilage: collagens (mostly collagen type II); proteoglycans (primarily aggrecan); and other noncollagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein) and the smaller proteoglycans (biglycan, decorin and fibromodulin). The interaction between highly negatively charged cartilage proteoglycans and collagen type II fibrils is responsible for the compressive and tensile strength of the tissue. Abbreviation: COMP, cartilage oligomeric matrix protein. Modified from Chen et al, 2006 [15].

Proteoglycan plays a crucial role in the stiffness, hydration and pore size of cartilage. Aggrecan is the primary proteoglycan found in articular cartilage. Smaller amounts of other proteoglycans such as biglycan, decorin and fibromodulin are also present in the ECM. Aggrecan is a core protein to which GAG chains attach to form a bottlebrush-like formation. Negatively charged GAGs (primarily keratin and chondroitin sulphate) are covalently linked to the core protein, while the end-terminal regions link to the non-sulphated GAG hyaluronic acid. When cartilage is compressed, the resulting high concentration of negatively charged proteoglycan provides resistance to loading and adds to the stiffness [16]. The proteoglycans also provide the tissue with osmotic pressure swelling drawing back in the water. However, collagen fibres intermingling the ECM prevent free swelling. Thus, proteoglycan helps the cartilage expand to its original shape after compression.

Collagen type II is the most abundant type of collagen in articular cartilage and its function is to make the tissue able to resist tensile and shear forces [16]. Small amounts of collagen type V, VI, IX, X and XI are also present in cartilage [14, 16]. Aggrecan and collagen type II are markers for differentiated chondrocytes. The presence of collagen type I or X is indicative of dedifferentiated or phenotype modulated

chondrocytes producing fibrocartilage or hypertrophic cartilage [1, 17]. Other markers of early chondrogenesis include gene expression of the transcriptional factor SOX9, secretion of CD-RAP and the transforming growth factor (TGF) activity modulator Delta Like-1 factor (Dlk1) [18-22].

3.2 Cartilage injuries and treatment

Cartilage defects are highly prevalent, and impairment caused by this condition has a severe socioeconomic impact. Due to inconsistency in inclusion criteria accurate incidence of cartilage injuries are unavailable but it is estimated that 16% to 60% of patients admitted to knee arthroscopic procedures have severe articular cartilage defects/injuries ranging from 0.5 to 12 cm^2 [23-25]. As previously mentioned, cartilage is an avascular tissue incapable of regeneration. Non-surgical therapies solely provide palliative pain relief and do not halt or reverse tissue damage.

Surgeons treat small-sized chondral lesions with a variety of proposed cartilage-stimulatory techniques, e.g., abrasion arthroplasty, subchondral drilling and microfracture. These procedures are widely used, arthroscopically easy to perform, low-cost and with minimal risk for the patient. However, the repair tissue does not obtain the same characteristics as native hyaline cartilage [6]. Autologous chondrocyte implantation (ACI) and related techniques are two-step procedures that are often used to treat medium- and large-sized chondral lesions. Cartilage from non-weight-bearing areas is first harvested and multiplied in the laboratory and then implanted into the cartilage defect. ACI is widely used in Europe and the USA with overall acceptable results [26, 27]. MACI is an enhanced ACI technique [28] in which in vitro-expanded chondrocytes are embedded in a supportive three-dimensional (3D) scaffold and cultured prior to implantation. The theoretical advantages of MACI over ACI are: (a) a more homogeneous distribution of cells (inhibiting leakage), (b) a less invasive surgical procedure, (c) reduced periosteal hypertrophy, (d) decreased post-operative adhesions and (e) enhanced maintenance of the chondrocyte phenotype in scaffolds [29]. However, studies explicitly supporting these benefits are lacking. Though ACI and MACI have shown good clinical results, these techniques also have disadvantages such as donor site morbidity [30]. Furthermore, chondrocytes harvested from joints are limited in number and proliferates slowly *in vitro*, which increases the time needed between chondrocyte harvesting and re-implantation; *in vitro* expansion is also associated with chondrocyte phenotype modulation, causing loss of phenotype [1, 17, 30-32]. In severe chondral lesions or end stage OA, a joint prosthesis is often the only treatment that can relieve the patient of his or her symptoms [33]. However, a joint prosthesis may not be a life-long solution, especially not in younger patients.

In summary, the limited intrinsic healing capacity of cartilage and the lack of effective treatments constitute significant health problems with economic and social implications. Current treatments have achieved acceptable clinical results, but the repair tissue never attains the same characteristics as native hyaline cartilage. Furthermore, some treatment options directly induce secondary cartilage lesions. A low proliferation rate and significant issues of phenotype modulation limit *in vitro* culturing of chondrocytes. Alternative cell sources and technologies promoting the development of robust and functional cartilage tissue are thus being intensively investigated [3]. MSCs are such a source of cells with the capability of chondrogenic differentiation and simplicity of isolation. Please refer to Section "3.3.3 Cells sources for cartilage tissue engineering" for further elaboration.

3.3 Cartilage tissue engineering approaches

3.3.1 Concept of tissue engineering

Tissue engineering (TE) is a relatively new field that utilises cells, biocompatible materials, suitable biochemical (e.g., growth factors) and physical (e.g., cyclic mechanical loading, hypoxic culturing) factors as well as combinations of such factors to create tissue-like structures (Figure 3) [34]. The primary objective of tissue engineering cartilage is to induce and promote complete regeneration of native articular cartilage.

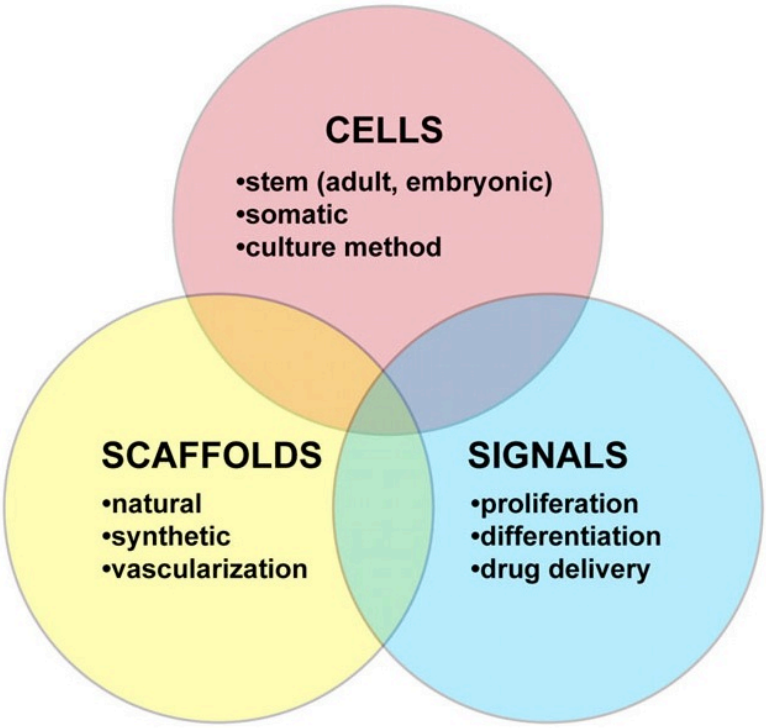

Figure 3 Schematic presentations of the three important factors involved in tissue engineering. Adapted from Ohba et al, 2009[35].

The challenge in tissue engineering is to identify the appropriate cell source, identify biocompatible materials and expose the cells to an optimal culture environment to produce and maintain healthy, functional cartilage. Figure 4 outlines the concept and flowchart of tissue engineering. Cells are first isolated and expanded *in vitro* to achieve a sufficient number of cells. Cells are then seeded on a biocompatible material (e.g., scaffold) together with a variety of growth and stimulatory factors and cultured in an appropriate bioreactor. Finally, the engineered tissue can be transplanted to the defect to restore its function. Figure 4 demonstrates the interdisciplinary approach of tissue engineering in which the centre of rotation is to expose cells to a multifaceted environment to mimic the *in vivo* native tissue, e.g., to provide scaffolds that mimic ECM to maintain the cell phenotype, to supplement the culturing medium with growth factors and to provide scaffolds that allow appropriate 3D alignment of cells.

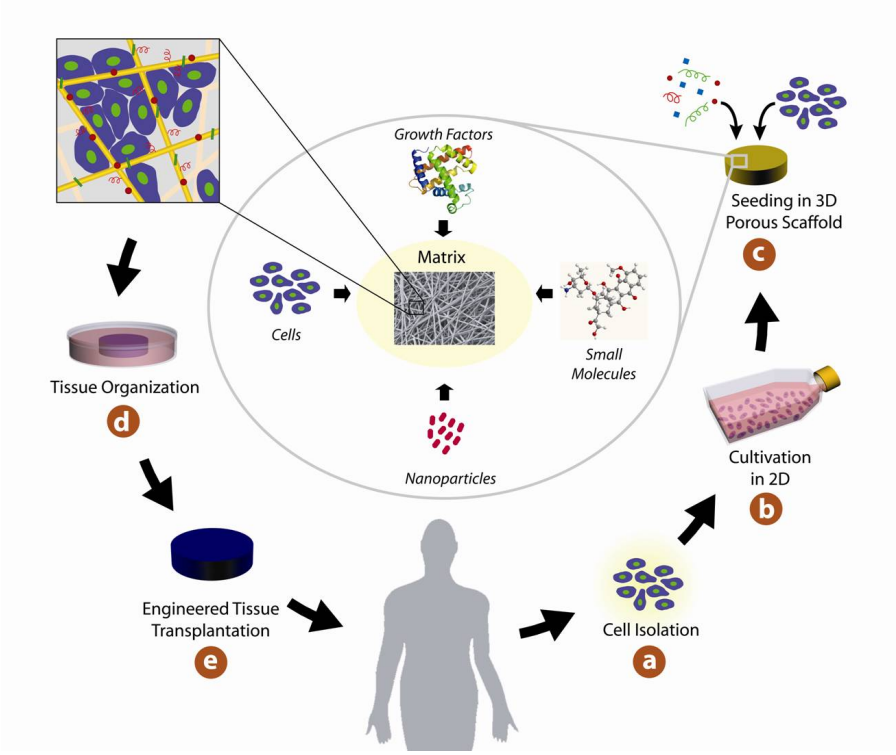

Figure 4 The concept and flowchart of tissue engineering. Adapted from Dvir et al, 2010 [36].

3.3.2 The role of scaffolds for cartilage tissue engineering

Scaffold requirements

Scaffolds function to provide cells with an environment for growth and differentiation and should ideally meet the following requirements:

1. Be non-toxic, biocompatible and biodegradable.
2. Allow cell attachment, proliferation and migration, and deposition of ECM.
3. Possess a porous structure to allow adequate nourishment.
4. Have adequate mechanical properties that mimic compressive stiffness and elasticity of *in vivo* cartilage.
5. Allow retention and presentation of biochemical factors (e.g., growth factors, ECM proteins).

Scaffold composition and fabrication techniques

Hydrogels of collagen type I and II [37, 38], agarose [39, 40], alginate [41, 42], hyaluronan [43] and polyethylene glycol (PEG) [44] have been used to encapsulate cells within a 3D environment and they all support the rounded morphology of the chondrocytic phenotype. However, studies demonstrating stable, long-term cartilage growth are lacking. Although hydrogels allow accumulation of ECM, their clinical usability is limited by lack of compressive stiffness and uneasy handling.

Recent technological advances have made the production of highly customised scaffolds possible and have made it possible to produce scaffolds with the above-mentioned properties. These techniques include fused deposition modelling (FDM) [45, 46], thermally induced phase separation combined with freeze-drying (TIPS-FD) [47] and electrospinning [48, 49]. Scaffolds fabricated by (a) FDM and (b) FDM combined with TIPS-FD were used in Study II.

A variety of materials can be utilised in the fabrication of scaffolds, e.g., natural polymers (collagen type I and II, chitosan, hyaluronic acid) [50, 51] or synthetic polymers such as polycaprolactone (PCL) [47], poly-lactic acid (PLA) [52, 53], poly-glycolic acid (PGA) [54] and copolymers of these two (PLGA) [55].

Scaffolds made of naturally derived materials, such as cartilage-derived matrix (CDM), have the potential advantage of biological recognition whereby inflammation is avoided and the advantage that they mimic the natural ECM environment. Their biocompatibility provides a substrate with specific ligands for cell-ECM-interaction enhancing cell-adhesion and migration, and regulates cellular proliferation and function [56, 57]. Even though natural polymers have shown promising results, drawbacks such as maintaining sterility and difficulties in controlling the batch-to-batch variation are critical issues in their applications.

Example of a scaffold made of naturally derived polymer

The FDA-approved scaffold Chondro-Gide® (Geistlich Pharma, Switzerland) is a clinically well-accepted and frequently used scaffold for ACI procedures [50, 58-62]. It is made of naturally derived collagen. Study I in this thesis made use of this scaffold. *In vitro* and *in vivo* studies have demonstrated that its structure permits cell attachment, proliferation and enrichment of the ECM which maintains the phenotype of chondrocytes, that it limits leakage of cells from the defect and that it limits migration of inflammatory proteins into the repair site [63-67]. The scaffold's matrix material consists of natural porcine non cross-linked collagen type I and type III fibres. It is a patented bilayer collagen-membrane artificially designed as to treat cartilage defects. The bilayer construction has two functions: (a) a compact surface provides mechanical strength and entrapment of cells, (b) whereas the inner porous surface provides cell-adhesive capacity (Figure 5) [29, 68-70]. Moreover, the scaffold has the additional advantage of a high tensile strength and excellent handling properties, which makes it clinically well-accepted and frequently used in ACI surgery [29, 58, 59, 68-72].

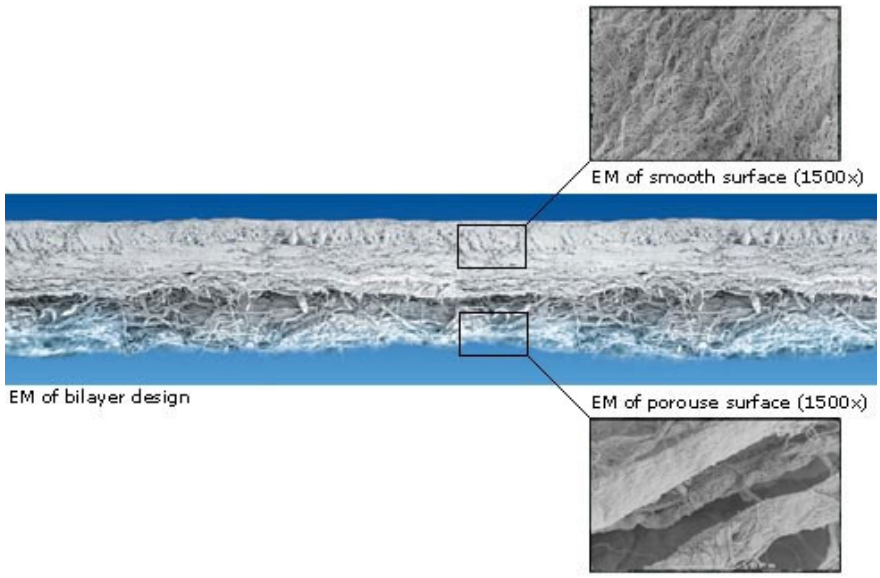

Figure 5 SEM picture of the dual-layered scaffold Chondro-Gide®. Picture adapted from Geistlich Pharma.

Example of scaffolds made of synthetically derived polymer

Scaffolds made of synthetic polymers (e.g., PCL, PLA, PLGA) have an advantage over natural polymers because they may be reproduced in large quantities with complete control of their: (a) mechanical properties, (b) microstructure (e.g., defined pore sizes), (c) degradation rate and (d) the possibility of modification and functionalisation (e.g., surface modification/coating, controlled release of incorporated growth factors/cytokines) [44, 73-75]. Studies have shown that chondrocytes attached to these polymers retain their differentiated cell function, multiply and create cartilage in the shape of the scaffold [47, 76-78]. Furthermore, some of these polymers have been approved by the American Federal Drug Agency (FDA) and are used commercially for biomedical applications, e.g., PLA is a component in absorbable sutures and stents. PCL has attracted many researchers' attention owing to its non-toxic degradation products (degraded by enzymatic hydrolysis of its ester), slow degradation rate (slower than PLA) and mechanical properties [79]. The FDA has approved PCL for use in sutures (Monocryls®) and approval for long-term implantable PCL-devices is pending. Scaffolds made of PCL have shown to maintain the chondrocytic phenotype with varying levels of success [47, 80-83]. Unfortunately, many polymers (including PCL) are hydrophobic, which diminishes cell attachment and infiltration. Additionally, without any supplementary cellular or biological component, these scaffolds lack chondro-conductive properties. Modification of the surface chemistry as well as coating the polymer surface with collagen, chondroitin sulphate, ECM/CDM, chitosan, TGF-β and bone morphogenetic protein (BMP) has been shown to improve cellular attachment to the polymer and to improve chondrogenic MSC differentiation [45, 84-92].

3D customised PCL scaffolds were fabricated with the FDM technique and used in Study II. The FDM technique is based on layered deposition of biomaterial fibre by a dispensing nozzle mounted on a robotic arm. The selected biomaterial must be floating during dispensing and keep its shape once deposited. Modification of the dispensing nozzle makes PCL handling possible for FDM. In this case, a heating element mounted on the dispensing nozzle assures the free flowing of the heated PCL which upon cooling solidifies and keeps its deposited shape. PCL fibres are deposited layer-by-layer according to a model loaded and controlled by a computer. This technique allows the fabrication of scaffolds of virtually any shape and 3D geometry. However, because production of the FDM scaffolds of PCL requires elevated temperatures, direct functionalization of the PCL fibres is not possible. For instance, loading of growth factors, such as TGF and BMP in the PCL fibre is not possible, and TGF and BMP therefore have to be added to the scaffold by other techniques at a later point in time.

3.3.3 Cells sources for cartilage tissue engineering

The chondrocyte for cartilage tissue engineering

The primary obstacle in cartilage engineering is to obtain a sufficient supply of chondrocytes to generate a functional cartilage matrix. As mentioned briefly in Section "3.2 Cartilage injuries and treatment", mature chondrocytes are presently favoured for the treatment of cartilage injuries. The chondrocyte is the native cell in hyaline cartilage, and it thus inherently possesses the specialised cell functions required to maintain a healthy cartilage turnover. However, exposing chondrocytes to an *in vitro* environments is associated with a number of shortcomings, e.g., chondrocytes exhibit low *in vitro* proliferation rates in monolayer (2D) cultures accompanied by phenotype modulation which results in a steady loss of the collagen type II and aggrecan synthesis; and harvesting of healthy cartilage is associated with donor site morbidity [1, 5, 17, 30-32, 93-98]. These outcomes may to some extent be explained by the expression and release of inflammatory cytokines, apoptosis, *in vitro* exposure to high oxygen tension, changes in cell morphology and cytoskeleton and the use of non-optimised protocols for expansion due to the choice of media and seeding density, etc.

Stem cells for cartilage tissue engineering

Recent research has therefore turned to the study of alternative sources of cells that have an inherent capacity to produce articular cartilage matrix. Such sources include, among others, multipotent mesenchymal stem cell or, more accurately, mesenchymal stromal cell (MSC). MSCs have attracted much interest as a consequence of its potential of chondrogenic differentiation and the simplicity of isolation and expansion. MSCs have been isolated from numerous tissues, e.g., bone marrow, adipose tissue, dental

cells, orbital fat, umbilical cord blood, placenta, skin, thymus and menstrual blood [7, 8, 10, 99-104].

According to the International Society for Cellular Therapies (ISCT), human MSCs must satisfy at least three important criteria to be characterised as MSC. First, they must have the ability to adhere to tissue culture flasks when cultured under standard conditions. Second, the expression or lack of expression of MSC-specific surface markers must be present, i.e. specifically the MSC population of investigation must have positive (≥95%+) surface markers for CD73, CD90 and CD105, and negative markers (≤2%+) for CD14, CD34, CD45, CD106 and HLA-DR. Finally, these cells must show multipotent differentiation potential to at least osteoblasts, adipocytes and chondroblasts (all tissues of mesodermal-mesenchymal origin) [105].

Bone marrow-derived stromal cells (BM-MSCs) are presently the cells most intensively investigated and best characterised MSC for tissue engineering of cartilage tissue, but the harvesting of BM-MSCs is, unfortunately, an invasive and painful procedure [106]. The literature is not unanimous in terms of recommending which MSC source or cell niche is optimal for cartilage tissue engineering (or other stem cell based therapies). The divergence between reports likely reflects differences in species, donor age, cell source, *in vitro* isolation and culture methods [107, 108]. In the context of the present PhD project, particular attention is addressed to adipose-derived stem cells (AT-MSCs), which were used in Study I, and to umbilical cord blood-derived stem cells (CB-MSCs), which were applied in experiments related to Study II and III.

AT-MSCs may have advantages over BM-MSCs as the harvesting procedure is cheaper, less invasive and, most importantly, yields a higher number of multipotent MSCs [109, 110]. In some studies, the chondrogenesis is reported to be superior in AT-MSCs to that of BM-MSCs [111, 112]. Other papers suggest that BM-MSCs have higher chondrogenic potential than AT-MSCs [113-115].

CB–MSCs may be even more favourable than AT-MSCs and BM-MSCs because they may be collected entirely non-invasively from new-borns. Moreover, CB-MSCs are devoid of ethical controversy and have increased self-renewal and differentiation capacity owing to a more immature phenotype compared with stem cells derived from adult tissues [8, 116, 117]. CB-MSCs have been reported to have a chondrogenic potential superior to that of BM-MSCs [118, 119].

In summary, MSCs are of special interest for cartilage tissue engineering (and other cell-based regenerative therapies) because they can be easily isolated, efficiently expanded *in vitro* and demonstrate differentiation to multiple phenotypes.

3.3.4 Biochemical induction of MSC chondrogenesis

TGF-β, BMP, dexamethasone, and insulin, transferrin and selenium (ITS) are cytokines or hormones routinely used in a so-called induction culture medium to stimulate *in vitro* MSC chondrogenesis [120-124]. These biochemical factors in general and TGF and BMP in particular are essential to trigger *in vitro* chondrogenesis of MSCs and to prevent phenotype modulation (dedifferentiation) of *in vitro* cultures of isolated chondrocytes. Furthermore, developmental studies provide evidence for the significant role of these signalling molecules in the embryonic cartilage maturation [125].

Even though other factors such as oxygen tension, scaffold characteristics, and mechanical stimulation all enhance MSC chondrogenesis, the supplementation of these exogenous biochemical to the culturing medium is a standard procedure. However, co-culturing mature chondrocytes with MSC may make it unnecessary to add exogenous growth factors to culture medium. Grässel and colleagues co-cultured cartage chips in non-direct contact with MSC encapsulated in alginate and showed secretion of TGF and BMP in the culturing medium [126].

Many researchers have focused on investigating the response of the TGF-β superfamily (e.g., TGF-β, BMP). The TGF-β superfamily modulates the chondrogenic differentiation, growth and ECM synthesis in pellet cultures of MSCs [127-131]. The TGF-β superfamily initiates a cascade of intracellular protein phosphorylation (e.g., Smad proteins) from a transmembrane receptor complex. Phosphorylated and thus activated Smad proteins interact with transcriptional factors that regulate the expression of cartilage-related genes such as SOX9, collagen type II and aggrecan [132-135]. TGF-β has been shown to regulate chondrogenesis during development [136] and to promote chondrogenic differentiation of AT-MSCs and BM-MSCs *in vitro* [108, 112, 137]. When cultured as pellet culture or hydrogels, TGF-β stimulates BM-MSC chondrogenesis by increasing collagen and proteoglycan production [108, 138]. Moreover, the addition of dexamethasone, a synthetic glucocorticoid, has been shown to enhance TGF-β stimulated differentiation [112].

3.3.5 The role of oxygen for inducing MSC chondrogenesis

The oxygen tension may be a key factor to achieve enhanced MSC chondrogenesis or to maintain the native phenotype of mature chondrocytes [9, 139-147].

Throughout embryonic chondrogenesis, hypoxia as well as various growth factors are believed to play a role in the prechondrogenic condensation of MSCs and the activation of various transcriptional factors that cause chondrogenesis of the mesenchyme, which forms a transient hyaline cartilage that is degraded and replaced by bone via a process known as endochondral ossification [125, 148, 149]. Hypoxia-inducible factor 1α (HIF-1α) is a survival factor that is activated by hypoxia and is

transported to the nucleus to target gene transcription of cartilage-related markers such as SOX9, TGF-β and collagen type II [149, 150]. A targeted deletion of the gene coding for HIF-1α results in apoptosis and inhibits chondrogenesis in AT-MSCs [149, 151]. In BM-MSCs, AT-MSCs and mature chondrocytes, hypoxic culturing results in decreased proliferation, but increased lifespan and synthesis of ECM [151-161]. It has been shown that expanding AT-MSCs in 5% O_2 initially inhibits proliferation, but subsequent culturing in chondrogenic medium increased the deposition of ECM [159, 162]. A similar pattern of enhanced chondrogenesis is reported when AT-MSCs are induced in 1%, 5% or 15% O_2 tension [142, 145]. Likewise, BM-MSCs also display enhanced chondrogenesis in 5% O_2 and 2% O_2 culturing [144, 163, 164].

However, the role of oxygen for *in vitro* MSC chondrogenesis remains a contentious issue in the literature as evidenced, among others, by conflicting reports indicating that low oxygen inhibits or decreases the chondrogenesis of MSCs [157, 165, 166].

It is believed that the positive effect of culturing MSC in reduced oxygen tension can be attributed to two factors. One explanation is that culturing in low oxygen tension mimics the low physiologic oxygen tensions observed in embryonic development of cartilage [167]. The other explanation is that a local oxygen tension of 2-10% is reported in mature cartilage and bone marrow [168-173], which would seem to suggest that chondrocytes are adapted to an environment with a low oxygen level. Krinner and colleges mathematically modelled and estimated the optimal *in vitro* oxygen tension for MSC chondrogenesis and found that 11% O_2 would be optimal [174]. A similar finding was recently reported in AT-MSCs in a study which investigated the chondrogenic potential under various oxygen tension levels [142]. This study found that the investigated AT-MSCs reached optimal chondrogenic differentiation at 15% O_2.

In summary, culturing in low oxygen tension may enhance and maintain the chondrocytic phenotype in primary chondrocytes and MSCs.

3.3.6 Biomechanical stimulation for cartilage tissue engineering

The previous sections have outlined different conditions and circumstances of significance for cartilage tissue engineering such as the importance of selecting an optimal cell source, scaffold compositions, the role of oxygen and biochemical inducers. Likewise, biomechanical stimulation in the form of hydrostatic pressure and fluid shear stress may also influence the growth and differentiation of MSCs and chondrocytes [175-177]. Compressive stimulation enhances the chondrogenic phenotype of both mature chondrocytes and BM-MSCs [178-182]. Some authors' work suggests that the combination of chondrogenic medium and dynamic compressive loading may enhance chondrogenesis of BM-MSCs even more than the addition of

exogenous factors alone [183]. Furthermore, combined low-oxygen culturing and compressive stimulation facilitates ECM deposition in BM-MSCs compared with compressive stimulation alone [67]; and low-oxygen culturing may be a more potent promoter of chondrogenic differentiation than co-stimulation with compressive loading [140]. Dynamic compressive loading increases the gene expression of chondrogenic markers SOX9, aggrecan and collagen type II, and increases the synthesis of sGAG and collagen in BM-MSCs and AT-MSCs [178, 183-186].

However, most studies evaluating the effect of mechanical stimulation apply hydrostatic pressure in their experiments and demonstrate that this gives rise to sustained chondrogenic phenotype of mature chondrocytes, enhanced chondrogenic differentiation and increased ECM synthesis of BM-MSCs and AT-MSCs [178, 180, 187-192].

4 Materials & Methods

4.1 Design overview and approach

The original plan for this PhD project was ambitious, i.e., I wanted to make an implantable cartilage-construct *in vitro* and to test it in an equine cartilage-defect model. My approach was to utilise MSCs and to differentiate the MSCs in 3D scaffolds. Unfortunately, I encountered numerous problems in the *in vitro* experiments. This is reflected in the change in cell source and scaffold composition, and eventually, no *in vivo* experiments were performed. Table 1 outlines the study setups and the major differences between the different setups.

	Study I	Study II	Study III
Hypotheses	Reduced oxygen tension increases chondrogenesis of AT-MSCs in collagen type I/III scaffold cultures	Reduced oxygen tension increases chondrogenesis of MLPCs in scaffold cultures of porous polycaprolactone	3D cultures on membranes and CPP increase chondrogenesis of MLPCs
Cell source	Human adipose tissue-derived stem cells	Human umbilical cord blood-derived stem cells	Human umbilical cord blood-derived stem cells
FBS content for cell expansion	Alfa-MEM supplemented with 10% FBS (Invitrogen, Denmark)	Low-glucose DMEM supplemented with 30% FBS (Invitrogen, Denmark)	Low-glucose DMEM supplemented with 30% FBS (PAA laboratories, Canada)
Chondrogenic differentiation medium	DMEM-HG, 0.1 µM dexamethasone, 50 µg/mL ascorbic acid 2 phosphate, 40 µg/mL proline, 1x ITS+, 10 ng/mL TGF-β3 and 1% streptomycin/penicillin	Commercial available hMSC Chondrogenic BulletKit®; Lonza, Walkersville, MD; supplemented with 10 ng/mL of TGF-β3	DMEM-HG, 0.1 µM dexamethasone, 100 µg/mL ascorbic acid 2 phosphate, 2 mM L-glutamine, 1 mM sodium pyrovate, 1x ITS+, 10 ng/mL TGF-β3 and 1% streptomycin/penicillin
Scaffold type	Collagen type I/III	Polycaprolactone	Polytetrafluoroethane (teflon) membrane

			inserts and CPP substrate
Functionalised scaffolds	No	Partly	Membranes coated with collagen type I, II and IV. CPP substrates not functionalised
Scaffold fabrication technique	Patented manufacturing procedure by Geistlich Pharma. Information not available	FDM + combination of FDM&TIPS-FD	Membranes: Produced by Millipore Corp, no further fabrication details available. CPP: solid state high-temperature particle gravity sintered
Culturing in reduced oxygen tension	Yes	Yes	No
Histology	H&E & Toluidine blue	H&E & Alcian blue	H&E & Toluidine blue
RNA extraction method	TRIzol®	GenElute™-kit	GenElute™-kit
qPCR method	Fluorescein-labelled probe	Fluorescein-labelled probe	SYBR green-based qPCR
Quantification of ECM	No	DMMB assay for sGAG and PicoGreen for DNA	DMMB assay for sGAG, hydroxyproline for collagen and Hoechst for DNA
Study collaborator	Laboratory for Stem Cell Research, Aalborg University, Denmark	Interdisciplinary Nanoscience Center, Aarhus University, Denmark	Department of Pathology and Laboratory Medicine, Toronto, Canada

Table 1 Outline of study setups and major differences between setups.

All of the three studies were designed as experimental cell studies with the primary aim to investigate the chondrogenic potential of MSCs under different conditions and scaffold systems and in the end to produce an implantable cartilage construct. During

the PhD project, I had different collaborators, which is reflected in the diversity of applied methods (Table 1 & Table 3). This involves a number of scientific shortcomings because the results of the studies cannot easily be compared.

Study I evaluated the effect of reduced oxygen culturing on the chondrogenic differentiation of AT-MSCs in pellet cultures and porous collagen type I/III scaffold cultures.

In Study II, both the cell source and scaffold was changed. Study II evaluated the effect of reduced oxygen culturing on the chondrogenic differentiation of CB-MSCs seeded on two different types of porous scaffolds based on PCL.

Study III was performed during a three-month exchange stay in the laboratory of Prof. Rita Kandel, University of Toronto, Canada. My aim in Study III was to produce a biphasic, transplantable osteochondral plug of CPP utilising CB-MSCs.

The individual studies, which are described on the following pages, start with a brief presentation of the hypothesis and design followed by a presentation of the methods applied.

4.2 Study I

Study design, aim and hypotheses

The aim of Study I was to evaluate the suitability of a collagen type I/III scaffold seeded with human AT-MSCs cultured in 5% O_2 for the *in vitro* production of cartilage-construct. I hypothesised that in reduced oxygen tension, culturing would increase the chondrogenesis of adipose-derived stem cells in collagen type I/III scaffold (Chondrogide®, Geistlich Pharma, Switzerland)) cultures. The collagen type I/III scaffold was chosen because it has previously been used for ACI procedures [29, 58, 59, 68-72], which would allow easy implementation of the scaffold for MSCs-based therapy.

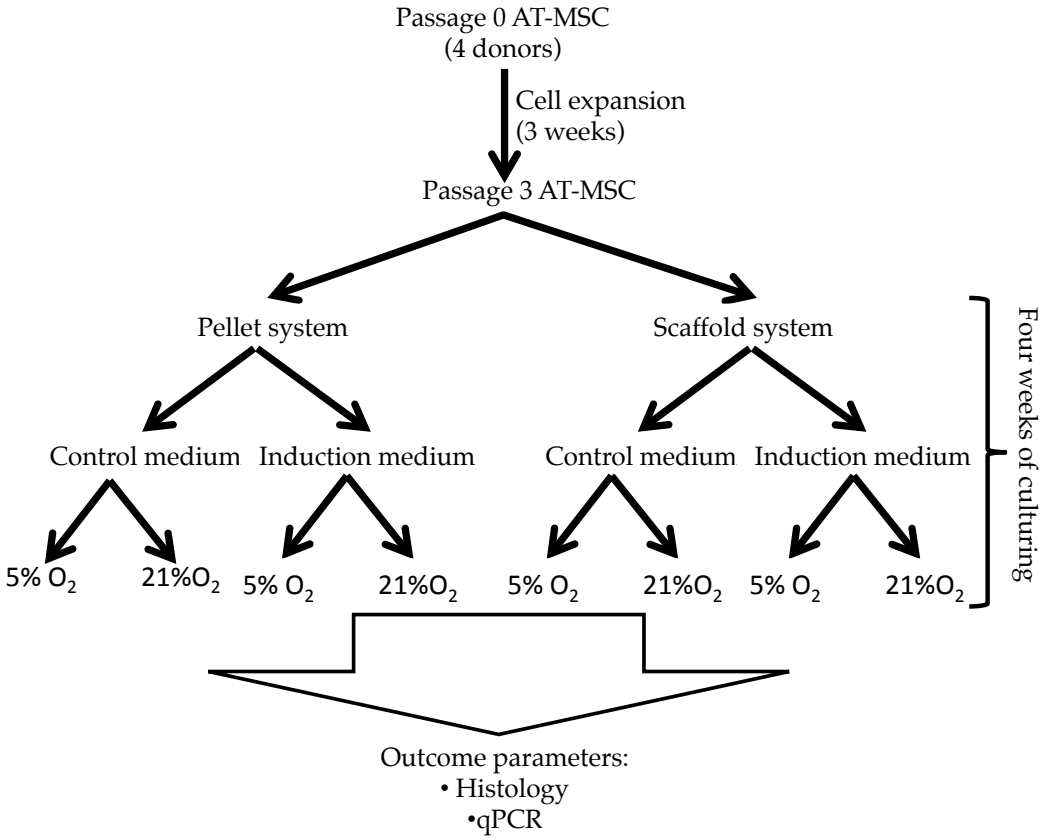

Figure 6 Schematic presentation of study design (I). AT-MSC: Adipose-derived mesenchymal stem cell.

Cells grown in 3D pellet cultures maintain their rounded cellular shape and will stop dividing and begin to express and deposit ECM proteins [193]. Differentiation (i.e., in the presence of growth-factors, etc.) in pellet cultures is thus an established standard procedure for evaluating the chondrogenic potential of specific MSC sources [193, 194].

Figure 6 is a schematic presentation of the study design. Human AT-MSCs from four donors were first expanded for three weeks and chondrogenic differentiation was then performed in standard pellet cultures or seeded on collagen type I/III scaffolds. Cell cultures were divided into subgroups as presented in Figure 6, i.e., cell cultures were divided into culturing in control vs. chondrogenic induction medium and allocated to 5% O_2 or 21% O_2 culturing. Cartilage neogenesis was evaluated by gene expression analysis of aggrecan, SOX9, and collagen type I, II and X. Histological analysis was done with H&E and toluidine blue staining.

Cells source and culturing

AT-MSCs from four donors were isolated by a slightly modified method described previously [142, 195]. Three out of the four used AT-MSCs have previous been characterized using differentiation assays and flow cytometric analysis and were found positive for MSC marker, CD74, CD90 and CD105, and negative for hematopoetic

progenitor cell marker, CD34, CD45 and HLA-DR [196-199]. This fulfils the minimum criteria of The International Society for Cellular Therapy for defining multipotent MSCs [105, 200, 201]. Adipose tissue was collected after informed consent had been obtained from three females and one male undergoing liposuction surgery at the Grymer Private Hospital (Skejby, Denmark). Subcutaneous fat from the abdomen and hips was the primary tissue source (Table 2). Tissue was digested (to release cells) with a buffer solution of collagenase containing 0.28 Wünsch U/mL crude collagenase mix (Lot no. LTQ5230; Wako, Neuss, Germany) and 20 mg/mL bovine serum albumin (BSA) (Roche Applied Science, Hvidovre, Denmark) in prewarmed Dulbecco's phosphate-buffered saline (PBS). Tissue was first digested at 37°C for 1 hour and the released cells were then separated by centrifugation at 400*g* for 10 min. Residual erythrocytes were lysed by resuspension of the pellet in 160 mM NH_4Cl and incubated at room temperature for 10 minutes following a final round of centrifugation. After this final round of purification, the cells were resuspended in dulbecco's modified essential medium-F12 (DMEM/F12) supplemented with 10% FBS and seeded at a density corresponding to 1500 cells/cm^2 and grown to 80% confluence (P0 cells). Finally, the cultures from each patient were cryopreserved for further experiments. Hence, AT-MSC cultures were not pooled inter-individually. Cryopreserved AT-MSCs were kindly provided by the Laboratory for Stem Cell Research (Aalborg University, Denmark).

For cell expansion, P0 AT-MSCs were thawed and seeded at 1000 cells/cm^2 in culturing flasks and grown to 80% confluence in a growth medium consisting of alfa-MEM (Invitrogen, Taastrup, Denmark) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Taastrup, Denmark) in a standard-humidified atmosphere containing 5% CO_2 and 21% oxygen at 37°C. The growth medium was changed twice a week. Upon reaching 80% confluence, the cells were detached by trypsinization (1.25% trypsin and 5 mM EDTA) and reseeded at 1000 cells/cm^2. After three passages, enough AT-MSCs were retrieved for the experiments. Consequently, P3 AT-MSCs were used in Study I.

Donor ID	Age (years)	Gender	BMI	Donor site
AT-MSC13	33	Female	21.6	Abdomen, hip, loin, inner thigh
AT-MSC21	52	Male	28.0	Abdomen, hip
AT-MSC23	42	Female	20.9	Inner thigh
AT-MSC24	28	Female	21.4	Abdomen, hip

Table 2 Characteristic of donors. AT-MSC: Human adipose tissue-derived mesenchymal stem cell; BMI: Body mass index.

AT-MSC chondrogenesis in pellet cultures

Pellet cultures were established by spinning 2x10^5 P3 AT-MSCs from each of the four donors separately at 500*g* for 5 minutes in V-type 96-well plates (cci3896; Corning, Schiphol-Rijk, The Netherlands). Pellets were randomly split (as outlined in

Figure 6) for culturing into either control (non-inducing) medium (alfa-MEM supplemented with 10% FBS and 1% streptomycin/penicillin) or chondrogenic-inductive culture medium (DMEM-HG supplemented with 0.1 μM dexamethasone, 50 μg/mL ascorbic acid 2 phosphate, 40 μg/mL proline, 1x ITS^{+}, 10 ng/mL TGF-β3 and 1% streptomycin/penicillin). The culturing plates containing the pellets were split into culture in either 21% O_2 or 5% O_2. Culturing in 5% O_2 was done in a designated hypoxia workstation (Xvivo System, BioSpherix, NY). Culture medium was equilibrated in 5% or 21% O_2 for two hours before change three times a week. The pellets were cultured for 4 weeks.

AT-MSC chondrogenesis in scaffold cultures

Scaffold cultures were constructed using the double-layer collagen type I/III scaffold (Geistlich Pharma, Switzerland). For a detailed description of this scaffold, please refer to the section "3.3.2 The role of scaffolds for cartilage tissue engineering" p.14. Scaffolds were cut into discs with a diameter of 6.0 mm using a disposable skin biopsy punch (Miltex, York, PA) under aseptic conditions and placed into 24-well culturing plates (one scaffold/well) prior to cell seeding. Vehicle drops of 2x10^5 P3 AT-MSCs suspended in 25 μL of growth medium (8x10^6 AT-MSC/mL) were positioned on the porous scaffold surface (Figure 5 & Figure 7A). A gentle liquid-drag method was used to ensure a uniform initial distribution of cells. Cell-seeded scaffolds were left in standard culture conditions (5% CO_2, 21% O_2 and 37°C) for two hours to allow the cells to adhere. One mL/well proliferation (non-inducing) medium consisting of alfa-MEM supplemented with 10% FBS and 1% streptomycin/penicillin was then gently added. After four days of proliferation, the cell-loaded scaffolds were randomly split into groups as outlined in Figure 6 and cultured as described above for the pellet cultures.

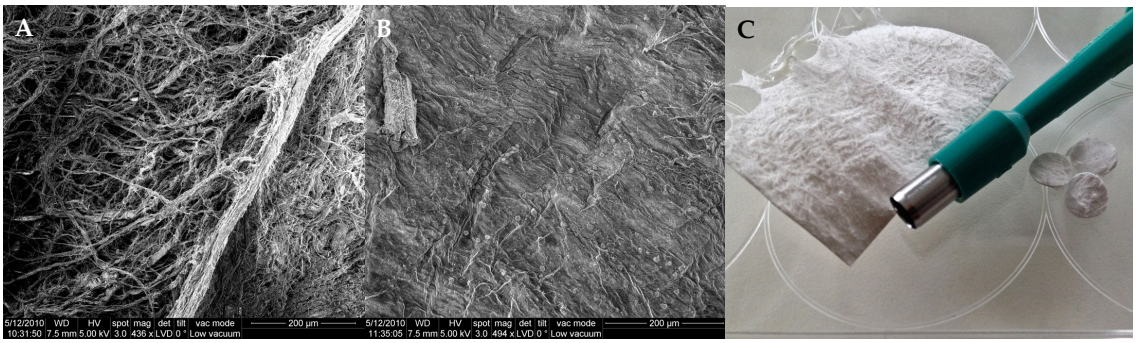

Figure 7 SEM of the collagen type I/III scaffold. A: The porous side with 20-100-μm large pores. This part of the scaffold was seeded with AT-MSC. B: The compact side of the scaffolds ensured cell-entrapment. C: Skin biopsy punch for the cutting of scaffolds.

Histology

Pellets and scaffolds were briefly counter-stained with Mayer's haematoxylin and then dehydrated in graded series of ethanol (70-96%) before being embedded in

methylmethacrylate (MMA) as described by Erben [202]. After polymerisation, 7 µm sagittal sections were prepared with Polycut E microtome (Reichert & Jung, Heidelberg, Germany). Cross sections were cut from the central part of the pellet and scaffold samples. Cells were visualised by staining first with haematoxylin for 15 minutes and then with eosin for 2 minutes (H&E). Sulphated proteoglycans and acidic polysaccharides were visualised by staining with 0.05% toluidine blue pH 4.0 for 20 minutes. Stained slides were mounted in DPX. Images were made on an Olympus BX51 microscope with the use of image acquiring software PixeLINK.

RNA extraction and real-time qPCR

Total RNA from samples was extracted in 1-mL TRIzol® (Invitrogen, Taastrup, Denmark) using a mixer mill (MM301; Retsch, Germany) to homogenise the scaffolds. Chloroform was added and samples were vortexed and centrifuged. RNA was precipitated with isopropanol, washed twice in 75% ethanol and finally dissolved in RNase-free, DEPC-treated water (Ambion, Cambridgeshire, UK). The concentration of total RNA was assessed by Quant-iT™ RiboGreen® RNA Kit (Molecular Probes) according to the manufacturer's instructions. Each sample was measured in a 96-well plate using a microplate reader. Samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm. Standards were prepared according to the manufacturer's instruction using the reagents provided (ribosomal RNA standard, concentration range: 0 to 50 ng/mL). Technical duplicates were run for each biological sample. RNA purity was measured by calculating the $A_{260\ nm}/A_{280\ nm}$ ratio using a spectrophotometer (values ranging from 1.92 to 2.06 indicated a high level of purity). RNA integrity was verified by agarose electrophoresis. Total RNA from pellets was extracted as described above, but preceded by digestion in 1 mg/mL collagenase (Gibco) at 37°C for one hour prior to homogenisation with the mixer mill.

Polymerase chain reaction (PCR) is used to amplify a specific region of a DNA strand. The specific gene region of interest is targeted with specific probes, which are primer sequences that determine the starting point for DNA replication by the DNA polymerase. Quantitative PCR (qPCR) is used to measure the accumulation of the DNA product after PCR. It quantitatively measures starting amounts of DNA, cDNA or RNA. Real time qPCR is a precise method for measuring the amount of amplified product in real time. In reverse transcription polymerase chain reaction (RT-PCR), a RNA strand is first reverse transcribed into complement DNA (cDNA) using reverse transcriptase and the cDNA is then amplified using real-time qPCR.

First-strand cDNA was synthesised according to the manufacturer's instructions using the High Capacity cDNA Archive Kit (Cat no 4322171, Applied Biosystems, Naerum, Denmark). The qPCR was performed at standard enzyme and cycling conditions on a 7500 fast real-time PCR system using TaqMan® gene expression assays

(Cat no 4352042, Applied Biosystems, Naerum, Denmark) Hs99999908_m1 (beta-glucuronidase [*Gusb*]), Hs00237047_m1 (tyrosine 3-monooxygenase /tryptophan 5-monooxygenase activation protein [*Ywhaz*]), Hs00427621_m1 (TATA box binding protein [*Tbp*]), Hs00153936_m1 (aggrecan [*Agc*]), Hs00165814_m1 (chondrocyte-specific transcription factor [*Sox9*]), Hs00164004_m1 (collagen type I alpha-1 [*Col1a1*]), Hs00264051_m1 (collagen type II alpha-1 [*Col2a1*]), Hs00166657_m1 (collagen type X alpha-1 [*Col10a1*]) and a custom-designed TaqMan assay for human RNA polymerase II [*RpII*]. Assays included one FAM™ dye-labelled TaqMan® MGB probe spanning exon boundaries in the gene of interest (GOI) and two unlabelled hot-start primers. The amplicon size was <170 bp for all primer sets to maximise the amplification efficiency. cDNA corresponding to 8 ng (i.e. pellets) or 20 ng (i.e. scaffolds) of mRNA was used in the qPCR reactions. Technical triplicates were used for each biological sample. qPCR reactions were run in 96-well plates at a final reaction volume of 20 µL in 40 cycles consisting of a denaturation step at 95°C for 3 seconds and annealing/extension step at 60°C for 30 seconds. Data analysis was performed using 7500 Fast System Sequence Detection Software, version 1.3. Gene expression levels of the GOI were normalised to the BestKeeper Index [203], which was determined by the geometric mean of the cycle threshold (Ct) from *Gusb*, *Ywhaz* and *Tbp*. *Gusb*, *Ywhaz* and *Tbp* have been reported to be the most stable genes for AT-MSCs cultured in different oxygen conditions and were used as references genes [204]. Gene expressions were normalised to the BestKeeper Index using the following equation:

$$\text{Relative gene expression} = 2^{\text{Ct (BestKeeper Index)} - \text{Ct (gene of interest)}}.$$

The delta delta Ct method [205] was used to compare the inductive effect of 5% O_2 with 21% O_2, i.e, gene expressions from each sample were first normalised to the endogenous housekeeping genes and data are then presented as the control-adjusted fold change of gene expression in the induced relative to the non-induced samples setting the control value to 1.0. RT. qPCR for pellets was done as described above, apart from cDNA corresponding to 8 ng of total mRNA was used in each qPCR reaction.

Data analysis and statistics

Data were checked for normal distribution and variance homogeneity. Two-way analysis of variance (ANOVA) was performed with culture medium and oxygen tension as independent variables (medium*oxygen). Data are presented as means ± standard errors of means for four donors (n = 4) in three biological replicates and measured in two technical duplicates. Differences between means were considered statistically significant when $p<0.05$. All statistical evaluation was done using STATA 10.1 software (Stata Corporation, College Station, TX).

4.3 Study II

Study design, aim and hypotheses

The aim of Study II was to evaluate the suitability of two different types of PCL scaffolds seeded with human umbilical cord blood multilineage progenitor cells (MLPC) cultured in 5% O_2 for the *in vitro* production of cartilage constructs. I hypothesised that reduced oxygen tension increases chondrogenesis of MLPCs in porous and biologically compatible scaffold cultures of polycaprolactone.

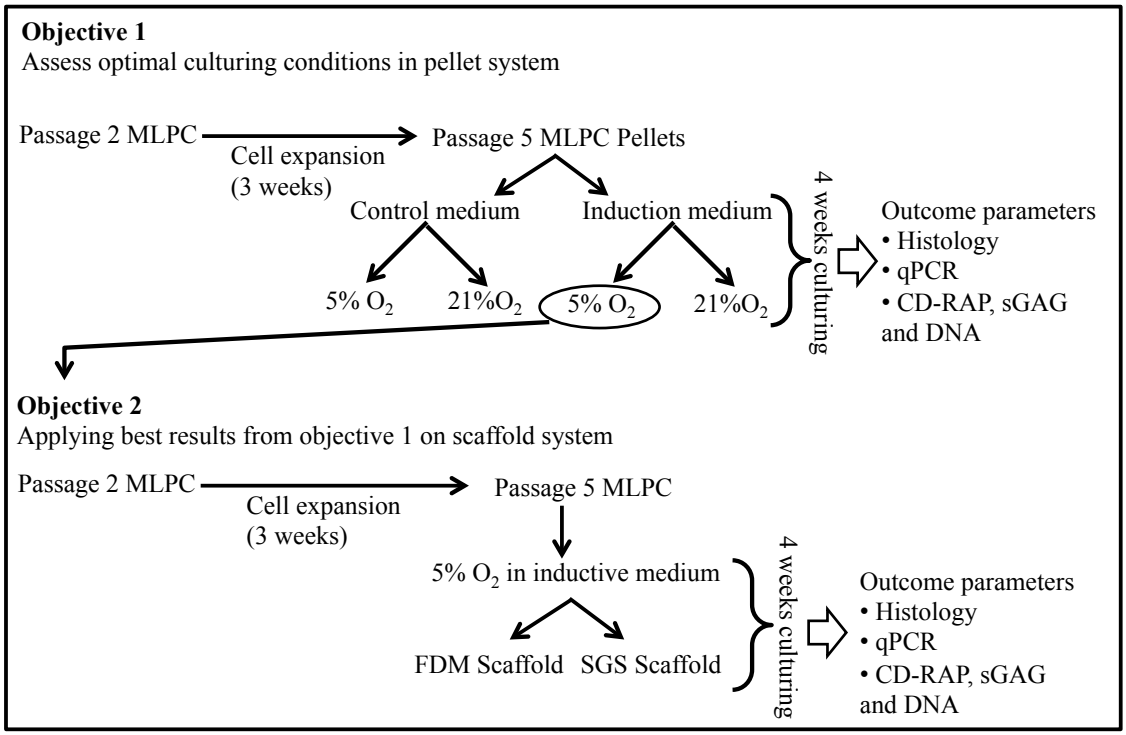

Figure 8 Schematic presentation of study design (II). FDM: Fused deposition-modelled scaffolds; SGS: structurally graded scaffolds fabricated by combined FDM and TIPS-FD.

Figure 8 is a schematic presentation of the study design and my objectives. The first objective was to determine the optimal oxygen tension for chondrogenic differentiation of MLPC pellet cultures. The results of this investigation were then applied in the scaffold cultures. MLPCs were first expanded for three weeks and differentiation was then done in standard pellet cultures in chondro-inductive or control medium in both 5% O_2 and 21% O_2. An additional vial of MLPC was then thawed, expanded and cultured in two different scaffold cultures (i.e., FDM scaffolds and FDM combined with TIPS-FD structurally graded scaffolds) for four weeks. Evaluation of chondrogenesis in Study II was extended compared to the evaluation performed in Study I and additionally included evaluation of the mRNA gene expression for CD-RAP, and ECM quantification (i.e., sGAG and CD-RAP). Histological analysis was done with H&E and Alcian blue.

Cells source and culturing

In this study (and Study III), human umbilical cord blood-derived Multi-Lineage Progenitor Cells (MLPC™) were used. The MLPC clone number 081704-1G1 was kindly provided by BioE Inc. (Saint Paul, MN). These MLPCs are a clonally expanded cell line that has previously been shown to possess osteogenic, adipogenic and chondrogenic potential (trilineage potential) [200]. MLPCs have been validated positive for surface markers CD73, CD90 and CD105, and negative for CD34, CD45, CD106 and HLA-DR. This fulfils the minimum criteria of The International Society for Cellular Therapy for defining multipotent MSCs [105, 200, 201]. The entire contents of one cryopreserved vial containing 1.0 x 10^5 P2 cells was first thawed in a 37°C water bath and then seeded into 75 cm^2 culture flask with expansion medium consisting of low-glucose DMEM (Lonza, Walkersville, MD) supplemented with 30% FBS (Invitrogen, Taastrup, Denmark) and 2mM L-glutamine (Lonza, Walkersville, MD). Cells were grown to 40-50% confluence at standard humidified atmospheric conditions containing 5% CO_2 and 21% O_2 at 37°C. The expansion medium was changed completely twice weekly. Upon reaching 40-50% confluence, cells were trypsinized (0.5% trypsin and 1.2 mM EDTA) and reseeded at 1500 cells/cm^2. After three passages, enough MLPCs were retrieved for the experiments. Hence, P5 MLPCs were used in the studies described.

MLPC chondrogenesis in pellet cultures

Pellet cultures were established by spinning 2.5x10^5 MLPCs at 150*g* for 5 minutes at room temperature in 15 mL conical polypropylene tubes. Pellets were randomly split (as outlined in Figure 8) for culturing into either 0.5 mL non-inductive control (expansion medium as stated above) medium or chondrogenic inductive medium (hMSC Chondrogenic BulletKit®; Lonza, Walkersville, MD; supplemented with 10 ng/mL of TGF-β3) and subsequently cultured in either 5% or 21% O_2. A 0.5-mL volume of culture medium was changed twice weekly. The culture medium was equilibrated for at least 2 hours at the relevant oxygen tension before change. The caps of culturing tubes were untightened to allow gas exchange. Pellets were cultured for a total of four weeks.

MLPC chondrogenesis in scaffold cultures

3D cell-scaffold cultures were constructed using either a fused deposition-modelled (FDM) or a novel structurally graded scaffold (SGS) of polycaprolactone (PCL). Detailed descriptions of the scaffolds are given below. Following expansion in monolayer culture, cells were detached by trypsin/EDTA and resuspended in chondrogenic inductive medium (hMSC Chondrogenic BulletKit®; Lonza, Walkersville, MD; supplemented with 10 ng/mL of TGF-β3). A total of 2.5 x 10^5 MLPCs in 10-μL

cell suspensions was seeded on top of the scaffolds. The cell-loaded scaffolds were placed in standard culture conditions (5% CO_2, 21% O_2 and 37°C) for 2 hours to allow cells to adhere. One mL of chondrogenic induction medium supplemented with 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin (Invitrogen, Taastrup, Denmark) were then added to the wells and the culture plates were transferred to the 5% O_2-containing hypoxia workstation. Culture time was four weeks with culture medium change twice a week.

Polycaprolactone (PCL) scaffold manufacturing and surface treatment

Two different PCL scaffold compositions were used: Macroporous FDM and structurally graded scaffolds (SGS). FDM-PCL scaffolds were made from PCL (MW=50 kDa, Perstorp, UK) by FDM at a processing temperature of 100°C with a BioScaffolder (SYS + ENG GmbH, Germany). Cylindrical scaffolds, 4.0 mm in diameter, were punched out from 2.0 mm thick porous PCL mats using a sterile biopsy punch (Miltex, York, PA). Deposited fibres had a thickness of 200 μm. However, the thickness of each individual layer was set to 120 μm to ensure solidly fused junctions between layers. The centre–centre fibre distance in each deposited layer was 1.0 mm and the fibre orientation of each consecutive layer was angled at 105° and shifted 0.17 mm [45]. Scaffold pores were designed to be completely interconnected having macro-pores of approximately 800-1000 μm (Figure 9 & Figure 10).

The microporous SGSs were made by embedding FDM scaffolds with a second PCL microporous network using thermally induced phase separation (TIPS). This approach enables scaffolds with a finely structured polymeric matrix and increased surface area for cell loading by using a solvent-based technique in combination with a much stiffer FDM component, which ensures the overall mechanical stability of the scaffold [206, 207]. A solution of dissolved PCL (MW=50 kDa, 40 mg PCL/g dioxane) in a dioxane-water (0.04% water/dioxane) mixture was poured onto the FDM scaffolds and phase-separated at -30°C followed by freeze-drying (FD). This produced interconnecting micro-pores of 100-250 μm within the larger macro-pores (Figure 10B). The controlled lowering of temperature to -30°C (2.5°C/min) causes dioxane and water to freeze and the initially dissolved PCL to precipitate and aggregate. The dioxane-PCL mixture will thus contain domains of PCL separate from domains of frozen dioxane-water crystals. Dioxane and water was then extracted by FD (i.e. controlled lowering of both temperature and pressure) in which dioxane-water sublimates (i.e. transition from solid phase to gas phase without becoming liquid) and leaves a porous PCL scaffold.

Scaffolds were sterilised in series of ethanol washes and sterile water followed by surface modification with 1.25 M NaOH, neutralisation in 1 M HCl and rinsed with sterile water, which increases surface hydrophilicity and subsequently improves cell attachment.

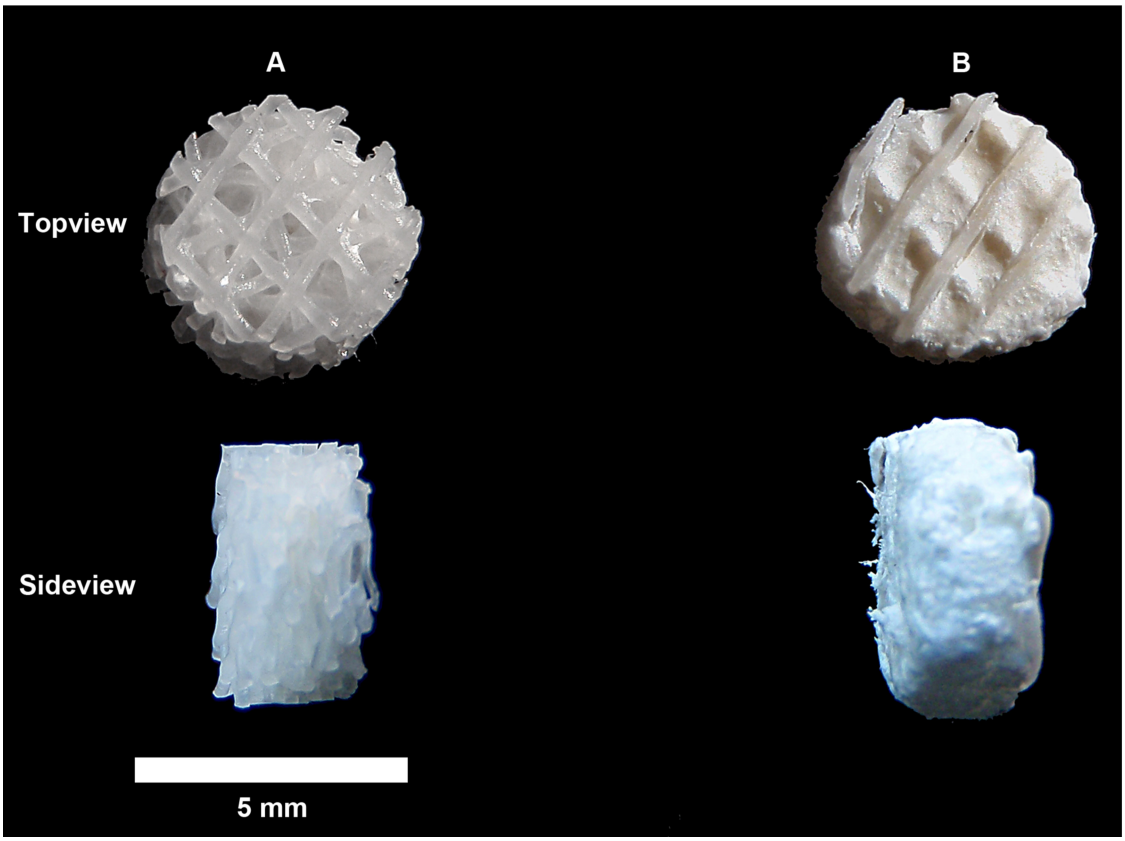

Figure 9 Macroscopic pictures of (A) FDM scaffold. Note that the shift in fibre orientation of 105° between layers creates the grid-shaped scaffold structure. (B) SGS made by combined FDM and TIPS-FD. Scale bar indicates 5 mm.

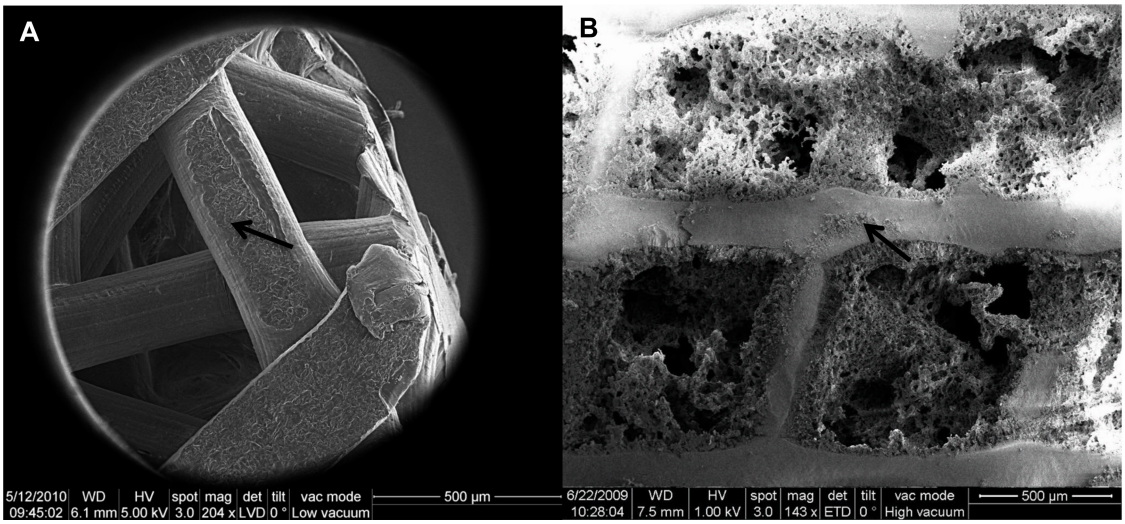

Figure 10 SEM images of (A) FDM scaffold. Note that the shift in fibre orientation of 105° between layers creates the grid-shaped scaffold structure. (B) SGS made by combined FDM and TIPS-FD. Note that TIPS of the solvent PCL creates micro-pores of 100-250 µm within the larger macro-pores. (↗) Arrow indicates PCL fibre. Scale bar indicates 500 µm.

Histology

For histological analysis, samples were first stained with Mayer's haematoxylin and then dehydrated in graded series of ethanol (70-96%). Pellet samples were embedded in MMA and cut transversely into 7-µm sections as described in Study I. Scaffold samples were embedded in Technovit® 7100 (Axlab, Vedbæk, Denmark) and cut transversely into 10-µm sections using a Sawing Microtome KDG 95 (Meprotech, Heerhugowaard, the Netherlands). Staining of slides and microscopic evaluation was done as described for Study I with the exception of determining extracellular glycosaminoglycan deposition with staining of 0.5% Alcian Blue (pH 3) for 20 minutes.

RNA extraction and real-time qPCR

Total RNA for quantitative qPCR was extracted using the GenElute™ Mammalian Total RNA Kit (Sigma-Aldrich, Missouri, USA) as described by the manufacturer with the following modifications (please note that in Study I RNA was extracted by the TRIzol-method). Samples were lysed in lysis buffer supplemented with β-mercaptoethanol and glass beads (0.7-1.0 mm diameter, Retsch GmBH, SKANLAB Aps, Denmark) for pellets or tungsten carbide balls (3 mm diameter, Retsch GmBH, SKANLAB Aps, Denmark) for scaffold constructs, respectively. Samples were homogenised using a mixer mill (Retsch GmBH MM 301, SKANLAB Aps, Denmark) for three cycles of 30 seconds at a frequency of 30.0 Hz with a 30-second rest period in between cycles. All subsequent RNA extraction steps were performed according to the GenElute™ protocol. The concentration and quality of isolated RNA was quantified using a nanophotometer (Implen, Munich, Germany).

First-strand cDNA was synthesised by the same procedure as described for Study I. qPCR was performed with the same protocol as described for Study I with the exception of including one additional gene, Hs00197954_m1 [*Cd-rap*]. Template cDNA corresponding to 4 ng mRNA was used in each PCR reaction and run in technical duplicates for each gene. Gene expression levels were normalised to reference genes in a similar way as in Study I.

Quantification of proteoglycan and DNA content

Samples were digested in 150 µL (i.e. pellets) or 450 µL (i.e. scaffolds) solutions of 300 µg/mL papain in 20 mM PBS, pH 6.8, 5 mM EDTA, 2 mM DTT at 60°C for 18 hours (Sigma-Aldrich, Broendby, Denmark).

The proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycan (sGAG) using the dimethylmethylene blue (DMMB) dye-binding assay (Blyscan™, Nordic BioSite ApS, Copenhagen, Denmark) with a chondroitin sulphate standard. The manufacturer's instructions were followed. DMMB dye is used to bind sGAG, which causes a colour change (metachromasia) from blue to purple upon

binding to sGAG. One mL of DMMB was allowed to form purple-coloured precipitate with 50 µL papain-digested extract for 30 minutes. The precipitate was separated from the unbound dye by centrifugation at 12,000*g*. The purple dye-sGAG precipitate was then re-solubilised with 1 mL dye dissociation reagent. Dye absorbance was measured at 650 nm on 200 µL sGAG samples, chondrotin-4-sulfate standards and blanks using a spectrophotometer (Victor^3 1420 Multilabel Counter, PerkinElmer Life Sciences, Hvidovre, Denmark) as technical duplicates in 96-well clear plates. The sGAG accumulation was normalised to corresponding DNA content.

Total DNA was quantified using the Quant-iT™ PicoGreen® dsDNA assay (Invitrogen, Denmark). DNA was released from papain-digested extract by vortexing 1:4 Tris–EDTA (TE) buffer-diluted samples. Fifty µL diluted samples were allowed to react with 50 µL PicoGreen (diluted 1:200 in TE buffer) for 5 minutes in the dark following measurement in a 96-well plate using a microplate reader (Victor3 1420 Multilabel Counter, PerkinElmer Life Sciences, Denmark). Samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm. Standards were prepared according to the manufacturer's instruction (lambda DNA, concentration range: 0–1 µg/mL). Technical duplicates were used for each biological sample.

CD-RAP ELISA

Cartilage-derived retinoic acid-sensitive protein (CD-RAP), also known as melanoma inhibitory activity (MIA), was included since it has recently been promoted as a novel marker for chondrogenic differentiation in MSCs [19, 21, 118, 208]. CD-RAP is primarily expressed and secreted by cartilage cells, including both developing and mature cartilage. CD-RAP is a secreted, soluble protein. Its secretion into conditioned culture medium allows continuous quantitative monitoring of the chondrogenic anabolism without cell culture interference or termination, which is a more favourable assay than qualitative immunostaining against collagen type II. Culture medium collected weekly was analysed for CD-RAP concentrations using the MIA ELISA (enzyme-linked immunosorbent assay) assay (Roche Diagnostics, Naerum, Denmark) according to the manufacturer's instructions. This kit is a one-step sandwich ELISA assay based on two murine monoclonal antibodies raised against human CD-RAP. One of the antibodies is conjugated with peroxidase enzyme, the activity of which is developed using ABTS as a substrate. The absorbance of ABTS was detected after 15 minutes of incubation at 405 nm. CD-RAP protein concentrations were interpolated from the standard curve generated from included standards containing CD-RAP derived from transfected Chinese hamster ovary cells in a serum-analogue matrix containing BSA.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to visualise the surface-morphology and distribution of cells and PCL matrix within the scaffolds. A SEM is a high-magnification microscope that uses a focused beam of high-energy electrons to probe the morphology of the surface. When the electron beam interacts with atoms on the surface (or near the surface), it loses energy. The lost energy is converted into other forms such as heat, low-energy secondary electrons and high-energy backscattered electrons. Scattered electrons are detected and carry information about the properties of the surface, i.e., its topography and morphology. SEM is excellent for producing high-resolution images with a high depth of field, which is important when examining 3D porous structures.

For scanning electron microscopy (SEM), the scaffolds were fixed in 2.5% glutaraldehyde containing 0.1 M sodium cacodylate buffer (pH 7.4) and dehydrated in a graded series of ethanol (50-99%) before being transferred to an exicator for air drying. Scaffolds were analysed using a low-vacuum secondary electron detector (Nova NanoSEM 600, FEI Company).

Data analysis and statistics

Data were ln-transformed (RT-qPCR), checked for normal distribution and variance homogeneity. Two-way ANOVA was performed with culture medium and oxygen tension as independent variables (medium*oxygen) for the pellet cultures; and culturing time and scaffold composition as independent variables (time*scaffold composition) for the scaffold cultures. Data are presented as means ± standard errors of means for three (n = 3) biological replicates. The level of significance and tools for statistical analysis were similar to those used in Study I.

4.4 Study III

Study design, aim and hypotheses

This study was performed during a three month long exchange stay in the laboratory of Prof. Rita Kandel, University of Toronto, Canada. New tissue engineering strategies and methods were introduced, which had great educational value.

The Canadian group had previously constructed and demonstrated good clinical outcome of a biphasic osteochondral cartilage-plug utilising porous calcium polyphosphate (CPP) as a substrate for chondrocyte attachment and growth (Figure 11) [209, 210]. In this model, isolated chondrocytes are first cultured on CPP for 8 weeks and then implanted in a sheep cartilage defect model. CPP consists of a porous bone substitute biomaterial with high values for compressive strength within the group of

porous ceramics and its osseointegrative properties as demonstrated *in vivo* and *in vitro* make CPP a suitable bone substitute material [211-213].

Figure 11 Histologic appearance of a biphasic implant harvested after 3 months (sheep model). Before implantation, the CPP is cultured *in vitro* with isolated chondrocytes for 8 weeks. The CPP is covered by a continuous layer of cartilage which is fused with the adjacent surrounding articular cartilage (toluidine blue stain). Adapted from Kandel et al, 2006 [209].

The aim of Study III was to engineer a biphasic cartilage implant formed *in vitro* from MLPCs and integrated on the articulation surface of CPP. Lee et al. have demonstrated the formation of such a biphasic osteochondral CPP construct [138]. However, they also reported the necessity of employing an intermediate step in which ovine BM-MSCs are first differentiated to chondroblasts on collagen IV coated semi-permeable culture plate inserts and subsequently cultured on CPP producing an biphasic osteochondral construct for implantation. In detail, $2.0*10^6$ ovine BM-MSCs were first pre-differentiated on collagen IV coated membranes for 2 weeks resulting in a disc-shaped cartilage-like tissue. The pre-differentiated cells were released by collagenase A and $2.0*10^6$ cells were then seeded on CPP and cultured for 8 weeks in 5% FBC containing medium. The two-week long period of pre-differentiation on membranes allows high-density culturing of BM-MSCs in 3D-like fashion thereby priming the chondroblastic differentiation [214].

I hypothesised that MLPCs are a suitable cell source for *in vitro* engineering of a biphasic osteochondral construct. The pre-chondrogenesis of MLPCs was evaluated in three culture systems: as pellets, on CPP, and on collagen coated and non-coated semi-permeable culture plate inserts.

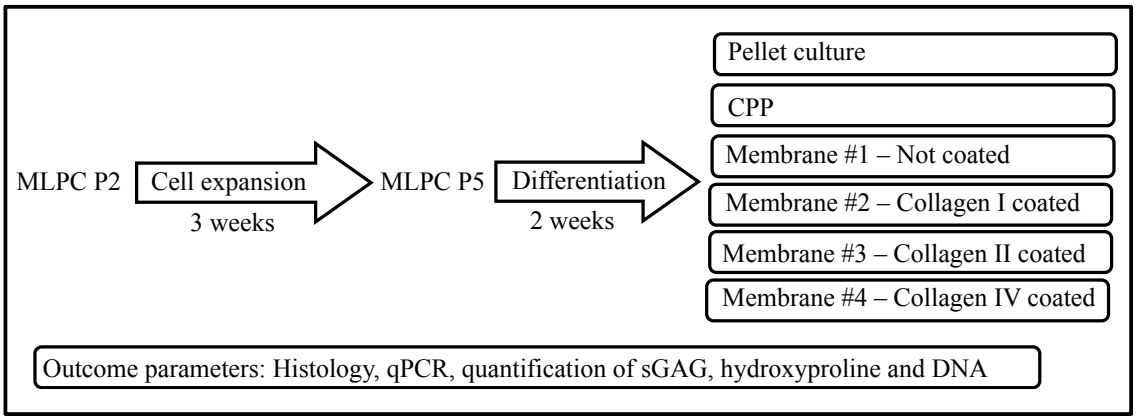

Figure 12 Schematic presentation of study design (III).

Figure 12 is a schematic presentation of the study design (III). P2 MLPCs were first expanded for 3 weeks to obtain sufficient cells. The resultant P5 MLPCs were then pre-differentiated for two weeks in three culturing systems, i.e., MLPCs were cultured (1) as pellets, (2) on the surface of CPP, and (3) on uncoated or collagen type I-, II- and IV-coated semi-permeable polytetrafluoroethane membrane inserts. Due to differences related to species, donor ages, cell source and *in vitro* isolation and culture methods membrane coated with collagen type I, II and IV were screened for the pre-differential chondrogenesis of MLPC. The mRNA gene expression of aggrecan, SOX9 and collagen type II was assed by qPCR to quantify chondrocytic differentiation. Histology evaluated the deposition of ECM. Quantification of accumulated sGAG and hydroxyproline (i.e. collagen) served as functional markers of cartilage anabolism.

Cells source and culturing

The expansion procedure and the cell source for this study was the same as those described for Study II. However, I was unable to obtain approval for export of FBS to Canada. The expansion medium was therefore supplemented with 30% FBS from a local supplier (PAA Laboratories, Ontario, Canada).

MLPC chondrogenesis in pellet cultures

Pellet cultures were established by spinning $2.5x10^5$ MLPCs at 200*g* for 5 minutes at room temperature in 15 mL conical polypropylene tubes. Pellets were cultured in chondrogenic inductive medium consisting of DMEM-HG supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 100 μg/mL ascorbic acid 2 phosphate (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyrovate, 1x ITS^+ (BD Biosciences, Bedford, MA), 10 ng/mL TGF-β3 (R&D Systems, Minneapolis, MN) and 1% streptomycin/penicillin (Invitrogen). The total culturing time was two weeks. Culture medium was changed three times a week.

MLPC chondrogenesis on CPP and semipermeable membranes

Biodegradable CPP rods were produced from CPP powder (particle sizes of 75–106 μm). The CPP powder was gravity-sintered at 950 °C in cylindrical tubes to form porous CPP [212]. The resulting rods characterised by 35 vol.% interconnected porosity were cut to form discs 2.0 mm in height and 4.0 mm in diameter. A non-leaking culture well was made by enclosing a silicone tube around the CPP disc (Figure 13) (Tygon 3350, Saint-Gobain Performance Plastics Corp., Aurora, OH, USA). CPP constructs were sterilised by gamma irradiation (2.5 Mrad). Please note that membrane cultures were seeded with cell densities similar to those of the PCL scaffolds in Study II, but no enclosing silicone was used to prevent cell leakage from PCL cultures.

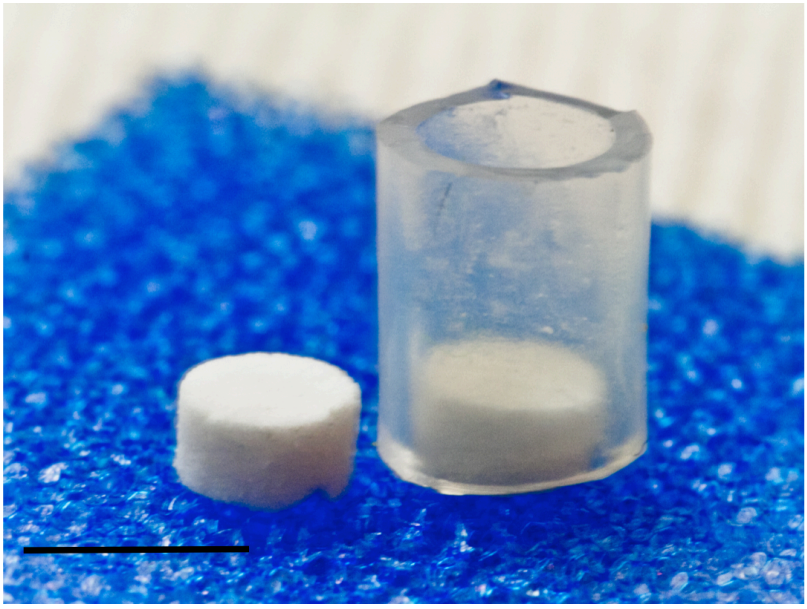

Figure 13 CPP disc enclosed by silicone tube forming a culture well preventing cell leakage. Scale bar indicates 5 mm.

Semipermeable polytetrafluoroethane membrane culture plate inserts 12 mm in diameter (0.2 μm pore size, Millicell-CM®, Millipore Corp., Bedford, MA, USA) were coated with collagen type I, II and IV (0.5 mg/mL in 0.1 N acetic acid; Sigma Chemical Co., St Louis, MO, USA) and dried overnight. Coated inserts were UV sterilised for 30 minutes prior to cell culturing.

A cell suspension of $2x10^6$ MLPCs were gently seeded on top of CPP constructs (30 μL) or inserts (400 μL) and left for three hours in the incubator to allow cells to adhere following gentle addition of medium to a total volume of 2.0 mL. Cultures were grown for two weeks in 24-well culture plates in chondrogenic inductive medium. The culture medium was changed three times a week. In Study I&II, and the literature have demonstrated the necessity of biochemical induction of MSCs with growth factors and cytokines (e.g. TGF-β, BMP and dexamethasone etc.) to drive differentiation towards

chondrogenesis [120-124]. These biochemical factors are routinely used, and due to shortage of cells and supplies no control medium (e.g. expansion medium) was included in Study III. MLPCs cultured on non-coated inserts served as controls for mutual comparison between the six different culture groups.

Histology

In vitro-formed neocartilage constructs were harvested and washed twice in PBS. Tissue was removed from the CPP, fixed in 10% formalin and embedded in paraffin. Tissue formed on membrane inserts was processed without the membrane insert. 5-µm transversely cut sections were stained with either toluidine blue (pH 3.0) to demonstrate the presence of proteoglycans or H&E to visualise cells and tissue by light microscopy.

Please note that in Study II, proteoglycans were visualised by Alcian blue stain, while in Study I&III toluidine blue was used. Both dyes are metachromatic, i.e., the dye changes colour upon binding to highly electronegative molecules such as sGAG. The staining of charged molecules is dependent on the pH and in this context the metachromatic of toluidine blue is less sensitive to the pH value than Alcian blue. Specific immunostaining directed against aggrecan, biglycan, collagen type I, II and X could accordingly have been utilised to evaluate the zonal layout of ECM components.

RNA extraction and real-time qPCR

The GenElute™ (same kit as used in Study II) Mammalian Total RNA Kit (Sigma-Aldrich, Missouri, USA) was used to extract RNA for qPCR. However, the following modifications of the protocol were made: Samples were first lysed in lysis buffer supplemented with β-mercaptoethanol. Tissue was then snap-frozen in liquid nitrogen and homogenised in a pestle mortar. After each processing, pestle was cleaned in series of 100% ethanol washing, SDS- and DEPC-treated water. All subsequent RNA extraction steps were performed according to GenElute™ protocol. Isolated RNA concentration and purity was measured with the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware USA). cDNA was synthesised from 150 ng of total RNA using Superscript® III reverse transcription kit (Invitrogen) at a total reaction volume of 20 µl containing FirstStrand buffer and 0.01 M dithiothreitol for 60 minutes at 42°C followed by a 15-minutes extension period at 70°C. Each reaction contained 40 units of recombinant ribonuclease inhibitor RNase OUT™ (Invitrogen, Carlsbad, CA), 200 ng of random primers, 0.5 mM dNTPs and 200 units of SuperScript® III enzyme.

Gene expression was determined with qPCR and sequence-specific primers for collagen type II, aggrecan, SOX9 and 18S (reference gene for normalisation). For collagen type II, a forward primer of 5´-GTG TCA GGG CCA GGA TGT C-3´ and a reverse primer of 3´-GCA GAG GAC AGT CCC AGT GT-5´ were used. For aggrecan,

a forward primer of 5′-TGG GAC TGA AGT TCT TGG AGA-3′ and a reverse primer of 3′-GCG AGT TGT CAT GGT CTG AA-5′ were used. For SOX9, a forward primer of 5′-GTA CCC GCA CTT GCA CAA C-3′ and a reverse primer of 3′-GTG GTC CTT CTT GTG CTG C-5′ were used. For 18S, a forward primer of 5′-GTA ACC CGT TGA ACC CCA TT-3′ and a reverse primer of 3′-CCA TCC AAT CGG TAG TAG CG-5′ were used. qPCR was performed using SYBR green dye on Realplex^2 Master Cycler (Eppendorf, Hamburg, Germany) according to instructions provided by the manufacturer and data were analysed with Mastercycler EP Realplex. Briefly, template cDNA corresponding to 10 ng RNA was amplified in 10 μL final volume with 1 μM of each primer suspended in 2X QuantiFast SYBR Green Master Mix (Qiagen, Hilden, Germany) and run in technical triplicates. Amplification parameters were identical for each primer pair (10 minutes at 95°C to activate the enzyme, 15 s denaturation at 95°C was followed by 30 s annealing at 60°C), and amplification data were collected for 40 cycles. Relative mRNA expression levels were normalised to 18S using the following equation:

Relative gene expression=$2^{Ct\ (18s)\ -\ Ct\ (gene\ of\ interest)}$.

Please note that for Study I&II, the qPCR was based upon a TaqMan® fluorescein-labelled RNA-probe, while Study III used the DNA intercalator (i.e. the binding of molecules in-between the DNA double helix) SYBR green for qPCR detection. SYBR is the original method developed for PCR quantification, it is cheaper than the TaqMan®-assays and its performance in terms of sensitivity is equal to that of newer methods, but unspecific detection of DNA after PCR in RNA-contaminated samples may occur in SYBR-based assays as may unspecific detection in artefact-annealed RNA and DNA. The TaqMan®-assays are sensitive owing to the use of a 5'-fluorescein dye-labelled probe than binds to exon parts in the gene of interest. The labelled dye is only fluorescent in its free form and will therefore only emit a signal if cleaved of during strand elongation by polymerases. Hence, the specificity in the TaqMan® lies in a combination of the starting primers and the dye-labelled probe.

Quantification of proteoglycan, collagen and DNA content

In vitro-formed tissue was removed from CPP or membrane inserts and snap frozen at -80°C. Frozen samples were digested using papain (Sigma; 40 μg/mL in 20 mM ammonium acetate, 1 mM EDTA and 2 mM dithiothreitol) for 48 hours at 65°C and stored at -30°C until further analysis. Aliquots of the digest were analysed separately for proteoglycan, hydroxyproline and DNA content.

The proteoglycan content was estimated by quantifying the amount of sGAG using the DMMB binding assay (Polysciences Inc., Washington, PA, USA) and spectrophotometry (wavelength 525 nm) [215]. The standard curve for the analysis was generated using bovine trachea chondroitin sulfate A (Sigma-Aldrich, Missouri, USA).

Please note the DMMB assay used in this study differed slightly from that used in Study II: the soluble sGAG-DMMB complex was stabilised with bovine serum albumin (BSA). This allowed a number of samples to be evaluated, while in Study II, the precipitated sGAG-DMMB complex (sGAG-DMMB precipitate due to the lack of stabilising BSA) was re-solubilised with a dissociation reagent prior to the spectrophotometer measurements. The two approaches yield comparable results. The sGAG content was normalised to corresponding DNA content.

Since collagen contains approximately 8-10% hydroxyproline (HP), a colourimetric hydroxyproline assay was used to determine the approximate amount of collagen. Hydroxyproline was released from protein and peptides by acid hydrolysis with 6 N HCl at 110°C for 18 hours. The hydroxyproline was then oxidized into a pyrrole with chloramine T. This intermediate becomes pink when Ehrlich's Reagent (4-dimethylaminobenaldehyde) is added and was detected with a spectrophotometer (wavelength 560 nm). The standard curve was generated using L-hydroxyproline (Sigma-Aldrich, Missouri, USA). The collagen content was normalised to corresponding DNA content.

The amount of DNA was quantified with the fluorescent dye Hoechst 33258 (Polysciences Inc., Washington, PA, USA) [216]. The Hoechst 33258 dye is a bisbenzamine DNA intercalator that has an excitation maximum of 356 nm and an emission maximum of 458 nm when bound to DNA. Specifically, the Hoechst 33258 dye binds to the adenosine- and thymidine-rich regions of double-stranded DNA. The standard curve was generated using calf thymus DNA (Sigma-Aldrich, Missouri, USA).

Please note that PicoGreen® was used as a DNA intercalator in Study II, which has a different excitation and an emission maximum than that of the Hoechst 33258.

Data analysis and statistics

Data were checked for normal distribution, variance homogeneity and one-way analysis of variance (ANOVA) was performed with the culturing system as the independent variable. Data are presented as means ± standard errors of means for three replicates (n = 3). The level of significance and tools for statistical analysis was similar to those used in Study I.

5 Results

5.1 Summary of results

This chapter contains the most important results of the studies. The studies were designed to investigate whether AT-MSCs and MLPC could play a future role in the clinical treatment of chondral injuries. The three studies consisted of *in vitro* experiments where MSCs from two different sources were cultured in scaffold-free (i.e., pellet cultures) as well as scaffold systems in different oxygen tensions. Because of unsatisfactory and unexpected results in the first study (I) and because the experiments were performed in collaboration with different research groups each with their specific expertise, the experiments in Study II and Study III were differently designed. This is reflected in the diversity of the applied methods, and evidently invites scientific shortcomings because the presented results cannot easily be compared across the studies. Table 3 outlines the key findings or "issues" that prompted a change in the experimental design of the subsequent studies.

	Study I	Study II	Study III
Hypotheses	Reduced oxygen tension increases chondrogenesis of AT-MSCs in pellet and collagen I/III scaffold cultures	Reduced oxygen tension increases chondrogenesis of MLPCs in scaffold cultures of porous PCL	MLPC chondrogenesis is increased in scaffold-free membrane and CPP cultures
Result/issue	• Contraction/curling and deformation of scaffold cultures • Unsatisfactory histology • Lack of biochemistry evaluation	• Histology improved	• Tissue of neocartilage produced in membrane cultures • Contraction of coated membrane cultures • Results supported by biochemistry
Change in subsequent experiment (or future research)	• Change to a well-characterized MSC source (➔MLPC) • Change of scaffold (➔PCL)	• Change of culturing system	• Culture pre-differentiated MLPC on CPP (to produce biphasic osteochondral

			plug) • Optimise differentiation of MLPC, e.g, with supplementary growth factors such as BMP2, cross-linking collagen, and inhibition of SMA.

Table 3 Outline of results/issue outcome in the presented experiments that prompted changings in subsequent study.

5.2 Study I

AT-MSC pellet cultures

Pellets cultured in chondrogenic inductive medium increased in size (diameter ≈ 2 mm after four weeks of culturing), whereas pellets cultured in control medium retained their initial size. Induced pellets had a robust and firm tissue texture (assessed by forceps), and their surface was smooth and yellow in contrast to non-induced pellets, which were non-uniform, fragile and in a state of disintegration. Histological examination of induced cultures revealed increased cellularity and ECM deposition as a function of oxygen tension, i.e., higher levels and intensity of matrix deposition was observed peripherally in the 5% O_2 cultures compared with 21% O_2 in which matrix deposition was located centrally (Figure 14). At high magnification of toluidine blue stained slides, induced cultures showed focal spots resembling the morphology of chondrocytes (i.e. rounded cells in lacunae surrounded by chondrocyte-like matrix). Haematoxylin and eosin staining displayed a similar pattern in zonal distribution of cellularity, i.e., higher levels of cellularity was observed peripherally in 5% O_2 cultures compared with 21% O_2 in which cultured cells were centrally located. Pellets cultured in control medium generated no proteoglycan and had low cellularity (Figure 14).

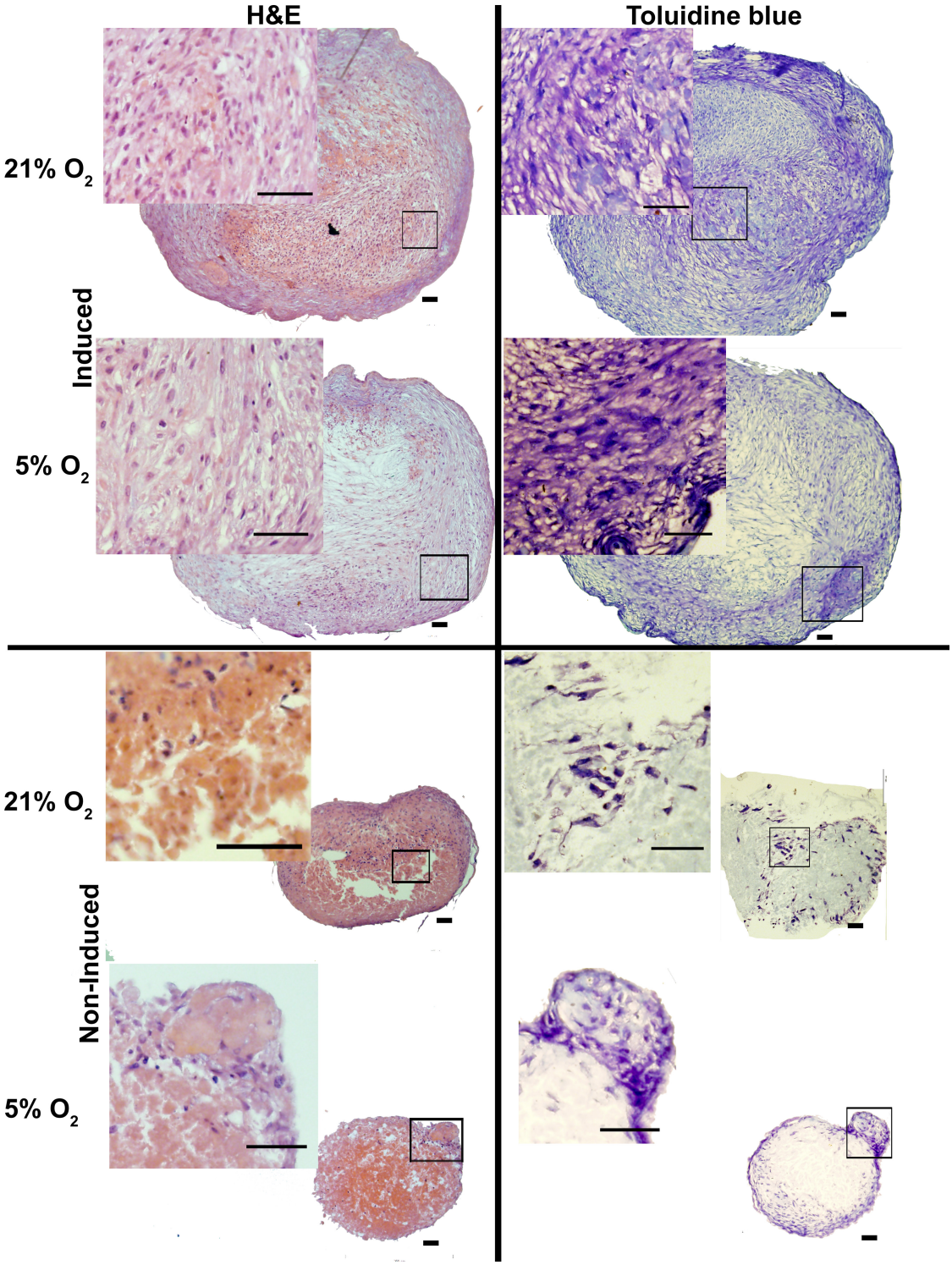

Figure 14 Histology of chondrogenically induced AT-MSCs pellets. AT-MSCs were cultured in 21% O_2 and 5% O_2 in chondrogenic inducing medium (upper panel) or non-inducing control medium (lower panel) for four weeks. Sections stained with H&E (left column) and toluidine blue (right column). ECM deposition shows zonal layout determined by the oxygen concentration, i.e., higher levels of cellularity and matrix deposition are observed in the peripheral parts for 5% O_2 cultures compared with 21% O_2 cultures in which the deposition of ECM is centrally located. Toluidine blue-stained slides show focal spots resembling the morphology of chondrocytes within lacunae surrounded by matrix. Non-induced cultures displayed decreased cellularity with pyknotic nuclei indicating apoptosis and generated no proteoglycan deposition. Scale bar indicates 50 µm at x10 (overview images) and x20 (inserted images) magnification.

The mRNA expression of aggrecan, SOX9, collagen I, II and X was higher for induced pellets compared with non-induced control pellets ($p<0.05$) (Figure 15). Cultures induced in 5% O_2 showed increased mRNA levels of SOX9 ($p<0.05$), collagen I ($p<0.05$), ratios of collagen II/I ($p<0.05$) and collagen II/X, and decreased collagen X ($p<0.05$) compared with 21% O_2 (Figure 15). No significant differences in mRNA expression of collagen II ($p=0.20$) and aggrecan ($p=0.75$) was noted in 5% O_2 induced cultures compared with 21% O_2. Higher control-adjusted fold increase was observed in 5% O_2 cultures for aggrecan ($p<0.001$), SOX9 ($p<0.001$) and collagen I ($p<0.001$), and decreased fold change of collagen X compared with 21% O_2 (Figure 15, bottom right).

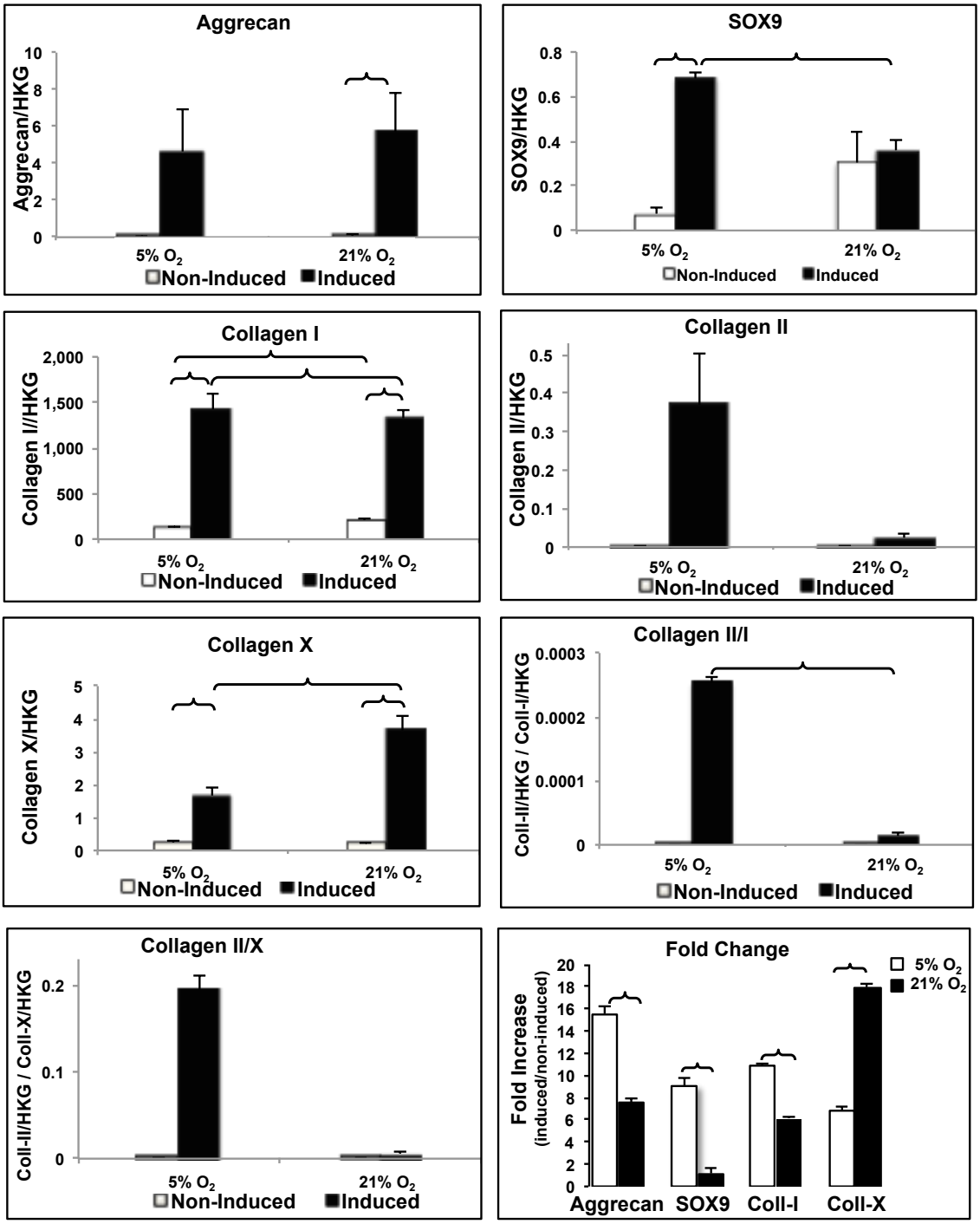

Figure 15 qPCR and adjusted fold change of chondrogenic gene markers aggrecan, SOX9, and collagens I, II and X in AT-MSC pellet cultures. Pellets were cultured in either 5% or 21% O_2, both in control medium (white bars) or chondrogenic inductive medium (black bar). Control-adjusted fold change is not reported for collagen II due to lack of mRNA expression in non-induced cultures. Values shown represent means ± SEM (n=4 donors). Brackets indicate significant difference between columns ($p<0.05$).

AT-MSC scaffold cultures

Regardless of the oxygen tension, induced collagen type I/III scaffolds cultures of AT-MSCs contracted and achieved a 3D spherical shape after four weeks culturing (Figure 16A). The rough and initially cell-seeded surface created a central, non-preformed cavity, referred to as an acquired cavity hereon, in which cells formed the observed tissue. Non-induced AT-MSC scaffold cultures maintained their native flat scaffold shape.

Chondrogenically induced AT-MSC-loaded scaffold cultures demonstrated a zonal deposition of ECM determined by the oxygen tension (Figure 16B), which was similar to that of induced pellets. But as previously mentioned, induced scaffolds contracted/curled up and formed an acquired cavity (Figure 16B); an arrow indicates the boundary between the native scaffold and the acquired cavity.

Scaffolds induced in 5% O_2 showed higher cellularity and matrix deposition peripherally compared with 21% O_2, in which cells and matrix was accumulated centrally in the acquired cavity (Figure 16B). At high magnification of toluidine blue stained slides, focal spots resembling the morphology of chondrocytes could be detected (i.e. rounded cells in lacunae surrounded by chondrocyte-like matrix); however, this was more distinctive in 5% O_2 cultures than in 21% O_2 cultured scaffolds. Non-induced scaffold cultures demonstrated limited cellularity and had no GAG deposition, and the seeded cells did not colonise deeper parts of the scaffolds.

Higher gene expression levels of aggrecan ($p<0.05$), SOX9 ($p<0.05$), collagen I ($p<0.05$) and collagen X ($p<0.05$) were observed for induced scaffold cultures compared with non-induced scaffolds (Figure 17). Scaffold cultures induced in 5% O_2 showed higher mRNA expressions of SOX9 ($p<0.05$), ratios of collagen II/I ($p<0.05$) and II/X ($p<0.05$) compared with 21% O_2 (Figure 17). No significant difference in mRNA expressions of aggrecan ($p=0.12$), collagen II ($p=0.23$), collagen I and collagen X ($p=0.13$) was noted is in 5% O_2 induced cultures compared with 21% O_2. In 5% O_2 compared with 21% O_2 mRNA expressions relative to controls were increased for aggrecan, collagen I, and SOX9 ($p<0.001$), and decreased for collagen X ($p<0.001$) (Figure 17, bottom right).

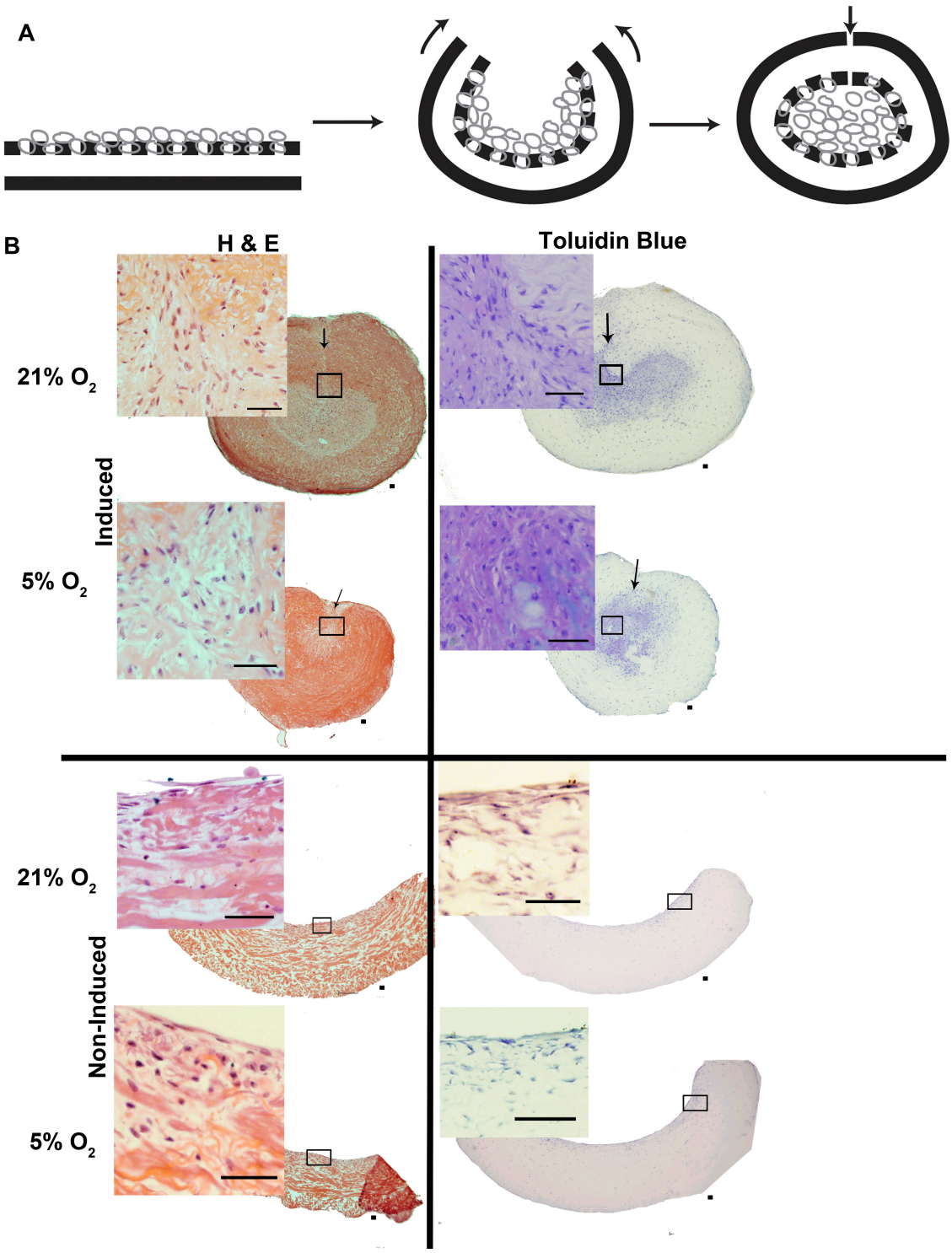

Figure 16 Schematic illustration demonstrating the contraction and creation of an acquired central cavity in chondrogenic induced AT-MSC scaffold cultures. (B) Histological appearance in collagen I/III scaffold cultures of AT-MSCs. Scaffolds were cultured in 21% O_2 and 5% O_2 in chondrogenic inducing medium (upper panel) or non-inducing control medium (lower panel) for four weeks. Sections stained with H&E (left column) and toluidine blue (right column). Arrow (↗) indicates the boundary between the native scaffold and the acquired cavity as illustrated in panel A. Scale bar indicates 50 µm at x4 (overview images) and x20 (inserted images) magnification.

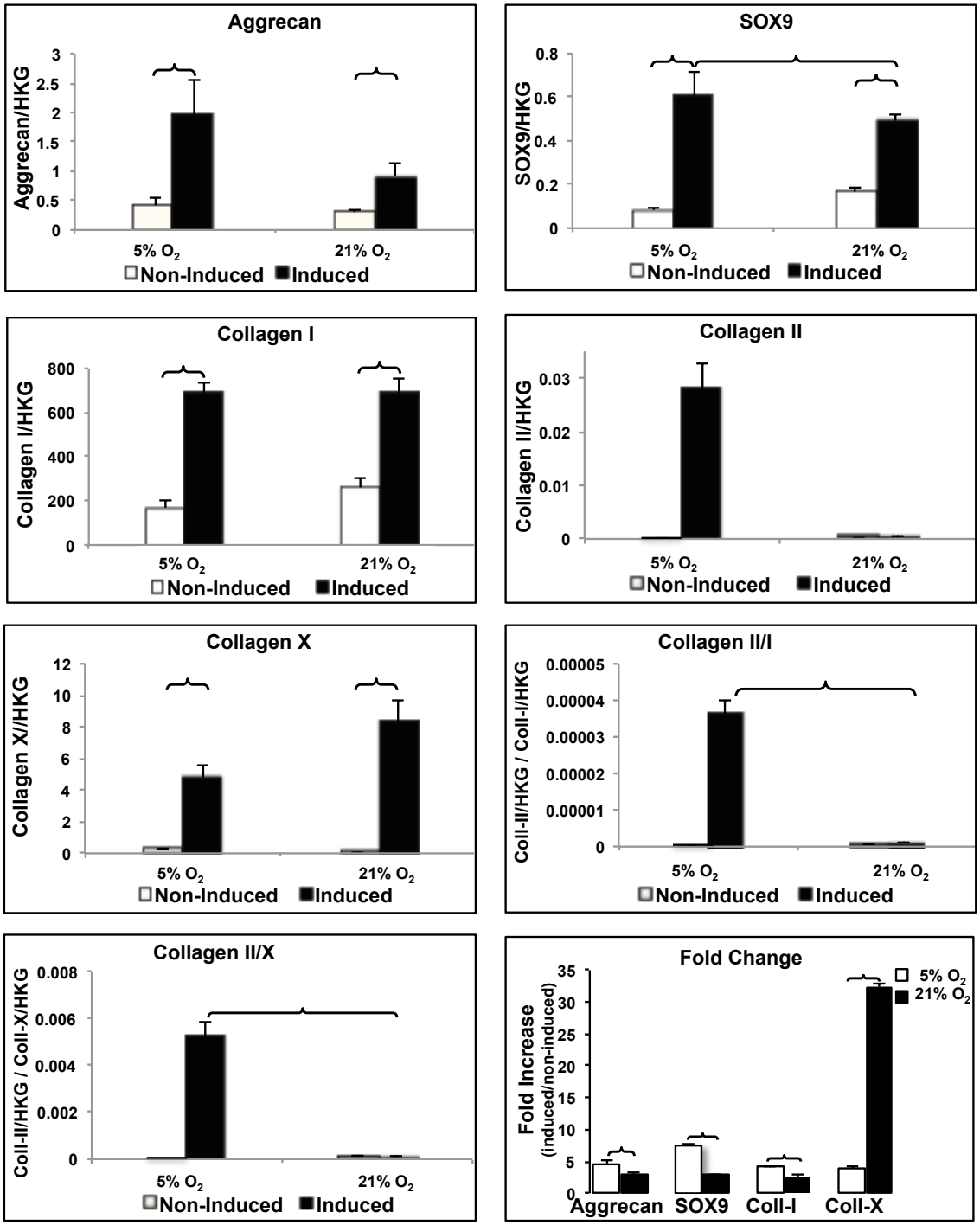

Figure 17 qPCR and adjusted fold change of chondrogenic gene markers aggrecan, SOX9, and collagens I, II and X in scaffold cultures of AT-MSCs. Scaffold cultured in either 5% or 21% O_2, both in control medium (white bars) or chondrogenically induced medium (black bar). Control-adjusted fold change is not reported for collagen II due to no mRNA expression in non-induced cultures. Values shown represent means ± SEM (n=4 donors). Brackets indicate significant difference between columns ($p<0.05$).

5.3 Study II

MLPC pellet cultures

Similar to AT-MSC pellet cultures (I) chondrogenic induced MLPC pellet cultures increased in size compared with control pellets which. Induced pellets were spherical with a diameter of approximately 2.0 mm and had a smooth yellow surface compared with non-induced control pellets, which were fragile and in a state of disintegration. Pellets cultured in chondrogenic induction medium supported the deposition of proteoglycan. The deposition was further facilitated by culturing in 5% O_2 as assessed from analysis of Alcian blue-stained sections (Figure 18), i.e., the morphology of cells in 5% O_2 cultures more closely resembled that of chondrocytes compared with 21% O_2 (i.e., cell residing within lacunae surrounded by matrix) (Figure 18). Cells and proteoglycan were depositions as a function of the oxygen tension, i.e., cells and matrix were located more peripherally in 5% O_2 than in 21% O_2 cultures in which cells were located centrally. Pellets cultured in non-inductive medium did not stain positive for proteoglycans and displayed decreased cellularity with pyknotic nuclei in H&E-stained sections (Figure 18).

Higher mRNA gene expressions were observed in induced pellets than in non-induced pellets, i.e., mRNA expressions of aggrecan ($p<0.01$), SOX9 ($p<0.01$), collagen type I ($p<0.05$), collagen type X ($p<0.01$) and CD-RAP ($p<0.01$) were significantly increased in induced 5% O_2 pellet cultures compared with non-induced control pellets (Figure 19). Similar observations were made in 21% O_2 for induced pellet cultures.

No differences were observed between induced 5% O_2 and 21% O_2 cultures, i.e., no-differences in mRNA expressions for aggrecan ($p<0.13$), SOX9 ($p<0.11$), collagen type I ($p<0.35$), collagen type X ($p<0.14$), CD-RAP ($p<0.48$) and collagen type II ($p=0.89$) for 5% O_2 induced pellet cultures compared with 21% O_2 cultures.

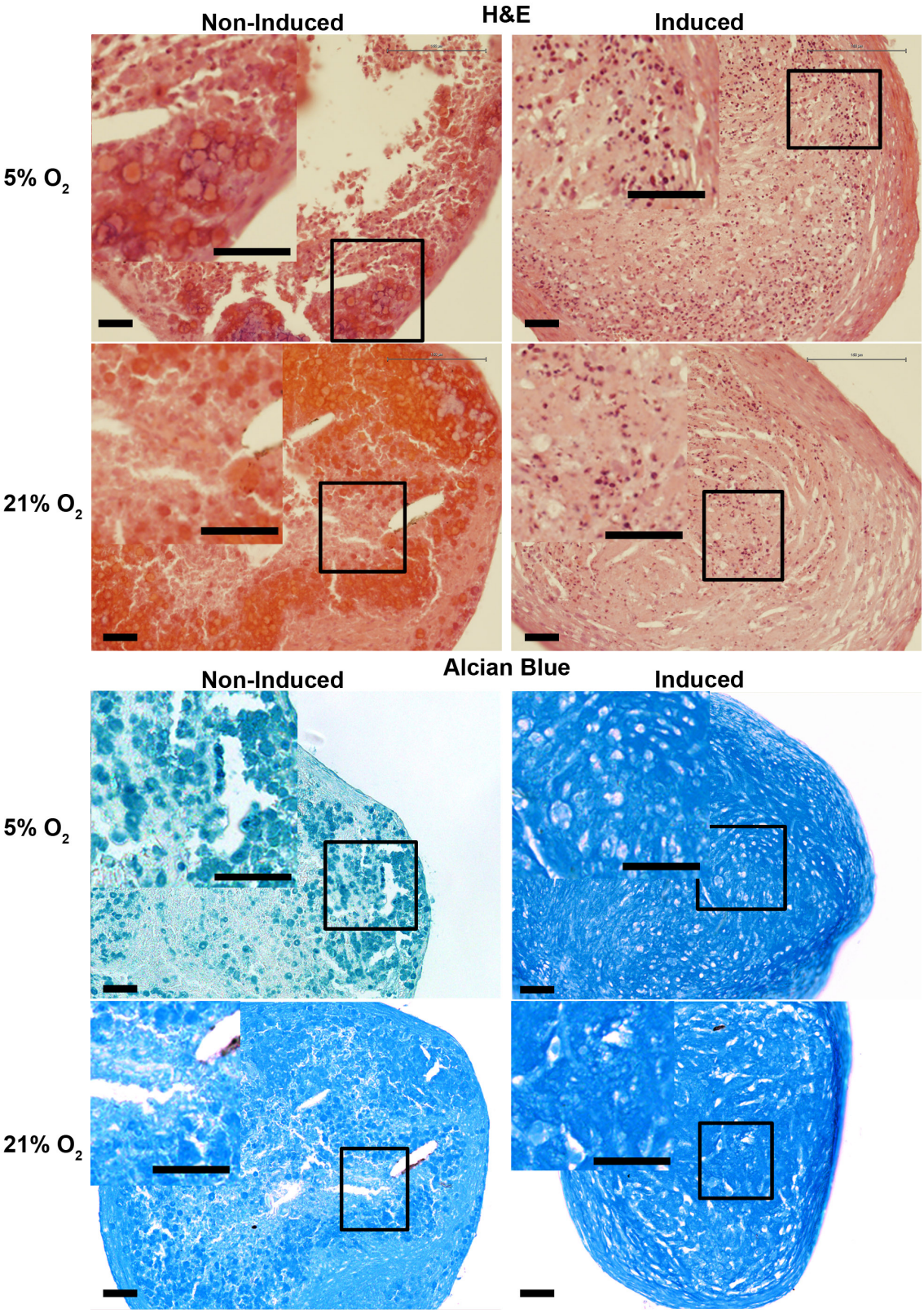

Figure 18 Histological appearance in pellet cultures of MLPCs. MLPCs were cultured in 5% O_2 and 21% O_2 in non-inducing control and chondrogenic inducing medium. Sections stained with H&E (upper panel) and Alcian Blue (lower panel). Scale bar indicates 50 µm.

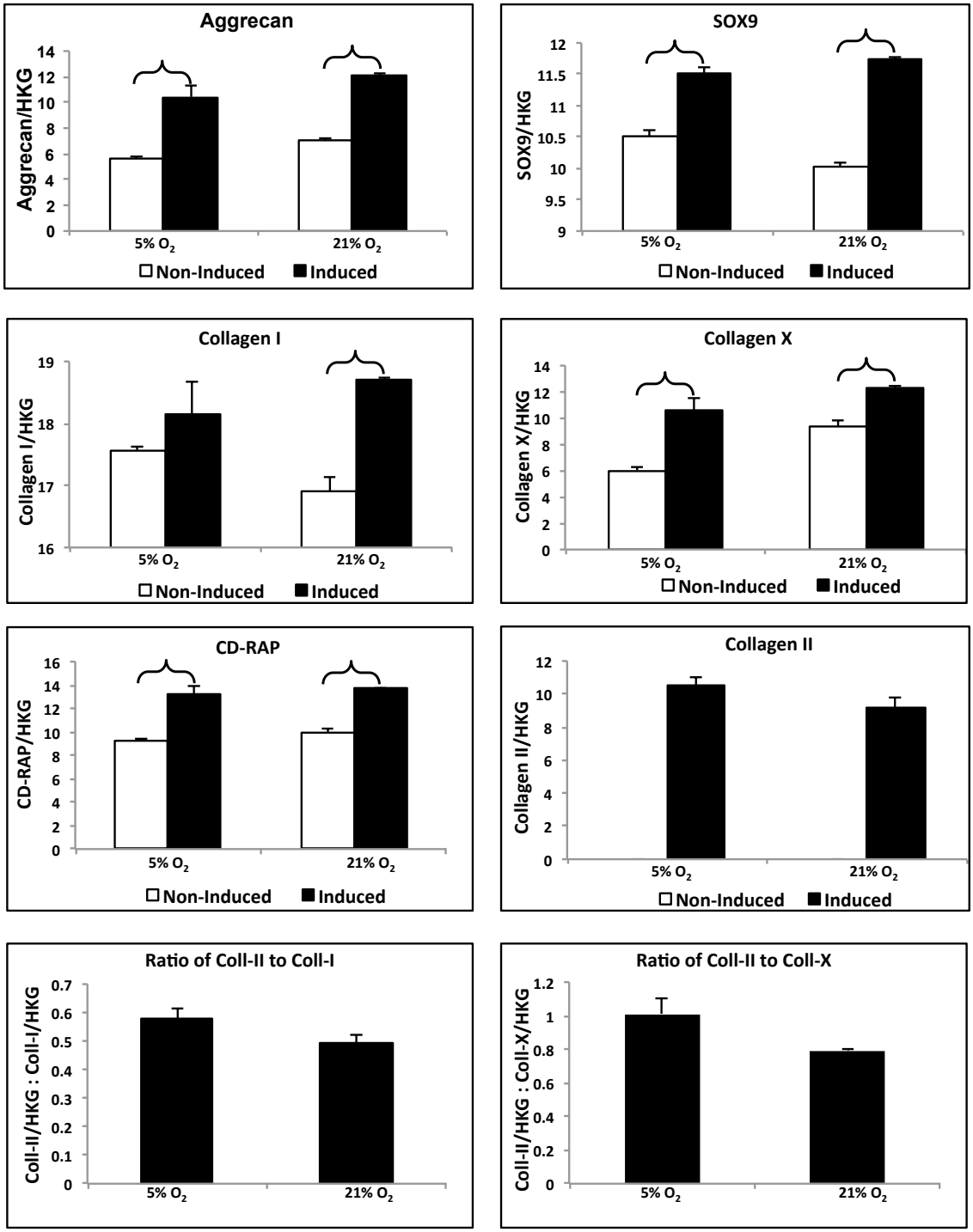

Figure 19 qPCR of chondrogenic gene markers aggrecan, collagen I, collagen II, collagen X, SOX9 and CD-RAP in pellet cultures MLPCs. Pellets cultured for four week in 5% or 21% O_2 in non-inductive control medium (white bars) or chondrogenic inductive medium (black bar). Values shown represent means ± SEM, n=3. Brackets indicate significant difference between columns ($p<0.05$).

Induced pellets showed significantly increased accumulation of sGAG compared with non-induced pellets in both 5% O_2 and 21% O_2 cultures ($p<0.01$) (Figure 20A). A significantly higher level of sGAG synthesis was observed in induced pellets cultured in 5% O_2 than in pellets cultured in 21% O_2 ($p<0.05$).

CD-RAP protein determination on serially collected conditioned culture medium showed detectable CD-RAP from day 14 in induced pellets and at day 28 in control pellets (Figure 20B). For 5% O_2-induced pellet cultures, CD-RAP concentrations increased significantly from day 14 to 28 ($p<0.05$). A similar trend in temporal CD-RAP increase was observed for induced pellets cultured in 21% O_2 ($p<0.05$). 5% O_2-induced pellets showed increased CD-RAP concentrations compared with 21% O_2-induced pellets both at day 14 ($p<0.01$) and day 28 ($p<0.05$), respectively.

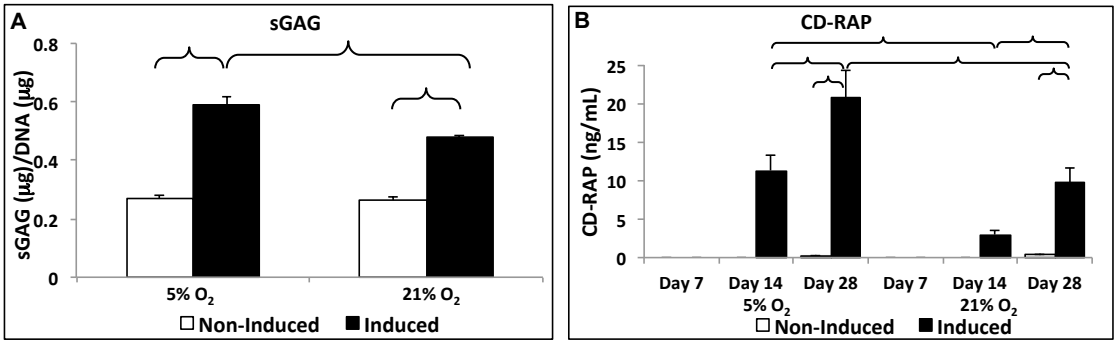

Figure 20 Accumulation of sGAG/DNA (A) and secreted CD-RAP (B) in pellet cultures of MLPCs. Pellets were cultured in either 5% O_2 or 21% O_2 in non-inductive control medium (white bars) or chondrogenic inductive medium (black bar). Values shown represent mean levels ± SEM, n=3. Brackets indicate significant difference between columns ($p<0.05$).

MLPC scaffold cultures

Tissue preparation for the scaffold histology lead to PCL dissolving that precluded optimal histological assessment. With this caveat, histology images of the cell-scaffold-constructs are provided in Figure 21A. Both FDM and SGS scaffolds supported chondrogenic differentiation of MLPCs and deposition of proteoglycans (Figure 21A). The cells were heterogeneous distributed in the scaffolds and were primarily located at the superficial scaffold regions and adherent to scaffold fibres. It is noted that cells were more dispersed in the SGS (Figure 21A). In between the fibres clustering of cells and deposition of proteoglycans is observed. In SEM cells spanning PCL fibre and FD-PCL could be demonstrated (Figure 21B).

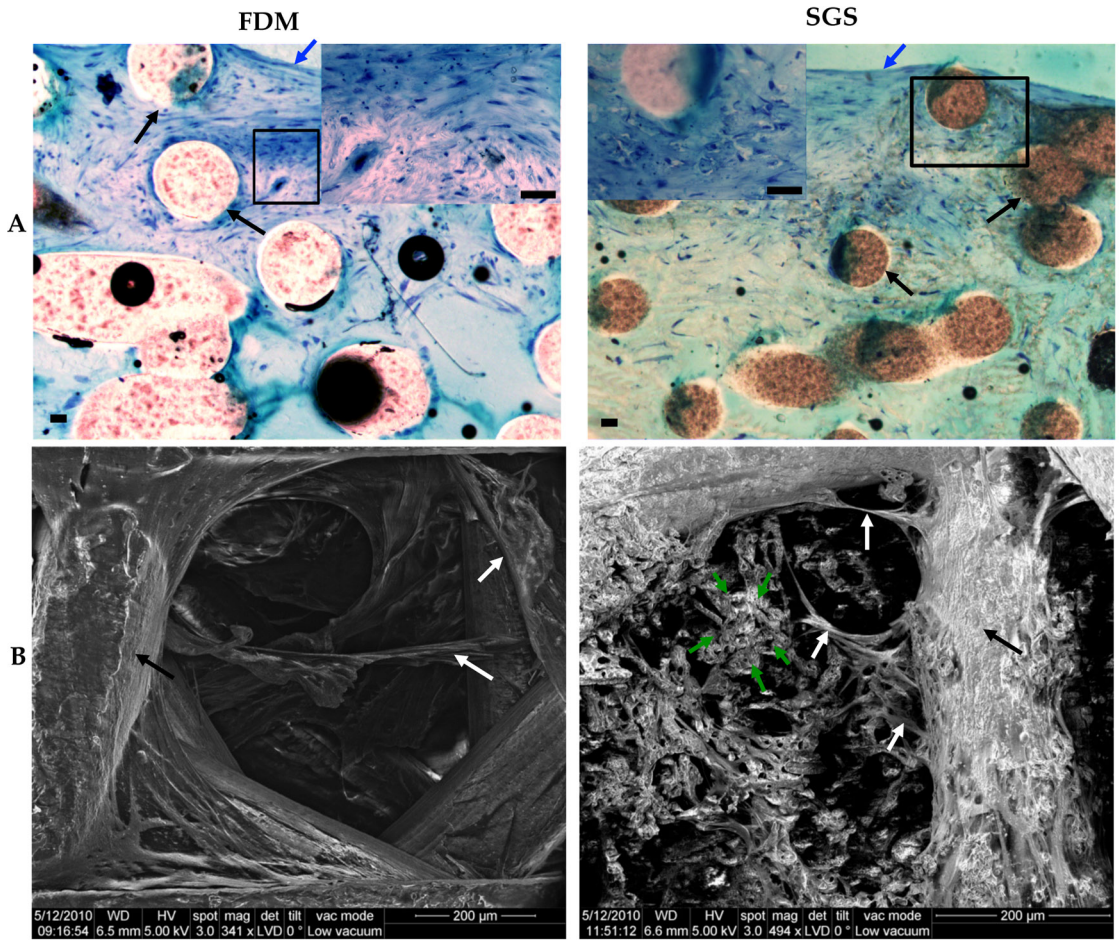

Figure 21 Panel A: Alcian blue sections for chondrogenically induced MLPCs cultured on PCL scaffolds in 5% O_2. Black arrow (↗) Indicates cross-sections of PCL fibre surrounded by cells. In between the fibres clustering of cells and deposition of proteoglycans is observed. Blue arrow (↗) indicates the surface of the scaffold. Please note suboptimal image quality due to laboratory error, see result section for further details. Scale bar indicates 50 µm. Panel B: SEM images for chondrogenically induced MLPCs cultured on PCL scaffolds. Black arrow (↗) indicates PCL fibre. Green arrow (↗) indicates FD-PCL. White arrow (↗) indicates cells spanning over PCL fibre or FD-PCL. FDM, Fused deposition-modelled scaffold. SGS, Structurally graded scaffold.

Some differences in gene expression were noted after 14 days, but on day 28 increased mRNA expressions of aggrecan ($p<0.01$), SOX9 ($p<0.01$), collagen type II ($p<0.01$), collagen type X ($p<0.01$) and CD-RAP ($p<0.05$) were observed in SGS cultures compared with FDM cultures (Figure 22). Collagen type I mRNA expression was similar in the different cell-scaffold groups at day 14 and 28, but on day 28 collagen type I was down-regulated in both cell-scaffold constructs compared with day 14 (FDM, $p<0.01$; SGS, $p<0.01$). Higher ratios of collagen type II/I ($p<0.01$) and collagen type II/X ($p<0.01$) were observed in SGS than in FDM scaffolds. SGS cultures displayed increased collagen type II/I and collagen type II/X ratios at day 28 compared with day 14 ($p<0.05$).

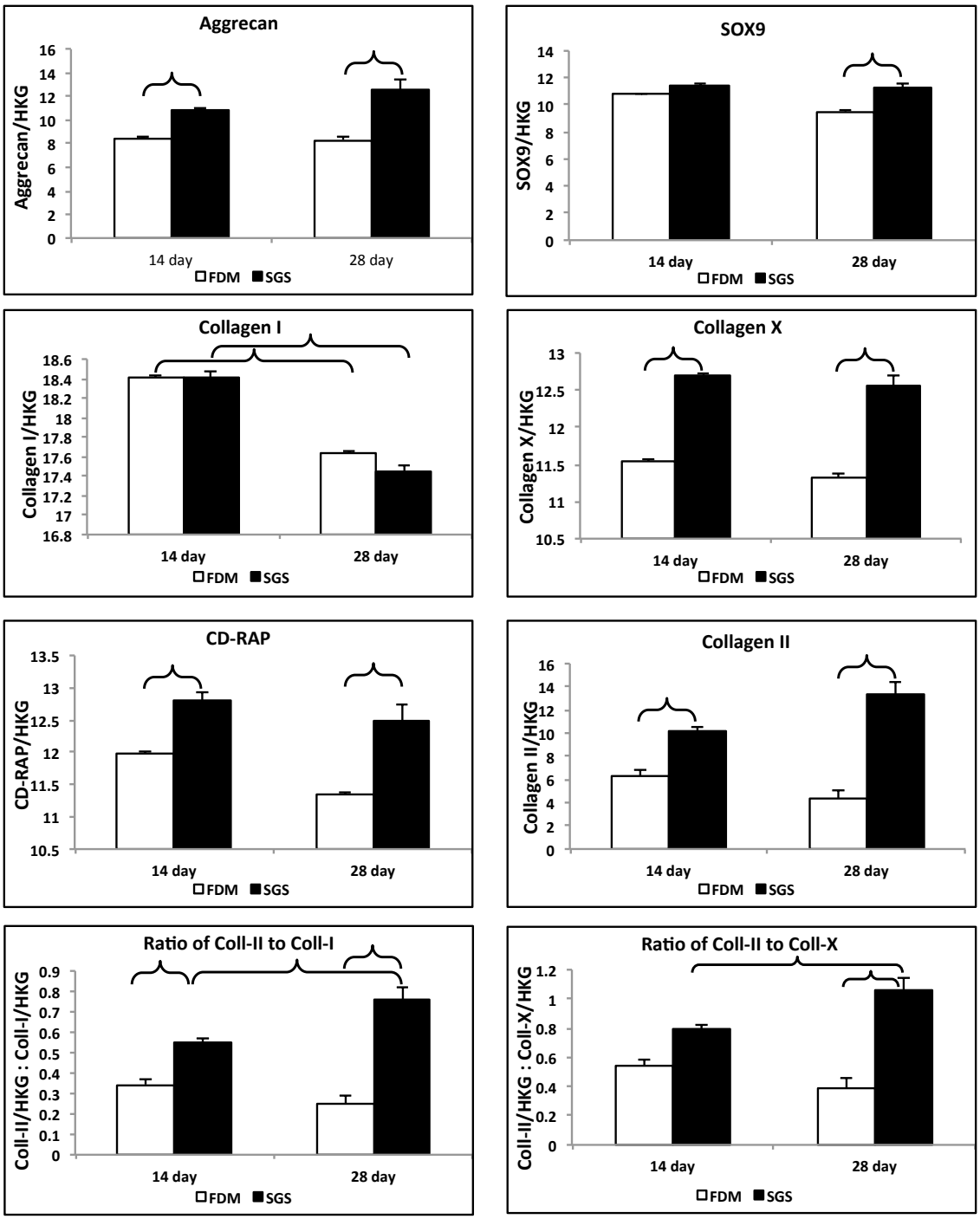

Figure 22 qPCR of chondrogenic gene markers aggrecan, collagen I, collagen II, collagen X, SOX9 and CD-RAP of MLPCs cultured on scaffolds at 5% O_2 in chondrogenic induction medium. FDM, Fused deposition-modelled scaffold (white bars). SGS, Structurally graded scaffold (black bars). Values shown represent means ± SEM, n=3. Brackets indicate significant difference between columns ($p<0.05$).

sGAG accumulation was significantly higher in MLPC-loaded SGS cultures than in FDM cultures ($p<0.05$) (Figure 23A). sGAG accumulation increased significantly in SGSs from day 14 to day 28 ($p<0.05$). CD-RAP could be detected from day 7 (Figure 23B). Over time (day 7, 14, 28), CD-RAP secretion significantly increased for both FDM and SGS cultures ($p<0.01$ and $p<0.01$, respectively). No significant differences in

CD-RAP concentrations between FDM and SGS-based cell-scaffold constructs were noted.

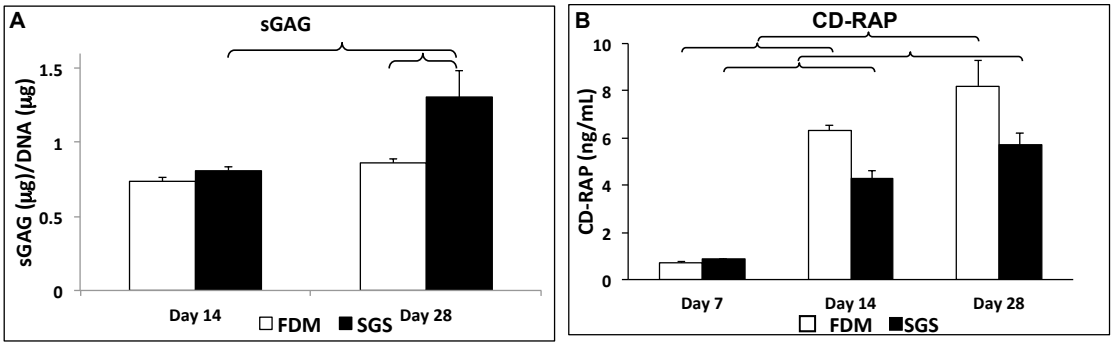

Figure 23 Accumulation of sGAG (A) and secreted CD-RAP (B) in scaffold cultures of MLPCs. Scaffolds were cultured at 5% O_2 in chondrogenic inductive medium in FDM (fused deposition-modelled scaffold; white bars) or SGS (structurally graded scaffold; black bars). Values shown represent mean levels ± SEM, n=3. Brackets indicate significant difference between columns ($p<0.05$).

5.4 Study III

Macroscopic and microscopic appearance of MLPC cultures

During the two weeks of chondrogenic differentiation of MLPCs, the tissue formed on CPP progressively detached from the edges of the CPP, i.e., the MLPCs formed a convex accumulation of tissue located centrally that had a smooth yellow surface with a gelatinous texture (Figure 24A&F). Non-coated membrane cultures maintained uniform covering the membrane, i.e., MLPCs produced an ivory-whitish disc-shaped tissue whose thickness was uniform (≈1.0 mm and 12.0 mm in diameter). The disc was easily removed intact from the membrane support (Figure 24B&G). Inversely, collagen type I-, II- and IV-coated membrane cultures progressively contracted and detached from the membrane producing a tissue whose morphology was non-uniform (Figure 24C-E, H). Contraction from the membrane support was most prominent for collagen type II-coated membrane cultures, which showed complete contraction and detachment after five days of culturing (Figure 24D1&D2). All collagen-coated membrane cultures contracted and detached from the membrane support during the two weeks of culture (Figure 24H). Tissue from coated membrane cultures was smooth, yellow and had a dense texture.

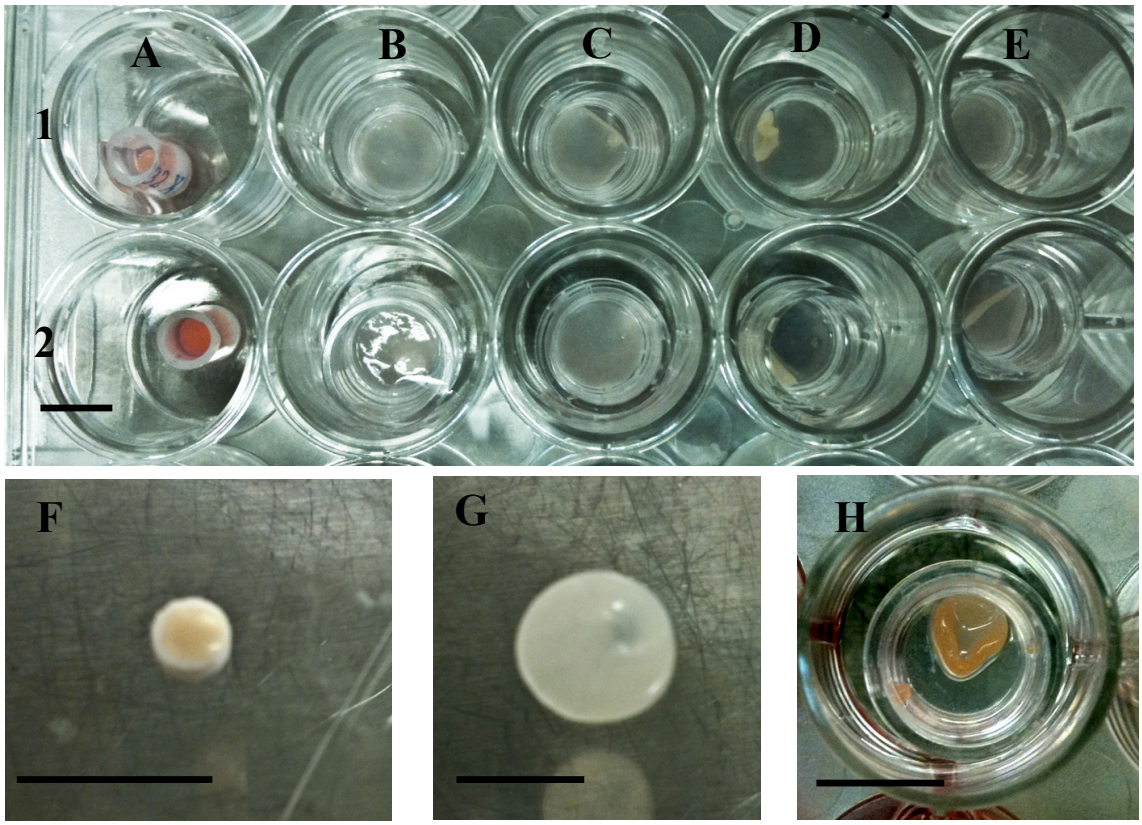

Figure 24 Macroscopic tissue constructs of 3D MLPC cultures on day 5 (A-E) and day 14 (F-H). Row 1 and 2 represent biological replicas of A-E. MLPCs cultured on CPP (A, F), non-coated membrane inserts (B, G), collagen type I-coated membrane inserts (C), collagen type II-coated membrane inserts (D) and collagen type IV-coated membrane inserts (E, H). Scale bar indicates 10 mm.

Pellet cultures of MLPCs showed cellularity throughout the pellet, and cells demonstrated different degrees of chondrocyte-like morphology. The metachromasia in toluidine blue-stained sections indicating secretion of proteoglycans was primarily located peripherally in pellets (Figure 25A & Figure 26A). In contrast, CPP cultures had a more spindle-shaped cell morphology, had a decreased level of cellularity and metachromasia was less intense (Figure 25B). Non-coated membrane cultures formed a tissue disc, which upon histologic evaluation displayed a multicellular-layered tissue of approximately 20 cells in thickness. Cellularity and ECM were abundant and uniformly distributed throughout the disc. Cells in the intermediate layer had round chondrocyte morphology, whereas the culture medium and membrane interphases contained cells with a more flattened morphology (Figure 25C). Collagen type I-, II- or IV-coated membrane cultures all displayed a similar pattern of matrix deposition. The most intense staining was observed for collagen type IV cultures and the least for collagen type I cultures, while collagen type II cultures were intermediate in terms of matrix staining (Figure 25D-F).

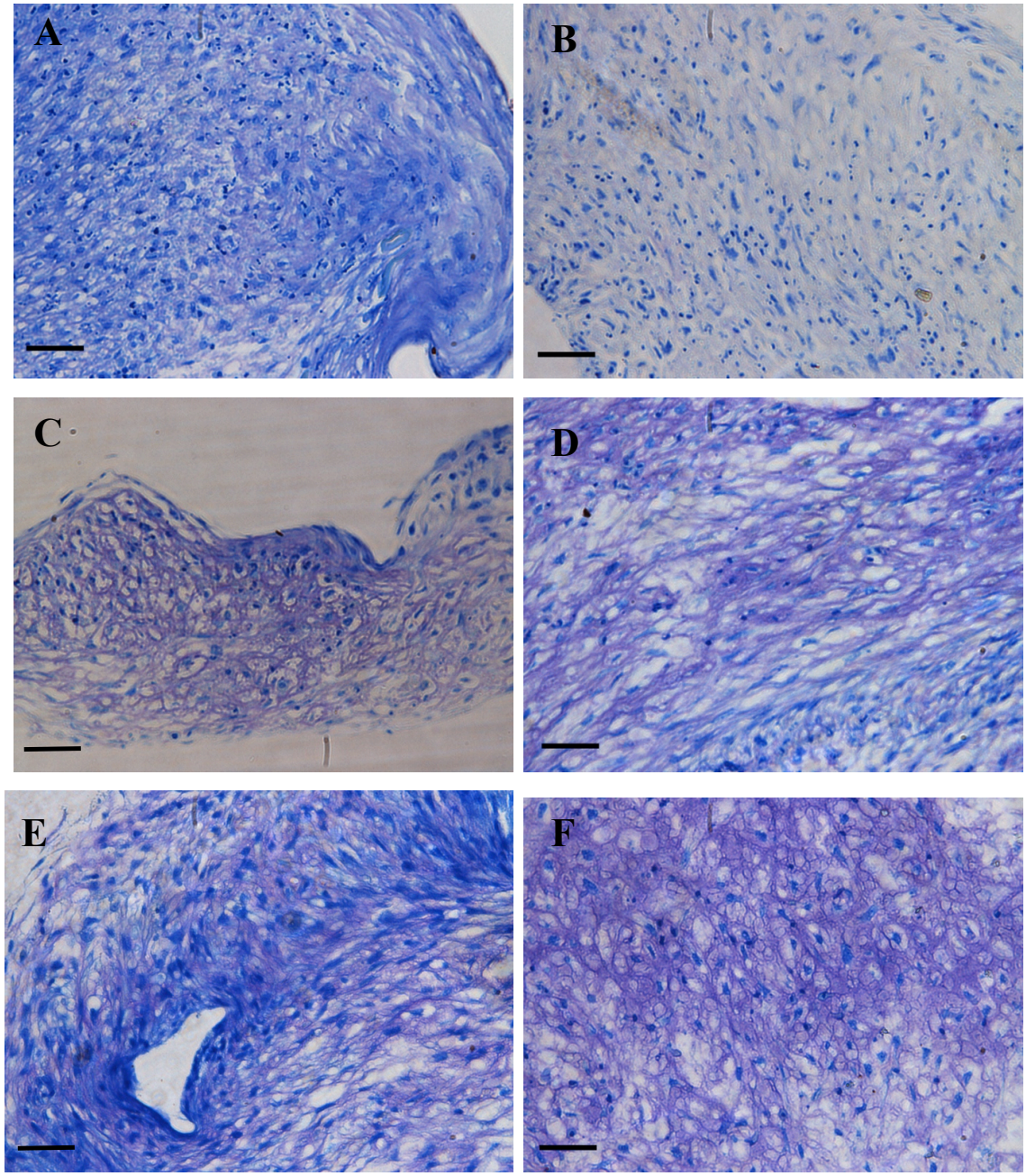

Figure 25 Histological distribution of sGAG in toluidine blue-stained sections for MLPCs cultured as pellet (A), on CPP (B), non-coated membrane insert (C), collagen type I-coated membrane insert (D), collagen type II-coated membrane insert (E) and collagen type IV-coated membrane insert (F). Scale bar indicates 50 µm.

Figure 26B demonstrates that MLPCs pellets cultured in medium supplemented with a double dose of TGF-β3 (20 ng/mL) were larger in size, had more uniformly distributed sGAG and a more pronounced intensity of metachromasia than pellets cultures in standard dose TGF-β3 (10 ng/mL) (supplementary data).

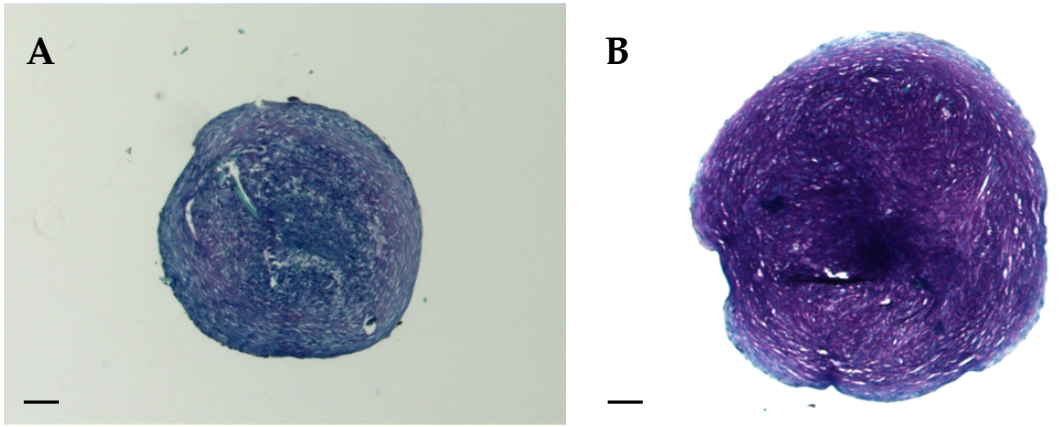

Figure 26 Distribution and intensity of sGAG in toluidine blue-stained sections for MLPC pellets cultured in medium supplemented with (A) 10 ng/mL TGF-β3 and (B) 20 ng/mL TGF-β3. Uniform distribution and more intensive metachromasia for sGAG was observed in pellets cultured in 20 ng/mL TGF-β3 compared with pellets cultured in 10 ng/mL TGF-β3. Scale bar indicates 100 µm.

Gene expression in MLPC cultures

Aggrecan, SOX9 and collagen type II mRNA expression was increased in membrane cultures compared with pellet and CPP cultures (Figure 27, $p<0.05$). Aggrecan expression was significantly increased 26-fold in non-coated membrane cultures compared with pellet and CPP cultures ($p<0.05$). Likewise, aggrecan expression was significantly increased in non-coated membrane cultures compared with collagen-coated membrane cultures ($p<0.05$). Non-coated membrane cultures displayed the most pronounced aggrecan difference in the form of a 22-fold increase ($p<0.05$) compared with collagen type II-coated membrane cultures, while the difference compared with type I- and type IV-coated membrane cultures was less outspoken (2-fold; $p<0.05$). Significant differences in aggrecan expression were also found when mutually comparing the coated membrane cultures. The most elevated mRNA expression was seen in type IV-coated membrane cultures followed by type I- and type II-coated cultures ($p<0.05$).

Collagen type II mRNA expression had a profile similar to that of aggrecan. Membrane cultures showed increased collagen type II mRNA expression compared with pellet and CPP cultures, and the expression was 20-fold higher in non-coated membrane cultures ($p<0.05$). Collagen type II was most profoundly expressed in non-coated membrane cultures, followed in descending order by collagen type IV, collagen type I, pellet, CPP and collagen type II-coated cultures.

Transcriptional factor SOX9 mRNA expression was increased in membrane cultures compared with pellet and CPP cultures ($p<0.05$). Collagen type IV-coated cultures displayed a significant 7-fold increase compared with CPP cultures ($p<0.05$), but with a 5-fold increase in non-coated and collagen type I-coated membrane cultures ($p<0.05$).

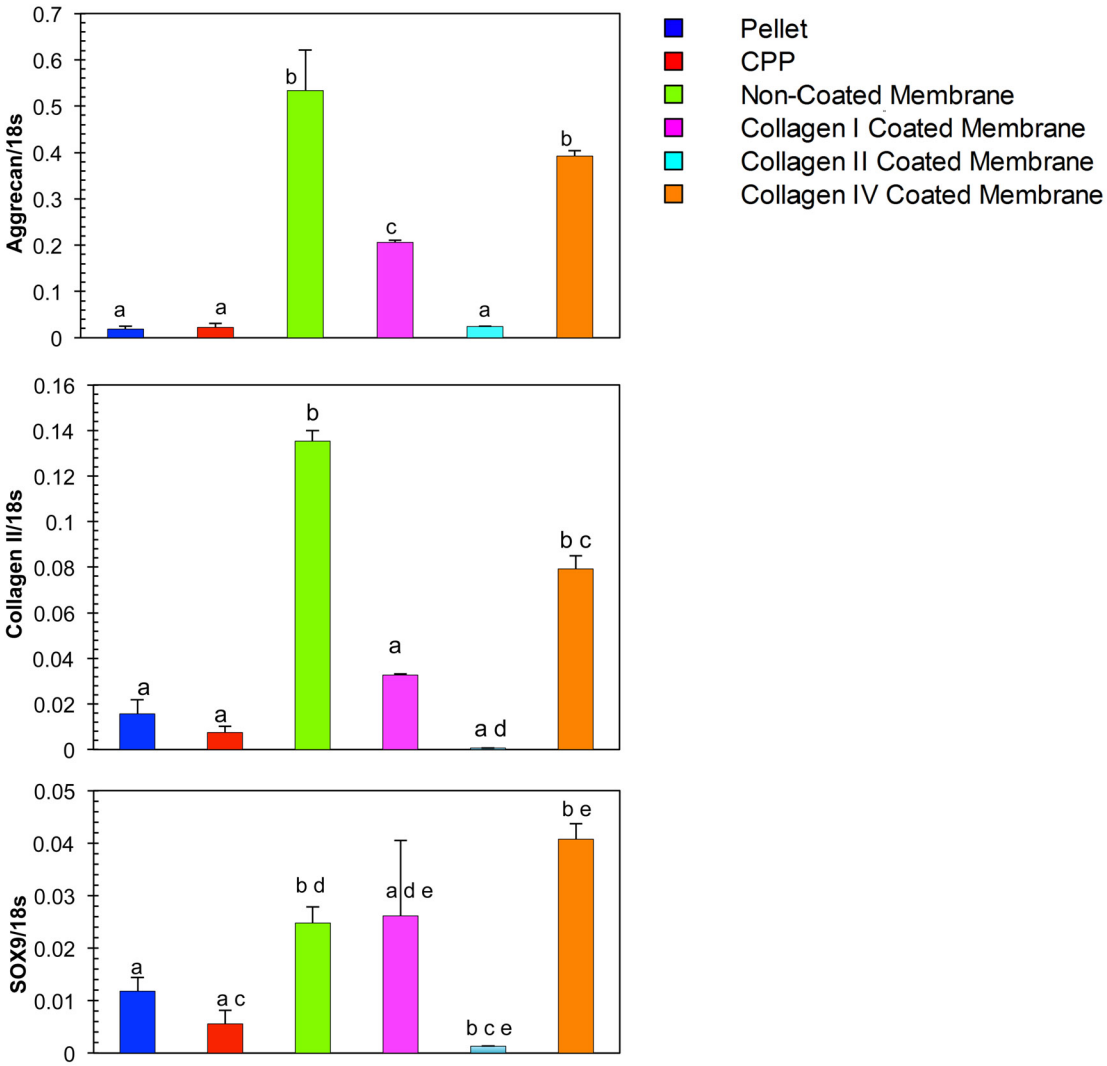

Figure 27 Gene expression for chondrogenic markers (aggrecan, collagen type II and SOX9) in tissue derived from MLPCs cultured as pellets (blue bars), on CPP (red bars), non-coated (green bars), collagen type I (purple bars), collagen type II (turquoise bars) and collagen type IV (orange bars) coated membrane inserts. Data shown as means ± SEM, n=3. Bars not sharing same letter indicate a significant difference ($p<0.05$).

Proteoglycan and collagen production in MLPC cultures

The amount of accumulated sGAG was significantly higher in membrane cultures than in pellet and CPP cultures ($p<0.05$) (Figure 28A). The amount of accumulated sGAG was significantly (i.e. 4-fold) higher in non-coated cultures compared with pellet and CPP cultures ($p<0.05$). Non-coated membrane cultures deposited the highest level of sGAG, followed in descending order by collagen type IV, collagen type I, collagen type II, pellet and CPP. No difference in proteoglycan production was observed between pellet and CPP cultures.

Likewise, hydroxyproline synthesis was significantly (i.e. 2-fold) higher in non-coated membrane cultures than in coated membrane and CPP cultures ($p<0.05$) (Figure 28B). No difference in hydroxyproline deposition was observed when collagen-coated membrane cultures were mutually compared to each other.

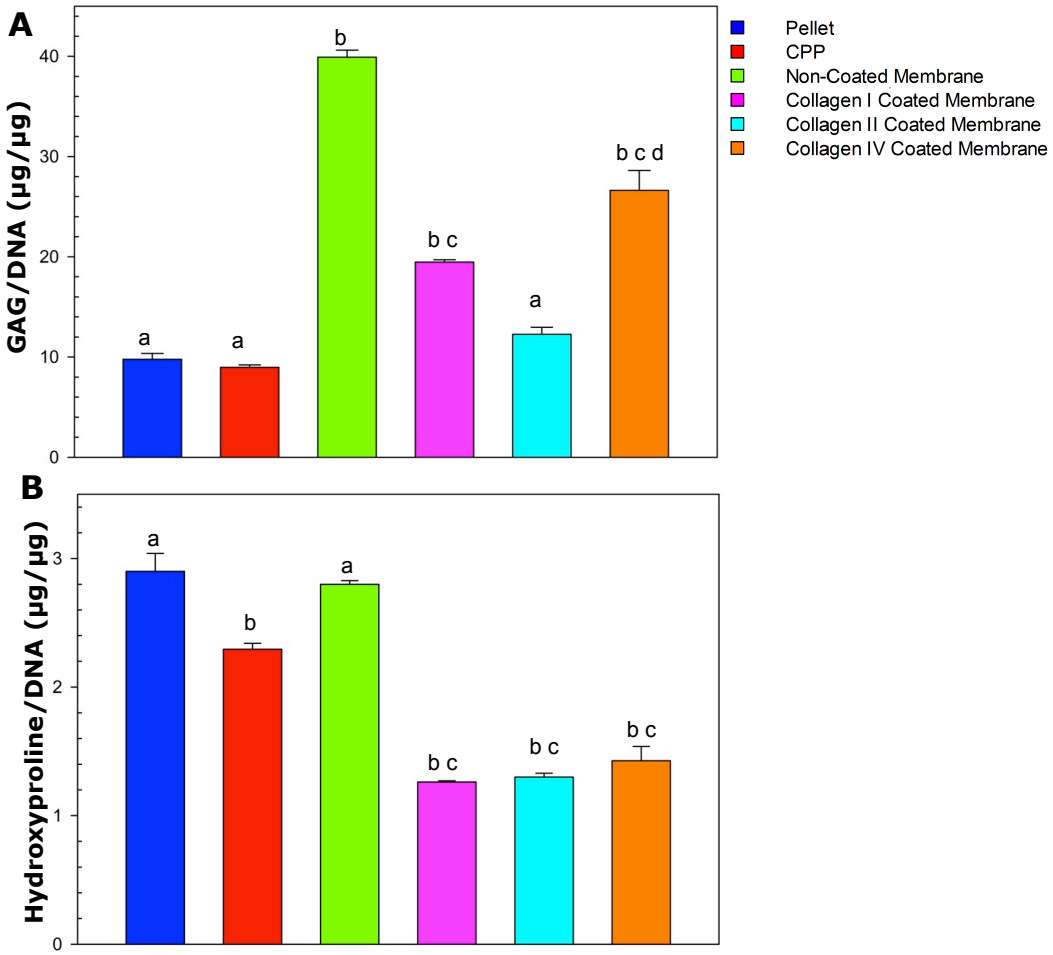

Figure 28 Accumulation of ECM in tissue derived from 2 weeks of culture of MLPCs as pellets (blue bars), on CPP (red bars), non-coated (green bars), collagen type I (purple bars), collagen type II (turquoise bars) and collagen type IV (orange bars) coated membrane inserts. (A) sGAG and (B) hydroxyproline. Data shown as means ± SEM, n=3. Bars not sharing same letter indicate a significant difference ($p<0.05$). GAG, glycosaminoglycan.

6 Discussion

In this thesis, my initial aim was to develop an MSC-based cartilage implant and to evaluate its use in the treatment of cartilage defects in an equine animal model. I wanted to utilise MSCs because of their promising clinical potential in terms of the ease with which they may be isolated, the efficiency with which they may be expanded and, most importantly, their capability to differentiate towards multiple phenotypes, including chondrocytes. The studies were designed to investigate whether AT-MSCs and MLPCs have a future role to play in the clinical treatment of chondral injuries. However, as outlined in section "4.1 Design overview and approach" and Table 1, I encountered numerous problems and therefore had to change the study design in the three described studies. The studies are different from each other and they share only few elements in materials and methods. The studies of the present thesis should therefore be seen as separate studies and their results will accordingly first be discussed separately; this is followed by a discussion of general issues that cut across the separate studies.

6.1 Study I

Exposure to 5% O_2 combined with culture in chondrogenic medium stimulated the chondrogenesis in AT-MSC pellets and AT-MSC-seeded collagen I/III scaffolds. I found that AT-MSC pellet cultures had up-regulated mRNA expression of cartilage-related genes aggrecan, SOX9, collagen type II and down-regulated mRNA expression of collagen type X in 5% O_2 compared with 21% O_2 culturing. These observations are consistent with the findings by Betre et al. who demonstrated increased collagen type II and SOX9 expression in AT-MSCs embedded in elastin hydrogels cultured in 5% O_2 [217]. The increased mRNA expression of collagen type II, ratios of collagen II/I and II/X, and decreased collagen X suggest that the formed tissue is neither fibrocartilage nor hypertrophic cartilage.

The contraction/curling of the scaffolds is obviously not a favourable feature when making a clinically transplantable cartilage construct. Other studies on the collagen type I/III scaffold report no similar findings; e.g., Scherer et al. showed that this scaffold has a chondrogenic potential when BM-MSCs are differentiated in 5% O_2 [67]. The same normoxia trend is reported in the work by Dickhut et al. [123]. Furthermore, work from our group confirms that the collagen type I/III scaffold remains unchanged when cultured with primary human chondrocytes [47]. In spite of this, comparison is difficult because other cell sources such as human BM-MSCs and mature bovine chondrocytes were investigated.

A lack of cross-linked collagen and presence of TGF-mediated α-smooth muscle actin may explain contraction of the scaffold cultures. For further elaboration, please refer to Section "6.4 General discussion", p.67.

6.2 Study II

This study confirmed that MLPCs have chondrogenic potency as also previously reported [200]. Furthermore, it was shown that chemical induction of MLPCs in pellet culture directed towards the chondrogenic cell fate is enhanced under reduced oxygen tension (5% O_2) compared with normoxic oxygen tension (21% O_2) as demonstrated by the chondrocyte-like morphology, the increased ECM production, the increased CD-RAP concentration and the up-regulated gene expression of collagen type II and the down-regulated gene expression of collagen type I and X. Importantly, the decreased gene expression of collagen type I and X suggests that the formed cartilage is not fibrocartilage or hypertrophic cartilage, respectively. Transcriptional factor SOX9 and CD-RAP expressions were decreased in 5% O_2 culturing, which is in accordance with observations of BM-MSCs cultured in hypoxia [20].

Gene expression of collagen type II/I and collagen type II/X ratios increased in 5% O_2-induced pellets compared with 21% O_2-induced pellets; however this was insignificant. Both of these two ratios provide indications that the chondrocytic phenotype of MLPC is favoured in a reduced oxygen environment. My finding of an increased collagen type II/I ratio in 5% O_2 is supported among others by Kurtz and colleagues (2004) who reported that mature chondrocytes cultured on a collagen type I/III scaffold showed increased collagen type II/I ratios in 5% O_2 culturing [63].

Another main finding of Study II was that culturing in 5% O_2 supported MLPC chondrogenesis better in cell-loaded SGS cultures than in FDM scaffold cultures. Higher mRNA expression levels were observed for aggrecan, collagen type II, SOX9 and CD-RAP in MLPS-loaded SGS cultures than in FDM scaffold cultures. I also demonstrated a time-dependent down-regulation of collagen type I in both types of scaffold constructs, which suggests cartilage maturation over time. The occurrence of cartilage maturation is supported by the increased gene expression of aggrecan, collagen type II and SOX9 in the SGS cultures from day 14 to day 28. The finding of increased collagen type II/I and collagen type II/X ratios in the SGS scaffolds compared with FDM scaffolds also indicate that SGS provides a more chondrogenic environment for the MLPCs.

I was unable to demonstrate that an increased sGAG accumulation in pellets cultured in 5% O_2 was followed by a corresponding increase in aggrecan and CD-RAP gene expression. It is possible that a low oxygen tension more quickly induces an initial aggrecan and CD-RAP up-regulation, thus accelerating sGAG and CD-RAP synthesis. I

believe that the increased protein synthesis then triggers gene down-regulation through a negative feedback loop mechanism. The opposite is observed in 21% O_2, i.e., aggrecan and CD-RAP gene expression levels were higher, but the downstream translated products were significantly lower than in 5% O_2. In this case, aggrecan and CD-RAP gene may still be up-regulated because the amount of protein had not reached the threshold levels that trigger the feedback loop. However, I do not have data on qPCR or quantification of ECM for earlier time points.

In spite of an increased mRNA expression of CD-RAP in SGS scaffolds, there was no significant difference between FDM and SGS scaffolds in terms of concentrations of CD-RAP released into the media. A probable reason for this discrepancy is that much less CD-RAP can be transported from the inside the SGS scaffold to the sampled media due to the tighter porous network, i.e., the denser porous structure limited diffusion of CD-RAP to a greater extent in SGS than in FDM scaffolds. In contrast to this, I detected higher deposition of glycosaminoglycans within the SGS compared with FDM scaffolds and this was followed up by a corresponding increase in aggrecan mRNA expression suggesting that the SGS provides a more chondrogenic environment for the chondrogenic MLPC differentiation. Furthermore the positive correlation in the measured sGAG to mRNA levels of aggrecan versus inverse correlation in measured CD-RAP concentration to mRNA levels of CD-RAP is justified by the differences in data collection, e.g., CD-RAP was measured by media collection in which the diffusion is compromised by the porous network while glycosaminoglycans was measured in the extract of papain digested scaffolds.

6.3 Study III

The focus in Study III was to learn and evaluate new scaffolds/methods and to a lesser extent to characterize and investigate cell biology. The chosen MLPC cell line was clonal-derived and would in theory allow for less cell variability than more non-clonal heterogeneous cultures such as the AT-MSC in Study I. This would in turn, allow more robust comparison between scaffold materials. Although a less than expected chondrogenic potency of MLPC was observed in Study II, the same cell source was maintained for Study III since it is recognized that cell culture environment can greatly influence cell behaviour. For instance, minor changings in microenvironments such as the choice upon expansion culture medium, differentiation medium, and culture method (e.g. pellet vs. membrane culture) significantly alters the chondrogenic potential of MSCs [196, 218, 219]. For instance, Lee et al. demonstrated that ovine BM-MSCs cultured on collagen-IV coated membranes had higher levels of sGAG compared with pellet cultures [138], while Buechli et al. demonstrated higher chondrogenesis in fibronectin-coated membrane cultures of equine CB-MSCs compared with pellet

cultures [220]. Finally, the MLPCs used in Study II&II are a clonally expanded cell line with a well-defined cell characterization (including minimum criteria defining MSCs [105]) which eliminated the uncertainty in culturing subpopulations of heterogeneous cells with decreased chondrogenic potential as used in Study I. For further elaboration, please refer to "Table 1, p. 23" and sections: "Growth factors, p. 68" and "MSC for in vitro cartilage tissue engineering, p. 71".

Membrane cultures facilitated the chondrogenic differentiation of MPLCs; and to my surprise, the non-coated membrane cultures performed better than the collagen-coated cultures in terms of no contraction and higher levels of sGAG and hydroxyproline. This is in contrast to my expectations since the non-coated membrane lacks natural ligands for cell-ECM-interaction to enhance cell adhesion and regulation of cellular proliferation, function and differentiation [56, 57, 191]. The functionalization of synthetic biomaterials in terms by coating with natural ECM components such as hyaluronan and collagen has proven to enhance chondrogenic differentiation of MSCs [85, 86]. Lee and colleagues demonstrated optimal chondrogenesis of ovine BM-MSCs on collagen type IV-coated membrane inserts which produced a similar disc-shaped cartilage tissue in cultures pre-differentiated for two weeks [138]. The differences between these studies are likely related to differences between species, donor ages, cell sources and *in vitro* isolation and culture methods [107, 108]. But they may also result from differences between the culturing systems, i.e., the semipermeable membrane insert provided a 3D organisation of cells, which promoted the cell-cell contact necessary for MSC chondrogenesis [137]. This allows cells close and easy access to nutrient supply from both above and below. Collagen coated membrane cultures performed better in terms of matrix staining and higher levels of sGAG than pellet and CPP cultures, but tissue contracted and detached from the membrane surface. The unfavourable contraction of the membrane cultures is discussed in section: "6.4 General discussion", p.67.

An enhanced differentiation of MLPCs towards chondroblastic tissue was observed in membrane cultures compared with pellet and CPP cultures. This is reflected by the chondrocyte-like morphology with a multicellular-layered organisation of cells, a 4-fold significant increase in ECM production (increased sGAG and hydroxyproline), and significantly increased aggrecan, collagen type II and SOX9 mRNA expression in membrane cultures. The most pronounced difference was observed in non-coated membrane cultures which synthesised more total sGAG compared with pellet and CPP cultures. Tissue in non-coated membrane cultures was much more uniform, i.e., the distribution and strong staining for proteoglycans was uniform throughout the disc tissue and had correspondingly increased sGAG and hydroxyproline accumulation compared with pellet cultures. The uniformity of the deposited matrix is most likely

caused by the influence of the culture conditions. The more uniform matrix in the discs may promote a more even exposure of differentiation signals to the cells with short diffusion distances from both sides of the multicellular-layered disc tissue. The membrane cultures likely provided better mass transport properties, i.e., the surface area available for nutrient diffusion in the 12.0 mm diameter (and 1.0 mm thick) disc was several fold higher than the 2.0-mm in diameter spherical pellets; and, moreover, the semipermeable membrane ensured tissue nourishment from both above and below.

6.4 General discussion and limitations

Reduced oxygen tension

Hypoxia is believed to trigger the embryonic condensation of mesenchymal cells and the activation of various transcriptional factors resulting in cartilage formation [149]. However, the role of oxygen tension for chondrogenic differentiation of MSCs *in vitro* is incompletely understood. A number of studies support that low oxygen tension favours the chondrogenic phenotype of both mature chondrocytes, AT-MSCs and BM-MSCs [151-153, 155, 162]. Other studies have been unable to demonstrate convincing benefits in hypoxic culturing [157, 165, 166].

Chondrogenesis of AT-MSCs and MLPC cultures was more pronounced in 5% O_2 culturing than in 21% O_2 culturing (I&II) regardless of the culturing system (pellet vs. scaffold culture). The cellularity and the ECM in pellet and scaffold cultures were organised in a zonal manner as a function of the oxygen tension, i.e., cells were located peripherally in 5% O_2 pellet cultures, whereas cells were located more centrally in 21% O_2 cultures. In the scaffold cultures, cells and ECM were located adjacent to the surface and around the scaffold fibres, which demonstrates that differentiation takes place in a gradient-like fashion or in areas with high cell densities. This zonal arrangement of cells and ECM is similar to findings in AT-MSC pellet cultures [142] and ovine BT-MSCs [174]. The zonal organisation of cells and ECM may indicate the existence of an oxygen gradient throughout the pellets and scaffolds, and optimal oxygen tension may be present within this gradient. Due to the effect of the oxygen gradient, some studies have suggested that chondrogenesis in pellet cultures is optimally carried out in 15% O_2 [142] or 10-11% O_2 [174].

Previous work measuring the actual oxygen concentration in cartilage constructs has demonstrated an oxygen gradient ranging from 21% at the surface down to 10% at a depth of 0.5 mm [171]. This oxygen gradient may be more pronounced in the pellet cultures because of the large pellet size (1.5 to 2.0 mm in diameter) and the lack of an open porous network, and this is further exacerbated by the lack of compressive forces to drive the nutrient and oxygen distribution throughout the tissue as observed in normal joint loading. The passive diffusion of the oxygen molecule is theoretically limited to a

maximum of 200 μm, which may explain the absence of cells throughout the pellet and scaffold cultures [157].

The supply of oxygen and nutrients needs to be considered more carefully when differentiation is carried out in scaffolds. Instead of a compacted aggregation of cells, the cell distribution in a scaffold can be much more heterogeneous and may comprise regions with highly confluent cells under pellet-like conditions and regions with more dispersed cells. In the macroporous FDM scaffold (II), oxygen diffusion into the scaffold is most likely more pronounced than in the SGS scaffold. Solely by virtue of its decreased nutrient transport, the SGS scaffold can thus generate hypoxic conditions for the seeded MLPCs that are conducive for chondrogenic differentiation. Likewise, it seems plausible that contraction of the collagen type I/III (I) scaffold and collagen coated membrane cultures (III) promoted hypoxic conditions imitating pellet-like conditions in terms of cell density and zonal oxygen gradient and thus enhanced the AT-MSC and MLPC chondrogenesis.

Growth factors

As mentioned in "Section 3. Background", no scaffolds or MSCs in themselves possess chondrogenic potency: addition of growth factors is essential to trigger MSC differentiation towards the chondroblastic cell lineage in *in vitro* cultures. In Study I-III, dexamethasone and TGF-β3 were supplemented to the culturing medium to induce chondrogenesis of AT-MSCs and MLPC because these biochemicals are the most established growth factors for MSC chondrogenesis.

The importance of growth factors for differentiation has recently been discussed [124]. Because different species, cell types and culturing systems are investigated, the literature often provides contradictory evidence [221]. However, of particular interest are BMP-2, BMP-4 and BMP-6 owing to their natural endogenous expression in the embryonic cartilage development. Ronziere et al. reported decreased hypertrophy of AT-MSCs in 5% O_2 with a combination of BMP-2/TGF-β added to the culture medium [165]. A similar finding of increased BM-MSC chondrogenesis has been reported by others [222]. Guilak's group demonstrated the synergistic effect of BMP-6 on the chondrogenesis of AT-MSCs [112, 223]. The positive effect of BMP-6 is also supported by a lower level of TGF receptors in AT-MSCs than in BM-MSCs that may be compensated for by adding BMP-6 to the culturing medium [224]. It should also be noted that BMP-6 could have a dual role of being both an osteogenic and a chondrogenic inducer. This dual effect is determined by the culturing system where BMP-6 induces alkaline phosphatase (osteogenic cell fate) in monolayer cultures of AT-MSCs and, correspondingly, enhances proteoglycan accumulation in pellet cultures [130]. Finally, parathyroid hormone-like peptide (PtHrP) inhibits the TGF-β-induced hypertrophy (e.g. collagen type X expression) in BM-MSC pellet cultures, i.e., PtHrP

seems to stall the MSC population in the chondrogenic phase by preventing their progression towards the hypertrophic phase [225].

The differentiation medium included no BMPs, but it seems plausible that the chondrogenesis and the cartilaginous appearance of AT-MSCs and MLPCs (primarily in Study I&II) could be improved through the use of selected BMPs. Furthermore, addition of 20 ng/mL (double dose) TGF-β3 (Study III) resulted in a more intensive matrix staining of MLPCs pellet cultures (Figure 26B), which suggests that the optimal chemical cocktail composition for the chondrogenesis of MLPCs has yet to be defined. This is particular important when the aim is to perform chondrogenesis in other conditions than the standard pellet culture. Furthermore, the usage of different compositions of chondrogenic induction medium in the three presented studies may also alter the chondrogenic potential of the studied cell cultures. Please refer to Table 1, p. 23 and section "MSC for in vitro cartilage tissue engineering", p. 71 for further elaboration.

Culturing time

AT-MSCs and MLPCs in the pellet, scaffold and membrane cultures produced cartilage-like tissue based on morphology and matrix formation. But, it did not meet my expectations of maturity, particularly in Study I&II; whereas the most convincing cartilage- and chondrocyte-like tissue in terms of zonal cell organisation and ECM accumulation was achieved in Study III.

According to Liu et al., a stable cartilage phenotype of BM-MSCs is achieved after 8-12 weeks of differentiation [226]. This may explain the weak matrix staining seen in my study, which suggests incomplete chondrogenesis in Study I&II; and it is likely that a longer induction time would have produced more mature tissue. Furthermore, AT-MSCs may have less chondrogenic potential than other sources of MSCs after only four weeks of culturing [113-115].

Based on comparative chondrogenic potency studies of human and equine umbilical cord blood-derived MSCs with that of BM-MSCs and AT-MSCs, I expected the MLPCs to exhibit high chondrogenic potency within the allowed induction time period [118, 119]. Biological variation between donors with respect to chondrogenic potency is a common finding and it is possible that the MLPCs studied were isolated from an individual or primary cell clone with reduced chondrogenic potential. However, this statement is not in line with the results reported in Study III in which membrane cultures from the same MLPC clone produced excellent cartilage tissue in just two weeks. This is most likely explained by the culturing system that allowed high-density and 3D-layered organisation of the cells to maintain the rounded chondrocyte cell shape guiding the MLPCs into the chondrocytic lineage [227, 228]. Additionally, the membrane system provided tissue nourishment from above and below.

Scaffold and surface interaction with MSC

Interaction of cells with scaffolds involves surface contact, which tends to mimic monolayer culture, which inhibits differentiation. Cell shape, cytoskeleton composition and scaffold stiffness can guide MSCs into a specific lineage [227-229]. In this context, MSCs cultured in hydrogels or pellets (i.e. soft substrates) maintain a round cell morphology, which supports chondrogenesis, while cells cultured on stiff substrates tend to adhere and spread on surfaces and to differentiate into osteoblast or smooth muscle cells.

Though I have no data on the hardness of the scaffolds in Study I&II, I speculate that the relatively higher stiffness of the collagen type I/III scaffold compared with that of the pellets modulates the AT-MSC cytoskeleton and increases smooth muscle cell differentiation, which may have led to the contraction of the cell-loaded scaffolds. Likewise, although PCL scaffolds (II) prevented contraction of the scaffold due to their higher stiffness, a round cell morphology was not completely obtained and most of the cells adhered to and were located adjacent to scaffold fibres. This is also reflected in their histology by tissue that focally resembled cartilage.

The 3D membrane cultures (III) enhanced the effect of hydrogels by keeping MLPCs in high-density and assured 3D alignment of the cells, which supported the chondrogenesis as demonstrated by the tissue resembling the zonal configuration of cartilage in terms of cell alignment and ECM deposition.

Indention test (test of material hardness), cell surface phenotyping and qPCR for additional markers such as smooth-cell, osteogenic cells (e.g. actin, myosin, Runx2, COMP, IHH, PTHrP and alkaline phosphatase) were not included in the studies, but would have allowed us to determine the degree of MSC differentiation towards non-chondroprogenitor cells. Likewise, justified by the highly chondrocyte-like histological appearance of the membrane cultures (III), additional, specific immunostaining directed to collagen type I, II and X, biglycan and aggrecan would have revealed the degree of zonal distribution of ECM components that are distinctive for native cartilage.

Scaffold contraction during culturing

In both Study I and Study III cultures of MSCs contracted. In Study I AT-MSC collagen I/III scaffold cultures contracted, while in Study III collagen coated MLPC cultures contracted resulting in unfavourable tissue.

Mature chondrocytes and MSCs express genes for α-smooth muscle actin (SMA), which plays a major role in cell cytoskeleton [230-232]. Chondrocytes cultured on porous type I collagen-glycosaminoglycan scaffolds contracts [231], and this effect is inhibited by staurosporine, an antibiotic known to disrupt cytoskeletal structure [233]. Furthermore, addition of TGF-β1 increased the expression of SMA and contraction in

chondrocyte-seeded collagen-glycosaminoglycan scaffolds [234]. Scaffold fabricated from cartilage-derived extracellular matrix (CDM) may provide an environment that stimulates chondrogenic differentiation and cartilage matrix synthesis in a variety of cell types including AT-MSCs and MSCs (for further elaboration, please refer to Section "3.3.2 The role of scaffolds for cartilage tissue engineering", p.14) [111, 235]. However, scaffolds of CDM may provide potential benefits for cartilage tissue engineering, they have generally shown to contract during culture due to low compressive moduli [235]. This contraction has been demonstrated in scaffolds of collagen-glycosaminoglycans *in vitro* culture [236], and the contraction is reduced by cross-linking collagen fibrils [237].

The scaffolds used in Study I&III is fabricated of natural porcine or shark derived collagen type I, II, III and IV and the collagen fibres were not cross-linked.

The condensation of MSCs mediated by hypoxia, the increased expression of SMA mediated by TGF, and the lack of cross-linking of collagen fibres may explain the unfavourable contraction of the scaffold cultures in Study I&III.

MSC for *in vitro* cartilage tissue engineering

From a theoretical point of view, the chondrocyte is optimal for cartilage tissue engineering because it is programmed to fulfil functions required to maintain the turnover of cartilage tissue. However, the harvesting of chondrocytes and their handling in the laboratory is associated with shortcomings. Human MSCs are easily isolated, have high proliferation rates and have the potential of multilineage differentiation and would thus be ideal for cartilage tissue engineering.

To address the issue of biological and donor-site variation, an ideal experimental setup would include multiple (a) donors of different age, sex and comorbidity, etc., (b) different tissues and (c) sufficient biological replicas. However, such a setup can easily become unrealistic. The inclusion of four donors in Study I allowed us, to some extent, to account for biological variability. However, at the time the experiments were carried out, the AT-MSCs were not characterised in terms of CD surface phenotype and trilineage differentiation potential. However, three out of the four used AT-MSCs have subsequently been characterized using differentiation assays and flow cytometric analysis and were found to fulfil the minimum criteria for defining multipotent MSCs [105, 196-201]. In contrast, MLPCs (II&III) are intensively characterised and fulfil the minimum criteria for multipotent MSCs defined by The International Society for Cellular Therapy. The same clone was used in Study II&III. It is possible that the MLPCs studied were isolated from an individual cell clone with a reduced chondrogenic potential.

MSCs gradually lose their proliferation rate and differential potential in *in vitro* expansion [238]. I addressed this issue by employing low passage cells for the experiments, but this accordingly limited the possible number of replicas and laboratory assays.

Defining the optimal culturing conditions and microenvironment need to be assessed broadly and with an open mind which accordingly adds up in complexity. For instance, a parameter that one would initially recognise as minor and even negligent is the choice of basal expansion medium. But the fact is that the choice of basal expansion medium modulates growth characteristics, surface marker distribution and chondrogenic differentiation of human MSCs [196, 218]. Lund et al. showed that proliferations rates of AT-MSCs were three fold higher in cells initially expanded in alfa-MEM compared with DMEM-F12; but, interestingly, the subsequent chondrogenic assay demonstrated a two-fold increased chondrogenesis for pellet cultures expanded in DMEM-F12 compared with alfa-MEM-expanded cultures [196]. Likewise, supplementing culture medium with the commonly used pH indicator, phenol red, changes the microenvironment and inhibits the chondrogenesis of human MSCs [219]. The four fold higher levels of sGAG for pellet cultured at 21% O_2 in Study III compared with Study II may likely reflect such differences in materials & methods used, and availability of laboratory supplies (Table 1, p.23), e.g., due to import regulations two different FBS batches were used for cell expansion. Furthermore, in Study II a commercial available and classified chondrogenic inducing medium was used, while in Study III the collaborator's verified chondrogenic medium for ovine BM-MSC [138] was used for MLPC pre-differentiation, which included double the dose of ascorbic acid 2 phosphate (100 µg/mL) and the addition of sodium pyrovate (1 mM) compared with Study I. Increased complexity in comparing across the three presented studies is plausible owing to differences in cell source and cell-subpopulation, cell expansion medium, and chondrogenic differentiation medium used in the studies (Table 1, p.23).

7 Conclusion, perspectives and future research

The aim of this PhD project was to construct a clinical-grade cartilage implant to aid in the surgical repair of focal joint cartilage defects. Important knowledge of different cells and scaffold material has been generated which will serve as foundation for the continued quest to develop such clinical grade cell-scaffold constructs. AT-MSCs and MLPCs were chondrogenically induced in different scaffold and culturing systems. Overall, the employed stem cells possess chondrogenic potential when cultured under particular conditions. The process of stem cell chondrogenesis *in vitro* is complex, and our knowledge of promoting and inhibiting factors is continuously expanding.

AT-MSCs and MLPCs chondrogenesis is improved when cells are exposed to reduced oxygen tension and this effect is further enhanced in 3D culturing systems such as pellets or by culturing on scaffolds or membranes. However, a characteristic zonal layout of cells and ECM similar to that of native cartilage was not completely obtained in my tissue-engineered cartilage. The most cartilage-like tissue in terms of histology, ECM production and gene expression was produced in non-coated membrane cultures of MLPC.

AT-MSCs and MLPCs hold great clinical potential for restoring cartilage defects if settings are wisely selected. In this context, further optimisation is needed prior to clinical implementation. Study I demonstrated the chondrogenic potential of AT-MSC pellet and scaffold cultures exposed to reduced oxygen tension. In Study II, FDM and SGS scaffolds demonstrated similar findings for MLPCs. Study III offered the most interesting findings, namely that chondrogenesis of MLPCs was improved in scaffold-free membrane cultures compared with pellet or CPP cultures. Study III highlights the core issue of optimising and how careful consideration is required in terms of selecting optimal culturing systems, functionalisation of culturing systems, growth factors, evaluation methods, etc.

The work carried out and the obtained results justify further research to illuminate the full potential of the employed MSCs and scaffolds. Of particular interest is to optimise and evaluate the chondro-conductive potential of additional growth factors, inhibition of SMA, cross-linking collagen and reduced oxygen tension on membrane cultures of MLPC. Evaluating the effect of culturing differentiated MLPCs on CPP would be an obvious next step.

8 References

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9 Appendices

9.1 Theses from the Orthopaedic Research Group

PhD and Doctoral Theses from the Orthopaedic Research Group, ***www.OrthoResearch.dk***, University Hospital of Aarhus, Denmark

PhD Theses

1. In vivo and vitro stimulation of bone formation with local growth factors
   Martin Lind, January 1996
2. Gene delivery to articular cartilage
   Michael Ulrich-Vinther, September 2002
3. The influence of hydroxyapatite coating on the peri-implant migration of polyethylene particles
   Ole Rahbek, October 2002
4. Surgical technique's influence on femoral fracture risk and implant fixation. Compaction versus conventional bone removing techniques
   Søren Kold, January 2003
5. Stimulation and substitution of bone allograft around non-cemented implants
   Thomas Bo Jensen, October 2003
6. The influence of RGD peptide surface modification on the fixation of orthopaedic implants
   Brian Elmengaard, December 2004
7. Biological response to wear debris after total hip arthroplasty using different bearing materials
   Marianne Nygaard, June 2005
8. DEXA-scanning in description of bone remodeling and osteolysis around cementless acetabular cups
   Mogens Berg Laursen, November 2005
9. Studies based on the Danish Hip Arthroplasty Registry
   Alma B. Pedersen, 2006
10. Reaming procedure and migration of the uncemented acetabular component in total hip replacement
    Thomas Baad-Hansen, February 2007
11. On the longevity of cemented hip prosthesis and the influence on implant design

Mette Ørskov Sjøland, April 2007

12. Combination of TGF-β1 and IGF-1 in a biodegradable coating. The effect on implant fixation and osseointegration and designing a new in vivo model for testing the osteogenic effect of micro-structures in vivo
    Anders Lamberg, June 2007
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Paper I

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REGULAR ARTICLE

Hypoxia enhances chondrogenic differentiation of human adipose tissue-derived stromal cells in scaffold-free and scaffold systems

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Abstract Human adipose-derived stromal cells (hASCs) possess the potential for chondrogenic differentiation. Recent studies imply that this differentiation process may be enhanced by culturing the cells in low oxygen tension in combination with three-dimensional (3D) scaffolds. We report the evaluation of the chondrogenic potential of hASC pellets in 5 and 21 % O_2 and as cell-scaffold constructs using a collagen I/III scaffold with chemical induction using TGF-β3. hASCs from four human donors were cultured both in a micromass pellet system and in 3D collagen I/III scaffolds in either 5 or 21 % O_2. Chondrogenesis was evaluated by quantitative gene expression analysis of aggrecan, SOX9, collagen I, II and X and histological evaluation with H&E and toluidine blue staining. Induced pellets cultured in 5 % O_2 showed increased peripheral cellularity and matrix deposition compared with 21 % O_2. Induced pellets cultured in 5 % O_2 had increased control-adjusted gene expression of aggrecan, SOX9 and collagen I and decreased collagen X compared with 21 % O_2 cultures. Induced pellets had higher gene expression of aggrecan, SOX9, collagen I, II and X and increased ratios of collagen II/I and collagen II/X compared with controls. As for pellets, scaffold cultures showed cellularity and matrix deposition organized in a zonal manner as a function of the oxygen tension, with a cartilage-like morphology and matrix deposition peripherally in the 5 % O_2 group and a more centrally located matrix in the 21 % O_2 group. There were no differences in histology and gene expressions between pellet and scaffold cultures. Five percent O_2 in combination with chondrogenic culture medium stimulated chondrogenic differentiation of hASCs in vitro. We observed similar patterns of differentiation and matrix disposition in pellet and scaffold cultures.

Keywords Cartilage neogenesis · Adipose-derived stem cell · Chondro-Gide · Hypoxia · Porous collagen I/III

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Introduction

Articular cartilage defects are highly prevalent and impairment caused by this condition has a severe socioeconomic impact (Arøen et al. 2004). Recent decades have seen intensive research into the development of bioactive three-dimensional

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(3D) constructs to enhance healing of articular cartilage defects and this research has paved the way for new surgical treatment options. Surgical treatment relies on cellular activation or transplantation of cells capable of healing chondral defects (Hunziker 2002; Ulrich-Vinther et al. 2003). However, methods that involve transplantation of mature articular chondrocytes, e.g., autologous chondrocyte implantation (ACI) and related techniques, suffer from a number of shortcomings. For example, chondrocytes cultured in vitro exhibit low proliferation rates, which increase the time needed for in vitro expansion and the time between chondrocyte harvesting and reimplantation (Behrens et al. 2006; Clar et al. 2005; Kon et al. 2009; Ulrich-Vinther et al. 2003). Furthermore, in vitro chondrocyte expansion may be associated with dedifferentiation, which entails phenotype modulation and loss of the cartilage-specific phenotype (Benya and Shaffer 1982; Binette et al. 1998; Darling and Athanasiou 2005; Foldager 2013; Hui et al. 2008; Mandl et al. 2004; Mankin 1982; Schulze-Tanzil 2009).

To address these issues, researchers are currently focusing on the investigation of alternative sources of cells with an inherent capacity for production of articular cartilage matrix; e.g, multipotent mesenchymal stromal cells (MSCs). MSCs have attracted much interest because they are easily isolated, readily expanded and are capable of multi-lineage differentiation. MSCs have been isolated from numerous tissues, e.g., bone marrow, adipose tissue, dental cells, orbital fat, umbilical cord blood, placenta, skin and thymus (Bieback et al. 2008; Ho et al. 2011; Koch et al. 2007; Musina et al. 2005; Petrovic and Stefanovic 2009; Richter 2009; Tapp et al. 2009; Tarle et al. 2011). Bone marrow-derived stromal cells (BM-MSCs) are presently the most intensively investigated and best characterised MSC for engineering cartilage tissue (Kuhbier et al. 2010a). However, their collection is an invasive procedure and the cell number, differentiation potential and maximal proliferative life span may decline with increasing donor age and cell passage number (Digirolamo et al. 1999; Kern et al. 2006; Kretlow et al. 2008). In contrast, adipose-derived stromal cells (ASCs) may have advantages over BM-MSCs as the harvesting procedure is cheaper, less invasive and, most importantly, yield higher numbers of multipotent MSCs (Kuhbier et al. 2010b). In some studies, the chondrogenic differentiation potential in ASCs is reported to be superior to that of BM-MSCs (Diekman et al. 2010b; Estes et al. 2006). Other findings suggest that BM-MSCs have a higher chondrogenic potential than ASCs (Afizah et al. 2007; Huang et al. 2005; Sakaguchi et al. 2005). However, there is a consensus that ASCs are capable of chondrogenic differentiation.

Reduced oxygen may be a key factor in achieving optimal chondrogenic ASC differentiation (Chen et al. 2006; Fink et al. 2004; Foldager et al. 2009, 2011; Koay and Athanasiou 2008; Meyer et al. 2010; Pilgaard et al.2009). The chondrocyte phenotype has been maintained by culturing mature articular chondrocytes at 1.5 % O_2 (Egli et al. 2008). It has been speculated that such maintenance may result because of the closer mimicing of a low physiological oxygen tension as reported in embryonic cartilage development (Brighton 1984). Local oxygen tension down to 2–10 % has been described in normal mature cartilage and bone marrow (Falchuk et al. 1970; Gaber et al. 2005; Lund-Olesen 1970; Malda et al. 2003; Pennathur-Das and Levitt 1987; Zhou et al. 2004).

Another aspect of the in vivo chondrogenic niche is the spatial arrangement surrounding the chondrocyte. Chondrogenesis is enhanced in 3D cultures such as the commonly used micromass pellet system or through the use of various 3D scaffolds/matrices (Engler et al. 2006; Grigolo et al. 2002; Hutmacher 2000; Lisignoli et al. 2005; Seda Tigli et al. 2009; Wise et al. 2009). These studies have shown that 3D scaffolds provide cellular niches more suitable for cell attachment, cellular arrangement, proliferation and differentiation. In addition to scaffold structure, the use of soluble factors such as TGF-β and BMP-6 has shown promising results for chondrogenic induction of MSCs (Lisignoli et al. 2005; Seda Tigli et al. 2009).

The purpose of the present study was to evaluate the effect of reduced oxygen tension on the chondrogenic differentiation of human adipose-derived stromal cells (hASCs) in micromass pellets and to evaluate a macroporous scaffold of collagen I/III for its supportive effect of hASC chondrogenesis. We hypothesize that hASCs exhibit enhanced chondrogenesis under reduced oxygen tension conditions and that they maintain their chondrogenic potency when cultured on a collagen-based scaffold.

Materials and methods

Study design

The study design is visualised in Fig. 1. Passage 0 hASCs from four donors were first expanded for 3 weeks and chondrogenic culturing was then performed in standard pellet cultures or scaffolds. Cell cultures were divided into subgroups as presented in Fig. 1, i.e., cell cultures were further divided into culturing in control versus chondrogenic induction medium and allocated to 5 or 21 % O_2 culturing.

Isolation and culture of hASCs

hASCs from four human donors were isolated by a slightly modified method as described previously (Pilgaard et al. 2009; Zuk et al. 2001). Three of the four used hASCs have previously been characterised using differentiation assays and flow cytometric analysis and were found positive for MSC marker, CD74, CD90 and CD105 and negative for haematopoetic progenitor cell markers, CD34, CD45 and HLA-DR (Chase et al. 2012; Lund et al. 2009; Pilgaard et al. 2008; Yang et al. 2012). This fulfils the minimum criteria of The International

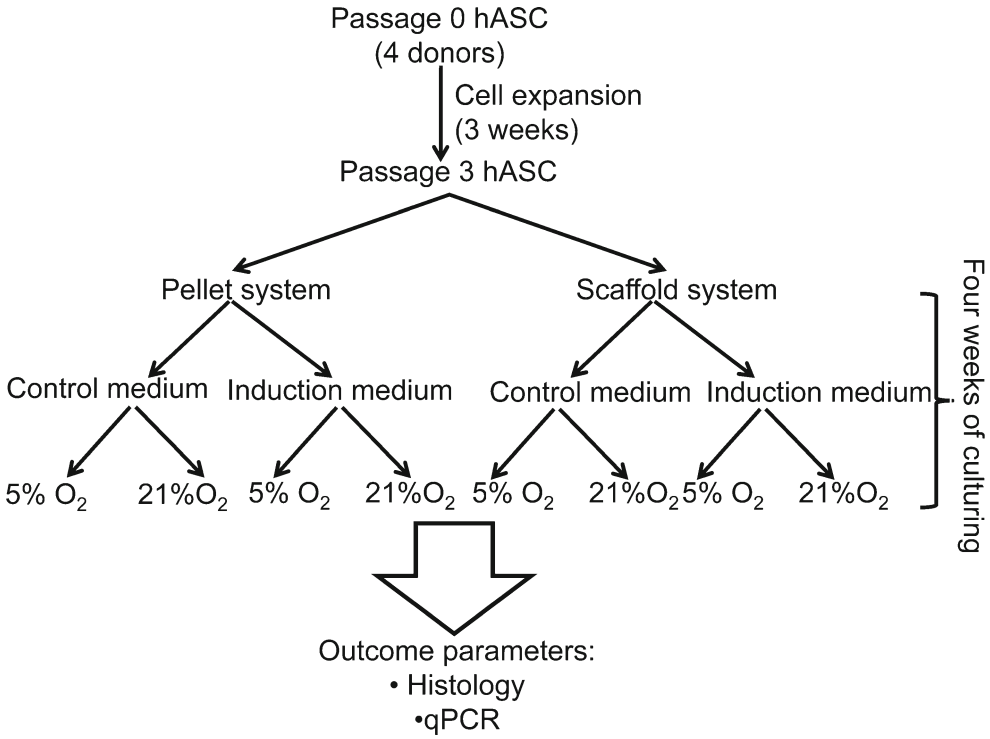

Fig. 1 Study design and outcome parameters. Passage 0 hASCs were first expanded for 3 weeks and chondrogenic culturing was then performed in standard pellet cultures or cultured within collagen type I/III scaffolds. Cells were cultured in either 5 or 21 % O_2 and TGF-β3 was the main chemical inducer of chondrogenesis. Chondrogenesis was determined by quantitative real-time PCR (RT-qPCR) and histological analysis

Society for Cellular Therapy for defining multipotent MSCs (Berger et al. 2006; Choi et al. 2008; Dominici et al. 2006). In brief, adipose tissue was obtained after informed consent had been obtained from three females and one male undergoing liposuction surgery at the Grymer Private Hospital (Skejby, Denmark). Subcutaneous fat were collected from one female and three male donors, with fat from the abdomen and the hips as the primary source (Table 1). Fat tissue was digested with a buffer solution of collagenase containing 0.28 Wunsch U/mL crude collagenase mix (Lot no. LTQ5230; Wako, Neuss, Germany) and 20 mg/mL bovine serum albumin (Roche Applied Science, Hvidovre, Denmark) in Dulbecco's phosphate-buffered saline. Following enzymatic digestion, released cells were harvested by centrifugation and subsequently filtered. Residual erythrocytes were lysed by resuspension of the pellet in 160 mM NH_4Cl and incubated at room temperature for 10 min following a final round of centrifugation and filtration. After this final round of purification, the cells were seeded at a density corresponding to 0.15 mL adipose tissue/cm^2 in growth medium and grown to 80 % confluence (P0). Finally, the cultures from each patient were cryopreserved for further experiments. Hence, hASC cultures were not pooled inter-individually. For expansion of cell-cultures, the P0 hASCs were thawed and seeded at 1,000 cells/cm^2 in culturing flasks and cultured to 80 % confluence in a growth medium consisting of alfa-MEM (Invitrogen, Taastrup, Denmark) supplemented with 10 % fetal calf serum (Invitrogen) in a standard humidified atmosphere containing 5 % CO_2 and 21 % O_2 at 37 °C. Growth medium was changed twice a week. Upon 80 % confluence, the cells were detached by trypsinisation (1.25 % trypsin and 5 mM EDTA) and reseeded at 1,000 cells/cm^2. After three passages, enough hASCs were retrieved for the experiments. Consequently, P3 hASCs were used in the studies described.

Table 1 Donor characteristics

Donor ID	Age (years)	Gender	BMI	Donor Site
ASC13	33	Female	21.6	Abdomen, hip, loin, inner thigh
ACS21	52	Male	28.0	Abdomen, hip
ASC23	42	Female	20.9	Inner thigh
ASC24	28	Female	21.4	Abdomen, hip

ASC Human adipose tissue-derived stromal cell, *BMI* body mass index

Human adipose stromal cell pellet cultures

Three-dimensional pellet cultures were established by spinning 2×10^5 P3 hASCs from each of the four patients separately at 500*g* for 5 min in V-type 96-well plates (cci3896; Corning, Schiphol-Rijk, The Netherlands). Pellets from each of the four donors, the corresponding culturing conditions and the measured outcome parameters were made in three biological replicates. Pellets were then immediately randomly split for culturing into either control medium (alfa-MEM supplemented with 10 % FCS and 1 % streptomycin/penicillin) or chondrogenic-inductive culture medium (DMEM-HG supplemented with 0.1 μM dexamethasone, 50 μg/mL ascorbic acid 2 phosphate, 40 μg/mL proline, 1× ITS^+, 10 ng/mL TGF-β3 and 1 %

streptomycin/penicillin). The culturing plates containing the pellets were split into culture in either 5 or 21 % O_2. Culture medium was equilibrated in the relevant oxygen tension for 2 hours and changed three times a week. The pellets were cultured for a total of 28 days.

Human adipose stromal cell scaffold cultures

Three-dimensional cell-scaffold cultures were constructed using the double-layer collagen I/III scaffold Chondro-Gide® (Geistlich Pharma, Switzerland). This scaffold has two proposed functions: it consists in a compact surface providing mechanical strength and entrapment of cells and an inner porous surface providing cell-adhesive capacity (Fuss et al. 2000). The scaffold's compact surface is designed to face the synovial cavity. In vitro and in vivo studies have demonstrated that the scaffold's structure permits cell attachment, proliferation and enrichment of the extracellular matrix maintaining the phenotype of chondrocytes and that it limits leakage of cells from the defect and migration of inflammatory proteins into the repair site (Ehlers et al. 1999; Frenkel et al. 1997; Fuss et al. 2000; Kurz et al. 2004; Nehrer et al. 1997, 1998; Scherer et al. 2004; Schlegel et al. 2008). Moreover, the scaffold has the additional advantage of a high tensile strength and excellent handling properties, which makes it clinically well accepted and frequently used in ACI surgery (Bentley et al. 2003; Briggs et al. 2003; Ehlers et al. 1999; Fuss et al. 2000; Gooding et al. 2006; Haddo et al. 2004; Iwasa et al. 2009; Niemeyer et al. 2008). The membrane scaffold was purchased from Interface Biotech (Hoersholm, Denmark).

Scaffolds were cut into discs with a diameter of 6 mm using a disposable skin biopsy punch (Miltex, York, PA, USA) under aseptic conditions and placed into 24-well culturing plates (one scaffold/well) before cell seeding. Vehicle drops consisting of 2×10^5 P3 hASCs suspended in 25 μL of growth medium (8×10^6 hASC/mL) were positioned on the porous scaffold surface. A gentle liquid-drag method was used to ensure an uniform distribution of cells throughout the scaffold. Cell-seeded scaffolds were left in standard culture conditions (5 % CO_2, 21 % O_2 and 37 °C) for 2 h to allow the cells to adhere. One mL/well control medium consisting of alfa-MEM supplemented with 10 % FCS and 1 % streptomycin/penicillin was then gently added. After 4 days in culture, the scaffolds were divided into two groups and cultured in either control medium (alfa-MEM supplemented with 10 % FCS and 1 % streptomycin/penicillin) or chondrogenically inductive culture medium (DMEM-HG supplemented with 0.1 μM dexamethasone, 50 μg/mL ascorbic acid 2 phosphate, 40 μg/mL proline, 1× ITS$^+$, 10 ng/mL TGF-β3 and 1 % streptomycin/penicillin). Both groups were further subdivided and cultured in either 5 or 21 % O_2. The culture medium was equilibrated in 5 or 21 % O_2 for 2 h and gently changed twice a week. Scaffolds were cultured for a total of 28 days.

Histology

Histology was used for evaluation of the extra-cellular matrix and cellularity. Pellet and scaffold samples were briefly stained with Mayer's haematoxylin and then dehydrated in graded series of ethanol (70–96 %) before being embedded in methylmethacrylate as described by Erben (1997). After polymerisation, 7-μm sagittal sections were prepared with Polycut E microtome (Reichert & Jung, Heidelberg, Germany). Cross-sections were cut from the central part of the pellet and scaffold samples. Standard staining procedures were performed for haematoxylin and eosin. Sulphated proteoglycans and acidic polysaccharides were visualised by staining with 0.05 % toluidine blue (pH 4.0) for 20 min. Stained slides were mounted in DPX. Images were made on an Olympus BX51 microscope with the use of the image-acquiring software PixeLINK.

Total RNA extraction

Total RNA for quantitative real-time RT-PCR (RT-qPCR) was extracted in 1-mL TRIzol® (Invitrogen, Taastrup, Denmark) using a mixer mill (MM301; Retsch, Germany) to homogenise the scaffolds. Chloroform was added and samples were vortexed and centrifuged. RNA was precipitated using isopropanol, washed twice in 75 % ethanol and finally resuspended in RNase-free, DEPC-treated water (Ambion, Cambridgeshire, UK). Concentrations of total RNA were assessed by Quant-iT™ RiboGreen® RNA Kit (Molecular Probes) according to the manufacturer's instructions. Each sample was measured into a 96-well plate using a microplate reader. Samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm. Technical duplicates were run for each biological sample. RNA purity was measured by calculating the $A_{260\ nm}/A_{280\ nm}$ ratio via a spectrophotometer (values ranging from 1.92 to 2.06 indicating a high level of purity). RNA integrity was verified by agarose electrophoresis. Total RNA was extracted from pellets as described above but preceded by digestion in 1 mg/mL collagenase (Gibco) at 37 °C for 1 h before homogenisation with the mixer mill.

RT-qPCR

The differentiation of the hASCs towards chondroblasts was determined by RT-qPCR to assess gene expressions of five cartilage markers: aggrecan, SOX9 and collagen I, II and X. First-strand cDNA was synthesised according to the manufacturer's instructions using the High Capacity cDNA Archive Kit (Cat no. 4322171, Applied Biosystems, Naerum, Denmark). The RT-qPCR was performed at standard enzyme and cycling conditions on a 7500-fast real-time PCR system using TaqMan® gene expression assays (Cat no. 4352042, Applied Biosystems): Hs99999908_m1 [beta-glucuronidase (*Gusb*)], Hs00237047_m1 [tyrosine 3-monooxygenase/tryptophan 5-monooxygenase

activation protein (*Ywhaz*)], Hs00427621_m1 [TATA box binding protein (*Tbp*)], Hs00153936_m1 [aggrecan (*Agc*)], Hs00165814_m1 [chondrocyte-specific transcription factor (*Sox9*)], Hs00164004_m1 [collagen type I alpha-1 (*Col1a1*)], Hs00264051_m1 [collagen type II alpha-1 (*Col2a1*)], Hs00166657_m1 [collagen type X alpha-1 (*Col10a1*)] and a custom-designed TaqMan assay for human RNA polymerase II (*RpII*). Assays included one FAM™ dye-labelled TaqMan® MGB probe spanning exon boundaries in the gene of interest (GOI) and two unlabelled hot-start primers. Amplicon size was <170 bp for all primer sets to maximise the amplification efficiency. cDNA corresponding to 20 ng of mRNA was used in each PCR reaction. Technical triplicates were used for each biological sample. The gene expression levels were normalised to the BestKeeper index (Pfaffl et al. 2004), which was determined by the geometric mean of the cycle threshold (Ct) from *Gusb*, *Ywhaz* and *Tbp*. *Gusb*, *Ywhaz* and *Tbp* have been reported to be the most stable genes for hASCs cultured in different oxygen conditions and were used as reference genes (Fink et al. 2008). Gene expressions were normalised to the BestKeeper index using the following equation: relative gene expression=$2^{\text{Ct (BestKeeper Index)} - \text{Ct (gene of interest)}}$. The delta delta Ct method (Livak and Schmittgen 2001) was used to compare the inductive effect of 5 % O_2 with 21 % O_2; gene expressions from each sample were normalised to the endogenous housekeeping genes and data are presented as the control-adjusted fold change of gene expression in the induced relative to the non-induced samples setting the control value to 1.0. RT-qPCR for pellets was done as described above, apart from the cDNA corresponding to 8 ng of mRNA for each PCR reaction.

Data analysis and statistics

Data were checked for normal distribution and variance homogeneity. Two-way analysis of variance (ANOVA) was performed with culture medium and oxygen tension as independent variables (medium × oxygen). Data are presented as means±standard errors of means for four donors ($n=4$) in three biological replicates and measured in two technical duplicates. Differences between means were considered statistically significant when $p<0.05$. All statistical evaluation was done using STATA 10.1 software (Stata, College Station, TX, USA).

Results

Human adipose stromal cell pellet cultures

Induced pellets cultured in both 5 and 21 % O_2 increased in size and were spherical with a diameter of approximately 2 mm, whereas pellets cultured in control medium retained their original size. The pellet tissue texture was robust and firm to the touch for induced cultures and had a smooth and yellow surface in contrast to control pellets, which were non-uniform, fragile and in a state of disintegration.

Culturing in both 5 and 21 % O_2 supported the deposition of proteoglycan synthesis as shown in toluidine blue-stained sections (Fig. 2b, d, f, h). Cultures in 5 and 21 % O_2 showed zonal extracellular matrix deposition as a function of oxygen tension, i.e., higher levels and intensity of matrix deposition were observed peripherally in the 5 % O_2 (Fig. 2h) cultures compared with 21 % O_2 (Fig. 2d) in which matrix deposition was located centrally. At high magnification of toluidine blue-stained slides, induced cultures showed focal spots resembling the morphology of chondrocytes (Fig. 2b, f), i.e., rounded cells in lacunae surrounded by hyaline-like matrix. Haematoxylin and eosin staining displayed a similar pattern in zonal distribution of cellularity as a function of the oxygen tension, i.e., higher levels of cellularity were observed peripherally in 5 % O_2 (Fig. 2g) cultures compared with 21 % O_2 (Fig. 2c) in which cultured cells were centrally located. Pellets cultured in control non-inducing medium displayed decreased cellularity with pyknotic nuclei indicating apoptosis and generated no proteoglycan deposition (Fig. 2i-p).

The mRNA expression of aggrecan, SOX9, collagen I, II and X was higher for induced pellets compared with non-induced control pellets ($p<0.05$) (Fig. 3a–e). Cultures induced in 5 % O_2 showed increased mRNA levels of SOX9 ($p<0.05$), collagen I ($p< 0.05$), ratios of collagen II/I ($p<0.05$) and collagen II/X and decreased collagen X ($p<0.05$) compared with 21 % O_2 (Fig. 3b, c, e–g). No significant differences in mRNA expression of collagen II ($p=0.20$) and aggrecan ($p=0.75$) were noted in 5 % O_2-induced cultures compared with 21 % O_2 (Fig. 3a, d). Higher control-adjusted (induced relative to non-induced) fold increase was observed in 5 % O_2 cultures for aggrecan ($p<0.001$), SOX9 ($p<0.001$) and collagen I ($p<0.001$) and decreased fold change of collagen X compared with 21 % O_2 (Fig. 3h).

hASC scaffold cultures

Regardless of the oxygen tension (5 or 21 % O_2), induced scaffold cultures curled up, contracted and achieved a 3D spherical shape after 28 days of culture (Fig. 4a). hASCs were seeded on the scaffolds' rough and porous surface. The rough and initially cell-seeded surface created a non-preformed central cavity, referred to hereafter as an acquired cavity, in which cells formed the observed tissue (Fig. 4a). Scaffolds cultured in non-inducing control medium maintained the native flat scaffold shape.

Scaffold cultures demonstrated a similar pattern of an oxygen-dependent zonal distribution of cells and extracellular matrix deposition as observed in induced pellet cultures (Fig. 4b–i). However, as illustrated in Fig. 4a, induced scaffolds contracted and formed an acquired cavity; an arrow indicates the boundary between the native scaffold and the acquired cavity. Scaffolds

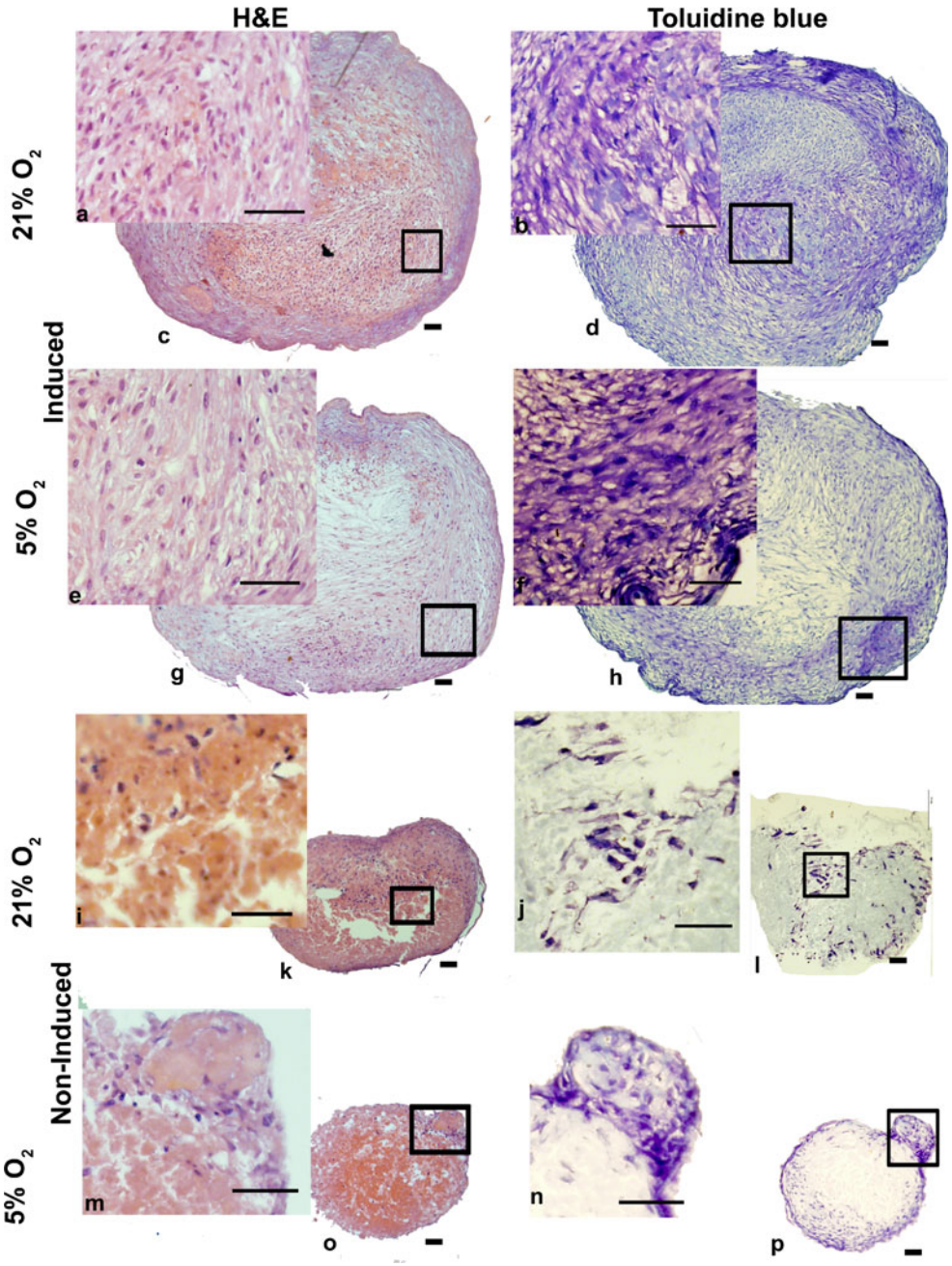

Fig. 2 Histological appearance in pellet cultures of human adipose tissue-derived stromal cells (hASCs). hASCs were cultured in 21 % O_2 (**a**–**d**, **i**–**l**) and 5 % O_2 in chondrogenic inducing medium (**a**–**h**) or non-inducing control medium (**i**–**p**) for 28 days. Sections stained with H&E (**a**, **c**, **e**, **g**, **i**, **k**, **m**, **o**) and toluidine blue (**b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**). *Scale bar* 50 μm at ×10 (overview images) and ×20 (inserted images) magnification

induced in 5 % O_2 showed higher cellularity and matrix deposition peripherally (Fig. 4h, i) compared with 21 % O_2, in which cells and matrix were accumulated centrally (Fig. 4d, e) in the acquired cavity. At high magnification of toluidine blue-stained slides, focal spots resembling the morphology of chondrocytes could be detected, i.e., rounded cells in lacunae surrounded by hyaline-like matrix (Fig. 4c, g); however, this was more distinctive in 5 % O_2 (Fig. 4g) cultures than in 21 % O_2 (Fig. 4c) cultured scaffolds. Non-induced scaffold cultures demonstrated limited cellularity and had no GAG deposition and the seeded cells did not colonise deeper parts of the scaffolds (Fig. 4j–q).

Higher gene expression levels of aggrecan ($p<0.05$), SOX9 ($p<0.05$), collagen I ($p<0.05$) and collagen X ($p<0.05$) were observed for induced scaffold cultures compared with non-induced scaffolds (Fig. 5a–c, e). Scaffold cultures induced in 5 % O_2 showed higher mRNA expressions of SOX9 ($p<0.05$), ratios of collagen II/I ($p<0.05$) and II/X ($p<0.05$) compared with 21 % O_2 (Fig. 5b, f, g). No significant differences in mRNA expressions of aggrecan ($p=0.12$), collagen II ($p=0.23$), collagen I and collagen X ($p=0.13$) were noted in 5 % O_2 induced cultures compared with 21 % O_2. In 5 % O_2 compared with 21 % O_2 mRNA expressions relative to controls were increased for aggrecan, collagen I and SOX9 ($p<0.001$) and decreased for collagen X ($p<0.001$) (Fig. 5h).

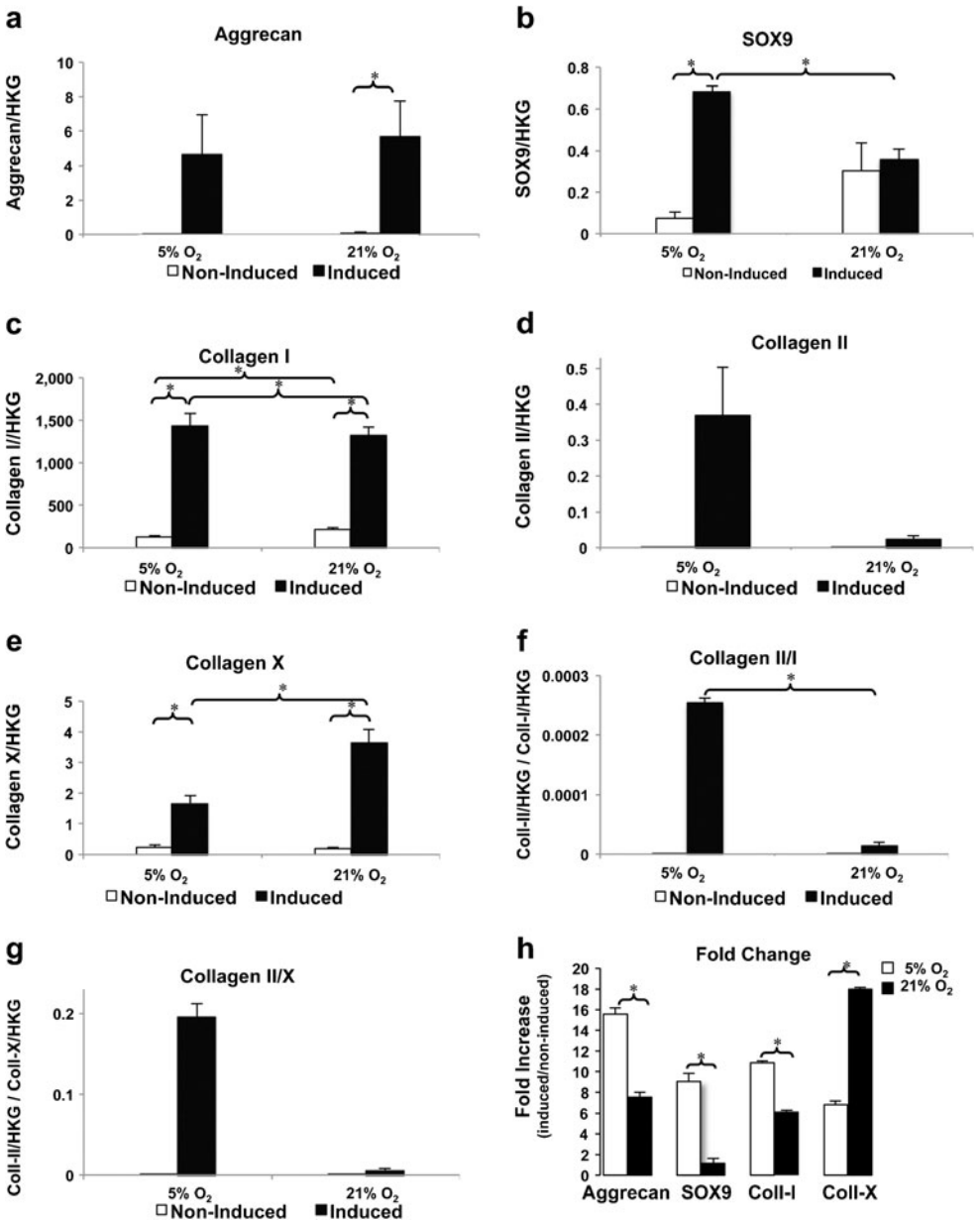

Fig. 3 RT-qPCR (**a**–**h**) and adjusted fold change (**h**) of chondrogenic gene markers aggrecan (**a**), SOX9 (**b**) and collagen I (**c**), II (**d**), X (**e**) and ratios of collagen I/II (**f**) and collagen II/X (**g**) in hASC pellet cultures. Pellets were cultured in either 5 or 21 % O^2, both in control medium (*white columns*) or chondrogenic inductive medium (*black columns*). Control-adjusted fold change is not reported for collagen II due to lack of mRNA expression in non-induced cultures. Values shown represent means±SEM (n=4 donors). *Asterisk* (*) indicates a significant difference ($p < 0.05$) by ANOVA

Discussion

We determined that chondrogenic induction of hASCs in pellet and scaffold cultures is enhanced in reduced oxygen tension (5 % O_2) compared to normoxic oxygen tension (21 % O_2). Despite the low to moderate chondrogenic potency of primary hASC cultures under the conditions provided, a number of interesting findings were made. We found that culturing in 5 % O_2 up-regulated the mRNA expression of the cartilage-related genes aggrecan, SOX9 and collagen II and down-regulated the expression of the hypertrophic cartilage gene collagen X. These observations are consistent with the findings by Betre et al. (2006). The increased gene expression of collagen II, ratios of collagen II/I and II/X and decreased collagen X suggest that the cartilage formed is neither fibrocartilage nor hypertrophic cartilage.

Our study culturing hASCSs in 5 % O_2 on a cell-scaffold-based system as well as pellets favoured chondrogenic differentiation compared with 21 % O_2 culturing. Most authors' work demonstrate that a low oxygen tension favours the chondrogenic phenotype for both mature chondrocytes and MSCs of different origin (Buckley et al. 2010; Guilak et al. 2010; Kanichai et al.

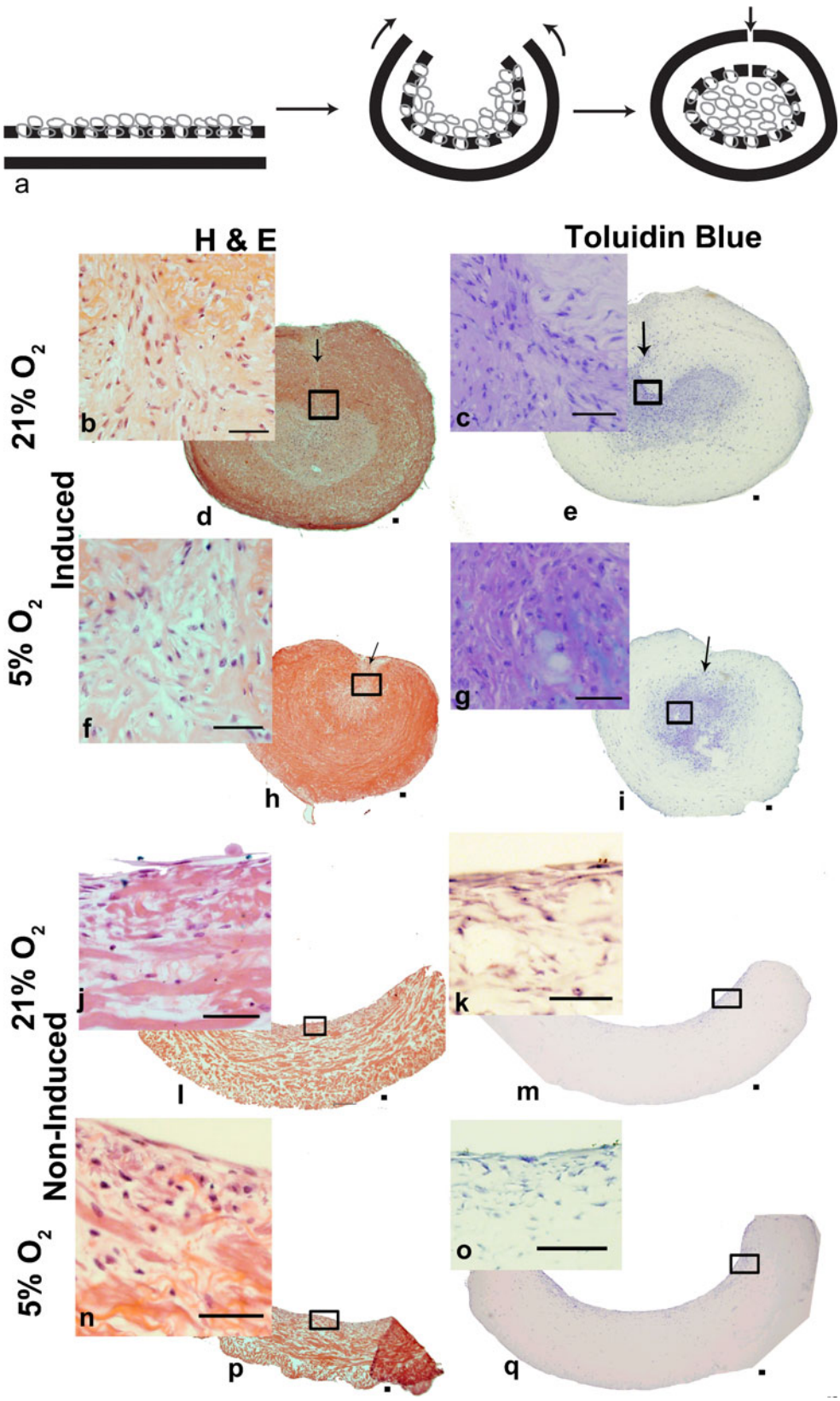

Fig. 4 **a** Schematic illustration demonstrating the curling up, contraction and creation of an acquired central cavity in the chondrogenic induced human adipose tissue-derived stromal cell (hASC) scaffold cultures. Histological appearance in collagen I/III scaffold cultures of hASCs. Scaffolds were cultured in 21 % O_2 (**b**–**e**, **j**–**m**) and 5 % O_2 (**f**–**i**, **n**–**q**) in chondrogenic inducing medium (**b**-**i**) or non-inducing control medium (**j**-**q**) for 28 days. Sections stained with H&E (**b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**) and toluidine blue (**c**, **e**, **g**, **i**, **k**, **m**, **o**, **q**). *Arrows* indicate the boundary between the native scaffold and the acquired cavity as illustrated in (**a**). *Scale bar* 50 μm at ×4 (overview images) and ×20 (inserted images) magnification

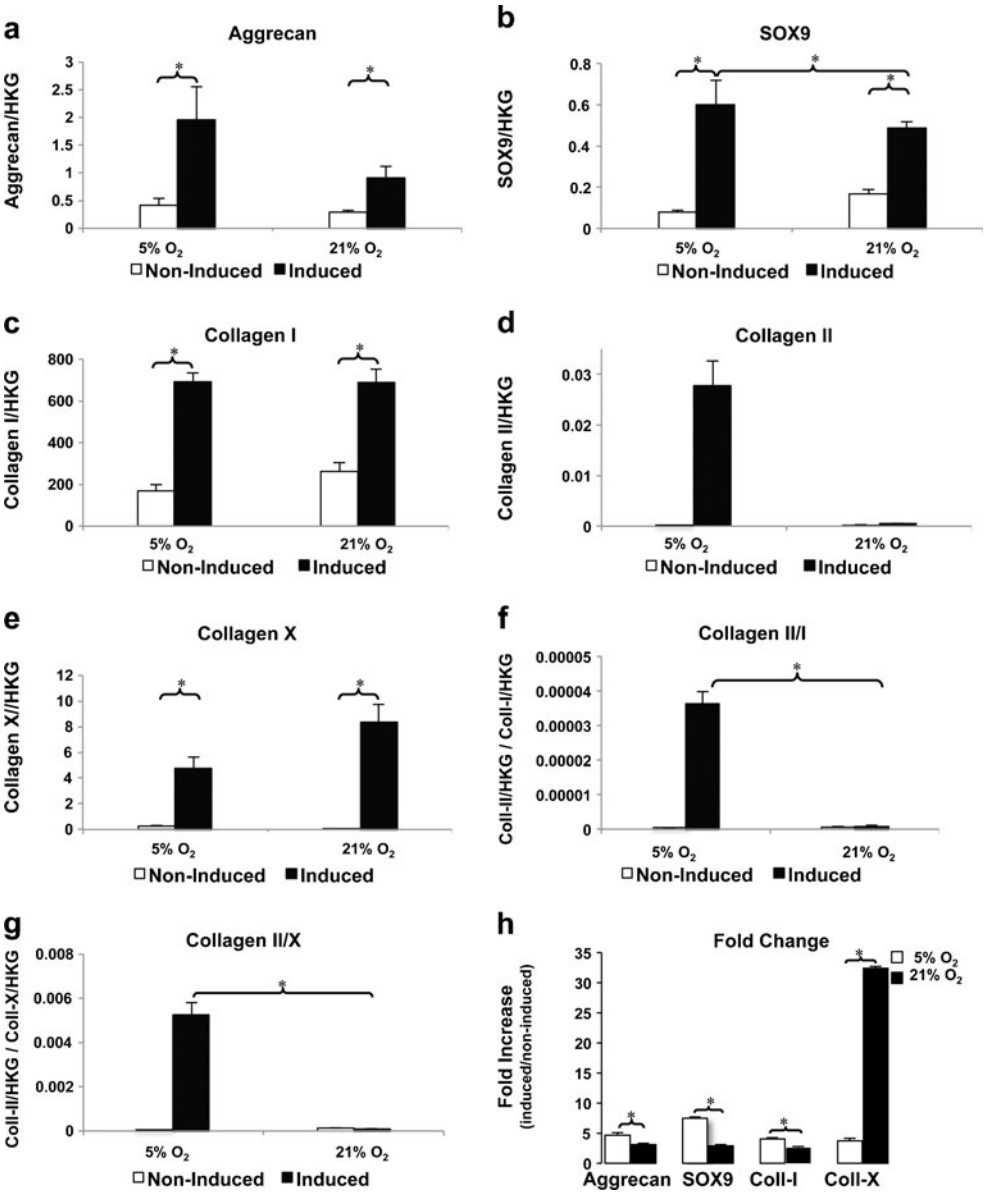

Fig. 5 RT-qPCR (**a**–**h**) and adjusted fold change (**h**) of chondrogenic gene markers aggrecan (**a**), SOX9 (**b**) and collagen I (**c**), II (**d**), X (**e**) and ratios of collagen I/II (**f**) and collagen II/X (**g**) in scaffold cultures of hASCs. Scaffold cultured in either 5 or 21 % O_2, both in control medium (*white columns*) or chondrogenically induced medium (*black columns*). Control-adjusted fold change is not reported for collagen II due to no mRNA expression in non-induced cultures. Values shown represent means±SEM (n=4 donors). *Asterisk* (*) indicates a significant difference (p<0.05) by ANOVA

2008; Khan et al. 2007; Lafont et al. 2008; Zscharnack et al. 2009). Other authors have been unable to demonstrate convincing benefits of using hypoxia for committing ASCs to the chondrocytic lineage (Malladi et al. 2006; Merceron et al. 2010; Ronzière et al. 2010). It is noteworthy that only a little research has been devoted to the study of differentiation of ASCs under hypoxic conditions (Ma et al. 2009).

In the present study, the cellularity and ECM were organized in a zonal manner as a function of the oxygen tension in both induced pellets and collagen I/III scaffolds (Figs. 2d, h, 3e, i). In 5 % O_2 cells, ECM were located more peripherally, while in 21 % O_2, the cells were clustered more centrally. These findings are consistent with the work byBuckley et al. (2012) and Pilgaard et al. (2009). This indicates that in terms of chondrogenesis MSCs seem to prefer an environment with relative hypoxia. The zonal organization of cells and ECM in the pellet and scaffold cultures indicates that the oxygen gradient imitates the low oxygen tension found in native hyaline cartilage. (Krinner et al. 2009; Pilgaard et al. 2009). The zonal organization of cells and ECM may also indicate that an oxygen gradient in the pellets and scaffolds exists and that within this gradient optimal oxygen tension for chondrogenesis may be present. The role of oxygen supply to cells is critical when attempting to differentiate cells within scaffolds (Buckley et al. 2012). Scaffolds are thick structures whose deeper parts therefore have a limited supply of oxygen and nutrients. The supply of oxygen and nutrients is further compromised when scaffold cultures contract as demonstrated in our study. The passive diffusion of the oxygen molecule is limited to a maximum of 200 μm, which may

explain the absence of cells throughout the scaffold (Merceron et al. 2010).

The relatively short culture period of 4 weeks in our study may explain the weak staining of the ECM components and incomplete cartilage-cell morphology. Liu et al. (2008) reported that human BM-MSCs made the most stable cartilage phenotype when differentiated for a period of 8–12 weeks. It is likely that a longer induction time would produce more mature tissue; however, the goal of our experiments was to assess the chondrogenic potential of hASCs differentiated in 5 % O_2. Within this context, 28 days of differentiation was sufficient to clarify this objective. Furthermore, at 28 days, ASCs may have less chondrogenic potential than other sources of MSCs (Afizah et al. 2007; Huang et al. 2005; Sakaguchi et al. 2005). Biological variation between donors with respect to chondrogenic potency is a common finding and it is possible that the hASCs studied were from individuals with reduced chondrogenic potential. The increased number of population doubling times required for cell cultures to reach sufficient cell numbers may also have selected cells with lower chondrogenic potency.

Finally, the culture medium compositions for chondrogenic differentiation of human ASCs may be further ameliorated. For instance, the knowledge that BMP-2, BMP-4 and BMP-6 are supplementary driving forces in the differentiation of ASCs is currently being investigated. Ronziere et al. (2010) reported decreased hypertrophy of ASCs when differentiation was carried out at 5 % O_2 with a combination of BMP-2/TGFβ added to the culture medium. Toh et al. (2005) reported similar findings of increased chondrogenesis in BM-MSCs. Guilak's group have demonstrated the synergistic effect of BMP-6 on the chondrogenesis of ASCs (Diekman et al. 2010a; Estes et al. 2006). The positive effect of BMP-6 is also supported by a lower level of TGF receptors in ASCs that may be compensated by adding of BMP-6 to the differentiation medium (Hennig et al. 2007). It should also be noted that BMP-6 could have a dual role of being both an osteogenic and a chondrogenic inducer. This dual effect is determined by the culturing system in which BMP-6 induces alkaline phosphatase (osteogenic cell fate) in monolayer cultures of ASCs and correspondingly enhances proteoglycan accumulation in pellet cultures (Kemmis et al. 2010). Our differentiation medium included no BMP.

The consistency of the substrate also determines the MSCs commitment (Park et al. 2011). MSCs cultured on stiff substrates differentiate into smooth muscle cells, while culturing in soft substrates such as gels or pellets promotes chondrogenic differentiation. Furthermore, cell shape and cytoskeleton composition can guide MSCs into a specific lineage (Lu et al. 2010; McBeath et al. 2004). Cells kept in a round morphology differentiate into chondrocytes but if they are allowed to adhere and spread on surfaces, they flatten and differentiate into osteoblasts. A Rho-ROCK signalling pathway mediates the shape of the cells (McBeath et al. 2004). We speculate that a relatively softer scaffold than the studied collagen-I/III scaffold would enhance chondrogenic differentiation even further.

Hypoxia is believed to trigger the embryonic condensation of mesenchymal cells and the activation of various transcriptional factors resulting in cartilage formation (Zuscik et al. 2008). Mature chondrocytes and MSCs express genes for α-smooth muscle actin (SMA), which plays a major role in cell cytoskeleton (Kim and Spector 2000; Kinner and Spector 2001; Ng et al. 2011). Chondrocytes cultured on porous type I collagen-glycosaminoglycan scaffolds contract (Kinner and Spector 2001) and this effect is inhibited by staurosporine, an antibiotic known to disrupt cytoskeletal structure (Lee et al. 2003). Furthermore, addition of TGF-β1 increased the expression of SMA and contraction in chondrocyte-seeded collagen-glycosaminoglycan scaffolds (Zaleskas et al. 2001). Scaffold fabricated from cartilage-derived extracellular matrix may provide an environment that stimulates chondrogenic differentiation and cartilage matrix synthesis in a variety of cell types including ASCs and MSCs (Cheng et al. 2009; Diekman et al. 2010b). However, scaffolds of cartilage-derived matrix may provide potential benefits for cartilage tissue engineering, they have generally shown to contract during culture due to low compressive moduli (Cheng et al. 2009). This contraction has been demonstrated in scaffolds of collagen-glycosaminoglycans in in vitro culture (Vickers et al. 2010) and the contraction is reduced by cross-linking collagen fibrils (Rowland et al. 2013). The scaffolds used in our study were fabricated from natural porcine collagen I and III and the collagen fibres were not cross-linked. The condensation of MSCs mediated by hypoxia, the increased expression of SMA mediated by TGF and the lack of cross-linking of collagen fibres may explain the unfavourable contraction (Fig. 4a) of the collagen I/III scaffold used in our study. The pleiotropic nature of hypoxia was recently demonstrated by the observation that hypoxia promotes collagen cross-linking within neocartilage if introduced at specific time points (Makris et al. 2013). Chondrocyte cultures exposed to hypoxia at the 3rd and 4th weeks of incubation accumulated higher levels of collagen and exhibited a higher content of cross-linked collagen compared with continuous exposure to hypoxia (Makris et al. 2013).

Gross comparisons of gene expression levels in 5 % O_2-induced pellet cultures with collagen I/III cultures showed higher levels of aggrecan, SOX9, collagen II and ratios of collagen II/I and II/X and decreased levels of collagen X. This indicates that the most stable hASC chondrocyte phenotype is obtained in the pellet cultures. The curling of the scaffold is obviously not favourable when making a clinically transplantable cartilage construct. Studies on a similar collagen I/III scaffold have not reported similar findings, e.g., Scherer et al. showed that this scaffold has a chondrogenic potential when BM-MSCs are differentiated on the scaffolds in 5 % O_2 (Scherer et al. 2004). The same trend in normoxia is reported by the work of Dickhut et al. (2010). In spite of this,

comparison is difficult because other cell sources such as BM-MSCs and mature chondrocytes were investigated (Dickhut et al. 2010; Fuss et al. 2000; Kurz et al. 2004; Scherer et al. 2004).

Conclusions

In conclusion, 5 % O_2 in combination with chondrogenic culture medium stimulated chondrogenic differentiation of human ASCs in vitro. We have demonstrated similar patterns of differentiation and ECM disposition in both pellet culture systems and in a clinically approved 3D collagen I/III scaffold.

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Paper II

Culturing in Reduced Oxygen Enhance Chondrogenic Differentiation of Human Cord Blood Multilineage Progenitor Cells in Structurally Graded Scaffolds of Polycaprolactone

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Abstract

Background/Purpose: Bone marrow and adipose tissue-derived mesenchymal stromal cells are favoured alternatives to articular chondrocytes for joint cartilage tissue-engineering approaches. Potential advantages of human umbilical cord blood multilineage progenitor cells (MLPC) include non-invasive collection, time for propagation of more homogenous cell populations and selection of cells with enhanced properties compared to primary cell cultures. We report evaluation of the chondrogenic potential of MLPC pellets in 5% and 21% O_2 and as cell-scaffold constructs using two novel scaffolds of polycaprolactone (PCL): Fused deposition modelling (FDM) versus structurally graded scaffolds (SGS) at 5% O_2 following chemical induction with TGF-β3.

Materials and Methods: Chondrogenic assessment consisted of histological evaluation, quantitative gene expression analysis of aggrecan, SOX9, CD-RAP, collagen type I, II and X, and determination of sulphated glycosaminoglycans (sGAG) and CD-RAP secretion.

Results: Induced pellets cultured in 5% O_2 showed increased cellularity and matrix deposition compared with 21% O_2. Induced pellets had higher gene expression of aggrecan, SOX9, CD-RAP, collagen type I, II, X and higher ratios of collagen type II/I and II/X compared with controls. Pellets in 5% O_2 accumulated significantly higher levels of sGAG and CD-RAP compared with 21% O_2. SGS cultures had significantly higher gene expression of aggrecan, SOX9, CD-RAP, collagen type II and X, higher ratios of collagen type II/I and II/X compared with FDM. SGS cultures had higher sGAG and CD-RAP protein expression than FDM cell-scaffold constructs.

Conclusion: MLPC possess chondrogenic potential which was enhanced in 5% O_2 in pellet cultures. SGS scaffolds supported chondrogenesis in 5% O_2 better than FDM

scaffolds. The novel SGS scaffold shows promise as a chondrogenic template in cartilage tissue engineering. The chondrogenic potency of the tested MLPC was less than expected and future work should include use of higher potency MLPC.

Introduction

Articular cartilage has limited ability for intrinsic repair and insufficient repair of focal defects often progress to osteoarthritis, which is a major cause of disability worldwide. Surgical treatment relies on cellular activation or cell transplantation with capability of healing chondral defects [1]. A number of shortcomings are related to methods based on transplantation of mature articular chondrocytes, e.g., autologous chondrocyte implantation (ACI) and scaffold-supported ACI. Chondrocytes cultured in vitro exhibit low proliferation rates, which increases the time needed for in vitro expansion and the time between chondrocyte harvesting and reimplantation [1,2,3,4]. Furthermore, in vitro chondrocyte expansion often induce dedifferentiation entailing loss of cartilage-specific phenotype [5,6]. To address these issues, researchers are currently focusing on tissue engineering strategies to improve the quality and longevity of regenerated articular cartilage. Cartilage tissue engineering employs the use of chondrocytes or multipotent mesenchymal stromal cells (MSCs) cultivated with biomaterials under specialized culture conditions. The chondrogenic differentiation potential of MSCs make them an appealing source for musculoskeletal tissue engineering [7,8]. MSCs have been isolated from numerous tissues e.g. bone marrow, adipose tissue, dental cells, orbital fat, umbilical cord blood, placenta, skin and thymus [8,9,10,11,12,13,14,15,16]. Bone marrow-derived stromal cells (BM-MSCs) are currently the most extensively investigated and best characterized multipotent cell for engineering cartilage tissue [17]. However, its collection is an invasive procedure, and the cell number, differentiation potential and maximal proliferative life span may decline with increasing donor age and cell passage number [18,19,20]. On the contrary, harvesting umbilical cord blood-derived mesenchymal stromal cells (CB–MSCs) is non-invasive in nature and these cells may express increased self-renewal and

differentiation capacity due to an immature phenotype compared with cells derived from adult tissues [13,21,22]. Equine and human CB-MSCs have been reported to exhibit increased chondrogenic potency compared to those of equine and human bone marrow and adipose tissue-derived MSCs [23,24].

Reduced oxygen tension may be a key factor in achieving optimal chondrogenic-differentiation of MSCs [25,26,27,28,29]. MSC condensation and activation of various transcriptional factors resulting in chondrogenesis during embryonic chondrogenesis are believed to be triggered by hypoxia [30]. The chondrocyte phenotype has been maintained by culturing mature articular chondrocytes at 1.5% O_2 [31]. It is speculated that such maintenance may result because of in vivo cell adaptation to low physiologic oxygen tensions as well as more closely mimicking of the hypoxic environment during the embryonic cartilage development [32]. In normal mature cartilage and bone marrow, local oxygen tensions down to 2-10% have been reported [33,34,35,36,37,38].

Another aspect of the in vivo chondrogenic niche is the spatial arrangement surrounding the chondrocyte. Chondrogenesis is enhanced in three-dimensional (3D) cultures such as the commonly used micromass pellet system or through the use of various 3D scaffolds/matrices [39,40,41,42,43,44]. These studies have shown that 3D scaffolds provide cellular niches more suitable for cell attachment, cellular arrangement, proliferation, and differentiation. Likewise, scaffold characteristics such as elasticity can direct stem cells to differentiate towards a specific stem cell lineage [44]. For example, nanofibrous polycaprolactone (PCL) scaffolds appear to enhance the chondrogenic differentiation of human MSCs [43]. In addition to scaffold structure, the use of soluble factors such as TGF-beta and BMP-6 showed promising results for chondrogenic induction of MSCs [40,41].

The purpose of this present study was to evaluate the effect of reduced oxygen tension on the chondrogenic differentiation of human clonal-derived CB-MSCs, termed multilineage progenitor cells (MLPC) in micromass pellets and to evaluate two novel macro and micro-porous scaffolds of PCL for their supportive effect of MLPC chondrogenesis. We hypothesized that commercially available human cord blood-derived MLPC exhibit enhanced chondrogenesis under reduced oxygen tension conditions and that they maintain their chondrogenic potency when cultured within novel PCL scaffolds.

Materials and Methods

Study Design

The study design is visualized in **Figure 1**. The influence of reduced oxygen tension was first studied on MLPC in conventional 3D micromass pellet cultures in 5% O_2 and 21% O_2. The favorable oxygen tension, as determined in the pellet study, was applied to the scaffold culturing system. MLPC were therefore cultured on top of and within novel 3D porous PCL scaffolds in induction medium at 5% O_2 tension. Chondrogenesis was determined by quantitative real-time PCR (RT-qPCR) to assess gene expression of six cartilage-related markers: aggrecan, SOX9, collagen type I, II and X and CD-RAP relative to reference genes GUSB and TBP. Histology was used to evaluate the deposition of extra-cellular matrix. Amount of sulphated glycosaminoglycans (sGAG) and secreted CD-RAP were used as functional markers of cartilage anabolism.

Human umbilical cord blood-derived Multi-Lineage Progenitor Cells (MLPC)

Human umbilical cord blood-derived Multi-Lineage Progenitor Cells (MLPC™, clone number 081704-1G1) were kindly provided by BioE Inc. (Saint Paul, MN). These MLPC are a clonally expanded cell line that has previously been shown to possess

osteogenic, adipogenic and chondrogenic potentials [45]. Choi et al. and Berger et al. have demonstrated that these MLPC are positive for surface markers CD73, CD90, CD105 and negative for CD34, CD45, CD106 and HLA-DR [45,46]. These phenotype characteristics and trilineage differentiation capacity of MLPC suggests that MLPC are a subpopulation of multipotent mesenchymal stromal cells (MSCs) as defined by The International Society for Cellular Therapy [47]. The cells were received in cryovials each containing 1.0 x 10^5 MLPC. The entire content of one cryopreserved vial containing 1.0 x 10^5 undifferentiated second passage (P2) cells was quickly thawed in a 37°C water bath and then seeded into a 75 cm^2 culture flask with expansion medium consisting of low glucose DMEM (Lonza, Walkersville, MD) supplemented with 30% FBS (Invitrogen, Taastrup, Denmark) and 2mM l-glutamine (Lonza, Walkersville, MD). Cells were cultured to 40-50% confluence in a standard humidified atmosphere containing 5% CO_2 and 21% O_2 at 37°C. Expansion medium was changed twice a week. Upon 40-50% confluence, cells were trypsinized (0.5% trypsin and 1.2 mM EDTA) and reseeded at 1,500 cells/cm^2. After three passages, enough MLPC were retrieved for the experiments. Hence, passage 5 MLPC was used in the studies described.

Pellet cultures of human cord blood-derived multi-lineage progenitor cells

Three-dimensional pellet cultures were established by sedimentation of 2.5x10^5 MLPC at 150*g* for 5 minutes at room temperature in 15 mL conical polypropylene tubes. Pellets were randomly split to culturing in either 0.5 mL non-inductive control (expansion medium as stated above) medium or chondrogenic inductive medium (hMSC Chondrogenic BulletKit®; Lonza, Walkersville, MD; supplemented with 10 ng/mL of TGF-β3). The pellets were cultured at 5% or 21% O_2, 5% CO_2, 37°C and humidified atmosphere with the lid loosened to facilitate gas exchange. A volume of 0.5

149 mL culture medium was changed twice a week. The culture medium was equilibrated
150 for at least 2 hours in the relevant oxygen tension before change. Pellets were cultured
151 up to 28 days.

152 **Three dimensional scaffold cultures of human cord blood-derived multi-lineage**
153 **progenitor cells**

154 Descriptions of the scaffolds are detailed below. Following expansion in monolayer
155 culture, cells were detached by trypsin/EDTA and resuspended in chondrogenic
156 inductive medium (hMSC Chondrogenic BulletKit®; Lonza, Walkersville, MD;
157 supplemented with 10 ng/mL of TGF-β3). A total of 2.5 x 10^5 MLPC in 10-μL cell
158 suspension were seeded on top of the scaffolds. The cell-loaded scaffolds were placed
159 in 1% agarose-coated 24-well culture plates without culture medium and left in standard
160 culture conditions (5% CO_2, 21% O_2 and 37°C) for 2 hours to allow cells to adhere.
161 Then, 1 mL of chondrogenic induction medium supplemented with 100 IU/mL
162 penicillin and 0.1 mg/mL streptomycin (Invitrogen, Taastrup, Denmark) was added to
163 the wells and the culture plates were transferred to a 5% O_2 containing cell culture
164 incubator. Culture time was 28 days with culture medium change twice a week.

165 **Polycaprolactone (PCL) scaffold manufacturing and surface treatment**

166 Two different PCL scaffold compositions were used: Macroporous fused deposition
167 modelled (FDM) and a novel structurally graded scaffold (SGS) of PCL. FDM-PCL
168 scaffolds were made by FDM at a processing temperature of 100°C with a
169 BioScaffolder (SYS + ENG GmbH, Germany) from PCL (MW=50 kDa, Perstorp, UK).
170 4.0 mm in diameter cylindrical scaffolds were punched out from 2.0 mm thick porous
171 PCL mats using a sterile biopsy punch (Miltex, York, PA). Deposited fibres had a
172 thickness of 200 μm. However, the thickness of each individual layer was set to120 μm
173 to ensure solidly fused junctions between layers. The center–center fibre distance in

each deposited layer was 1.0 mm and the fibre orientation of each consecutive layer was angled 105° and shifted 0.17 mm [48]. Scaffold pores were designed to be completely interconnected having macro-pores of approximately 800-1000 μm (**Figure 2**).

The microporous SGS scaffolds were made by embedding FDM scaffolds with a second PCL microporous porous network using thermally induced phase separation (TIPS). This approach enables scaffolds with a finely structured polymeric matrix and increased surface area for cell loading by using a solvent-based technique in combination with a much stiffer FDM component, which ensures the overall mechanical stability of the scaffold [49,50,51]. The SGS scaffolds were produced as described previously [52]. Briefly, a solution of 50 kDa PCL in a dioxane-water mixture was poured into the FDM scaffolds and phase separated at -30°C followed by freeze-drying, which produced interconnecting micro-pores of 100-250 μm within the larger macro-pores. Scaffolds were sterilized in series of ethanol washes and sterile water followed by surface modification with 1.25 M NaOH, neutralization in 1 M HCl and rinsed with sterile water, which increases surface hydrophilicity and subsequently improves cell attachment.

Histology

Samples were first stained with Mayer's haematoxylin and then dehydrated in graded series of ethanol (70-96%). Pellet samples were embedded in cold methylmethacrylate as described by Erben [53], and cut transversely into 7 μm sections using a microtome (Reichert-Jung Polycut E, Heidelberg, Germany). Scaffold samples were embedded in Technovit® 7100 (Axlab, Vedbæk, Denmark), and cut transversely into 10 μm sections using a Sawing Microtome KDG 95 (Meprotech, Heerhugowaard, the Netherlands). Cross sections were cut from the central part of the pellet and scaffold samples. Standard staining procedures were performed for haematoxylin and eosin. Sections

were stained with 0.5% Alcian Blue (pH 3) for 20 minutes to detect extracellular sGAG and proteoglycan synthesis. Stained slides were mounted in DPX. Sections were viewed and photographed on an Olympus BX51 (Olympus, Denmark) microscope with the use of image acquiring software PixeLINK.

Total RNA extraction

Total RNA for RT-qPCR was extracted using the GenElute™ Mammalian Total RNA Kit (Sigma-Aldrich, Missouri, USA) as described by the manufacturer with the following modifications. Samples were lysed in lysis buffer supplemented with β-mercaptoethanol and glass beads (0.7-1.0 mm diameter, Retsch GmBH, SKANLAB Aps, Denmark) for pellets or tungsten carbide balls (3 mm diameter, Retsch GmBH, SKANLAB Aps, Denmark) for scaffold constructs, respectively. Samples were homogenized using a mixer mill (Retsch GmBH MM 301, SKANLAB Aps, Denmark) for three cycles of 30 seconds at a frequency of 30.0 Hz with a 30 second rest period in between cycles. All subsequent RNA extraction steps were performed according to GenElute™ protocol. Isolated RNA concentration and quality was quantified using a nanophotometer (Implen, Munich, Germany).

Quantitative real-time RT-qPCR

First-strand cDNA was synthesised from 100 ng of extracted total RNA using the High Capacity cDNA Archive Kit (Cat no 4322171, Applied Biosystems, Naerum, Denmark). The RT-qPCR was performed at standard enzyme and cycling conditions on a 7500 Fast Real-Time PCR system using TaqMan® gene expression assays (Cat no 4352042, Applied Biosystems, Naerum, Denmark) for Hs99999908_m1 (beta-glucuronidase [*Gusb*]), Hs00427621_m1 (TATA box binding protein [*Tbp*]), Hs00153936_m1 (aggrecan [*Agc*]), Hs00165814_m1 (chondrocyte-specific transcription factor [*Sox9*]), Hs00164004_m1 (collagen type I alpha-1 [*Col1a1*]),

Hs00264051_m1 (collagen type II alpha-1 [*Col2a1*]), Hs00166657_m1 (collagen type X alpha-1 [*Col10a1*]), Hs00197954_m1 [*Cd-rap*] and a custom designed TaqMan assay for human RNA polymerase II [*RpII*]. These two assays included one FAM™ dye-labelled TaqMan® MGB probe spanning exon-exon boundaries in the gene of interest (GOI) and two unlabelled hot-start primers. Amplicon size was less than 170 bp for all primer sets to maximize the amplification efficiency. Template cDNA corresponding to 4 ng mRNA was used in each PCR reaction and run in technical duplicates for each gene. PCR reactions were run in 96-well plates in a final reaction volume of 20 µL at 40 cycles consisting of a denaturation step at 95°C for 3 seconds and annealing/extension step at 60°C for 30 seconds. Data analysis was performed using 7500 Fast System Sequence Detection Software, version 1.3. GUSB and TBP have been reported to be the most stable genes for MSCs cultured in different oxygen conditions and were used as reference genes (housekeeping genes) [54]. Gene expression levels were normalized to a BestKeeper index [55] based on the geometric mean of cycle's threshold (Ct) from GUSB and TBP using following equation:

Relative gene expression=$2^{\text{Ct (BestKeeper Index) - Ct (gene of interest)}}$.

Sulphated glycosaminoglycans and DNA extraction and quantification

Samples were digested in 150 µL (i.e. pellets) or 450 µL (i.e. scaffolds) solutions of 300 µg/mL papain in 20 mM PBS, pH 6.8, 5 mM EDTA, 2 mM DTT at 60°C for 18 hours (Sigma-Aldrich, Brøndby, Denmark).

Proteoglycan content was estimated by quantifying the amount of sGAG using the dimethylmethylene blue dye-binding assay (Blyscan™, Nordic BioSite ApS, Copenhagen, Denmark), with a chondroitin sulphate standard. Manufacturer's instructions were followed. One mL of dimethylmethylene blue was allowed to form purple coloured precipitate with 50 µL papain-digested extract for 30 minutes. The

precipitate was separated from the unbound dye by centrifugation at 12,000x*g*. The purple dye-sGAG precipitate was re-solubilised with 1 mL dye dissociation reagent. Dye absorbance was measured at 650 nm on 200 µL sGAG samples, chondrotin-4-sulfate standards, and blanks using a spectrophotometer (Victor^3 1420 Multilabel Counter, PerkinElmer Life Sciences, Hvidovre, Denmark) as technical duplicates in 96-well clear plate.

Total DNA was quantified using the Quant-iT™ PicoGreen® dsDNA assay (Invitrogen, Denmark). DNA was released from papain-digested extract by vortexing 1:4 Tris–EDTA (TE) buffer diluted samples. Fifty µL of diluted samples were allowed to react with 50 µL PicoGreen (diluted 1:200 in TE buffer) for 5 min in dark followed by measurement in 96-well plate using a microplate reader (Victor3 1420 Multilabel Counter, PerkinElmer Life Sciences, Denmark). Samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm. Standards were prepared according to the manufacturer's instruction (lambda DNA, concentration range: 0–1 µg/mL). Technical duplicates were used for each biological sample.

CD-RAP ELISA

Cartilage-derived retinoic acid-sensitive protein (CD-RAP), also known as melanoma inhibitory activity (MIA), was included since it has recently been promoted as a novel marker for chondrogenic differentiation in MSCs [23,56,57,58]. CD-RAP is primarily expressed and secreted by cartilage cells, including both developing and mature cartilage. CD-RAP is a secreted soluble protein and its secretion into conditioned culture medium allows continuous monitoring of MSCs chondrogenic anabolism, differentiation and the chondrocyte phenotype development without cell culture interference or termination. Weekly collected culture medium was analysed for CD-RAP concentrations using the MIA ELISA assay (Roche Diagnostics, Naerum,

Denmark) according to the manufacturer's instructions. The absorbance was detected after 15 min at 405 nm (reference: 495 nm). CD-RAP protein concentrations were interpolated from the standard curve generated from included standards containing CD-RAP derived from transfected Chinese hamster ovary cells in a serum analogue matrix containing BSA.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to visualize the surface-morphology and distribution of cells and PCL matrix within the scaffolds. The scaffolds were fixed in 2.5% glutaraldehyde containing 0.1 M sodium cacodylate buffer (pH 7.4) and dehydrated in a graded series of ethanol (50-99%) before being transferred to an exsiccator for air drying. Scaffolds were analysed using a low-vacuum secondary electron detector (Nova NanoSEM 600, FEI Company).

Statistical analysis

Data were ln-transformed (RT-qPCR), checked for normal distribution and variance homogeneity. Two-way analysis of variance (ANOVA) was performed with culture medium and oxygen tension as independent variables (medium*oxygen) for the pellet cultures; and culturing time and scaffold composition as independent variables (time*scaffold composition) for the scaffold cultures. Data are presented as means ± standard errors of means for three (n = 3) biological replicates and two technical duplicates. A two-way ANOVA (Bartlett's test for equal variances) was performed using STATA version 10.1 (STATA Corp., TX). For all analyses, a 95% confidence interval was used and significance level was set to $p<0.05$.

Results

Micromass pellet cultures of human cord blood-derived multi-lineage progenitor cells

Pellets cultured for four weeks in chondrogenic inductive medium in either 5% O_2 or 21% O_2 both increased in size compared with control pellets which did not increase in size. Induced pellets were spherical with a diameter of approximately 2 mm. Induced pellets cultured in either 5% O_2 or 21% O_2 developed a firm tissue texture and had a smooth yellow surface compared with non-induced control pellets which were non-uniform, fragile and in a state of disintegration. Culturing in 5% O_2 supported the deposition of proteoglycan synthesis as shown in Alcian Blue stained sections (**Figure 3**). Pellets cultured in 5% O_2 more closely resembled the morphology of chondrocytes compared with 21% O_2 cultured pellets (i.e. rounded cells in lacunae surrounded by hyaline-like matrix) (**Figure 3**). In 5% O_2, cells and proteoglycan depositions were located peripherally in the pellets. This is in contrast to 21% O_2 in which cells and matrix were located centrally in the pellets. Regardless of the oxygen tension non-induced control pellets did not stain positive for proteoglycans (**Figure 3**). Haematoxylin and eosin staining of control pellets displayed decreased cellularity with pyknotic nuclei indicating apoptosis (**Figure 3**).

Pellets induced in 5% O_2 showed increased mRNA expression of aggrecan ($p<0.01$), SOX9 ($p<0.01$), collagen type I ($p<0.05$), collagen type X ($p<0.01$) and CD-RAP ($p<0.01$) compared with non-induced control pellets (**Figure 4**). Similarly, pellets induced in 21% O_2 showed increased mRNA expression of aggrecan ($p<0.01$), SOX9 ($p<0.01$), collagen type I ($p<0.01$), collagen type X ($p<0.01$) and CD-RAP ($p<0.01$) compared with control pellets. No differences in gene expression were noted between induced pellet cultures at 5% versus 21% O_2.

Induced pellets showed increased accumulation of sGAG compared with non-induced control pellets at both 5% O_2 and 21% O_2 ($p<0.01$) (**Figure 5A**). Pellets induced in 5% O_2 demonstrated higher sGAG levels compared with induced pellets in 21% O_2 ($p<0.05$). CD-RAP protein determination on serially collected conditioned culture medium showed detectable CD-RAP from day 14 in 5% O_2 and 21% O_2 induced pellet cultures and at day 28 in control pellets (**Figure 5B**). CD-RAP concentrations increased from day 14 to 28 ($p<0.05$) in induced pellets cultured in 5% O_2. Similar temporal CD-RAP increase was observed in 21% O_2 ($p<0.05$). Induced pellets in 5% O_2 showed increased CD-RAP concentrations compared with 21% O_2 both at day 14 ($p<0.01$) and day 28 ($p<0.05$), respectively.

Three dimensional scaffold cultures of MLPC

Both FDM and SGS scaffolds supported chondrogenic differentiation of MLPC. On day 28 SGS scaffolds showed increased mRNA expressions of aggrecan ($p<0.01$), SOX9 ($p<0.01$), collagen type II ($p<0.01$), collagen type X ($p<0.01$) and CD-RAP ($p<0.05$) compared with FDM scaffolds (**Figure 6**). Collagen type I mRNA expression was similar between the cell-scaffold groups at day 14 and 28, but on day 28 collagen type I was down regulated in both cell-scaffold constructs compared with day 14 (FDM, $p<0.01$; SGS, $p<0.01$). SGS scaffolds showed higher ratios of collagen type II/I ($p<0.01$) and collagen type II/X ($p<0.01$) compared with FDM scaffolds. On day 28, SGSs showed increased collagen type II/I and collagen type II/X ratios compared with day 14 ($p<0.05$).

On day 28, SGSs had accumulated higher amounts of sGAG compared with FDM scaffolds ($p<0.05$) (**Figure 7A**). sGAG accumulation increased in SGS scaffolds from day 14 to day 28 ($p<0.05$). CD-RAP was detectable in conditioned medium from day 7 (**Figure 7B**). There was a continuous temporal increase during the culture period in CD-

346 RAP secretion for both FDM and SGS based cell-scaffold constructs ($p<0.01$). No
347 differences in CD-RAP concentrations between FDM and SGS based cell-scaffold
348 constructs were noted.

349 Tissue preparation for the scaffold histology lead to PCL dissolving that precluded
350 optimal histological assessment. With this caveat, histology images of the cell-scaffold-
351 constructs are provided (**Figure 8A**). Both FDM and SGS scaffolds supported
352 chondrogenic differentiation of MLPCs and deposition of proteoglycans. The cells were
353 heterogeneous distributed in the scaffolds and were primarily located at the superficial
354 scaffold regions and adherent to scaffold fibres. It is noted that cells were more
355 dispersed in the SGS scaffolds. Between the fibres clustering of cells and deposition of
356 proteoglycans is observed (**Figure 8A)**. In SEM cells spanning PCL fibre and TIPS-
357 PCL could be demonstrated (**Figure 8B**).

358 ## Discussion

359 We determined that chondrogenic induction of MLPC in pellet culture is enhanced in
360 reduced oxygen tension (5% O_2) compared with normoxic oxygen tension (21% O_2).
361 Furthermore, we demonstrated that SGS scaffolds supports MLPC chondrogenesis
362 better than FDM scaffold in vitro.

363 The decreased gene expression of collagen type I and X suggest that the formed
364 cartilage is neither fibrocartilage nor hypertrophic cartilage. Transcriptional factor
365 SOX9 and CD-RAP expressions were decreased in 5% O_2 culturing, which is in
366 accordance with observations for BM-MSCs cultured in hypoxia [59,60]. We did not
367 investigate the time-course of gene expressions, but such studies may have revealed
368 increased expression of SOX9 and CD-RAP in the initial chondrogenic phase as
369 reported elsewhere [60]. Collagen type II/I ratios increased in 5% O_2 induced pellets
370 compared with 21% O_2; however, this was insignificant. Both of these ratios provide

371 indications that a hyaline chondrocytic phenotype of MLPC is favoured in hypoxic
372 environment. Our finding of increased collagen type II/I ratios in 5% O_2 is supported by
373 others such as Kurtz and colleagues who reported increased collagen type II/I ratios in
374 mature chondrocytes cultured on a collagen type I/III scaffold at 5% O_2 [61].

375 SGS MLPC-scaffold constructs had significantly higher expression levels of
376 aggrecan, collagen type II, SOX9 and CD-RAP compared with FDM MLPC-scaffold
377 constructs. We also demonstrate a time-dependent downregulation of collagen type I in
378 both types of MLPC-scaffold constructs suggestive of cartilage maturation over time.
379 The occurrence of cartilage maturation is supported by the increased gene expression of
380 aggrecan, collagen type II and SOX9 in the SGSs from day 14 to day 28. Increased
381 collagen type II/I and collagen type II/X ratios in the SGS scaffolds compared with
382 FDM scaffolds also indicate that SGS provides a more chondrogenic environment for
383 the MLPC.

384 The role of oxygen tension in chondrogenic differentiation of MSCs is incompletely
385 understood. A number of studies show that low oxygen tension favours the
386 chondrogenic phenotype for both mature chondrocytes and MSCs from different origins
387 [62,63,64,65,66]. Other studies have been unable to demonstrate convincing benefits in
388 hypoxic culturing [67]. In the present study, the cellularity and ECM were organized in
389 a zonal manner in both induced pellets and MLPC-seeded scaffolds. In 5% O_2 cultured
390 pellets differentiated cells were located peripherally compared with 21% O_2 where
391 differentiation was most prominent centrally. In the scaffolds, increased cell density and
392 ECM deposits were present close to the surface between the scaffold fibres
393 demonstrating that differentiation takes place in a gradient-like fashion similar to the
394 observation made in the pellet cultures and by others [28,68]. The graded distribution of
395 cells and ECM in the pellet and scaffold cultures may indicate that an optimal oxygen

tension for chondrogenesis may be present. Previous work measuring the actual oxygen concentration in in vitro cartilage constructs after 2 weeks of dynamic culturing revealed an oxygen gradient ranging from 21% at the surface down to 10% at 0.5 mm depth [36]. This oxygen gradient may be more pronounced in our pellet study due to a large pellet size (1.5 to 2 mm in diameter) and lack of an open porous structure. The passive diffusion of the oxygen molecule is theoretically limited to a maximum of 200 μm for pellet culture [67]. Due to the effect of oxygen gradient, some studies have suggested that chondrogenic induction in pellet cultures is optimally carried out in 10-15% O_2 [28,68].

Gas diffusion and protein retention differences are to be expected between various scaffold materials. For the macroporous FDM scaffold, oxygen diffusion into the scaffold is most likely higher than for the more compact SGS scaffold. SGS scaffold may thus generate hypoxic conditions for the seeded cells that are more conducive for chondrogenic differentiation. The SGS scaffolds' increased density may also contribute to decreased macromolecular transport. In accordance with the argument of compromised diffusion in molecules, we observed increased expression of CD-RAP on the mRNA level in SGS scaffolds while there was no significant difference between the measured concentrations of CD-RAP released into the media when comparing the FDM and SGS scaffolds. A probable explanation for this discrepancy is that the transport of CD-RAP from the inside of the SGS scaffold to the sampled media is compromised due to the tighter porous network limiting the passive diffusion of CD-RAP. In contrast, we detected higher deposition of sGAG within the SGS versus FDM scaffolds and this was followed up by a corresponding increase in aggrecan gene expression suggesting that the SGS provides a more chondrogenic environment for the chondrogenic differentiation of MLPC. Furthermore the positive correlation in the measured sGAG to

aggrecan mRNA levels versus inverse correlation in measured CD-RAP concentration to CD-RAP mRNA levels is justified by the differences in data collection; CD-RAP was measured by media collection in which the diffusion is compromised by the porous network while sGAG was measured in the extract of papain-digested scaffolds.

We were unable to detect that increased matrix accumulation in pellets cultured in 5% O_2 was followed by a corresponding increase in aggrecan and CD-RAP gene expressions. We speculate that a low oxygen tension more quickly upregulates aggrecan and CD-RAP initially, thus accelerating sGAG and CD-RAP synthesis. A negative feedback loop mechanism-triggering gene down regulation in response to increased protein synthesis may be present [69,70]. It is possible that aggrecan and CD-RAP gene expressions were higher in 21% O_2, but the downstream translated products were significantly lower compared to 5% O_2. In this case, aggrecan and CD-RAP gene expressions may still be upregulated because the amount of protein had not reached threshold levels triggering the feedback loop. However, we do not have qPCR data for earlier time points. It would be of interest that future studies investigate the association between gene expression and protein synthesis at various time points to evaluate their temporal relationship.

Although hyaline-like cartilage was produced by MLPC in both pellet and scaffold studies it did not meet our expectations of maturity based on morphology and matrix formation. According to Liu et al., stable cartilage phenotype of BM-MSCs is achieved after 8 to 12 weeks of differentiation [71]. This may explain the weak matrix staining suggestive of incomplete chondrogenesis in the present study and it is likely that a longer induction time would produce more mature tissue. Based on side-by-side comparative studies of human and equine umbilical cord blood-derived MSCs with that of MSC derived from bone marrow and adipose tissue, we expected the MLPC to

446 exhibit higher chondrogenic potency [23,24]. However, clonally expanded cell cultures
447 are unlikely to be comparable to primary heterogeneous cell cultures for a number of
448 reasons and the MLPC properties should not be extrapolated to primary cord blood
449 MSC cultures. Biological variation between donors with respect to chondrogenic
450 potency is a common finding and it is possible that the MLPC studied were isolated
451 from an individual or primary cell clone with reduced chondrogenic potential. The
452 increased number of population doubling times required for clonal-derived cell cultures
453 to reach sufficient cell numbers may also have selected for cells with lower
454 chondrogenic potency.

455 Since the optimal differentiation medium for MLPC has yet to be defined, an
456 improved chondrogenic differentiation of MLPC may also be obtained through the use
457 of different chemical inducers in the culture medium. The importance of growth factors
458 for differentiation has recently been discussed [72]. The effect of BMP-2, BMP-4 and
459 BMP-6 as a supplementary driving force for MSC differentiation is a subject of very
460 active investigation. Decreased hypertrophy of AD-MSCs have been reported with a
461 combination of BMP-2/TGF added to the culture medium [73]. Synergistic effect of
462 BMP-6 in the chondrogenesis of AD-MSCs has been reported [74,75]. However, it is
463 also noted that BMP-6 might have a dual role being both an osteogenic inducer as well
464 as a chondrogenic inducer [76]. Our differentiation medium did not include BMPs but it
465 seems plausible that enhanced chondrogenic differentiation of MLPC can be obtained
466 through the use of selected BMPs.

467 In conclusion, MLPC possess chondrogenic potency when differentiated in a pellet
468 micromass system as well as in PCL scaffolds in a defined chondrogenic inductive
469 culture medium. This differentiation is enhanced when culture is carried out in an
470 oxygen tension of 5% O_2. Furthermore, SGS scaffolds of PCL supports MLPC

chondrogenesis better than a FDM produced scaffold. Future studies of SGS cell-scaffold constructs using MSCs with high chondrogenic potency could provide further insight into the possible clinical utility of this novel scaffold in cell-based cartilage repair approaches.

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Author Contributions

Conceived and designed the experiments: SM DQSVL CBF JVN KS TGK. Performed the experiments: SM RJF. Analysed the data: SM TGK. Contributed reagents/materials/analysis tools: CBF KS. Manuscript preparation: SM RJF TGK.

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Figure legends

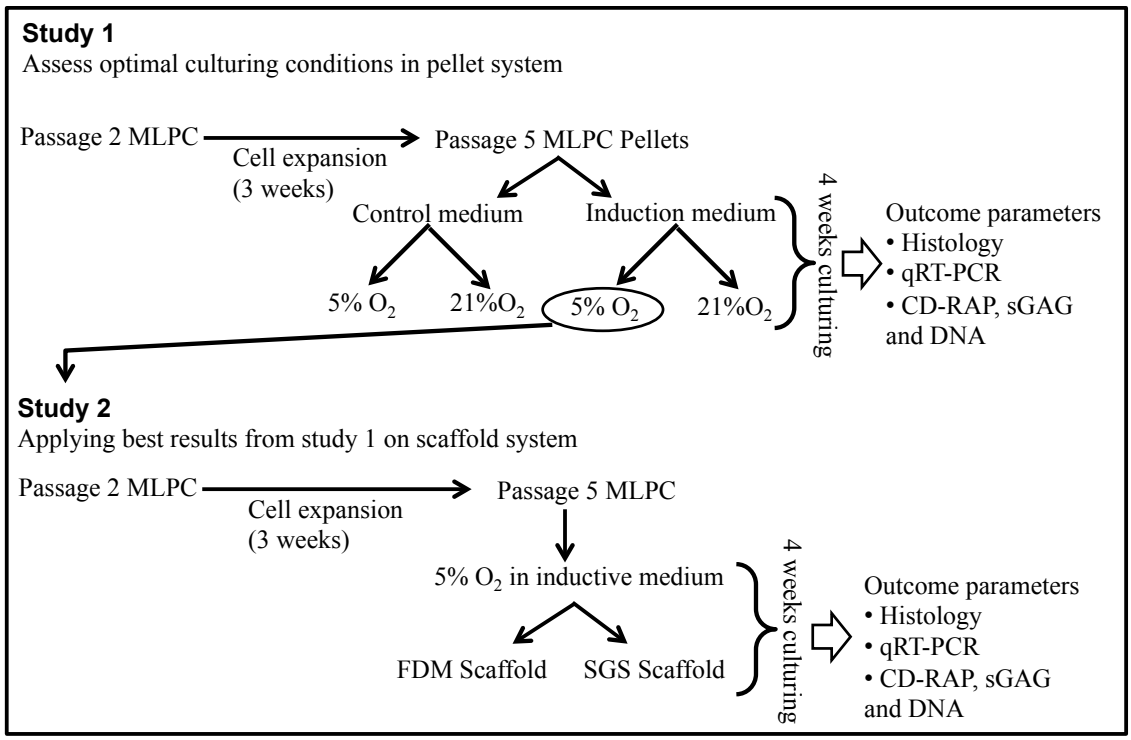

Figure 1. Study design and outcome parameters. Chondrogenic differentiation of multilineage progenitor cells (MLPC) evaluating the effect of oxygen tension and medium was assessed in study one. Study two evaluated the chondrogenic potential of MLPC cultured on two scaffold systems. FDM, Fused deposition modeled scaffold. SGS, Structurally graded scaffold.

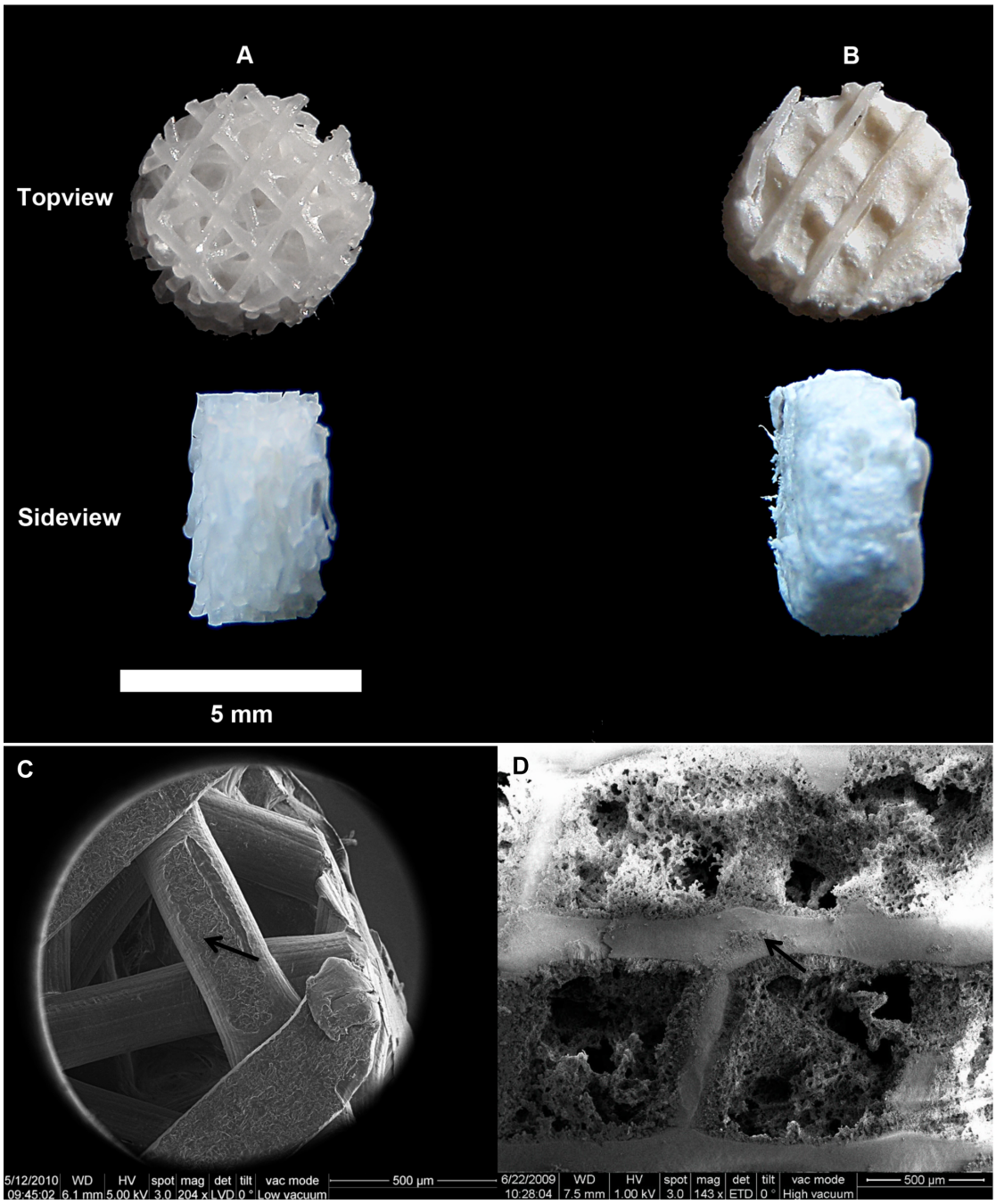

Figure 2. Macroscopic pictures of (A) Fused deposition modeled (FDM) scaffold. Note that the shift in fibre orientation of 105° between layers create the grid-shaped scaffold structure. (B) Structural graded scaffold made by combined FDM and TIPS-FDM (thermally induced phase separation). Scale bar indicate 5 mm. SEM images of (C) FDM and (D) SGS scaffolds. Note that TIPS of the solvent PCL creates micro-pores of 100-250 µm within the larger macro-pores. (↗) Arrow indicates PCL fibre. Scale bar indicates 500 µm.

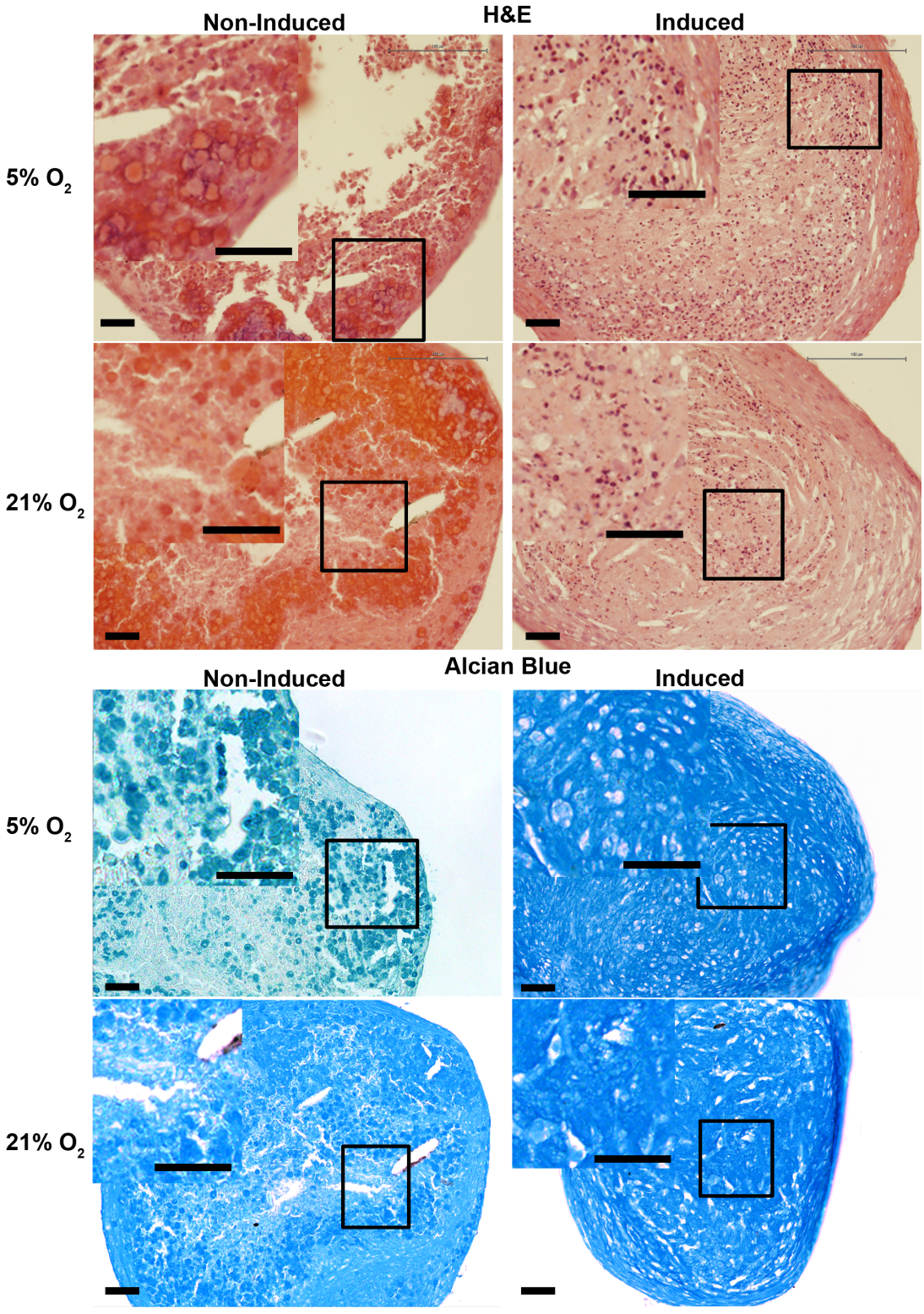

Figure 3. Histological appearance in pellet cultures of multilineage progenitor cells (MLPC). MLPC were cultured in 5% O_2 and 21% O_2 in non-inducing control and chondrogenic inducing media. Sections stained with H&E (upper panel) and Alcian Blue (lower panel). Scale bar indicate 50 µm.

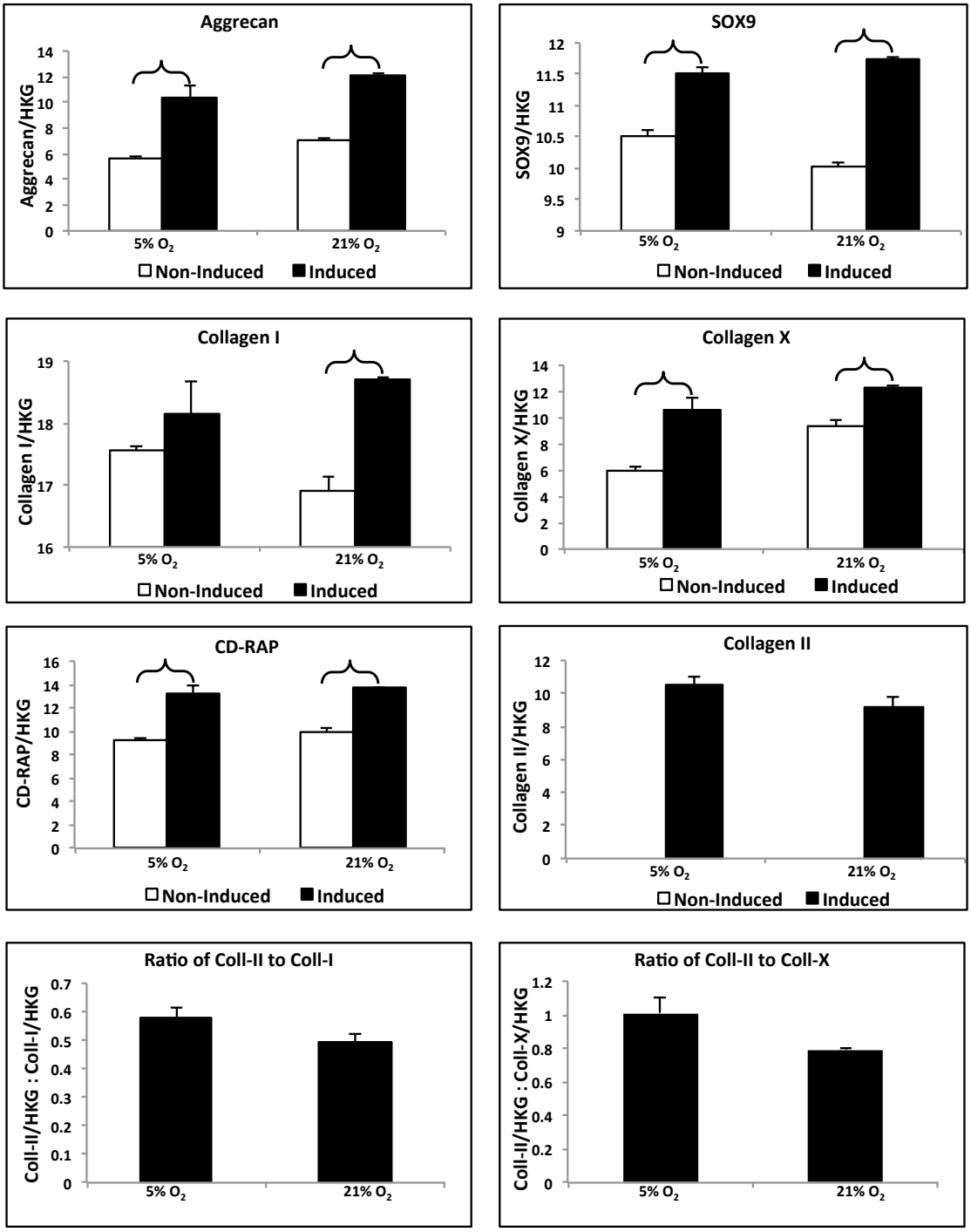

Figure 4. RT-qPCR of chondrogenic gene markers aggrecan, collagen I, collagen II, collagen X, SOX9 and CD-RAP in pellets of multilineage progenitor cells. Pellets cultured in 5% or 21% O_2 in non-inductive control medium (white bars) or chondrogenic inductive medium (black bar). Values shown represent means ± SEM normalized to two housekeeping genes, n=3. Brackets indicate significant difference between columns ($p<0.05$).

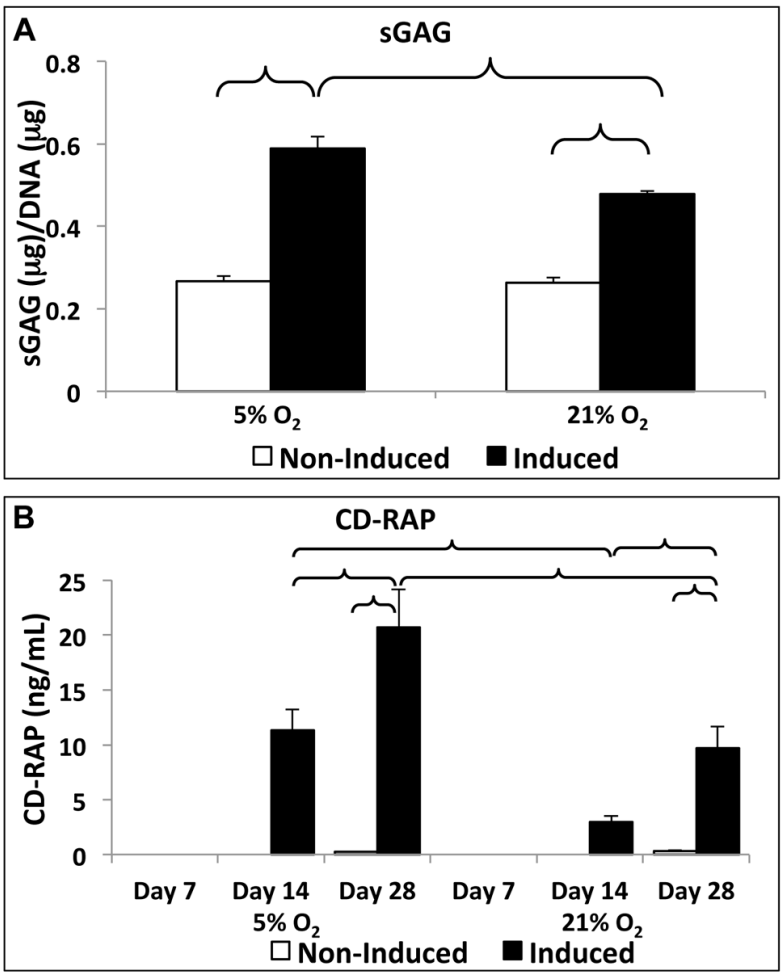

Figure 5. Intra-pellet accumulation of proteoglycan and secreted CD-RAP in pellet cultures of human umbilical cord blood-derived multi-lineage progenitor cells (MLPC). Pellets were cultured in either 5% O_2 or 21% O_2 in non-inductive control medium (white bars) or chondrogenic inductive medium (black bar). (A) Sulphated proteoglycan accumulation was normalized by corresponding DNA content of the pellet mass. (B) Secreted CD-RAP content was measured on the conditioned medium, e.g. medium exposed to the pellets for 2-3 days. Values shown represent mean levels ± SEM, n=3. Brackets indicate significant difference between columns ($p<0.05$).

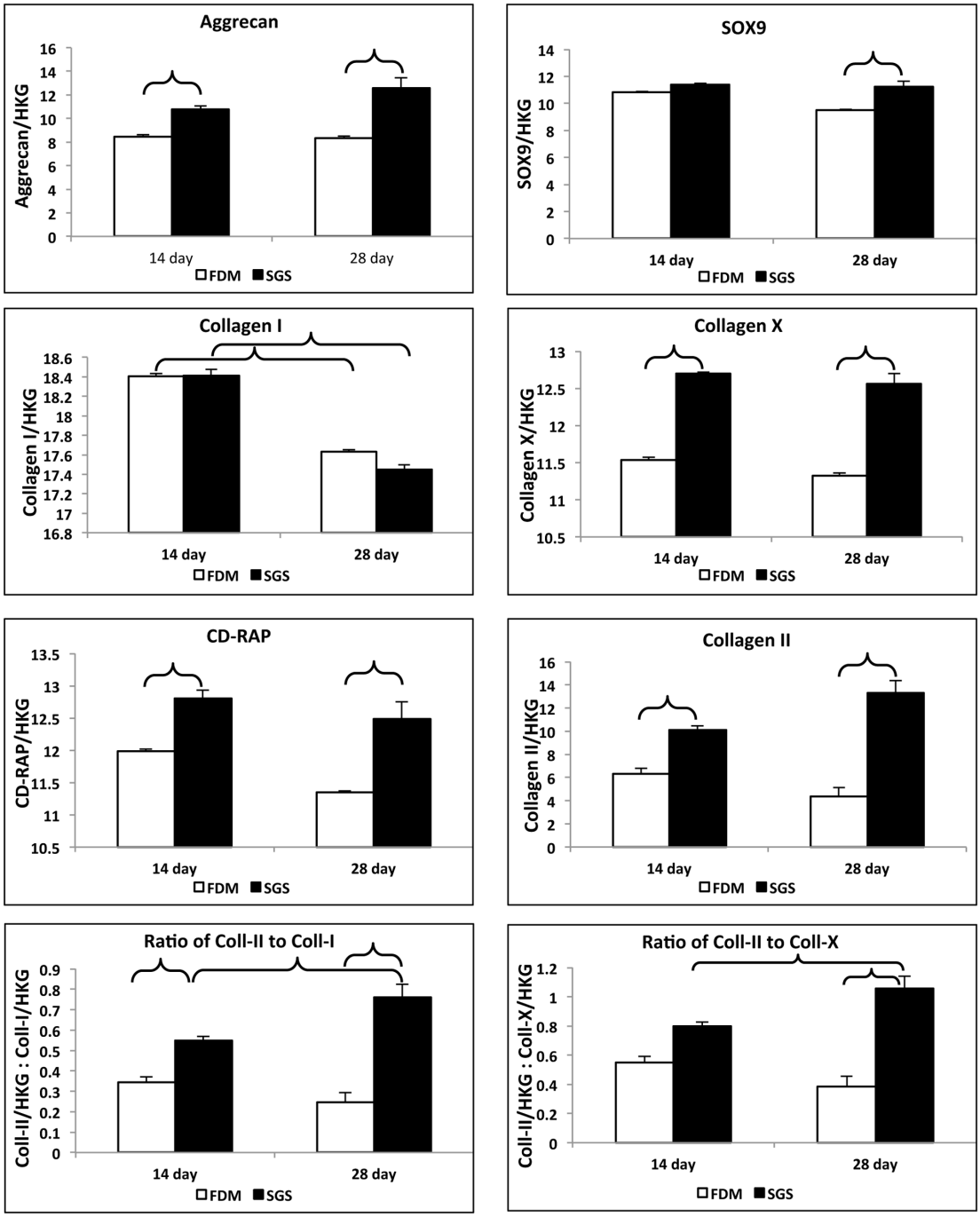

Figure 6. RT-qPCR of chondrogenic gene markers aggrecan, collagen I, collagen II, collagen X, SOX9 and CD-RAP of multilineage progenitor cells cultured on scaffolds at 5% O_2 in chondrogenic induction medium. FDM, Fused deposition modeled scaffold (white bars). SGS, Structurally graded scaffold (black bars). Values shown represent means ± SEM normalized to two housekeeping genes, n=3. Brackets indicate significant difference between columns ($p<0.05$).

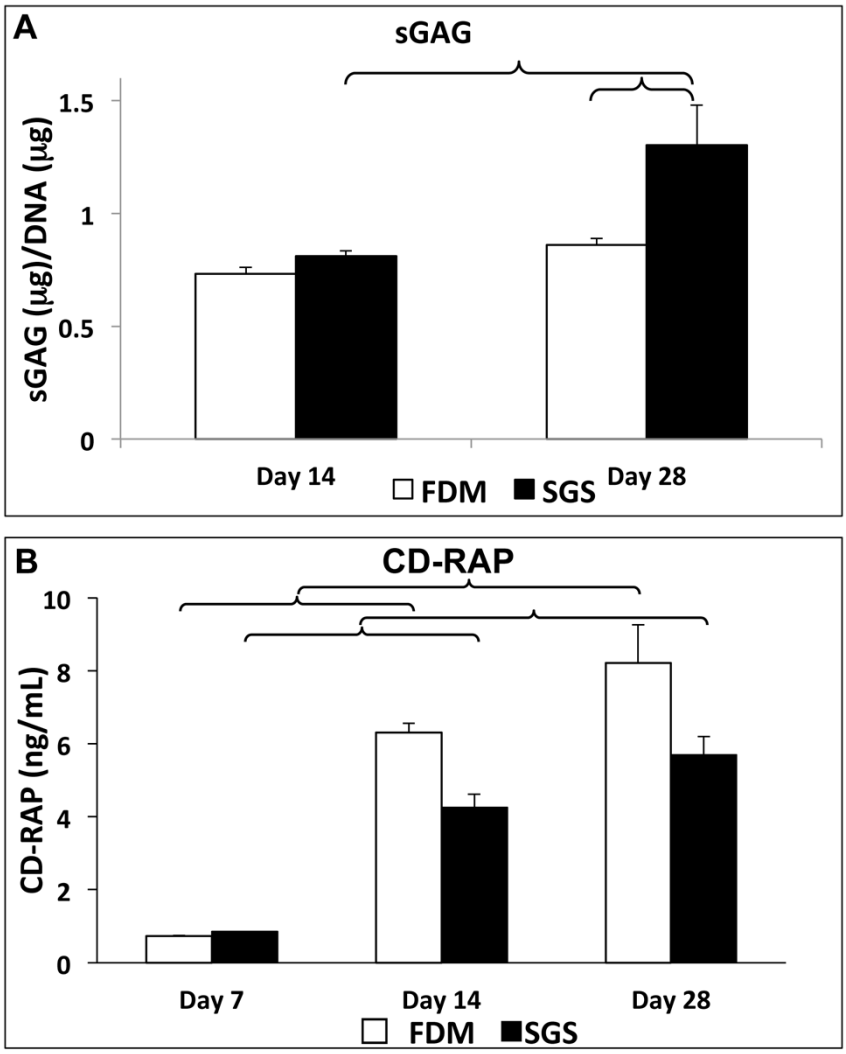

Figure 7. Accumulation of proteoglycan and secreted CD-RAP in scaffold cultures of human umbilical cord blood-derived multi-lineage progenitor cells (MLPC). Scaffolds were cultured at 5% O_2 in chondrogenic inductive medium on FDM, Fused deposition modeled scaffold (white bars) or SGS, Structurally graded scaffold (black bars). (A) Sulphated proteoglycan accumulation was normalized by corresponding DNA content. (B) Secreted CD-RAP content was collected from the culturing medium. Values shown represent mean levels ± SEM, n=3. Brackets indicate significant difference between columns ($p<0.05$).

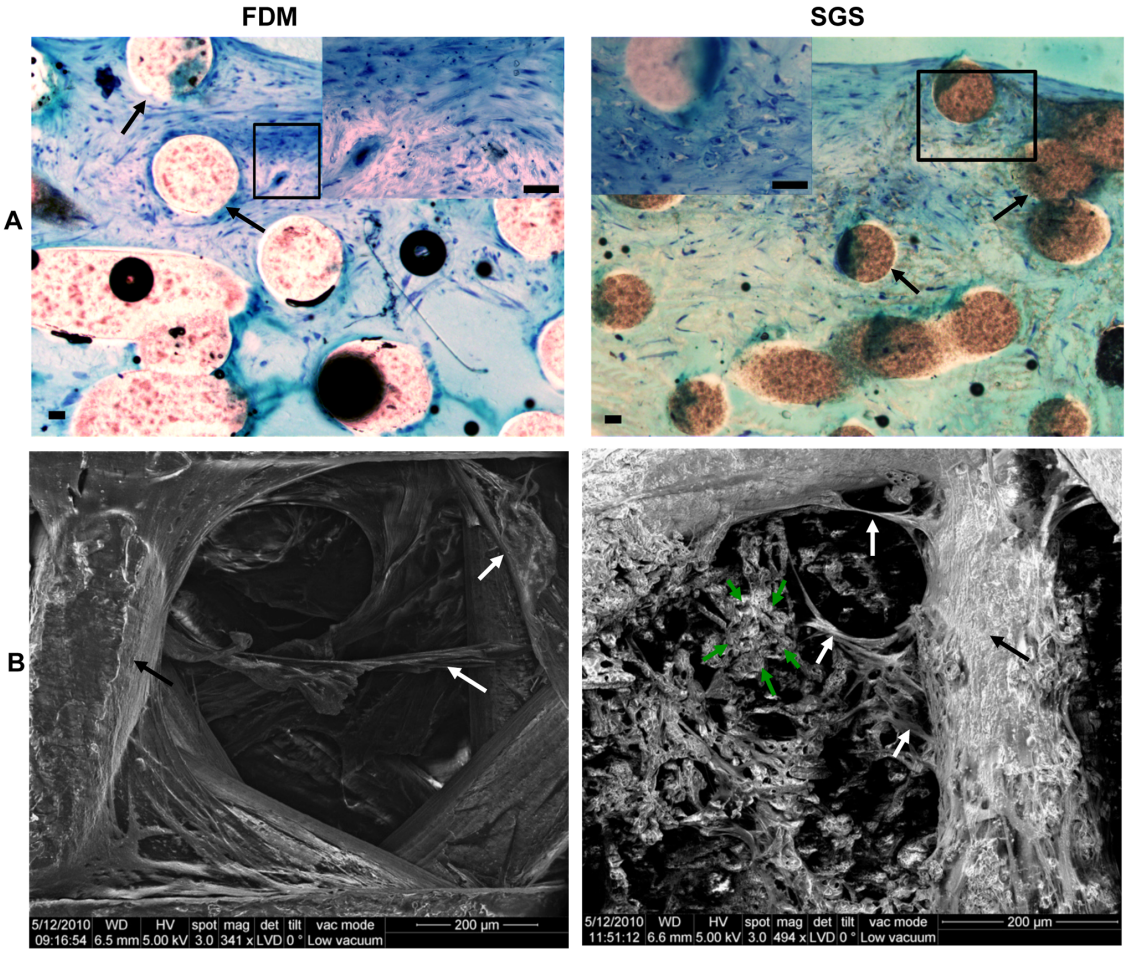

Figure 8. Panel A: Alcian Blue stained sections for chondro-induced MLPC cultured on PCL scaffolds in 5% O_2. FDM, Fused deposition modeled scaffold. SGS, Structurally graded scaffold. Both FDM and SGSs supported the deposition of proteoglycans. Black arrow (↗) indicates cross-sections of polycaprolactone (PCL) fibre surrounded by cells and in between fibre clustering of cells and deposition of proteoglycans is observed. Cell density is lower in SGS. In the displayed images, the surface of the scaffold is orientated upwards. Please note suboptimal image quality due to laboratory error, see result section for further details. Scale bar indicate 50 µm. Panel B: SEM images for chondrogenically induced MLPCs cultured on PCL scaffolds. Black arrow (↗) indicates PCL fibre. Green arrow (↗) indicates TIPS-PCL. White arrow (↗) indicates cells spanning over PCL fibre or TIPS-PCL.

Paper III

Collagen-Coated Polytetrafluoroethane Membrane Inserts Enhances Chondrogenic Differentiation of Human Cord Blood Multi-Lineage Progenitor Cells

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Abstract

Articular cartilage defects are common and may progress to osteoarthritis. High incidence makes osteoarthritis a major cause of disability for which there is no definitive cure. Articular chondrocytes and bone marrow-derived multipotent mesenchymal stromal cells (MSCs) are presently the favoured cells for cartilage tissue engineering. Umbilical cord blood multilineage progenitor cells (MLPC) have proven an alternative source of MSCs that may be more potent chondroprogenitor cells than bone-marrow MSCs. MLPCs are clonal-derived cells and may therefore provide a cell source with more reproducible outcomes compared to heterogeneous primary MSC cultures. This in turn would allow development of cell-based therapies with a higher positive predictive value of the treatment modality. We evaluated chemically directed chondrogenic potency of MLPCs in standard micromass pellet system, MPLCs layered on calcium polyphosphate (CPP) scaffolds, and on semi-permeable polytetrafluoroethane membranes with and without pre-coating of collagen type I, II or IV. Membrane cultures resulted in a multicellular layer tissue with formation of cartilage-like tissue compared with micromass and CPP cultures. Membrane cultures of MLPCs produced pellucid discs, 12 mm in diameter by 1 mm in thickness from $2x10^6$ cells. The discs had chondrocyte-like extracellular matrix (ECM), with 4-fold greater proteoglycan content compared with pellet cultures of MLPCs. The mRNA expression of cartilage related genes for aggrecan, collagen II and SOX9 were significantly increased in membrane cultures compared with micromass and CPP cultures. In conclusion, we have demonstrated that MLPCs possess' chondrogenic potency, which increased in scaffold-free membrane cultures resulting in multicellular-layered neocartilage tissue. Evaluating the effect of culturing pre-differentiated MLPCs on CPP is an obvious next step since direct seeding of MLPCs on CPP did not yield satisfactory biphasic constructs.

Introduction

Articular cartilage is distinguished by a limited ability for intrinsic repair, making the tissue vulnerable to further degeneration and resulting in osteoarthritis if left untreated[1]. Non-surgical therapies provide palliative pain relief and do not halt or reverse tissue damage. Surgical treatment relies on cellular activation of cells or transplantations of cells and/or scaffolds to halt, or even heal, chondral defects[2,3]. Chondrocyte, the resident cell of articular cartilage, has been used extensively to repair focal cartilage defects though methods such as autologous chondrocyte implantation (ACI) and scaffold-supported ACI. A number of shortcomings are present in methods that rely on transplantation of mature articular chondrocytes. Chondrocytes harvested from joints are limited in number and proliferates slowly *in vitro*, which increases the time needed between chondrocyte harvesting and re-implantation[2,4-6]. Furthermore, *in vitro* expansion is associated with chondrocyte dedifferentiation entailing phenotype modulation[7-12]. An alternative, rapidly-proliferating cell source with chondrogenic potential would circumvent these challenges.

Multipotent mesenchymal stromal cells (MSCs) have attracted many researchers' attention as a consequence of its chondrogenic potential and the simplicity of isolation and expansion. MSCs have been isolated from numerous tissues, including bone marrow, adipose tissue, dental cells, orbital fat, umbilical cord blood, placenta, skin and thymus[13-20]. Bone marrow-derived stromal cells (BM-MSCs) are presently the most extensively investigated and best characterized cell for tissue engineering cartilage tissue[21], but harvesting from patients is invasive. On the other hand, harvesting umbilical cord blood-derived mesenchymal stromal cells (CB–MSCs) is non-invasive. Additionally, CB-MSCs express increased self-renewal and differentiation capacity owing to a more immature phenotype compared to stem cells derived from adult tissues[17,22,23]. Superior chondrogenic potential of CB-MSCs compared to BM-MSCs has

been reported[24,25]. In an unpublished, but submitted manuscript, we report chondrogenic potential of human umbilical cord blood-derived multilineage progenitor cells (MLPCs).

Three-dimensional culturing allow cells to achieve a spherical morphology that enhances chondrogenesis, i.e., culturing in pellets, in various scaffolds/matrices, and scaffold free 3D cultures maintains round cell morphology and thus enhances chondrogenic differentiation[26-32]. Biphasic osteochondral constructs have been made from calcium polyphosphate (CPP). CPP consists of a porous bone substitute biomaterial with relatively high values for compressive strength within the group of porous ceramics and its excellent osseointegrative properties as demonstrated *in vivo* and *in vitro* makes CPP a suitable bone substitute material[33-35]. Cell-anchoring of chondrocytes to CPP, entailing a firm and transplantable biphasic osteochondral construct, has been reported[36,37].

In a recent study Lee et al. demonstrated the formation of a biphasic osteochondral construct consisting of hyaline-like cartilage on top of porous CPP[32]. They reported the necessity of employing an intermediate step in which ovine BM-MSCs are first chemically induced to chondroblasts on collagen IV coated semi-permeable culture plate inserts and then cultured on porous CPP producing an excellent biphasic osteochondral construct for implantation. In detail, $2.0*10^6$ ovine BM-MSCs were pre-differentiated on collagen IV coated membranes for 2 weeks resulting in a disc-shaped cartilage-like tissue. The pre-differentiated cells were released by collagenase A and $2.0*10^6$ cells were then seeded on CPP and cultured for 8 weeks in 5% FBC containing medium which produced a biphasic osteochondral construct with superior hyaline cartilage in terms of histology and mechanical properties.

The hypothesis of this present study is that human cord blood-derived MLPCs is a suitable cell source for engineering a biphasic osteochondral construct *in vitro*. The

chemically directed pre-chondrogenic potential of human MLPCs was evaluated for 2 weeks in three culture systems; as micromass pellets, on porous biodegradable CPP, and on uncoated or collagen I, II and IV coated semi-permeable Millicell-CM® culture plate inserts.

Materials and Methods

Study Design

The study design is summarized in Fig. 1. Cryopreserved passage 2 MLPCs were expanded for 3 weeks on monolayer to obtain a sufficient number of cells for the experiments. The resultant passage 5 MLPCs were differentiated in serum-free chondrogenic induction medium for 2 weeks in three culturing systems: micromass pellets, on the surface of CPP, and on uncoated or collagen-coated polytetrafluoroethane membrane inserts. After 2 weeks of culturing the differentiation towards chondrocytes was determined by RT-qPCR to assess gene expression of three cartilage related markers: aggrecan, SOX9 and collagen type II. Histology was used to evaluate the cellularity and accumulation of extracellular matrix. Quantification of sulphated glycosaminoglycans (sGAG) and hydroxyproline (HA) were used as functional markers of cartilage anabolism.

Human umbilical cord blood-derived Multi-Lineage Progenitor Cells (MLPCs)

Human umbilical cord blood-derived Multi-Lineage Progenitor Cells (MLPC™, clone number 081704-1G1) were kindly provided by BioE Inc. (Saint Paul, MN). MLPCs are clonally expanded cell lines, which has previously shown to possess osteogenic, adipogenic, and chondrogenic potential[38]. MLPCs have been validated positive for surface markers CD73, CD90 and CD105, and negative for CD34, CD45, CD106 and HLA-DR, which fulfil The International Society for cellular Therapy minimum criteria for defining multipotent human MSCs[38-40]. Entire content of one vial

containing 1.0 x 10^5 undifferentiated passage 2 cells were thawed and cultured in a 75 cm^2 culture flask with expansion medium consisting of low glucose DMEM (Lonza, Walkersville, MD) supplemented with 30% FBS (PAA Laboratories, Ontario, Canada) and 2mM L-glutamine (Sigma Aldrich, St. Louis, MO, USA). Cells were grown to 40-50% confluence at standard humidified atmosphere containing 5% CO_2 and 21% O_2 at 37°C. Expansion medium was changed twice weekly. Upon reaching 40-50% confluence, cells were detached by trypsinization (1% trypsin-EDTA, Invitrogen, Burlington, ON, Canada) and replated at a cellular density of 1500 cells/cm^2. Passage 5 MLPCs were was used in the studies described below.

MLPC chondrogenesis in micromass (pellet) culture

Three-dimensional pellet cultures were established by spinning 2.5x10^5 MLPCs at 200x*g* for 5 minutes at room temperature in 15 mL conical polypropylene tubes. Pellets were cultured for 2 weeks in chondrogenic inductive medium consisting of DMEM high glucose supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 100 μg/mL ascorbic acid 2 phosphate (Sigma-Aldrich), 2 mM l-glutamine (Invitrogen), 1 mM sodium pyrovate, 1x ITS^+ (BD Biosciences, Bedford, MA), 10 ng/mL TGF-β3 (R&D Systems, Minneapolis, MN) and 1% streptomycin/penicillin (Invitrogen). The pellets were cultured at 5% CO_2, 21% O_2, 37°C and humidified atmosphere with the lid loosened to facilitate gas exchange. Culture medium was changed three times a week.

MLPC chondrogenesis on CPP or membrane inserts

Biodegradable CPP rods were produced as described previously[34]. In brief, CPP powder with particle sizes of 75–106 μm were gravity sintered at 950 °C in cylindrical tubes to form porous CPP. The resulting rods characterized by 35 vol.% interconnected porosity were cut to form disks 2 mm in height and 4 mm in diameter. Enclosing a silicone tube around the CPP disk formed a culture well preventing cell leakage (Tygon

3350, Saint-Gobain Performance Plastics Corp., Aurora, OH, USA) (Fig. 2). CPP constructs were sterilized by gamma irradiation (2.5 Mrad).

Semipermeable polytetrafluoroethane membrane culture plate inserts 12mm in diameter (0.2 μm pore size, Millicell-CM®, Millipore Corp., Bedford, MA, USA) were coated with collagen type I, II and IV (0.5 mg/ml in 0.1 N acetic acid; Sigma Chemical Co., St Louis, MO, USA) and dried overnight. Coated inserts were UV sterilized for 30 minutes prior to cell culturing.

$2x10^6$ MLPCs were seeded on top of CPP constructs (30 μL suspension) or inserts (400 μL suspension) and left for three hours in the incubator to allow cells to adhere. Following that, media was carefully added to a total volume of 2 mL. Cultures were grown for 14 days in 24-wells culture plates in chondrogenic inductive medium with medium change three times a week. $2x10^6$ MLPCs grown on non-coated inserts under the same conditions served as controls for mutual comparison between the six different culture groups.

Histology

In vitro formed tissue was harvested and washed twice in PBS. Tissue was removed from the CPP, fixed in 10% formalin and embedded in paraffin. Tissue formed on membrane inserts was processed without the membrane insert, i.e., the tissue was removed from the membrane with forceps before further processing. Five micron transversely cut sections was stained with either toluidine blue (pH 3.0) to demonstrate presence of proteoglycans or haematoxylin-eosin to visualize cells and tissue by light microscopy.

Total RNA extraction and reverse transcription

Total RNA was extracted using the GenElute™ Mammalian Total RNA Kit (Sigma-Aldrich, Missouri, USA) as described by the manufacturer with following

modifications. Samples were first lysed in lysis buffer supplemented with β-mercaptoethanol. Tissue were then snap-frozen in liquid nitrogen and homogenized by a pestle mortar. After each processing pestle was cleaned in series of 100% ethanol washing, SDS and DEPC treated water. All subsequent RNA extraction steps were performed according to GenElute™ protocol. Isolated RNA concentration and purity was measured with NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware USA). cDNA was synthesized from 150 ng of total RNA using Superscript® III reverse transcription kit (Invitrogen) in a 20 μL of total reaction volume containing FirstStrand buffer and 0.01 M dithiothreitol for 60 min at 42°C followed by a 15 min extension period at 70°C. Each reaction contained 40 units of recombinant ribonuclease inhibitor RNase OUT™ (Invitrogen, Carlsbad, CA), 200 ng of random primers, 0.5 mM dNTPs and 200 units of SuperScript® III enzyme.

Quantitative real-time RT-PCR

Gene expression was determined with quantitative reverse-transcriptase polymerase chain reaction (qPCR) and sequence specific primers for type II collagen, aggrecan, SOX9, and 18S rRNA (reference gene for normalization). For type II collagen, a forward primer of 5′-GTG TCA GGG CCA GGA TGT C-3′ and a reverse primer of 3′-GCA GAG GAC AGT CCC AGT GT-5′ were used. For aggrecan, a forward primer of 5′-TGG GAC TGA AGT TCT TGG AGA-3′ and a reverse primer of 3′-GCG AGT TGT CAT GGT CTG AA-5′ were used. For SOX9, a forward primer of 5′-GTA CCC GCA CTT GCA CAA C-3′ and a reverse primer of 3′-GTG GTC CTT CTT GTG CTG C-5′ were used. For 18S rRNA, a forward primer of 5′-GTA ACC CGT TGA ACC CCA TT-3′ and a reverse primer of 3′-CCA TCC AAT CGG TAG TAG CG-5′ were used. qPCR was performed using SYBR green dye on Realplex^2 Master Cycler (Eppendorf, Hamburg, Germany) according to instructions provided by the manufacturer and data were analysed with Mastercycler EP Realplex. Briefly, template

cDNA corresponding to 10 ng RNA was amplified in 10 μL final volume with 1 μM of each primer suspended in 2X QuantiFast SYBR Green master mix (Qiagen, Hilden, Germany) and run in technical triplicates. Amplification parameters were identical for each primer pair (10 min at 95°C to activate the enzyme, 15 s denaturation at 95°C was followed by 30 s annealing at 60°C), and amplification data were collected for 40 cycles. Relative gene expression levels were normalized to 18S using following equation:

Relative gene expression=$2^{Ct\ (18s)\ -\ Ct\ (gene\ of\ interest)}$.

Quantification of proteoglycan, collagen and DNA content

In vitro formed tissue was removed from CPP or membrane inserts and snap frozen at -80°C. Frozen samples were digested using papain (Sigma; 40 μg/mL in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM dithiothreitol) for 48 hours at 65°C and stored at -30°C until further analysis. Aliquots of the digest were analysed separately for proteoglycan, hydroxyproline and deoxyribonucleic acid (DNA) content.

The proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans (sGAG) using the dimethylmethylene blue dye binding assay (Polysciences Inc., Washington, PA, USA) and spectrophotometry (wavelength 525 nm)[41]. The standard curve for the analysis was generated using bovine trachea chondroitin sulfate A (Sigma-Aldrich, Missouri, USA). Proteoglycan amount is reported as value adjusted to the DNA content.

Collagen content was estimated from the determination of hydroxyproline (HP) content. Aliquots of the papain digest were hydrolysed in 6 N HCl at 110°C for 18 hours. Hydroxyproline content of the hydrolysate was determined using chloramine-T/Ehrlich's reagent assay and spectrophotometry (wavelength 560 nm). The standard curve for this analysis was generated using L-hydroxyproline (Sigma Chemical Co.). Collagen amount is reported as value adjusted to the DNA content.

Tissue cellularity was determined by quantifying the total DNA content using the Hoechst dye 33258 assay (Polysciences Inc., Washington, PA, USA) and fluorometry (emission wavelength 458 nm; excitation wavelength 358nm) [42]. The standard curve for the analysis was generated using calf thymus DNA (Sigma-Aldrich, Missouri, USA).

Statistical analysis

Data was checked for normal distribution, variance homogeneity and one-way analysis of variance (ANOVA) was performed with the culturing system as the independent variable. Data are presented as means ± standard errors of means for three replicates (n = 3). sGAG and HP content are reported as DNA adjusted values. One-way ANOVA (Bartlett's test for equal variances) was performed using STATA version 10.1 (STATA Corp., TX). For all analyses, a 95% confidence interval was used and significance was assigned at $p< 0.05$.

Results

Macroscopic and microscopic appearance of tissue derived from chondrogenic differentiation of human cord blood-derived multi-lineage progenitor cells

During the 14 days of chondrogenic differentiation MLPCs pellets grew to a final size of 2 mm in diameter and maintained their spherical shape. Pellet tissue texture was relatively firm to the touch and had a smooth yellow surface. On CPP substrates, the MLPCs formed a convex accumulation of tissue located centrally, which had a smooth yellow surface with a gelatinous texture (Fig. 3A, F).

Non-coated membrane cultures maintained uniform covering of the membrane, i.e., MLPCs produced an ivory-whitish disc-shaped tissue uniform in thickness (≈1.0 mm and 12 mm in diameter). The disk was easily removed intact from the membrane support (Fig. 3B, G). On the contrary, coated membrane cultures of collagen type I, II and IV progressively contracted and detached from the membrane producing non-

uniform tissue (Fig. 3C-E, H). Detachment from the membrane support was most prominent for collagen II coated membrane cultures, which showed complete contraction and detachment after five days culturing (Fig. 3D1&2). All collagen coated membrane cultures contracted and detached from the membrane support during the two weeks of culture (Fig. 3H). Tissue from coated membrane cultures was smooth, yellow and had a dense texture.

Pellet cultures of MLPCs showed cellularity throughout the pellet and cells resembled different degrees of chondrocyte-like morphology, i.e., cells with round morphology in lacunae surrounded by neocartilage matrix. The metachromasia in toluidine blue stained sections indicating secretion of proteoglycans was primarily located peripherally in pellets (Fig. 4A). In contrast, cells in the CPP cultures resembled more spindle-shaped cell morphology, had decreased level of cellularity and the metachromasia was less intense (Fig. 4 B). Non-coated membrane cultures formed a tissue-disc, which upon histologic evaluation displayed a multicellular-layered tissue of approximately 20 cells in thickness. Cellularity and extracellular matrix was abundant and uniformly distributed throughout the disc. Most cells had round chondrocyte morphology, whereas culture medium and membrane interphases contained cells with more flattened morphology (Fig. 4C). Collagen type I, II or IV coated membrane cultures all displayed a similar pattern of matrix-deposition. The most intense staining was observed for collagen type IV cultures and least for collagen I cultures, while collagen type II cultures were intermediate in matrix staining (Fig. 4D-F).

Gene expression of chondrogenic markers in tissue derived from cultures of human cord blood-derived multi-lineage progenitor cells

Overall, membrane cultures had increased mRNA expression of aggrecan, SOX9 and collagen type II compared with pellet and CPP cultures ($p<0.05$) (Fig. 5). Non-coated membrane cultures had a significant increase in aggrecan expression by 26-fold

compared to pellet and CPP cultures ($p<0.05$). Likewise, non-coated membrane cultures had increased aggrecan expression compared with collagen type I and II coated membrane cultures ($p<0.05$). Non-coated membrane cultures displayed the most pronounced difference by 22-fold increase ($p<0.05$) compared with collagen II coated membrane cultures, while the difference with type I and type IV coated membrane cultures were modest to an increase by 2-fold ($p<0.05$). Coated membranes cultures had significant differences in aggrecan expression when compared mutually to each other, this with the most elevated expression in type IV coated membrane cultures followed by type I and type II coated membrane cultures ($p<0.05$).

Gene expression of collagen type II had a similar profile as aggrecan. Membrane cultures showed significantly increased mRNA expression of collagen II compared with pellet and CPP cultures, and this was significantly 20-fold higher in non-coated membrane cultures ($p<0.05$). The most elevated collagen II expression was observed in non-coated membrane cultures, followed in descending order by collagen type IV, collagen type I, pellet, CPP and collagen type II coated cultures.

Non-coated membrane cultures, collagen type I and collagen type IV coated membrane cultures had significantly increased mRNA expression for the transcriptional factor SOX9 compared with pellet and CPP cultures ($p<0.05$). A significant increase by 7-fold was observed in collagen type IV coated cultures compared with CPP cultures ($p<0.05$), while non-coated and collagen type I coated membrane cultures were modest in difference by 5-fold increase ($p<0.05$).

Proteoglycan and collagen production in tissue derived from cultures of human cord blood-derived multi-lineage progenitor cells

Membrane cultures accumulated significantly higher amounts of sGAG compared with pellet and CPP cultures ($p<0.05$). This was significantly 4-fold higher in non-coated membrane cultures ($p<0.05$). Non-coated membrane cultures deposited the

highest level of proteoglycans, followed in descending order by collagen type IV, collagen type I, collagen type II, pellet and CPP cultures. No difference in proteoglycan production was observed between pellet and CPP cultures. Likewise, non-coated membrane cultures accumulated significantly 2-fold higher amounts of HP compared with coated membrane and CPP cultures ($p<0.05$). No difference in HP deposition was observed comparing collagen coated membranes cultures mutually to each other.

Discussion

This study confirms that MLPCs possess' chondrogenic potency as previously reported[38] and that culturing cells three-dimensionally on permeable membranes results in neocartilage tissue. Additionally, work from our group confirms MLPCs chondrogenic differentiation, which is enhanced by culturing in reduced oxygen tension (5% O_2) (manuscript submitted and in the process of revision).

Membrane cultures facilitated the chondrogenic differentiation of MPLCs and to our surprise the non-coated membrane cultures performed better than collagen coated cultures in terms of no contraction and higher levels of sGAG and HP. This is in contrast to our expectations since the non-coated membrane lacks natural ligands for cell-ECM-interaction enhancing cell-adhesion, regulation of cellular proliferation, function, and differentiation[43-45]. Functionalization of synthetic biomaterials in terms of coating with natural ECM components such as hyaluronan and collagen has proven to enhance chondrogenic differentiation of MSCs[46,47]. Lee and colleagues demonstrated optimal chondrogenesis of ovine BM-MSCs on collagen IV coated membrane inserts producing a similar disc-shaped cartilage tissue[32]. Although using a different membrane material, namely polycarbonate, chondrogenic differentiation of human BM-MSCs on non-coated membrane inserts has been reported[48,49]. These contrasting reports are likely related to differences in varying species, age of donor, cell source, *in vitro* isolation and culture methods[50,51].

Our membrane cultures produced moderate neocartilage within a 14 day period. We demonstrate enhanced differentiation of MLPCs towards chondroblastic tissue in membrane cultures compared to the well-established pellet cultures. This is reflected by the chondrocyte-like morphology with a multicellular-layered organization of cells, a 4-fold significant increase in extracellular cartilage matrix production, and significantly increased aggrecan, collagen type II and SOX9 mRNA expression in membrane cultures. The most pronounced distinction was observed in non-coated membrane cultures that synthesized more total proteoglycan compared with pellet and CPP cultures. Non-coated membrane cultures were much more uniform, distribution and stained strongly for proteoglycans throughout the disc-tissue and had a correspondingly increased cell to matrix ratio compared with pellet cultures. The uniformity of the deposited matrix is most likely caused by the influence of the culture conditions. The more uniform matrix in the discs may promote a more even exposure of differentiation signals and nutrients to the cells with short diffusion distances from both sides of a the multicellular-layered disc-tissue. The membrane cultures likely provided better mass transport properties, i.e., the surface area available for nutrient diffusions in the 12-mm in diameter (and 1.0 mm thick) disc was several folds higher compared to the 2.0 mm in diameter spherical pellets, and secondly the semipermeable ensure tissue-nourishment from above and below. Collagen type I, II and IV coated membrane cultures performed better than pellet and CPP cultures but tissue contracted and detached from the membrane surface.

Embryonic condensation and contraction of mesenchymal cells and the activation of various transcriptional factors results in cartilage formation[52]. Mature chondrocytes and MSCs express genes for α-smooth muscle actin (SMA), which plays a major role in cell cytoskeleton[53-55]. Chondrocytes cultured on porous type I collagen-glycosaminoglycan scaffolds contracts[54], and this effect is inhibited by staurosporine, an antibiotic known to

disrupt cytoskeletal structure[56]. Furthermore, addition of TGF-β1 increased the expression of SMA and contraction in chondrocyte-seeded collagen-glycosaminoglycan scaffolds[57] while supplementation of 5% FCS or basic fibroblast growth factor (bFGF) to TGF containing induction medium antagonized the TGF mediated contraction in scaffold-free membrane cultures of BM-MSCs[58,59]. Scaffold fabricated from cartilage-derived extracellular matrix may provide an environment that stimulates chondrogenic differentiation and cartilage matrix synthesis in a variety of cell types including MSCs[60,61]. However, exposure of cartilage-derived matrix may provide potential benefits for cartilage tissue engineering, they have generally shown to contract during culture due to low compressive moduli[60]. This contraction has been demonstrated in scaffolds of collagen-glycosaminoglycans *in vitro* culture[62], and the contraction is reduced by cross-linking collagen fibrils[63]. The collagen used in our study is fabricated of natural collagen I, II and IV and the collagen fibres were not cross-linked. The increased expression of SMA mediated by TGF, lack of bFGF, and cross-linking of collagen fibres may explain the unfavourable contraction of the collagen-coated membrane cultures compared with the non-coated cultures.

The relatively short culture period of two weeks may explain the moderate staining of the ECM components as seen in our study. Liu et al. reported that human BM-MSCs made the most stable cartilage phenotype when differentiated for a period of 8 to 12 weeks[64]. Improved chondrogenic differentiation of the MLPCs may also be obtained through the use of different chemical inducers in the culture medium since the optimal differentiation medium for MLPCs has yet to be defined. The effect of BMP-2, BMP-4 and BMP-6 as a supplementary driving force for MSC differentiation is a subject of active investigation since different species and cell sources reported in the literature often provides contradictory evidence. Ronziere and colleagues have reported decreased hypertrophy of AT-MSCs in hypoxic culturing supplemented with the addition of BMP-

2/TGF to the culture medium[65]. Similar findings are reported in BM-MSCs[66]. Synergistic effect of BMP-6 in the chondrogenesis of AT-MSCs has med reported by Guilak and colleagues[67,68]. It is also noted that BMP-6 might have a dual role being both an osteogenic inducer as well as a chondrogenic inducer[69]. Our differentiation medium did not include BMPs, but it seems plausible that enhanced chondrogenic differentiation of MLPCs can be obtained through the use of selected BMPs. Furthermore, addition of 20 ng/ml TGF-β3 resulted in intensively matrix staining of MLPCs pellet cultures (data not shown). Gene expression of collagen type I and X, immunohistochemistry on collagen type I, II and X and aggrecan where not included in this study but would have allowed us to determine the degree, if any, of formation of collagen I rich fibrocartilage and collagen X rich hypertrophic cartilage.

Conclusion

In conclusion, we have demonstrated that MLPCs possess' chondrogenic potency, which increased when cultured scaffold-free on membrane inserts resulting in multicellular-layered neocartilage tissue. Evaluating the effect of culturing pre-differentiated MLPCs on CPP is an obvious next step since direct seeding of MLPCs on CPP did not yield satisfactory biphasic constructs.

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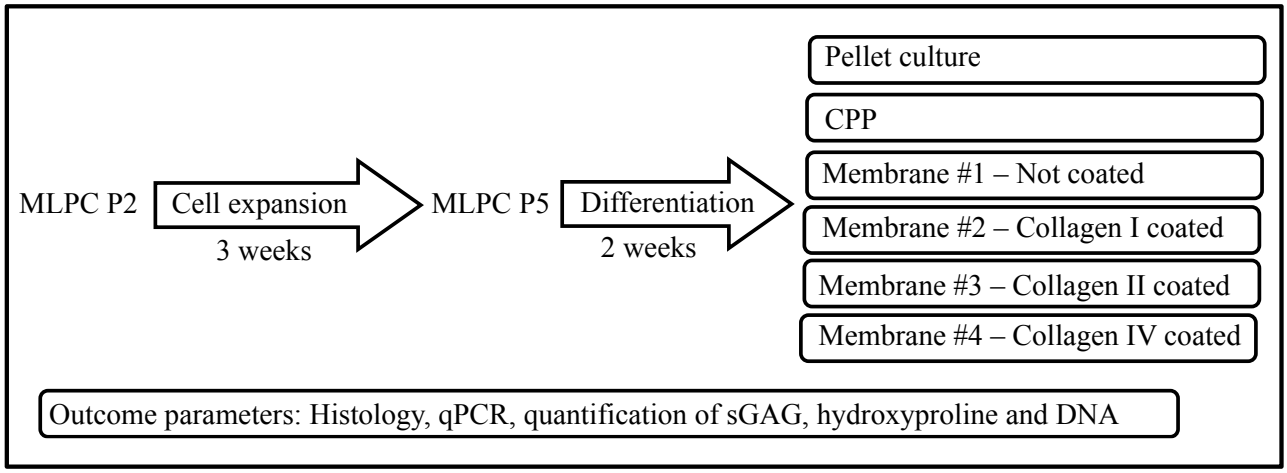

Fig. 1. Study design objectives. Multilineage progenitor cells (MLPCs) were expanded for three weeks following chondrogenic differentiation in three culturing systems, i.e., as pellets, directly on the surface of CPP, and non-coated and collagen coated semi-permeable polytetrafluoroethane membrane inserts for 14 days. CPP, calcium polyphosphate.

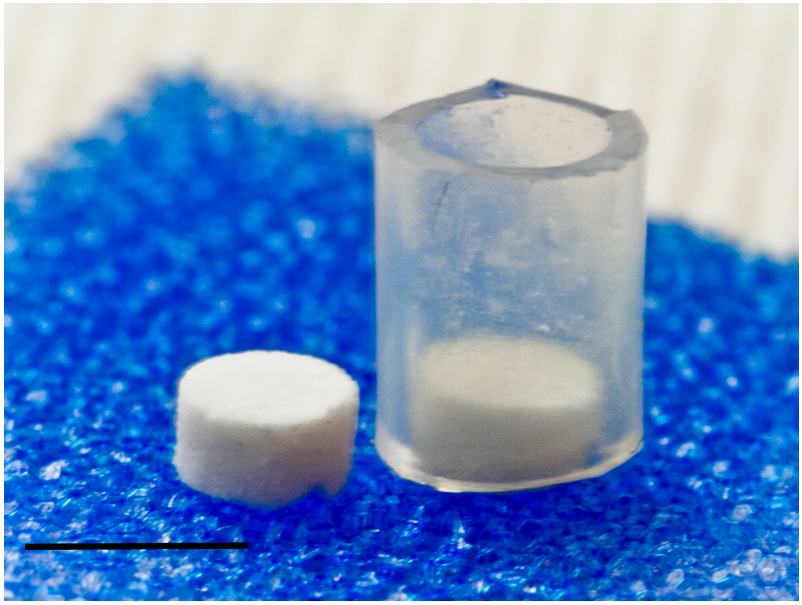

Fig. 2. Calcium polyphosphate (CPP) disc enclosed by silicone tube forming a culture well preventing cell leakage. Scale bar indicate 5 mm.

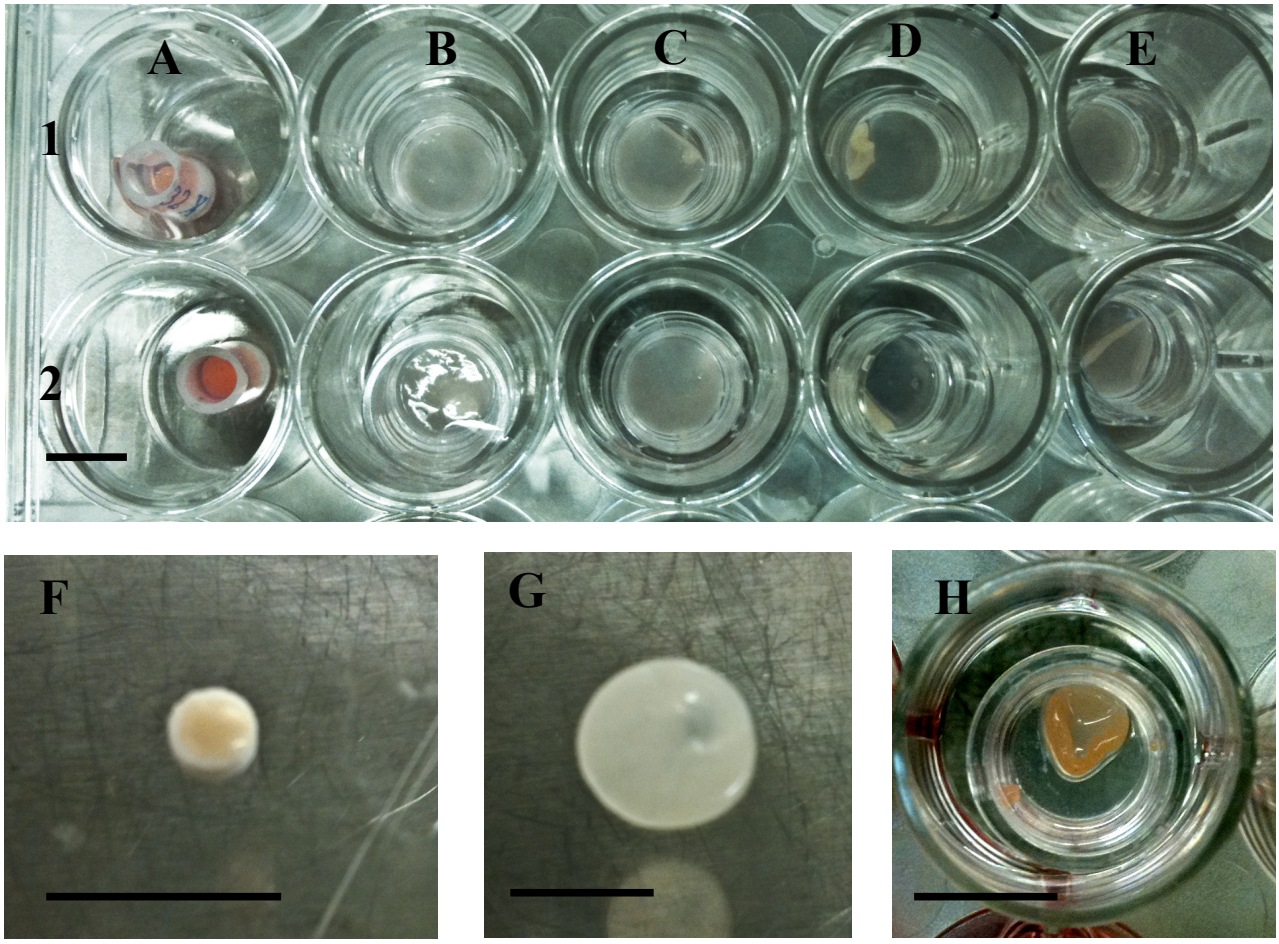

Fig. 3. Macroscopic tissue constructs multilineage progenitor cells (MLPC) cultures after five days **(A-E)** and 14 days **(F-H)**. Row 1 and 2 represent biological replicas of A-E cultures. MLPCs cultured on calcium polyphosphate **(A, F)**, non-coated membrane inserts **(B, G)**, collagen I coated membrane inserts **(C)**, collagen II coated membrane inserts **(D)** and collagen IV coated membrane inserts **(E, H)**. Scale bar indicate 10 mm.

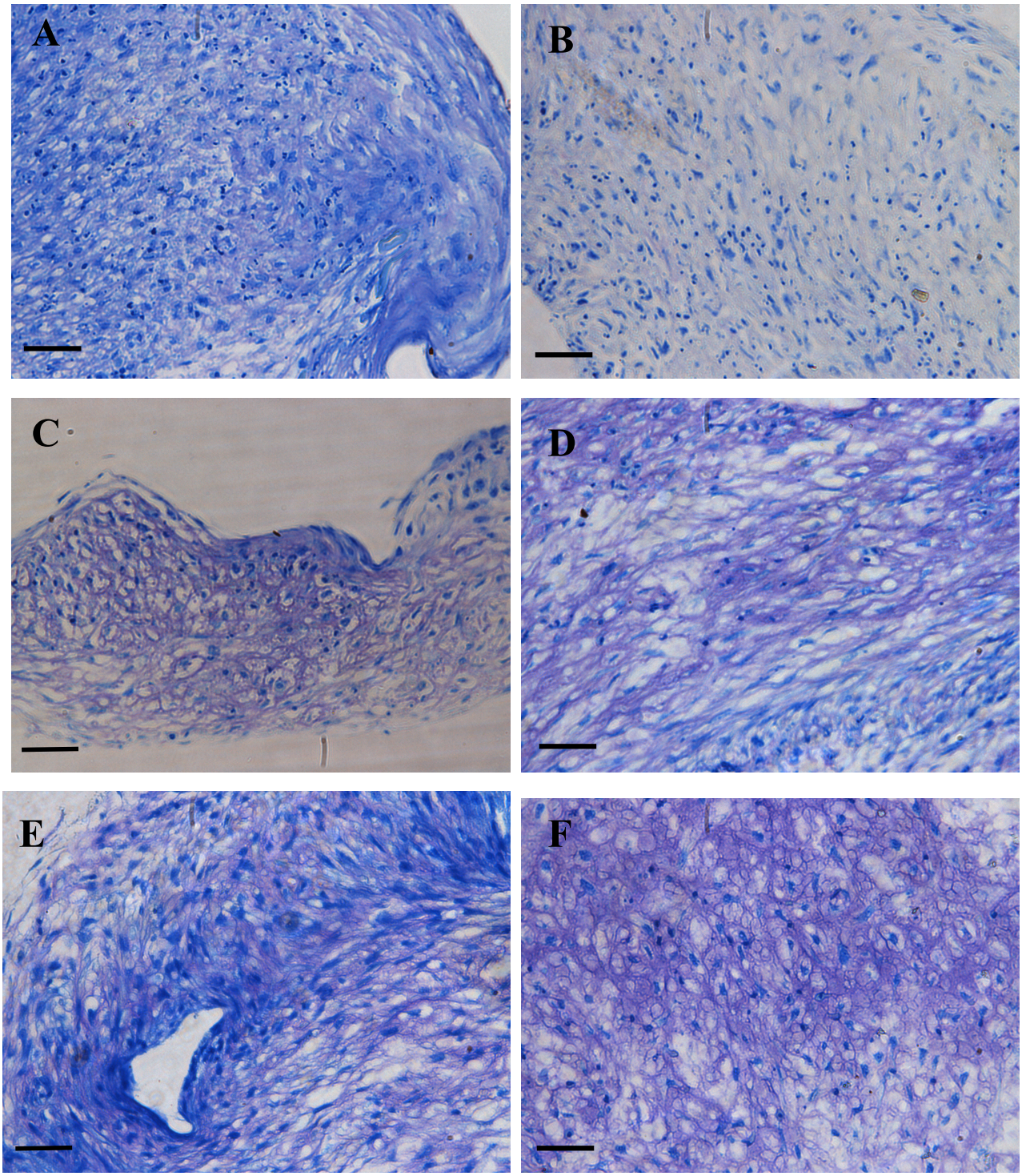

Fig. 4. Histological distribution of sulfated polyanion in toluidine blue stained sections of multilineage progenitor cells cultured as pellets **(A)**, on calcium polyphosphate **(B)**, non-coated membrane inserts **(C)**, collagen I coated membrane inserts **(D)**, collagen II coated membrane inserts **(E)** and collagen IV coated membrane inserts **(F)**. Scale bar indicate 50 μm.

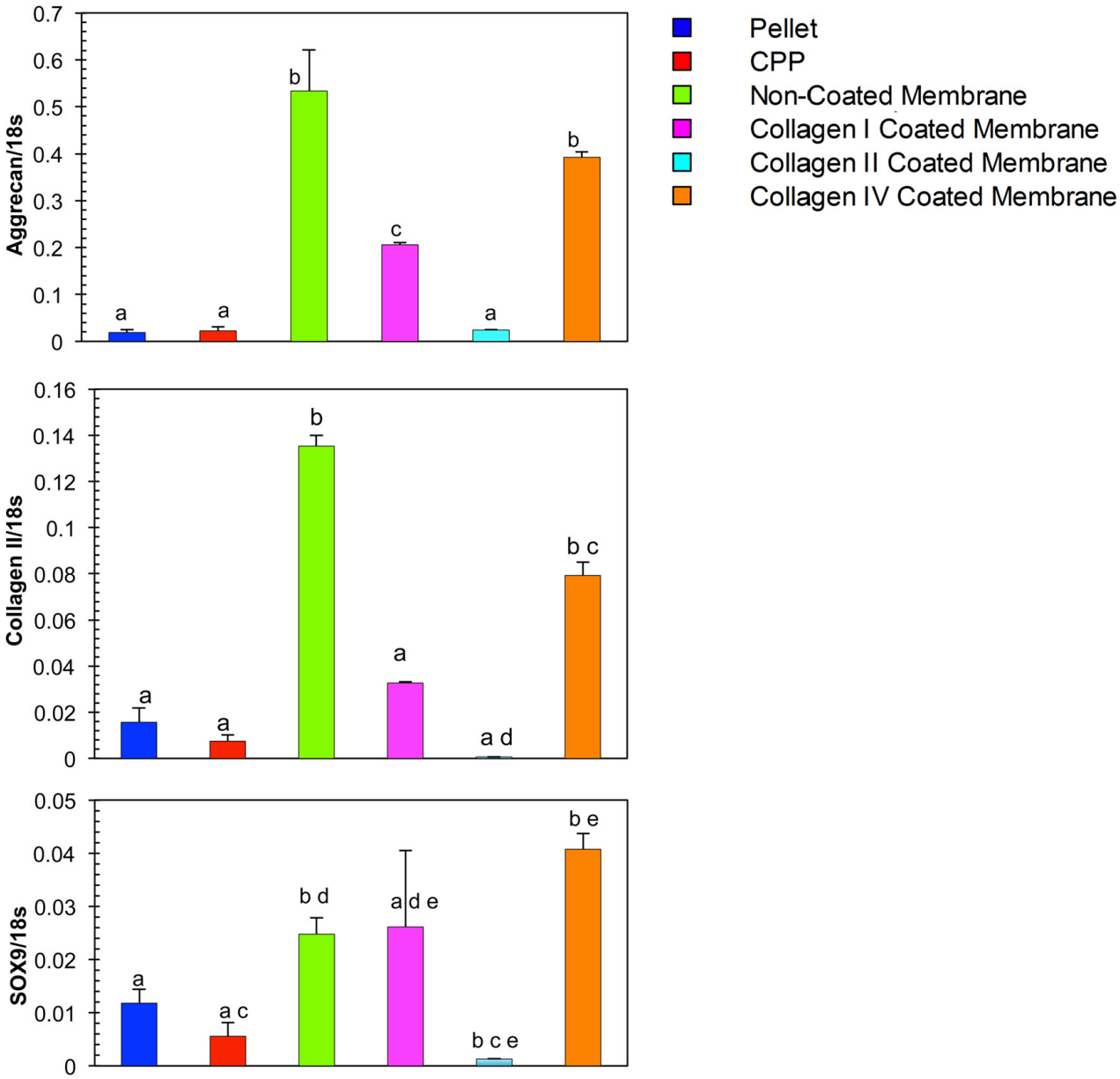

Fig. 5. RT-qPCR of chondrogenic markers (aggrecan, collagen II and SOX9) in multilineage progenitor cells cultures of pellets **(blue bars)**, on calcium polyphosphate **(red bars)**, non-coated **(green bars)**, collagen I **(purple bars)**, collagen II **(turkis bars)** and collagen IV **(orange bars)** coated membrane inserts. Data shown as means ± SEM, n=3. Bars not sharing same letters indicate a significant difference ($p<0.05$).

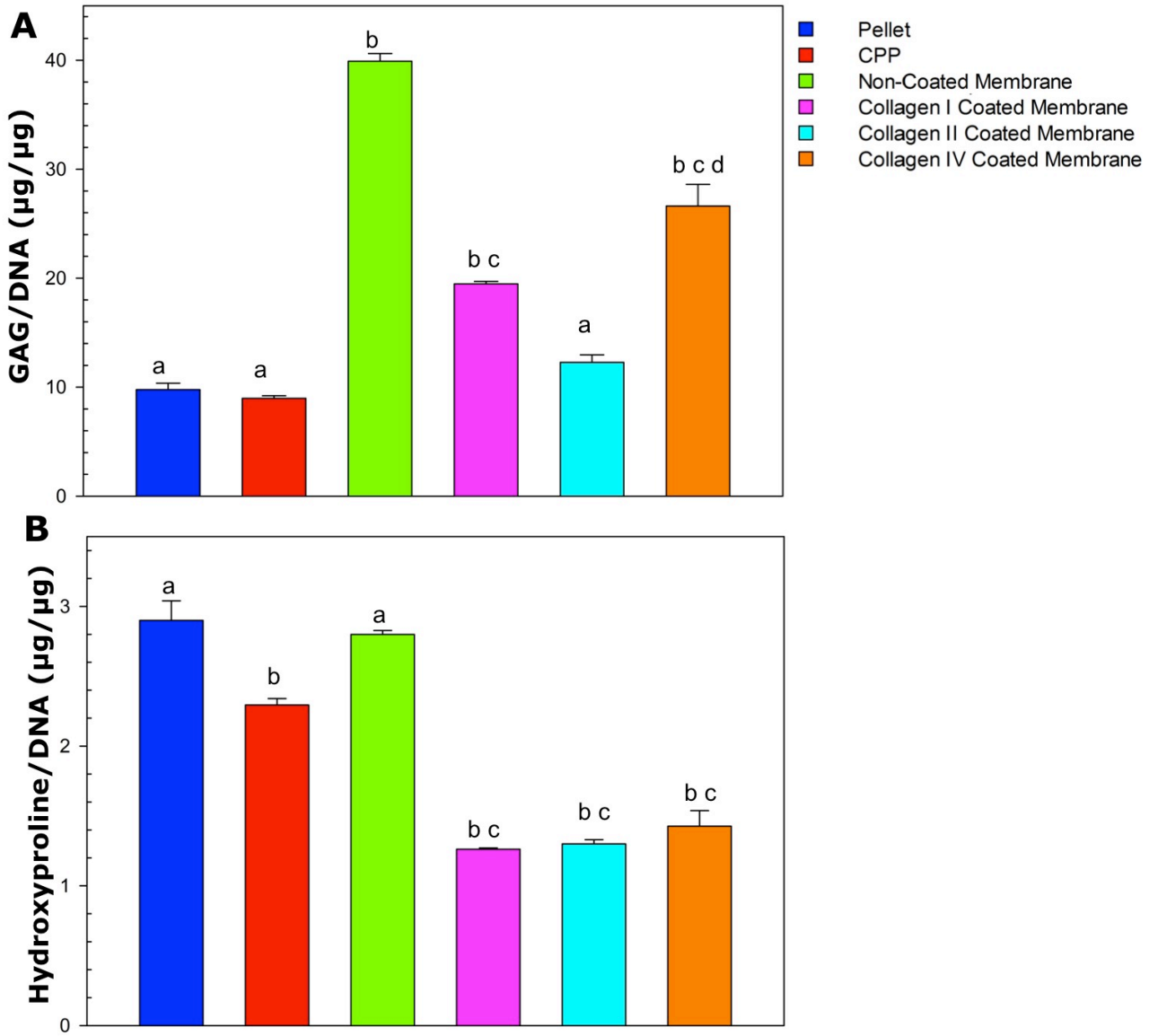

Fig. 6. Accumulation of extracellular matrix in tissue derived from 14 days culture of multilineage progenitor cells as pellets **(blue bars)**, on calcium polyphosphate **(red bars)**, non-coated **(green bars)**, collagen I **(purple bars)**, collagen II **(turkis bars)** and collagen IV **(orange bars)** coated membrane inserts. Sulfated proteoglycan **(A)** and hydroxyproline **(B)**. Data shown as means ± SEM, n=3. Bars not sharing same letter indicate a significant difference ($p<0.05$). GAG, glycosaminoglycan.