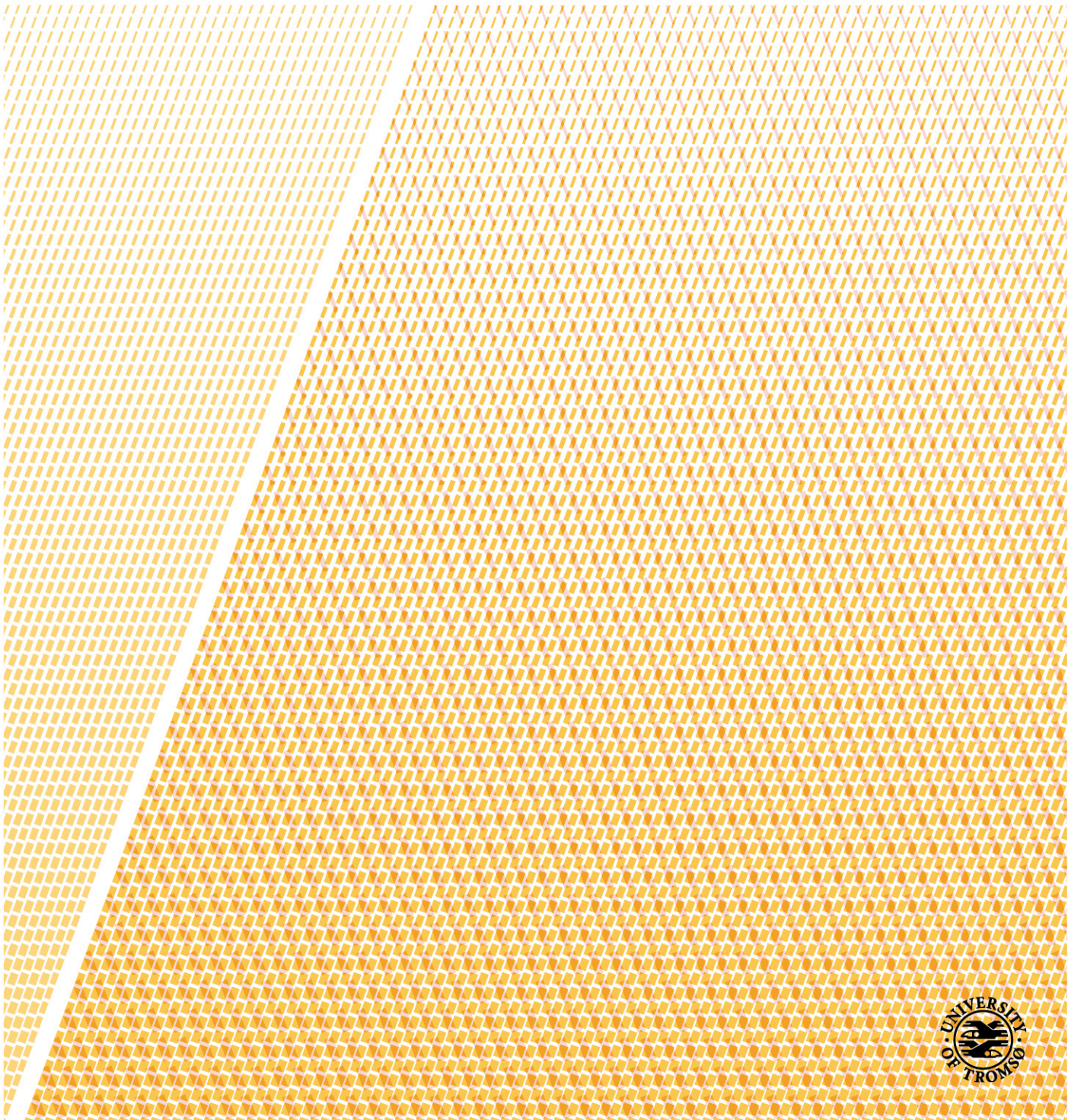UiT

THE ARCTIC
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Department of Clinical Medicine

Biology of mesenchymal stromal cells: Chondrogenesis, paracrine signalling and cartilage repair

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Ashraful Islam

A dissertation for the degree of Philosophiae Doctor – July 2018

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"In the middle of every difficulty lies opportunity."

Albert Einstein

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Acknowledgements

Summary

The management of articular cartilage lesions is one of the weighty challenges for orthopaedic surgeons. Gradual deterioration of articular cartilage from trauma or degenerative pathophysiology leads to swelling of the synovial joint, debilitating pain, functional impairment, and eventually osteoarthritis. Cell-based repair techniques have been extensively investigated in last few decades to improve the treatment regime for cartilage repair. The use of mesenchymal stem/stromal cells (MSCs) has demonstrated as an alternative cell source for cartilage repair due to their multilineage differentiation potential and hypoimmunogenic properties. Despite the advances in MSC-based cartilage repair techniques, there is no consensus relating to the most suitable cell type for cartilage repair or osteoarthritis treatment. The overall aim of this thesis was to investigate and compare the *in vitro* chondrogenic potential, and paracrine signalling potential of MSCs to find a suitable source for cartilage repair. Additionally, we also used efforts to gather new knowledge about cell-based biomarkers to predict clinical outcomes after cell transplantation procedures.

In the paper I, we characterised and compared *in vitro* chondrogenic capacity of stromal cells harvested from Hoffa's fat pad (HFPSCs), synovial membrane (SMSCs), umbilical cord (UCSCs) and articular cartilage. We demonstrated poorer *in vitro* chondrogenesis of MSCs from umbilical cord compared to cells harvested from adult joint tissues. The reason for poor chondrogenic capacity is yet to be elucidated. However, the study of TGF-β receptors revealed low expression of TGF-β receptor type II in umbilical cord stromal cells (UCSCs). This finding may explain the reason for poor chondrogenesis of UCSCs. In the paper II, we investigated the secretomes of HFPSCs, SMSCs, UCSCs and chondrocytes (ACs) to unveil *in vitro* secretory protein profiles that contribute to paracrine signalling and immunomodulatory characteristics. We found that UCSCs secretes less catabolic factors and less pro-inflammatory factors compared to cells from the adult origin. Considering the anti-inflammatory and pro-anabolic

paracrine effects of secreted soluble molecules, UCSCs could be used as an adjuvant therapy for cartilage repair.

In the paper III, we investigated if *in vitro* chondrogenic potential of donor-matched surplus chondrocytes from Autologous Chondrocyte Implantation (ACI)-treated patients could predict clinical outcomes. Counterintuitive, we did not observe any correlation between *in vitro* chondrogenic capacity of cultured cells and short-term clinical outcomes. Additionally, constitutive expression of previously proposed and novel chondrogenic markers had no value to predict clinical outcomes. Of interest, high-throughput LC-MS/MS protein analysis revealed prolyl 4-hydroxylase 1, an enzyme involved in collagen biosynthesis, as a novel biomarker linked to superior chondrogenic capacity.

List of papers

Paper I:

Islam, A., Hansen, A. K., Mennan, C., and Martinez-Zubiaurre, I. **Mesenchymal stromal cells from human umbilical cords display poor chondrogenic potential in scaffold-free three dimensional cultures**. European Cells and Materials, 2016. 31: p. 407-24.

Paper II:

Islam, A., Urbarova, I., Bruun, J. A., and Martinez-Zubiaurre, I. **Large-scale secretome analyses unveil a superior immunosuppressive phenotype from umbilical cord stromal cells compared to other adult mesenchymal stromal cells.** May 2018. Submitted.

Paper III:

Islam, A., Fossum, V., Hansen, A. K., Urbarova, I., Knutsen, K., and Martinez-Zubiaurre, I. ***In vitro* chondrogenic potency of surplus chondrocytes from autologous transplantation procedures do not predict short-term clinical outcomes.** June 2018. Submitted.

Other publications where the author contributed:

Islam, A., Romijn, E. I., Lilledahl, M. B., and Martinez-Zubiaurre, I. **Non-linear optical microscopy as novel quantitative and label-free imaging modality to improve the assessment of tissue-engineered cartilage.** Osteoarthritis and Cartilage, 2017. 25: p. 1729-37.

List of abbreviations

ACI	Autologous chondrocyte implantation
ADAMTS	A disintegrin and metalloprotease with thrombospondin motifs
ACs	Articular chondrocytes
ALCAM	Activated-leukocyte cell adhesion molecule, CD166
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
CFSE	Carboxyfluorescein succinimidyl ester
CD44	Hyaluronan receptor
CD271	Low-affinity nerve growth factor receptor, LNGFR
CM	Conditioned medium
COL1A1	Collagen type 1
COL2A1	Collagen type 2
ECM	Extracellular matrix
ESCs	Embryonic stem cells
GAGs	Glycosaminoglycans
HFPSCs	Hoffa's fat pad derived stromal cells
iPSCs	Induced pluripotent stem cells
ICAM-1	Intercellular adhesion molecule-1, CD54
IL-6	Interleukin-6
IL-8	Interleukin-8
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloproteinase

MSCs	Mesenchymal stem/stromal cells
OA	Osteoarthritis
PBMCs	Peripheral blood mononuclear cells
PDGFD	Platelet-derived growth factor D
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
qPCR	Quantitative real-time polymerase chain reaction
SDF-1	Stromal cell-derived factor 1 (CXCL12)
SMAD	SMA (small body size gene and protein in C. Elegans) + MAD (mothers against decapentaplegic, protein in Drosophila) = SMAD
SMSCs	Synovial membrane derived stromal cells
TEC	Tissue-engineered cartilage
TGF-β	Transforming growth factor-β
TGFBR	Transforming growth factor-β receptor
TMT	Tandem-mass-tag
UCSCs	Umbilical cord-derived stromal cells
VCAM-1	Vascular cell adhesion molecule -1

1 Introduction

1.1 Articular cartilage

Articular cartilage is a specialised connective tissue that covers the ends of bones of the diarthrodial joint. It is an aneural and avascular type tissue, which obtains nutrients by diffusion from the surrounding synovial fluid and the subchondral bone [1]. The primary function of articular cartilage is to provide frictionless movement of load bearing surfaces and to absorb and distribute the mechanical loading generated during locomotion. The thickness of juvenile articular cartilage is approximately 2.7-4 mm [2], while the thickness decreases in adult articular joints and it ranges between 2-2.5 mm [3, 4]. Articular cartilage is sparsely populated with cells called chondrocytes, which constitute approximately 2 % of total tissue volume [4]. The main bulk of the cartilage tissue volume comprises extracellular matrix made of collagen type II, proteoglycans and glycosaminoglycans (GAGs), which provides structural integrity and the capacity to retain water molecules.

Mature cartilage contains primarily water, which makes up approximately 70-80 % of its weight [5]. **Hyaline cartilage** tissue is organised into four different zones from the articular surface down to the subchondral bone that facilitates its specific biological and mechanical functions (Fig. 1A). The **superficial zone** (also known as a tangential zone) lines the surface of articular cartilage and comprises 10-20 % of the tissue. It is characterised by densely packed collagen fibrils and flattened cells that oriented horizontally to the articular surface [6]. This zone has low proteoglycan content and low permeability that facilitates to handle the sheer forces during locomotion. However, chondrocytes produce lubricin (also known as proteoglycan 4) that serves as a lubricant and provides frictionless movement of knee joint [7]. It has been reported that superficial layer contains progenitor/stem cells that are responsible for appositional growth during development [8]. The **middle zone** or transitional zone is characterised by rounded cells in the perpendicular direction and randomly oriented collagen type II fibrils/ fibres.
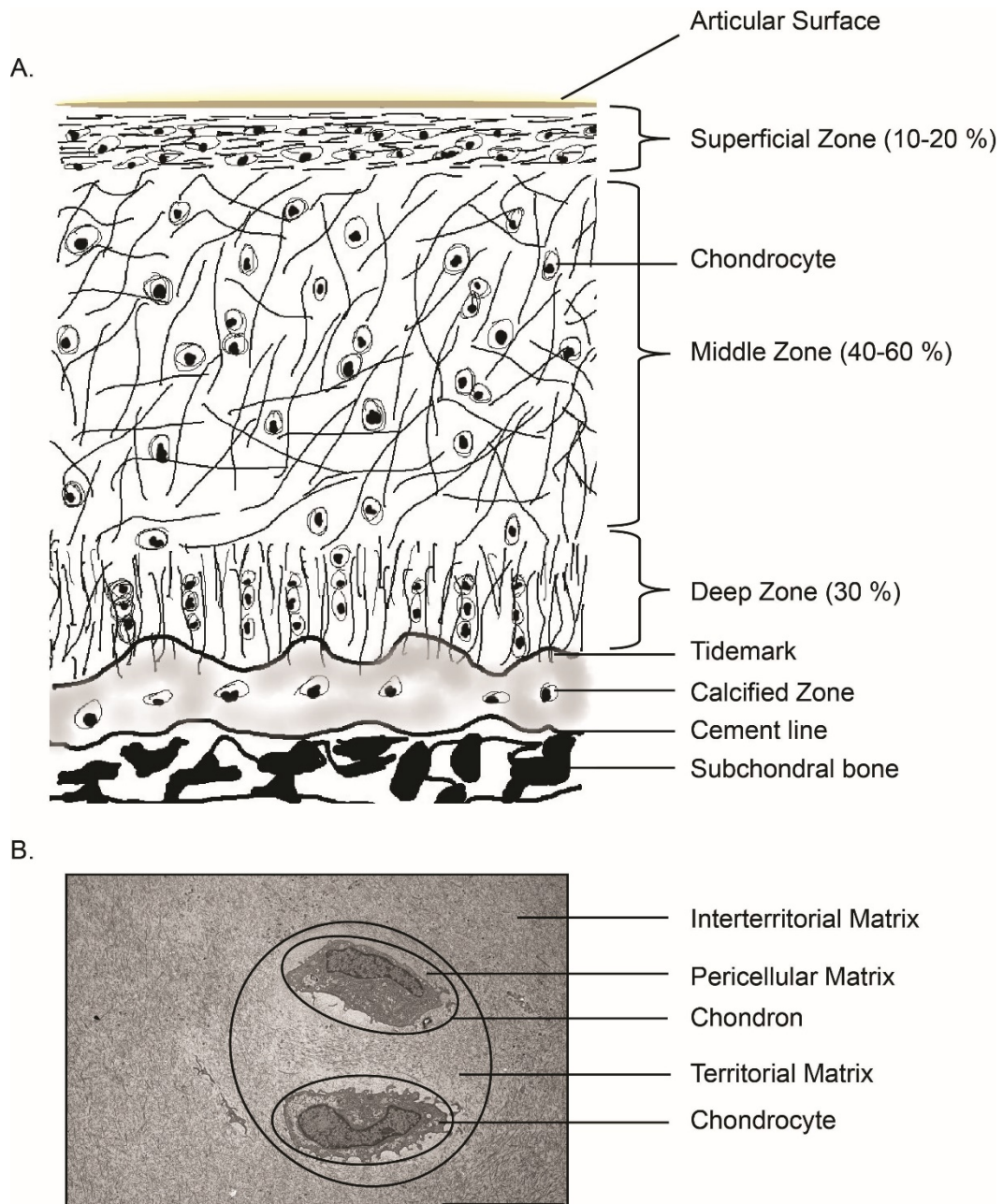
Figure 1: Structure of human articular cartilage. A. The zonal organisation of articular cartilage showing the organisation of chondrocytes and collagen fibrils in a different layer. B. Regional organisation of articular cartilage showing chondrons and proximity of ECM from the chondrocytes. Scale bar: 10 µm.

Unlike the superficial zone, this zone has more proteoglycan content and lower cell density. The **deep or radial zone** is rich in thick collagen fibrils oriented perpendicularly to the articular surface. In the deep zone, cells often group in columnar orientation along with collagen fibres.

A thin line below the deep zone called "**the tidemark**" distinguishes between the non-calcified and calcified zone. In this zone, cells are scarce and hypertrophic. The calcified zone serves as an anchor for the cartilage tissue that is fused with the underlying subchondral bone via the cement line [9].

Based on the proximity of chondrocytes and surrounding extracellular matrix (ECM), cartilage can also be distinguished into several regions (Fig. 1B). The pericellular region is the immediate surrounding matrix of chondrocytes. The so-called "**chondron**" consists of chondrocytes and its pericellular region, which represents the simplest metabolic and functional structure of cartilage [10]. The area surrounding the pericellular matrix is termed as the **territorial matrix**. It is mainly composed of chondroitin sulphated proteoglycans and collagen type VI [11, 12]. The **interterritorial matrix** represents the bulk of ECM, which are most distant from the cells and contains mainly collagen type II and keratin sulphate-rich proteoglycans [13].

In articular cartilage, there are two major load-bearing macromolecules: **collagens and proteoglycans**. The collagen serves as a scaffold and forms the ECM framework to withstand tensile forces during movement. **Collagen type II** is the predominant (~ 90 %) collagen type in the ECM matrix of articular cartilage. Collagen type IX and XI associate with collagen type II and mediate between collagen fibrils and other ECM macromolecules [14]. Other collagens such as collagen type VI contribute to the mechanical function of chondrons and maintain direct interaction between chondrocytes and ECM [15], while collagen type X mediates cartilage mineralisation [16]. The proteoglycan network consists of core protein and **glycosaminoglycans** (GAGs) that links to a long chain of hyaluronan molecule. This long chain interlaces throughout the collagen network and forms a large polymer chain with many proteoglycans (Fig. 2). **Aggrecan** is the most abundant proteoglycan and contains negatively charged chondroitin sulphate and keratan sulphate [17]. This strong negative charge causes the matrix to absorb water, which creates an osmotic pressure in the joint that equilibrates the

compressive loading forces. The avascular, alymphatic and aneural nature of cartilage is behind the low healing potential once the tissue is injured or diseased.
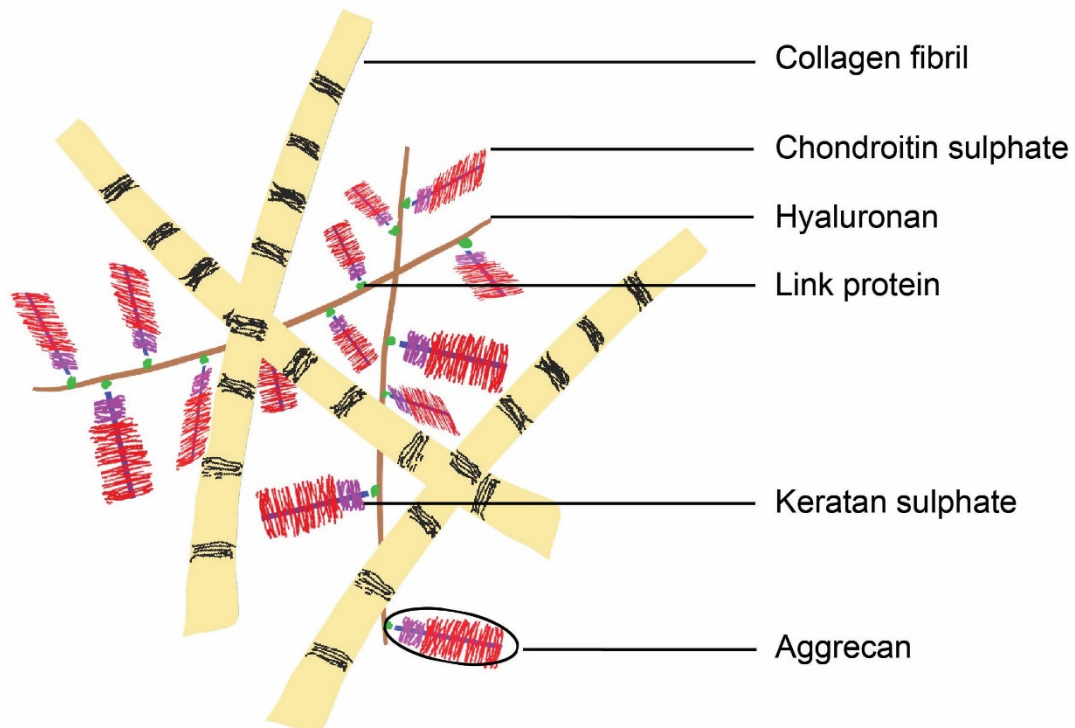
Figure 2: Collagen network and proteoglycan polymer chain form ECM backbone of articular cartilage.

1.2 Cartilage injuries and Osteoarthritis

Forces transmitted at the knee joint during normal physiological activity range from 1.9 to 7.2 times of body weight [18]. An imbalance between ECM mechanobiology and the loading forces transmitted across the joint can result in deterioration of the cartilage [19]. The primary causes of articular cartilage injuries are mechanical trauma or chronic degenerative diseases. Cartilage injuries caused by mechanical trauma can be classified into three types based on the kind of tissue damage [20]: chondral defects, osteochondral defects, and intra-articular fractures. Approximately 20 % of patients undergoing knee arthroscopy are reported to have chondral or osteochondral defects [21]. Like major knee traumas, repetitive microtraumas from sports activities could also lead to localised cartilage damage. These cartilage lesions if left untreated

may enlarge and contribute to the development of degenerative diseases. Progressive degeneration of knee cartilage leads to osteoarthritis (OA). It is the most common type of degenerative joint disease affecting globally over 250 million people and expected to be the fourth leading cause of disability by 2020 [22].
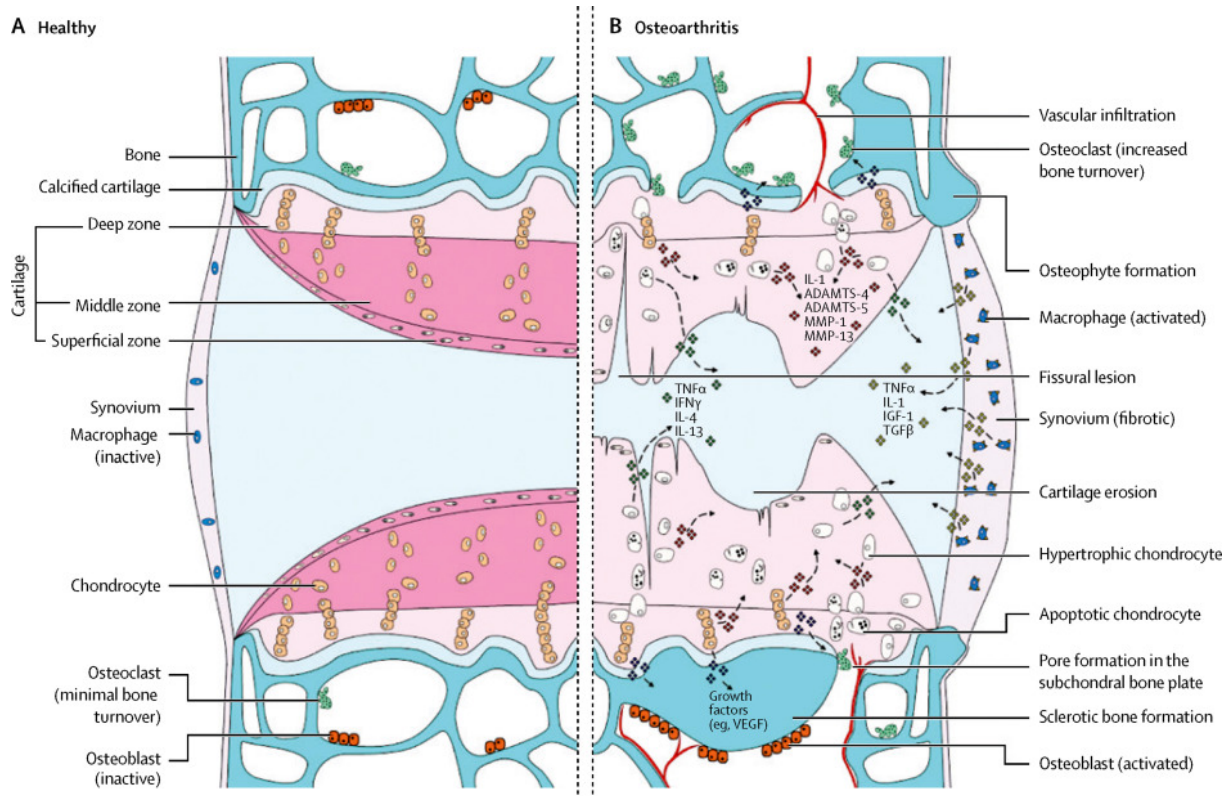
Figure 3: Pathophysiology of osteoarthritis. A. Healthy articular cartilage and B. Signalling pathways and structural changes in osteoarthritis (reproduced with permission from [23]).

OA is a multifactorial chronic disease of the whole synovial joint and is characterised by ECM degradation and impaired joint microenvironment due to maladaptive repair responses to cartilage injuries [24]. There are many risk factors identified for OA progressions, such as age, joint trauma, joint overload, obesity and inflammation, but the exact reasons of OA are still unknown [23]. Age is considered as the most influential risk factor for OA development [25], whereas traumatic knee injuries increase the risk of developing of OA by more than four times [26]. Early events during OA development are the activation of quiescent chondrocytes to form

clusters and increased non-aggregated proteoglycan, and collagen type I production [27, 28]. Initial tissue injury triggers the production of several inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. As a consequence, articular cartilage degenerates by the acceleration of catabolic activities such as proteolysis of aggrecan by aggrecanases (ADAMTS 4 and ADAMTS 5) and degradation of collagen type II by matrix metalloproteinases (MM1, MMP3, and MMP13) (Fig. 3) [24, 29, 30]. As the OA progresses, water retention ability of articular cartilage decreases. Therefore, the resistance of knee cartilage to compression decreases and transmits mechanical loading towards the subchondral bone. Commonly used surgical and nonsurgical OA treatment modalities include intra-articular injections of soluble materials such as corticosteroids or hyaluronate, autologous blood products, joint realignment, nonsteroidal anti-inflammatory drugs (NSAIDs), weight loss, and joint replacement. These procedures improve OA symptoms to a certain degree but do not completely heal the progressive loss of joint functions [31].

1.3 Cartilage repair techniques

Several cartilage repair techniques have been developed for the treatment of focal cartilage defects. The most frequently used methods are microfracture [32], mosaicplasty [33], and autologous chondrocyte implantation [34]. The ultimate aim of these techniques is to regenerate native-cartilage type tissue for symptomatic relief of pain and functional recovery of cartilage integrity. The choice of these treatments is dependent on the defect size and location, and the health of surrounding cartilage. However, these methods have limited application for treatment of OA joints. Pros and cons of most commonly used cartilage repair techniques are briefly discussed in the following section.

1.3.1 Microfracture

Microfracture is a bone marrow stimulation method for cartilage repair. This technique creates a network of the holes in the subchondral bone at the base of the injured cartilage that permits the access of bone marrow stem cells and growth factors to form a fibrin clot in the cartilage

lesions (Fig. 4) [32]. It is probably the most commonly used cartilage repair method given its minimal invasiveness, low cost and technical ease. However, the repaired tissue is in most cases fibrocartilage in nature containing collagen type I, which make it less durable compared to native cartilage [35]. Microfracture is not recommended to treat large defects, for elderly patients or diseased joints [36]. This technique has not been exclusively studied for OA treatment; however, few studies demonstrated worsen outcomes in patients with OA [37, 38]. A detailed description, application, and outcomes of microfracture are outside the scope of this thesis and discussed elsewhere [39].

Cartilage lesion   Subchondral bone drilling
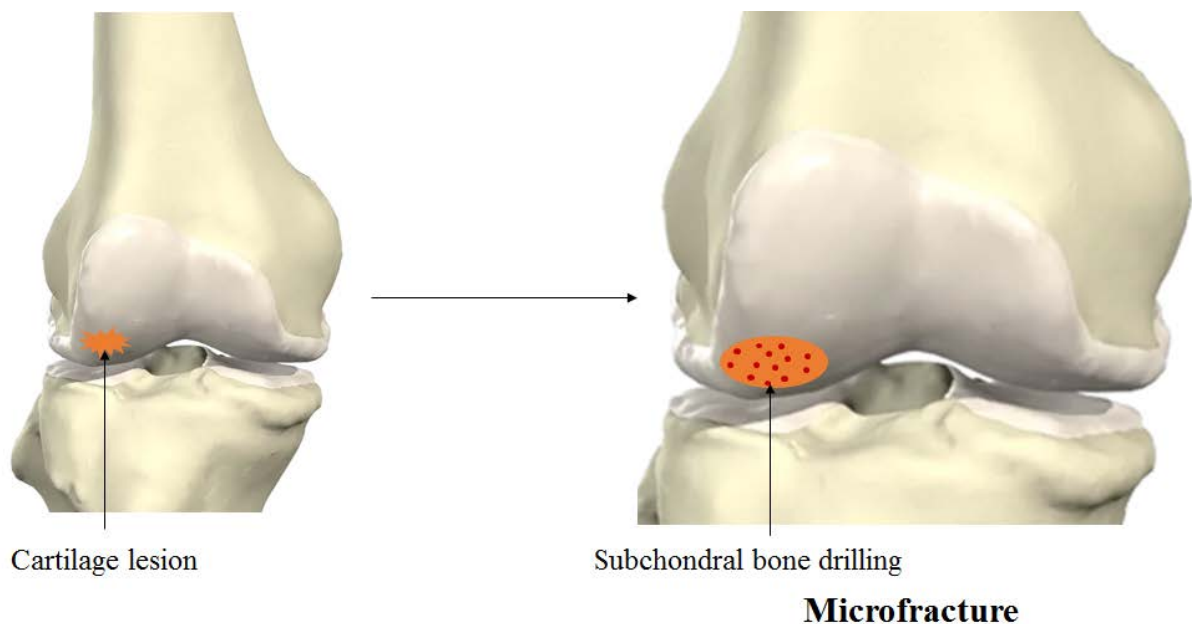
Microfracture

Figure 4: Illustration of microfracture technique.

1.3.2 Mosaicplasty

Mosaicplasty (osteochondral autograft transfer) involves harvesting healthy cartilage and bone plugs from a low-weight-bearing site of the joint and transplantation into the cartilage lesion [33]. This method is less associated with fibrocartilage formation and capitalises bone-to-bone recovery from patient's joint (Fig. 5). The main advantage of this method is faster recovery potential than other methods due to graft stability [40]. This technique is best suited for smaller defects (≤ 4 cm^2). It has been reported that patients treated with mosaicplasty had superior

athletic activity than patients treated with microfracture [41]. However, no significant differences in clinical outcomes are observed between mosaicplasty and microfracture after long-term follow-up [42]. The use of mosaicplasty in OA cartilage repair is rare, but it has been reported in patients with signs of OA [43].
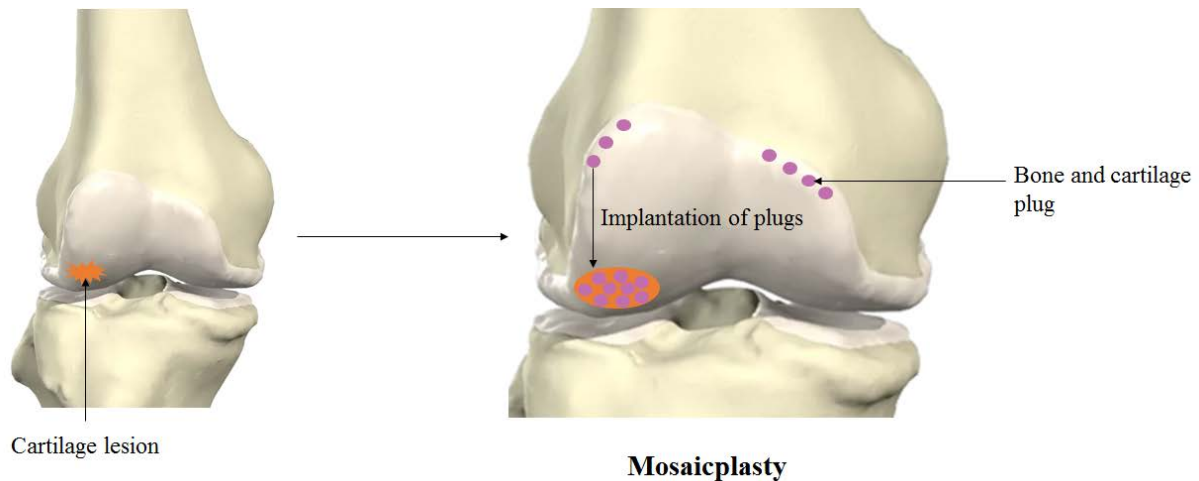
Figure 5: Illustration of the mosaicplasty procedure.

1.3.3 Autologous chondrocyte implantation

Autologous chondrocyte implantation (ACI) has been available as a method to ameliorate impairing localised cartilage defects since the early 90's [34]. It is a two-step procedure using patient's chondrocytes to treat the defect (Fig. 6). The first arthroscopic operation involves collecting a small biopsy from a low-weight-bearing region of joint and culturing the cells *in vitro* to increase cell yield. The culture-expanded chondrocytes are implanted into the debrided cartilage defect and covered with a membrane during the second operation. The first reported technique has experienced refinements such as the introduction of collagen membranes instead of periosteum to cover the defect (second generation ACI or ACI-C), the use of characterized chondrocytes to improve the quality of the repair tissue, or the so-called matrix-assisted chondrocyte implantation (MACI) where the chondrocytes are seeded in a collagen matrix before implantation (third generation ACI) [44, 45]. This technique has been demonstrated as an effective treatment method for large ($\geq 2 \text{ cm}^2$) cartilage defects [46, 47].

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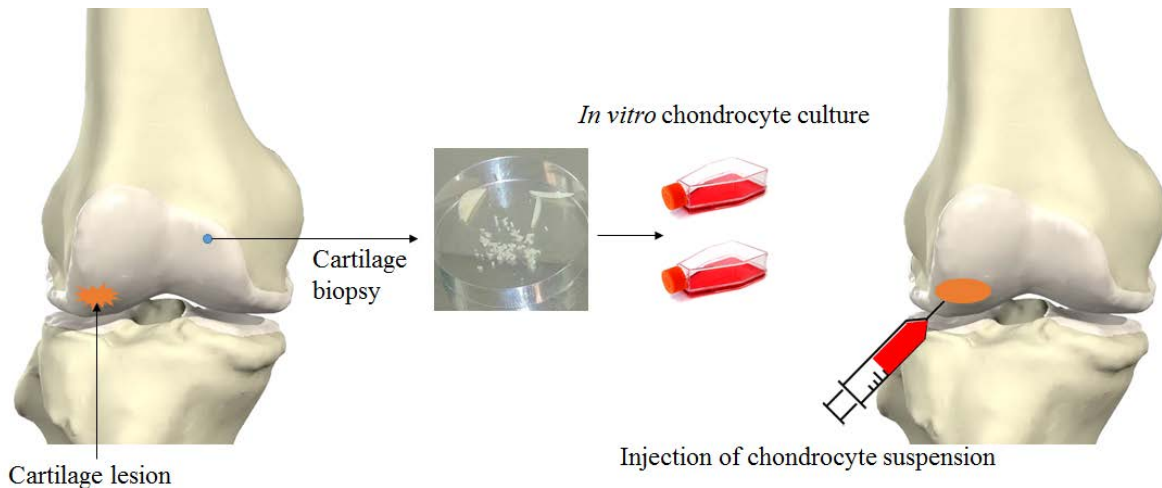
Figure 6: Illustration of ACI procedure.

The major drawback of this technique is the two-step operational procedure, high costs and the dedifferentiation (loss of function) of chondrocytes during the *ex-vivo* expansion phase. Implantation of dedifferentiated chondrocytes demonstrated to have worsened the outcomes of ACI [48]. Also, this method results in fibrocartilage formation, while only 15-30% of patients develop hyaline-like cartilage tissue [48, 49]. This technique is the least cost-effective surgical method compared to microfracture and mosaicplasty [50]. Although successful clinical outcomes have been reported for ACI, the long-term failure rate ranges between 20-40 % [47, 51]. The use of this method is in degenerative cartilage lesions showed significant improvement in all scores in early OA patients treated with second-generation ACI [52]. Although a substantial improvement observed in the studied population, the number of failures was higher than what had been reported earlier in non-arthritic populations [53].

1.3.4   Other cell-based and cell-free cartilage repair approaches

Considering the advantages and limitations of first and second generation of ACI, several cell-based and cell-free methods have been developed for cartilage repair. The third generation ACI is scaffold-based cell therapy involving two operational steps. Chondrocytes are seeded on

absorbable porcine collagen membrane for three days (MACI) or chondrocytes are cultured inside the 3D scaffolds (Bioseed-C, NeoCart® 3D, Hyalograft® C, Cartipatch®, and Biocart™II) before implantation into the cartilage defects [54]. Although MACI had promising clinical results [55, 56], the problem with fibrocartilage tissue formation and longer rehabilitation time still exist [57, 58].
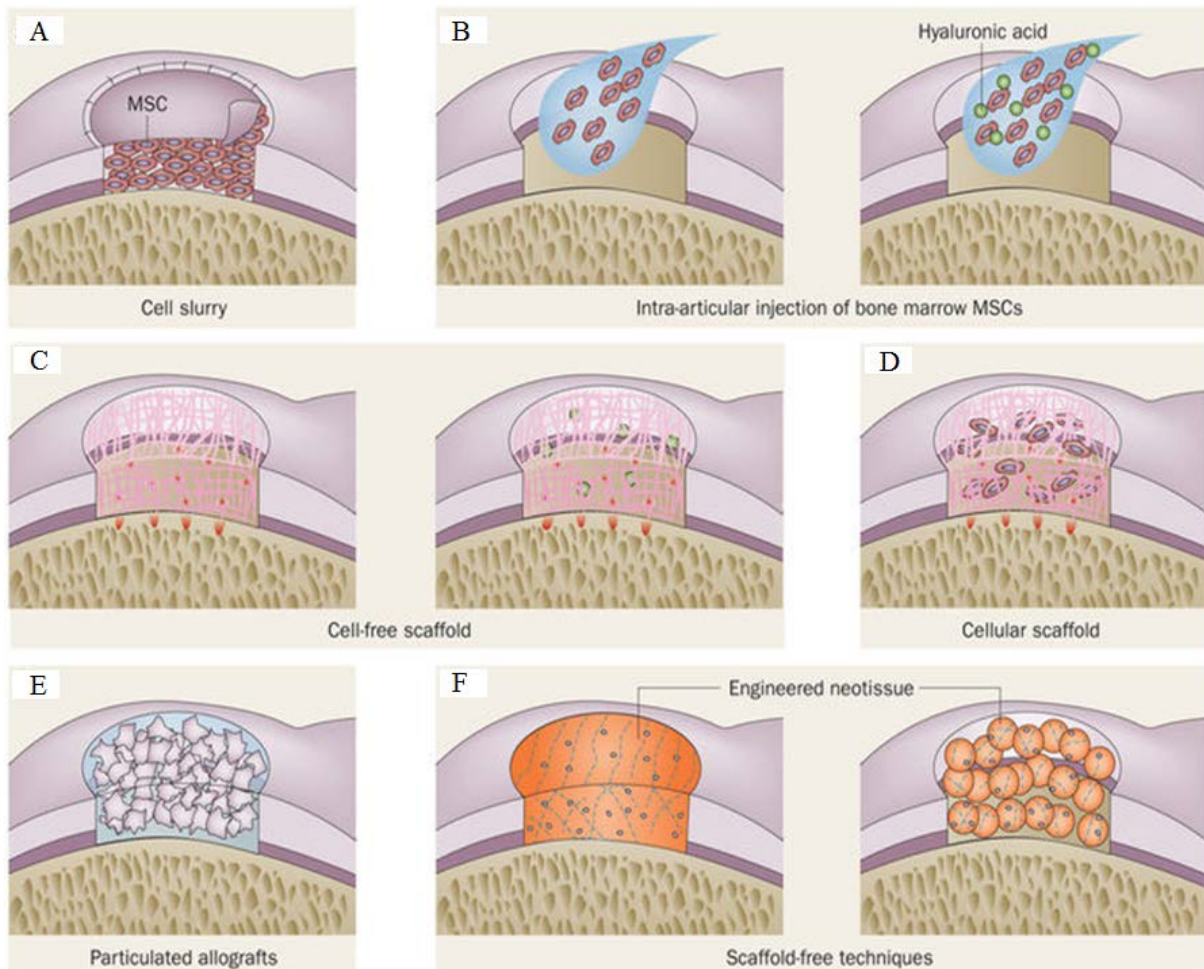
Figure 7: Different cell-based and cell-free approaches to mimic ACI. A. Autologous bone marrow-MSCs implantation, B. Intra-articular injection of MSCs, C. AMIC, a cell-free scaffold-based surgery, D. MACI uses scaffolds with primary chondrocytes, E. Small particulated native cartilage approach and F. Scaffold-free chondrospheres or engineered neotissue (reproduced with permission from [59]).

Autologous matrix-induced chondrogenesis (AMIC) is single step procedure involving microfracture, to supply bone marrow stem cells and blood elements, and covering the defects with a collagen membrane (Fig. 7) [60]. The use of autologous serum or platelet-rich plasma, hyaluronic acid, and chitosan-glycerol phosphate with AMIC have emerged as a novel *in situ* approaches to treat cartilage lesions [61, 62]. No significant differences in short-term clinical outcomes have been observed between microfracture alone and *in situ* AMIC [63]. Unlike scaffold-based cell therapy, scaffold-free neotissue known as chondrosphere® has been developed to enhance cartilage regeneration [64]. It is composed of spheroids of neocartilage containing expanded chondrocytes and generated matrix. Chondrosphere® technique was reported to significantly improve the clinical scores after one-year follow-up [64]; however still lacking the long-term randomised control study. Other 3D scaffold-based chondrocyte therapies show some extent of improvement in the treated joints, but requiring the long-term randomised control clinical study. A detailed description and outcomes are reviewed elsewhere [54], which is outside the scope of the thesis.

1.4 Alternative cell sources

Autologous chondrocytes have been used as an intuitive cell source for cell-based therapy due to their direct implication in cartilage homeostasis. However, their use is limited to cell-based treatment by several issues, such as donor site morbidity, a limited number of cells that need expansion and the loss of phenotypic traits during monolayer expansion [65, 66]. Alternative cell sources have advantages over these commonly raised problems with chondrocytes. Cell sources that are being investigated in this field include embryonic stem cells (ESCs), adult mesenchymal stem/stromal cells (MSCs), and induced pluripotent stem cells (iPSCs).
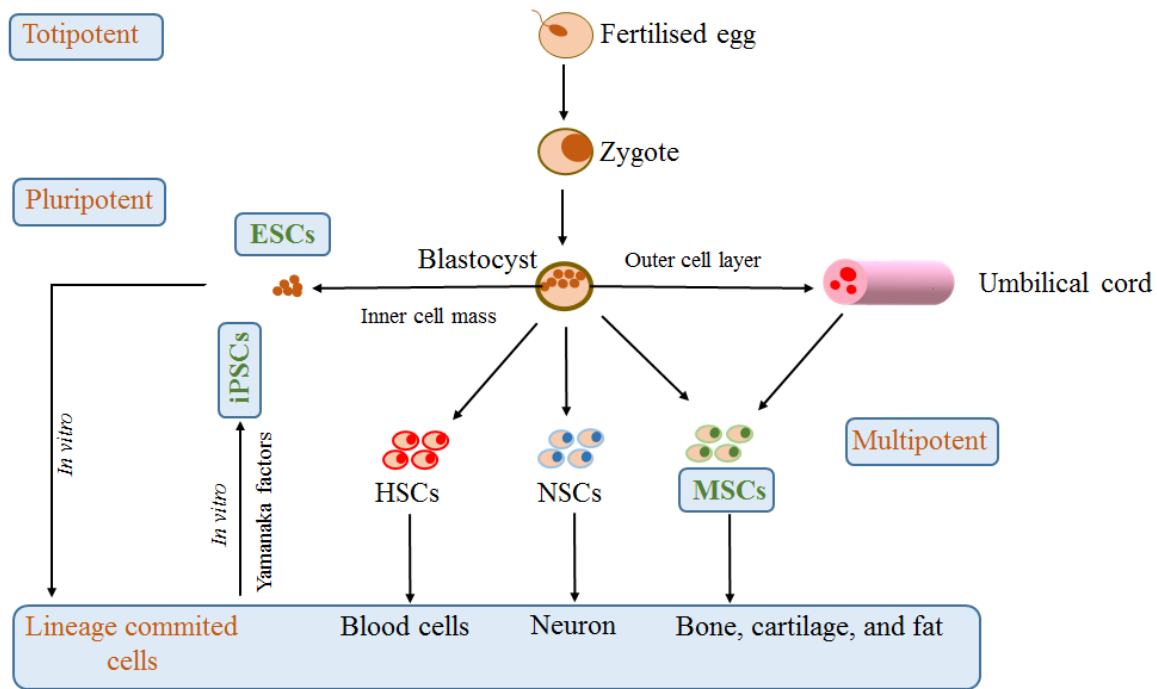
Figure 8: Flowchart illustrating the hierarchy of stem cells.

1.4.1 Mesenchymal stem/stromal cells (MSCs)

MSCs are multipotent cell types with self-renewal and multi-lineage potential to differentiate into mesoderm cell types (Fig. 8). MSCs can be isolated from multiple tissues and organs including bone marrow, adipose tissue, synovial membrane, umbilical cord, muscle, and dental pulp [67-70]. These cells are heterogeneous cell populations with varying differentiation and proliferation potentials [70, 71]. Many scholars in the field support the notion that MSCs represent a defined population of multipotent progenitor cells residing in the perivascular niche of nearly all human tissues, [72, 73] although different views exist [74]. To improve the characterisation, The International Society of Cellular Therapy (ISCT) has set guidelines to define the traits of human MSCs [75]. These criteria are plastic adherence, expression of surface markers CD73 (ectonucleotidase), CD90 (thy-1) and CD105 (endoglin), and the ability to differentiate towards multiple cell types of mesenchyme origin, such as adipocytes, chondrocytes and osteocytes. In addition, to avoid contamination of MSCs from other cell types, these cells should not express hematopoietic and other immune cells markers such as

CD34, CD45, CD14, and CD19. MSCs do not express HLA-DR; however, priming of cells with inflammatory cytokines can induce expression of this receptor [76]. Bone marrow and adipose tissue MSCs are most frequently used for cartilage repair. In addition, patients treated with MSCs from synovial membrane reported having superior clinical outcomes compared to MACI [77]. Although MSCs are considered as a suitable alternative cell source, their proliferation and differentiation potential were reported to be affected by ageing [78, 79].
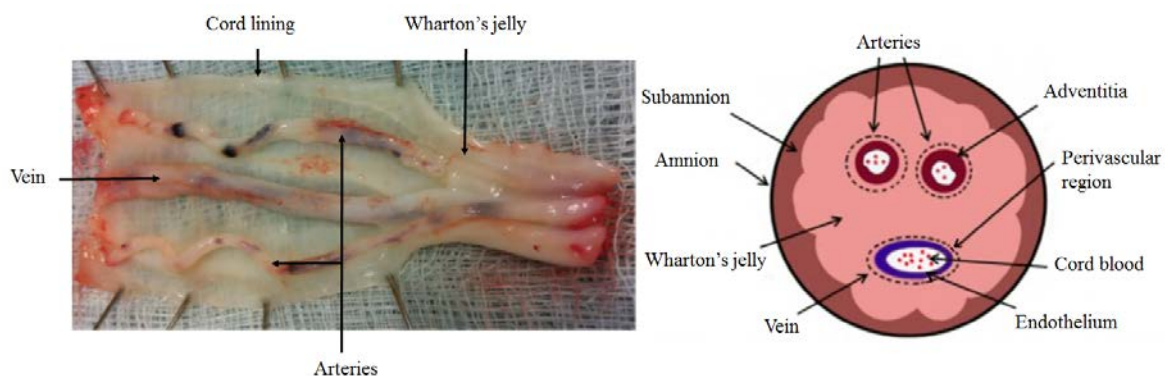
Figure 9: Dissection of human umbilical cord showing Wharton's jelly, cord lining, vein, and arteries (reproduced with permission from [80, 81]).

1.4.2   Umbilical cord stem/stromal cells (UCSCs)

To avoid aforementioned problems with adult MSCs, cells have been isolated from perinatal extraembryonic sources, such as umbilical cord, placenta, and amniotic fluid [80, 82]. Umbilical cord derives from the epiblast during embryonic stage; therefore, it retains some embryonic characteristics [83]. Umbilical cord stem/stromal cells (UCSCs) are immature and collected from what is considered as medical waste, which makes it easily accessible with minimal ethical constraints to use a suitable source of allogeneic MSCs. UCSCs can be isolated from different regions of the cord, such as Wharton's jelly, vein, arteries, and cord lining. MSCs from different regions possess comparable proliferation and differentiation potential (Fig. 9) [80, 84]. In addition to MSCs from solid parts of cords, MSCs derived from cord blood have also been isolated and demonstrated to have the multi-lineage potential [85, 86]. Like adult

counterparts, UCSCs also possess high proliferative and multi-lineage differentiation potentials [80, 87]. In addition to these characteristics, UCSCs possess pro-angiogenic, anti-inflammatory and low immunogenic characteristics compared to other MSCs [88-90]. Low immunogenic properties of UCSCs allow these allogeneic cells to evade immune rejection after transplantation [91, 92]. However, their chondrogenic potential has been studied with divergent outcomes, such as immature cartilage forming, and poor chondrogenic ability compared to other cell types [70, 93-95].

1.4.3   Embryonic stem cells

ESCs are pluripotent and have the potential to differentiate into any type of cells in the adult body. ESCs are isolated from the blastocyst stage of embryos by removing the inner cell mass and subsequently, expanded in culture [96]. The outer cell layer known as trophectoderm forms the umbilical cord and placenta (Fig. 8). This pluripotent cell type has been demonstrated to differentiate into chondrogenic lineage [97]. However, due to the high risk of tumourigenicity, it is essential to growing ESCs in stable culture conditions for chondrogenic differentiation [98]. In addition to teratoma formation, ethical constraint limits the use of ESCs in clinical application.

1.4.4   Induced pluripotent stem cells

iPSCs are genetically reprogrammed stem cell types derived from any somatic adult cell type by transfecting cells with Oct3/4, Sox-2, Klf4 and c-Myc (Fig. 8) [99]. This technique provides new insight into cartilage repair by reprogramming cells into chondrogenic lineage [100]. iPSCs generated from chondrocytes demonstrated to have superior chondrogenic potential compared to iPSCs from other sources [101]. One of the major challenges involves incomplete reprogramming of iPSCs [102]. It has been reported that iPSCs retain epigenetic memory and genetic background [103, 104]. These characteristics cause the variation during reprogramming of iPSCs. In addition, there still other challenges that need to be addressed, such as safety, tumourigenicity, regulatory validation, and chondrogenic efficacy [105].

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1.5 Cartilage Tissue-engineering

Chondrogenesis is a complex process of cartilage development initiated by MSCs condensation during embryonic development. This condensation process is regulated by a series of cell-cell and cell-matrix interactions. During foetal development, cartilage serves as a template for bone formation and is subsequently replaced via endochondral ossification except for the end layer of bones [106, 107]. A detailed description of signalling pathways can be found elsewhere [108]. Appositional growth of articular surfaces continues until skeletal maturity [109]. Understanding the process of endochondral bone formation has played a pivotal role in the development of chondrogenic medium for tissue-engineered cartilage (TEC-here understood as laboratory made cartilage tissue). The development of artificial TEC encompasses several fundamental elements. These are cell sources, culture conditions, scaffolds, and biochemical/biomechanical stimuli. Although chondrocytes are considered as the intuitive source, MSCs, ESCs, and iPSCs are all demonstrated to be a suitable source for cartilage repair (Section 1.4). TEC using ACs and MSCs will be discussed in the following section due to relevance to this thesis.

The scientific community has not reached a consensus on the ideal cell source for TEC. Bone marrow-derived MSCs have been considered as the gold standard. However, few noteworthy comparative studies showed SMSCs as a superior cell source for TEC (Table 1). The ability of cells to induce chondrogenesis is mostly dependent on the exogenous stimuli and signalling molecules. Important environmental elements considered in cartilage tissue engineering can be divided into two categories: 1) signalling molecules and factors that facilitate cell proliferation and expansion in monolayer and 2) signalling molecules or exogenous stimuli that facilitate chondrogenic differentiation in 3D culture to promote ECM production.

1.5.1 2D culture phase (cell expansion)

The serum is essential for monolayer expansion of cells to enhance proliferation [110, 111]. Although serum supplementation of media is most commonly used for *ex-vivo* culture

expansion, there is an argument on avoiding exogenous FBS. It has been shown to be affected by source and batch-to-batch composition. To avoid these issues, autologous serum or suitable anabolic factors, such as dexamethasone, and ITS have been used as media supplement for culture expansion of cells [112, 113]. Ascorbic acid, a water-soluble antioxidant, induces *in vitro* cell proliferation and collagenous matrix deposition upon addition to the culture medium [114, 115]. Monolayer culture of adherent cells is often supplemented with additional growth factors to promote cell proliferation. Among all growth factors, bFGF is the most commonly used anabolic factor in monolayer expansion of cells to promote proliferation, stem cell renewal and to keep the chondrogenic potential [116, 117]. However, varying concentration of bFGF might have a different effect on proliferation and matrix production [118].

1.5.2   3D culture phase (matrix formation)

In vitro expansion of chondrocytes in monolayer cultures leads to undesirable loss of function. This characteristic was first reported in the late 60's by observing changes in cell morphology and reduction of chondroitin sulphate synthesis [119]. This fact promoted the development of culture systems that preserve the chondrogenic potential such as the pellet culture [120]. Passaged cells are integrated into either a scaffold-based or a scaffold-free 3D construct to induce chondrogenesis. Scaffold-based 3D construct provides ECM niche for seeded cells to grow and differentiate into the scaffold to form TEC. Ideal characteristics of scaffolds are biocompatible, biodegradable, porous, and supportive for chondrogenesis [121, 122]. There are two main types of scaffolds used in cartilage tissue engineering: natural biopolymers and synthetic biopolymers. Natural scaffolds facilitate cellular adhesion, and synthetic scaffolds improve structural integrity. Poly L-lactic acid and polyglycolic acid are the most commonly used synthetic scaffolds in cartilage tissue engineering [123]. Commonly used natural scaffolds include agarose, alginate, hyaluronic acid, and collagen [124, 125].

Table 1: List of some comparative studies analysing the chondrogenic potential of human MSCs from various sources. AC: Articular chondrocytes, AT: Adipose tissue, BM: Bone marrow, SM: Synovium, FP: Fat pad and UC: Umbilical cord.

Sources of MSCs								Best source (Superior chondrogenesis)	Ref
AC	AT	BM	SM	FP	UC		Other sources (periosteum, skeletal, and dental)		
					Matrix	Blood			
	✓	✓	✓				✓	SM	[126]
		✓	✓					SM	[127, 128]
✓	✓	✓		✓				AC>FP>BM>AT	[71]
	✓	✓			✓			Similar chondrogenesis	[129]
✓			✓	✓	✓			AC>SM>FP>UC matrix	[70]
✓		✓	✓					SM	[130]
	✓			✓				FP	[131]
	✓	✓		✓	✓			FP>BM>AT>UC matrix.	[132]
✓	✓	✓					✓	AC	[133]
✓	✓	✓					✓	Nasal septum	[134]
	✓				✓			AT	[93]
	✓	✓				✓	✓	AT and BM	[135]

Scaffold-free 3D constructs have certain advantages over scaffold-based approaches for clinical applications. Scaffold-free 3D constructs do not involve complicated processing steps or toxic degradation, and it provides a natural microenvironment compared to scaffold-based approaches [136, 137]. It has also been reported to affect the morphology of chondrocytes due to crosslinking density of polyethyleneglycol scaffold [138]. Detailed techniques and

comparisons about scaffolds used for TEC can be found in some reviews [139, 140]. In scaffold-free approaches, cells are cultured in dense cell formations to facilitate TEC formation by cell-cell interactions [70, 141]. There are different techniques for preparing scaffold-free cartilage 3D construct. Cell aggregation and cell self-assembly are the most commonly used techniques for cartilage tissue engineering. Pellet culture is a cell aggregation method in which cells (0.5-2.5×10^5) are centrifuged in small well or tube to form small tissue-like structures under 1 mm in diameter (Fig. 10) whereas in self-assembly technique TEC is formed without applying any centrifugal forces.
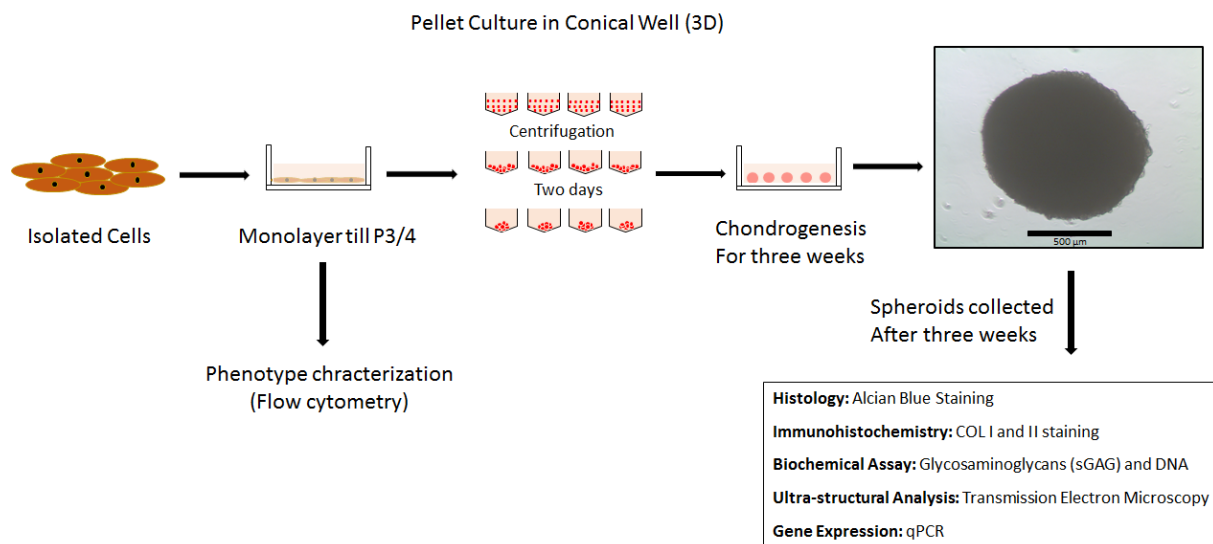
Figure 10: Schematic of pellet culture method for chondrogenesis.

Supplementation of anabolic growth factors to promote chondrogenesis during 3D growth, is a normal practice to achieve TEC. TGF-β is the master regulator of chondrogenesis and ECM production in pellet culture [142]. The first well-established TGF-β chondrogenic medium in the 1990s still influences presently used chondrogenic medium [143]. Along with TGF-β supplementation, the chondrogenic medium is often enhanced by BMPs, PTHrP, ITS, dexamethasone, ascorbic acid, glucose, and pyruvate. However, it has been demonstrated that the presence of serum reduces ECM production during *in vitro* chondrogenesis [120]. ITS and

dexamethasone, on the other hand, have been shown to enhance chondrogenic differentiation and matrix production during 3D chondrogenesis [112, 144].

The TGFβ superfamily includes the TGFβ and BMP subgroups and plays a central role in articular cartilage development and homeostasis. TGFβ subfamily acts by binding of receptor type II (TGFβRII) with ligand and activates receptor type I (TGFβRI, ALK1 or ALK5), which mediate SMAD signalling by phosphorylation. This signalling cascade is important during cartilage development. Ligand binding to ALK5 activates SMAD2/3 signalling while ALK1 triggers the SMAD1/5/8 downstream cascade [145, 146]. TGFβRIII receptor enhances ligand binding for TGFβRII and TGFβRI. The ALK1 pathway becomes activated mostly with ageing and in OA cartilage compared to the ALK5 pathway that is active in healthy cartilage [147]. On the other hand, the BMP subfamily binds with BMPRII and activates BMPRIA (ALK1, ALK2 and ALK3) or BMPR1B (ALK6) to mediate downstream signalling by SMAD1/5/8. A detailed description of their pathway can be found in this review [148].

BMPs are included in the chondrogenic medium to exert synergistic effects along with TGF-β [149, 150]. They also involve in promoting chondrogenesis, maturation of chondrocytes and terminal differentiation. To reverse hypertrophic phenotype, PTHrP is added to the chondrogenic medium [151]. Low glucose has been reported to be beneficial for chondrogenesis of culture-expanded chondrocytes [152]. In contrast, high-glucose was demonstrated to promote cells survival and proteoglycan synthesis in pellet culture [153], which was also observed in our studies [70]. In addition, hypoxia is another essential factor that provides microenvironment niche with low oxygen tension during 3D culture. It facilitates chondrogenesis and reduces apoptosis in the 3D culture [154]. However, static culture condition often results in poor ECM development. This leads to the development of bioreactors involving mechanical stimulation, such as compressive, shear, and hydrostatic forces, to mediate collagen

production and improve the orientation of collagen in TEC [155-157]. Following review is recommended for detailed description bioreactors and its mechanism [158].

1.6 New concepts on MSCs regenerative potential

The tissue regenerative potential of MSCs was thought to rely on the capacity of MSCs to migrate and engraft in damaged tissues, and transdifferentiating into tissue forming cells to promote tissue repair [159]. However, the fate of implanted cells during biological repair of cartilage is mostly unknown. The presence of cells of unknown origin in the repaired tissue has also been documented [160, 161]. Importantly, considering the effects of soluble signalling molecules from cultured MSCs, Arnold Caplan first proposed MSCs as trophic mediators in tissue regeneration [162]. This change of paradigm in understanding MSCs mechanism of action involves paracrine signalling and trophic effects exerted by the released bioactive molecules from MSCs, which in turn leads to support tissue microenvironment and reconstruction of the damaged tissue [163, 164]. Co-culture studies have demonstrated that MSCs facilitate proliferation and ECM enrichment of chondrocytes in a paracrine fashion, irrespective of sources of MSCs [165, 166]. A human clinical trial using allogeneic bone marrow MSCs demonstrated the trophic effects of this cell population during cartilage repair [167]. MSCs secrete a spectrum of bioactive soluble factors known as the secretome, comprising growth factors, cytokines, and chemokines that work in an omnidirectional way to regulate angiogenesis, apoptosis, and inflammation (Fig. 11).

Inflammation during OA disease progression is prompted by resident cells in the synovial joints such as chondrocytes or synoviocytes [168, 169]. The bioactive molecules released by local tissue resident cells could have a pleiotropic effect at the disease site, which could trigger inflammatory cascades. Importantly, it has been demonstrated that MSCs secretomes may be influenced by inflammatory conditions at the damaged tissue [170, 171]. Therefore, for experimental purposes, pre-activation of MSCs with pro-inflammatory cytokines is often

considered to reveal immunosuppressive effects [172, 173]. However, it has also been demonstrated that non-activated MSCs also exert similar immunosuppressive effects [174]. Of note, *ex vivo* pre-activation of cells has been shown to cause immunogenic effects upon transplantation [175, 176]. Therefore, it is likely that MSCs undergo a phenotypic activation upon exposure to the inflammatory environment.
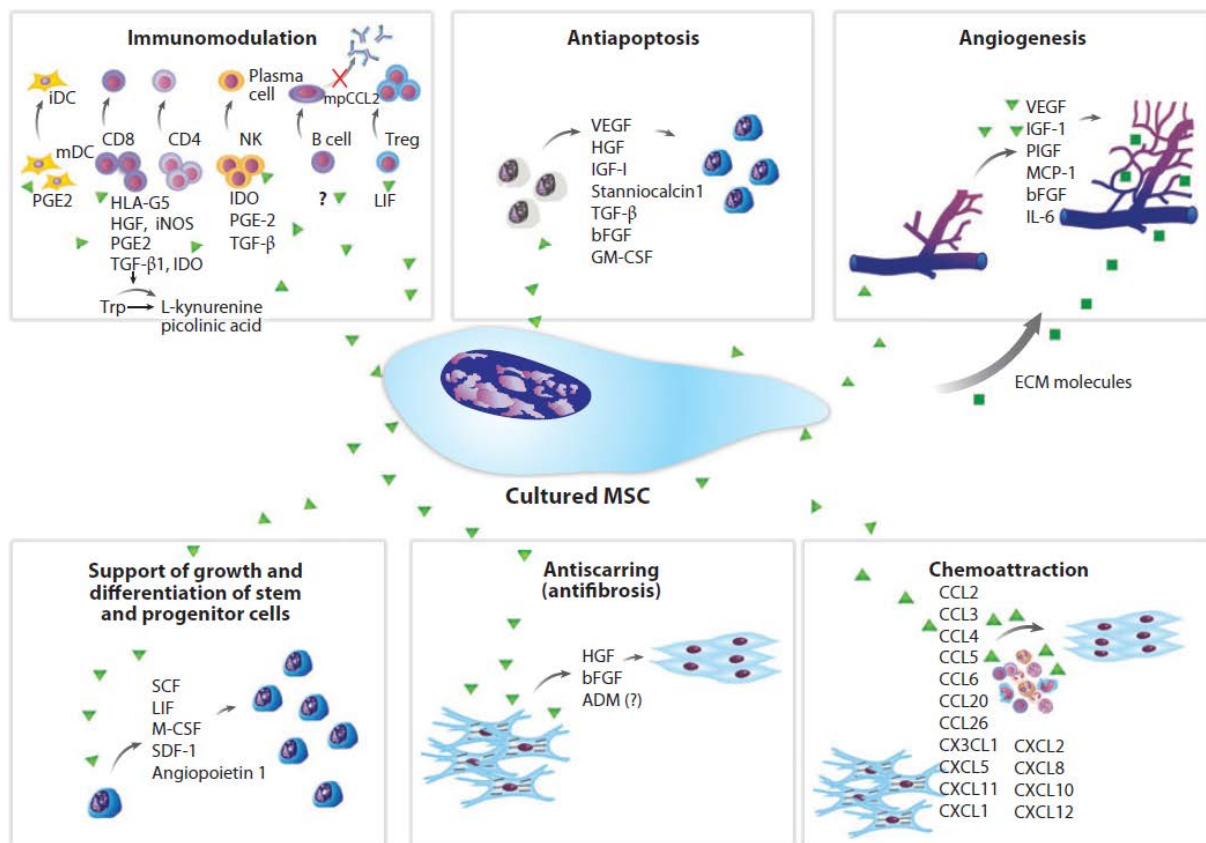
Figure 11: Schematic of multifunctional effects of MSCs through bioactive soluble factors (reproduced with permission from [177]).

Safety is the major concern when considering MSCs-based therapy for disease management. Like autologous MSCs, it has also been demonstrated that allogeneic MSCs are safe and promote immunosuppressive effects during cartilage repair (Table 2). Both autologous and allogeneic MSCs have shown similar efficacy in bone regeneration in a preclinical study [178]. Although it has become well established concerning the equivalent efficacy of autologous and allogeneic MSCs, there are controversies that evidently showing immunogenic responses after

allogeneic transplantation [176, 179]. Importantly, routes of administration may influence the therapeutic efficacy of MSCs. Allogeneic MSCs are currently administered via systemic route for the treatment of diseases such as graft-versus-host disease, Crohn's disease, and respiratory disease in the clinical trials because of the immunosuppressive properties [180]. Systemic administration results in rapid clearance of MSCs, which decreases the number of MSCs delivered to the injured site [181]. It results in poor therapeutic effects of administered MSCs. In addition, it may raise the concern of losing immunomodulatory property and may initiate immune response [182]. Local administration of MSCs for cartilage repair, which is an immunoprivileged tissue, often avoids these complications. Intra-articular injection of MSCs in induced OA in preclinical models have shown that MSCs could inhibit OA progression [183, 184]. Similar findings have also been reported in clinical studies (Table 2). However, there is still no evidence about the suitable source of MSCs or superiority MSCs over chondrocytes for cartilage repair or OA management. One comparative clinical study demonstrated the superior effect of MSCs from synovial membrane compared to chondrocytes in the treatment of chondral defects [77].

Table 2: List of few clinical studies using MSCs for articular cartilage repair and OA. AT: Adipose tissue, BM: Bone marrow, FP: Fat pad, HA: Hyaluronic acid, IA: Intra-articular, MF: Microfracture, PRP: Platelet rich plasma and UCB: Umbilical cord blood.

Cell source/ number of cells	Sample size/type of lesions	Delivery system	Control groups	Follow-up period	Outcomes	Ref
Autologous MSCs for articular cartilage repair and OA						
BM/ 1.3×10^7	24/OA	Implantation (MSCs + collagen sheet)	Cell free	95 weeks	Better arthroscopic and histological grading score	[185]
AT/ 2×10^6 10×10^6	18/OA	Single IA injection	No	6 months	Safe & improved pain levels	[186]

20×10^6						
AT/ 5×10^6	80/Symptomatic cartilage defect (≥ 3 cm^2)	Implantation (MF + fibrin glue)	MF	24 months	Improved KOOS pain and symptom subscore	[187]
FP/ 1.18×10^6	18/OA	IA Injection	No	26 months	Improved knee function	[188]
FP/ 1.89×10^6	25/OA	IA Injection	PRP	18 months	Safe & improved knee function	[189]
SM/ 4×10^6	14/Chondral defect (≥ 2 cm^2)	Implantation (MSCs + collagen sheet)	MACI	24 months	SM>MACI	[77]
BM/ 1-1.5×10^6	72/Chondral defect (≥ 3.6 cm^2)	Implantation (Fibrin glue)	ACI	24 months	As effective as ACI	[190]
BM/ 8-9×10^6	4/OA	Single IA injection	No	60 months	Advanced OA	[191]
AT/ 14×10^6	6/OA	Single IA injection	No	12 months	Safe and improved pain levels	[192]
BM/ 40×10^6	12/OA	IA injection	No	12 months	Improved cartilage quality	[193]
Allogeneic MSCs for articular cartilage repair and OA						
BM/ Not specified	10/Symptomatic cartilage defect (2-8 cm^2)	Implantation (Chondrons + fibrin glue)	No	12 months	Successful tissue regeneration	[194]
BM/ 40×10^6	30/OA	Single IA injection	HA	12 months	Safe	[195]
BM/ 5×10^7 1.5×10^8	55/Partial medial menisectomy	IA injection	Cell free	24 months	Safe	[196]
UCB/ 5×10^6	6/OA & full-thickness cartilage defects	Implantation	No	7 years	Safe and effective	[197]

1.7 Biomarkers for cartilage repair

Although ACI has been clinically adopted for cartilage repair since the 1990s, the procedure has a long-term failure rate ranging between 20-40 % [47, 51]. Some patient characteristics including demographic and injury-associated risk factors have been identified [198-200]. Along with these risk factors, Osteoarthritis Research Society International (OARSI) highlighted the importance of identifying soluble biomarkers to predict the clinical outcome before ACI intervention and to improve the decision-making process for patients with cartilage injuries

[201]. The investigation of biomarkers can be broadly divided into two source types: 1) liquid biopsies including synovial fluid, blood and urine and 2) cell quality (Table 3). Few putative biomarkers, such as CD14, and ADAMTS-4, have been identified from synovial fluids [202-204].

Acknowledged markers of chondrogenesis including cell adhesion molecules, integrins, chondrogenic signalling pathways and matrix proteins have been explored to find suitable cell-based biomarkers with predictive potential in the clinics. Cell adhesion molecules and cell-cell contact receptors play a pivotal role in initial cell condensation and differentiation during chondrogenesis. Previously proposed chondrogenic biomarkers include surface receptors such as CD44, CD151, CD146, FGFR, CD29 or CD49. CD44 (Hyaluronan receptor) plays a crucial role in cartilage homeostasis and structural orientation of pericellular matrix by retaining aggrecan aggregates [205, 206]. Also, CD44 has been reported to positively correlate with chondrogenesis and short-term clinical outcome [207, 208]. Like the CD44 marker, CD146 (melanoma cell adhesion molecule/MCAM), CD151 (tetraspanin) and CD166 (activated leukocyte cell adhesion molecule/ALCAM) are also associated with enhanced chondrogenic potential [207] [209]. On the other hand, CD54 (intercellular cell adhesion molecule 1/ICAM-1) has shown a divergent outcome when comparing chondrogenic potentials in different stem cells [210, 211].

Like cell adhesion molecules, integrins are also known for their effects on cartilage homeostasis. Integrins are small molecule heteromeric cell-surface receptors that mediate cytoplasmic kinase and cytoskeleton signalling cascades in response to different stimuli, mechanical load, and differentiation. Change in cartilage homeostasis affects their expression and vice versa. The expression of all alpha subunits and the beta-1 subunit increase in OA chondrocytes [212, 213]. Therefore, change in the expression of integrins is considered an important regulator in cartilage repair. Integrins such as ITGA3 (CD49c), ITGA5 (CD49e), and

24

ITGA6 (CD49f) have been linked to improved chondrogenesis [207], while ITGB1 (CD29) has been associated with the inhibition of early chondrogenesis [214]. Although cell adhesion molecules, integrins, and cell-cell receptors have been considered as potential key players in chondrogenesis, the relevance of their expression in clinical outcomes is still questionable [215, 216].

Table 3: List of some human studies investigating biomarkers to forecast either chondrogenic potential of chondrocytes or clinical outcomes of ACI.

Biomarkers	Cell type/sample type	Donor	Outcomes	References
CD44, Aggrecan and Collagen type II	Chondrocyte	ACI patients	**Clinical vs chondrogenesis:** No patient-specific parameter identified but younger patients displayed higher expression compared to older patients.	[216]
CD44, Aggrecan and Collagen type II	Chondrocytes	ACI patients	**Clinical vs chondrogenesis:** CD44 and Collagen type II expression associated with the postoperative score.	[208]
CD44, CD166, Aggrecan, Collagen type II, BMP2, FGFR3 and ACVRL1	Chondrocyte	ACI patients	**Clinical:** No differences observed between success and failure groups.	[215]
CD44, CD49c, CD49e, CD49f and CD151	Chondrocyte	Human	**Chondrogenesis:** These markers expression displayed a positive correlation with chondrogenesis.	[207]
CD166	OA and normal chondrocyte	Human	**Chondrogenesis:** CD166 associated with high chondrogenic potential.	[217]
CD146	Total knee arthroplasty		**Chondrogenesis:** CD146 associated with high	[209]

	OA chondrocyte and ATMSCs		chondrogenic potential.	
MMP-3 and IGF-1	Synovial fluid	ACI patients	**Clinical:** These markers elevated than control.	[203]
ADAMTS-4, COMP, Hyaluronan, and soluble CD14	Synovial fluid and plasma	ACI patients	**Clinical:** Absence of ADAMTS-4 in synovial fluid displayed predictive value of ACI.	[202]
BMP2, Collagen type II, FGFR3 and ACVRL1	Chondrocyte	Human	**Chondrogenesis:** BMP2, Collagen type II and FGFR3 downregulated with loss of chondrogenic potential whereas ACVRL1 upregulated.	[218]

2 Aims of the thesis

The aims of the work presented in this thesis were twofold: I) to study *in vitro* properties of mesenchymal stromal cells in search of the most suitable cell source for cartilage repair and II) to explore if the *in vitro* chondrogenic potency of cells used for ACI could predict clinical outcomes.

The specific goals related to each presented work were:

1. To characterise and compare the *in vitro* chondrogenic capacity of culture-expanded cells harvested from articular cartilage, synovial membrane, Hoffa's fat pad and umbilical cord matrix.

2. To characterise the secretory protein profiles of culture-expanded cells harvested from articular cartilage, synovial membrane, Hoffa's fat pad and umbilical cord matrix, and to compare the immunoregulatory potential of the different cell secretomes.

3. To investigate if the *in vitro* chondrogenic capacity of patient-matched chondrocytes from ACI procedures could predict clinical outcomes. Additionally, to search if molecular biomarkers of chondrogenesis from cells could predict clinical outcomes.

3 Methodological considerations

In this section, I will briefly discuss strengths and limitations of some of the methodology included in this thesis.

3.1 Biological material

Regional Ethical Committee (REK Nord) evaluated and approved the research project. In papers I and II, articular cartilage as well as pieces of synovium and infrapatellar fat pad were collected from patients undergoing total knee replacements. Umbilical cords were collected during normal (non-cesarean) child-deliveries. Although we collected cartilage tissue from a macroscopically healthy looking area of the knee joints, the tissue source should be regarded as diseased tissue due to the general joint disease prompting a knee joint replacement. However, it has been demonstrated that chondrocytes from OA cartilage possess similar properties compared to cells from healthy donors when used for tissue-engineered cartilage [219]. In paper III, we used leftover chondrocytes from patients undergoing ACI, diagnosed with focal cartilage lesions but not OA.

3.2 Cell isolation

We used a mixed enzymatic-explant method to isolate cells from the tissue biopsy as described in the materials and methods of each paper. This approach increases the number of viable cells quickly in the culture flask compared to explant culture [220]. The tissue biopsies were minced and digested with collagenase XI. For cartilage, we used 3 h of digestion followed by washing and plating partially digested tissue for outgrowth culture (Fig. 12). For Hoffa's fat pad, synovium membrane and umbilical cord matrix digestion period was only 1 h. We decided to minimise the exposure of collagenase to avoid any detrimental effects on quantity and quality of cells isolating from Hoffa's fat pad, synovium and umbilical cord [221]. However, we needed

at least 3 h digestion in collagenase to reach the recommended 90% digestion of cartilage biopsies.
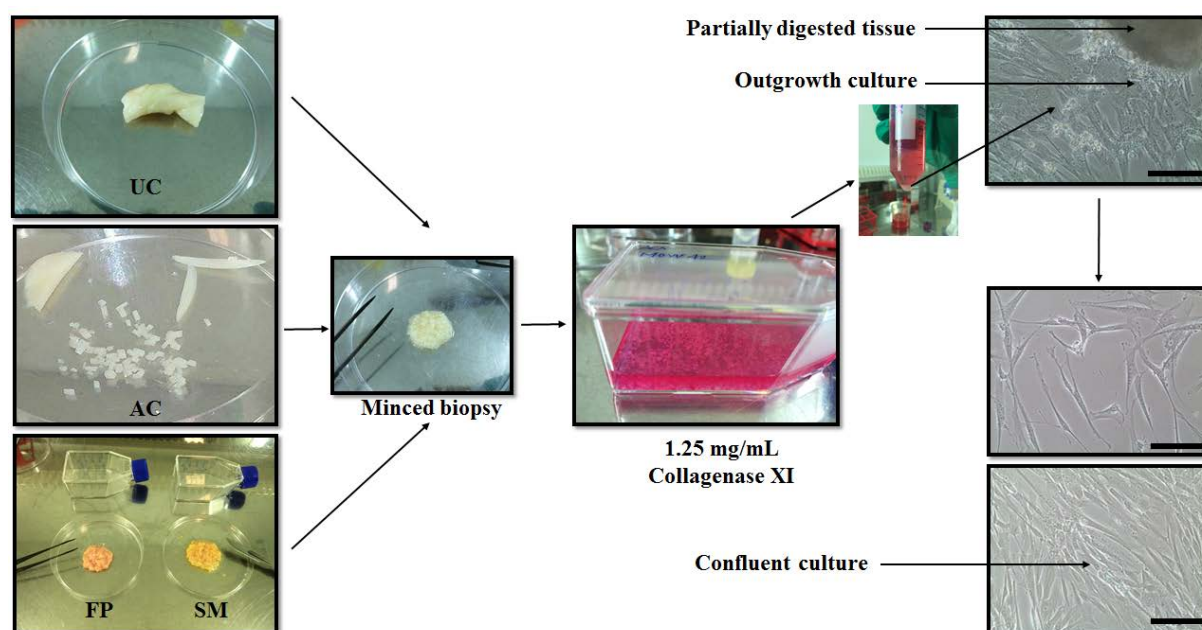
Figure 12: Schematic of cell isolation process. Scale bar: 5 µm.

3.2.1   3D cultures

The 3D culture was carried out using a scaffold-free pellet culture and hanging-drop culture approach in our laboratory. We used this approach to facilitate TEC formation by aggregation and cell-cell interactions, providing biomimetic microenvironment compared to scaffold-based approaches such as alginate, agarose, and collagen. The scaffold-free approach also avoids complicated processing steps, and interferences that may arise between degradation of biomaterials and cells own matrix formation [136, 138]. We used both methods in paper III but used only pellet culture system in paper I. During our pilot experiments we found that the pellet culture was more easily reproducible and less time consuming than the hanging-drop method (Fig. 13). Spheroids prepared by hanging-drops were unsuccessful in a higher number of donors. The pellet culture is the most commonly used 3D culture method since the 1980s to induce chondrogenesis [120]. We used quite some efforts at the beginning to find the optimal combination of growth factors to induced chondrogenesis with each cell source. (Fig. 14).

Pellet culture (Conical well)   Hanging-drop culture
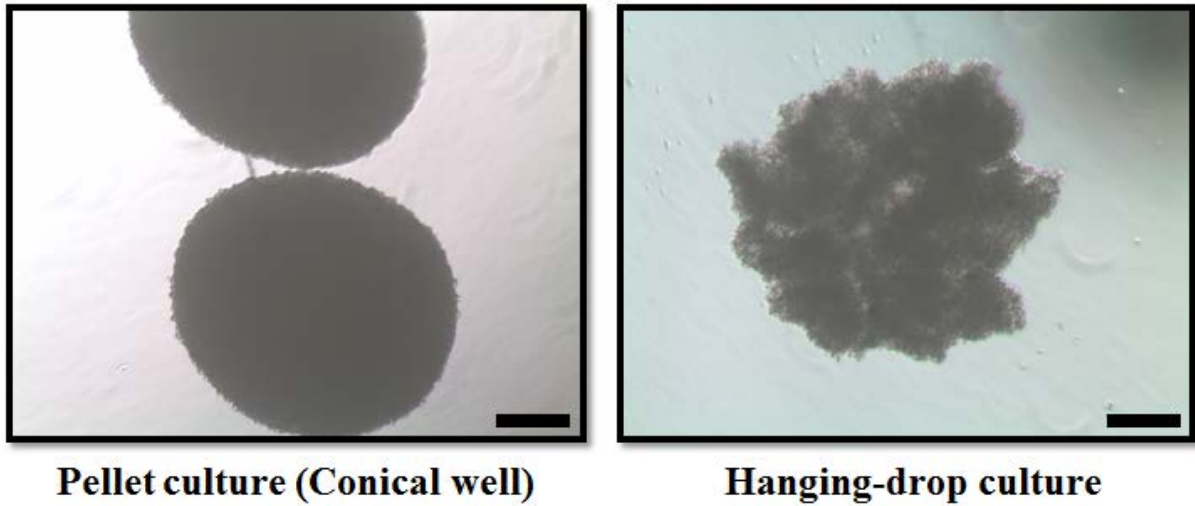
Figure 13: Comparison of spheroids prepared by pellet culture and hanging-drop culture from same chondrocyte donor. Scale bar: 200 μm.

TGF-β1   TGF-β1 and BMP-2
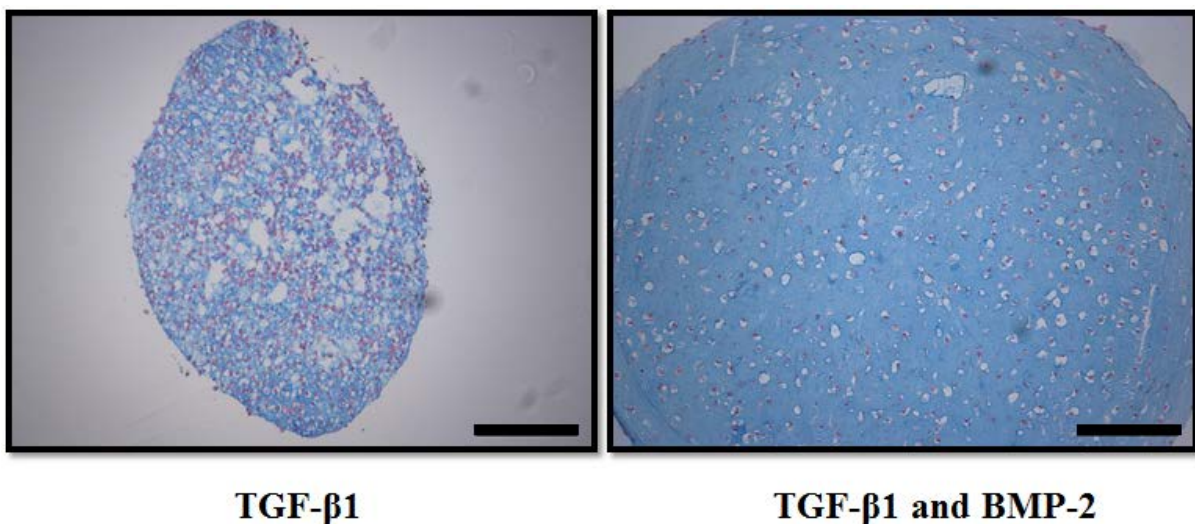
Figure 14: Metachromatic staining (Alcian blue) of spheroids in the presence of only TGF-β1 and combination of TGF-β1 and BMP-2. Scale bar: 200 μm.

3.2.2 Serum

We used the FBS-supplemented medium for expansion of cells in monolayers. The use of FBS is associated with the possible risk of contamination and may vary from batch to batch production. On the other hand, the use of human serum or platelet products avoid the risks associated with animal serum [222]. In addition, human serum or platelet products have been

demonstrated to enhance proliferation of cultured cells compared to FBS. However, expansion of cells monolayer in the presence of FBS and human serum has shown no differences when comparing differentiation potential [222, 223]. In 3D culture, we used a serum-free medium to induce chondrogenesis. To compensate for serum deficiency during chondrogenesis, we enriched the medium with ITS and dexamethasone. These anabolic factors have been shown to successfully promote chondrogenesis in the absence of serum [112, 144]. Additionally, in paper II, proteomics and multiplex protein assays were performed with the same serum-free conditioned medium (CM), which allowed us to make direct comparisons of results. However, functional assays with immune cells were done with serum-supplemented CM, as serum deprivation has been shown to affect proliferation and induce apoptosis in lymphocytes and macrophages, respectively [224, 225]. Short periods of serum deprivation have not affected the cell viability in previous studies [226]. We have analysed in parallel the expression of TNF-α, IFN-γ, IL-6 and IL-12 in both serum-containing and serum-free CM from all four cell types and only the expression of IL-6 was considerably changed in the presence of serum (Fig. 8 of paper II). Although we expect only minor phenotypic changes in cells associated with serum presence, alterations in the expression of some bioactive molecules could occur and should be taken into consideration.

3.2.3 Glucose

In the paper I and III, basal DMEM medium containing high glucose was used for chondrogenesis. During pilot experiments, we found spheroids prepared in high glucose chondrogenic medium had improved spheroid morphology and enhanced matrix production compared to low glucose chondrogenic medium (Fig. 15). Similar findings were observed by Mackay *et al.* [153].
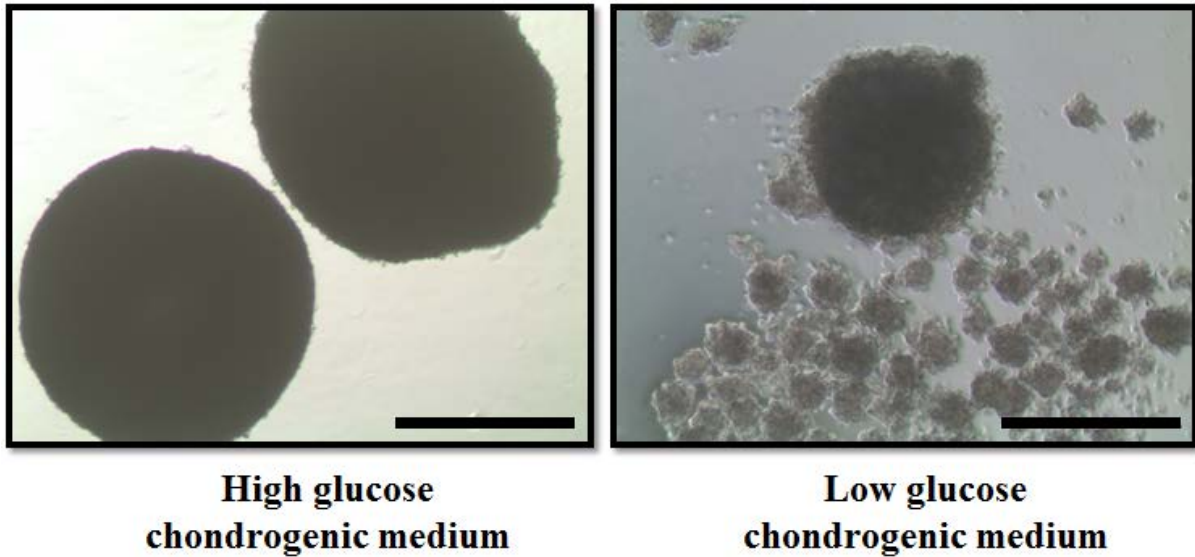
Figure 15: Comparison of spheroids from SMSCs from the same donor prepared in high glucose chondrogenic medium and low glucose chondrogenic medium. Scale bar: 500 µm.

3.3 Flow cytometry

Flow cytometry, a laser-based fluidics platform, is based on the principle of light scattering from individual particles in the liquid suspension. After hydrodynamic focusing of single cell suspension into a stream of fluid, each particle or cell in the suspension passes through the beam of a laser. The emitted light in the forward direction from the passing cells provides information about the size of the cells whereas the side scattered light gives information about complexity or granularity. The use of fluorescent conjugated-antibodies or dyes makes it a powerful tool, which provides a quantitative measure of the cell proliferation, enzyme activity, drug uptake, intracellular proteins, and surface proteins [227]. We employed this technique to analyse the surface marker expression of the protein of interests in the studied cell types. Flow cytometry is sophisticated and required multiple controls for analysis [228]. To determine cellular autofluorescence and set negative gates in the analysed cell population, we used antibody-free controls. On the other hand, isotype controls were used to check non-specific binding of antibodies. However, the use of isotype control is controversial when it uses as gating control. This is because the isotype control does not contain similar fluorescence-to-protein ratio as the

antibody. Fluorescence minus one (FMO) fluorophore control is considered as the suitable approach for multicolour complex immunophenotyping. In a multicolour assay, it shows how fluorophore spread over other channels while comparing with others, therefore allows setting right gate accordingly [228, 229]. Another approach involves compensating spectral overlap in multicolour flow cytometry by counting 5000 events in both positive and negative cell population. We used this approach to avoid fluorescence spill over in multicolour flow cytometry in the paper I.

3.4 Real-time quantitative polymerase chain reaction

Quantitative PCR (qPCR) is one of the widely used tools for quantification of RNA in a biological sample due to its robustness and specificity. RNA extraction is the first step of the process, which was important for us due to different sample types. In the paper I, we extracted RNA from both monolayer culture and tissue-engineered cartilage, whereas we only extracted RNA from monolayer culture in paper III. Extracting RNA from monolayer is straightforward and does not involve additional step. It is more challenging to extract RNA from spheroids. We collected few spheroids in an Eppendorf tube containing a stainless steel ball (5 mm) and disrupted the constructs in a TissueLyser for 2.5 min at 25 Hz. We used QIAshredder columns to homogenise and clean the RNA extract from spheroids [230]. To avoid DNA contamination, we performed on-column DNase digestion of the samples. A dye is incorporated in the qPCR reaction that results in the emission of fluorescence as cDNA doubled during each cycle. Therefore, fluorescence increase exponentially, which is detected by qPCR platform and the reaction can be monitored in real-time. The qPCR reaction slows down as reagents get limited followed by entering the plateau phase.

The amount of cDNA produced during reverse transcription reflects the quality of starting RNA material [231]. Contaminants in the sample will also be exponentially amplified during the qPCR reaction. In addition, using too much RNA input in reverse transcription phase often left

out RNA that are not being reverse transcribed. These can be controlled by a series of dilution of RNA [231]. Therefore, we performed validation experiments with a five-step 1:10 dilution series to avoid such contaminations. Each dilution contained cDNA reverse transcribed in the presence of probes with both high expressing genes and low expressing genes. Results from validation experiments confirmed the efficiency of dilution curves within 90-110 % as recommended when excluding the undiluted samples. In addition, interpretation of qPCR results is based on the normalisation of expression of internal reference known as a reference gene. Therefore, it is important to include a reference gene that has a constant expression in all the studied samples [232]. We performed validation experiments with potential reference genes to find a suitable one with constant expression in our study. In the paper I, we found that YWHAZ was the stable reference gene while studying cartilage signature genes expression in 3D culture. For the monolayer cultures studied in Paper III, RPL13A proved to be the more stable reference gene.
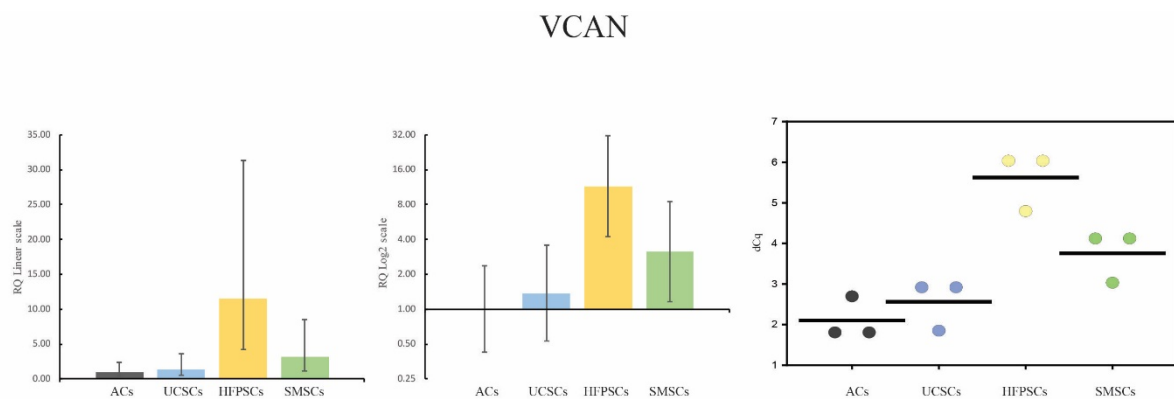
VCAN

Figure 16: Comparison of VCAN expression using a linear scale, log scale and dCq in 3D culture.

Fold change of expression of target gene compared to control gene is a commonly used method to present the qPCR data. In the paper I, we used fold change to present our qPCR data. It was calculated from 2^{ddCq} formula in which ddCq= Mean (dCq treated) – Mean (dCq control). However, the major drawback of using fold change as it shows upregulation nicely with positive

value but downregulation restricts between 1 and 0, which is unequally weighted the visualisation of results on a linear scale (Fig. 16). Therefore, we transformed y-axis to log scale in the paper I, which eliminates the problem of the unequal weight of up-and downregulation (Fig. 16). The use of dCq to represent qPCR data eases the interpretation of result and avoids the complications of using fold change and log scale [233, 234]. It is calculated by subtracting the Cq value of gene of interest from the Cq value of reference gene (dCq= Cq reference gene – Cq gene of interest). This method is straightforward and represents result with higher values as a higher expression of the gene of interest and vice versa (Fig. 16). We employed this technique to interpret qPCR data in paper II.

3.5 Histological evaluation of spheroids

We performed metachromatic staining of proteoglycan contents of spheroids to evaluate the chondrogenic potential of different cell types. The Bern score, a visual histological grading system, was used for semi-quantitative assessment of cartilage tissue constructs [235]. This method uses three categories that include the intensity of proteoglycans staining, cell and matrix density and morphology of cells in the tissue construct. One of the major limitations of this grading system is not including collagen content for evaluation of chondrogenesis. Therefore, the quality of tissue-engineered cartilage often misinterpreted while only using this scale. The inclusion of collagen content could solve this discrepancy. This discrepancy has been demonstrated in a study by our group in which we included the score for collagen contents using second harmonic generation microscopy [236]. We also proposed a further modification of this grading based on specific collagen type I and type II contents in engineered cartilage.

In the paper I, we performed immunohistochemistry to compare expression and distribution of collagen type I and II in spheroids. We used formalin fixed 4 µm sections of spheroids that were prepared through series of ethanol washing, antigen retrieval, blocking of unspecific binding, peroxidase quenching before incubating with primary antibody. Antigen retrieval was

carried out using the enzyme-based method, which provided better antigen recovery than heat-induced antigen recovery. Using later method, the sections were destroyed and resulted in poor antigen recovery. We performed validation experiments with cartilage and tendon as positive and negative control respectively, to determine the suitable dilution factor for antibodies. Validation studies confirmed that a dilution of 1:500 of collagen type I and 1:100 of collagen type II antibodies was suitable for our tissue-engineered cartilage.

3.6 Proteomics

Liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) has emerged as a comprehensive tool for characterisation of protein profiles in a high-throughput manner. Quantitative mass spectrometry can be divided into two broad categories: label-free quantification methods and label-based quantification method [237]. Different label-based methods including metabolic labelling, chemical labelling, and enzymatic labelling have been developed to improve the quantification of proteins [238]. We performed 'shotgun' proteomics in which the whole proteome was digested without prior separation of proteins. Protein separation using gel electrophoresis is often associated with restricted sample throughput, and limited quantifiable proteins in a gel [237, 239].

In the paper III, we used 6-plex tandem mass tag (TMT) based chemical labelling technique to look for differentially expressed proteins between samples with extreme scores. Unlike metabolic labelling (SILAC), TMT allows multiplexing of several samples in a single LC-MS/MS run (Fig. 17). It has also been reported to provide more precise and reproducible quantification of peptides compared to metabolic labelling [238]. Each TMT tag in 6-plex contains a specific reporter ion of *m/z* 126, 127, 128, 129, 130 and 131, which can tag six different samples [240]. The relative intensities of each reporter ion are used to generate quantitative information of labelled peptides among different samples. In our studied materials, six samples with highest and lowest scores (3 in each group) were tagged with six different

reporter ions to derive quantitative information of differentially expressed proteins between two groups (Fig. 17).
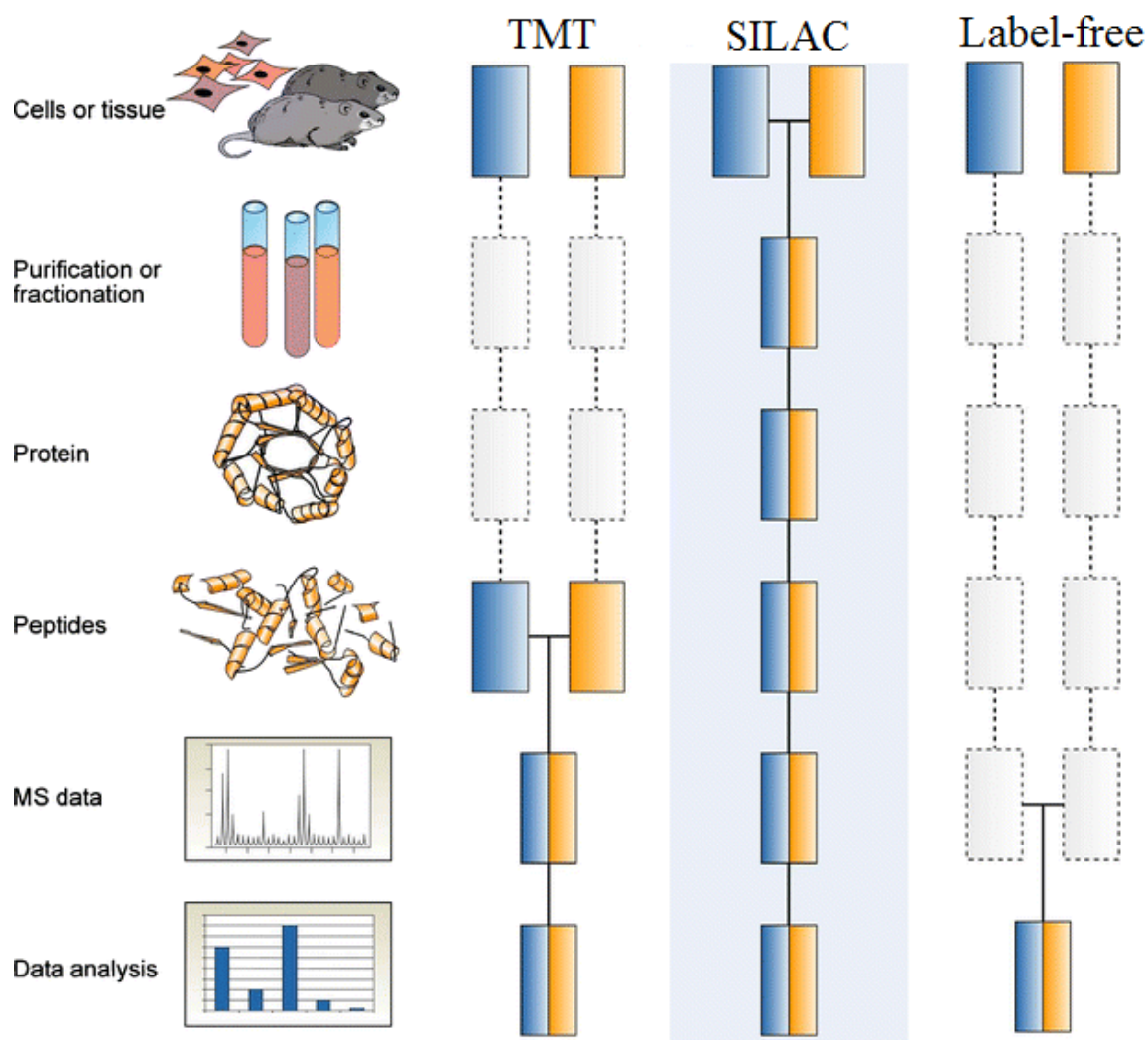
Figure 17: Schematic of different label-based and label-free protein quantification methods (Adapted with permission from [241]).

Although label-based quantification provides data reproducibility, one of the major limitations of this technique is a restricted number of samples for analysis. It also requires complex sample preparation steps. Label-free protein quantification, on the other hand, is not restricted to a number of samples to be analysed. It is cost-efficient and involves simple sample preparation steps. In paper II, we analysed 16 samples using label-free protein quantification which allowed comparison of protein expression among four different sample types. TMT analysis does not

offer such flexibility of data analysis in a large number of samples. In addition, the label-free technique has been reported to provide broader proteome coverage and increased number of identified proteins [237]. However, each sample needs to be run separately in label-free quantification (Fig. 17). This data-dependent acquisition has been reported to associate with low reproducibility and to bias to picking the strongest signal for fragmentation, which could affect the analysis of low-abundance peptides. This approach could overcome by implementing data-independent acquisition in which all peptides are fragmented and not limited to the predefined peptides of interest [242]. In this thesis, our collaborator at the proteomics platform performed part of experimental procedures and proteomics data acquisition.

3.7 Multiplex protein array

We performed multiplex protein array in paper III to complement our findings from LC-MS/MS. Some relevant cytokines, enzymes and growth factors may be expressed at a very low concentration in culture supernatants, which might fall below the detection limit by mass spectrometric analysis [243]. This antibody-based detection technique allows quantitative measurement of pre-determined proteins (up to 100) simultaneously from a small volume of sample. The antibody against the protein of interest is incorporated with beads with defined colour intensities, which binds with the respective protein in the sample and finally, detected by the fluorescent-conjugated detector antibody. One laser detects the protein of interest (colour of beads) and the second laser determines the fluorescence intensity, which is proportional to the bound protein of interest. It has been reported to have a similar sensibility and reproducibility like ELISA, but unlike ELISA, it is simple and less time consuming [244]. However, the quality of assays may vary from different suppliers [245]. Comparative analysis of our findings from LC-MS/MS and multiplex protein array confirmed the reproducibility of results obtained from the later method.

3.8 ELISA

ELISA is the "gold standard" for quantitative measurement of proteins. However, it allows measuring only one protein at the time. It has the similar principle like multiplex protein array but differs in the detection system. It uses streptavidin-HRP-conjugate that binds to detection antibody and results in colourimetric detection of a protein of interest. We used this technique to check the concentration of targeted cytokine of interest, which was not included in the multiplex protein array. We did not encounter any problem while measuring the concentration of cytokines in our studied samples. However, we ran a few samples several times, such as TGF-β1, IL-6, and TNF-α to adjust the dilution factor. For TGF-β1 analysis, we diluted our samples as supplier's instruction but failed to detect TGF-β1 in the supernatants of all other cell types except UCSCs. The concentration was below the detection limit, and we performed the analysis without any dilution and detected TGF-β1 in all cell types.

3.9 Functional assay of immune cells

We performed functional assays of immune cells in paper II. To perform these assays, we isolated PBMCs from whole blood of healthy donors.

3.9.1 Lymphocytes proliferation assay
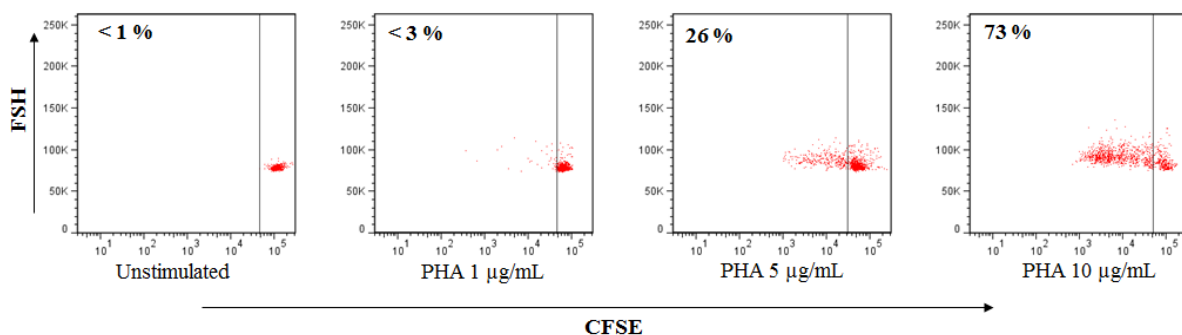
Figure 18: Activation of lymphocytes with different concentration of PHA.

We analysed lymphocytes proliferation using carboxyfluorescein succinimidyl ester (CFSE) dye dilution assay in paper II. CFSE, a membrane-permeable dye, covalently binds with

intracellular molecules. During cell division, CFSE also divides equally between daughter cells. A flow cytometer was used to analyse the CFSE labelled cells to measure the proliferation of lymphocytes. We used phytohemagglutinin (PHA), a widely used lymphocytic mitogen to activate lymphocytes. In our previous studies, we used 1 µg/mL of PHA to activate lymphocytes [246]. Surprisingly, we did not manage to achieve any activation using this concentration with a new batch of PHA. After a few pilot experiments with the new batch of PHA, we found that 10 µg/mL of PHA was the necessary concentration to activate lymphocytes (Fig. 18). At this concentration, no cell death was observed. In our study, we did not perform CD3+/CD4+ marker characterisation of T-lymphocytes. However, most gated cells correspond to CD4+ and CD8+ cells as demonstrated by us in earlier studies [246]. Both characterised, and non-characterised lymphocytes had similar proliferation pattern, and presence of B cells (<10 %) did not interfere with the proliferation assay.

3.9.2 Macrophage polarisation assay

In the paper II, we checked for macrophage activation to investigate the immunomodulatory effect of culture supernatants from different cell types. We isolated CD14+ monocytes from PBMCs using magnetic-activated cell sorting. We incubated CD14+ monocytes with M-CSF for 6 d to induce macrophage differentiation (M0) (Fig. 19). M0 macrophages were then polarised into M1 and M2 phenotypes using LPS and IFN-γ and dexamethasone, respectively [247]. The detailed experimental procedure is written in paper II. We used dexamethasone induced M2 polarisation as a control to compare surface expression of a few costimulatory molecules with M1 polarised macrophages. We found discrepancies while going through literature regarding activation and phenotype changes of macrophages. To address a few of the issues, we performed several pilot experiments to decide on surface markers to include in the paper II. During our pilot experiments, we observed differential expression of co-stimulatory molecules among different cell types and stimulants used to induce polarisation. IL-10 usually studied as a marker for M2 polarised macrophages [248]. In our pilot study, we found that LPS

and IFN-γ stimulation increased the production of IL-10 compared to dexamethasone or TGF-β and IL-4 stimulation. Previous studies also have shown these type of discrepancies [249-251]. IL-10 has also been reported as an irrelevant marker for M2 polarised macrophages [247]. After pilot experiments, we decided not to include this as a marker for M2 polarised macrophages.
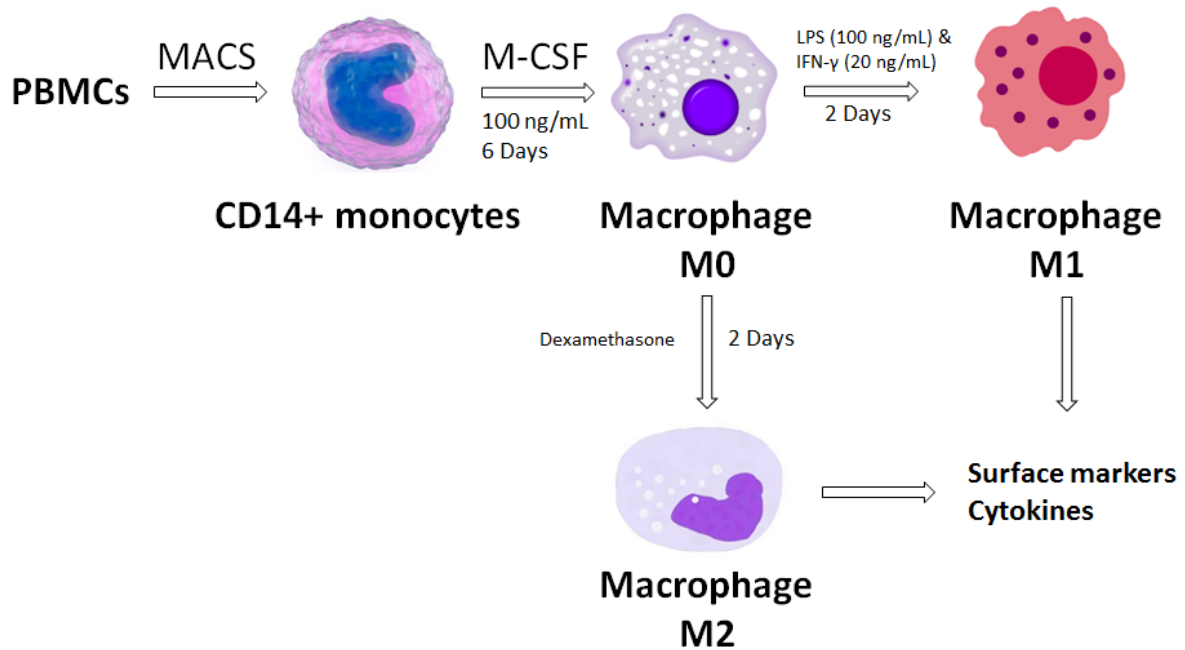
Figure 19: Illustration of macrophage polarisation assay.

In addition, CD206 is included as a marker for M2 polarised macrophages. We found CD206 expression did not discriminate between M1 and M2 polarised macrophages in our pilot experiments using LPS and IFN-γ and dexamethasone, respectively (Fig. 20). Similar findings have been reported in an earlier study using an IL-4 stimulant for M2 polarised macrophages [248]. On the other hand, we found that CD163 was a suitable marker to discriminate between M1 and M2 polarised macrophages in our studied populations (Fig. 20), which has also been demonstrated in a previous study [247]. In our pilot experiments, CD206 expression was found to be a suitable marker to discriminate between M1 and M2 polarised macrophages when stimulated with TGF-β1 and IL-4 (Fig. 20). Since we used dexamethasone to induce M2 polarisation of macrophages, we included CD163 as a marker for M2 polarisation.
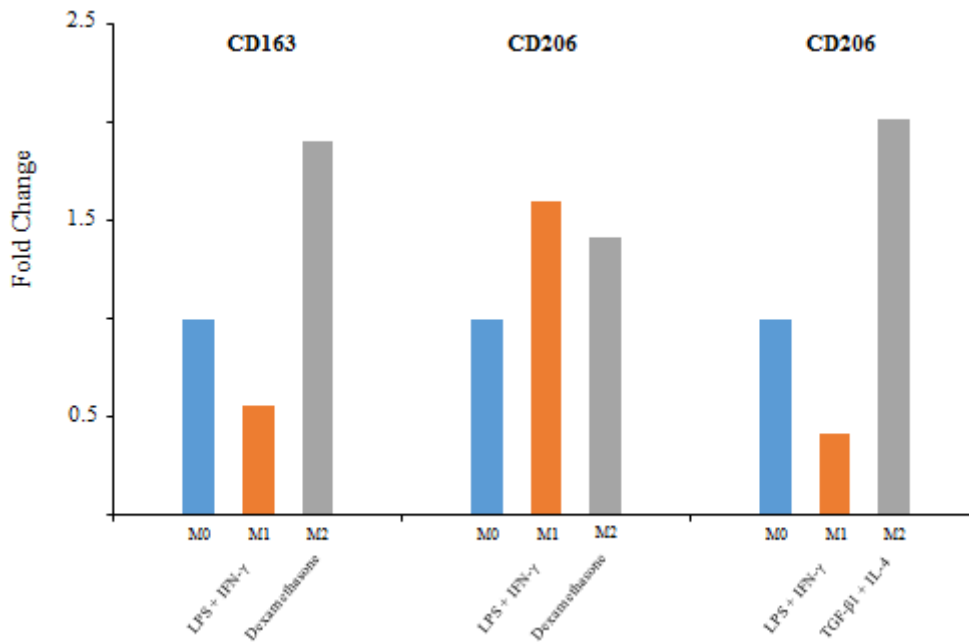
Figure 20: Surface marker expression of CD163 and CD206 in non-polarised and polarised macrophages in the presence of different stimulants.

4 Summary of results

4.1 Paper I

In this study, we isolated and characterised mesenchymal stromal cells from Hoffa's fat pad, synovial membrane, and umbilical cord with the aim of comparing the *in vitro* chondrogenic capacity of culture-expanded cells. We also isolated chondrocytes from cartilage to use them as the gold standard. Cells from all sources maintained fibroblast-like and plastic adherent characteristics. UCSCs had a slow growth rate after initial plating compared to HFPSCs and SMSCs. However, the proliferation rate of UCSCs increased after first sub-culturing with homogeneous morphology. Unlike MSCs, chondrocytes had poor proliferation rate. All MSCs, including chondrocytes, were positive for classical surface markers such as CD73, CD90 and CD105, but did not express any haematopoietic, macrophage or endothelial markers. MSCs were also checked for expression of previously proposed markers of chondrogenesis including CD44, CD146, CD166, and CD271. None of the MSCs or ACs was positive for CD106 and CD271. We did not observe any differences in surface expression of CD44 or CD166 and only

a subtle change in expression of CD146. Additionally, we found no correlation between any surface markers expression and chondrogenesis.

When studying *in vitro* chondrogenic potential by metachromatic staining of proteoglycan, we found that ACs underwent chondrogenesis in all six studied growth factor combinations. UCSCs, on the other hand, displayed poor chondrogenesis in all studied combinations. HFPSCs and SMSCs displayed the best chondrogenesis in the presence of TGF-β3 and BMP-2 combination and TGF-β1 and BMP-2 combination, respectively. These findings were also reproducible at collagen level. When investigating cartilage signature genes expression in spheroids, we found significant downregulation of COL2A1, ACAN and SOX-9 in UCSCs compared to ACs. The relative expression of VCAN was significantly upregulated in spheroids from HFPSCs. To induce chondrogenesis in UCSCs, we performed co-culture of spheroids with cartilage pieces or cultured synoviocytes. However, we did not manage to induce proper chondrogenesis of UCSCs in these conditions.

To investigate whether UCSCs remained undifferentiated in 3D culture, we studied stemness-related transcriptional factors (SRTF). The results confirmed that UCSCs did not express any SRTF genes in 3D spheroids whereas they maintained the expression of these genes in monolayer culture. This supported that UCSCs underwent transition towards a differentiated state, which certainly was not cartilage type. Importantly, after publication, we investigated receptors from TGF-β superfamily in all cell types during the expansion phase. We found both TGFβRII and TGFβRIII were significantly downregulated in UCSCs compared to other cell types (Fig. 21; unpublished data).
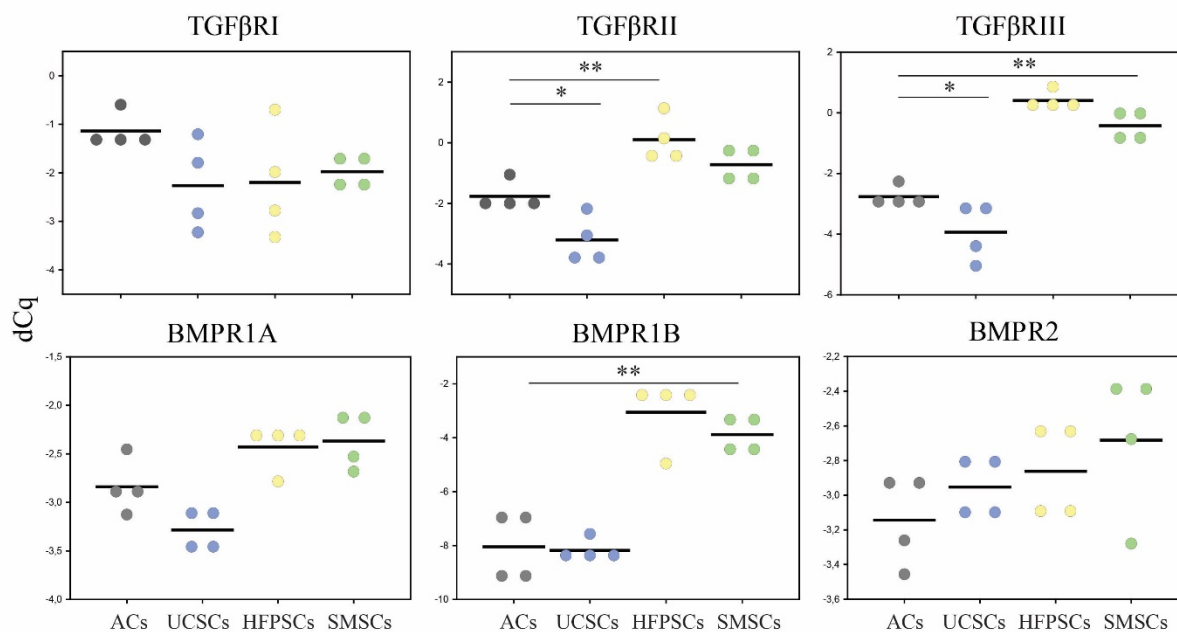
*Figure 21: Relative expression of receptors from TGF-β superfamily in different cell types before inducing chondrogenesis. Level of significance, * and ** with p-value < 0.05 and <0.005, respectively.*

4.2 Paper II

In this paper, we studied and compared the secretory profiles of ACs, HFPSCs, SMSCs, and UCSCs from four unrelated donors using label-free LC-MS/MS. While comparing the protein profiles of each donor, the samples distributed in two major clustering; one cluster corresponding to the four donors of UCSCs and other cluster contained rest of the donors from adult cell sources. Qualitative comparisons of identified proteins showed that ACs (709) secreted more proteins in the culture medium compared to HFPSCs (641), SMSCs (567) and UCSCs (653). Among these identified proteins, 472 proteins were present in the supernatants of all cell types. UCSCs had more uniquely expressed proteins (50) than other cell types. In quantitative analyses, we found that cell signalling proteins such as TGF-β1, PDGFD, and MCP-1 were significantly upregulated in UCSCs, whereas catabolic proteins such as MMPs, serpins, and complement factors were downregulated compared to cells from the adult origin. Determination of growth factors and MMPs using multiplex protein arrays also confirmed the

findings from LC-MS/MS. When analysing results from multiplex protein arrays, we observed significant downregulation of MMP3 and MMP7 production by UCSCs compared to ACs. The concentration of TGF-β1 and PGE2, on the other hand, was significantly elevated in the supernatants of UCSCs compared to HFPSCs.

To elucidate the immunosuppressive effect of supernatants from the different cell sources, we performed lymphocytes proliferation assays. We observed that UCSCs significantly blocked the proliferation of PHA-activated lymphocytes compared to other cell types. Additionally, the production of inflammatory cytokines TNF-α and IFN-γ by lymphocytes was only suppressed by UCSC supernatants. When comparing results from M1 polarised macrophages, we found that surface expression of different co-stimulatory molecules varied distinctly upon incubation of M1 polarised macrophages with supernatants from different cell types. Supernatants from all stromal cell types reduced surface expression of HLA-DR on activated macrophages. When comparing inflammatory cytokines productions by M1 polarised macrophages, we found that supernatants from all cell types suppressed the production of TNF-α, IL-6, and IL-12. Importantly, among all cell types, only UCSCs significantly reduced the production of IL-6 and IL-12 by M1 polarised macrophages.

4.3 Paper III

In the paper III, we investigated *in vitro* chondrogenic potential of surplus chondrocytes from 14 ACI procedures with the aim of establishing a functional bioassay to predict clinical outcomes. Chondrocytes from different donors displayed distinct chondrogenic potential, which allowed categorisation of donors into two groups using Bern score. Donors in "Group A" and "Group B" represented spheroids with good and bad cartilage-like characteristics, respectively. Lysholm score 65 at two-year follow-up was used as the cut-off value to group patients into clinical success and failure. When comparing Lysholm scores at two-year follow-up after ACI surgery with donor-matched *in vitro* chondrogenic capacity of chondrocytes, we

could not find a significant correlation between *in vitro* chondrogenic potentials and clinical outcomes. We also evaluated a number of previously reported markers in our studied material to predict the clinical outcomes and chondrogenic potentials. We observed significant upregulation of CD166 surface expression in clinical success group compared to failure group. When investigating surface expression in chondrogenic groups, the CD106 marker was significantly high in the chondrogenic group B.

In gene expression analysis, we found significant upregulation of ITGA1 (CD49a) and ITGB1 (CD29) in the good chondrogenic group, whereas TGFβRIII was significantly downregulated in this group. Relative expression of COMP was significantly higher in the clinical failure group compared to clinical success group. Additionally, we performed an unbiased approach to look for predictive biomarkers in both chondrogenic and clinical groups using quantitative 6-plex TMT proteomics. We identified 2113 and 2034 proteins from chondrocytes extracts in the chondrogenic and clinical groups, respectively. Seven proteins were significantly downregulated (FDR = 0.05) in the bad chondrogenic group B compared to group A. Importantly, prolyl-4-hydroxylase 1 (P4HA1), an enzyme that plays a pivotal role in triple helix formation of collagens, was the only differentially expressed protein in the chondrogenic groups when FDR set at 0.01. This finding was also validated using western blots. We did not observe any differentially expressed proteins when comparing clinical groups.

5   General discussion

Articular cartilage lesions in synovial joints result in pain and discomfort, which may promote degeneration of cartilage and prolong sufferings due to the poor healing capacity of this specialised tissue. Several treatment strategies such as microfracture, mosaicplasty, ACI, transplantation of chondrocytes or stem cells with or without scaffolds, have been used to treat localised cartilage defects [59]. The ultimate goal of these methods is to develop durable articular cartilage. However, in most instances, the repair tissue results in fibrocartilage

formation or a mix of hyaline and fibrocartilage development [35, 49, 57, 252]. None of these treatment procedures has been proven superior to each other [253]. Additionally, these treatment procedures are not useful in advanced osteoarthritis [38, 43, 52, 53]. The management of cartilage lesions represents a weighty clinical challenge worldwide for younger patients who may require joint replacement procedure. This is because of the increasing prevalence of this debilitating disease due to prolongation of life expectancy and the absence of effective treatment strategies for articular cartilage regeneration. Therefore, it is of ample importance to developing new treatment procedures that can circumvent the problems and limitations associated with currently used methods. In this thesis, we tried to enrich our knowledge around cartilage neotissue formation by investigating different cell sources, gather new knowledge about cell-based biomarkers for chondrogenesis and clinical outcomes, and to explore the anti-inflammatory and immunomodulatory potential of supernatants of MSCs obtained from different tissues. The role of transplanted MSCs as "drug stores" and signalling agents that promote healing by modulating the microenvironment rather than forming new tissue is gaining momentum in recent years.

Articular cartilage repair using culture-expanded autologous chondrocytes has been adapted in the clinics during the last decades since its first intervention in the 90s [34, 44, 45]. One of the major limitations of this technique is that culture-expanded cells become dedifferentiated and loss of phenotypic traits [48, 119]. To ameliorate this characteristic of chondrocytes, MSCs have been introduced as an alternative cell source due to their cartilage tissue regeneration potential [126, 254]. Still, there is no consensus on the optimal cell source for cartilage repair. Stromal cells bone marrow and adipose tissue, in addition to chondrocytes, are the most widely used cell sources in both clinical and preclinical settings. Importantly, the previous study showed no differences in clinical outcomes in patients treated with ACs and BMSCs [190]. Another study comparing ACs and SMSCs demonstrated that SMSCs improved clinical

outcomes in treated patients compared to ACs [77]. However, autologous MSCs have several limitations. One of the difficulties involves donor site morbidity and limited availability of donor tissue from some patients, for example, autologous bone marrow-MSCs from myelofibrosis patients. It has also been demonstrated that autologous MSCs harvested from elderly donors have decreased regenerative potential and biological activities [78, 79, 255, 256]. In addition, systemic diseases, such as rheumatoid arthritis, diabetes, and systemic lupus erythematosus, have been shown to alter the intrinsic functional properties of autologous MSCs [257-259]. UCSCs overcome the above-mentioned constraints. This primitive cell type can be stored and readily available for use in the clinics. In addition, UCSCs have also been demonstrated to exert immunosuppressive effects [260, 261]. These features make this cell source an attractive candidate for allogeneic transplantation. However, it has been studied in the context of cartilage tissue engineering with divergent outcomes [84, 87, 93]. In this study, we investigated *in vitro* chondrogenic potential of MSCs harvested from the umbilical cord and compared the outcomes with other cells harvested from the adult knee joint.

We used histological scoring (Bern score) of Alcian blue stained spheroids to evaluate the chondrogenic potential of each cell types. This semi-quantitative scoring method has been routinely used in the field of cartilage tissue engineering and validated for GAG measurements in pellet cultures [235, 236]. We included six most commonly featured combinations of growth factors to investigate the chondrogenic potential. Each cell type displayed distinct chondrogenesis in the presence of different growth factors. UCSCs showed poor chondrogenic potential in all six combinations of growth factors. Only a few comparative studies reported similar findings and argued about their differentiation potential towards chondrogenic lineages [93, 95]. We performed cartilage signature gene expression analysis, GAG analysis, TEM and Collagen type I and II immunostaining to validate this finding and compared with ACs, which is considered the "gold standard." All these analyses revealed the poor chondrogenic capacity

of UCSCs. On the other hand, HFPSCs and SMSCs underwent better chondrogenesis in the presence of TGF-β3 and TGF-β1, respectively, in combination with BMP-2 and dexamethasone. Other studies have also been reported similar findings [132, 262]. We observed significantly higher expression of COL10A and VCAN in spheroids from HFPSCs. These markers were reported to associate with hypertrophy and bone formation [263, 264]. The use of PTHrP in the chondrogenic medium was reported to inhibit these characteristics [151].

It has been reported that hypoxia maintained undifferentiated phenotype of UCSCs [265]. To answer this question, we investigated the expression of SRTF genes including OCT4A, NANOG, and SOX2 in our studied material. The results from this analysis indicated that hypoxia was not involved in restraining chondrogenic potential of UCSCs. Low receptor expression has been demonstrated to affect the chondrogenic potential of MSCs using BMP-2 stimulation [266]. However, we did not see any differences in UCSCs while comparing BMP-2 and BMP-7 stimulation. Importantly, when we looked at gene expression of receptors from TGF-β superfamily, we observed both TGFβRII and TGFβRIII were significantly downregulated in UCSCs. TGFβRII binds with ligands and activates TGFβRI, which mediates downstream SMAD signalling and chondrogenesis [145, 146]. These results indicate that UCSCs are not a suitable source for cartilage neotissue formation. It could be due to their low expression of TGF-β receptor type II. Therefore, the use of TGF-β based stimulation for chondrogenesis of these cell types might become redundant. A complementary study investigating specific receptor type and their signalling pathway could provide a mechanistic insight regarding the poor chondrogenesis of UCSCs.

The mechanisms used by MSCs in tissue regeneration are not yet well established. Earlier it was believed that MSCs engraft to the injured tissue and promote tissue regeneration [159]. Newer studies, on the other hand, have demonstrated paracrine signalling and secretory bioactive molecules that promote tissue repair rather than direct cell engraftment and

differentiation [163, 164, 267]. No human study has investigated quality and fate of implanted cells due to ethical constraints. A recent clinical trial demonstrated that allogeneic BMSCs orchestrated cartilage tissue repair through trophic mediation rather than differentiating into the new host tissue [167]. Based on the new way of understanding the mechanism of MSCs, we investigated secreted trophic factors, and paracrine signalling of MSCs harvested from HFPSCs, SMSCs, and UCSCs, and we compared these factors with culture-expanded chondrocytes.

Results from both LC-MS/MS and multiplex protein array indicated that UCSCs constitutively release higher levels of soluble bioactive molecules promoting anti-inflammatory and anabolic activities compared to mesenchymal cells harvested from adult tissues. These molecules include TGF-β1, PDGFD, and PGE2 that were detected at high concentration in the supernatants of UCSCs; whereas MMPs, IL-17, and complement factors were detected at very low concentration. TGF-β1 is a master regulator of chondrogenesis and has been shown to ameliorate OA pathogenesis [142, 268]. Like TGF-β1, it has been shown that PGE2 secreted from MSCs, mediated inhibition of arthritic inflammation in an IL-6 dependent manner [269]. On the other hand, MMPs are key catabolic factors that are involved in ECM homeostasis and proteolytic processes [270]. Mechanistically, IL-17 has been reported to inhibit chondrogenesis and promote MMPs in chondrocytes [271, 272]. Despite the omnidirectional role of soluble bioactive molecules, our observations from the global expression of released factors in culture media indicated that UCSCs displayed a favourable secretory protein profile for tissue repair.

We also performed functional assays to investigate immunomodulatory effects of conditioned medium from different cell types on activated immune cells. We observed that supernatants from UCSCs had superior effect in blocking lymphocytes proliferation and the M1 polarisation of macrophages. Articular chondrocytes, bone marrow, and adipose tissue stromal cells are the most commonly used sources for cartilage repair [180]. Importantly, there is no consensus on

which cell source is beneficial in the context of modulating inflammation. We found only one clinical study that demonstrated the superior healing power of SMSCs over ACs [77]. In line with our study, MSCs from cords have been shown to exert superior immunomodulatory effects compared to BMSCs [273]. Similar findings have also been documented in animal models [174, 274]. However, MSCs have been shown to be differentially stimulated upon exposure to different stages of disease [170]. Therefore, the findings from this study need to be validated in suitable animal models. Collectively, these results displayed better secretome profiles of UCSCs compared to MSCs from the adult origin. Due to their intrinsic immunosuppressive functions, UCSCs might be used as an adjuvant therapy in combination with chondrocytes to promote cartilage regeneration as shown using allogeneic BMSCs [167, 194].

Biological repair of articular cartilage lesions using ACI can not only promote cartilage regeneration but also prevent secondary OA progression [275] and delay the need for total knee arthroplasty. Although successful clinical outcomes of ACI have been reported for up to 20 years [46, 276]; their long-term failure rate range between 20-40 % [47, 51]. This indicates that the ACI procedure is only beneficial to a sub-group of patients. Such findings have led researchers to look for predictive tools that can identify patients who are likely to obtain an optimal outcome from ACI procedure. Some risk factors such as age, sex, and previous surgery to the index knee have been identified to predict clinical outcome [198, 199]. Others have proposed putative biomarkers in synovial fluid or serum to predict the clinical outcome of ACI [202, 204]. Additionally, the quality of cells and their influence on cartilage repair have also been investigated to identify potential biomarkers [207, 218]. Nonetheless, direct comparison of *in vitro* chondrogenic potency of patient-matched cells with clinical outcomes has not been made hitherto. From a cohort of 14 ACI patients, we observed distinct chondrogenic abilities from chondrocytes of different donors. Based on this finding, we investigated whether *in vitro* chondrogenic potential can be used as a functional bioassay to predict the clinical outcomes of

ACI. However, we found no correlations between two-year clinical outcome after ACI surgery and *in vitro* chondrogenic abilities of culture-expanded chondrocytes. It is uncertain to what degree implanted cells participate in rebuilding damaged tissue. Results from a few preclinical studies demonstrated that the majority of cells in the repaired tissue are of unknown origin [160, 161]. In patients, on the other hand, it has been reported that the quality of the repaired tissue assessed by histology does not always correlate with the clinical outcomes [198, 277].

A number of biomarkers associated with cell quality and chondrogenic potential have been proposed. In our study, molecular biomarkers associated with chondrogenesis had no value as predictors of clinical outcomes and vice versa. Stenberg *et al*. reported similar findings when comparing clinical success and failure groups after ACI [215]. Collectively, these results indicate that markers associated with chondrogenic abilities have limited or no value in clinical settings. It is likely that chondrogenic ability or cell quality is one of many other factors that affect clinical outcomes. Probably, we need to use a sophisticated approach by combining biomarkers from patients' clinical parameters, synovial fluid, and cell quality to predict the clinical outcomes for ACI procedure. However, one of the limitations of this study was small sample size which is because of the discontinuation of ACI procedure at the University Hospital of Northern Norway. Since there was no correlation between *in vitro* chondrogenic potential and clinical outcomes, therefore, the use of an additional parameter, such as magnetic resonance observation of cartilage repair tissue (MOCART) score would be interesting. It would provide a probable link if there exists any between *in vitro* cell quality and the structural quality of the repaired tissue.

In addition, when we investigated protein expression in clinical success and failure groups. From the over 2100 proteins identified in cell extracts, not a single protein was differentially expressed at FDR = 0.05. In chondrogenic groups, on the other hand, we found just seven differentially expressed proteins including P4HA1, P4HA2, and P4HB at FDR = 0.05. These

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proteins are involved in biosynthesis and triple helix formation of collagen. In line with our study, upregulation of P4HB has been reported in chondrogenically differentiated human BMSCs [219]. However, only P4HA1 was significantly upregulated in the good chondrogenic group at FDR = 0.01, which was also validated by western blot. This finding indicates that P4HA1 could represent a true biomarker to distinguish chondrogenic population from the culture-expanded chondrocytes. This potential new tool could help to improve the scaffold-free neotissue approach known as chondrosphere® for cartilage repair.

6 Conclusion and implications

In the first paper, we demonstrated poor chondrogenic ability of human UCSCs compared to cells harvested from the adult joint. We performed different approaches and quantitative measurements; however, we were not able to induce chondrogenesis from UCSCs. On the other hand, ACs, HFPSCs, and SMSCs underwent good chondrogenesis by pellet cultures. UCSCs might not be a suitable source for generation of tissue-engineered cartilage. In an attempt to find an explanation, we investigated receptor expression of TGF-β receptor family. We observed significant downregulation of TGF-β receptor type II before inducing chondrogenesis in UCSCs. This suggests that use of TGF-β based stimulation in our studied materials could be redundant to induce chondrogenesis of UCSCs.

In the second paper, we demonstrated that UCSCs display more favourable secretory protein profiles compared to cells harvested from adult joints. Additionally, our data also showed superior immunosuppressive effects of UCSCs. Although these cells displayed poor cartilage tissue forming ability, findings from paper II suggest that considering the pro-anabolic and immunomodulatory potential of UCSCs, this cell source can still be considered as an adjuvant therapy in combination with chondrocytes to modulate tissue microenvironment.

In the third paper, we explored the *in vitro* chondrogenic capacity of patient-matched chondrocytes from ACI procedures as a functional bioassay to predict clinical outcomes.

However, we found no correlations between donor-matched *in vitro* chondrogenesis and short-term (2 years) clinical outcomes. We also argued on the limitations of using cell-based markers and the chondrogenic potential as predictors of clinical outcomes. Additionally, we found prolyl hydroxylase enzymes as a potential biomarker that could predict *in vitro* chondrogenic ability of culture-expanded chondrocytes. Further analysis of these markers in chondrocytes population could take chondrosphere® treatment for cartilage lesions a step ahead.

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Paper I

MESENCHYMAL STROMAL CELLS FROM HUMAN UMBILICAL CORDS DISPLAY POOR CHONDROGENIC POTENTIAL IN SCAFFOLD-FREE THREE DIMENSIONAL CULTURES

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Abstract

Many researchers world over are currently investigating the suitability of stromal cells harvested from foetal tissues for allogeneic cell transplantation therapies or for tissue engineering purposes. In this study, we have investigated the chondrogenic potential of mesenchymal stromal cells (MSCs) isolated from whole sections of human umbilical cord or mixed cord (UCSCs-MC), and compared them with cells isolated from synovial membrane (SMSCs), Hoffa's fat pad (HFPSCs) and cartilage. All MSCs were positive for surface markers including CD73, CD90, CD105, CD44, CD146 and CD166, but negative for CD11b, CD19, CD34, CD45 and HLA-DR in addition to CD106 and CD271. Chondrogenic potential of all cell sources was studied using 3D pellet cultures incubated in the presence of different combinations of anabolic substances such as dexamethasone, IGF-1, TGF-β1, TGF-β3, BMP-2 and BMP-7. BMP-2 and dexamethasone in combination with TGF-β1 or TGF-β3 excelled at inducing chondrogenesis on SMSCs, HFPSCs and chondrocytes, as measured by glycosaminoglycans and collagen type II staining of pellets, quantitative glycosaminoglycan expression, quantitative PCR of cartilage signature genes and electron microscopy. In contrast, none of the tested growth factor combinations was sufficient to induce chondrogenesis on UCSCs-MC. Moreover, incubation of UCSCs-MC spheroids in the presence of cartilage pieces or synovial cells in co-cultures did not aid chondrogenic induction. In summary, we show that in comparison with MSCs harvested from adult joint tissues, UCSCs-MC display poor chondrogenic abilities. This observation should alert researchers at the time of considering UCSCs-MC as cartilage forming cells in tissue engineering or repair strategies.

Keywords: Chondrogenesis; 3D pellet culture; Mesenchymal stromal cells, Umbilical cord derived stromal cells; Hoffa's fat pad derived stromal cells; Synovial membrane derived stromal cells.

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Introduction

Articular cartilage is a specialised avascular and hypo-cellular load-bearing tissue that covers the terminal edges of adjacent bones and provides frictionless movement of the joints (Hunziker, 2002). Lesions in articular cartilage evolving from traumatic or pathological processes represent a very common clinical condition in both developed and developing countries, and show increasing prevalence due to the steady prolongation of life expectancy (Beris *et al.*, 2005). During the last decades biological repair of cartilage has been attempted by both the direct implantation of autologous cells, (Brittberg, 2008) and by the *ex-vivo* generation of tissue engineering-based implants (Filardo *et al.*, 2013). Despite promising achievements in the laboratory and in animal models, clinical translation of these techniques remains very limited.

One of the main obstacles encountered is the limited availability of donor tissue, along with some ethical constraints associated with painful harvesting and possible donor site morbidity (Horas *et al.*, 2003). Furthermore, articular chondrocytes used in autologous chondrocyte implantation (ACI) or bone marrow-derived MSCs (bMSCs) have been shown to gradually lose proliferative and differentiation potential *in vitro* in relation to increasing patient age (Mueller and Glowacki, 2001; Roobrouck *et al.*, 2008; Smeriglio *et al.*, 2015). In the clinic, the procedures mentioned above are often associated with the formation of biomechanically inferior fibrocartilage in the repair zone (Horas *et al.*, 2003; Wright *et al.*, 2013). Hence, approximately 25 % failure rate has been observed in both ACI and micro-fracture in a randomised control clinical study after 5 years (Knutsen *et al.*, 2007), which is expected to increase in longer follow up studies.

To overcome the afore mentioned constraints, researchers have started exploring the use of allogeneic cell sources such as foetal-derived stromal cells from placenta and umbilical cords which are young and immature, easily accessible, abundant and not associated to ethical concerns (Baksh *et al.*, 2007; Fong *et al.*, 2012). MSCs can be isolated from different compartments of the umbilical cord including Wharton's jelly (WJ), the perivascular region (PV), cord lining (CL), artery, and from the whole cord (MC, without separating each compartment) (Mennan *et al.*, 2013; Subramanian *et al.*, 2015). Like other adult MSCs, umbilical cord-derived stromal cells (UCSCs) have been shown to display high proliferative rate and multilineage differentiation potential (Mennan *et al.*, 2013; Nirmal and Nair, 2013). They also express markers typical for MSCs, whilst being negative for haematopoietic,

macrophage and endothelial cell markers. In addition, UCSCs are thought to have an immune privileged status and to exert immunosuppressive effects over different immune cell types *in vitro*, which makes them an attractive candidate for allogeneic based therapies (Subramanian *et al.*, 2012; Troyer and Weiss, 2008).

The use of UCSCs for cartilage repair or cartilage tissue engineering have been studied to some extent in the past. Most available literature refers to the chondrogenic abilities displayed by MSCs collected from the blood compartment of cords; however, much less information is available on cells isolated from cord matrix or stroma (Park *et al.*, 2015; Zhang *et al.*, 2011). The intrinsic chondrogenic potential of MSCs isolated from the solid parts of umbilical cords has been studied mostly *in vitro* with divergent outcomes. While some studies are showing differentiation of UCSCs towards immature cartilage-forming cells (Wang *et al.*, 2009a; Wang *et al.*, 2009b), other studies argue poorer chondrogenic ability of UCSCs when compared with MSCs from other adult tissues (Bailey *et al.*, 2007; Hildner *et al.*, 2010; Mennan *et al.*, 2013). In line with published original papers, many review articles also underscore several contradictory differences including multi-lineage potential and phenotypic profiles, which could be due to many factors, notably, isolation and handling techniques of MSCs in different laboratories, culture medium, scaffolds or cell carriers and use of various growth factors for chondrogenesis (El Omar *et al.*, 2014; Troyer and Weiss, 2008). Of importance, many of the available studies are showing only qualitative outcomes and provide insufficient evidence for expression of cartilage tissue signature genes and proteins quantitatively, or the development of tissue-like structures that clearly resemble cartilage.

In our study, we aim to shed some light on this still uncertain topic by studying the chondrogenic potential of UCSCs-MC isolated from whole cords. Here we have used multiple quantitative approaches for measuring cartilage genes and proteins. Chondrogenic potency has been investigated by exposing cells to multiple combinations of growth factors, including co-culture trials with cartilage explants, and the outcomes have been compared with MSCs collected from different sources. Our data revealed intriguing differences in chondrogenic potential among different MSCs. Of note, UCSCs-MC are found to have poor differentiation ability towards the chondrogenic lineage *in vitro* when compared to Hoffa's fat pad derived stromal cells (HFPSCs), synovial membrane derived stromal cells (SMSCs) and articular chondrocytes (ACs).

Materials and Methods

Human Material

All human samples were collected from the University Hospital Northern Norway (UNN) with patients' informed consent, and the Regional Ethical Committee (REK Nord) at the University of Tromsø approved the study. Human ACs, SMSCs and HFPSCs were isolated from the knee joints of three patients aged 45 to 60 years undergoing total knee replacement. UCSCs-MC were isolated from

three umbilical cords collected immediately after birth and processed within 3 h of collection.

Isolation and culture of UCSCs-MC, HFPSCs, SMSCs and ACs

All cell types were isolated using a mixed enzymatic-explant method in which tissue sections were minced and digested in collagenase XI solution (Cat. no. C9407; Sigma Aldrich) at a final concentration of 1.25 mg/mL on a shaker at 37 °C. Isolation of UCSCs-MC was conducted as previously described with minor modifications (Mennan *et al.*, 2013). Briefly, whole cord was washed three times with sterile Dulbecco's phosphate buffered saline (DPBS; Cat. no. D8537; Sigma-Aldrich) to remove blood, followed by immersion in 90 % ethanol for 30 s and immediately washed and stored in DPBS for immediate use. Approximately 2 cm sections of whole cord were cut into small pieces, and further subjected to enzyme digestion for 1.5 h at a concentration previously indicated. During knee replacement operations, pieces of synovium were collected and the membrane fraction (synovial membrane) was carefully separated from the fat tissues (Hoffa's fat pad). HFP and SM tissues were washed three times with DPBS, followed by cutting into small pieces prior to enzymatic digestion for 1.5 h. On the other hand, cartilage biopsies were washed in DPBS and minced carefully into 1-1.5 mm^3 pieces to avoid any bone fraction with the biopsies, prior to enzymatic digestion for 4 h at a concentration previously indicated.

Partially digested tissues were centrifuged for 10 min at 800 ×g and re-suspended in high glucose Dulbecco's Modified Eagle Medium (DMEM; Cat. no. D5796; Sigma-Aldrich) supplemented with L-ascorbic acid (62 mg/L) (Cat. no.103033E; BDH Laboratory), penicillin and streptomycin (1 %) (P/S; Cat. no. P4333; Sigma-Aldrich) and 20 % foetal bovine serum (FBS; Cat. no. S0115; Biochrom) and subsequently, plated in 75 cm^2 culture flasks (Cat. no. 156499; Thermo Scientific) at 37 °C in humidified atmosphere containing 5 % CO_2. Partially digested cartilage allowed more cells to attach to the culture flask. For isolation of HFPSCs, only the pelleted fraction of cells were collected after centrifugation, whereas the fat layer on top was discarded. After cell attachment, cultures of UCSCs-MC, HFPSCs and SMSCs were expanded in high glucose DMEM supplemented with 10 % FBS and basic fibroblast growth factor (25 ng/mL) (bFGF; Cat. no. 100-18C; Peprotech), whereas de-differentiated ACs were expanded with only 10 % FBS as medium supplement. Primary cultures were further expanded in monolayers, followed by dissociation from culture flasks using PBS based enzyme-free dissociation solution (Cat. no. S-014-B; Merck Millipore) and plated in 175 cm^2 culture flasks (Cat. no. 159910; Thermo Scientific). Medium was changed every 3 to 4 d, and the cells were used for experimentation at passage 3-4, which correspond to 6-8 cumulative population doublings, measured by direct cell counting.

Immunoprofiling of stromal cells by flow cytometry

Expression of cell surface molecules from cultured UCSCs-MC, HFPSCs and SMSCs on the third passages

were analysed by flow cytometry using the BD stemflow hMSC analysis kit (Cat. no. 562245; BD Biosciences) following the manufacturer's instructions. Briefly, cells from each tissue source were harvested and pelleted at $400 \times g$ for 3 min at 4 °C. The pellet was washed three times with cold stain buffer (Cat. no. 554656; BD Biosciences), filtered through a cell strainer (70 µm) and re-suspended in cold stain buffer to a concentration of 5×10^6 cells/mL. Cells were incubated with directly conjugated antibodies against classical MSCs characterisation markers described by the International Society for Cellular Therapy (ISCT) (Dominici *et al.*, 2006). In addition, antibodies for CD44, CD106, CD146, CD166, and CD271 cell surface markers and appropriate isotype control antibodies (BD Biosciences, USA) were used in the analysis. Samples were analysed using a BD FACSAria flow cytometer and FlowJo software (Tree Star Inc., USA).

3D cell culture and induction of chondrogenesis

Chondrogenic differentiation potential of all cell sources used in this study was done using the pellet system as described previously with few modifications (Ivascu and Kubbies, 2006). Confluent cell cultures at passage 3-4 were harvested and re-suspended in high glucose DMEM supplemented with L-ascorbic acid (62 mg/L), P/S (1 %), 10 % FBS and bFGF (25 ng/mL). A volume of 150 µL containing 5×10^4 cells was transferred to each well of a poly-HEMA (Cat. no. P3932; Sigma-Aldrich) coated conical-bottom 96-well culture plate (Cat. no. 249935; Thermo Scientific). Plates were centrifuged for 10 min at $1100 \times g$ to form aggregates, and then transferred into a low oxygen atmosphere incubator set up at 5 % CO_2, 3 % O_2 and 37 °C. After 48 h, spheroids were collected and transferred to a 24-well ultra-low attachment cell culture plate (Cat. no. 3473; Corning) containing 1 mL of complete chondrogenic medium per well (8-10 spheroids/well). Spheroids were cultured in different serum-free chondrogenic media for three weeks at low oxygen (3 % O_2), and half of the medium was changed with fresh chondrogenic medium twice a week.

Chondrogenic media

Basal chondrogenic medium was composed of high glucose DMEM, L-ascorbic acid (62 mg/L), P/S (1 %), dexamethasone (1 µg/mL) (Cat. no. PZN-3103491; Galenpharma), Insulin-transferrin-selenium supplement (ITS) (1:1000) (Cat. no. 354351; BD Biosciences) (Tang *et al.*, 2015). Five different anabolic growth factors were used to induce chondrogenesis in 3D cultures. These include transforming growth factor β1 (10 ng/mL) (TGF-β1; Cat. no. 100-21C), transforming growth factor β3 (10 ng/mL) (TGF-β3; Cat. no. 100-36E), bone morphogenic protein 2 (100 ng/mL) (BMP-2; Cat. no. 120-02C), insulin like growth factor 1 (20 ng/mL) (IGF-1; Cat. no. 100-11) and bone morphogenic protein 7 (100 ng/mL) (BMP-7; Cat. no. 120-03). All growth factors were purchased from Peprotech, UK. Three dimensional cell aggregates were grown in complete chondrogenic medium consisting of basal chondrogenic medium supplemented with one of the six different combination of growth factors: 1) TGF-β1 + IGF-1; 2) TGF-β1 + BMP-2; 3) TGF-β1 + BMP-7; 4) TGF-β3 + IGF-1; 5) TGF-β3 + BMP-2 and 6) TGF-β3 + BMP-7.

Chondrogenesis of UCSCs-MC in co-culture with cartilage explants and cultured synovial cells

Co-culture experiments were carried out using 0.4 µm transwell inserts (Cat. no. 3413; Corning) and the best growth factor mixture to induce chondrogenesis among the six different conditions tested. On one hand, fresh cartilage biopsies were washed three times with sterile DPBS and minced into 1-1.5 mm³ pieces. The minced cartilage pieces were transferred to ultra-low attachment 24-well cell culture plates containing complete chondrogenic medium supplemented with TGF-β3+BMP-2 (Fig. 10**a**). On the other hand, synovial cells, consisting of a mixed culture of primary HFPSCs and SMSCs (1:1) were seeded at a density of 2×10^4 cells in 24-well cell culture plates (Cat. no. 353047; BD Falcon) containing complete chondrogenic medium supplemented with TGF-β3+BMP-2 (Fig. 10**b**). On the top chamber, four or five UCSCs-MC spheroids collected 48 h after initial cell aggregation were placed in each insert and incubated in separated co-culture during three weeks (see diagram in Fig. 10**a,b**) at low oxygen (3 % O_2), and half of the medium was changed with fresh chondrogenic medium twice a week.

Histology and immunohistochemistry

Spheroids from all cell sources were harvested after three weeks incubation, washed three times with ice cold DPBS and fixed in 4 % formalin overnight. Fixed spheroids were washed with DPBS embedded in agarose blocks (1 %) and transferred into paraffin. Paraffin embedded sections (4 µm) were heated at 60 °C for 30-60 min prior to de-waxing and immersing in xylene twice for 10 min and rehydrated in a series of ethanol washes followed by washing twice in water for 3 min each. Sections were stained with Alcian blue solution (Cat. no. A5268; Sigma-Aldrich) for 30 min followed by washing in distilled water for 2 min and counter stained with nuclear fast red solution (Cat. no. N3020; Sigma-Aldrich) for 5 min, ending by washing in distilled water for 2 min. The sections were dehydrated in a series of ethanol wash followed by cleaning in xylene twice and a cover slip with Histokit (Cat. no. 1025/500; Glaswarenfabrik Karl Hect) mounted over stained sections. The image was analysed using bright field light microscopy for metachromatic staining of proteoglycans with Alcian blue.

For immunostaining of collagen type II and type I, rehydrated paraffin sections were treated with hyaluronidase (2 mg/mL) (Cat. no. H3506; Sigma-Aldrich) and pronase (1 mg/mL) (Cat. no. 10165921001; Roche) for 15 min and 30 min, respectively at 37 °C for antigen retrieval. The slides were then rinsed in distilled water followed by blocking with 2 % BSA for 10 min, prior to overnight incubation at 4 °C using rabbit antihuman collagen type II antibody (Cat. no. T59104R; Meridian Life Science) and rabbit antihuman collagen type I antibody (Cat. no. LS-B3653; LifeSpan Biosciences) at a dilution of 1:100 and 1:500 respectively. The sections were treated with peroxidase block solution for 5 min, followed by rinsing with distilled water. The slides were then wiped gently

Table 1. Scoring categories for histological evaluation of chondrogenesis of Alcian blue stained spheroids.

Scoring Categories	Score
1. Uniformity and intensity of Alcian blye stain	
No stain	0
Weak stain of matrix	1
Moderate stain	2
Strong stain of matrix	3
2. Matrix formation based on proteoglycan staining	
No matrix formation	0
Little matrix formation with high cell density	1
Moderate matrix formation with relatively low cell density	2
High matrix formation with low cell density	3
3. Cell morphology	
Highly condensed and elongated cells	0
Less condensed and elongated/rounded cells	1
Mixed elongated/rounded cells with lacunae	2
Rounded Cells with Lacunae/cartilage morphology	3

and incubated with peroxidase labelled polymer-HRP (Cat. no. K4010; Dako) for 30 min followed by washing three times with PBS. The sections were incubated with diaminobenzidine (DAB) chromogen and substrate-buffer solution (1 drop of DAB + 1 mL of substrate-buffer) (Cat. no. K4010; Dako) for 5-10 min, followed by rinsing off with distilled water. Sections were then counterstained with haematoxylin (Cat. no. RBA-4213-00A; Cell Path) for 45 s followed by washing with distilled water and incubation in Scotts solution for 20 s. Finally, sections were dehydrated and mounted with Histokitt.

Quantitative analysis of histology
We have used "The Bern Score" with a few modifications as a semi-quantitative scoring method to evaluate the chondrogenicity of 3D spheroids, based on the staining of matrix proteoglycans with Alcian blue (Grogan *et al.*, 2006). The Bern score evaluates the cartilage formation by three categories and each of the categories have a scoring range from 0 to 3 with an overall score of 9 for each spheroid (Table 1).

Biochemical analysis of GAGs
Spheroids from ACs and UCSCs-MC were subjected to biochemical analysis to determine the GAG (glycosaminoglycan) and DNA content. Spheroids were harvested after three weeks in chondrogenic conditions and washed with PBS three times followed by digestion with papain (125 µg/mL) (Cat. no. P3125; Sigma-Aldrich) buffer for 18-24 h at 65 °C. Papain digested samples were centrifuged at 16,000 ×*g* for 10 min and supernatants were used immediately or stored at −70 °C for biochemical analysis. Sulphated-GAG content was measured spectrophotometrically at 655 nm by 1, 9 dimethylmethylene blue dye assay using the Blyscan s-GAG assay kit (Cat. no. B1000; Biocolor). In addition, DNA was measured using Quant-iT PicoGreen dsDNA Assay Kit (Cat. no. P7589; Life Technologies) as described previously (Toh and Cao, 2014). Fluorescence readings

were taken using the CLARIOstar microplate reader (BMG LABTECH, Germany) with excitation and emission at 485 nm and 535 nm, respectively. The amount of s-GAG content was normalised to DNA content for each sample.

Transmission electron microscopy
Spheroids were harvested after three weeks and processed as previously described (Meknas *et al.*, 2012). Briefly, spheroids were fixed in McDowell's fixative (McDowell and Trump, 1976) overnight followed by post-fixation in 1 % OsO_4 for 1 h. Samples were stained *en bloc* in 2 % uranyl acetate prior to dehydration in a graded series of ethanol washes and embedded in Epon according to standard procedure. Sections were cut on a Leica Ultracut S (Vienna, Austria) with a diamond knife from Diatome (Switzerland). Micrographs were taken using a JEOL 1010 (Tokyo, Japan) electron microscope with a Morada camera system (Olympus Soft Imaging Systems, Germany).

Stemness-related transcriptional factors (SRTF) gene expression analysis
RNA from monolayer cultures was extracted using the Perfect Pure RNA Cultured Cell Kit (Cat. no. 2900319; 5 prime) according to the manufacturer's instructions. RNA from spheroid cultures was extracted using the RNeasy® Micro Kit (Cat. no. 74004; Qiagen). In brief, the spheroids were harvested into 2 mL PCR clean tubes containing one stainless steel ball (Cat. no. 69989; Qiagen), washed once with PBS before disruption in buffer RLT in a Qiagen TissueLyser machine (Retch) and homogenisation using QiaShredder columns (Cat. no. 79654; Qiagen). RNA was cleaned using MinElute columns including on-column DNase digestion. Concentration was measured using spectrophotometry (Nano Drop ND-1000), and samples were diluted to a final concentration of 8.25 ng/L before reverse transcription using the qScript cDNA Synthesis Kit (Cat. no. 95047; Quanta Biosciences) according to the manufacturer's protocol. Specific primers for stemness-related transcriptional factors (Drela *et al.*,

Table 2. Primers used for RT-PCR.

Gene	Product size	Primer sequence
OCT3/4A	144 bp	Forward: 5'-GCA GAG GGA TAC GCC CTA AGT-3'
		Reverse: 5'-CAA GAG TAC AGC CAT GAT TCC AAA-3'
SOX2	221 bp	Forward: 5'-ACA CCA ATC CCA TCC ACA CT-3'
		Reverse: 5'-GCA AAC TTC CTG CAA AGC TC-3'
NANOG	148 bp	Forward: 5'-AAT ACC TCA GCC TCC AGC AGA TG-3'
		Reverse: 5'-TGC GTC ACA CCA TTG CTA TTC TTC-3'
APRT	300 bp	Forward: 5'-CCC GAG GCT TCC TCT TTG GC-3'
		Reverse: 5'-CTC CCT GCC CTT AAG CGA GG-3'

Table 3. Hydrolysis probes.

Gene name	Gene symbol	Assay ID
Collagen, type I, alpha 1	COL1A1	Hs00164004_m1
Collagen, type II, alpha 1	COL2A1	Hs00264051_m1
Collagen, type IX, alpha 1	COL9A1	Hs00932129_m1
Collagen, type X, alpha 1	COL10A1	Hs00166657_m1
Versican	VCAN	Hs00171642_m1
Aggrecan	ACAN	Hs00153936_m1
SRY(sex determining region Y)-box 9	SOX9	Hs00165814_m1
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activating protein, zeta	YWHAZ	Hs00237047_m1

2014) were ordered from Invitrogen (Table 2). Adenine phosporibosyltransferase (APRT) was included for RNA quality control as an 800 bp product would yield in the case of contaminating DNA. The RT-PCR reaction mix comprised of 12.5 µL JumpStart™ REDTaq® ReadyMix® Reaction Mix, 0.5 µL forward primer (20 nM), 0.5 µL reverse primer (20 nM), 9.5 µL H_2O and 2 µL cDNA. The 25 µL reaction was run on a MJ Research PTC-200 thermal cycler using the following conditions: initial denaturation for 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 2 min at 72 °C; final extension for 5 min at 72 °C. Products were separated on a FlashGel (Cat. no. 57023; Lonza) and photographed using the ImageQuant LAS4000 system.

Cartilage specific gene expression analysis
Quantitative (qPCR) reactions were performed for spheroids culture on StepOnePlus Real-Time PCR detection system (Applied Biosystems). The relative quantification experiment was run with hydrolysis probes targeting cartilage signature genes (Table 3, Life Technologies). Based on validation experiments YWHAZ alone proved as the most stable reference gene. The reaction volume of 10 µL included 5 µL TaqMan Fast Universal PCR Mastermix No AmpErase UNG (Cat. no. 4366072; Applied Biosystems), 2.5 µL water, 2 µL cDNA and 0.5 µL of the respective assays. Three technical replicates of each sample were applied to a MicroAmp Fast Optical 96-well reaction plate (Cat. no. 4346906; Applied Biosystems), and water and no-RT samples were added for the negative control. The plates were run on a StepOnePlus (Applied Biosystems), using the following cycling conditions: hold at 95 °C for 20 s, 40 cycles at 95 °C for 1 s and then at 60 °C for 20 s. Expression relative to chondrocyte spheroids

was calculated using the delta delta Cq method and 95 % confidence intervals were generated in Excel.

Statistical analysis
For the biochemical analysis, the Student's *t*-test was used to compare the mean differences between experimental and control groups (three different biological replicates per group). The result was expressed as mean ± SD and values of $p < 0.05$ were considered as statistically significant. In addition, for histological scoring, the Kruskal-Wallis was used followed by *post hoc* Bonferroni correction with Mann-Whitney U comparisons for statistical analysis using IBM SPSS statistics 22 (Chicago, USA). Values of $p < 0.05$ were considered as statistically significant. Last, for gene expression analysis, relative gene expression normalised to ACs was shown as 95 % confidence intervals using Microsoft Excel.

Results

Outcomes of cell isolation and features of cell growth in monolayers
Cells with fibroblastic appearance were successfully isolated from all four tissues sources. Isolated cells from umbilical cords, Hoffa's fat pads and synovial membranes were adherent to plastic and had colony-forming abilities. For umbilical cord tissue, cells normally took 5-10 d to attach to the culture plate, and the yield of cells after initial cell seeding was relatively low. In primary cultures, UCSCs-MC cultures presented a heterogeneous morphology and had a slow growing rate, taking 15-20 d to reach confluence (Fig. 1**b**). However, after first sub-culturing, UCSCs-MC acquired a more homogeneous
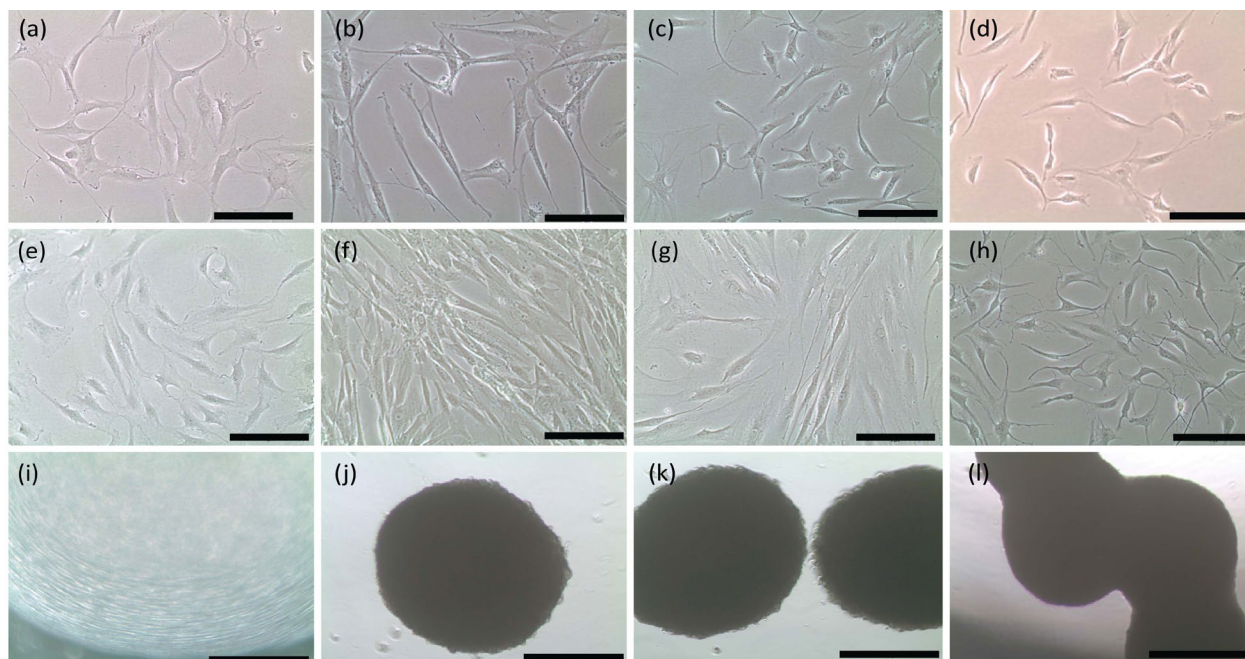
Fig. 1. Light microscopy images of different stages of cell culture and in 3D spheroids. (**a-d**) Monolayer culture of (**a**) ACs, (**b**) UCSCs-MC, (**c**) HFPSCs and (**d**) SMSCs after first plating. Scale bar: 5 µm. (**e-h**) Confluent culture of (**e**) ACs, (**f**) UCSCs-MC, (**g**) HFPSCs and (**h**) SMSCs. Scale bar: 5 µm. In the lower row (**i-l**), 3D Cell culture of UCSCs-MC in micro-aggregates to induce chondrogenesis: (**i**) 50,000 cells after initial centrifugation in conical bottom wells, (**j**) single UCSCs-MC spheroid after 1 week, (**k**) UCSCs-MC spheroids prior to fuse after 1 week and (**l**) fused spheroids after 2 weeks in 3D culture. Scale bar: 500 µm.

morphology and higher proliferation rate (Fig. 1**f**). In contrast, isolated HFPSCs and SMSCs contained a high number of adherent MSC-like cells, which attached to the culture plate during the first 3-5 d (Fig. 1**c,d**). In addition, HFPSCs and SMSCs grew initially faster than UCSCs-MC, reaching 100 % confluence in 10-14 d in 75 cm^2 culture flasks (Fig. 1**g,h**). Unlike stromal cells, chondrocytes attached to plastic faster (2-4 d) but grew at a slower rate (18-25 d to reach confluence). In addition, the de-differentiated chondrocytes looked less elongated and more polygonal than the mesenchymal cells from the other sources (Fig. 1**a**). In 3D conditions, one week after cell aggregation, spheroids from all cell sources looked similar, displaying a perfectly round and compact morphology with sharp edges (Fig. 1**j-l**)). However, sometimes spheroids from all cell sources had fused with others after 2-3 weeks and turned into a larger structure (Fig. 1**i-l**).

Mesenchymal stromal cell phenotypic characterisation

Immunophenotypic characterisation of cells using flow cytometry demonstrated that UCSCs-MC, HFPSCs and SMSCs remained positive for classical mesenchymal markers such as CD73, CD90 and CD105, with expression levels above 99 %. In contrast, the same cells lacked expression for haematopoietic, macrophage and endothelial markers *i.e.* CD11b, CD19, CD34, CD45 and HLA-DR (Fig. 2**a**). In this study, we wanted to further investigate the phenotype of isolated MSCs for additional cell surface markers related to cell differentiation potential (Fig. 2**b**). Hence, it has been shown that subpopulations of MSCs

may express cell surface markers which could predict differentiation potential towards a chondrogenic lineage (Arufe *et al.*, 2010; Baksh *et al.*, 2007; Maleki *et al.*, 2014; Pretzel *et al.*, 2011) including CD44, also called homing cell adhesion molecule (hyaluronate receptor), CD106 or vascular cell adhesion molecule (VCAM-1), CD146 or melanoma cell adhesion molecule (MCAM), CD166 or activated leukocyte cell adhesion molecule (ALCAM), and CD271 also named low-affinity nerve growth factor receptor. In this study, CD44 was expressed in 99 % of the population in the three MSCs, whereas none of the cell types expressed CD106 and CD271. Furthermore, UCSCs-MC showed positivity for both CD146 and CD166 with expression levels above 90 %. However, a subtle difference in expression of CD146 was observed among the three cell types. Whereas UCSCs-MC cultures showed a uniform positivity for CD146, HFPSCs and SMSCs showed two distinct populations, indicating the existence of a subgroup of cells in the cultures that were negative for this marker (Fig. 2**b**).

Chondrogenic potential of ACs, UCSCs-MC, HFPSCs and SMSCs in 3D cultures

Chondrogenic potential of UCSCs-MC and other cell sources was tested in scaffold-free 3D cultures originated by cell condensation or pellets. To take a more unbiased approach, chondrogenesis of all cell types was tested under the influence of different mixtures of growth factors (GF). Metachromatic staining with Alcian blue was used for early screening of spheroids in different chondrogenic medium, and the best growth factors combinations were used to

Fig. 2a. Characterisation for stromal cell-like phenotype by flow cytometry. Cells in passage 3 were prepared from the three different tissue sources, stained with specific cell surface markers and analysed by flow cytometry. The classical set of positive and negative markers characteristic for mesenchymal stromal cells following requirements from the International Society for Cellular Therapy (ISCT). Red peaks represent the isotype control and blue peaks represent the tested markers.

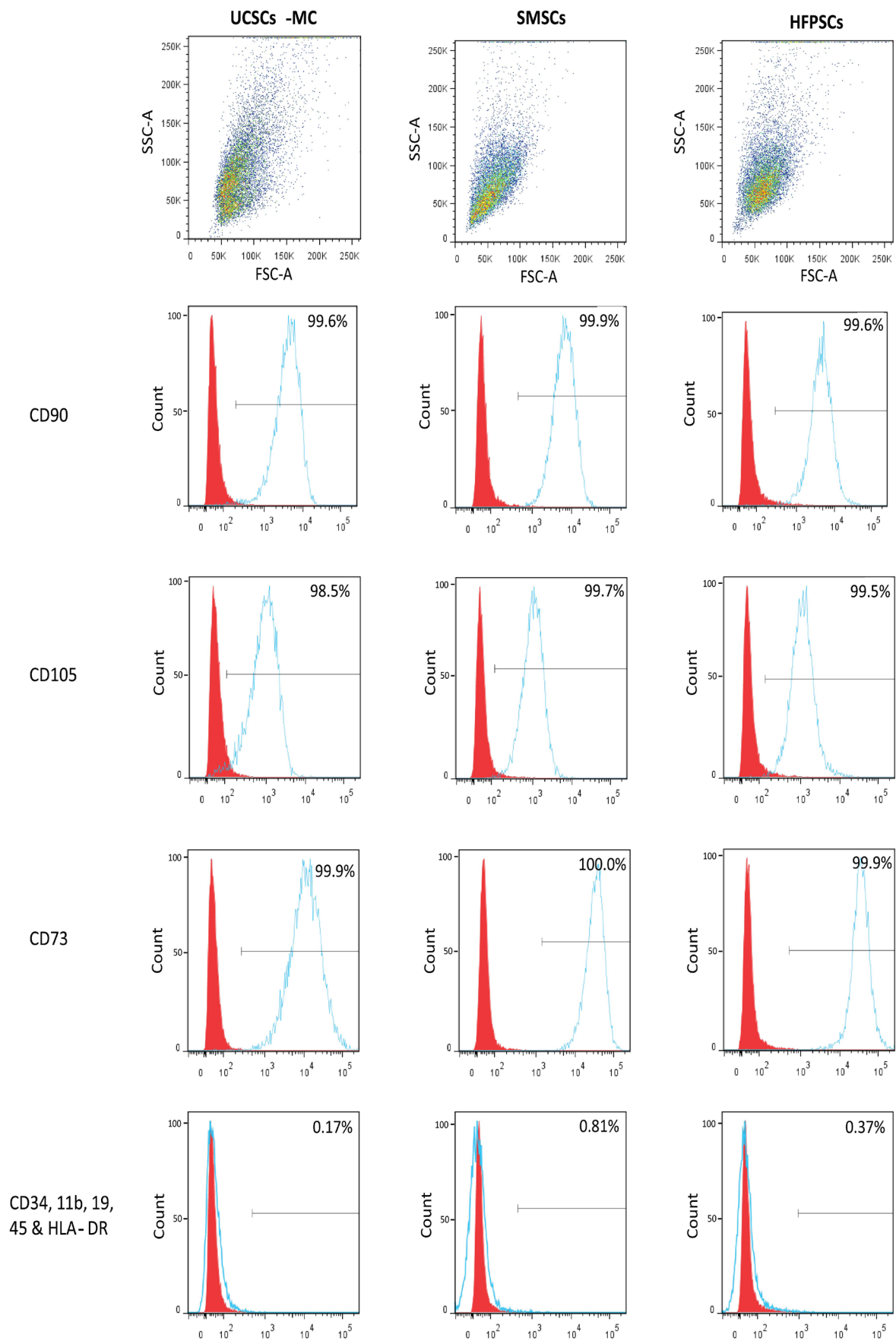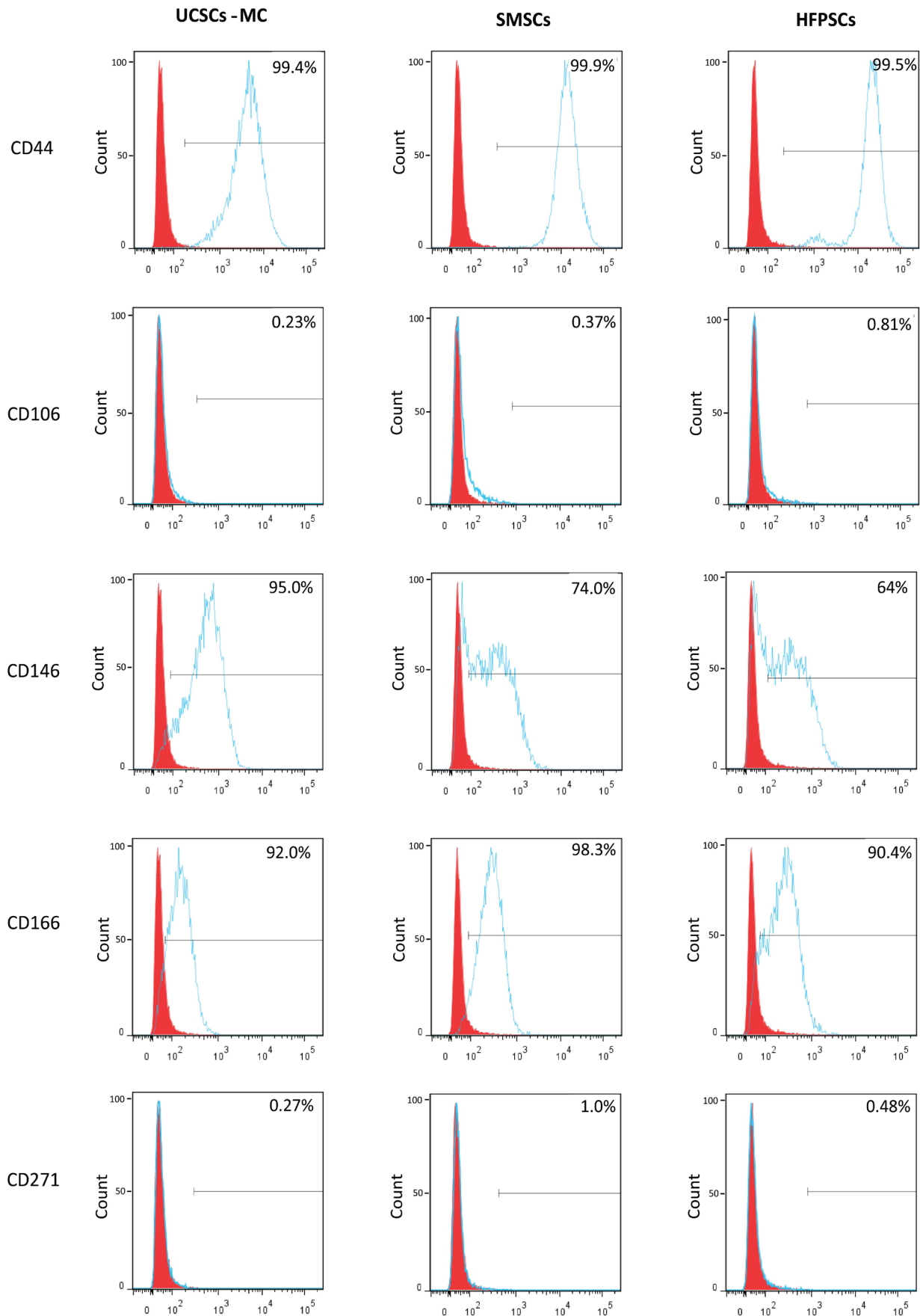**Fig. 2b**. Characterisation for stromal cell-like phenotype by flow cytometry. Cells in passage 3 were prepared from the three different tissue sources, stained with specific cell surface markers and analysed by flow cytometry. Additional cell surface markers related to stromal cells differentiation potential. Red peaks represent the isotype control and blue peaks represent the tested markers.

ACs
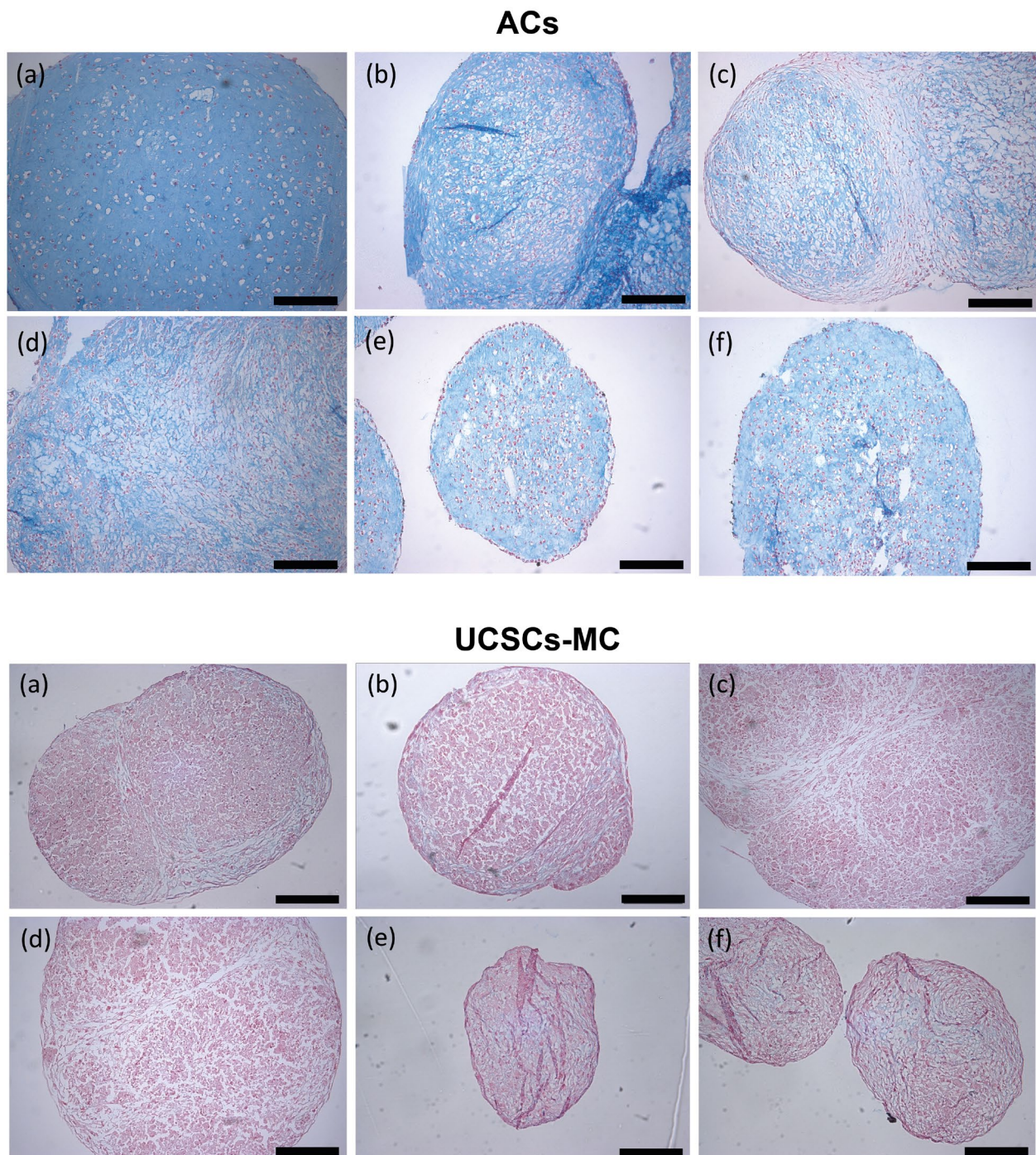
UCSCs-MC

Fig. 3. Chondrogenesis of ACs and UCSCs-MC in 3D spheroids exposed to different cocktails of growth factors. Images show bright light microscopy of thin sections from UCSCs-MC spheroids stained for proteoglycans with Alcian blue, and the nuclei counterstained with Sirius red. (**a**) Condition TGF-β1+BMP-2, (**b**) condition TGF-β3+BMP-2, (**c**) condition TGF-β1+IGF-1, (**d**) condition TGF-β3+IGF-1, (**e**) Condition TGF-β1+BMP-7 and (**f**) Condition TGF-β3+BMP-7. Scale bar: 200 μm and *n* = 3 donors.

further check for collagen type II and type I staining and gene expression analysis. Histomorphological outcomes with Alcian blue demonstrated that chondrogenic medium containing TGF-β3+BMP-2 was the best chondrogenic medium for HFPSCs, whereas the combination TGF-β1 and BMP-2 was the best alternative for SMSCs (Fig. 4**d**,**e**). ACs spheroids demonstrated good cartilage-like features with all combinations of growth factors (Fig. 3 and 4). None of the chondrogenic media tested was sufficient

to differentiate UCSCs-MC into cartilage-forming cells (Fig. 3 and 4). Chondrogenic medium containing TGF-β3 and BMP-2 was successful at inducing chondrogenesis with all three types of adult tissue cells and therefore this growth factor combination was used in further analyses, also with UCSCs-MC. All UCSCs-MC spheroids were characterised by low Alcian blue staining, low collagen type II staining, scant extracellular matrix and irregular cell shape. Semi-quantitative scoring for cartilage-like features

Alcian Blue	Collagen Type II	Collagen Type I
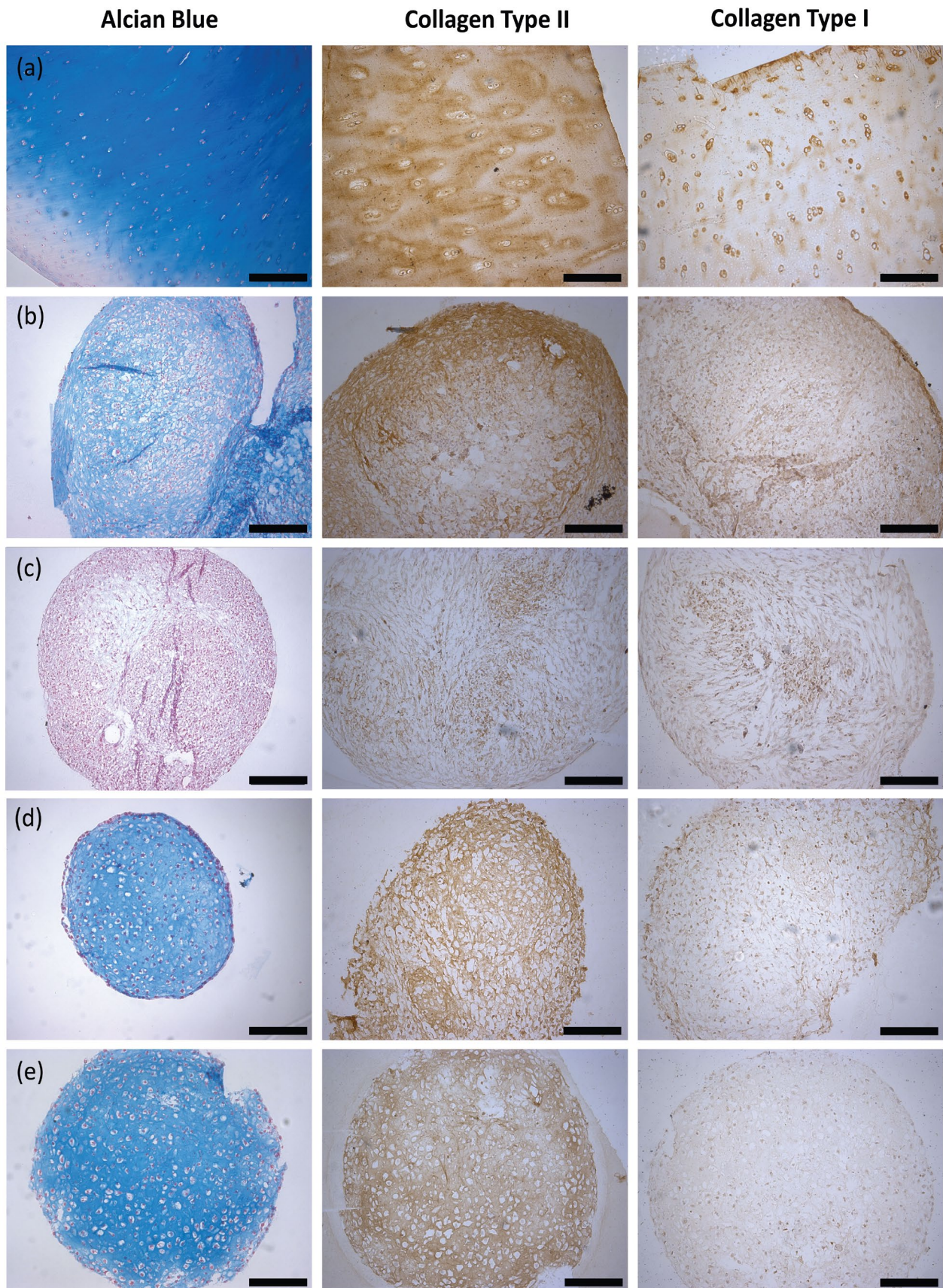
Fig. 4a-e. Bright light microscopy images of thin sections (4 µm) of 3D spheroids stained with Alcian blue, collagen-II and collagen-I. (**a**) Cartilage, (**b**) ACs in the presence of TGF-β3+BMP-2, (**c**) UCSCs-MC in the presence of TGF-β3+BMP-2, (**d**) HFPSCs in the presence of TGF-β3+BMP-2 and (**e**) SMSCs in the presence of TGF-β1+BMP-2. Scale bar: 200 µm.

Fig. 4f. Semi-quantitative analysis representing histological scoring of Alcian blue stained 3D spheroids. (*) Shows levels of significance with $p < 0.005$ and $n = 3$ donors (per donor 2 samples were analysed).
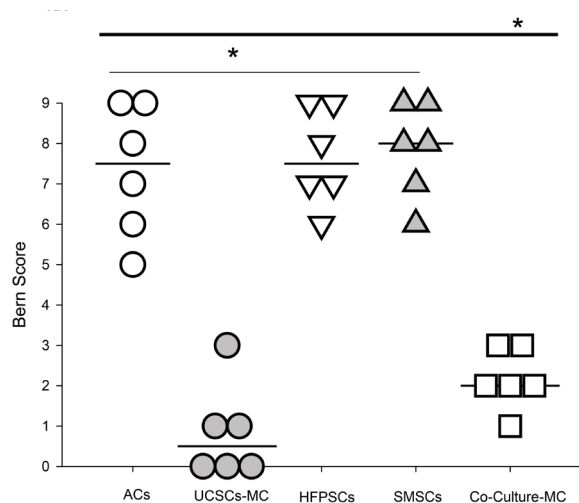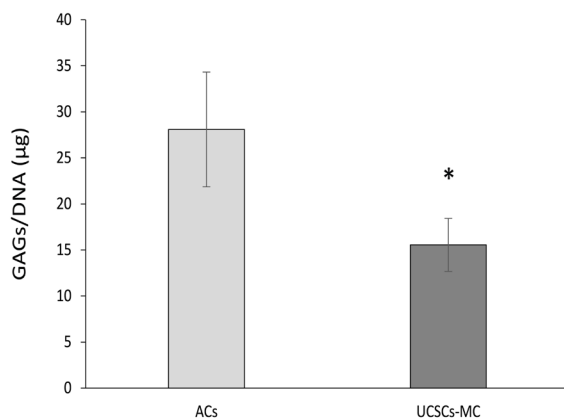

Fig. 5. Comparison of GAGs/DNA (μg) between ACs and UCSCs-MC of three weeks old pellets in the presence of TGF-β3+BMP-2. Data shown are Mean ± Standard Deviation from three different donors and (*) shows levels of significance with $p < 0.05$.

Table 4. Bern Score of Alcian blue stained spheroids.

Cell Source			Category 1	Category 2	Category 3	Total Score
ACs	1	A/B	3/2	3/3	3/3	9/8
	2	C/D	2/3	1/2	2/2	5/7
	3	E/F	2/3	2/3	2/3	6/9
UCSCs-MC	1	A/B	1/1	1/0	1/0	3/1
	2	C/D	0/1	0/0	0/0	0/1
	3	E/F	0/0	0/0	0/0	0/0
HFPSCs	1	A/B	3/3	2/3	3/3	8/9
	2	C/D	1/2	3/3	2/2	6/7
	3	E/F	3/3	2/3	2/3	7/9
SMSCs	1	A/B	3/3	2/3	2/2	7/8
	2	C/D	3/3	3/3	2/3	8/9
	3	E/F	2/3	2/3	2/3	6/9
Co-Culture-MC	1	A/B	1/1	1/0	1/1	3/2
	2	C/D	1/1	0/1	1/1	2/3
	3	E/F	0/1	0/1	1/0	1/2

scoring, similar differences in GAGs production were observed between ACs and UCSCs-MC ($p < 0.05$) after normalising content of GAGs to the amount of DNA (Fig. 5). Spheroids from ACs produced 28.1 ± 6.2 GAGs/DNA (μg), whereas spheroids from UCSCs-MC produced 15.1 ± 2.1 GAGs/DNA (μg). Furthermore, gene expression analysis by qPCR demonstrated that the expression of COL1A1 was similar for all cell types. However, COL2A1, aggrecan and the transcription factor SOX9 were significantly downregulated in UCSCs-MC compared to spheroids from ACs and the other two MSCs types (Fig. 6). Gene expression analyses revealed also that UCSCs-MC spheroids did not express COL9A1 and little or no COL10A1. Of note, HFPSCs had significantly higher expression levels of the proteoglycan versican and the hypertrophic chondrocyte marker COL10A than ACs spheroids, which could reveal a tendency of this MSCs source to form deep zone-type/hypertrophic cartilage. To check the differentiation status of UCSCs-MC before and after 3D culturing, we checked the expression of several SRTF genes including OCT4A, NANOG and SOX2 in monolayers and spheroids at different oxygen tensions. SRTF gene expression revealed that UCSCs-MC express these SRTF genes during monolayer cultures at both high and low oxygen (Fig. 7). Of note, expression of SRTF genes was downregulated in UCSCs-MC upon 3D culture, even when this incubation was carried out at low oxygen tensions.

Ultrastructural examination of 3D constructs
Transmission electron microscopy was used for ultrastructural evaluation of spheroids from ACs, UCSCs-MC, HFPSCs and SMSCs. This technique permits the visualisation of cellular and extracellular matrix characteristics that is not achievable with other microscopic techniques. Hence, spheroids from ACs, HFPSCs and SMSCs showed abundant inter-territorial matrix between cells, where randomly oriented collagen fibrils were easily identifiable (small arrows in images, Fig. 8). Of note, cells in spheroids from all cell sources had a round-shape morphology, had well organised cytoplasm with numerous organelles, and presented no signs of cell

after incubation with the best chondrogenic medium for each cell type showed no differences among ACs, HFPSCs and SMSCs (Table 4), but significant lower values for UCSCs-MC compared with the rest (Fig. 4**f**). Furthermore, immunostaining of spheroids against collagen type II and type I also demonstrated high expression of collagen type II in spheroids from ACs, HFPSCs and SMSCs, which resembles the collagen type II staining of native cartilage, and low expression in UCSCs-MC spheroids (Fig. 4). In contrast, collagen type I staining was relatively weak in spheroids from ACs, HFPSCs and SMSCs. For UCSCs-MC, due to low matrix formation in general, both collagen type II and type I staining were weak (Fig. 4).

Comparative analysis of GAGs and gene expression
To add further support to the observations gathered by histology and immunohistochemistry, chondrogenesis was assessed by measuring GAGs content in spheroids, and the expression of cartilage signature genes in best growth factors combination. In line with results from histological
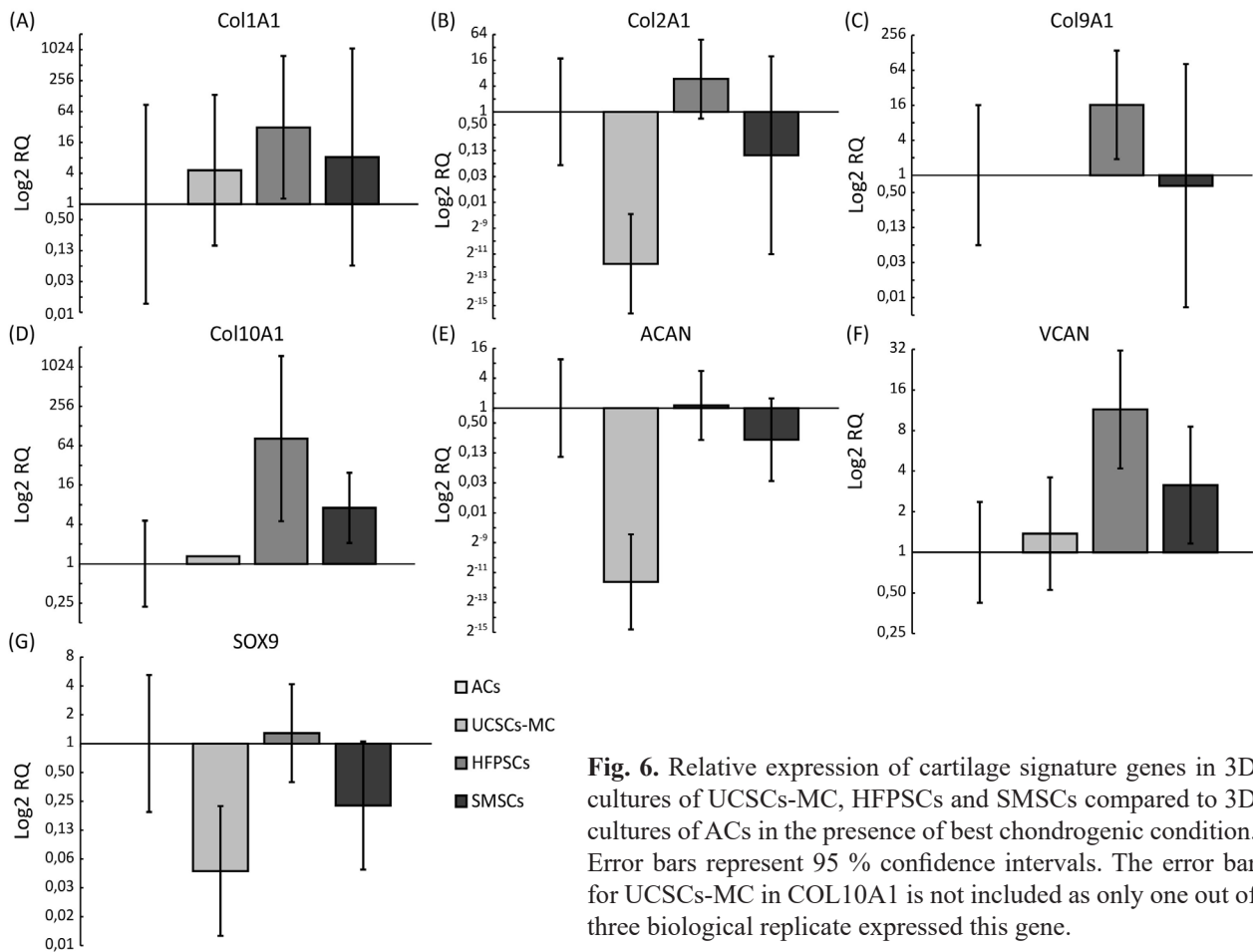
Fig. 6. Relative expression of cartilage signature genes in 3D cultures of UCSCs-MC, HFPSCs and SMSCs compared to 3D cultures of ACs in the presence of best chondrogenic condition. Error bars represent 95 % confidence intervals. The error bar for UCSCs-MC in COL10A1 is not included as only one out of three biological replicate expressed this gene.

death. On the other hand, cells in UCSCs-MC spheroids were more densely packed, had more irregular and less matrix, characterised by some electron dense structures, vacuole-like structures and few collagen fibrils (Fig. 8**b** and 9). In addition, HFPSCs and UCSCs-MC were rich in fat droplets (Fig. 8).

UCSCs-MCs chondrogenesis in co-culture conditions
Previous studies have suggested that the anabolic factor BMP-7, also known as osteogenic protein 1 (OP-1), may act synergistically with TGF-β1 inducing chondrogenesis of MSCs from different sources in pellet culture systems (Kim and Im, 2009; Kurth *et al.*, 2007). Based on these previous observations, and considering that our earlier attempts to induce chondrogenesis in UCSCs-MC were unsuccessful, we also studied the response of UCSCs-MC to alternative combinations of growth factors that included BMP-7 (Fig. 3). Once again, none of the new GFs combinations was able to significantly affect chondrogenesis of UCSCs-MCs, as all spheroids were characterised by high cell density, little extracellular matrix and weak Alcian blue staining (Fig. 3).

In a last attempt to induce chondrogenesis from UCSCs-MCs, spheroids were put into co-culture with either cartilage explants or freshly isolated synovial cells (Fig. 10**a,b**). Results from Alcian blue staining (Fig. 4**f**) again reveal poor chondrogenic capacity of UCSCs-MCs even in the presence of natural elements of the joint (Fig. 10**c,d**).
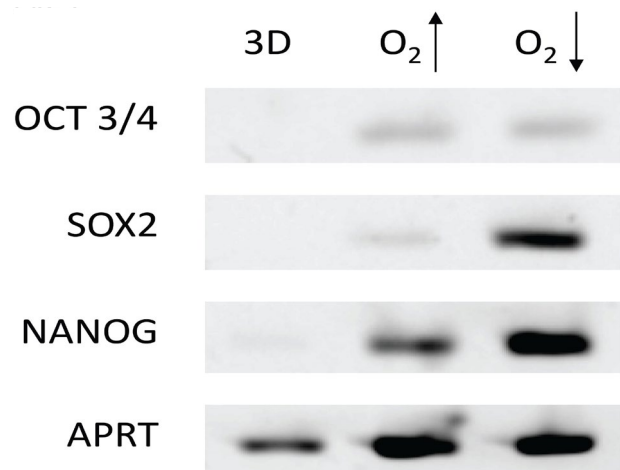
Fig.7. Gene expression of stemness-related transcriptional factors OCT3/4, SOX2 and NANOG in 3D spheroids (3 % O_2) and monolayer cultures at both atmospheric (21 %) O_2 levels and low (3 %) O_2 level. APRT is included for RNA quality control.

Discussion

The main goal in this study was to check the *bona fide* capacity of stromal cells isolated from umbilical cord to make cartilage *in vitro*, and to compare it with adult stromal cells harvested from other sources. By the use of different quantitative approaches and different culture conditions, we show here that, in contrast to what is observed with
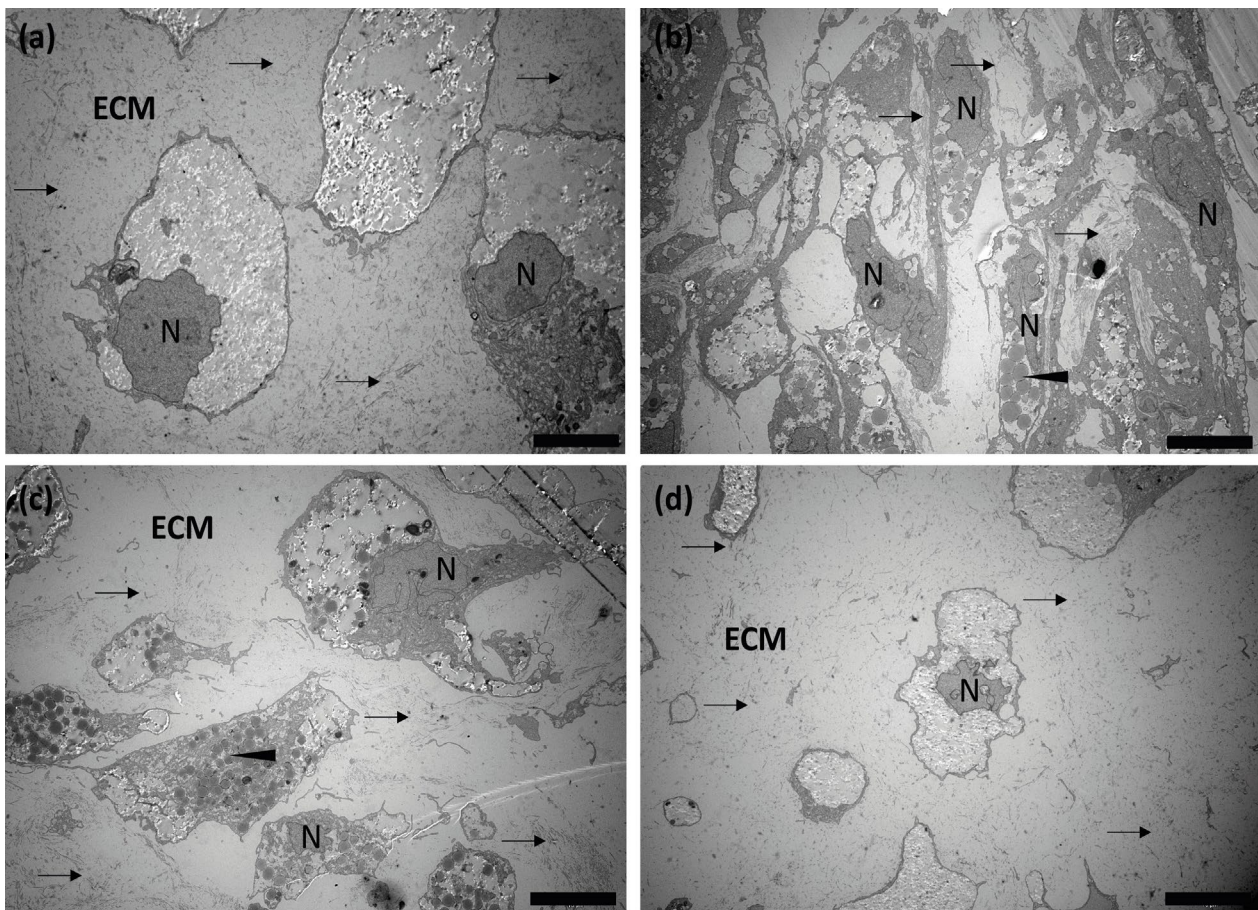
Fig. 8. Transmission Electron Microscopy (TEM) images of ultra-thin sections of 3D spheroids in the best chondrogenic conditions for each cell type. (**a**) ACs in the presence of TGF-β3+BMP-2, (**b**) UCSCs-MC in the presence of TGF-β3+BMP-2, (**c**) HFPSCs in the presence of TGF-β3+BMP-2 and (**d**) SMSCs in the presence of TGF-β1+BMP-2. Symbols: N = Cell nuclei, (ECM) = Extracellular matrix, (→) = Collagen fibres and (◀) = Fat droplets. Scale bar: 10 µm.

stromal cells collected from synovial compartments or with chondrocytes, UCSCs-MC display low chondrogenic potential in scaffold-free 3D cultures, even under the influence of differentiated cells from the joint.

In our study, we have used stromal cells harvested from whole cord. It has been shown that MSCs with multipotent abilities may be isolated from different regions of the cord. However, in comparative studies it has been demonstrated that cells from all regions display very similar growth kinetics and immunoprofiles (Mennan *et al.*, 2013; Subramanian *et al.*, 2015). Additionally, these studies reveal that differentiation potential towards adipogenic, osteogenic and to some extent chondrogenic lineages are better achieved by cells from Wharton's jelly and whole cord. At our laboratory, we have also tested and compared chondrogenic potential of MSCs from Wharton's jelly and whole cord, both cell sources exhibiting equally low chondrogenic differentiation potential (data not shown). Due to the easier processing, we decided to use stromal cells from whole cord in this study.

Different methods including enzymatic and non-enzymatic have been reported for successful isolation of MSCs from umbilical cords (Choudhery *et al.*, 2013; Mennan *et al.*, 2013). Here we have used a mixed

enzymatic-explant method in which tissue biopsies were briefly treated with enzyme to disrupt the membrane followed by explant culture. This method helps to avoid excessive enzymatic digestion and to reduce the time for explant culture. In line with other studies, isolated MSCs demonstrated fibroblast-like characteristics and plastic adherence in *in vitro* culture (Baksh *et al.*, 2007; Vinardell *et al.*, 2012). Unlike most published literatures, we observed UCSCs-MC grow slowly in early passages compared to HFPSCs and SMSCs and their proliferation rate increased after first sub-culture, which was also observed previously from stromal cells isolated from Wharton's jelly (Chen *et al.*, 2012).

Phenotypic comparison by surface markers of isolated MSCs also demonstrated that UCSCs-MC, HFPSCs and SMSCs positively expressed the classical stromal cell markers CD73, CD90, and CD105, and lack expression of CD11b, CD19, CD34, CD45 and HLA-DR as previously shown in the literature (Dominici *et al.*, 2006). Furthermore, we studied additional markers including CD44, CD106, CD146, CD166 and CD271 to investigate chondrogenic specific lineages. Arufe *et al.* reported that cell populations collected from human synovial membranes with high expression of CD271 and low expression of
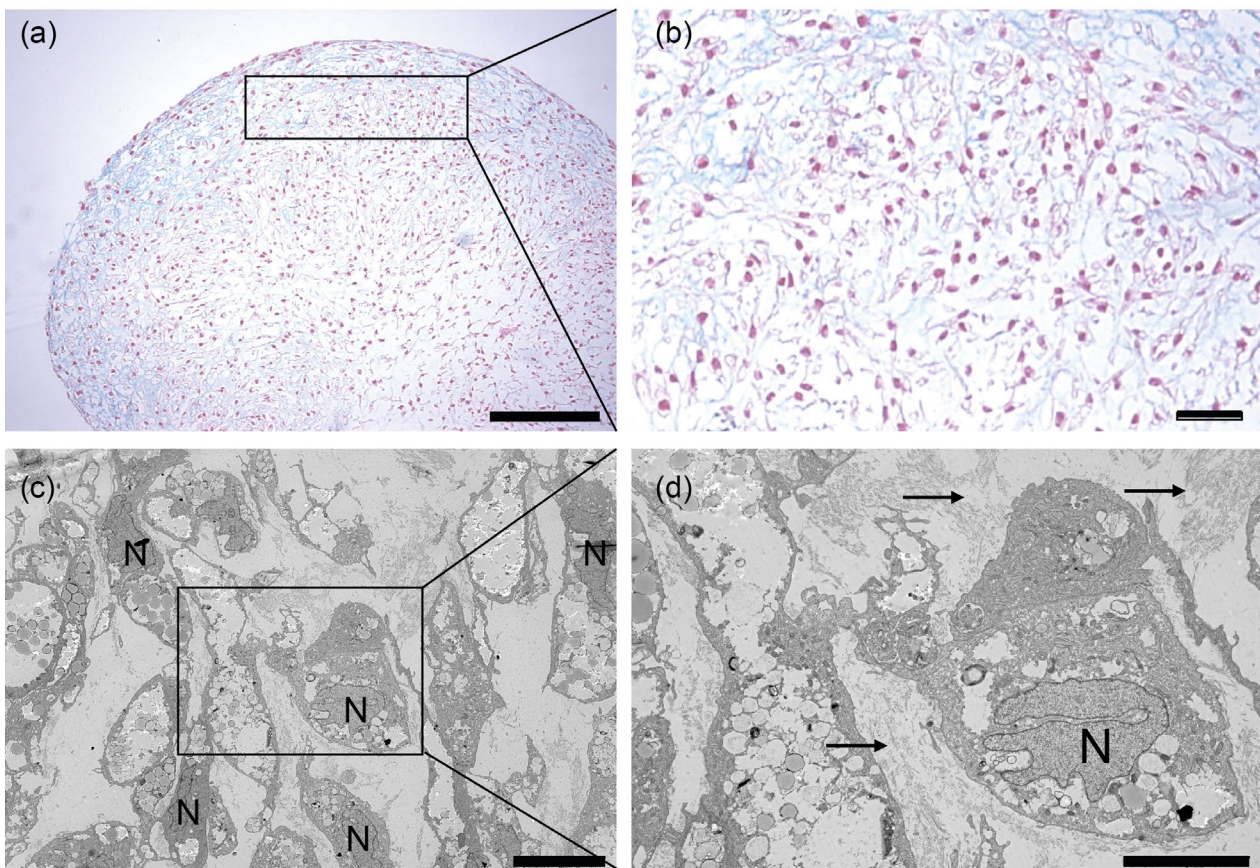
Fig. 9. Comparative Alcian blue and TEM images of UCSCs-MC spheroids at low and high magnification. (**a**) Alcian blue image at lower magnification (Scale bar: 200 µm), (**b**) Alcian blue image at higher magnification (Scale bar: 50 µm), (**c**) TEM image at low magnification (Scale bar: 10 µm) and (**d**) TEM image at high magnification (Scale bar: 5 µm). Symbols: N = Cell nuclei and (→) = Collagen fibres.
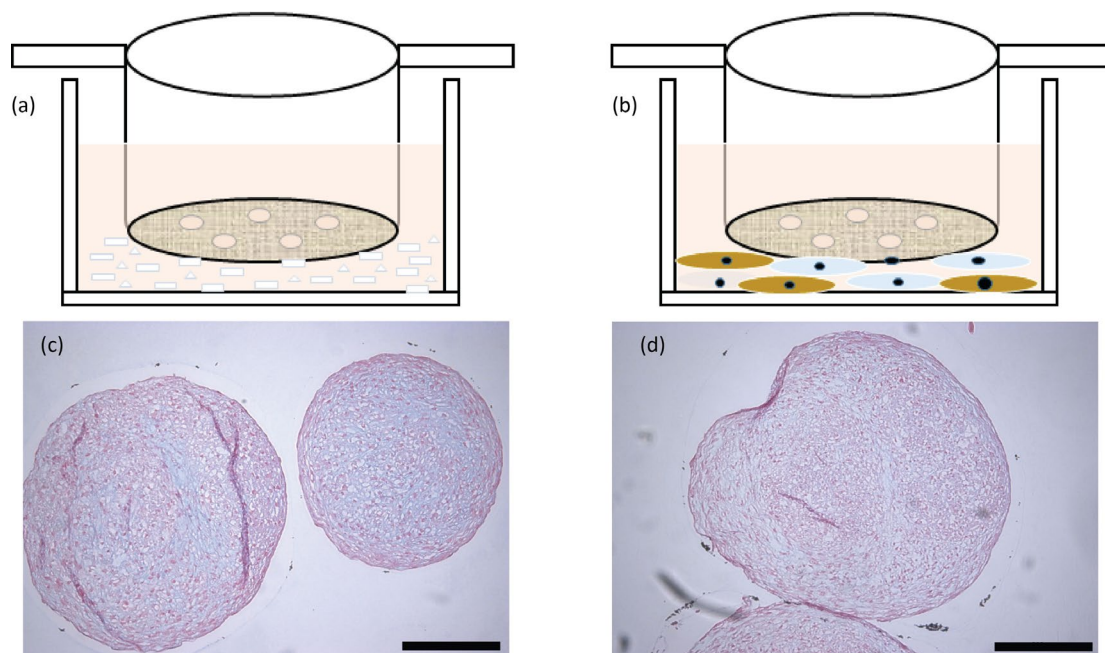
Fig. 10. Chondrogenesis of UCSCs-MC in co-culture with cartilage explants or cultured synovial cells in the presence of TGF-β3+BMP-2. Schematic images of experimental set up during co-culture (**a**) with cartilage explants; and (**b**) with mixed HFPSCs and SMSCs in monolayers. (**c**) and (**d**) bright light microscopy images of thin sections of UCSCs-MC spheroids stained with Alcian blue after co-culture. Scale bar: 200 µm and n = 3 donors.

CD106 possess higher chondrogenic potential (Arufe *et al.*, 2010). Additionally, other studies also revealed that expression of CD44, CD146 and CD166 in cell populations from umbilical cord showed differentiation ability towards chondrogenic lineage (Ali *et al.*, 2015; Baksh *et al.*, 2007; Lu *et al.*, 2006). Hence, we checked expression of these additional markers in all MSC cell types. Our results show uniform expression of CD44 and CD166 in all three cell sources, and absolute negative expression of CD106 and CD271. However, we have seen clear differences in the chondrogenic potential between the cells sources, altogether indicating that the set of markers included in this study were not good for predicting chondrogenic differentiation abilities. An exception could perhaps be done with CD146. We observed that over 99 % of UCSCS-MCs express this receptor; however, HFPSCs and SMSCs show a subpopulation of cells that are negative for CD146. The predictive value for chondrogenesis of this cell surface marker could be further investigated by sorting positive and negative cells within the HFPSCs or SMCSs populations followed by chondro-induction in 3D cultures.

For chondrogenesis, in our study we have used the pellet culture method in conical-bottom multiwell plates, which is relatively easy to perform and permits reproducible outcomes. Additionally, we have used different combinations of growth factors to test chondrogenic potential *in vitro* in hypoxic environment. The panel of anabolic factors chosen in this study are in line with the most widely used growth factors for chondrogenesis. In our hands, the best combination for chondro-induction of HFPSCs and SMCSs were TGF-β3 and TGF-β1 respectively, in combination with BMP-2 and dexamethasone. These outcomes are also in line with previous studies showing chondrogenic differentiation of HFPSCs and SMSCs (Ding *et al.*, 2015; Vinardell *et al.*, 2012).

In this study, UCSCs-MC showed poor differentiation potential toward the chondrogenic lineage compared with other adult stromal cells, irrespective of the presence of growth factors in the chondrogenic medium. Of note, we have deliberately used quantitative approaches to measure chondrogenesis in different ways, and we have used de-differentiated articular chondrocytes (ACs) as the "gold standard". When compared to spheroids from ACs, UCSCs-MC demonstrated a lack of matrix production, densely packed cells and poor cartilage like morphology, which was confirmed by Alcian blue staining, collagen type II immunostaining, GAG content and electron microscopy. Furthermore, we studied gene expression analysis of seven cartilage related genes in which favourable articular cartilage gene expression profile includes low levels of COL1A1, COL10A1 and VCAN, while upregulation of ACAN, SOX9, COL2A1 and COL9A1. Compared to ACs, spheroids from UCSCs-MC showed a significant downregulation of COL2A1, ACAN and SOX9, which in turn demonstrate their poor differentiation ability towards a chondrogenic lineage.

In line with our studies, Hildner *et al.* and Mennan *et al.* demonstrated poor chondrogenesis UCSCs-MC using pellet culture system, while Wang *et al.* showed similar results in scaffold-based cultures (Hildner *et al.*, 2010; Mennan *et al.*,

2013; Wang *et al.*, 2009b). Both Hildner *et al.* and Wang *et al.* question the hyaline cartilage transformation ability of umbilical cord stromal cells and rather emphasising on their fibrocartilage formation ability. Unlike these studies, chondrogenesis of umbilical cord stromal cells has been demonstrated in other studies using similar culturing conditions (Choudhery *et al.*, 2013; Nirmal and Nair, 2013; Subramanian *et al.*, 2015). Choudhery *et al.* demonstrated chondrogenesis of UCSCs isolated by explant method, in pellet culture condition using commercial chondrogenic medium, while Nirmal and Nair showed chondrogenesis of UCSCs isolated by enzymatic digestion, in scaffold-based culture using different combinations of TGF-β2 and BMP-2. Particular isolation or culturing procedures supporting chondrogenesis of stromal cells from UCSCs cannot be withdrawn from these studies.

Of relevance, none of these studies includes hypoxia during chondrogenesis. A recent study by Reppel *et al.* demonstrated better chondrogenesis of stromal cells from Wharton's Jelly in hypoxic condition (Reppel *et al.*, 2014). On the contrary, cell expansion in low oxygen environments has been suggested to maintain UCSCs in undifferentiated state (Drela *et al.*, 2014). In our study, expression of OCT4A, NANOG and SOX2 is elevated in UCSCs-MC during monolayer expansion in both normoxic and hypoxic environments (albeit at higher levels in low oxygen conditions), which evidently shows that cells are kept in undifferentiated state during monolayer growth. Intriguingly, there was no expression of SRTF genes in UCSCs-MC spheroids at low oxygen levels, suggesting a transition of cells towards a differentiated phenotype, which certainly was not cartilage. Taking together, these observations indicate that hypoxia is not responsible for restraining chondrogenesis of UCSCs-MC.

A potential rationale behind the poor chondrogenesis displayed by UCSCs-MC *in vitro* might rely on low TGF receptor expression induced by BMP-2 stimulation, as demonstrated in adipose tissue derived stromal cells (Hennig *et al.*, 2007). However, changing BMP-2 by BMP-7 as chondrogenic factor had no major effects (Fig. 3). On the other hand, CD105 (endoglin), a member of the TGF receptor complex which binds with high affinity to TGF-β1 and TGF-β3 (Robledo *et al.*, 1996), was found to be expressed by nearly all undifferentiated cell types at both high oxygen and low oxygen environments (data not shown). Further studies must be undertaken to explore expression of other receptors of the TGFβ family in UCSCs-MC before conclusions can be reached.

Spheroids from HFPSCs and SMSCs displayed an overall favourable cartilage gene expression pattern, much in line with spheroids from ACs. Of relevance, in our gene expression analyses we observe upregulation of genes such as COL10A1 and VCAN in differentiated HFPSCs. Higher expression of COL10A1 and VCAN was reported in earlier studies associated with cartilage hypertrophy and bone development, respectively (Nakamura *et al.*, 2005; Pelttari *et al.*, 2006). Such observations should be taken into consideration at the time of choosing HFPSCs as a cell source for cartilage tissue engineering.

In earlier studies, chondrogenesis of MSCs have been enhanced by co-culturing the stromal cells with

chondrocytes (Fischer *et al.*, 2010). Thus, in a new attempt to induce chondrogenesis from UCSCs-MC, we developed a co-culture system to mimic *in vitro* intra-articular microenvironments. Once again, UCSCs-MCs spheroids did not develop histo-morphological characteristics of articular cartilage as revealed by metachromasia. Poor chondrogenic potential of UCSCs-MC in our co-culture study could be due to the use of UCSCs-MC and ACs separately rather than using in the same spheroids as previous studies shown (Bian *et al.*, 2011).

Conclusions

Here we disclose poor chondrogenic potential of UCSCs-MC, at least under the conditions tested in the present study. However, it is becoming increasingly accepted that undifferentiated stromal cells might act as adjuvant elements during tissue healing, not by building new tissue themselves but rather by modulating the local environment, making it more favourable for anabolic processes (Liu *et al.*, 2012). Thus, despite their low chondrogenic differentiation potential, stromal cells from the umbilical cord might still be considered for transplantation strategies as facilitators of tissue repair. The precise mechanism of action and the potential therapeutic effects of these cells is yet to be elucidated.

Acknowledgements

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Discussion with Reviewers

Reviewer II: What do you think are the major differences between your work and the work in other groups that have already shown robust chondrogenic potential of umbilical cord-derived stromal cells?

Authors: We have made several allusions to this point throughout the manuscript. In our view, no previous publications are showing "robust" chondrogenic potential of UCSCs. Most of these reports focus primarily on showing multi-lineage differentiation potential of cells, do not focus on chondrogenesis, and the evidences provided are only qualitative, and at its best questionable. The few other reports that provide more quantitative approaches do not compare
expression of cartilage signature molecules with native cartilage or other cell sources, so it is not possible to ascertain the true degree of differentiation. For the rest of the reports, the conclusions are that at best UCSCs they are able to differentiate into fibrocartilage but not into hyaline cartilage.

Editor's Note: Scientific Editor in charge of the paper: Brian Johnstone

Paper II

Large-scale secretome analyses unveil a superior immunosuppressive phenotype from umbilical cord stromal cells compared to other adult mesenchymal stromal cells

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Abstract

Mesenchymal stromal cells (MSCs) with regenerative and immunomodulatory potential are being investigated as a potential therapeutic tool for cartilage lesions. MSCs express a wide variety of bioactive molecules including cytokines, trophic factors, and proteases, which act in a paracrine fashion to modulate the tissue microenvironment. Yet, little is known about the divergence of these signalling molecules between MSCs populations from adult or young tissues. This makes it challenging to decide the optimal source of MSCs for a specific clinical application. In this study, we investigated cell secretomes from cultured human stromal cells harvested from Hoffa's fat pad (HFPSCs), synovial membrane (SMSCs), umbilical cord (UCSCs) and cartilage (ACs) by quantitative LC-MS/MS proteomics. We also performed multiplex protein arrays and functional assays to compare the constitutive immunomodulatory capabilities of different MSCs. Proteins involved in extracellular matrix degradation and inflammation such as MMPs, IL-17, and complement factors were significantly downregulated in UCSCs compared to other cell types. Additionally, we found enhanced expression of TGF-β1 and PGE2 in UCSCs supernatants. UCSCs were superior in inhibiting peripheral blood mononuclear cells proliferation, migration and TNF-α and IFN-γ secretion compared to ACs, HFPSCs and SMSCs. Although all cell types could repress HLA-DR surface expression and cytokine release by activated macrophages, only UCSCs significantly blocked IL-6 and IL-12 production. Our data demonstrate that stromal cells from umbilical cords display superior anti-inflammatory and immunosuppressive properties than stromal cells from adult tissues. This Allogeneic cell source could potentially be considered as an adjuvant therapy for articular cartilage repair.

Introduction

Articular cartilage lesions associate with pain, discomfort, and inflammation in the synovial joint, which subsequently restrict the function of articular activities. Mechanical trauma or degenerative diseases are the major causes of articular cartilage injuries. Traumatic cartilage lesions, on the other hand, increase the risk of developing osteoarthritis (OA) by more than four times (Muthuri *et al.*, 2011). This fast-growing chronic disease is expected to be the fourth leading cause of disability by the year 2020 (Cross *et al.*, 2014). Commonly used surgical and nonsurgical OA treatment modalities include intra-articular injections of soluble materials such as corticosteroids or hyaluronate, autologous blood products, nonsteroidal anti-inflammatory drugs (NSAIDs), and arthroscopic lavage. These procedures improve OA symptoms to a certain degree but do not heal completely the progressive loss of joint functions (Lee and Wang, 2017; Wolfstadt *et al.*, 2015). Additionally, the treatment of localised cartilage injuries with cell-based therapies benefit patients from debilitative knee functions and also prevents the onset of developing secondary OA (Ogura *et al.*, 2017). Although autologous chondrocytes have been used as an intuitive source for cell-based therapy, in recent years, mesenchymal stromal cells (MSCs) are gaining attention as an alternative and potentially effective therapeutic tool for cartilage lesions.

MSCs have been successfully isolated and expanded *in vitro* from numerous tissues sources. Many scholars in the field support the notion that MSCs represent a defined population of multipotent progenitor cells residing in the perivascular niche of nearly all human tissues, although different views exist (Crisan *et al.*, 2008; Guimaraes-Camboa *et al.*, 2017). MSCs differentiation capacity and immunomodulatory properties have been demonstrated *in vitro* irrespective of tissue sources (Ghannam *et al.*, 2010). However, *in vitro* studies have shown that MSCs from different origins differ in their lineage-specific differentiation capacity and their functional potential (Garcia *et al.*, 2016; Islam *et al.*, 2016; Subramanian *et al.*, 2015). In

70   addition, a systematic review of intra-articular injection of bone marrow MSCs in humans

71   concluded that articular stem cells therapies are safe (Peeters *et al.*, 2013). Some preclinical

72   studies in animals have demonstrated the *in vivo* efficacy of MSCs from different sources in

73   OA management including autogenic, allogeneic and xenogeneic cell sources. However, none

74   of these studies has compared the optimal source of MSCs (Ozeki *et al.*, 2016; Saulnier *et al.*,

75   2015; Singh *et al.*, 2014; Yang *et al.*, 2015). Therefore, the choice of optimal source of MSCs

76   for a given clinical implication has yet to be elucidated.

77   Mechanistically, it is not well established how MSCs exert their effects *in vivo*. It was

78   previously believed that MSCs promote tissue regeneration by engraftment of cells in damaged

79   areas and transdifferentiating into tissue forming cells to promote repair (Liechty *et al.*, 2000).

80   Recently, the field has witnessed a paradigm shift in understanding the mechanism of action

81   elicited by MSCs, which highlights paracrine signalling and the release of potent bioactive

82   factors to modulate the microenvironment in benefit of tissue healing (Gnecchi *et al.*, 2016; Iso

83   *et al.*, 2007; Prockop, 2009). In the field of cartilage repair and OA, the fate of implanted cells

84   during biological repair procedures and their contribution to rebuilding the damaged tissue is

85   mostly unknown. Previous studies in animals suggest that most of the repaired tissue is

86   composed of cells of unknown origin migrating to the lesion (Dell'Accio *et al.*, 2003; Grande

87   *et al.*, 1989). A recent human clinical trial concluded that Allogeneic bone marrow MSCs

88   function as a source of stimulatory and trophic factors, which orchestrate tissue repair rather

89   than differentiating into the host tissue (de Windt *et al.*, 2017). However, clinical procedures

90   based on autologous MSCs transplantation, including bone marrow or adipose tissue MSCs,

91   may provide beneficial effects, but are associated with invasive harvesting procedures, two-

92   stage operations and long-time cell expansion ex *vivo*. Allogeneic MSCs harvested from

93   umbilical cords, amniotic membrane and placenta might represent alternative sources for one-

94   stage cell-based therapies. In addition to their pro-angiogenic properties, anti-inflammatory

95   phenotype, and multi-lineage differentiation potential, these Allogeneic MSCs are well

96   tolerated and elicit low immunogenic responses as their adult counterparts (Balasubramanian

97   *et al.*, 2012; Donders *et al.*, 2015). Unlike investigating neotissue forming ability, the role of

98   secreted bioactive molecules in the context of paracrine signalling and immunomodulation have

99   not been comprehensively explored between cells from adult joints and young cells. The aim

100   of this study was to find a suitable cell source that could serve as a potent immunomodulator to

101   mediate the tissue microenvironment.

102   In this study, we compared the secretome of culture-expanded cells harvested from four

103   different tissues sources comprising cartilage (ACs), Hoffa's fat pad (HFPSCs), synovial

104   membrane (SMSCs) and umbilical cords (UCSCs). For analyses, mechanisms and pathways

105   relevant to cartilage and joint physiology including inflammation and immune regulation,

106   extracellular matrix (ECM) remodelling, mitotic factors and chondro-inductive molecules have

107   been considered. Proteins involved in ECM remodelling such as MMPs, complement factors,

108   and serpins were significantly downregulated in UCSCs compared to other cell types, whereas

109   cell signalling molecules such as TGF-β1, MCP-1, and PDGFD were upregulated in UCSCs.

110   To evaluate the constitutive abilities of the different MSCs as immunomodulators, we compared

111   the immunoregulatory properties of supernatants from the different cell types by functional

112   immune assays. Our data revealed that UCSCs exhibit superior anti-inflammatory properties

113   and low catabolic phenotypes compared to ACs, HFPSCs and SMSCs.

114

Materials and Methods

116   **Human Materials and Ethical statements**

117   Human samples were collected from the University Hospital of Northern Norway (UNN). The

118   Regional Ethical Committee (REK Nord 2014/920 and 2010/586) at the University of Tromsø

119   approved the study. Adult stromal cells were isolated from knee joint tissues of 25 patients aged

120   45 to 75 undergoing total knee replacement (TKR) to complete this study. A macroscopic

121   observation was made by the operating surgeon to assess the inflammatory states of the

122   samples. Patients with inflammatory joint diseases and very advanced OA were excluded;

123   however, both secondary posttraumatic and primary osteoarthritis patients were included in the

124   study. Fat pad and synovial membrane tissues were harvested from TKR patients. UCSCs were

125   isolated from seven umbilical cords immediately after birth. Buffy-coats for isolation of

126   peripheral blood mononuclear cells (PBMCs) were collected from healthy donors from the local

127   blood bank (REK Nord 2014/401). All patients provided written informed consent.

128   **Isolation and culture of human stromal cells**

129   Macroscopically good-looking cartilage without any traces of bone, collected from femoral

130   heads during total knee replacements was used to isolate human chondrocytes. All cell types

131   were isolated using a mixed enzymatic-explant method as previously described (Islam *et al.*,

132   2016; Islam *et al.*, 2017). Briefly, all tissue specimens were washed three times with sterile

133   Dulbecco's phosphate buffered saline (PBS; Cat. no. D8537; Sigma-Aldrich) and minced into

134   small pieces for enzymatic digestion in collagenase XI solution (Cat. no. C9407; ≥ 800 units/mg

135   solid, Sigma-Aldrich) at a final concentration of 1.25 mg/mL on a shaker at 37 °C. Cartilage

136   tissue specimens were digested for 3-4 h, and other adult tissue specimens were digested only

137   for 1-1.5 h. UCSCs were isolated from cord matrix (also known as a mixed cord) using 1 h of

138   digestion. Partially digested tissues were centrifuged for 10 min at 800 x*g* and resuspended in

139   high glucose Dulbecco's Modified Eagle Medium (DMEM; Cat. no. D5796; Sigma-Aldrich)

140   before plating on a T-75 culture flask (Cat. no. 156499; Thermo Scientific). The culture medium

141   was supplemented with L-ascorbic acid (62 mg/L) (Cat. no.103033E; BDH Laboratory),

142   penicillin and streptomycin (1 %) (P/S; Cat. no. P4333; Sigma-Aldrich) and 20 % foetal bovine

143   serum to promote cell attachment (FBS; Cat. no. S0115; Biochrom). All cells were incubated

144 in a humidified atmosphere containing 5 % CO_2 at 37 °C. After the initial 24 h, primary cultures

145 were expanded in 10 % FBS supplemented medium, and the medium was changed every 3-4 d

146 until the cultures became confluent.

Preparation of conditioned medium

148 All cells were used for experimentation at passage 3-4. Serum-rich conditioned medium was

149 used in functional assays with peripheral blood mononuclear cells (PBMCs) and macrophages,

150 whereas serum-free conditioned medium was used for protein-arrays and secretomics. Upon

151 reaching 70-80 % confluence, culture flasks were thoroughly washed with warmed PBS, and

152 the medium was replaced with fresh medium containing high glucose DMEM and 1 % P/S

153 (with or without 10 % FBS). Serum-free medium was additionally supplemented with insulin-

154 transferrin-selenium supplement (1:1000) (ITS; Cat. no. 354351; Corning). Both serum-free

155 and serum-rich conditioned medium (CM) were collected after 48 h, centrifuged at 4500 x*g* for

156 10 min, filtered using 0.22 µm porous membrane and used immediately for experimentation or

157 stored at – 70 °C for further analysis. The number of cells was counted for each culture

158 condition and used to normalise the measured expression of cytokines and growth factors in

159 CM. In functional assays with PBMCs and macrophages, the fresh culture medium was diluted

160 with serum-rich CM (1:1) from different stromal cells.

Quantitative and qualitative LC-MS/MS analysis

162 Serum-free CM (6 mL) from all cultures were collected from T-75 culture flasks and

163 concentrated in PBS to a final volume of 500-800 µL using 5000 Da MWCO vivaspin column

164 (Cat. no. Z614440-25EA; Sigma-Aldrich) at 4500 x*g* for 20 min. Protein concentration was

165 measured using DC Protein Assay Kit (Cat. no. 5000116; Bio-Rad). Protein samples (100

166 µg/tube) were reduced in 5 mM dithiothreitol (Cat. no. D9779; Sigma-Aldrich) for 30 min at

167 70 °C. Samples were alkylated by incubation with 375 mM iodoacetamide (Cat. no. 90034;

168   Thermo Scientific) at room temperature for 30 min in the dark. Protein samples were collected

169   as dry pellets after overnight precipitation in pre-chilled acetone (Cat. no. 270725; Sigma-

170   Aldrich) at -20 °C. Dry pellets containing 100 µg protein were resuspended in 100 µL of 2 M

171   Urea (Cat. no. U1250; Sigma-Aldrich) with 50 mM TEAB. Only 25 µg of protein per sample

172   was taken for further analysis. Samples were pre-digested for 6 h with 1:100 (w/w) LysC

173   endopeptidase (Cat. no. 125-05061; Wako Chemicals) with 1 mM final concentration of $CaCl_2$,

174   followed by further dilution with 50 mM TEAB in 1 M Urea and digestion overnight in 1:20

175   (w/w) trypsin (Cat. no. V511A; Promega). A volume of 5 µL trifluoroacetic acid (10 %) (Cat.

176   no. 28904; Thermo Scientific) was added to each tube and centrifuged at 13000rpm for 10 min.

177   OMIX C18 tips were used for sample clean-up and concentration. Samples containing 0.2 %

178   formic acid (FA; Cat. no. 28905; Thermo Scientific) were loaded to a Thermo Fisher Scientific

179   EASY-nLC1000 system and EASY-Spray column (C18, 2 µm, 100 Å, 50 µm, 50 cm). Peptides

180   were fractionated using a 2-100 % acetonitrile (Cat. no. 51101; Thermo Scientific) gradient in

181   0.1 % FA at a flow rate of 250 nL/min over 180 min. The separated peptides were analysed

182   using a Thermo Scientific Q-Exactive mass spectrometer. Data were collected by a Top10

183   method in data-dependent mode. The raw data were processed using MaxQuant (v 1.5.6.0) for

184   label-free protein quantification (LFQ). MS/MS data were searched against the UniProt human

185   database from November 2016 to yield protein identification (false discovery rate (FDR) =

186   0.01). Parameters used for the search: fixed modification, carbamidomethylation of cysteines;

187   variable modifications, oxidation of methionine and acetylation of protein N-terminal; ion mass

188   tolerance, 4.5 ppm; fragment mass tolerance, 20 ppm; charge states, 2+, 3+ and 4+; Maximum

189   missed cleavages, 2; enzyme specificity, trypsin; and minimum number of unique peptides, 2.

190   Perseus 1.5.6.0 software was used for statistical analysis of identified proteins. All contaminants

191   were filtered out before log10-transformation of data for further analysis. The log10-

192   transformed intensities were normalised by subtracting the median. Data were grouped as ACs,

193   HFPSCs, SMSCs and UCSCs and analysed using a *t*-test, with a minimum of three valid values

194   in each group. Volcano plots for each comparison were generated to identify differentially

195   expressed proteins using FDR < 0.01.

Multiplex protein arrays

196

197   A panel of 36 specific proteins including cytokines, chemokines, matrix metalloproteinases

198   (MMPs) and growth factors was measured in the serum-free CM of all four stromal cell types

199   by immune-based protein arrays. A human cytokine magnetic 25-plex kit (Cat. no. LHC0009M;

200   Thermo Scientific) was used to measure the concentration of 18 cytokines (1:4 dilution)

201   involved in inflammation including GM-CSF, IFN-α, IFN-γ, IL-1β, IL-1RA, IL-2, IL-2R, IL-

202   4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17 and TNF-α and 7

203   chemokines (1:4 dilution) including Eotaxin, IP-10, MCP-1, MIG, MIP-1α, MIP-1β and

204   RANTES. Fluorokine MAP human MMP base kit (Cat. no. LMP000; R&D) was used to

205   measure the concentration of MMP-1, MMP-3, MMP-7, MMP-9 and MMP-13 (1:5 dilution).

206   Quantitative measurements (two replicates) were performed according to manufacturers'

207   guidelines using Luminex Bio-Plex 200 system (Bio-Rad, USA). In addition, quantification of

208   PGE2 (Cat. no. KGE004B) and five growth factors including TGF- β1 (Cat. no. DY240-05),

209   BMP-2 (Cat. no. DY355-05), IGF-1 (Cat. no. DY291-05), PDGF-AB (Cat. no. DY222) and

210   bFGF (Cat. no. DY233-05) was performed by ELISA. All ELISA kits were purchased from

211   R&D Systems and performed according to manufacturer instructions. Measured protein

212   concentrations were normalised with cell number at specific culture conditions and expressed

213   as $pg/mL/10^6$ cells.

Isolation and culture of human PBMCs

214

215   PBMCs were isolated from buffy-coats (*n* = 5) of whole blood using lymphoprep (Cat. no.

216   1114545; Alere Technologies) following manufacturer instructions. Briefly, peripheral blood

217 was diluted in sterile PBS (1:1) and slowly layered over a lymphoprep gradient in a 50 mL tube.

218 The tube was centrifuged without a break at 800 xg for 30 min at room temperature. PBMCs

219 were collected from the gradient-interface with a Pasteur pipette, followed by washing three

220 times for 10 min at 400 xg with PBSA containing PBS and 0.2 % bovine serum albumin (BSA;

221 Cat. no. 130-091-376; Miltenyi Biotec). PBMCs were cultured in growth medium containing

222 Rosewell Park Memorial Institute medium (RPMI-1640; Cat. no. R8758; Sigma-Aldrich), 1 %

223 P/S and 10 % FBS in a humidified atmosphere (5 % CO_2) at 37 °C.

PBMCs activation assays

225 PBMCs proliferation was assessed using the carboxyfluorescein succinimidyl ester (CFSE)

226 dilution assay (Cat. no. 10009853; Cayman). Cultured PBMCs were washed in pre-warmed

227 sterile PBS and centrifuged at 400 xg for 5 min before incubating cells with CFSE for 15 min

228 at 1:400 dilution. CFSE-stained PBMCs were cultured in RPMI, 1 % P/S and 10 % FBS in a

229 24-well plate at a density of 10^6 cells/well. PBMCs were stimulated with the mitogen

230 phytohemagglutinin (10 μg/mL) (PHA; Cat. no. 1249738; Roche) for 5 d to induce

231 proliferation. Half of the medium was replaced with fresh medium after the second day. The

232 proliferation assay was performed on a BD FACSAria III flow cytometer, and the data were

233 analysed by FlowJo software (Tree Star Inc., USA). CM from stromal cells was added to the

234 culture of PHA activated PBMCs from 1 d at 1:1 ratio with fresh culture medium. CM from

235 last 3 d during proliferation of PBMCs was collected to measure cytokine profiles. CM derived

236 from PBMCS was centrifuged at 4500 xg for 5 min and filtered by 0.22 µm porous membrane

237 before analysing TNF-α (Cat. no. DY210-05; R&D) and IFN-γ (Cat. no. DY285-05; R&D)

238 contents using ELISA.

PBMCs migration assay

240 PBMCs migration assay was performed by a Boyden chamber assay. PBMCs were activated

241 with PHA (10 µg/mL) for 48 h before performing the assay. Recombinant stromal cell-derived

242 factor-1 (100 ng/mL) (SDF-1; Cat. no. 300-28A; PeproTech) was used as a chemoattractant.

243 PBMCs (10^6 cells/100 µL) were added to the top chamber of Transwell culture inserts (6.5 mm

244 diameter, 8 µm pores, Cat. no. CLS3464; Sigma-Aldrich). Bottom chambers contained either

245 growth medium or serum-rich CM from different stromal cells. After 2 h, PBMCs that migrated

246 to the lower chamber were harvested and washed in PBSA by centrifugation at 400 xg for 4 min

247 followed by resuspending in 0.5 ml of PBSA. Lymphocytes population was gated using

248 forward, and side scatter and counted on a BD FACSAria III flow cytometer.

Isolation and culture of monocyte-derived macrophages

250 Monocytes were isolated from PBMCs using CD14+ magnetic-activated cell sorting (MACS)

251 (Cat. no. 130-050-201; Miltenyi Biotec) with minor modification from manufacturer

252 guidelines. Briefly, PBMCs were washed in MACs buffer containing autoMACS rinsing

253 solution (Cat. no. 130-091-222; Miltenyi Biotec) and BSA (20:1) for 10 min at 4 °C. PBMCs

254 were incubated with CD14+ microbeads at a concentration of 10 µL/10^7 in 40 µL of MACs

255 buffer for 15 min at 4 °C. PBMCs were rinsed in MACs buffer and resuspended in appropriate

256 volume before passing through MS column. CD14+ monocytes were eluted from the column

257 and washed with ice-cold MACs buffer. The purity of eluted monocytes was checked by flow

258 cytometry using anti-CD14-FITC conjugated antibody (Cat. no. 130-098-063; Miltenyi Biotec)

259 and its isotype control mouse IgG2a-FITC (Cat. no. 130-098-877). Fully transformed

260 macrophages (M0-M) were achieved after six days incubation of CD14+ monocytes in a

261 macrophage growing medium containing RPMI-1640, 1 % P/S, 10 % FBS and 100 ng/mL

262 Macrophage Colony Stimulating Factor (M-CSF; Cat no. 300-25; Peprotech) in a humidified

263 atmosphere (5 % CO_2) at 37 °C. The medium was replaced with fresh medium after 3 d.

Macrophage polarisation assays

265 For induction of M1 phenotype (M1-M), M0-M were plated in a 6-well plate at density of 2.5

266 x 10^6 cells/well and stimulated with lipopolysaccharides (100 ng/mL) (LPS; Cat. no. L6529-

267 1MG; Sigma-Aldrich) and IFN-γ (20 ng/mL) (Cat. no. 300-02; PeproTech) for 48 h. To

268 investigate the effects of cell secretomes on M1-M polarisation, macrophage culture medium

269 was diluted with serum-rich CM (1:1) from different stromal cells. Macrophages were

270 harvested after 48 h and washed in MACs buffer before analysing on BD FACSAria III flow

271 cytometer. FlowJo software was used for analysing surface markers expression.

272 M1-M polarization was characterised by surface marker expression of CD40 (Cat. no. 130-099-

273 385; Miltenyi Biotec), CD64 (Cat. no. 130-100-415; Miltenyi Biotec), CD80 (Cat. no. 130-110-

274 371; Miltenyi Biotec), CD86 (Cat. no. 560957; BD Biosciences) and HLA-DR (Cat. no.

275 560943; BD Biosciences). All antibodies were PE-conjugated and analysed with respective

276 isotype controls, including mouse IgG1 (Cat. no. 130-098-845; Miltenyi Biotec), REA control

277 (Cat. no. 130-104-612; Miltenyi Biotec) and mouse IgG2a (Cat. no. 555574; BD Biosciences).

278 For induction of M2 phenotype (M2-M), M0-M were stimulated with dexamethasone (4

279 µg/mL) (Cat. no. PZN-3103491; Galenpharma) for 48 h and characterised by surface marker

280 expression of CD163-FITC (Cat. no. 130-099-969; Miltenyi Biotec) and its isotype control

281 mouse IgG1-FITC (Cat. no. 130-098-847). To further investigate the effects of CM from all

282 cell types on M1-M polarisation, macrophage-CM was collected after 48 h, centrifuged at 4500

283 x*g* for 5 min and filtered by 0.22 µm porous membrane. The concentration of TNF-α, IL-6 (Cat.

284 no DY206-05) and IL-12 (Cat. no. DY1240-05) were measured by ELISA. All ELISA kits were

285 purchased from R&D Systems.

286 **Statistical analyses**

287 All statistical analyses were performed using IBM SPSS statistics version 24 (Chicago, USA).

288 Data were analysed using non-parametric Kruskal-Wallis test, and significance values were

289 adjusted by Bonferroni correction for multiple comparisons. The level of significance was set

290 at $p < 0.05$. Results were presented as density graphs, where each donor plotted as a dot in the

291 dataset.

Results

Comparative protein profiles in supernatants of different stromal cells by LC-MS/MS
proteomics

295 All cell types were characterised by MSCs surface markers and retained similar characteristics

296 as shown in our previous study (Islam *et al.*, 2016). The cell secretome established in serum-

297 free conditioned media from each cell type (four unrelated donors per cell type) was analysed

298 by LC-MS/MS proteomics. Only proteins identified in at least three donors of each cell type

299 were considered for further analyses. Results showed more proteins identified in the

300 supernatants of ACs (709) compared to HFPSCs (641), SMSCs (567) and UCSCs (653) (Fig.

301 1A). Comparative analysis of identified proteins revealed 472 proteins present in the

302 supernatants of all cell types. Only a minor fraction of proteins was exclusively found in

303 supernatants of specific cell types, including 50 differentially expressed by UCSCs, 44 by ACs,

304 22 by HFPSCs and two by SMSCs. Hierarchical clustering of identified proteins revealed two

305 major clusters, where one cluster comprised the four donors of UCSCs, and the second cluster

306 comprised all stromal cells from adult tissues (Fig. 1B). Furthermore, among the stromal cells

307 from adult tissues, the four ACs donors were clearly separated from HFPSCs and SMSCs

308 donors. Identified proteins were divided into six groups according to their functions using Gene

309 Ontology Biological Process (GOBP) terms (Fig. 2A). Qualitative comparison of proteins in

310 different pathways revealed no significant differences between cell sources. Proteins involved

311 in the catabolic process and ECM remodelling were abundant in the supernatants of all cell

312 types. In addition, all stromal cells released similar percentage of proteins involved in

313 immunoregulation ($\sim$ 20 %) and secretion ($\sim$ 13 %) (Fig. 2A).

314   Quantitative analyses of protein expression were performed using the LFQ approach (Fig. 2B).

315   Six volcano plots representing all possible comparisons show differentially expressed proteins

316   by plotting Log10 of the fold change on the X-axis and –Log10 of the *p*-value on the Y-axis for

317   each comparison (*e.g.* HFPSCs vs ACs). Results revealed largest differences in protein

318   expression between UCSCs and adult stromal cells ($p < 0.01$). Proteins involved in cell

319   signalling such as TGF-β1, PDGFD, and MCP-1 were significantly upregulated in UCSCs,

320   while catabolic proteins such as MMPs, serpins, and complement factors were downregulated

321   compared to stromal cells from the adult origin (Fig. 2B). Notably, minor differences

322   particularly in ECM remodelling proteins such as MMPs, and serpins were observed while

323   comparing stromal cells from cartilage and synovium (Fig. 2B). Protein profiles belonging to

324   specific pathways (ECM remodelling, cell communication, and inflammation) were compared

325   among the four cell types (Fig. 3). Several MMPs, serpins, some complement factors, and heat

326   shock proteins were less expressed in UCSCs. On the other hand, some cell signalling

327   molecules including MCP-1, ITG-β1, PDGFD, CSF-1, HLA-C and TGF-β1 were more

328   abundant in the supernatants of UCSCs.

Determination of cytokines and growth factors in supernatants by multiplex protein
329
330   **arrays**

331   A panel of 18 selected cytokines involved in inflammation and immunoregulation was

332   measured in supernatants of all stromal cells. Only IL-4, IL-6, IL-8, IL-12 and IL-17 were

333   detected in supernatants of all cell types, whereas GM-CSF, IFN-α, IFN-γ, IL-1β, IL-1RA, IL-

334   2, IL-2R, IL-5, IL-7, IL-13, IL-15 and TNF-α could not be detected in any of the supernatants.

335   From the panel of chemokines, MCP-1, MIP-1α and RANTES were detected in all

336   supernatants, but Eotaxin, IP-10, MIG and MIP-1β could not be detected. Overall, the levels of

337   IL-6, MPC-1 and PGE2 were increased in UCSCs supernatants compared to other cell types,

338   whereas the levels of IL-17, MIP-1α and RANTESs were decreased (Fig. 4). The concentration

339   of IL-17 and MIP-1α was significantly lower in the supernatants of UCSCs compared to ACs,

340   and the levels of PGE2 was significantly higher in UCSCs supernatants compared to HFPSCs

341   (Fig. 4). Regarding expression of proteases, only MMP-13 was not detectable, whereas MMP-

342   1, MMP-3, MMP-7 and MMP-9 were detected to some degree in all serum-free CM (Fig. 5).

343   The secretion of MMP-1, MMP-3, MMP-7 and MMP-9 was in general lower in UCSCs cultures

344   compared to all other cell types. Significant differences were found for MMP-3 and MMP-7

345   when comparing UCSCs and ACs. The anabolic growth factors TGF-β1, BMP-2 and bFGF

346   were detected at low levels in supernatants of the four cell sources, whereas IGF-1 and PDGF-

347   AB could not be detected. Importantly, TGF-β1 was significantly elevated by UCSCs compared

348   to HFPSCs (Fig. 5).

349   **UCSCs supernatants exert stronger immunosuppressive effects on mitogen-activated**

350   **PBMCs**

351   To investigate the immunomodulatory effects of cell supernatants on activated PBMCs, we

352   performed *in vitro* proliferation and migration assays (Fig. 6A). Mitogen (phytohaemagglutinin,

353   PHA)-activated PBMCs were incubated for 5 d in the presence or absence of serum-containing

354   CM from the different cell types. Proliferation assays revealed that UCSCs supernatants

355   blocked PBMCs proliferation ($p = 0.06$) when compared with PHA-treated controls (Fig. 6A

356   and B). CM from ACs did not block PBMCs proliferation, while HFPSCs (71 ± 5 %) and

357   SMSCs (68 ± 3 %) had a minor effect. In migration assays, both UCSCs and SMSCs blocked

358   the migration of activated PBMCs compared to positive controls (38 ± 2.5 % and 38 ± 1.2 %

359   vs 44.2 ± 0.5 %, respectively) (Fig. 6B). To further investigate the immunomodulatory effects

360   of MSCs supernatants, we measured the expression of TNF-α and IFN-γ in PBMCs-CM. Values

361   were normalised against residual expression levels present in supernatants of stromal cells.

362   Supernatants from HFPSCs and SMSCs stimulated the production of TNF-α and IFN-γ above

363   the levels achieved by PHA treatments. Importantly, supernatants from UCSCs suppressed the

364   production of both TNF-α and IFN-γ by activated PBMCs, reaching significant differences

365   when compared to HFPSCs (Fig. 6C).

366   **UCSCs supernatants exert superior anti-inflammatory effects on M1 activated**

367   **macrophages**

368   Macrophage polarisation assay was performed as previously reported (Ambarus *et al.*, 2012;

369   Vogel *et al.*, 2014). A panel of costimulatory molecules and cytokines to characterise

370   macrophage polarisation was chosen based on validation tests using different stimulants. We

371   observed divergent expression of these markers in the presence of different stimulants. In

372   addition, we found IL-10 production as an irrelevant marker for M2 polarised macrophages. In

373   the validation study, we observed increased IL-10 production in the presence of LPS and IFN-

374   γ compared to dexamethasone or TGF-β and IL-4 stimulation. These discrepancies have also

375   been reported in other studies (Chanteux *et al.*, 2007; Vogel *et al.*, 2014). Moreover, we also

376   observed CD163 as a suitable marker for dexamethasone-stimulated M2 polarised

377   macrophages. The discrepancy concerning the expression of CD206 has also been demonstrated

378   in a previous study (Jaguin *et al.*, 2013).

379   In this study, immunomodulatory effects on M1 polarised macrophages were investigated by

380   characterisation of surface markers expression of CD40, HLA-DR, CD64, CD80, CD86 and

381   inflammatory cytokines release (Fig. 7). Supernatants from all stromal cell types suppressed

382   the surface expression of HLA-DR on activated macrophages, whereas only UCSCs

383   supernatants were able to significantly suppress the expression of CD40. On the other hand,

384   supernatants from ACs were able to increase the expression of the co-regulatory receptors

385   CD80 and CD86 above the levels of M1 activation (Fig. 7B). In contrast to ACs and UCSCs,

386   supernatants from HFPSCs and SMSCs increased the surface expression of CD64 above M1

387   activation levels. None of the supernatants was able to alter the expression of the M2 phenotype

388   marker CD163. Regarding cytokines profiles, CM from all cell types was able to reduce the

389   production of TNF-α, IL-6 and IL-12 by M1-M. Of note, a significant reduction of IL-6 and IL-

390   12 concentration was only achieved by UCSCs (Fig. 7C).

391   **Discussion**

392   The main objective of this study was to ascertain which source of stromal cells possesses the

393   most favourable phenotype for the treatment of hyaline cartilage lesions or chronic

394   inflammatory joint disorders. Given the importance of paracrine signalling of MSCs, we

395   performed large-scale comparative analyses of cell secretomes and conducted functional

396   studies with cell supernatants on immune cells to compare the constitutive immunomodulatory

397   capabilities of different MSCs. Overall, our results demonstrate that stromal cells from

398   umbilical cord matrix exhibit better anti-inflammatory and trophic effects when compared with

399   ACs, HFPSCs and SMSCs.

400   In our study, all cell sources have been expanded in monolayer cultures in the serum-

401   supplemented medium for some weeks, as done in standard cell transplantation procedures. To

402   facilitate the analyses of secretory profiles by LC-MS/MS proteomics, the media were

403   conditioned under serum-free conditions. Multiplex protein assays were performed with the

404   same serum-free CM that was used for proteomics, which allowed us to make direct

405   comparisons of results. However, functional assays with immune cells were done with serum-

406   supplemented CM, as serum deprivation has been shown to affect proliferation and induce

407   apoptosis in lymphocytes and macrophages, respectively (Sato *et al.*, 2009; Wei *et al.*, 2006).

408   Short periods of serum deprivation have been shown to not affect the cell viability of

409   mesenchymal cells (Boraldi *et al.*, 2008). However, some changes in the secretome could occur

410   upon changes in serum supplementation. We have analysed in parallel the expression of TNF-

411   α, IFN-γ, IL-6 and IL-12 in both serum-containing and serum-free CM from all four cell types

412   and only the expression of IL-6 was considerably changed in the presence of serum (Fig. 8).

413   Although we expect only minor phenotypic changes in cells associated with serum presence,

                                                                                                17

414   alterations in the expression of some bioactive molecules could occur and should be taken into

415   consideration.

416   Currently, MSCs are viewed as "drugstores" with the potential to modulate the phenotype,

417   migration and activation of resident tissue and inflammatory cells (Caplan and Correa, 2011).

418   These have lead researchers to study MSC-mediated paracrine effects and profiles of secreted

419   proteins from different mesenchymal stromal cell types. Previous studies comparing secretory

420   profiles from different MSCs sources highlight the existence of differentially expressed factors

421   with impact on angiogenesis, matrix remodelling, inflammation and immunosuppression

422   (Amable *et al.*, 2014; Dabrowski *et al.*, 2017; Hsiao *et al.*, 2012; Li *et al.*, 2015). Our qualitative

423   analyses using large-scale proteomic approach reveal similar protein profiles, where the

424   majority of identified proteins are present in all cell supernatants. However, after hierarchical

425   clustering of protein profiles from all donors, UCSCs secretomes single out from the other adult

426   cell sources (Fig. 1B). Quantitative analyses of the secretome data reveal that proteins involved

427   in cell signalling such as TGF-β1 and PDGFD were significantly upregulated in UCSCs

428   supernatants, while catabolic proteins such as MMPs, serpins, and complement factors were

429   downregulated compared to stromal cells from the adult origin. TGF-β1 is a master driver of

430   chondrogenesis and has been shown to ameliorate OA pathogenesis (Tang *et al.*, 2015; Zhang

431   *et al.*, 2015). In addition, TGF-β1 has been shown to possess anti-apoptotic effects (Rehman *et*

432   *al.*, 2004). Observations from other studies are disparate and include cell sources that we have

433   not used; however, the superior anabolic phenotype of UCSCs, including highest expression of

434   TGF-β among the compared cell types, has also been observed recently by others (Dabrowski

435   *et al.*, 2017). Data from our multiplex protein analyses also confirmed the findings of LC-

436   MS/MS. It revealed a significant reduction of MMPs and increased expression of TGF-β1 by

437   UCSCs, thus reasserting observations made in the large-scale proteomic approach. Altogether,

438 these observations highlight less catabolic phenotype of UCSCs compared to the three other

439 adult MSCs in the context of cartilage repair.

440 The immunomodulatory profile of the different MSCs was also investigated by proteomics and

441 multiplex arrays. Secretome analyses revealed comparable expression of complement

442 components, heat shock proteins, galectins and immunoregulators such as CSF-1, MCP-1, MIF

443 and TGF-β1 among the different cell sources. In addition, our data from multiplex protein

444 arrays showed enhanced expression of the immunomodulators IL-6, MCP-1 and PGE2, and

445 reduced expression of IL-17 and MIP-1α by UCSCs. IL-6 has an omnidirectional role in

446 maintaining biological functions. It has been reported to have deleterious effects in the joint

447 (Poree *et al.*, 2008; Sui *et al.*, 2009). However, selective depletion of IL-6 in animals is

448 associated with accelerated joint degeneration upon ageing (de Hooge *et al.*, 2005). Other

449 authors have demonstrated IL-6-dependent inhibition of local inflammation in experimental

450 arthritis (Bouffi *et al.*, 2010).

451 MCP-1 (also called CCL2), MIP-1α (CCL3) and IL-17 are all potent inflammatory factors

452 mediating recruitment and activation of myeloid cells. Their presence is associated with

453 cartilage degeneration and progression of OA (Appleton *et al.*, 2015; Snelling *et al.*, 2017;

454 Wang *et al.*, 2017; Xu *et al.*, 2015; Zhao *et al.*, 2015). Mechanistically, IL-17 has been reported

455 to inhibit chondrogenesis and promote MMPs in chondrocytes (Benderdour *et al.*, 2002; Kondo

456 *et al.*, 2013). PGE2 is known to regulate the phenotype and functions of pro-inflammatory

457 macrophages and NK cells (Manferdini *et al.*, 2017), however, the overall role of this factor in

458 OA progression and cartilage homeostasis is still controversial (Bouffi *et al.*, 2010; Miwa *et al.*,

459 2000; Otsuka *et al.*, 2009). Despite the difficulty to reach general conclusions due to the

460 pleiotropic nature of many chemokines and cytokines, our observations based on the global

461 expression of released factors indicate that UCSCs display a more immunosuppressive and anti-

462 inflammatory phenotype than their adult counterparts.

463 To investigate further the paracrine potential of the different MSCs on immunomodulation we

464 conducted functional assays on lymphocytes and macrophages. Results follow the same trend

465 as the analyses made on protein profiles, highlighting the superior immunosuppressive

466 phenotype of UCSCs compared to the other MSCs. The ability of MSCs to regulate

467 inflammation and immunity has been the focus of intense research during recent years (Donders

468 *et al.*, 2018; von Bahr *et al.*, 2012). Many *in vitro* studies have shown that mesenchymal cells

469 from multiple sources, including differentiated connective tissue cells such as chondrocytes and

470 fibroblasts, have the potential to regulate inflammation and T-cell functions to some extent

471 (Bouffi *et al.*, 2011; Lohan *et al.*, 2016). Still, there is no consensus on which cell source is the

472 most powerful in this respect.

473 Most published studies have compared bone marrow, adipose tissue and foetal tissues with

474 different outcomes. Some comparative studies demonstrated bone marrow MSCs have slightly

475 superior immunosuppressive capacity than other MSCs (Heo *et al.*, 2016; Karaoz *et al.*, 2017).

476 In line with our observations, some groups have previously observed superior

477 immunosuppressive abilities by MSCs from cords (Jin *et al.*, 2013; Najar *et al.*, 2012). It is

478 important to mention that in our study MSCs were not primed with pro-inflammatory cytokines

479 during medium conditioning. Such experimental condition allowed us to investigate the

480 constitutive abilities of these cells without external stimuli. Priming of MSCs is extensively

481 documented in the published literature and has been recommended as a mandatory step to

482 unleash the full immunosuppressive potential of MSCs (Gomez-Aristizabal *et al.*, 2017; Najar

483 *et al.*, 2012; van Buul *et al.*, 2012). In agreement with our study, constitutive immunoregulation

484 by unstimulated MSCs has been previously observed (Saulnier *et al.*, 2015). In this context, *ex*

485 *vivo* priming of MSCs has been related with few controversial outcomes *in vivo* describing

486 increased immunogenicity of pre-stimulated MSCs (Papadopoulou *et al.*, 2012; Treacy *et al.*,

487 2014).

488   In clinical settings, MSCs from different sources have been investigated for the treatment of

489   focal cartilage lesions and OA (Lee and Wang, 2017). Articular chondrocytes, bone marrow

490   and adipose tissue stromal cells are the most commonly used sources for cartilage repair (Vonk

491   *et al.*, 2015). These studies mostly assessed safety and efficacy of used MSCs for specific

492   clinical implications. However, a comparative study in humans argued that autologous SMSCs

493   exert superior healing outcomes (Akgun *et al.*, 2015). On the other hand, in preclinical models,

494   MSCs from cords have been shown to exert immunosuppression and disease regression in

495   experimental models of OA and autoimmune disorders (Donders *et al.*, 2015; Saulnier *et al.*,

496   2015; Yang *et al.*, 2015). In contrast to MSCs from autologous sources, there are few ongoing

497   trials exploring the potential of allogeneic UCSCs for both OA management and focal cartilage

498   repair in humans (NCT02580695, NCT02291926, NCT03166865 and NCT03358654), without

499   published outcomes hitherto. Confirming the results of this comparative study in suitable

500   animal models would provide more insight into the use of UCSCs in the clinics.

Conclusions

502   Traditionally, the regenerative potential of MSCs has been directly linked to their multipotent

503   differentiation and tissue-forming capabilities. Nowadays, increasing attention is given to their

504   role as cellular modulators. In the field of articular cartilage lesions and degenerative joint

505   diseases, there is no consensus on the best cell source for treatment. Considering the relevance

506   of paracrine signalling, in this study we have compared the secretomes among MSCs from

507   different sources. Both the molecular analyses and the functional assays indicate that UCSCs

508   display superior anti-inflammatory and trophic effects compared to other MSCs from adult

509   tissues. The hypoimmunogenic nature of UCSCs, along with their high abundancy, simple

510   isolation and favourable protein profiles makes this cell source an attractive tool for off-the-self

511   allogeneic adjuvant therapy.

Availability of data and materials

The datasets used in this study are available from the corresponding author upon request.

Authors' Contributions

AI primarily conducted the laboratory work, planned the study and prepared the manuscript. IMZ participated in the conception of the study, data evaluation, edited and approved the final draft of the manuscript. IU and JAB performed LC-MS/MS. All authors contributed to the data interpretation of results, provided direction and comments on the manuscript.

Ethical statement

The Regional Ethical Committee of Northern Norway has approved the study (REK Nord 2014/920 and 2010/586).

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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759   **Figure legends**

760 **Figure 1. Hierarchical clustering of identified proteins from secretomes of ACs, HFPSCs,**

761 **SMSCs and UCSCs. A.** Venn diagram depicts the percentage of identified proteins shared

762 among four different stromal cell types. **B.** Dendrogram shows two major clusters of four

763 different stromal cell types. All cell sources from adult mesenchyme origin clustered together,

764 whereas the four donors of UCSCs from extra-embryonic origin clustered separately.

765 **Figure 2. Protein expression analysis by LC-MS/MS from conditioned medium of ACs,**

766 **HFPSCs, SMSCs and UCSCs. A.** Distribution of identified proteins into six main categories

767 according to their function annotated using GOBP terms. **B.** Volcano plot illustrates the results

768 of the six sets of statistical comparisons made between HFPSCs vs ACs, SMSCs vs ACs,

769 HFPSCs vs SMSCs, UCSCs vs ACs, UCSCs vs HFPSCs and UCSCs vs SMSCs. These plots

770 show each protein with $-$Log10 (*p*-value) and Log10 of fold change of the comparison on the

771 Y-axis and X-axis, respectively. Proteins with greater fold change and lower *p*-value are plotted

772 further away from zero on each axis. Proteins that are significantly up and down-regulated ($p <$

773 0.01) are presented in green and red colour, respectively.

774 **Figure 3. Comparative expression of selected proteins from conditioned medium of ACs,**

775 **HFPSCs, SMSCs and UCSCs.** The heat map shows proteins involved in **A.** ECM remodelling

776 (proteases and inhibitors), **B.** cell signalling (growth factors) and **C.** inflammation/immune

777   responses. Down-regulated proteins are indicated in red, whereas up-regulated proteins are in

778   blue.

779   **Figure 4. Comparison of identified cytokines and chemokines from conditioned medium**

780   **of ACs, HFPSCs, SMSCs and UCSCs.** Dot density show concentration of cytokines involved

781   in inflammation (IL-4, IL-6, IL-8, IL-12, IL-17 and PGE2) and chemokines (MCP-1, MIP-1α

782   and RANTES) detected in supernatants of four different stromal cell types. Level of

783   significance is p (*) < 0.05.

784   **Figure 5. Comparison of identified MMPs and anabolic factors from conditioned medium**

785   **of ACs, HFPSCs, SMSCs and UCSCs.** Dot density shows the concentration of MMPs (MMP-

786   1, MMP-3, MMP-7 and MMP-9) and growth factors (TGF-β1, bFGF and BMP-2) detected in

787   supernatants of four different stromal cell types. Level of significance is p (*) < 0.05.
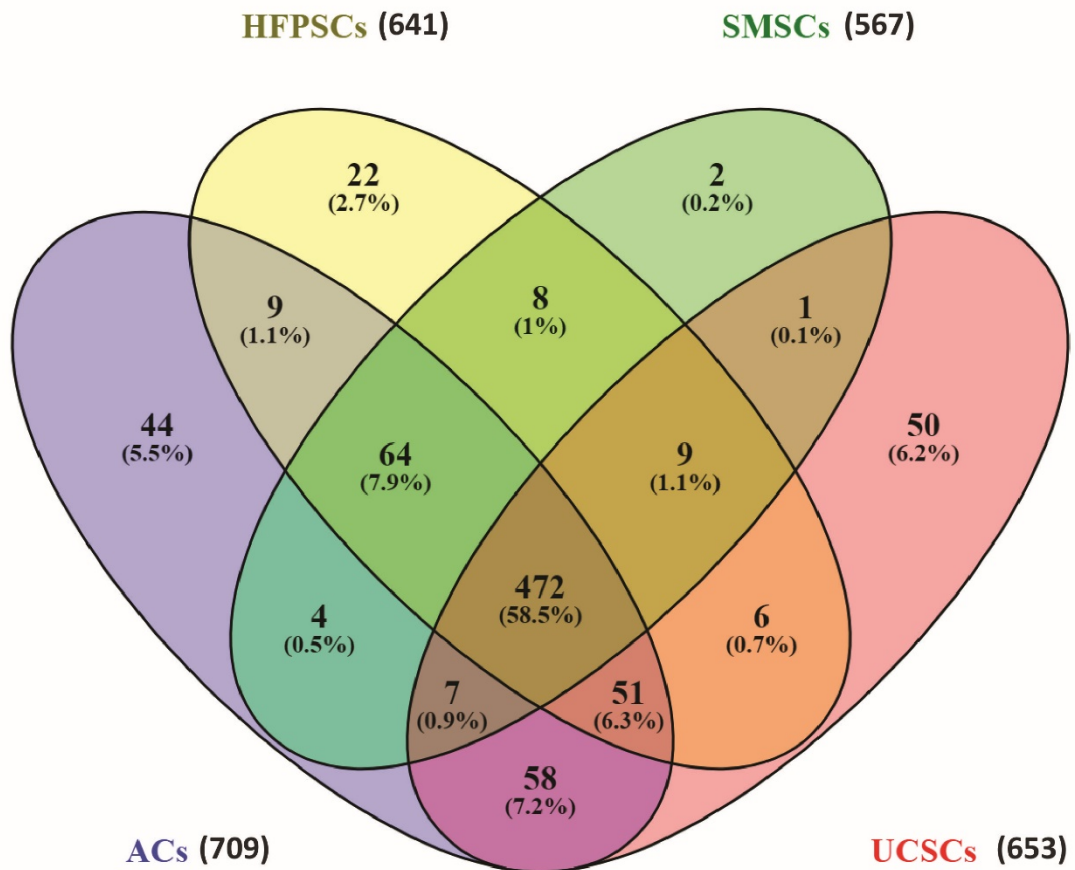
788   **Figure 6. Differential modulation of PBMCs activation by MSCs conditioned medium. A.**

789   PBMCs proliferation assay: representative flow cytometry dot plots depict the percentage of

790   CFSE labelled PBMCs stimulated with 10 ug/mL of PHA in presence and absence of CM from

791   the four different stromal cell types. **B.** Quantitative analyses of PBMCs proliferation and

792   migration in presence and absence of CM from four different stromal cell types. SDF-1 at 100

793   ng/mL was used for chemo-attraction in migration assays **C.** Total concentration of TNF-α, and

794   IFN-γ detected in PBMCs-CM (10^6 cells/well) after incubation with PHA and CM from four

795   different stromal cell types. Level of significance is p (*) < 0.05.

796   **Figure 7. UCSCs secretomes can modulate macrophage-mediated inflammation. A.**

797   Characterisation of surface molecules during polarisation of M0-M into M1-M (CD40, CD64,

798   CD80, CD86 and HLA-DR) and M2-M (CD163) by flow cytometry. Red, blue and orange peak

799   represents isotype control, M0-M and activated macrophages, respectively. **B.** Dot density

800   depicts M1-M activation and distinct blocking of M1-M activation by CM from ACs, HFPSCs,

801   SMSCs and UCSCs. M2-M polarisation was used as a negative control for surface expression

802   of CD40 and HLA-DR. **C.** Levels of TNF-α, IL-6 and IL-12 detected in macrophage culture

803   conditioned medium (2.5×10^6 cells/well) after incubation with CM from four different stromal

804   cell types. Level of significances are $p < 0.05$ (*) and $p < 0.005$ (**).

805   **Figure 8. Comparison between serum-free and serum-containing conditioned medium of**

806   **ACs, HFPSCs, SMSCs and UCSCs.** Presence of serum increases the production of IL-6 by
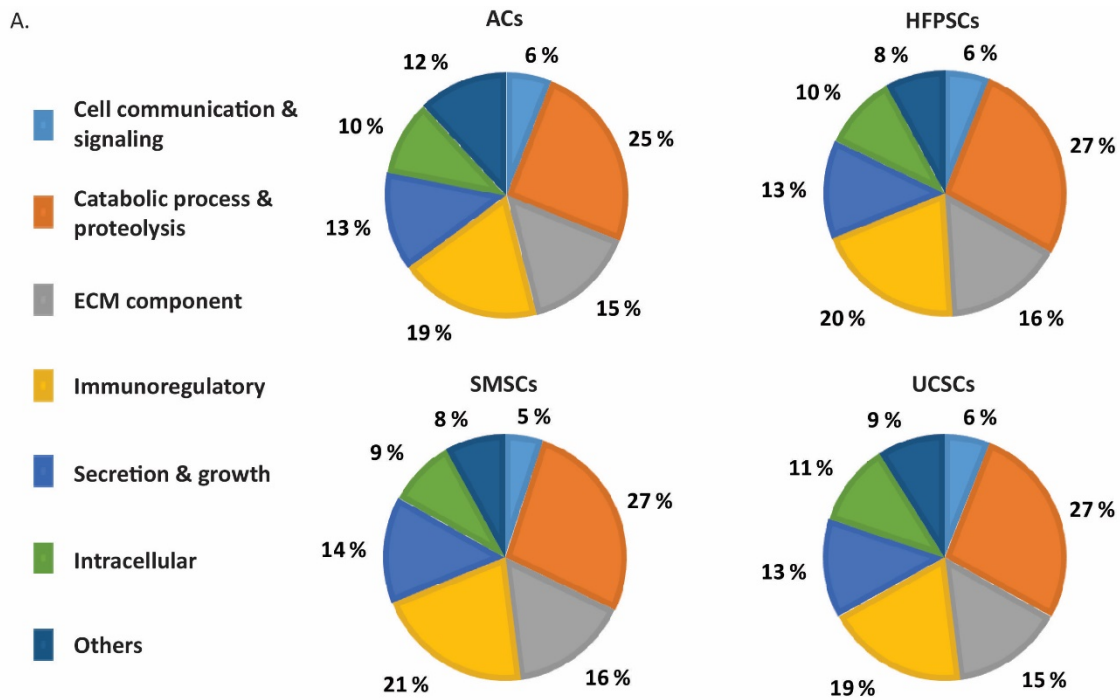
807   all cell types.

A.

HFPSCs (641)   SMSCs (567)

22 (2.7%)

2 (0.2%)

9 (1.1%)

8 (1%)

1 (0.1%)

44 (5.5%)

64 (7.9%)

9 (1.1%)

50 (6.2%)

472 (58.5%)

4 (0.5%)

6 (0.7%)

7 (0.9%)   51 (6.3%)

58 (7.2%)

ACs (709)   UCSCs (653)

B.

UCSCs 3
UCSCs 1
UCSCs 4
UCSCs 2
SMSCs 4
ACs 4
ACs 3
ACs 2
ACs 1
SMSCs 1
SMSCs 2
SMSCs 3
HFPSCs 3
HFPSCs 2
HFPSCs 4
HFPSCs 1

808

809   Figure 1: Hierarchical clustering of identified proteins from secretomes of ACs, HFPSCs,
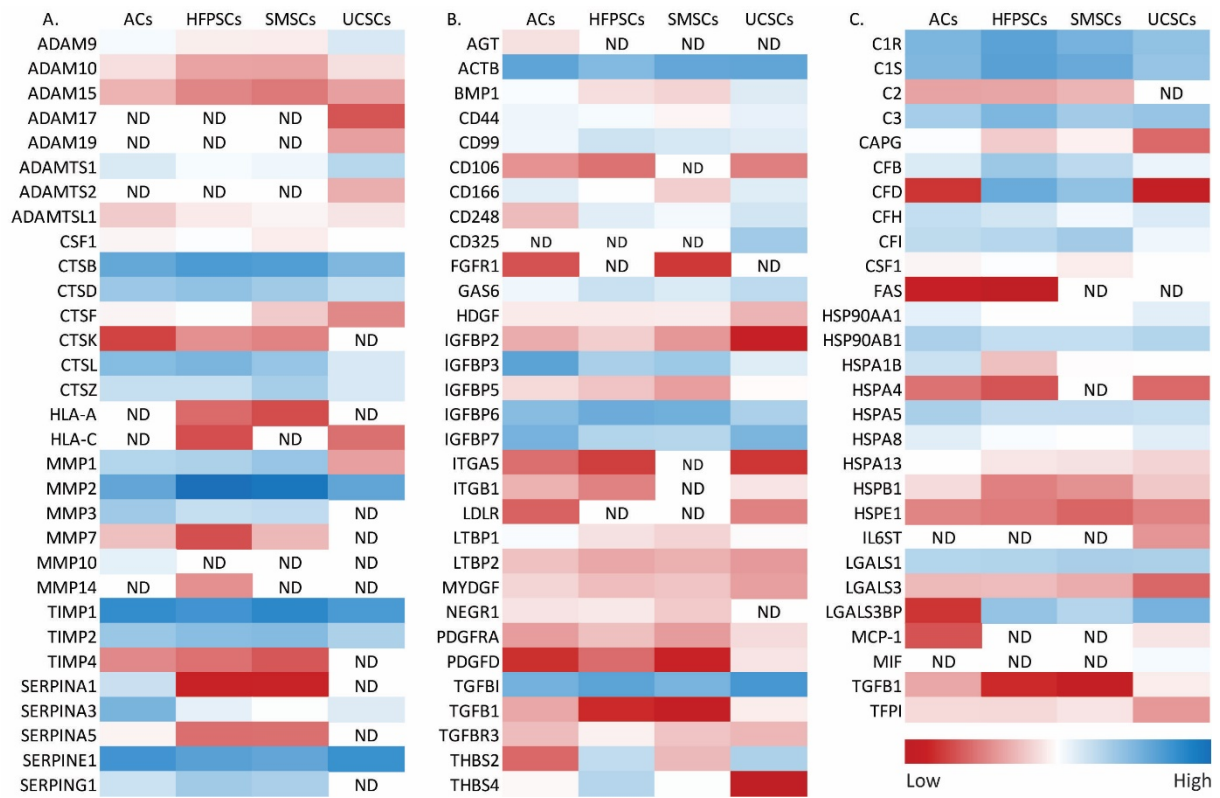
810   SMSCs and UCSCs.

811
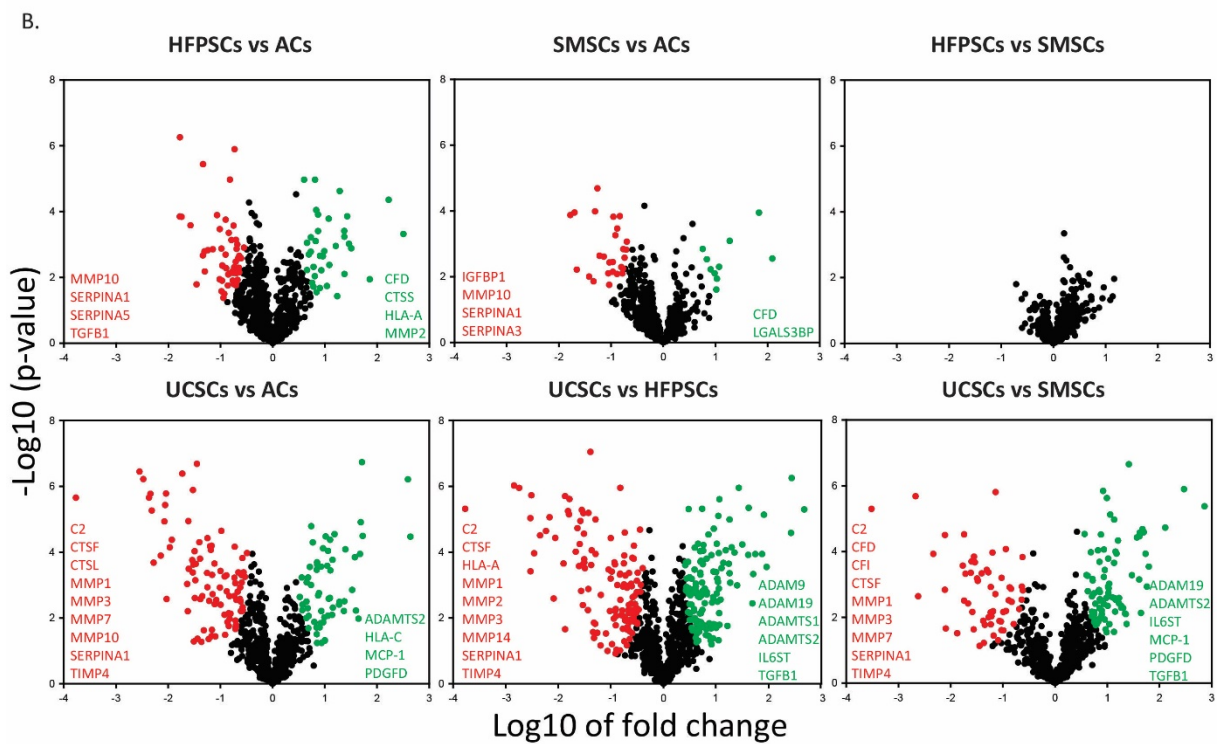
Figure 2. Protein expression analysis by LC-MS/MS from conditioned medium of ACs, HFPSCs, SMSCs and UCSCs.

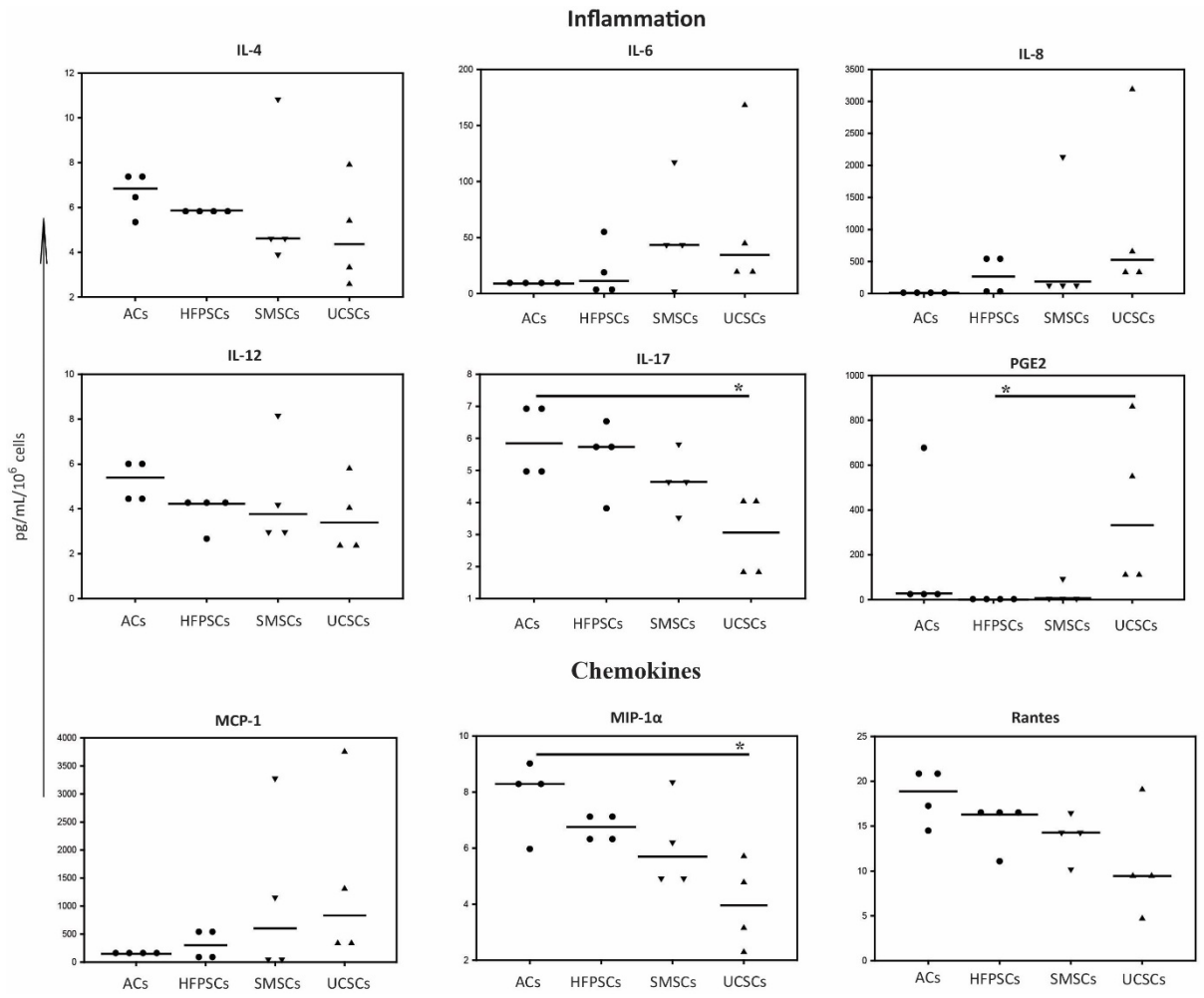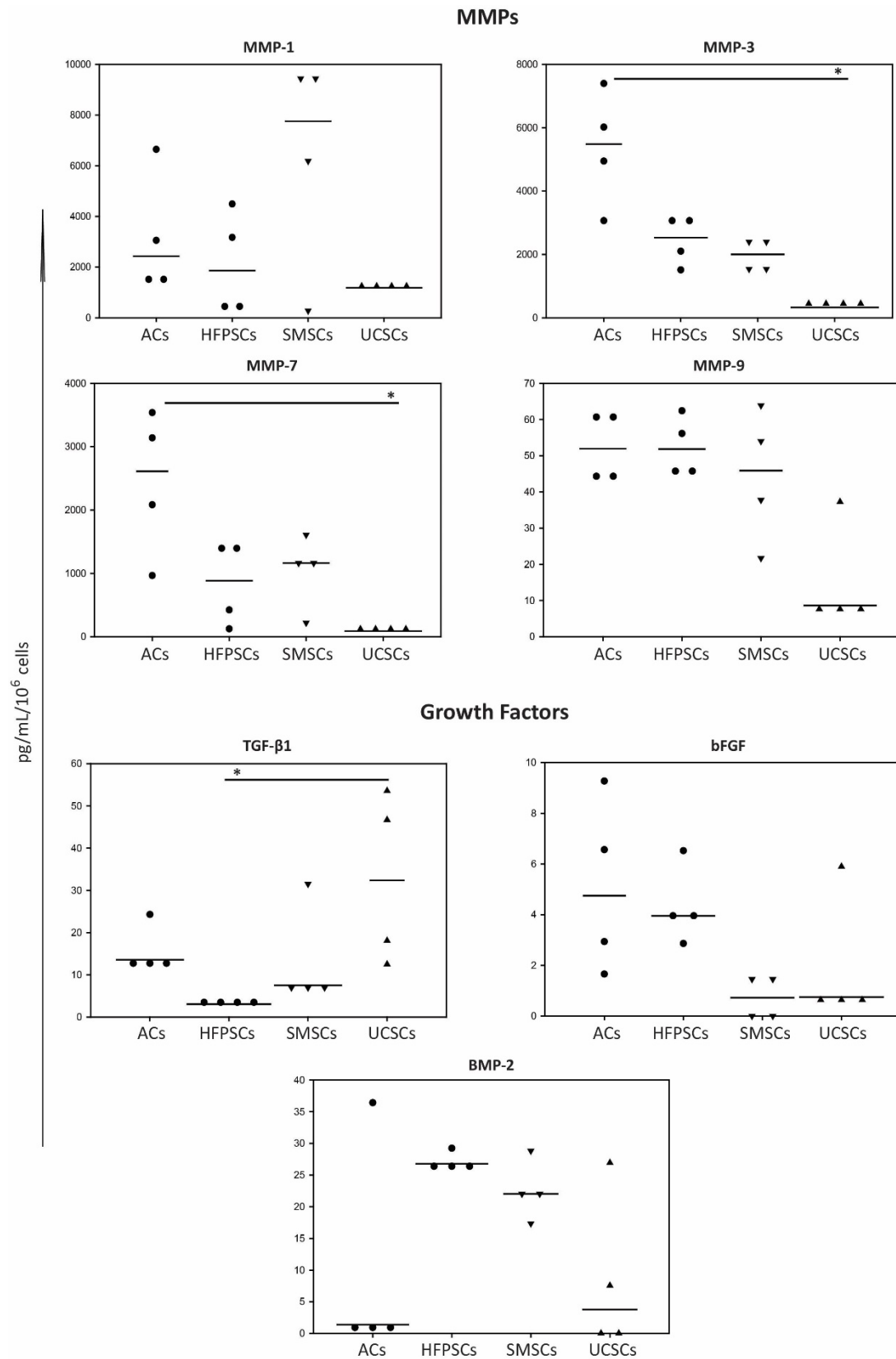Figure 3. Comparative expression of selected proteins from conditioned medium of ACs, HFPSCs, SMSCs and UCSCs.
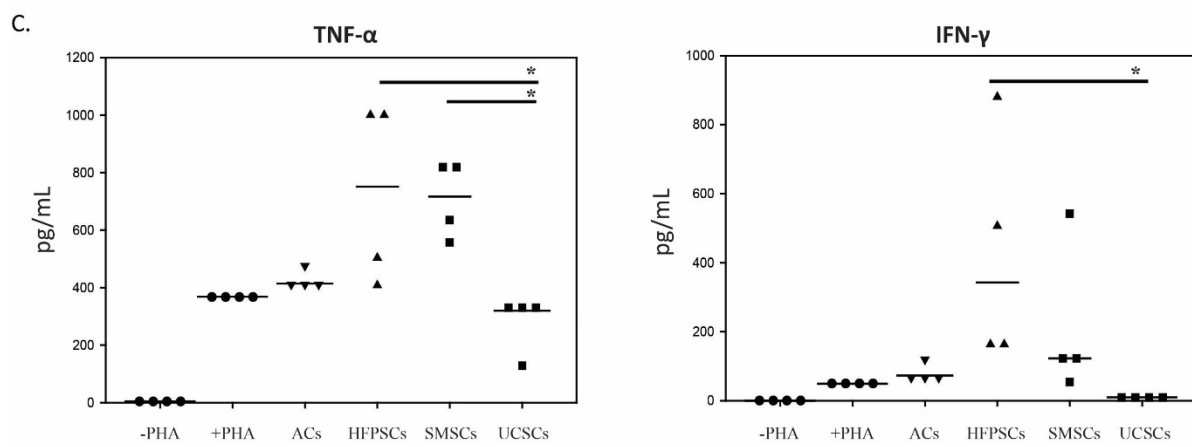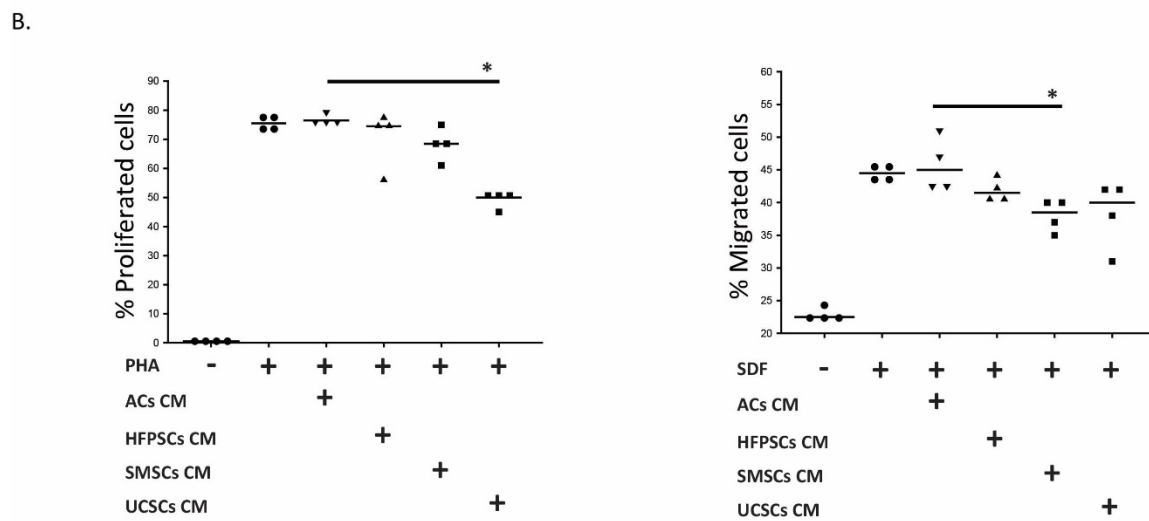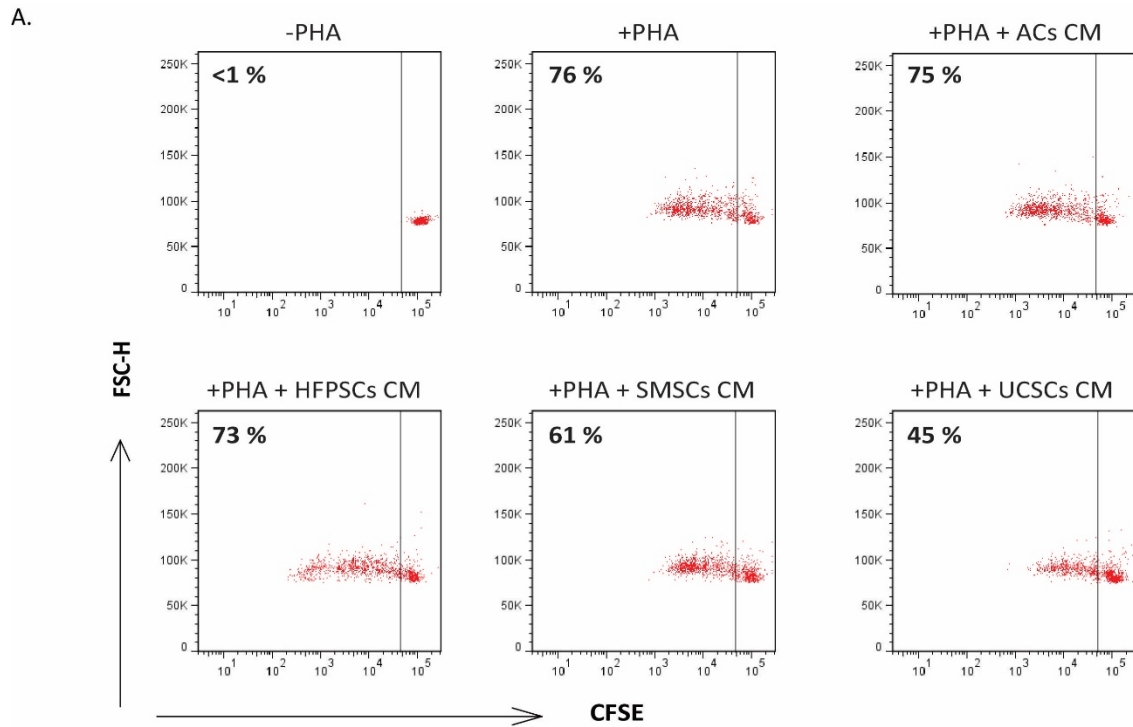
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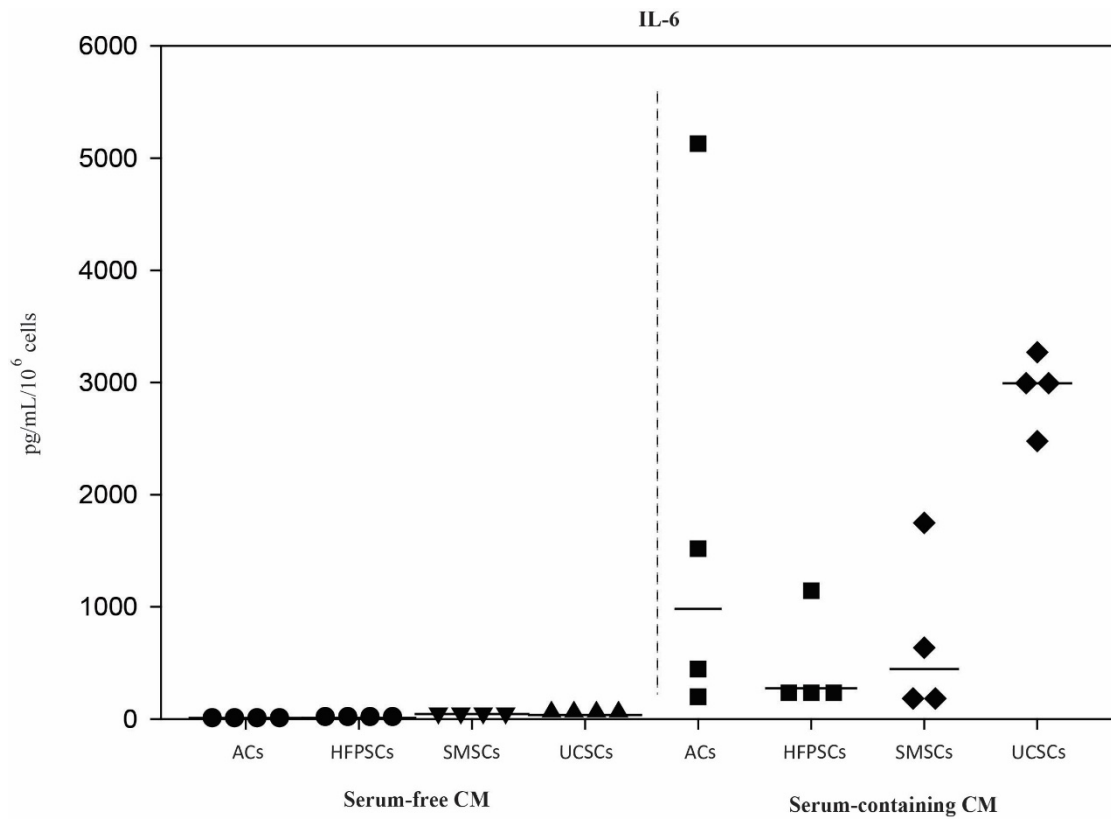
826

827   Figure 6. Differential modulation of PBMCs activation by MSCs conditioned medium.

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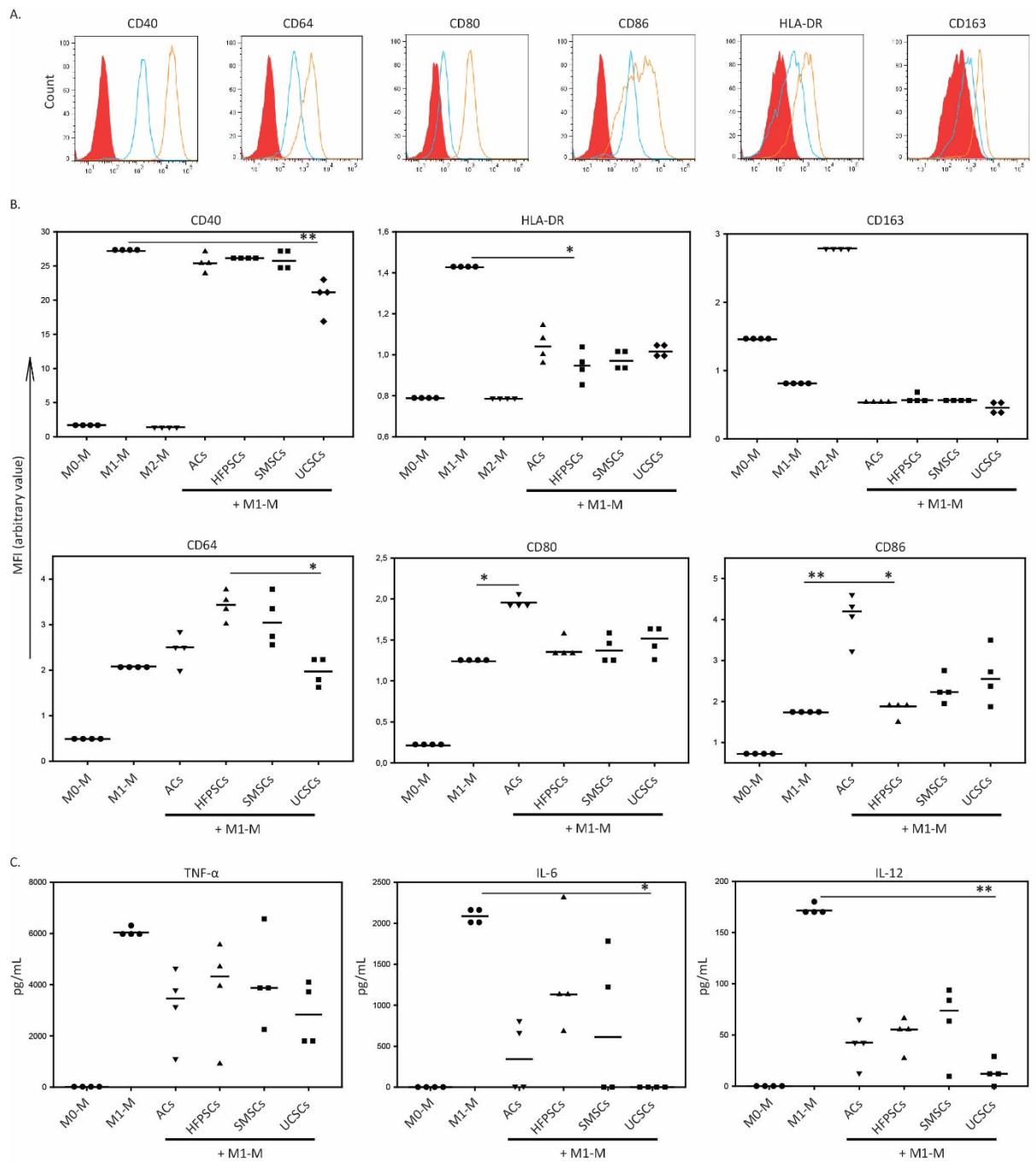
Figure 7. UCSCs secretomes can modulate macrophage-mediated inflammation.

830

831   Figure 8. Comparison between serum-free and serum-containing conditioned medium of ACs,

832   HFPSCs, SMSCs and UCSCs.

Paper III

1 ***In vitro* chondrogenic potency of surplus chondrocytes from autologous**

2 **transplantation procedures do not predict short-term clinical outcomes**

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Abstract

Background: Autologous chondrocyte implantation (ACI) has been used over the last two decades for the treatment of focal cartilage lesions to prevent the onset of osteoarthritis; however, some patients do not respond adequately to the procedure. A number of biomarkers that can forecast the clinical potency of the cells have been proposed, but evidence for the relationship between *in vitro* chondrogenic potential and clinical outcomes is missing. In this study, we explored if the ability of cells to make cartilage *in vitro* correlates with ACI clinical outcomes. Additionally, we evaluated previously proposed chondrogenic biomarkers and searched for new biomarkers in the chondrocyte proteome capable of predicting clinical success or failure after ACI.

Methods: The chondrogenic capacity of chondrocytes derived from 14 different donors was defined based on proteoglycans staining and visual histological grading of tissues generated using the pellet culture system. Lysholm score of 65 two years post-ACI was used as a cut-off to categorise "success" and "failure" clinical groups. A set of predefined biomarkers were investigated in the chondrogenic and clinical outcomes groups using flow cytometry and qPCR. High-throughput proteomics of cell lysates was used to search for putative biomarkers to predict chondrogenesis and clinical outcomes.

Results: Visual histological grading of pellets categorised donors into "good" and "bad" chondrogenic groups. Direct comparison between donor-matched *in vitro* chondrogenic potential and clinical outcomes revealed no significant associations. Comparative analyses of selected biomarkers revealed that expression of CD106 and TGFβR3 was significantly enhanced in the bad chondrogenic group, while expression of ITGA1 and ITGB1 was significantly upregulated in the good chondrogenic group. Additionally, significantly increased surface expression of CD166 was observed in the clinical success group, while COMP was significantly downregulated. High throughput proteomics revealed no differentially expressed

proteins from success and failure clinical groups, whereas only seven proteins including prolyl-4-hydroxylase 1 (P4HA1) were differentially expressed when comparing chondrogenic groups.

Conclusion: The present study indicates that the *in vitro* cartilage-forming capacity of donor-matched chondrocytes does not correlate with clinical outcomes, and argue on the limitations of using the chondrogenic potential of cells or markers for chondrogenesis as predictors of clinical outcomes.

Introduction

Articular cartilage injuries may develop into osteoarthritis (OA) [1]. However, the management of cartilage lesions in the synovial joints still represents a weighty clinical challenge. Since the mid 90's autologous chondrocyte implantation (ACI) has been available as a method to ameliorate these impairing localised cartilage defects [2]. Successful clinical outcomes of ACI have been reported for up to 20 years [3, 4]. The original technique has experienced refinements such as the introduction of collagen membranes to replace periosteum to cover the defect, the use of characterized chondrocytes to improve the quality of the repair tissue or the more recently matrix-assisted chondrocyte implantation (MACI) where the chondrocytes are seeded in a collagen matrix before implantation [5, 6]. The long-term failure rate of the first generation procedure is in the range between 20-40 % after 15 years [7, 8], while five-year failure rate of MACI is reported to be 11 % [9], mind that the definition of failure is not directly comparable between studies.

To improve the decision-making process around the choice of treatment for patients with localised cartilage defects, it would be of great advantage to have a tool to identify those likely to obtain an optimal outcome of the procedure. Some patient characteristics have been identified, and although the reports are not unanimous, most agree on patient age, preoperative Lysholm scores, previous surgeries to the index knee and defect location and age being linked to the surgical outcome [10-12]. Further stratification methods have been pursued by trying to identify biomarkers linked to clinical outcomes from liquid biopsies. Wright *et al*. reported that increased levels of CD14 and ADAMTS-4 in the preoperative synovial fluid was linked to the poor outcome of the ACI [13]. Some few other studies have assessed synovial fluid or serum for biomarkers of cartilage injury treatment from which limited putative predictive biomarkers have been identified [14, 15]. Additionally, molecular biomarkers to predict treatment outcomes have been explored from the cell sources used in the procedures. Thus, markers found

4

91 in monolayer cultures such as collagen type II A1 (COL2A1), aggrecan (ACAN), fibroblast

92 growth factor receptor 3 (FGFR-3) and bone morphogenic protein 2 (BMP-2) have been

93 associated with cartilage formation *in vivo* in a murine model [16]. On the contrary, Stenberg

94 *et al*. performed a global microarray analysis of surplus cells from ACI and found no links

95 between clinical outcomes and genes linked to cartilage formation *in vivo* [17].

96 In the past, it has been demonstrated that even after applying identical isolation and culture

97 conditions, human chondrocytes from different individuals display strikingly different *in vitro*

98 chondrogenic capacity [18, 19]. Based on such findings, researchers have tried to search for

99 markers that forecast cell chondrogenicity from *in vitro* expanded cells, in order to recognise

100 the quality of the cells from donors and possibly to improve the quality of the generated tissue

101 [20-23]. However, evidence to support the relationship between the *in vitro* chondrogenic

102 potency of cells before the implantation and clinical outcomes is lacking. Therefore, it is

103 uncertain whether markers of intrinsic chondrogenic potency could be used as prognostic and

104 quality measures in clinical practice.

105 In this study, we have explored first if the *in vitro* chondrogenic potency of leftover cells from

106 ACIs established in pellet cultures could be used as a convenient and reproducible functional

107 bioassay to predict clinical outcomes. Secondly, we evaluated if previously reported markers

108 have predictive clinical or chondrogenic value in our material. Finally, we investigated whole

109 cell lysates by quantitative high-throughput proteomics to identify yet unknown molecular

110 biomarkers that can predict chondrogenesis and clinical outcomes.

111

Materials and Methods

Human materials and cell isolation

114 Chondrocytes were surplus cells from 14 patients treated with autologous chondrocyte

115 implantation and were acquired after the written consent of the patients and approval from the

5

116   regional ethics committee (REK Nord 2014/920). The isolation protocol has been described

117   previously [24]. Briefly, the ~200 mg cartilage specimens were kept in 0.9 % NaCl for

118   maximum 2 hours before mincing to ~1 mm^3 pieces and enzymatic digestion for 3-4 hours in

119   DMEM/HAM's F-12 (Cat. no. T 481-50, BioChrom Labs) containing collagenase XI (Cat. no.

120   C-9407, Sigma-Aldrich) at a final concentration of 1.25 mg/mL. Chondrocytes released from

121   matrix were serially expanded in DMEM/HAM's F-12 supplemented with 10 % human

122   autologous serum until implantation (passage 3). Surplus cells used in the following

123   experiments were propagated in high glucose Dulbecco's Modified Eagle Medium (DMEM;

124   Cat. no. D5796; Sigma-Aldrich) supplemented with L-ascorbic acid (62 mg/L) (Cat.

125   no.103033E; BDH Laboratory), penicillin and streptomycin (1 %) (P/S; Cat. no. P4333; Sigma-

126   Aldrich) and 10 % foetal bovine serum (FBS; Cat. no. S0115; Biochrom) at 37 °C in humidified

127   atmosphere containing 5 % CO2. The medium was changed twice a week and passaged upon

128   reaching 70-80 % confluency.

129   **Chondrogenesis and 3D cultures**

130   Chondrogenic potential of dedifferentiated chondrocytes was achieved by using both hanging-

131   drop and pellet culture method. For pellet cultures, *ex vivo* expanded chondrocytes were

132   harvested and prepared at a final concentration of 5 x 10^4 cells/150 µL per pellet as previously

133   described [25]. Briefly, 5 x 10^4 cells/well were placed in poly-HEMA (Cat. no. P3932; Sigma-

134   Aldrich) coated conical-bottom 96 well culture plate (Cat. no. 249935; Thermo Scientific) and

135   centrifuged at 1100g for 10 min to form cell aggregates. For hanging-drops, chondrocytes were

136   dispensed as a 40 µL drop containing 2 x 10^4 cells/drop on the lid of a Petri dish. Aggregates

137   were formed by gravitational forces as the drop was hanging upside down. After 48 hours,

138   spheroids from conical-bottom plates or hanging-drops were collected and cultured on a 24 well

139   ultra-low attachment cell culture plate (Cat. no. 3473; Corning) containing a serum-free

140   chondrogenic medium for 21 d at low oxygen (3 % O$_2$). The chondrogenic medium contained

141 high glucose DMEM, L-ascorbic acid (62 mg/L), P/S (1 %), dexamethasone (1 µg/mL) (Cat.

142 no. PZN-3103491; Galenpharma), Insulin-transferrin-selenium supplement (ITS) (1:1000)

143 (Cat. no. 354351; BD Biosciences), transforming growth factor β1 (10 ng/mL) (TGF-β1; Cat.

144 no. 100-21C; Peprotech) and bone morphogenic protein 2 (100 ng/mL) (BMP-2; Cat. no. 120-

145 02C; Peprotech). Half of the chondrogenic medium was replaced with fresh chondrogenic

146 medium twice a week.

147 **Flow cytometry**

148 Monolayer cultured chondrocytes were harvested and prepared at passage 3-4 for surface

149 marker expression by flow cytometry as previously described [25]. Briefly, chondrocytes were

150 harvested and washed three times with cold stain buffer (Cat. no. 554656; BD Biosciences),

151 filtered through a 70 µm cell strainer and prepared on ice as single-cell suspensions to a final

152 concentration of $<1 \times 10^6$ cells/100 µL and incubated with antibodies at 1:10 dilution for 1 h.

153 Fluorochrome-conjugated antibodies targeting CD44 (Cat. no. 555479), CD106 (Cat. no.

154 561679), CD146 (Cat. no. 561013), CD166 (Cat. no. 560903), CD271 (Cat. no. 560927),

155 isotype control PE Mouse IgG2b (Cat. no. 555743) and isotype control PE Mouse IgG1 (Cat.

156 no. 555749) were purchased from BD Biosciences, USA. Samples were analysed using a BD

157 FACSAria III flow cytometer and FlowJo software (Tree Star Inc., USA). Data from three

158 donors were presented as the average of median fluorescence intensity (MFI) +/- standard error.

159 **Alcian blue staining and Bern score**

160 Metachromatic staining of proteoglycans by Alcian blue was done as previously described [25].

161 Spheroids from pellet cultures ($n = 14$, diameter ≈ 1 mm) and hanging-drops ($n = 4$, diameter

162 ≈ 0.5 mm) were harvested at day 21, washed in DPBS and fixed in 4 % formalin overnight.

163 Fixed spheroids were embedded in 1 % agarose and transferred into a paraffin block. Paraffin-

164 embedded sections (4 µm) were dewaxed and stained with Alcian blue solution (Cat. no.

165 A5268; Sigma-Aldrich) for 30 min. Sections were washed for 2 min in distilled water and

166   counterstained with a Nuclear fast red solution (Cat. no. N3020; Sigma-Aldrich) for 5 min.

167   Finally, the sections were washed and dehydrated by a series of ethanol and xylene wash, before

168   mounting a coverslip with Histokit (Cat. no. 1025/500; Glaswarenfabrik Karl Hect). Sections

169   were imaged by bright field light microscopy (Leica DMI6000B). To quantify the *in vitro*

170   chondrogenic potential, a visual semi-quantitative scoring of tissue sections (Bern score) was

171   applied independently by three different observers [26]. The chondrogenic potential was

172   classified into two groups according to histological outcomes: "Group A" with good

173   chondrogenic potential (Bern score 6-9) and "Group B" with bad chondrogenic potential (Bern

174   score <6) (Table 1).

175   **Clinical outcomes and score**

176   ACI procedure was done as previously described [7]. In this patient cohort, Chondro-Gide®

177   membranes were used to cover the defect [27]. Lysholm score and the knee injury and

178   osteoarthritis outcome score (KOOS) reporting patients' pain, symptoms and disability were

179   recorded at the preoperative stage, one-year and two-year follow-up and subsequently used to

180   evaluate patients' clinical outcomes. We have used Lysholm score of 65 at two-year follow up

181   as a cut-off to categorise clinically success group (>65) and failure group (<65) as suggested

182   by Knutsen *et al*. [7]. Besides, we evaluated clinical outcomes by minimal clinically important

183   difference (MCID), which confers with an increase of 10 points in the Lysholm score after one

184   year of post-treatment, to categorise clinically success group [28]. Both approaches resulted in

185   identical patient distribution between clinical success and failure groups. Patients' demographic

186   data, symptoms, history, functional score, clinical findings and pain as indicated on a visual

187   analogue scale (VAS) were recorded. Patients' demographic characteristics, as well as defect

188   location and size, are summarised in Table 2.

189

8

qPCR

Monolayer chondrocytes were harvested at passage 3-6 at the time of establishment of 3D cultures, and RNA was extracted using the RNeasy Plus Mini Kit (Cat. no. 74134; Qiagen) according to the manufacturer's procedure including DNase I treatment. The RNA concentration was measured using the NanoDrop 2000, and 285 ng of each sample was transcribed to cDNA using the qScript cDNA Synthesis Kit (Cat. no. 95047; Quanta Biosciences). The qPCR reaction included 5 µL PrecisionFAST mastermix (Cat. no. Precision-FAST-R; PrimerDesign), 0.5 µL hydrolysis probe (all from Applied Biosystems), 2.5 µL H_2O and 2 µL cDNA (diluted to 2 ng/µL) and was run in 96-well plates (Cat. no. BW-FAST; PrimerDesign) using the StepOnePlus Real-Time PCR system (Applied Biosystems). Hydrolysis probes are summarised in Table 3. The gene for ribosomal protein L13a (RPL13A) was used as the reference gene, and ΔCq was calculated by subtracting the gene of interest from the reference gene, making higher ΔCq reflect increased gene expression.

Protein extraction and LC-MS/MS analysis

Three donors with extreme scores from each chondrogenic groups and clinical groups were analysed by LC-MS/MS. Monolayer chondrocytes were harvested at passage 3-4, and whole protein was extracted using the TMTsixplex™ Isobaric Mass Tagging Kit (Cat. no. 90064; Thermo Scientific). Briefly, cells were washed 3 times with DPBS and lysed in buffer containing 1 % sodium deoxycholate (Cat. no. D6750; Sigma-Aldrich) and 100 mM triethylammonium bicarbonate (TEAB). Cell lysates were incubated with Pierce™ Universal Nuclease (Cat. no. 88700; Thermo Scientific) at room temperature for 15 min and centrifuged at 16000 g for 10 min at 4 °C. The supernatants were collected, and protein concentration was measured using a DC Protein Assay Kit (Cat. no. 5000116; Bio-Rad). Samples containing 100 µg/tube protein were reduced in 5 mM dithiothreitol (Cat. no. D9779; Sigma-Aldrich) for 30 min at 70 °C and followed by incubation with 375 mM iodoacetamide for 30 min in the dark at room temperature. Samples were precipitated overnight in pre-chilled acetone (Cat. no. 270725;

9

216   Sigma-Aldrich) at -20 °C and collected as dry pellet after centrifugation at 8000 g for 10 min

217   at 4 °C. Protein pellets (25 µg) were resuspended in 2 M Urea (Cat. no. U1250; Sigma-Aldrich)

218   with 50 mM TEAB. Proteins were digested for 6 hours with 1:100 (w/w) lysyl endopeptidase

219   (Cat. no. 125-05061; Wako Chemicals). The samples were further diluted to 1 M Urea and

220   digested overnight by 1:20 (w/w) trypsin (Cat. no. V511A; Promega). Peptides from each

221   sample were labelled with the TMTsixplex^{TM} Isobaric Mass Tagging Kit according to the

222   manufacturer's protocol.

223   OMIX C18 tips were used for sample clean-up and concentration. Peptide mixtures containing

224   0.1 % formic acid (Cat. no. 28905; Thermo Scientific) were loaded to a Thermo Fisher

225   Scientific EASY-nLC1000 system and EASY-Spray column (C18, 2 µm, 100 Å, 50 µm, 50

226   cm). Peptides were fractionated using a 2-100 % acetonitrile (Cat. no. 51101; Thermo

227   Scientific) gradient in 0.1 % formic acid over 180 min at a flow rate of 250 nL/min. The

228   separated peptides were analysed using a Thermo Scientific Q-Exactive mass spectrometer.

229   Data were collected in a data-dependent mode using a Top10 method. Raw data were processed

230   using MaxQuant (v 1.5.6.0) with the integrated Andromeda search engine. MS/MS data were

231   searched against the UniProt human database from November 2016. A false discovery rate

232   (FDR) of 0.01 was needed to yield a protein identification.

233   Statistical validation of protein regulation was performed using the Perseus 1.5.6.0 software.

234   All contaminants were filtered out, and intensity values were log2-transformed for subsequent

235   analysis. The log2-transformed intensities were normalized by adjustment. Data were grouped

236   as group "A (good) and B (bad)" for chondrogenesis and "success and failure" for clinical

237   outcomes. Data were then analysed with a minimum of two valid values in each group. A t-test

238   visualised as a volcano plot was generated to identify potentially regulated proteins in the

239   chondrogenic and clinical groups by a permutation-based FDR < 0.05.

240

Western blots

Three donors from each chondrogenic group were analysed by western blot. The protein input was 35 μg/lane in the TruPage gels (Cat. no. PCG2004; Sigma-Aldrich). The protein was separated along with BLUeye Prestained Protein Ladder (Cat. no. PM007-0500; Sigma-Aldrich) and MagicMark™ XP Western Protein Standard Ladder (Cat. no. LC5602; Novex). Proteins were transferred to PVDF membrane, blocked for 2 h in PBS-Tween (0.05 %) buffer containing BSA (2 %) and incubated with 0.1 μg/mL of prolyl 4-hydroxylase 1 antibody (P4HA1; Cat. no. NB100-57852; Novus Biologicals) overnight at 4 °C. The membrane was incubated with secondary donkey anti-goat antibody (Cat. no. HAF109; Novus Biologicals) for 1 h at room temperature. Finally, a chemiluminescence detection solution (Cat. no. 170-5040, BioRad) was applied to the membrane before acquiring the images using an ImageQuant LAS 4000 CCD camera. Beta-actin antibody (Cat. no. AB8227; Abcam) and goat anti-rabbit antibody (Cat. no. AB6721; Abcam) were used as loading control and secondary antibody for beta-actin, respectively. Relative density was assessed using ImageJ before comparing the two chondrogenic groups.

Statistical analysis

The Bern score between the two chondrogenic groups was plotted as dot density and analysed using Mann-Whitney U comparison. Differences in preoperative, one-year and two-year follow up scores of VAS, Lysholm and KOOS total between two chondrogenic groups were studied using Mann-Whitney U comparison. Differences in gene expression between the chondrogenic groups and clinical groups were analysed using linear regression and Benjamini-Hochberg p-value adjustment. Pearson correlation (*r*) was performed to investigate the relationship between *in vitro* chondrogenic potentials and clinical outcomes. The significance level for all tests was set to < 0.05.

Results

The donor-specific chondrogenic potential of surplus chondrocytes in 3D cultures

In vitro chondrogenic potential of culture-expanded chondrocytes was tested in scaffold-free

3D cultures originated by both pellet and hanging-drop cultures. Chondrocytes from different

donors displayed distinct *in vitro* chondrogenic potential in 3D cultures (Fig. 1A). Pellet

cultures were achievable with cells from all donors. Semi-quantitative assessments of

constructs by visual histological grading system (Bern score) allowed the categorisation of all

donors into two groups: "Group A" (8 donors) and "Group B" (6 donors) with good and bad

cartilage-like characteristics, respectively (Fig. 1B). Hanging-drop cultures were, on the other

hand, successful in half of the donors in group A and none in group B, indicating that the ability

of cells to form cartilage-like micro-tissues by hanging-drops had a positive correlation with

the intrinsic *in vitro* chondrogenic potential in pellets (Table 1). To exclude the possible

influence of passage number in chondrogenic outcomes, chondrogenesis was evaluated for

some donors across passages 3 to 6. Bern score demonstrated no differences in cartilage-like

features in constructs made by same donor-cells across different passages. Donor

characteristics, summarised in Table 1, showed that the distribution of age, gender and passage

is comparable between the two chondrogenic groups. Of note, chondrocytes from a young

patient (age: 19) at low passage number (3) obtained the lowest Bern score (Table 1).

In vitro **chondrogenic potential do not predict clinical outcomes**

To explore if the *in vitro* chondrogenic potency of surplus cells from ACIs could be used as a

functional bioassay to predict clinical outcomes, we compared VAS, total KOOS and Lysholm

score to the chondrogenic groups at baseline, one and two-year after ACI surgery. Patients'

demographic characteristics and defect location and size are summarised in Table 2 along with

the clinical outcomes. Preoperatively, the median VAS score for patients in chondrogenic

groups A and B was 50.50 (interquartile range (IQR) 15.75) and 45 (IQR: 35.75), respectively,

in a scale ranging from 0-100, with 100 representing worst imaginable pain. Median VAS score

at first-year follow-up for group A and B was 36 (IQR: 35.75) and 12.50 (IQR: 15.75), respectively. At one-year follow-up, significantly reduced VAS score was observed in patients from group B compared to group A. At the two-year follow-up, the median VAS score was 44 and 20.50 in group A (IQR: 57.75) and group B (IQR: 25.75), respectively (Fig. 2A). Both KOOS total and Lysholm scores range from 0-100, with 100 representing unimpaired knee function. The median KOOS total preoperatively was 63.30 (IQR: 27.05) and 65.50 (IQR: 36.90), for patients in chondrogenic groups A and B respectively. After one-year follow-up, the median KOOS total was significantly increased in group B (78, IQR: 18.13) compared to group A (54.15, IQR: 26.80). Median KOOS total at the two-year follow-up was 61.60 and 79.50 for group A and B, respectively (Fig. 2B). In addition, preoperative median Lysholm score was 56 (IQR: 3.50) and 57 (IQR: 13.75) in chondrogenic group A and B, respectively. Like VAS and KOOS total at the one-year follow-up, the median Lysholm score in group B (76.50, IQR: 12.25) was significantly improved than group A (60, IQR: 30). At the two-year follow-up, the median Lysholm score was 62.50 (IQR: 35.5) and 73.50 (IQR: 18.25) in group A and B, respectively (Fig. 2C). Of importance, none of the two-year follow-up scores resulted in significantly different scores between the two chondrogenic groups. Both 65 cut-off of Lysholm score and MCID revealed that four donors from chondrogenic group A fell in the category of clinical failure along with one donor from group B. Remarkably, five donors from the bad chondrogenic group (group B) were in the clinical success category (Fig. 2D). We did not notice a significant correlation ($r = -.308$, $p = 0.284$) between *in vitro* chondrogenic potentials and clinical outcomes.

Comparative expression of selected markers by the different chondrogenic and clinical outcome groups

Chondrocytes from three donors with extreme scores from each chondrogenic and clinical outcomes groups were investigated using flow cytometry to determine the expression of the surface markers CD44, CD106, CD146, CD166 and CD271 (Fig. 3 and 4). In addition, gene expression of selected integrins, TGF-β receptors and matrix molecules (Table 3) were explored using qPCR. Of note, 13 of the 14 donor-cells samples were included for qPCR analysis as one donor was excluded due to the bad quality of the extracted RNA. We found a significant upregulation of CD166 in the clinical success group compared to the failure group (MFI: 2160+/-250 vs 730+/-50) (Fig. 4A). The surface expression of CD44 was upregulated in the clinical success group in a near significant way ($p = 0.054$). Additionally, the expression of CD106 and CD146 was on average higher in the clinical success group compared to the clinical failure group (MFI: 1400+/-370 vs 500+/-100 and MFI: 1150+/-310 vs 500+/-30, respectively) (Fig. 4A), but the difference did not reach statistical significance.

When comparing the chondrogenic groups, the surface expression of CD106 (MFI: 2370+/-160) was significantly high in group B compared to group A (MFI: 1140+/-160), thus suggesting a negative association with *in vitro* chondrogenic potential. We did not see significant differences in the surface expression of CD44 and CD166 between two chondrogenic groups (Fig. 3A). On the other hand, the surface expression of CD146 was uneven among donors within the same chondrogenic group, and their expression was not indicative of chondrogenic potential (Fig. 3A). Notably, we also observed very low surface expression CD271 in both chondrogenic and clinical groups (Fig. 3 and 4). Relative gene expression, on the other hand, revealed significant upregulation of ITGA1 (CD49a) and ITGB1 (CD29) in the good chondrogenic group (A) compared to group B, whereas TGFBR3 expression was significantly downregulated in group A (Fig. 3B). In the clinical groups, the expression of cartilage oligomeric matrix protein (COMP) and integrin-β1 were elevated in the failure group

340   compared to the success group, but the expression of integrin-β1 ($p = 0.055$) was barely

341   significant (Fig. 4B). Otherwise, we did not detect significant differences in any of the studied

342   genes associated with chondrogenic and clinical outcome categories (Supplementary Fig. 1 and

343   2).

An unbiased search of predictive biomarkers for *in vitro* chondrogenesis and ACI clinical outcomes by large-scale proteomics

346   Three donors representing the highest and lowest scores from each chondrogenic and clinical

347   outcome groups were investigated using quantitative peptide-labelled TMT proteomics.

348   Differential expression of relevant candidate proteins was validated by western blots. A total of

349   2113 proteins were identified in cell extracts of chondrocytes from donors in the chondrogenic

350   groups, of which 76 and 66 were classified as cell adhesion molecules and cell surface receptors,

351   respectively, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. On the

352   other hand, 2034 proteins were identified in cell extracts of chondrocytes from the different

353   clinical outcome groups, of which 74 and 59 were categorised as cell adhesion molecules and

354   cell surface receptors. High throughput comparative analyses of identified proteins in the two

355   chondrogenic groups revealed seven proteins significantly downregulated in group B compared

356   to group A (Fig. 5 A and B). Of relevance, prolyl-4-hydroxylase 1 (P4HA1) (FDR < 0.01), an

357   enzyme involved in collagen biosynthesis, was among the differentially expressed proteins.

358   This outcome was validated in western blot analyses from all six donors (Fig. 5C). Moreover,

359   we found no differentially expressed proteins when comparing donor cells belonging to the two

360   clinical outcome groups (Fig. 5D).

361

Discussion

363   The main objective of this study was to address the question if *in vitro* chondrogenic potential

364   of donor-matched chondrocytes could predict clinical outcomes after ACI. Earlier studies have

365 investigated on the influence of cell quality on ACI clinical outcomes with divergent outcomes

366 [17, 22], and others have searched for novel biomarkers with predictive value in cultured cells

367 [16, 20]. However, the evidence is still lacking on whether the *in vitro* chondrogenic abilities

368 of patients' chondrocytes can predict clinical outcomes. The second objective of the current

369 study was to investigate if previously proposed biomarkers of chondrogenesis had predictive

370 value for clinical outcomes and vice versa, and we have searched for novel biomarkers in the

371 chondrocyte proteome capable of predicting chondrogenic potential and clinical success or

372 failure after ACI.

373 We prepared multicellular 3D pellets with chondrocytes from different donors and compared

374 their chondrogenic potential using visual histological grading system [26, 29]. Of note, it has

375 been demonstrated that histological grading of pellets by Bern Score correlates significantly

376 with biochemically assessed glycosaminoglycans content [29]. In line with other studies, we

377 have also demonstrated divergent *in vitro* chondrogenic potentials of culture-expanded

378 chondrocytes from different donors [18, 20]. Due to unavoidable circumstances external to the

379 experimental plan, the chondrocyte cultures included in this study were not synchronised at the

380 same passage, but from passage 3-6 when preparing the pellets and RNA extracts for qPCR. To

381 exclude the possible influence of passage number in chondrogenic outcomes, chondrogenesis

382 was evaluated for some donors across different passages (Table 1). Besides, other authors have

383 proposed that the loss of phenotypic traits occurs primarily during the first passages, and the

384 cell phenotype becomes more stable after passage 3-4 [30]. Moreover, we could verify that

385 neither patient's age nor gender were associated with good or bad *in vitro* chondrogenic

386 potential (Table 1).

387 To investigate the relationship between *in vitro* chondrogenic potential and clinical outcomes,

388 we compared cartilage-like tissue formation of donor-matched chondrocytes with short-term

389 (two-year follow-up) clinical outcomes. Remarkably, our results show a tendency to an inverse

390   correlation ($r = -.308$, $p = 0.284$) between *in vitro* chondrogenesis and clinical outcomes. Earlier

391   studies have proposed a number of patients' characteristics such as patient age, defect size, age

392   and location, preoperative Lysholm score, or prior knee surgeries to select patients that may

393   benefit from the procedure [10, 11, 31]. In parallel, others have proposed cell quality as one of

394   the multiple parameters that may influence clinical outcomes after ACI [16, 22, 23]. In these

395   later studies, cell quality was evaluated by expression of cartilage-specific differentiation

396   markers such as collagen type II and aggrecan, and other cell surface receptors such as fibroblast

397   growth factor receptor 3 (FGFR3) and CD44. In a more recent study, the predictive clinical

398   value of the suggested cell quality markers has been questioned [17]. In line with the later

399   mentioned study, we do not observe correlations between the *in vitro* chondrogenic potency

400   and clinical outcomes. There exist a number of possible circumstances that could explain our

401   finding. The fate of implanted chondrocytes and their contribution to rebuilding the damaged

402   tissue, compared with cells from surrounding tissues, is mostly unknown. Reports in pre-

403   clinical models show varying proportions of injected cells in the repaired tissue. However,

404   results demonstrate that most of the repair tissue is composed of cells of unknown origin

405   migrating to the lesion [32, 33]. Histologically, ACI repair tissue appears predominantly fibro-

406   cartilaginous [34]. In patients, it has been observed that the quality of the repair tissue after

407   ACI, from a histological point of view, does not always correlate with clinical outcomes [7, 10,

408   35]. Collectively, these observations and our results suggest that the cell quality and the intrinsic

409   chondrogenic capacity of the implanted cells may not play a major role in the outcomes of the

410   ACI procedure.

411   In previous studies aiming at identifying cell surface receptors that can predict chondrocytes

412   with an improved chondrogenic potential *in vitro*, CD44, CD151 and CD146 have singled out

413   at positively correlated with good chondrogenesis as judged by GAG content [20] or

414   histological evaluation of spheroid cultures [36]. The CD44 protein expression has also been

415 investigated in a clinical setting where a positive correlation between a clinical knee score at 24

416 months and CD44 protein expression in excess chondrocytes after ACI was found [22].

417 Stenberg *et al*. also analysed surplus chondrocytes from ACI, and found no correlation between

418 CD44 gene expression and clinical outcome after three years [17]. In our study, while all donors

419 were compared in qPCR analyses, only three donors from each group were used in flow

420 cytometry analyses. We observed no differences in expression of CD44 when analysing

421 chondrogenic groups. When comparing CD44 expression between the clinical groups, our

422 findings are in line with Stenberg's study, revealing no differences between the success and

423 failure groups (Fig. 4). Furthermore, in our cohort, CD146 surface expression did not correlate

424 with either chondrogenesis or clinical outcomes.

425 We found elevated surface expression of vascular cell adhesion molecule 1 (CD106) in

426 chondrocytes from donors displaying bad chondrogenesis. A previous study reported the

427 expression of CD106 in chondrocytes and their role as a marker for immunomodulation in

428 inflamed joint [37]. However, in an early study from our group comparing the chondrogenic

429 potential of stromal cells from different tissue sources, we observed no association of CD106

430 surface expression with the chondrogenic potential of cells *in vitro* [25]. Hence, the role of

431 CD106 in chondrogenesis may require further investigation. Importantly, we saw a significant

432 upregulation of CD166 in the clinical success group. CD166 has been used as a marker to

433 identify mesenchymal progenitor cells in cartilage [38, 39]. The expression of CD166 has been

434 reported to be upregulated upon dedifferentiation [40], and others have observed expression

435 changes also during redifferentiation [41]. However, there are no records of the predictive

436 potential of CD166 in clinical outcomes. Our findings on CD166 represent an interesting lead

437 with clinical relevance that deserves further validation.

438 Several studies have implied that integrins, a group of cell surface receptors facilitating

439 chondrocyte-matrix crosstalk, are central players in differentiation and chondrogenesis [20, 42].

440   Grogan *et al.* suggested ITGA3 (CD49c) as a marker for good chondrogenic potential, and also

441   showed upregulation of ITGA5 (CD49e) and ITGA6 (CD49f) in chondrogenesis [20]. Another

442   study investigating effect blocking of ITGA1, ITGA5 and ITGB1 on chondrogenesis reported

443   early chondrogenesis was only inhibited by blocking of ITGB1 [43]. Unlike their observations,

444   we found ITGA1 and ITGB1 expression associated with good chondrogenesis but no

445   correlations of other integrin alpha units with chondrogenesis or clinical outcomes (Fig. 3 and

446   4). Cartilage oligomeric protein (COMP), a matrix molecule, has previously been investigated

447   as a potential biomarker, unlike Wright *et al.* who found no correlation between COMP protein

448   level in synovial fluid and clinical outcome [13], we found that the gene expression of COMP

449   was significantly upregulated in the clinical failure group. Collectively, these observations

450   suggest that markers associated with chondrogenesis of cells have limited or no value in clinical

451   settings. Lastly, our gene expression analyses revealed significant upregulation of TGFBR3

452   gene in the poor chondrogenic group. We have not found any previous studies on TGFBR3 in

453   relation to chondrogenesis. However, an upregulation upon dedifferentiation of chondrocytes

454   has been suggested [44]. The clinical relevance of this finding is still uncertain.

455   The global proteomic approach to search for potential new biomarkers in cell-associated

456   material revealed no differences between clinical success and failure group (Fig. 5). Similar

457   observations were made by Stenberg *et al.* using global transcriptomics to compare clinical

458   success and failure groups [17]. Besides, we found seven proteins that were significantly

459   upregulated in the good chondrogenic group. In this reduced group of proteins, we found all

460   subunits of the enzyme prolyl-4-hydroxylase (P4HA) (FDR < 0.05, Fig. 5), a critical enzyme

461   involved in the biosynthesis of collagen. This finding was validated by western blots. Previous

462   studies have reported gene and protein expression of P4HA1, P4HA2 and P4HB in human

463   chondrocytes [45] and showed that they were induced by hypoxia. The role of P4HA1 in

464   chondrogenesis is not yet defined, but given the critical role of this enzyme in the triple helix

465 formation of newly formed collagens, our results suggest that P4HA1 (FDR < 0.01) could

466 represent a promising biomarker to predict the cells with superior *in vitro* chondrogenic

467 potential.

468 There are limitations of this study that need to be addressed. The relatively low number of

469 patients included in the study may not give sufficient statistical power to find differences

470 between the experimental groups. Hence the findings unveiled in the present study should be

471 validated in larger cohorts. The clinical data represent short-term (two-year follow up)

472 outcomes. A long-term follow-up in which the number of failures could increase might provide

473 different scenarios [7]. We used Lysholm scores with a cut-off of 65 at two years postoperative

474 to discern between clinical success and failure. However, we do not have records of factors that

475 might have influenced the healing process after ACI including lifestyle, bad joint homeostasis,

476 and compliance with previous medications. Finally, we do not have postoperative biopsies of

477 the repair tissue so we are unable to make direct comparisons between the *in vitro* chondrogenic

478 potential and the quality of the repaired tissue, which as mentioned earlier may not necessarily

479 have a direct correlation with clinical outcomes.

480

481 **Conclusions**

482 This is the first study evaluating the *in vitro* chondrogenic potential of donor-matched

483 chondrocytes and ACI clinical outcomes. The study shows that the cartilage-forming capacity

484 of cells *in vitro* does not correlate with clinical outcome for ACI. Additionally, the results reveal

485 disparities between predictive markers of chondrogenesis and predictive markers of clinical

486 outcomes. Furthermore, we provide insights on novel predictive biomarkers for chondrogenesis

487 and clinical outcomes. The data presented in this study needs to be validated in a larger cohort

488 of patients. However, our findings do not support the use of *in vitro* chondrogenic or molecular

489 markers for chondrogenesis as predictive tools to be used in patient stratification for ACI.

Declarations

Acknowledgements

Availability of data and materials

The datasets used in the current study are available from the corresponding author upon reasonable request.

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Authors' Contributions

AI primarily conducted the laboratory work and prepared the manuscript. AKH performed qPCR and edited the manuscript. VF collected clinical data. GK performed ACI and collected clinical data. VF, AKH and GK analysed clinical data. IU performed LC-MS/MS. All authors contributed to the data interpretation for the results, provided direction and comments on the manuscript. IMZ planned the study, edited and approved the final draft of the manuscript.

Ethical statement

The Regional Ethical Committee of Northern Norway has approved the study (REK Nord 2014/920).

Consent for publication

Not applicable

514 **Competing interests**

515 The authors declare no competing interests.

516

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663

Figure legends

Figure 1. Chondrogenesis of culture-expanded chondrocytes in 3D pellets incubated in chondrogenic medium. (A) Representative bright light microscopy images of histological sections, stained for proteoglycans with Alcian blue and the nuclei counterstained with Sirius red, corresponding to "Group A" and "Group B" with good and bad chondrogenic potential, respectively. (B) Semi-quantitative analysis representing histological scoring of Alcian blue stained 3D pellets demonstrated significant differences between two groups. Scale bar: 200 µm and significance level, p (**) = < 0.005.

Figure 2. Comparison of donor-matched chondrogenic potential with clinical outcomes. VAS score (A), KOOS total (B) and Lysholm score (C) were plotted against chondrogenic Group A and Group B at the preoperative stage, one-year and two-year follow-up after ACI. (D) Patient distribution using Lysholm score (cut-off < 65) at two-year follow-up demonstrated clinical success and failure groups and their no significant association (r = -.308, p = 0.284) with *in vitro* chondrogenic potentials. Significance level, p (*) = <0.05.

Figure 3. Comparison of selected molecular biomarkers between chondrogenic groups. (A) Surface protein expression of CD44, CD106, CD146, CD166 and CD271 by flow cytometry from donors with extreme good scores (n = 3; upper panels) and extreme bad scores (n = 3; low panels). Red peak represents the isotype control, and blue, orange and green peak represent expression by each independent donor. Average median fluorescence intensity (MFI) +/- standard error demonstrated differences in surface marker expression between two groups.

684 (B) Analysis of selected genes of interest by qPCR revealed their relative expression in the good

685 ($n = 8$) and bad ($n = 5$) chondrogenic groups. Plotted values represent each donor, and the error

686 bar represents standard deviation. Significance level, p (*) = <0.05.

687 **Figure 4. Comparison of selected molecular biomarkers between clinical groups.** (A)

688 Surface protein expression of CD44, CD106, CD146, CD166 and CD271 by flow cytometry

689 from donors with extreme good scores ($n = 3$, upper panels) and extreme bad scores ($n = 3$;

690 low panels). Red peak represents the isotype control, and blue, orange and green peak represent

691 the tested cell surface marker for each donor. Average median fluorescence intensity (MFI) +/-

692 standard error demonstrated differences in surface marker expression between two groups. (B)

693 Analysis of selected genes of interest by qPCR revealed their relative expression in the success

694 ($n = 8$) and failure ($n = 5$) clinical groups. Plotted values represent each donor, and the error

695 bar represents standard deviation. Significance level, p (*) = < 0.05 and (**) = < 0.005.

696 **Figure 5. Comparative global protein expression analysis by LC-MS/MS between**

697 **chondrocyte cultures associated with different chondrogenesis and clinical outcomes.** (A)

698 Volcano plot represents the expression of proteins in bad chondrogenic samples (Group B)

699 compared to good chondrogenic samples (Group A). Proteins underwent greater fold change,

700 and lower p-value in the comparison are plotted further away from zero on X-axis and Y-axis,

701 respectively.   The red dot shows significantly down-regulated proteins (FDR < 0.05) in

702 chondrogenic group B. (B) Heat map showing the differentially expressed proteins when

703 comparing chondrogenic groups. (C) Validation of P4HA1 protein expression by western blot.

704 (D) Volcano plot represents the expression of proteins in clinical failure group compared to

705 clinical success group. Significance level, p (*) = < 0.05.

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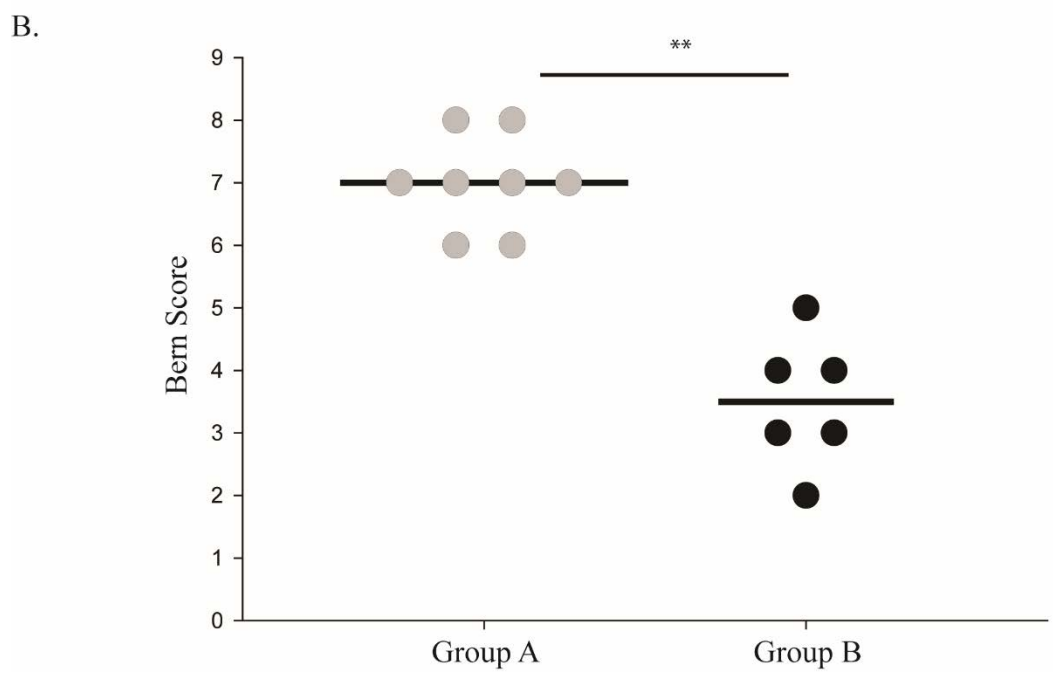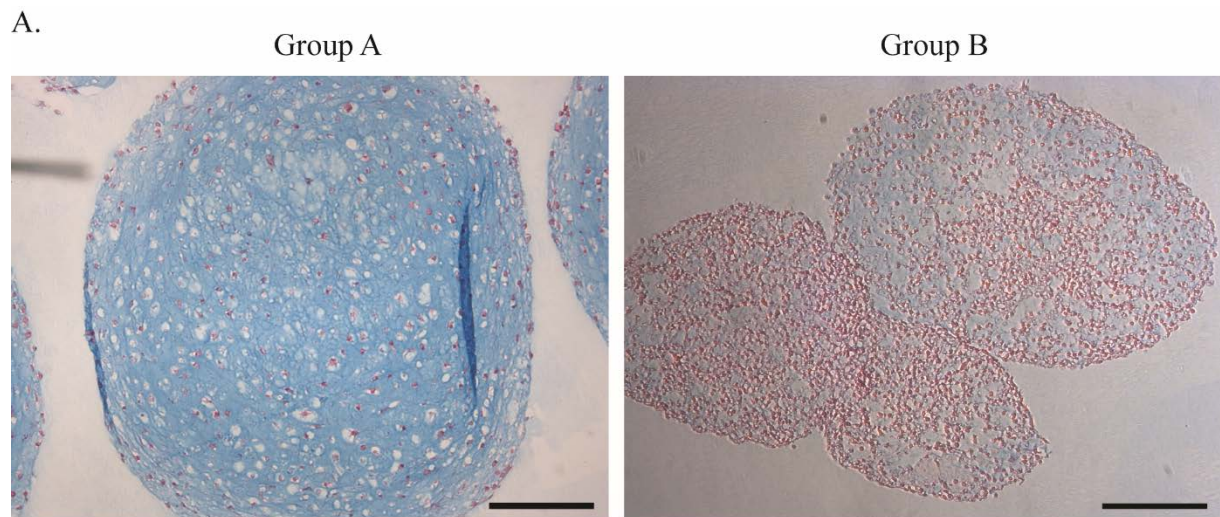
Figure 1. Chondrogenesis of culture-expanded chondrocytes in 3D pellets incubated in chondrogenic medium.

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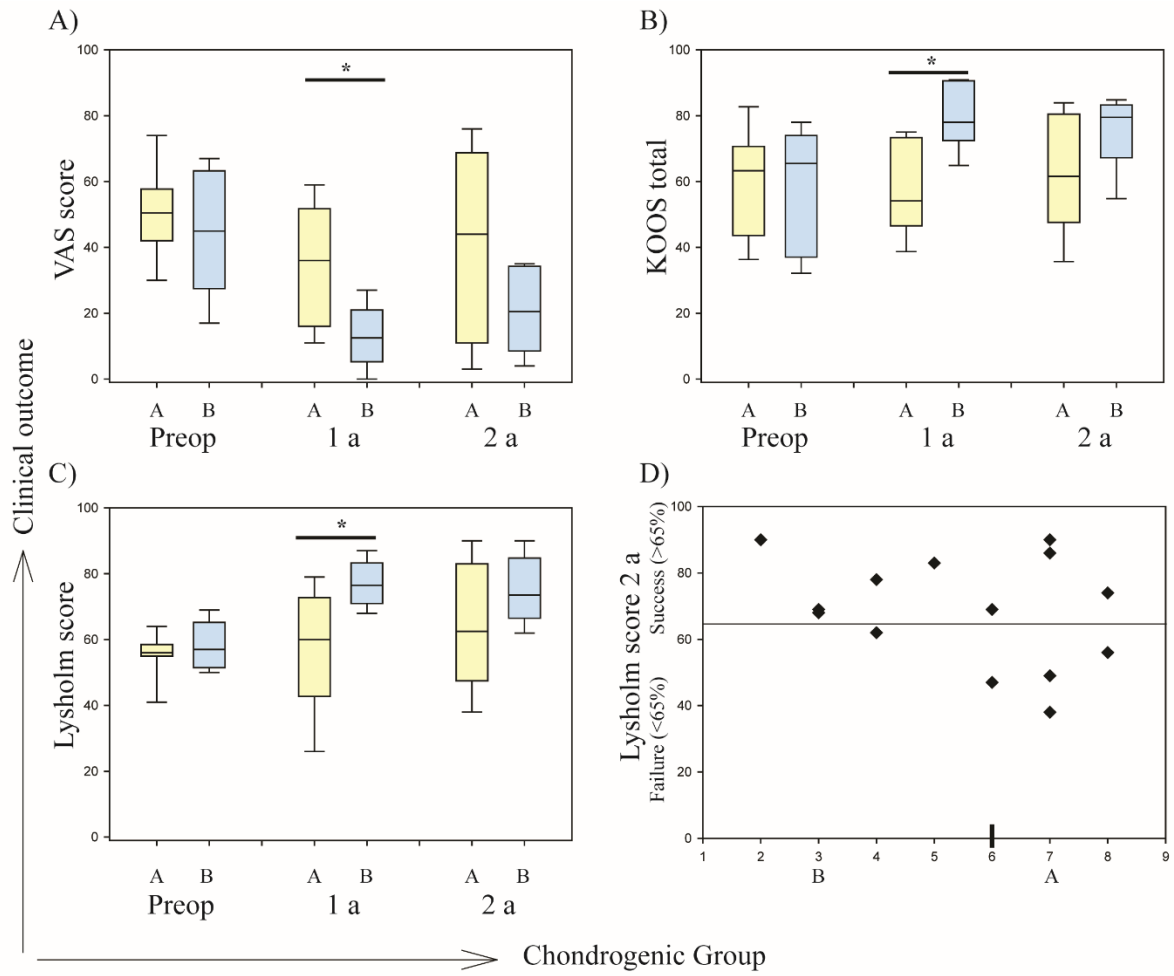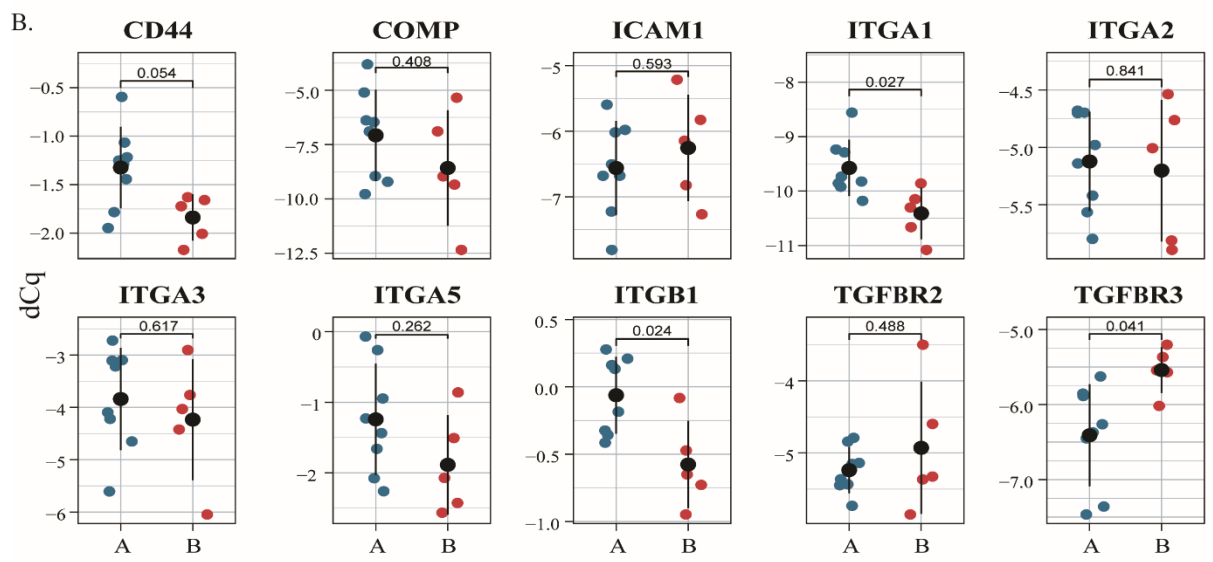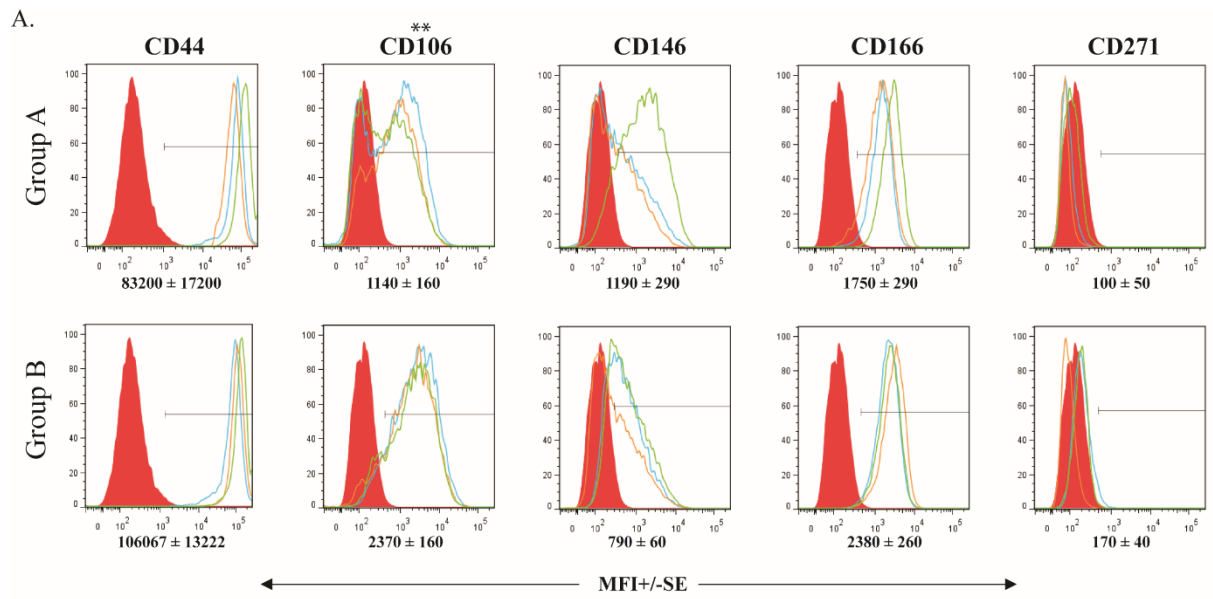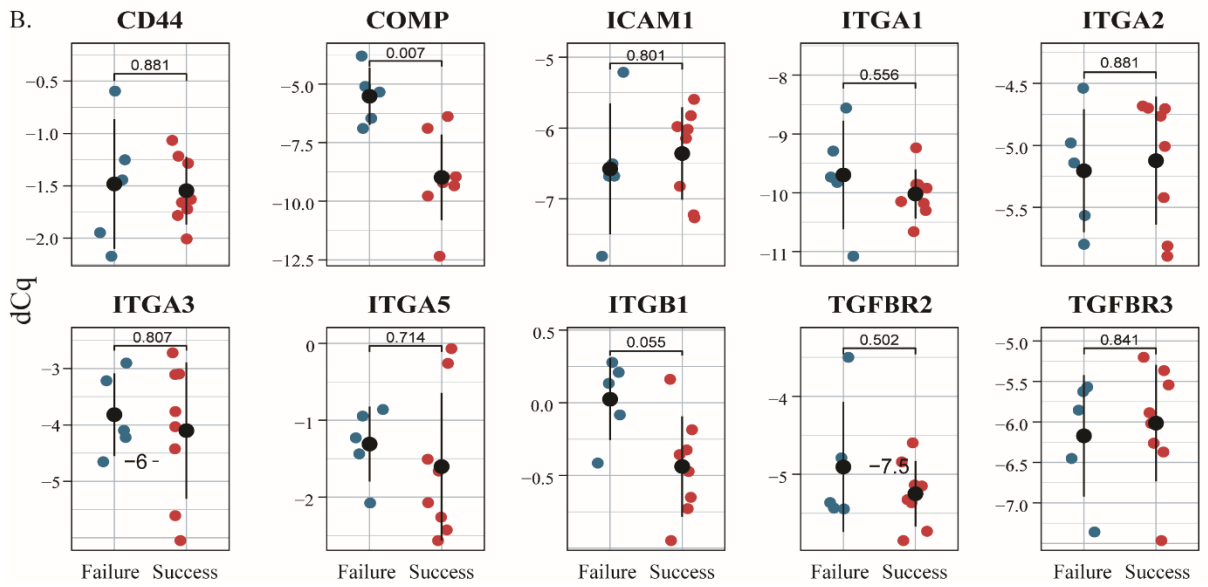717 Figure 2. Comparison of donor-matched chondrogenic potential with clinical outcomes.
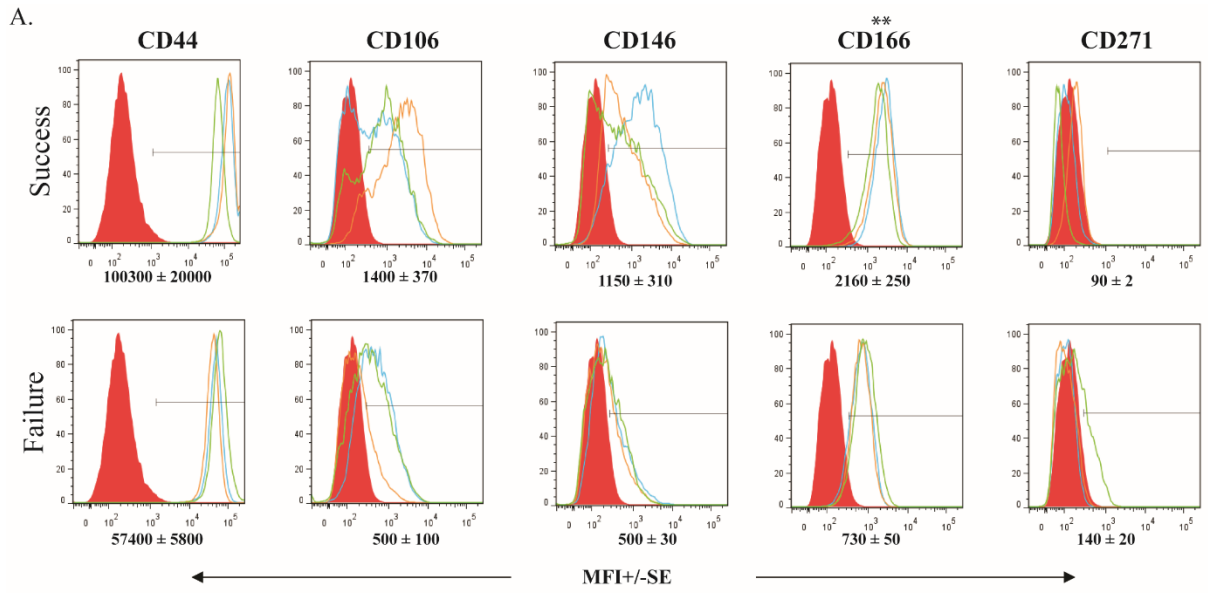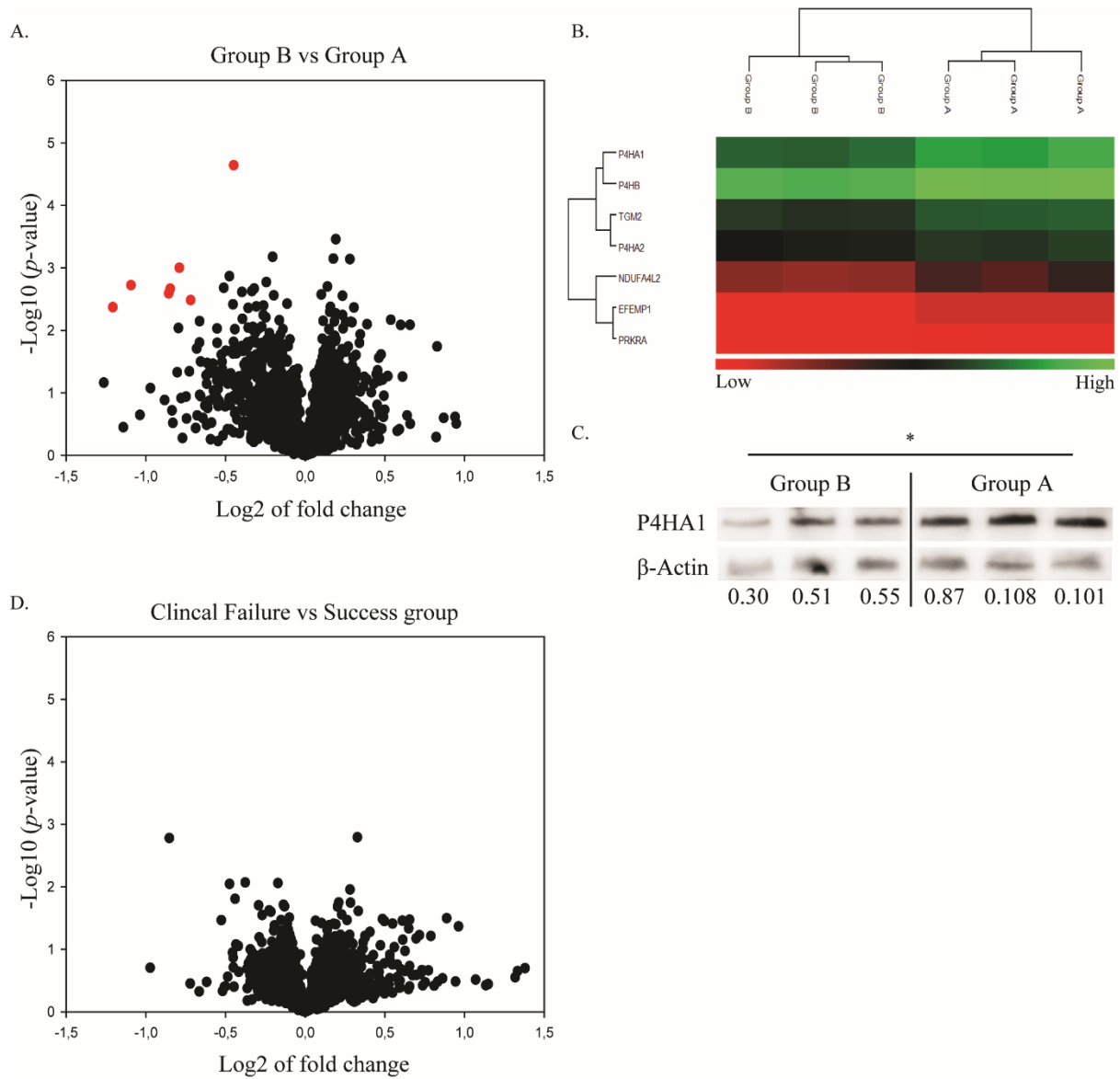
Figure 3. Comparison of selected molecular biomarkers between chondrogenic groups.

Figure 4. Comparison of selected molecular biomarkers between clinical groups.

Figure 5. Comparative global protein expression analysis by LC-MS/MS between chondrocyte cultures associated with different chondrogenesis and clinical outcomes.

Table 1. Donor characteristics and donor-specific chondrogenic potential of culture

expanded chondrocytes in 3D spheroids.

Group A (Bern Score 6-9)						
Source	**Age**	**gender**	**Passage**	**Hanging-drop culture**	**Pellet culture**	**Bern Score**
Donor 1	37	F	4	+	+	8
Donor 2	55	M	6	+	+	7
Donor 3	52	M	6	-	+	8
Donor 4	52	M	3	+	+	7
Donor 5	39	M	3	-	+	7
Donor 6	31	M	3	-	+	7
Donor 7	24	F	3	-	+	6
Donor 8	33	F	3	+	+	6
Group B (Bern Score <6)						
Source	**Age**	**gender**	**Passage**	**Hanging-drop culture**	**Pellet culture**	**Bern Score**
Donor 9	37	M	5	-	+	5
Donor 10	51	F	4	-	+	4
Donor 11	53	F	6	-	+	4
Donor 12	46	M	3	-	+	3
Donor 13	44	M	5	-	+	3
Donor 14	19	M	3	-	+	2

Table 2. Clinical outcome of patients after two years of ACI. Lysholm score (65% cutoff)

after two years was used to divide patients in success and failure group.

Success group (>65% Lysholm)				VAS		KOOS		Lysholm	
Source	**Age**	**gender**	**Defect size**	**Pre**	**2yr**	**Pre**	**2yr**	**Pre**	**2yr**
Donor 1	55	M	2.25	40	3	43.5	82.7	55	90
Donor 2	19	M	3	40	12	68.5	82.1	69	90
Donor 3	39	M	4.6	50	10	71.4	83.9	56	86
Donor 4	37	M	9.75	62	10	62.5	76.9	52	83
Donor 5	53	F	5.2	31	34	78	82.7	64	78
Donor 6	37	F	3.6	51	14	68.3	73.8	59	74
Donor 7	24	F	6	48	51	58.3	70.8	57	69
Donor 8	44	M	21.5	17	4	72.6	84.8	50	69
Donor 9	46	M	2.4	67	35	38.7	71.4	58	68
Failure group (<65% Lysholm)				VAS		KOOS		Lysholm	
Source	**Age**	**gender**	**Defect size**	**Pre**	**2yr**	**Pre**	**2yr**	**Pre**	**2yr**
Donor 10	51	F	1.82	50	69	32.1	54.8	56	62
Donor 11	52	M	5	51	37	82.7	52.4	64	56
Donor 12	52	M	3	30	56	36.3	47.6	56	49
Donor 13	33	F	3.1	60	73	68.5	47.6	55	47
Donor 14	31	M	1.2	74	76	44	35.7	41	38

739 **Table 3: Hydrolysis probes.**

ITGA1	Hs00235006_m1
ITGA2	Hs00158127_m1
ITGA3	Hs01076879_m1
ITGA5	Hs01547673_m1
ITGA6	Hs01041011_m1
ITGA10	Hs00174623_m1
ITGAV	Hs00233808_m1
ITGB1	Hs00559595_m1
ITGB3	Hs01001469_m1
ITGB4	Hs00236216_m1
ITGB5	Hs00174435_m1
COMP	Hs00164359_m1
MATN3	Hs00159081_m1
NCAM1	Hs00941830_m1
CD44	Hs01075861_m1
ICAM1	Hs00164932_m1
CDH2	Hs00983056_m1
BMPR1A	Hs01034913_g1
BMPR1B	Hs01010965_m1
BMR2	Hs00176148_m1
TGFBR1	Hs00610320_m1
TGFBR2	Hs00234253_m1
TGFBR3	Hs00234257_m1
RPL13A (reference gene)	Hs04194366_1g

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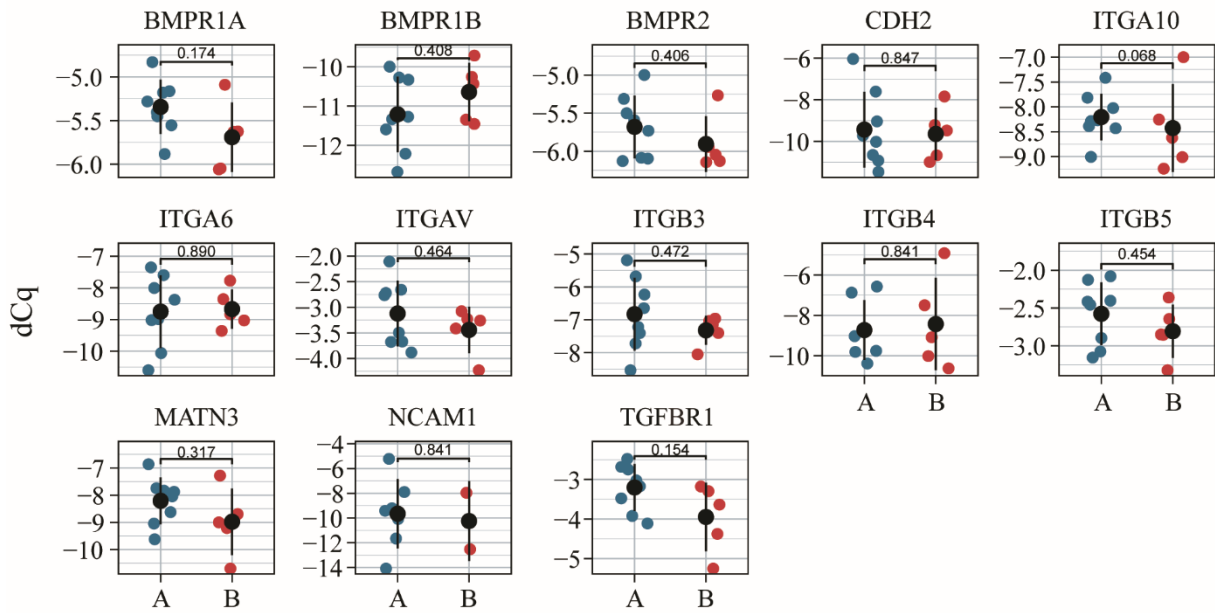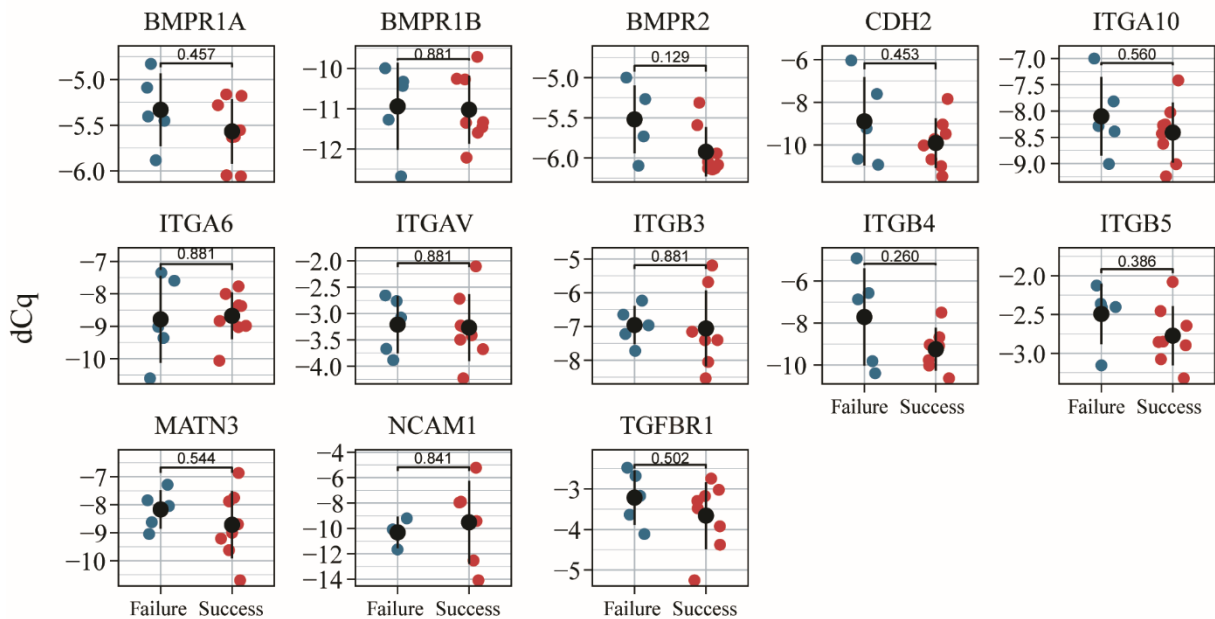
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Supplementary Fig. 1. Comparison of genes of interest by qPCR revealed their relative expression in the good ($n = 8$) and bad ($n = 5$) chondrogenic groups. Plotted values represent each donor, and the error bar represents standard deviation. Significance level, p (*) = <0.05.

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Supplementary Fig. 2. Comparison of selected genes of interest by qPCR revealed their relative expression in the success ($n = 8$) and failure ($n = 5$) clinical groups. Plotted values represent each donor, and the error bar represents standard deviation. Significance level, p (*) = < 0.05.