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THE ARCTIC
UNIVERSITY
OF NORWAY

Faculty of Health Sciences

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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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.
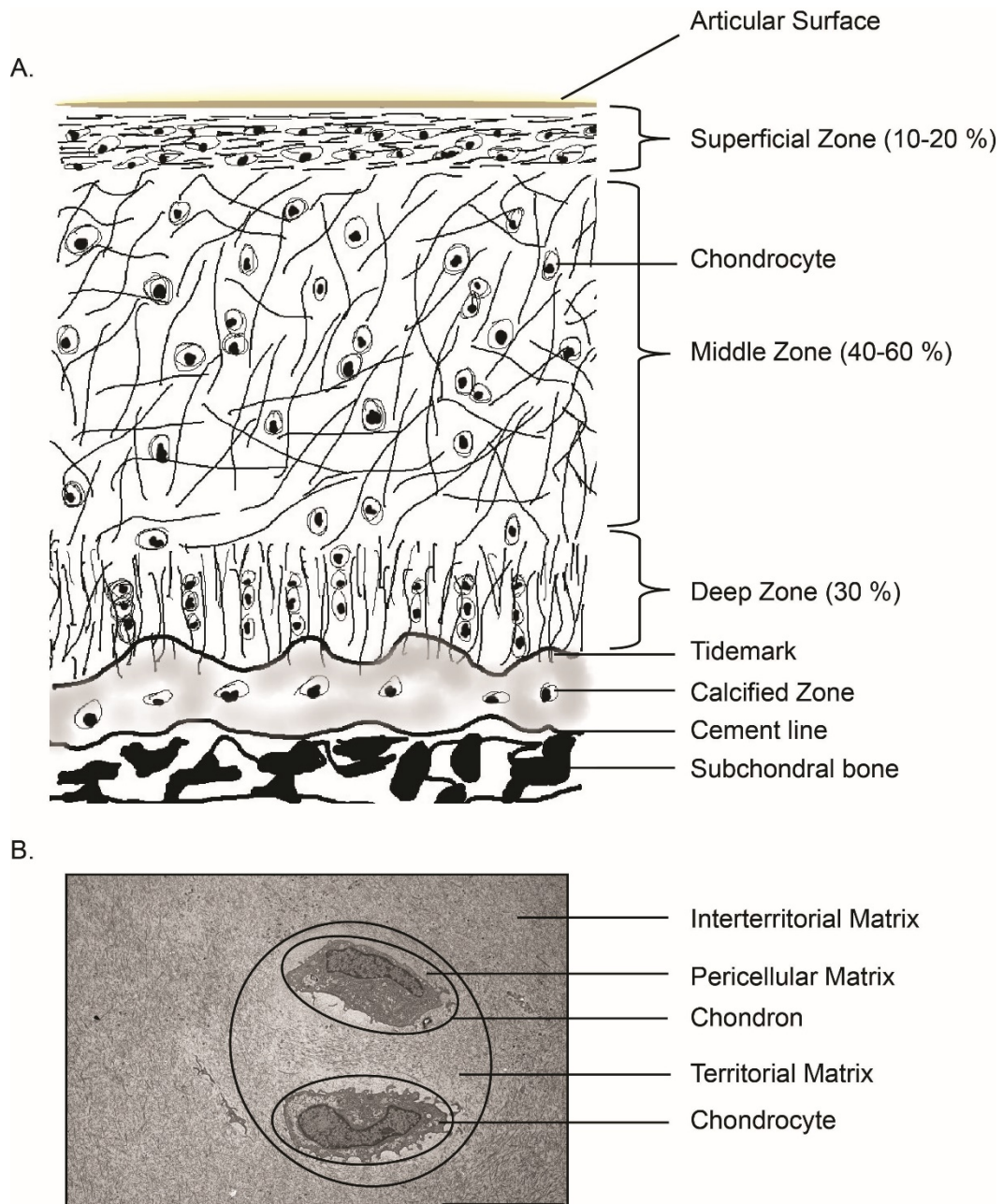
Figure 1: Structure of human articular cartilage. A. The zonal organisation of articular cartilage showing the organisation of chondrocytes and collagen fibrils in a different layer. B. Regional organisation of articular cartilage showing chondrons and proximity of ECM from the chondrocytes. Scale bar: 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 ($\sim$ 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.
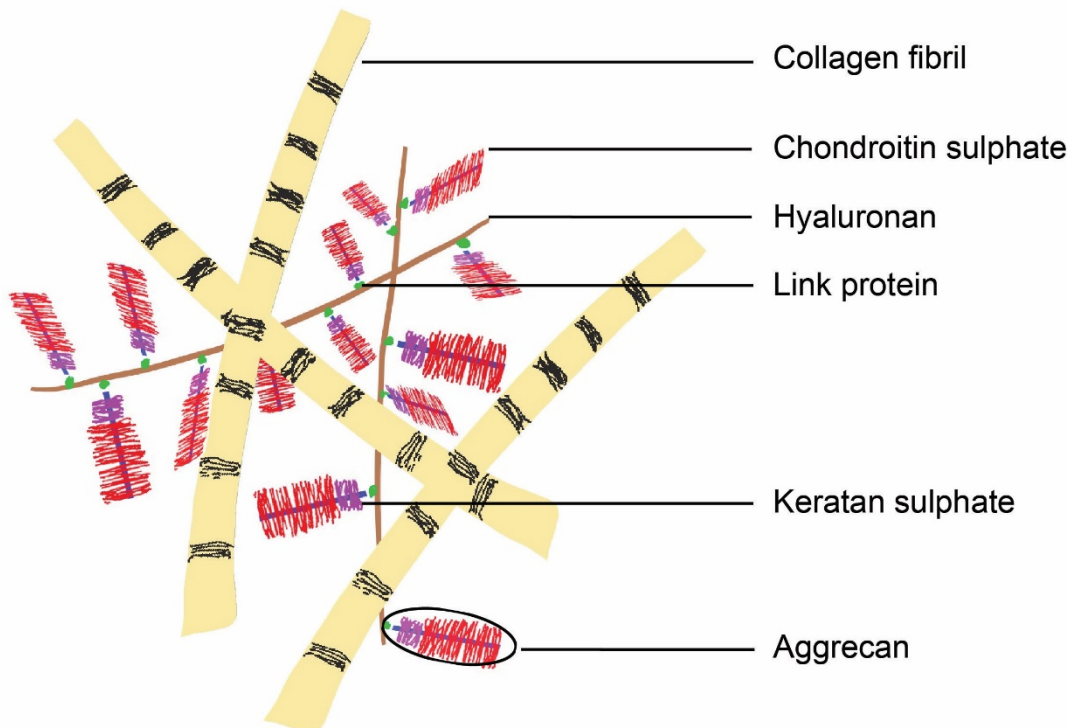
Figure 2: Collagen network and proteoglycan polymer chain form ECM backbone of articular cartilage.

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].
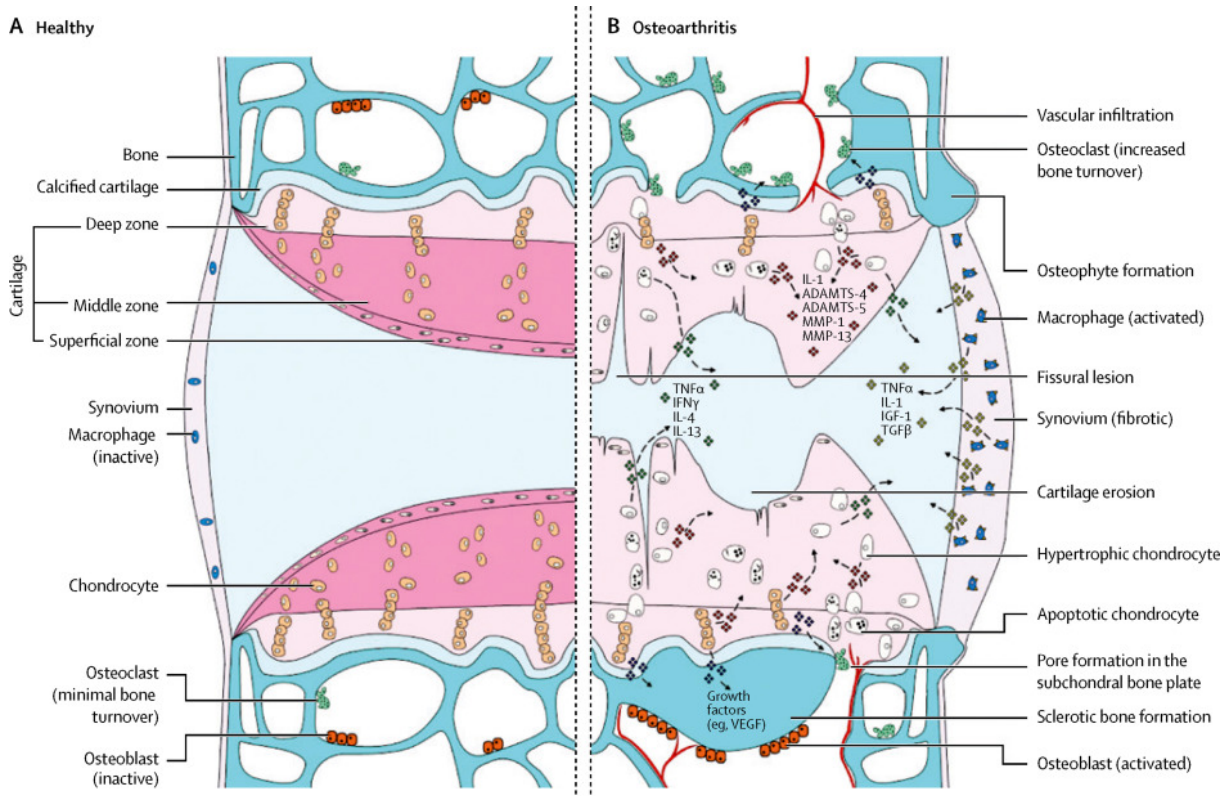
Figure 3: Pathophysiology of osteoarthritis. A. Healthy articular cartilage and B. Signalling pathways and structural changes in osteoarthritis (reproduced with permission from [23]).

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
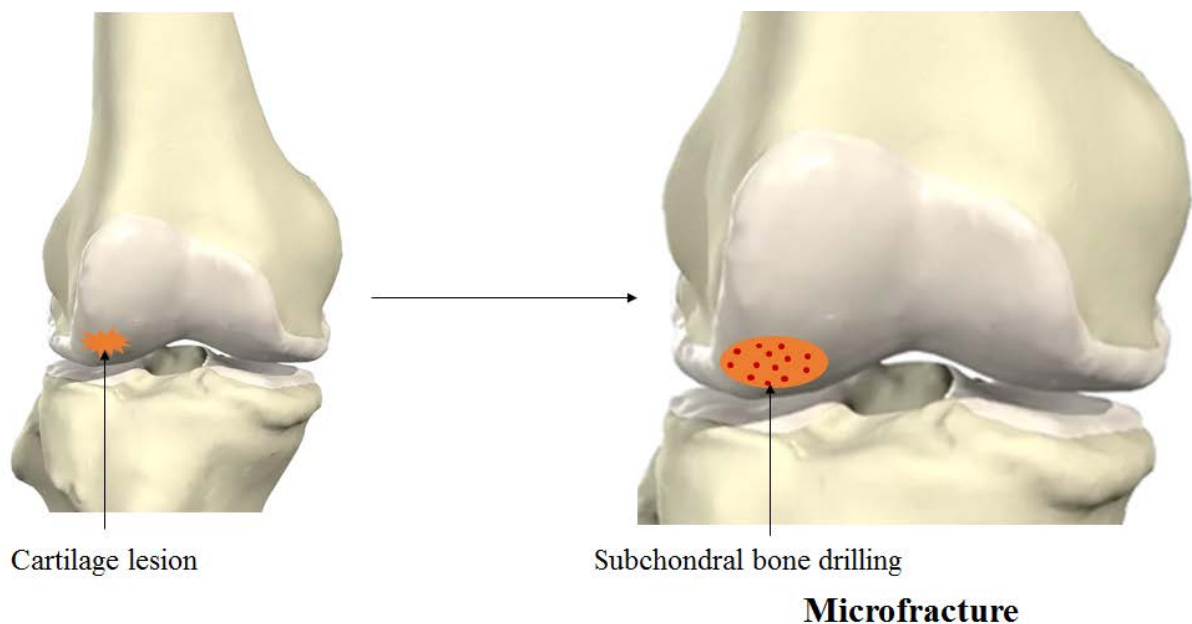
Microfracture

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].
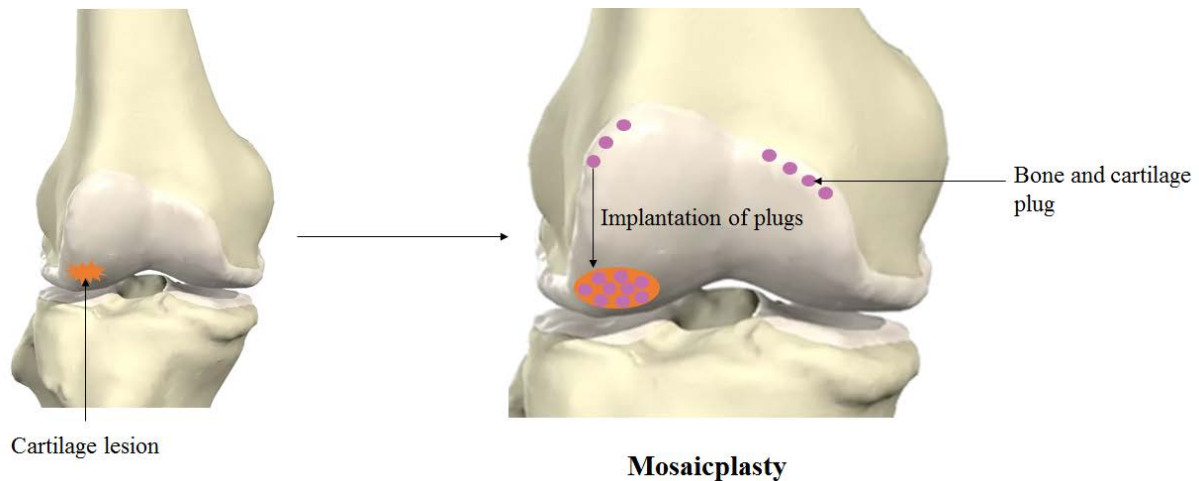
Figure 5: Illustration of the mosaicplasty procedure.

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 ($\geq 2 \ cm^2$) cartilage defects [46, 47].
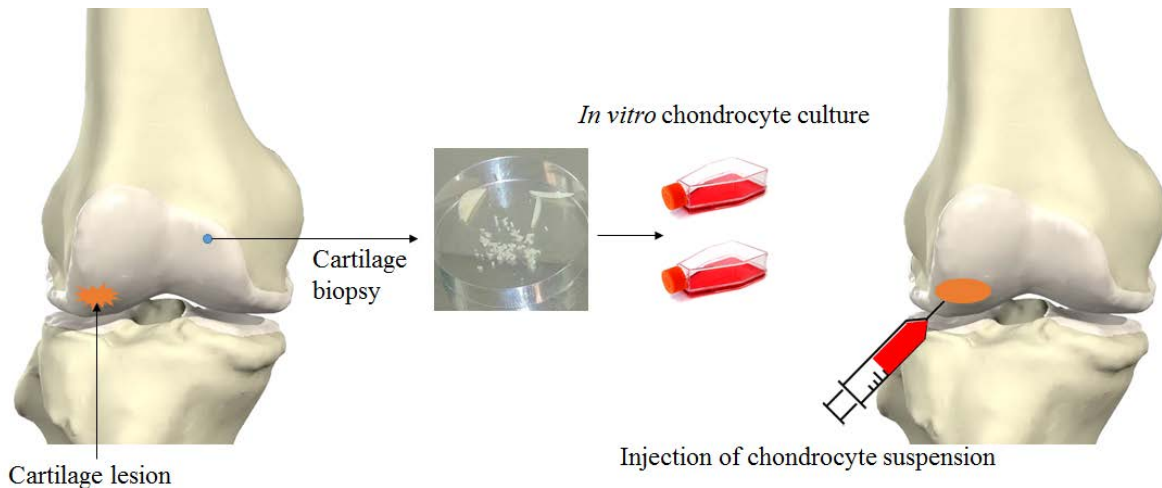
Figure 6: Illustration of ACI procedure.

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].
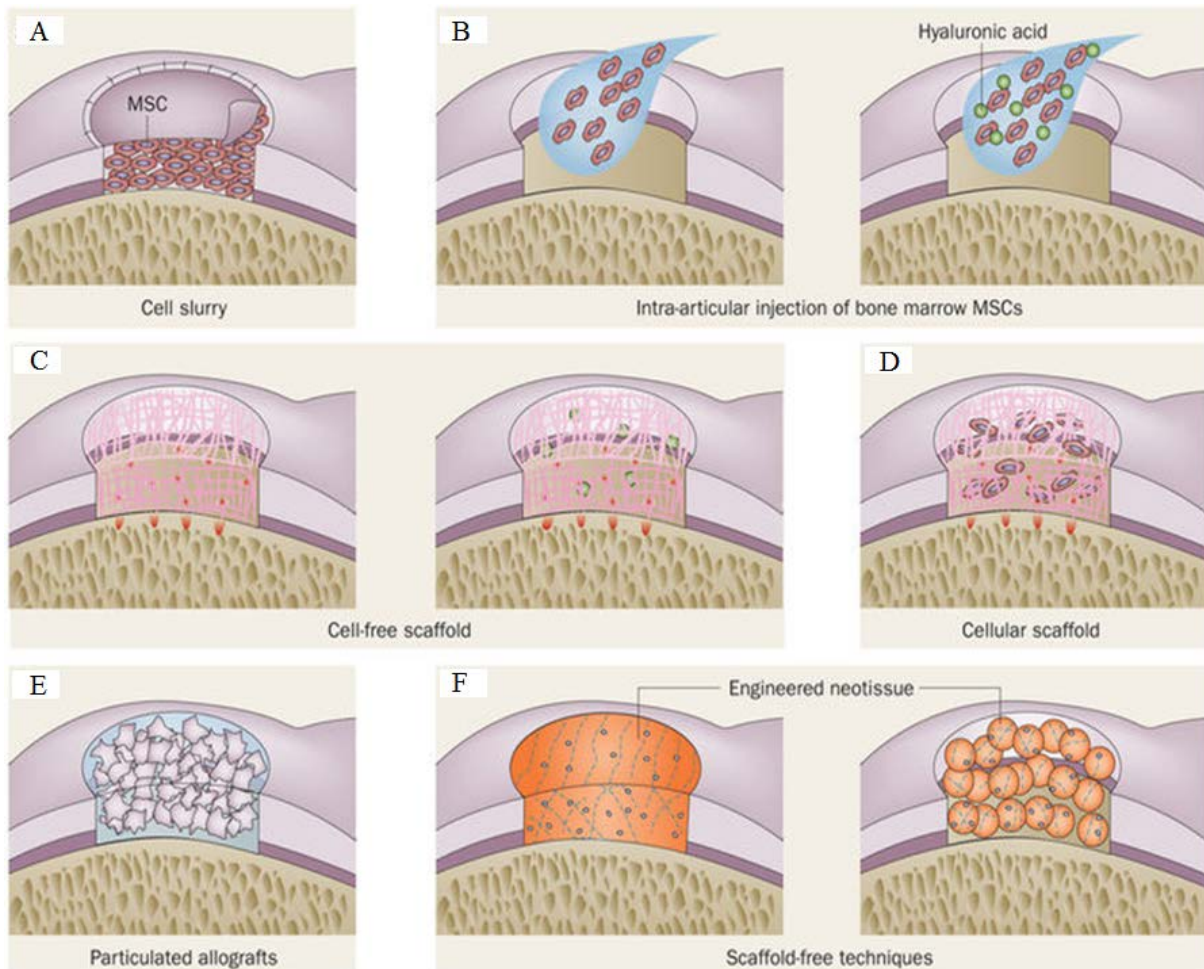
Figure 7: Different cell-based and cell-free approaches to mimic ACI. A. Autologous bone marrow-MSCs implantation, B. Intra-articular injection of MSCs, C. AMIC, a cell-free scaffold-based surgery, D. MACI uses scaffolds with primary chondrocytes, E. Small particulated native cartilage approach and F. 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).
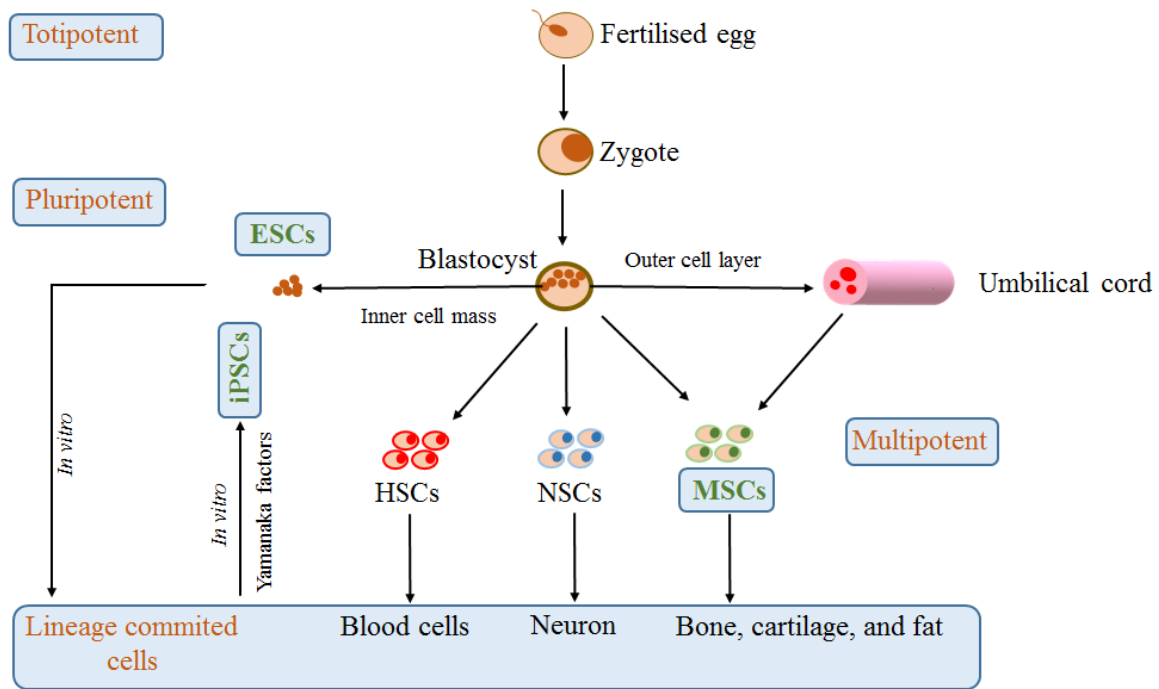
Figure 8: Flowchart illustrating the hierarchy of stem cells.

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

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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].
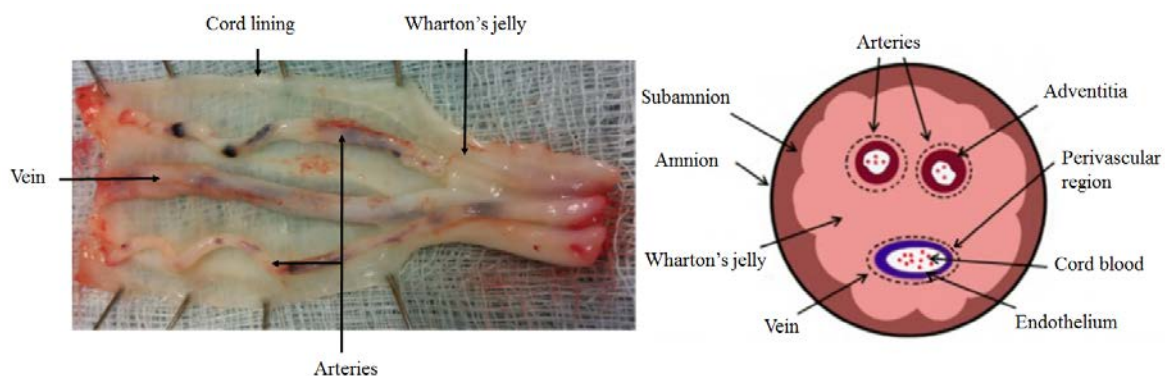
Figure 9: Dissection of human umbilical cord showing Wharton's jelly, cord lining, vein, and arteries (reproduced with permission from [80, 81]).

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.

Pellet Culture in Conical Well (3D)

Centrifugation

Two days

Isolated Cells — Monolayer till P3/4 — Chondrogenesis For three weeks

500 μm

Phenotype chracterization (Flow cytometry)

Spheroids collected After three weeks

Histology: Alcian Blue Staining
Immunohistochemistry: COL I and II staining
Biochemical Assay: Glycosaminoglycans (sGAG) and DNA
Ultra-structural Analysis: Transmission Electron Microscopy
Gene Expression: qPCR

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.
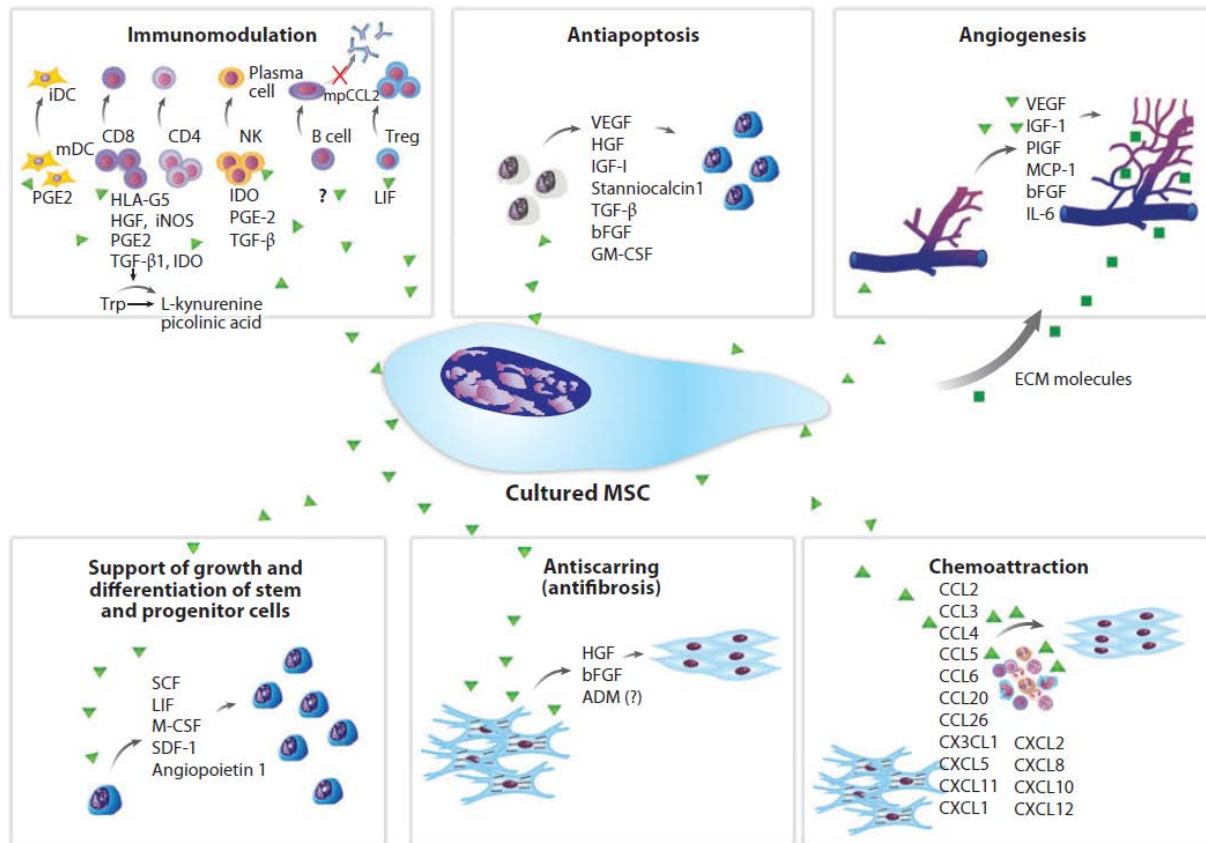
Figure 11: Schematic of multifunctional effects of MSCs through bioactive soluble factors (reproduced with permission from [177]).

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 x 10^6						
AT/ 5 x 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 x 10^6	18/OA	IA Injection	No	26 months	Improved knee function	[188]
FP/ 1.89 x 10^6	25/OA	IA Injection	PRP	18 months	Safe & improved knee function	[189]
SM/ 4 x 10^6	14/Chondral defect (≥2 cm^2)	Implantation (MSCs + collagen sheet)	MACI	24 months	SM>MACI	[77]
BM/ 1-1.5 x 10^6	72/Chondral defect (≥3.6 cm^2)	Implantation (Fibrin glue)	ACI	24 months	As effective as ACI	[190]
BM/ 8-9 x 10^6	4/OA	Single IA injection	No	60 months	Advanced OA	[191]
AT/ 14 x 10^6	6/OA	Single IA injection	No	12 months	Safe and improved pain levels	[192]
BM/ 40 x 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 x 10^6	30/OA	Single IA injection	HA	12 months	Safe	[195]
BM/ 5 x 10^7 1.5 x 10^8	55/Partial medial menisectomy	IA injection	Cell free	24 months	Safe	[196]
UCB/ 5 x 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

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.
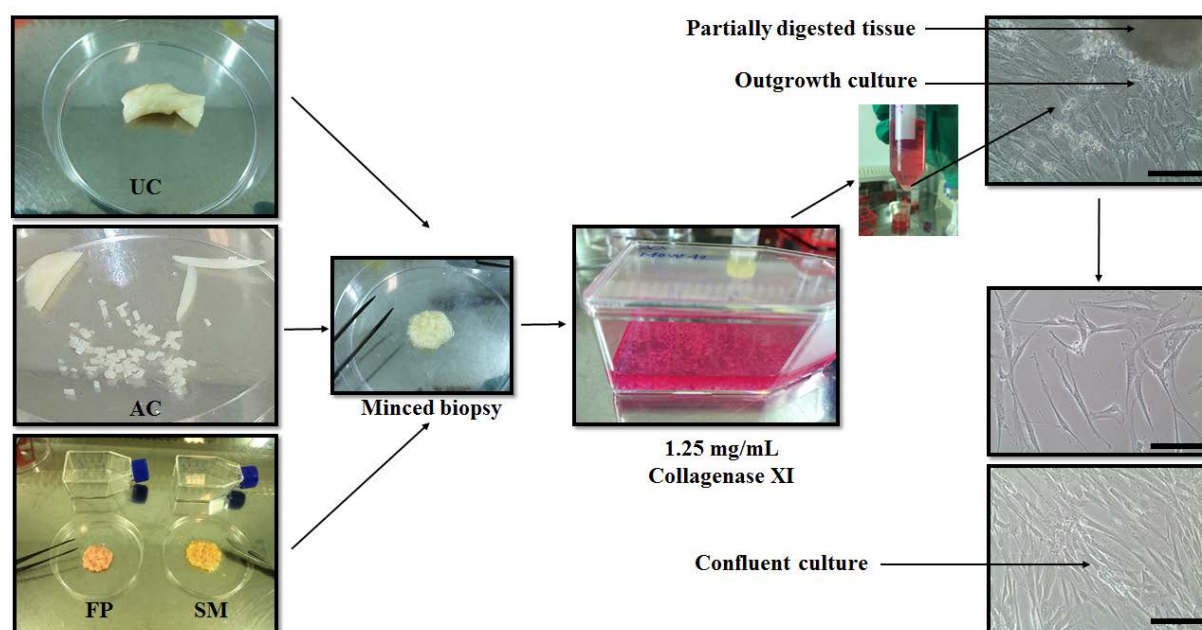
Figure 12: Schematic of cell isolation process. Scale bar: 5 µm.

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).

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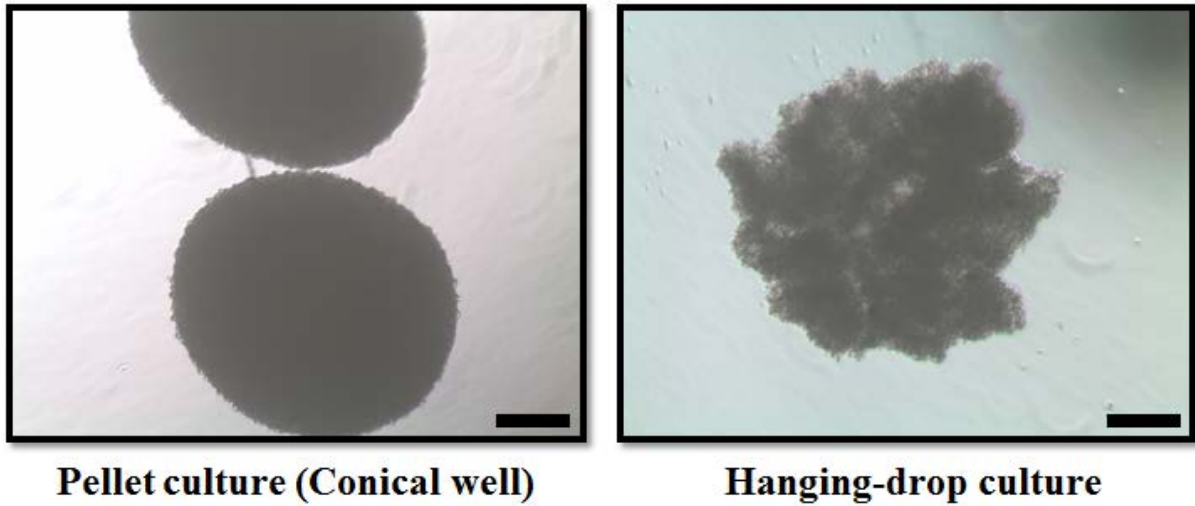
Figure 13: Comparison of spheroids prepared by pellet culture and hanging-drop culture from same chondrocyte donor. Scale bar: 200 μm.

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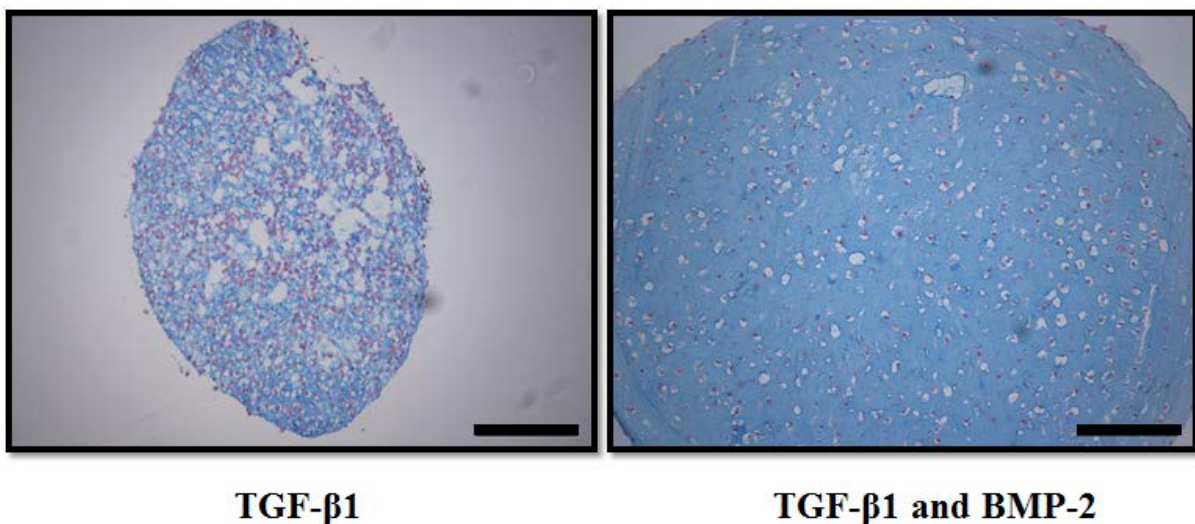
Figure 14: Metachromatic staining (Alcian blue) of spheroids in the presence of only TGF-β1 and combination of TGF-β1 and BMP-2. Scale bar: 200 μm.

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].
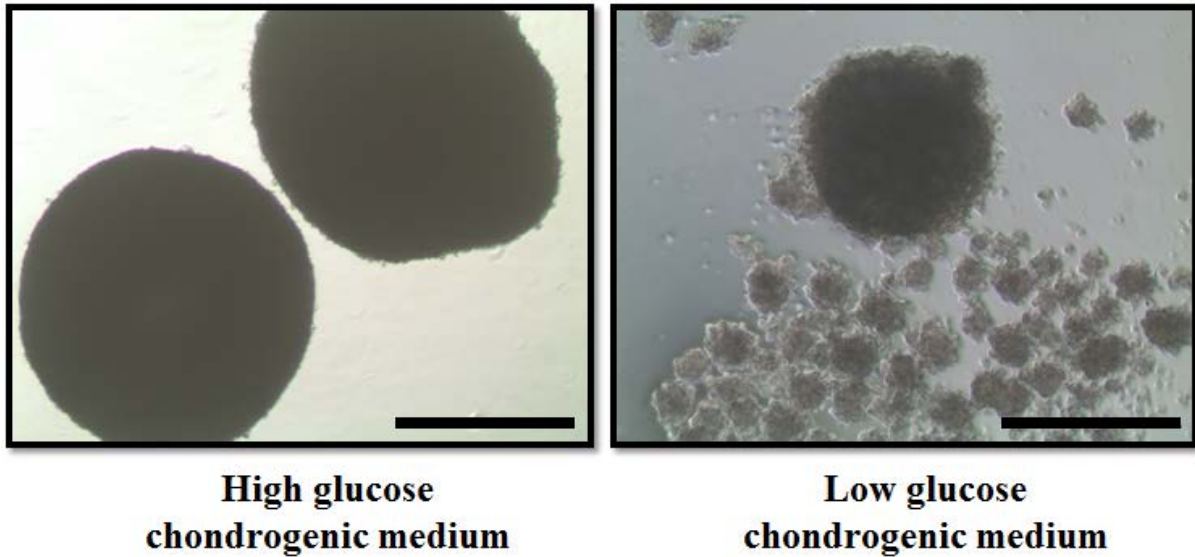
Figure 15: Comparison of spheroids from SMSCs from the same donor prepared in high glucose chondrogenic medium and low glucose chondrogenic medium. Scale bar: 500 µm.

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

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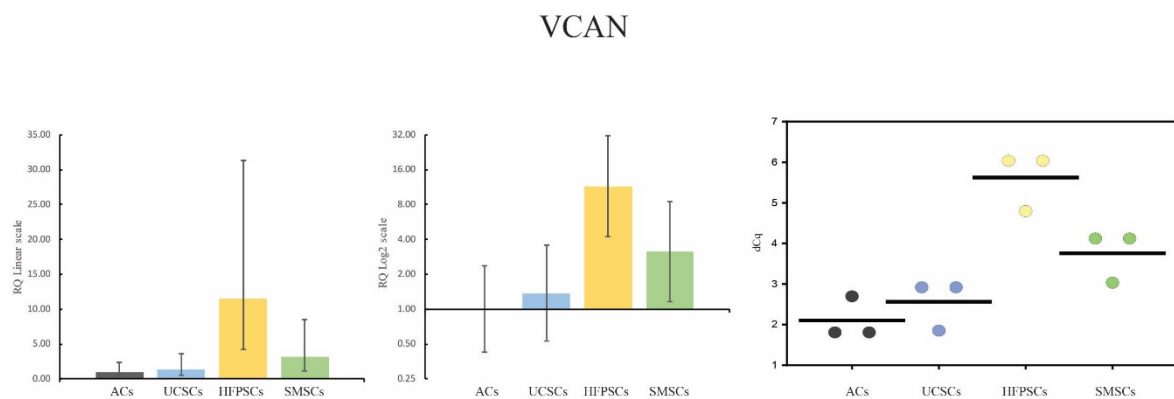
Figure 16: Comparison of VCAN expression using a linear scale, log scale and dCq in 3D culture.

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).
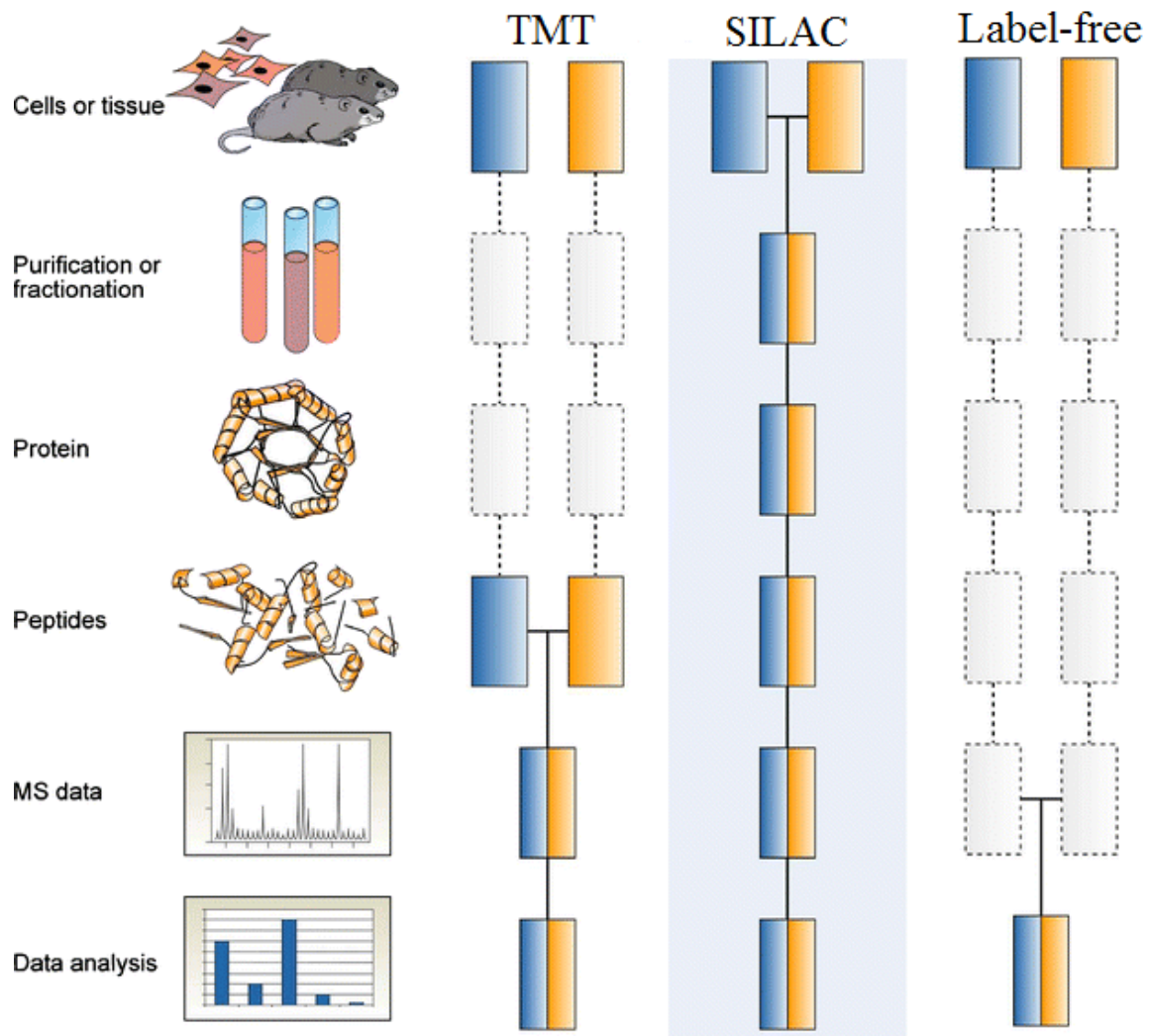
Figure 17: Schematic of different label-based and label-free protein quantification methods (Adapted with permission from [241]).

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
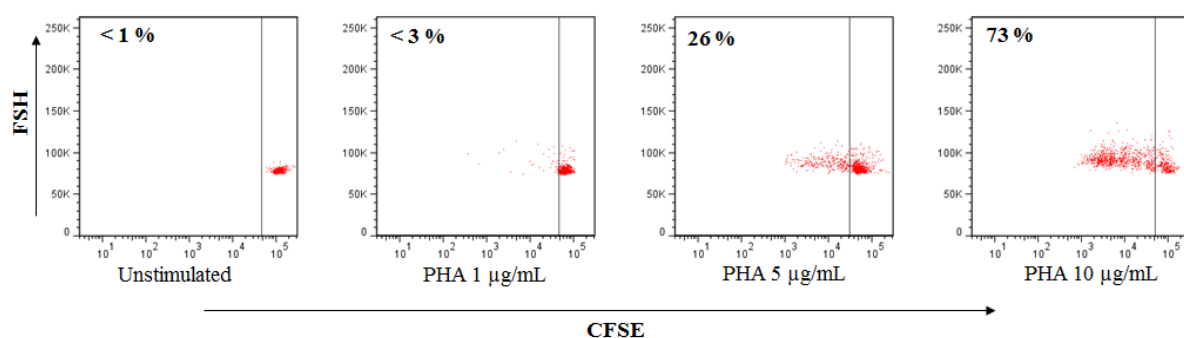
Figure 18: Activation of lymphocytes with different concentration of PHA.

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.
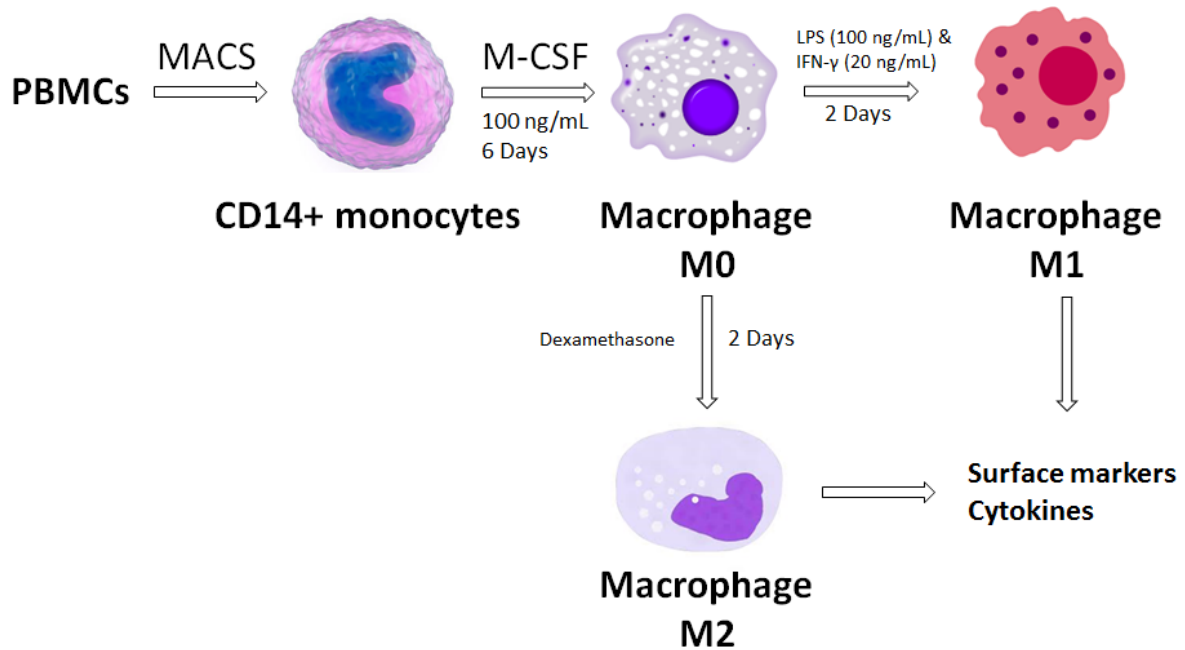
Figure 19: Illustration of macrophage polarisation assay.

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.
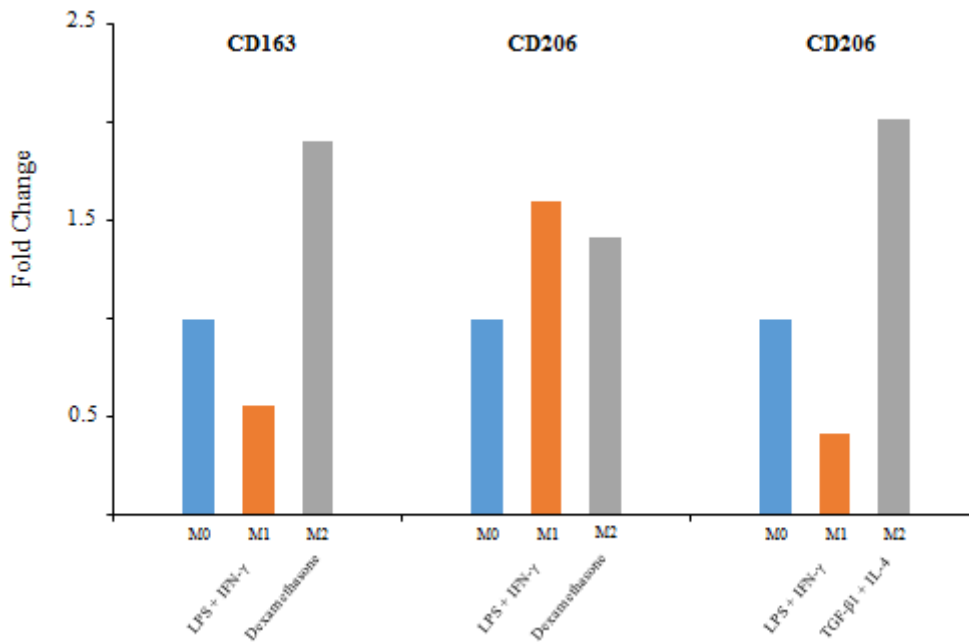
Figure 20: Surface marker expression of CD163 and CD206 in non-polarised and polarised macrophages in the presence of different stimulants.

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).
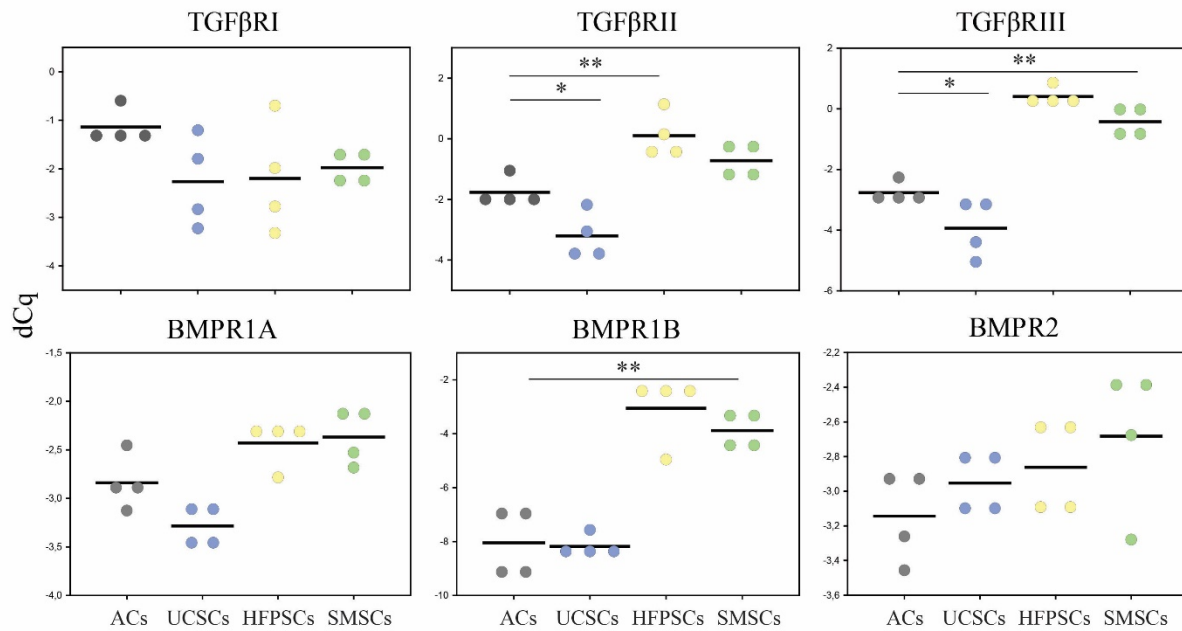
*Figure 21: Relative expression of receptors from TGF-β superfamily in different cell types before inducing chondrogenesis. Level of significance, * and ** with p-value < 0.05 and <0.005, respectively.*

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

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

Paper II

Paper III