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ARJEN GEBRAAD
Tissue Engineering Approaches for the Treatment of Degenerated Intervertebral Discs

CLINICUM
DEPARTMENT OF ORAL AND MAXILLOFACIAL SCIENCES
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DOCTORAL PROGRAMME IN ORAL SCIENCES
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Tissue Engineering Approaches for the Treatment of Degenerated Intervertebral Discs

Academic Dissertation

to be presented with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Lecture hall 3 of Biomedicum 1, Haartmaninkatu 8, Helsinki on February 16th 2018, at 12 noon

by

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To the little dragon
Abstract

This dissertation describes novel tissue engineering approaches for the treatment of intervertebral disc (IVD) degeneration.

Defects in the outer part of the IVD, the so-called annulus fibrosus (AF), cannot be restored using the current surgical methods. AF tissue cannot be regenerated using autologous AF cells, because of their limited availability and expansion capacity. We compared the potency of transforming growth factor-β (TGF-β) type 1 and type 3 to stimulate differentiation of adipose tissue derived-mesenchymal stem/stromal cells (AT-MSCs) towards an AF-like phenotype. We engineered AF tissue in vitro by seeding and stimulating AT-MSCs inside AF-mimetic polymer scaffolds. Several techniques to seed cells in the scaffolds were compared.

Cells release membrane vesicles called extracellular vesicles (EVs). EVs have gained interest as a biomimetic tool to induce lineage-specific differentiation of stem cells. We evaluated gene expression in AT-MSCs after exposure to EVs from activated monocytes or osteoclasts.

Neither TGF-β1 nor TGF-β3 increased matrix synthesis by AT-MSCs. Nevertheless, TGF-β3 supported cell proliferation and thereby matrix accumulation. Seeding of AT-MSCs into the scaffolds using fibrin gel resulted in superior cell distribution, proliferation and AF-like matrix production compared to other seeding strategies. The formed collagen was aligned into bundles within the pore channels of the scaffolds.

Activated monocytes promoted the chemotaxis of immune cells by AT-MSCs via EV-mediated signalling, showing the importance of EVs in controlling the function of the immune system. Monocyte-derived EVs upregulated the expression of matrix metalloproteinases in AT-MSCs.

The results show the potential of AT-MSCs as a multipotent cell source in tissue engineering applications for the treatment of degenerated IVDs. The extracellular matrix deposited in the cell-seeded scaffolds had a composition and structure similar to that in native AF tissue, demonstrating the high potential of this strategy in AF repair. Furthermore, the study sheds light on the mechanism by which EVs regulate the immune system and bone remodelling.
Samenvatting

Dit proefschrift beschrijft onderzoek naar weefseltechnologietoepassingen voor de behandeling van versleten tussenwervelschijven.

Beschadigingen aan het buitenste deel van de tussenwervelschijf, de zogenaamde annulus fibrosus (AF), zijn met de huidige chirurgische technieken niet te herstellen. In dit onderzoek vergeleken we het vermogen van groefactor TGF-β type 1 en type 3 om mesenchymale stamcellen uit vetweefsel te laten differentiëren naar cellen met kenmerken van cellen in de AF. Ook maakten we nieuw AF-weefsel in het laboratorium door stamcellen te zaaien in een kunststof draagconstructie (Engels: scaffold). De structuur van de draagconstructie was gebaseerd op die van AF-weefsel.

Cellen scheiden blaasjes af die extracellulaire vesikels (EV’s) worden genoemd. EV’s staan in de belangstelling als een mogelijk natuurlijk hulpmiddel om stamcellen te laten differentiëren naar een specifiek celtype. We analyseerden de genexpressie in stamcellen die waren blootgesteld aan EV’s van monocyten (een type witte bloedcel) en osteoclasten (botafbrekercellen).

TGF-β1 en TGF-β3 stimuleerden geen van beide de aanmaak van matrixcomponenten door de stamcellen. Desondanks ondersteunde TGF-β3 de celvermeerdering en daarmee de opbouw van een extracellulaire matrix. Het zaaien van de stamcellen in de draagconstructie door middel van fibrine-gel was de best geteste zaatechniek wat betreft de verdeling en vermeerdering van de cellen en de vorming van een AF-achtige matrix. De gevormde collageenbundels volgden de kanaaltjes in de draagconstructie.

Geactiveerde monocyten stimulerden via hun EV’s de aantrekking van immuuncellen door de stamcellen. EV’s van monocyten stimuleerden ook de expressie van genen die coderen voor matrix-metalloproteinases. Deze enzymen breken collageen af.

Dit proefschrift laat de mogelijkheden zien van mesenchymale stamcellen uit vetweefsel in weefseltechnologietoepassingen voor de behandeling van versleten tussenwervelschijven. We toonden aan dat onder de juiste omstandigheden de stamcellen een matrix afscheiden die zowel in samenstelling als in structuur sterke overeenkomsten vertoont met die van de AF. Verder werpt dit proefschrift licht op de manier waarop EV’s het afweersysteem regelen, en de opbouw en afbraak van bot controleren.
Väitöskirjan aiheena on kudosteknologiset sovellukset nikamaväilevyn rappuuman hoi- toon.

Nykyisillä kirurgisilla menetelmissä ei voida korjata nikamaväilevyn ulomman sitkeän syykehän eli niin sanotun annulus fibrosuksen (AF) vaurioita. Tässä tutkimuksessa vertasimme TGF-β-kasvutekijän tyyppein 1 ja 3 kykyä ja tehokkuutta rasvakudoksen mesenkymaalisten kantasolujen erilaistamisessa AF-soluiksi. Tuotimme uutta AF-kudosta laboratoriossa istuttamalla kantosoluja AF-kudosta jäljitteleviin polymeerimateriaalista valmistettuihin kehikkoihin (engl. scaffold). Vertailimme myös useita erilaisia solujen istutusmenetelmiä.


TGF-β1 ja TGF-β3 eivät kumpikaan lisänneet kantasolujen soluväliaineen synteesiä. TGF-β3 kuitenkin tuki solujen lisääntymistä ja siten soluväliaineen muodostumista. Kantasolujen istuttaminen kehikoihin fibrinigleelin avulla osoittautui parhaaksi testatuista istutusmenetelmiä solujen levineisyyden, lisääntymisen ja soluväliaineen muodostumisen suhteen. Muodostetut kollageenikimput seurasivat kehikkojen pieniä kanavia.

Aktivoidut monosyytit tukivat solunulkoisten vesikkeen avulla prosesseja, joissa kan tasolot vetävät valkosoluja puoleensa. Monosyyttien solunulkoiset vesikkelit lisääivät myös kollageenia hajottavien entsyymien, matriksin metalloproteinaasien, ilmentymistä.

Tämä väitöskirja esittelee rasvakudoksen mesenkymaalisten kantasolujen mahdollisuksia monipotentiaalisen solulähteenä kudosteknologisissa sovelluksissa nikamaväilevyn rappuuman hoidossa. Soveltuville olosuhteille kantasolut tuottivat soluväliainetta, jolla oli samanlainen koostumus ja rakenne kuin alkuperäisessä AF-kudoksessa. Lisäksi tutkimus valottaa sitä, miten solunulkoiset vesikkelit ohjaavat immuunipulositusjärjestelmän toimintaa sekä luukudoksen muodostumista ja hajoamista.
Preface

This work presented in this dissertation was carried out in the Adult Stem Cell group at the University of Tampere, at the Department of Biomaterials Science and Technology at the University of Twente and at the Department of Oral and Maxillofacial Diseases at the University of Helsinki. The research was financially supported by University of Helsinki project funding, the Finnish Cultural Foundation and Helsinki University Hospital State funding. I thank my supervisor Adj. prof. Suvi Haimi for her guidance and encouragement. It is thanks to her commitment that my work could lead to this thesis. Adj. prof. Susana Miettinen, Prof. Dirk Grijpma, Dr. André Poot, Prof. Riitta Seppänen-Kajiansinkko and Dr. Bettina Mannerström allowed me to work in their groups and I acknowledge their valuable advice. Dr. Sébastien Blanquer put a lot of time and effort in preparing the scaffolds and helping me out in the lab. I have gotten excellent technical assistance in all labs. Especially Anna-Maija Honkala, Miia Juntunen and Sari Kalliokoski are thanked for their contributions.

My work has benefited from the expertise of biologists, engineers, chemists, surgeons, dentists among others. I am happy to have shared many great experiences with my colleagues, friends and family in Capelle, Enschede, Tampere, Turku, Espoo, Helsinki and beyond. I appreciate that some of you came from far to join me in hiking, skiing, cycling and camping, making music, picking berries and mushrooms, swimming and going to sauna, drinking coffee, beer and wine, and eating pulla and apple pie. I want to thank my parents in particular for all their support over the years and for travelling the distance to spend time together. Finally, I want to thank Hanna for her love and for coping with my frustrations that have been part of this learning process. I could not do without you.

‘Hukka’, Mikkeli, Christmas Eve 2017
Arjen Gebraad

Als de hemel valt, de hemel faalt.
De druk op God wordt groter en Zie draagt het allemaal.
Maar als de hemel valt, zullen we het samen moeten dragen en kunnen Zij en Allah samen even weg.

from the song ‘Hemel valt’ by Typhoon
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The dissertation is based on the following publications, which are referred to in the text by their Roman numerals (I-III):

I. Gebraad A.W.H., Miettinen S., Grijpma D.W., Haimi S.P.
   Human Adipose Stem Cells in Chondrogenic Differentiation Medium without Growth Factors differentiate towards Annulus Fibrosus Phenotype in Vitro

    Differentiation of Adipose Stem Cells seeded towards Annulus Fibrosus Cells on a Designed Poly(Trimethylene Carbonate) Scaffold prepared by Stereolithography
    Journal of Tissue Engineering and Regenerative Medicine, 2017; 11: 2752-2762.
    Published online Jul 4 2016. 10.1002/term.2170

    Monocyte-derived Extracellular Vesicles Stimulate Immune Cell Chemotaxis and Collagen Removal by Mesenchymal Stem/Stromal Cells
    Submitted.

* These authors contributed equally.
Personal contribution

Publication I
I designed the experiment together with SH and SM. I carried out cell cultures, collection of samples and analysis. I performed the data interpretation, wrote the manuscript draft and revised the manuscript together with the other co-authors.

Publication II
I designed the experiment together with SH. I carried out cell cultures, collection of samples and analysis. I performed the data interpretation, wrote the manuscript draft together with SB and revised the manuscript together with the other co-authors.

Publication III
I designed the experiment and carried out cell cultures, collection of samples, isolation of EVs and analysis. As part of this project, I introduced new methodologies to our lab including EV uptake experiments, the isolation and culture of monocytes and their differentiation towards osteoclasts. I interpreted the data including analysis of the microarray data and gene ontology enrichment analysis. I wrote the manuscript draft and revised the manuscript together with the other co-authors.

For this dissertation, I revised the presentation and statistical analysis of the data from publication I and II. In addition, previously unpublished results are presented.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>AF</td>
<td>annulus fibrosus</td>
</tr>
<tr>
<td>AFC</td>
<td>annulus fibrosus cell</td>
</tr>
<tr>
<td>AT-MSC</td>
<td>adipose tissue-derived mesenchymal stem/stromal cell</td>
</tr>
<tr>
<td>BMU</td>
<td>basic multicellular unit</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>CM</td>
<td>chondrogenic medium</td>
</tr>
<tr>
<td>DM</td>
<td>differentiation medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EV</td>
<td>extracellular vesicle</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>ICAM1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IVD</td>
<td>intervertebral disc</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor 1</td>
</tr>
<tr>
<td>MC-EV</td>
<td>extracellular vesicle derived from a lipopolysaccharide-activated monocyte</td>
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<tr>
<td>MM</td>
<td>maintenance medium</td>
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</table>
MMP  matrix metalloprotease
MRI  magnetic resonance imaging
MSC  mesenchymal stem/stromal cell
MVE  multivesicular endosome
NTA  nanoparticle tracking analysis
NP   nucleus pulposus
NPCM nucleus pulposus culture medium
OCDM osteoclast differentiation medium
OC HA-EV extracellular vesicle derived from an osteoclast cultured on hydroxyapatite
OC TCPS-EV extracellular vesicle derived from an osteoclast cultured on tissue culture polystyrene
ODM  osteogenic differentiation medium
OPG  osteoprotegerin
PBS  phosphate buffered saline
PCL  poly(ε-caprolactone)
PTMC poly(trimethylene carbonate)
RANK receptor activator of nuclear factor κ-B
RANKL receptor activator of nuclear factor κ-B ligand
RT-qPCR reverse transcription quantitative polymerase chain reaction
sGAG sulphated glycosaminoglycan
S1P  sphingosine-1-phosphate
SMAD small mothers against decapentaplegic homolog
SVF  stromal vascular fraction
TCPS tissue culture polystyrene
TGF-β transforming growth factor beta
TR-AP tartrate resistant acid phosphatase
Wnt wingless-type protein

Gene symbols in italics correspond to the unique symbols approved by the HUGO Gene Nomenclature Committee.
Chapter 1

Introduction

The intervertebral discs (IVDs) connect the vertebrae of the spinal column. They serve as shock absorber and allow limited mobility of the spine. Degeneration of the IVD is a strongly associated cause of lower back pain and related diseases of the spine. These skeletal defects require effective and feasible new treatments as they remain one of the major musculoskeletal health issues in both Western industrialized nations and in the rest of the world (Walker, 2000).

The inner part of the IVD is a gelatinous structure called nucleus pulposus (NP). It is confined by a highly organized ring rich in collagen fibres called annulus fibrosus (AF). Degeneration of the IVD leads to tearing of the AF (Bron et al., 2009) ultimately leading to disc herniations in which the NP protrudes through tears in the AF. Disc herniation is a major cause of low back pain that frequently occurs in young and middle-aged patients (An et al., 2003). Even minor injuries to the AF can lead to permanent disc damage (Fazzalari et al., 2001). To date no convincing therapeutic approach has been able to treat AF defects effectively.

Although autologous AF cell transplantation therapies to regenerate AF tissue have had some success in animal models (Sakai and Andersson, 2015), an alternative for clinical therapy is required because of the limited availability and expansion capacity of autologous AF cells (Bron et al., 2009). The use of multipotent human adipose tissue-derived mesenchymal stem/stromal cells (AT-MSCs) differentiated towards an AF phenotype could avoid these problems as adipose tissue is an abundant and easily accessible cell source (Lindroos et al., 2011). Isoforms of transforming growth factor beta (TGF-β) have been used to stimulate the differentiation of animal- and human-derived AT-MSCs towards an AF phenotype (Tapp et al., 2008; Gruber et al., 2010b). However, a systematic comparison is lacking between the effect of the key isoforms of TGF-β, type 1 and type 3, on the differentiation of AT-MSCs towards an AF phenotype.

Repair of large AF defects would require a combination of mechanical and biological repair. An attractive approach is a tissue-engineered construct combining cells with a
supporting biomaterial scaffold. The biomaterial scaffold would provide immediate closure of the defect and restore the biomechanical properties of the disc, while the cellular component would repopulate the degenerated IVD and enable new extracellular matrix (ECM) synthesis at the defect site (Guterl et al., 2013). Several scaffold-processing techniques with different types of biodegradable biomaterials have been suggested for AF tissue engineering (Guterl et al., 2013). Unfortunately, none of the current strategies have been able to attain the biomechanical properties of the native AF tissue and restore its function. A major challenge has been the reproduction of the complex multilamellar structure and the biomechanical cues of the native tissue, which are prerequisites for efficient cell differentiation and ECM organization (See et al., 2011; Nerurkar et al., 2011). A scaffold for AF regeneration must preferably induce the specific orientation and direction of the collagen bundles (Nerurkar et al., 2008).

Spinal fusion is an established surgical technique that can be effective for disabling back pain due to a degenerated IVD. In spinal fusion, two adjacent vertebral bodies are fused together. After part of a disc or vertebra has been taken out, the surgeon creates an environment where the vertebrae will fuse together over time (usually over several months or longer) (Highsmith, 2016). As the surgery does not lead to successful fusion in a significant number of cases, tissue engineering approaches could enhance the bone regeneration process. In this context, AT-MSCs form an attractive multipotent cell source which has proven its therapeutic applicability for the repair and regeneration of bone tissue (Sándor, 2012; Mesimäki et al., 2009).

A typical construct for bone tissue engineering is a three-dimensional (3D) scaffold seeded with osteoprogenitor cells. Growth factors are used to induce osteogenic differentiation before the tissue-engineered construct is transplanted into the bone defect site. However, these constructs are still very different from native bone owing to lack of a more sophisticated structure and delicate organisation of multiple cell types, poor vascularisation, and difficulty in integrating with the host environment. Such drawbacks hamper their wider clinical application (Dong and Wang, 2013). Novel strategies are in high demand that aim to induce bone development in a way closer to the native scenario.

The immune system and the mononuclear phagocytic system strongly influence tissue repair and regeneration (Julier et al., 2017), including bone regeneration (Dong and Wang, 2013). Monocytes for example, play a central role in regulating bone development and repair: monocytes from the bloodstream are recruited to bone fracture sites, where they differentiate into macrophages and start to produce several pro-osteogenic factors affecting mesenchymal precursors present at the healing site (Pirracco et al., 2013; Champagne et al., 2002). Osteoclasts, which share precursors with monocytes/macrophages, couple their bone-resorbing activity to the activity of osteoblasts by providing signals that promote osteogenic differentiation of mesenchymal stem/stromal cells (MSCs) and coordinate osteoblastic bone formation (Boyce, 2013; Pederson et al., 2008; Henriksen et al., 2012). Signals from monocytes/macrophages and osteoclasts could play a pivotal role in biomimetically engineered bone regeneration.
Cells release membrane vesicles called extracellular vesicles (EVs) into the extracellular environment. EVs are capable of inducing specific cellular responses by transferring complex cargo of biologically active molecules to target cells (Ratajczak et al., 2006; Valadi et al., 2007). EVs have potential as biomimetic tools to induce lineage-specific differentiation of stem cells in regenerative medicine (de Jong et al., 2014; Narayanan et al., 2016). EVs derived from mononuclear phagocytes may contribute to bone regeneration through regulation of osteogenic differentiation of MSCs in the bone environment. In the context of tissue healing, there are few studies looking into the potential of EVs from cells of the mononuclear phagocytic system. Nevertheless, EVs from these cells most likely have a role in the crosstalk between immunity and tissue healing (Silva et al., 2017).
Chapter 2

Review of the literature

2.1 Anatomy and pathology of the intervertebral disc

The intervertebral discs (IVDs) lie in between the bodies of adjacent vertebrae in the spinal column (Figure 2.1). The IVD is a moderately moving joint that provides flexibility and elasticity, allowing for limited mobility between adjacent vertebrae and a wide range of movements to the spine as a whole. The IVDs transmit mechanical loads through the spinal column by providing strong resistance against pressure and tension (Moore, 2000; Pattappa et al., 2012). The IVD consists of a gelatinous centre called nucleus pulposus (NP), which is peripherally contained by the fibrous annulus fibrosus (AF; Figure 2.1). These tissues differ in function and consist of a specific matrix structure that is maintained by distinct cell populations (Pattappa et al., 2012; Chelberg et al., 1995).

The IVD is remarkably acellular. Cell density in the human disc is only around $6 \times 10^6$ cells/cm$^3$, much less than mature hyaline cartilage, which has a cell density of $1.4 \times 10^7$ cells/cm$^3$ (Roughley, 2004; Maroudas et al., 1975). Nevertheless, the cells are essential for disc function since they manufacture and turn over the macromolecules of the extracellular matrix (ECM) which regulate the mechanical response of the disc to load. The main structural components of the IVD are fibrillar collagens, aggrecan and water. Together these constitute 90-95% of the volume of the normal disc (Urban and McMullin, 1988). The collagen network anchors the disc to the vertebral bodies, and its intricate weave allows the disc to deform in flexion or torsion. The proportion of collagen, proteoglycans and water varies across the disc. Fluid and aggrecan concentrations are the highest in the NP and the lowest in the outer AF, whereas the reverse is true for collagen (Urban and McMullin, 1988). Changes in composition from the cervical to the lumbar part of the spine have also been described, though these have been less well characterised (Urban et al., 2000).

During development, tiny blood vessels penetrating the end-plates provide nutrition to the developing IVD. These blood vessels persist only until skeletal maturity, at which
Figure 2.1: Schematic representation of the intervertebral disc. The intervertebral disc is located in between the vertebral bodies of the spinal column (left). A cut out section of the disc showing the lamellar structure of the annulus fibrosus (AF) surrounding the gelatinous nucleus pulposus (NP) in the centre. Successive lamellae contain collagen fibres with an alternating orientation forming an angle-ply structure. In between the lamellae are the interlamellar septa that contain proteoglycans. Adapted from Smith et al. (2011) and Ngwa and Agyingi (2012).

time the IVD becomes almost completely avascular. In fact, the largest discs in the adult human lumbar spine can be up to 20 mm from the nearest blood vessels, making them the largest avascular structures in the body. Apart from a sparse blood supply in the outer AF, nutrients and metabolites reach the cells in the IVD by means of diffusion (Moore, 2000).

2.1.1 Nucleus pulposus

The NP develops embryologically from the notochord, a rod-like structure derived from the mesoderm (Stemple, 2005). At the level of the developing vertebrae, the notochord is replaced by bone. In between the vertebrae, the notochord enlarges to form the NP (Urban et al., 2000). In humans, notochordal cells are essentially replaced by chondrocyte-like cell by age 10, although in some species they persist throughout life. It is presently unclear whether this change in cell populations is due to dedifferentiation of the notochordal cells, or due to apoptosis of the resident cells and the subsequent invasion by mesenchymal cells (Errington et al., 1998; Urban et al., 2000; Hunter et al., 2004).

The NP has a cell density of about $4 \times 10^6$ cells/cm$^3$ at maturity (Roughley, 2004; Maroudas et al., 1975). Like hyaline cartilage, the NP is mostly made up of water
(70-90%, depending on the age). A high hydrostatic pressure gives the NP the ability to resist compression under load. The high level of hydration is maintained by the presence of proteoglycans (ca. 50% of dry weight). Proteoglycans consist of a positively charged protein core with a great number of negatively charged sulphated glycosaminoglycans (sGAGs). The proteoglycans aggrecan and biglycan are highly present within the NP (Pattappa et al., 2012; Melrose et al., 2001).

The cells and proteoglycans are embedded in a mesh of randomly oriented collagen fibres (mainly collagen type II), which makes up 20% of the dry weight of the NP of a younger person (Buckwalter, 1995). NP tissue can be distinguished from hyaline cartilage by measuring the collagen:sGAG ratio (Mwale et al., 2004). The collagen:sGAG ratio is 1:5 in the NP of young adults, while it is 5:2 in the hyaline cartilage endplate (Mwale et al., 2004).

2.1.2 Annulus fibrosus

The AF develops from the mesenchymal tissue surrounding the notochord. Around 5-6 weeks of foetal development, the mesenchymal tissue begins to segment and form regularly spaced condensations that give rise to the AF (Urban et al., 2000).

Being subjected to both tension and compression, the AF contains relatively large amounts of both collagen and GAG. The AF is therefore a fibrocartilage tissue, whose ECM is similar in composition to that of the knee meniscus (Nerurkar et al., 2011). The AF has complex mechanical properties, which are nonlinear, viscoelastic (rate-dependent) and anisotropic (direction-dependent). The tensile, compressive, and shear properties in the axial, circumferential, and radial directions may differ by an order of magnitude (Nerurkar et al., 2008; Sun and Leong, 2004). These mechanical properties originate from a highly organized, lamellar structure. Based on structural and cellular differences, the AF can be further distinguished into an inner and an outer part. The outer AF is the most fibrous part. The inner AF contains less collagens and lacks the organization of the outer AF. It forms a broad transition zone with the NP and consists of a mixture of the ECM components of both (Bron et al., 2009; Yu et al., 2007). The collagen:sGAG ratio in the AF is 3:1 on average. The collagen:sGAG ratio can be used as a specific parameter to distinguish AF from NP tissue (Mwale et al., 2004).

The fundamental tension-bearing elements in the AF are bundles of collagen. Collagens constitute the bulk of the ECM in terms of dry weight, comprising 40-60% of the outer AF and 25-40% of the inner AF (Smith and Fazzalari, 2009). Collagen types I and II are the most prevalent. The relative proportions of collagen type I and type II change gradually from the outer towards the inner AF. Collagen type I is most prevalent in the outermost regions and collagen type II is most prevalent close to the NP (Eyre and Muir, 1976). Bundles of collagen fibres form concentric, cylindrical lamellae around the spinal axis. In the lumbar AF, the number of distinct lamellae varies between 15 and 25, depending on the circumferential location, spine level and age. The orientation
of collagen fibres in successive lamellae alternates with respect to the spinal axis, thus forming a reinforced angle-ply structure (Figure 2.1). The angle with the vertical axis decreases from 62° at outer edge of the AF to 45° close to the NP (Cassidy et al., 1989).

The spaces between the lamellae, the so-called interlamellar septa, mainly contain proteoglycans (Figure 2.1). Proteoglycans constitute 58% of the outer AF and 11-20% of the inner AF (Smith and Fazzalari, 2009). They are responsible for the hydration of the tissue through their water-binding capacity, enabling the tissue to undergo rapid reversible deformation and distributing the force around the circumference of the AF (Cassinelli et al., 2001; Pattappa et al., 2012). The large aggregating proteoglycans include aggrecan and versican, whereas the small interstitial proteoglycans include biglycan, decorin, fibromodulin and lumican (Singh et al., 2009). Decorin expression in the IVD is confined to the AF (Melrose et al., 2001). Apart from proteoglycans, the interlamellar septa contain a complex structure of linking elements creating interlamellar cohesion (Pezowicz et al., 2006).

The cell density in the AF is about $9 \times 10^6$ cells/cm$^3$, which is over two times higher than in the NP (Roughley, 2004; Maroudas et al., 1975). Although all AF cells (AFCs) are derived from the mesenchyme, cells in the different regions of the AF differ in morphology and ECM components they produce. The main collagen type produced by outer AFCs is collagen type I. The outer AFCs are fusiform shaped and align with the collagen fibres. The cells are interconnected through very long membrane protrusions. The protrusions gradually become shorter and thicker towards the inner AF. AFCs closest to the NP closely resemble chondrocytes. Inner AFCs are spherically shaped with only one or two short protrusions. Cells in the inner AF mainly produce type II collagen (Bron et al., 2009; Bruehlmann et al., 2002).

2.1.3 Degeneration of the intervertebral disc

IVD degeneration is characterized by changes in composition of the ECM. The most significant change is the loss of proteoglycans in the NP. The loss of proteoglycans affects the penetration of molecules into the disc. In the normal disc, aggrecan prevents movement of uncharged molecules, due to its charge. Loss of aggrecan in a degenerated IVD allows growth factor complexes, cytokines and catabolic enzymes to penetrate into the disc, affecting cellular behaviour and progression of degeneration. Increased vascular and neural ingrowth seen in degenerated discs are associated with proteoglycan loss because aggrecan inhibits neural ingrowth.

As the proteoglycan content of the NP diminishes, it looses its hydrogel-like properties and becomes more fibrotic. The proportion and distributions of collagen types change, although there is only a small change in the absolute collagen quantity (Urban and Roberts, 2003). The AF looses its lamellar organization: the number of distinct lamellae in the AF decreases while the thickness of the individual lamellae increases. The collagen fibre bundles within the lamellae become more disorganised with increased
interbundle spaces (Bron et al., 2009).

IVD degeneration leads to a reduction of hydrostatic pressure and a decrease in disc height (Figure 2.2). Compromised height and stability of the disc can lead to pressure on the nerves of the spinal cord, causing radiating pain and affecting nerve function. In response to the loss of stability, bone spurs called osteophytes develop onto the normal bone structure of the vertebrae. Osteophytes may also impinge on nerves (NorthShore Encyclopedia, 2016).

IVDs degenerate far earlier than other musculoskeletal tissues; the first findings of degeneration in the lumbar discs are seen in teenagers. The degree and progression of degeneration varies between subjects for which genetic and environmental (e.g. physical loading, nutrition, smoking) factors are held responsible (Bron et al., 2009). Disc degeneration can result in lower back or upper neck pain, but this is not always the case. The degree of degeneration does not correlate well with the degree of pain that patients experience. Some people may experience no pain, while others, with the exact same amount of damage, have severe, chronic pain. It all depends on the location of the affected disc and how much pressure is being put on the spinal column and surrounding nerve roots. Nevertheless, degenerated discs are one of the most common causes of lower back pain (Lim, 2017).

2.1.4 Disc herniation

As a result of aging or degeneration, reduced height and stability of the disc lead to an increase of the strain on the fibres in the AF. Simultaneously, with the loss of lamellar organization, the AF looses its ability to withstand the forces from inside the disc (Bron et al., 2009). This can lead to tears and fissures in the AF and ultimately to protrusion of the NP, called disc herniations (Figure 2.2). Traumatic injury, possibly in combination with degenerative failure of the AF may provoke a disc herniation.

The herniations may impinge on nerves of the spinal cord or cause an inflammatory response, leading to back pain (Richardson et al., 2006) (Figure 2.2). Disc herniations are one of the major causes of low back pain that frequently occur in young and middle-aged patients (An et al., 2003). Often, herniated discs are not diagnosed immediately, as the first symptoms are undefined pains in the thighs, knees, or feet. Disc herniations were found in 50% of symptom-free participants in a small-sample study, suggesting that a considerable part of the population can have herniated discs that do not cause noticeable symptoms (Ernst et al., 2005).

Current classifications of disc herniations focus on the amount and localization of herniated NP material. Modern quantitative magnetic resonance imaging (MRI) techniques can detect degenerative changes at earlier stages by determining the morphology and degeneration of the AF (Hoppe et al., 2012; Zobel et al., 2012)
Figure 2.2: Pathology of the intervertebral disc (IVD). Degenerative changes in the IVD lead to a decrease in height and a loss of stability of the spinal column. The nucleus pulposus can press outwards on the weakened annulus fibrosus, causing the IVD to bulge outwards. A disc herniation occurs when the nucleus pulposus protrudes through tears in the annulus fibrosus. A disc bulge or herniation can pinch nearby nerves, causing pain or numbness in various parts of the body depending on its location. Adapted from The Spine Hospital at the Neurological Institute of New York (2017) and Servier Medical Art.

2.2 Strategies for treating degenerated intervertebral discs

Degenerated IVDs and/or disc herniations can often be successfully treated without surgery. The majority of patients are treated with rest, exercise, pain killers and/or steroid injections. The large majority of minor disc herniations heal on their own with conservative treatment. Surgery becomes an option when several months of conservative treatment - about six months - have not helped to reduce the pain. Surgery is usually only recommended for relatively young patients, as recovering from surgery is a tough process and older patients are more susceptible to complications. The two most common surgical procedures for patients with degenerated IVDs are discectomy and spinal fusion (Highsmith, 2016).

2.2.1 Discectomy

Discectomy is the surgical removal of the herniated disc material that impinges on nerves. Discectomies are the most common surgical procedure performed for patients suffering from back pain (Deyo and Weinstein, 2001). Discectomy is an effective treatment as it releases pain and improves physical function in the vast majority of patients. However, recurrent disc herniations are quite frequent and often require revision surgery. Progressive degeneration leading to instability and loss of disc height frequently occur after a discectomy (McGirt et al., 2009). The surgical outcome is particularly limited in cases of large disc protrusions with only minor disc degeneration, which
mainly occur in relatively young patients (Guterl et al., 2013). Advances in options have produced minimally invasive alternatives to traditional discectomy procedures, such as endoscopic discectomy and laser discectomy. These options have benefited patients, but still damage the disc structure and the progression of disc degeneration cannot be prevented (An et al., 2003).

2.2.2 Spinal fusion

Spinal fusion is a process of fusing two adjacent vertebral bodies together. Spinal fusion is often performed when the disc is reduced in height due to degenerative or age-related changes that decrease the swelling pressure in the disc. A spinal fusion will stabilize the spine and recover its height. After part of a disc or vertebra has been taken out, the surgeon creates an environment where the vertebrae will fuse together over time (usually over several months or longer). Bone grafts (usually an autograft) or degradable biomaterials are used to stimulate bone growth. Screws, rods, plates, or cages can be used to hold the bones in place while bone union between the two vertebrae takes place (Highsmith, 2016) (Figure 2.3).

The major drawback of these procedures is that the disc structure is highly compromised. Spinal fusion changes the normal motion of the spine. Spinal fusion is usually performed when the disc degeneration or disc herniation is restricted to just one or two levels. Multi-level spinal fusion may restrict the mobility of the spine too much. Rigidity at the fusion site has the potential to accelerate degeneration of IVDs on adjacent levels (Ekman et al., 2009).

In a significant number of cases, fusion of the vertebrae does not occur and the patient may require another operation. Depending on the technique used, fusion rates of 60 to 95% have been reported. Higher fusion rates are generally seen in the more invasive techniques with added surgical risk. Satisfactory reduction of pain occurs in 50-80% of patients. If the patients pain was wrongly assigned to a certain spinal level, the patient will still have pain after fusion (Spivak, 2006).

2.2.3 Biological repair

New biologic strategies that aim to reverse IVD degeneration and promote regeneration are on the rise. Given the high prevalence of degenerated IVDs and herniations in the young population, these new strategies are promising future treatment options.

The majority of current biological strategies aim to restore proteoglycan synthesis within the degenerated IVD, because the loss of proteoglycan in the NP is the first event in IVD degeneration. Proteoglycan synthesis can be increased by promoting cell viability and proliferation of NP cells, and by upregulating anabolic pathways (Leung et al., 2006; Fassett et al., 2009; Knezevic et al., 2017).
Various growth factors, such as bone morphogenic proteins (BMPs) and transforming growth factor beta (TGF-β), promote the viability of cells upon injection into the degenerated IVD. Growth factors, inflammatory inhibitors, proteinase inhibitors, and intracellular regulatory proteins have been studied as therapeutic agents for the treatment of degenerated IVDs (Fassett et al., 2009). Using these molecules in clinical therapies, however, may have limited success because of the limited therapeutic duration of molecular treatments. This strategy for regeneration will likely be limited in reversing disc degeneration (a chronic, progressive process) due to the limited therapeutic duration of the molecules. The normal in vivo half-life of many of these therapeutic molecules is on the order of minutes and therefore the effects will not last for significant periods of time. In order for these molecular therapies to be effective in long-term maintenance of the IVD, repeated or continuous infusions will be required and thus the clinical utility of direct molecular therapies seems limited (Fassett et al., 2009; Sobajima et al., 2004).

Gene therapies could overcome the limited therapeutic duration of molecular treatments. Transferring genes to the cells has the potential to facilitate a local, long-term production of therapeutic proteins. There have been encouraging results from animal studies and in vitro human cell cultures using adenovirus vectors with successful transfection rates and long-term gene expression (Sobajima et al., 2004). However, concerns exist regarding the safety of the vectors used for gene transfer, in particular injecting viral agents in close proximity to the central nervous system as would be required for IVD regeneration (Driesse et al., 2000; Fassett et al., 2009).

Despite the encouraging results of molecular and gene strategies to reverse disc degeneration, these therapies may be limited as the viable cell population within a degenerated
disc is depleted. Growth factors or gene therapies will only affect a decreased number of viable cells available and therefore their effects on the tissue as a whole will be limited.

2.2.4 Annulus fibrosus repair

The AF has only a very limited intrinsic regenerative capacity to cope with damage or degenerative changes. Healing is limited to the outer layer of the AF, where scar tissue with inferior mechanical properties is formed (Hampton et al., 1989; Osti et al., 1990; Fazzalari et al., 2001). Even small tears in the AF influence disc mechanics and pathology. Therefore, even relatively minor disruptions of the AF initiate a cascade of events that affects not only the disc, but also the vertebral body bone (Thompson et al., 2000; Fazzalari et al., 2001; Veres et al., 2008).

The most straightforward solution would be to close AF defects by direct suturing. Several attempts to close an AF defect with sutures after discectomy have been undertaken in animals, but outcomes have been inconsistent (Ahlgren et al., 2000; Heuer et al., 2008; Chiang et al., 2011; Bateman et al., 2016). Suturing of the AF is technically demanding, due to limited space and potential injury to the proximal neurological structures (Guterl et al., 2013). Suture alone is not sufficient for long-term reliable AF closure (Heuer et al., 2008; Bailey et al., 2013). Sutures do not compensate the loss of AF material nor reverse degeneration (Bron et al., 2009). For the same reasons, glues based on natural polymers, such as high-density collagen gel (Grunert et al., 2014) and fibrin-genipin glue (Guterl et al., 2014), are only capable of repairing small AF defects.

Bron et al. (2010) proposed an AF closure device based on polyethylene. The device was meant to interlock with the tissue to provide mechanical support and prevent extrusion of the NP. However, most implants revealed signs of severe plastic deformation and subsequent displacement, because of the mismatch in the elasticity modulus of the implant and the tissue.

The results of these studies indicate the need for a combined mechanical and biological repair to restore large AF defects. An attractive approach is a tissue-engineered construct combining cells with a supporting biomaterial scaffold. The biomaterial scaffold would provide immediate closure of the defect and restore the biomechanical properties of the disc, while the cellular component would repopulate the degenerated IVD and enable new ECM synthesis at the defect site (Guterl et al., 2013).

2.3 Cell sources for annulus fibrosus repair

2.3.1 IVD-derived cells

IVD-derived cells have been proposed as cell sources to repopulate the degenerated disc. Cells from the NP have the ability to increase the proteoglycan content and
viability upon transplantation and can be expanded in vitro (Sivakamasundari and Lufkin, 2013). Transplantation of NP cells delays the degenerative process and promotes regeneration in animal models of IVD degeneration (Fassett et al., 2009; Sakai and Andersson, 2015). The capacity of AFCs to reverse disc degeneration has been studied less often. To increase cell number, the AFCs are cultured in vitro first. In vitro expansion poses problems, since AFCs have only limited expansion capacity and lose differentiation markers during monolayer expansion (Chou et al., 2006). AFCs expanded in a 3D environment are more promising than monolayer expansion in terms of cell viability and matrix production (Sato et al., 2003). In addition, the use of either fibrin gel or collagen-enriched fibrin gel as a cell carrier for AFCs supports both the cell viability during in vivo implantation and the production of a fibrocartilaginous matrix by these cells (Colombini et al., 2015).

Harvesting autologous IVD cells could result in increased morbidity or further damage to the IVD. Removal of IVD cells will likely accelerate degeneration of the host disc and thus it may only be performed as part of a discectomy procedure. In addition, IVD cells obtained from an individual with a degenerative disc will have the same genetic predisposition for degeneration (Fassett et al., 2009). The use of stem cells in cell therapies could circumvent the problems associated with IVD-derived cells.

2.3.2 Stem cells

Stem cells are undifferentiated cells capable of proliferation, self renewal, conversion to differentiated cells, and regenerating tissues. There are two main types of stem cells: embryonic and nonembryonic. Embryonic stem cells are isolated from the inner cell mass of blastocysts, which forms several days after an egg is fertilised. Embryonic stem cells are pluripotent i.e. they can differentiate into all cell types. Non-embryonic stem cells are also known as adult stem cells. Non-embryonic stem cells act as a repair system for the body, replenishing adult tissues. Non-embryonic stem cells are multipotent: they have the potential to differentiate into a limited number of cell types. Unlike embryonic stem cells, the use of human adult stem cells in research and therapy is not considered controversial, as they are derived from adult tissue samples rather than human embryos designated for scientific research (Tuch, 2006).

Mesenchymal stem/stromal cells (MSCs) are a type of non-embryonic stem cells that can differentiate into lineages of mesenchymal tissues, including osteoblasts, chondrocytes, myocytes and adipocytes. MSCs reside in various locations in the body, e.g., in bone marrow, around blood vessels (as pericytes), in fat, skin, muscle, teeth and other locations (Pittenger et al., 1999; Caplan and Bruder, 2001; Lindroos et al., 2008).

Similar to other regenerative medicine procedures, MSCs are recognized as the best donor cell candidate for IVD regeneration because of their accessibility and proliferative capability (Sivakamasundari and Lufkin, 2013; Sakai and Andersson, 2015). MSCs can adopt a gene expression profile that is closer to that of native IVD tissue than to expression profiles in hyaline articular cartilage (Steck et al., 2005). In vivo studies
have demonstrated the ability of implanted MSCs to enhance matrix production, particularly GAG synthesis, and increase disc height and hydration (Sakai and Andersson, 2015; Richardson et al., 2016; Vadalà et al., 2016). Although results from animal studies are promising, concerns have arisen about MSCs migrating out of the IVD and causing undesirable bone formation in the osteophytes. In order to avoid side-effects, IVD regeneration strategies need to focus on cell carrier systems and sealing of the AF (Vadalà et al., 2012).

Harvesting MSCs from bone marrow aspirates has become a standard in the field of adult stem cell biology and regenerative medicine due to their high differentiation potentials (Pittenger et al., 1999; Caplan and Bruder, 2001). Nevertheless, there are drawbacks to using this source. Firstly, a bone marrow aspiration is a painful procedure, frequently requiring general or spinal anaesthesia. Secondly, the number of cells acquired is usually low: approximately 1 MSC per $10^5$ marrow cells (Rickard et al., 1996). Low stem cell numbers necessitate an ex vivo expansion step to obtain clinically significant cell numbers. Such a step is time consuming, expensive, and risks cell contamination (Zuk et al., 2001).

### 2.3.3 Adipose tissue-derived mesenchymal stem/stromal cells

In skeletal tissue engineering applications, adipose tissue has proven to be an attractive source of autologous stem cells. Adipose tissue can be easily obtained from either liposuction aspirates or subcutaneous adipose tissue fragments in substantial quantities with minimal risk. It is accessible at most sites used in surgical procedures, which eliminates the need of a separate harvest site. Adipose tissue consists of adipocytes surrounded by a heterogeneous population of cells, which is referred to as the stromal vascular fraction (SVF). The SVF is isolated from adipose tissue by digestion with collagenase type 1 and centrifugation enrichment. It includes cells from the microvasculature, leukocytes and an adherent cell fraction, referred to as adipose tissue-derived MSCs (AT-MSCs) (Lindroos et al., 2011). AT-MSCs are obtained as the plastic-adherent population after overnight culture. The incidence of MSCs in adipose tissue is estimated to be 1 per $10^3$ nucleated cells, which is 2 magnitudes higher than the number of MSCs in bone marrow (Zuk et al., 2001). Approximately $4 \times 10^5$ AT-MSCs can be obtained from one ml of lipoaspirate (Aust et al., 2004). AT-MSCs express cell surface markers similar to those observed on bone marrow-derived MSCs (Gimble et al., 2007). After lineage-specific stimulation, AT-MSCs differentiate into cells of mesenchymal lineages, including adipocytes, osteoblasts, chondrocytes and myocytes (Gimble and Guilak, 2003). But some evidence suggests AT-MSCs have the potential to differentiate towards non-mesenchymal lineages, such as endothelial cells, hepatocytes and neuronal cells (Tsuji et al., 2014). In addition, AT-MSCs have immunomodulatory effects and modulate tissue regeneration through the secretion of growth factors. A large number of clinical trials using AT-MSCs have been performed and many of them are ongoing (Tsuji et al., 2014).

The use of AT-MSCs differentiated towards an AF phenotype could evade the problems
of limited availability and expansion capacity of autologous AFCs (Bron et al., 2009; Steck et al., 2005). There is however, a lack of information about appropriate growth factors to differentiate ASCs towards an AF phenotype combined with a lack of conclusive phenotypic markers for AFCs (Bron et al., 2009; Hoogendoorn et al., 2008).

AT-MSCs are able to respond to soluble mediators from the IVD cells as was demonstrated by Li et al. (2005): AT-MSCs isolated from adult New Zealand white rabbits were co-cultured with NP and AF tissues. AT-MSCs co-cultured with AF tissue expressed collagen type II (COL2A1) and aggrecan (ACAN) at higher levels than AT-MSCs alone.

In order to direct AT-MSCs to an AF phenotype, Gruber et al. (2010b) and Tapp et al. (2008) assessed the effect of adding TGF-β3 in culturing of AT-MSCs in collagen sponges. AT-MSCs were isolated from the sand rat (Tapp et al., 2008) or human fat samples (Gruber et al., 2010b). Cultures exposed to TGF-β3 had significantly higher proteoglycan content than without TGF-β3. In both studies, a greater ECM production containing collagen type I and II, keratan sulphate and decorin was observed. Gruber et al. (2010b) found that genes encoding growth factors, such as BMP1, were upregulated in human AT-MSCs exposed to TGF-β3 compared to the control. Gene expression patterns were compared to the profiles of human AFCs cultured in collagen sponges. These findings support the appropriate differentiation of the human AT-MSCs towards an AF phenotype, although there were still major differences in gene expression profiles between human AFCs and AT-MSCs. Both Gruber et al. (2010b) and Tapp et al. (2008) also reported on coculture experiments of AT-MSCs with AFCs resulting in significantly increased GAG content compared to single culture of either cell type. The findings complement the work by Le Visage et al. (2006) who performed cocultures of AFC with MSCs and NP cells with MSCs. Only AFCs were associated with greater GAG content during MSC co-culture.

### 2.4 Scaffolds for annulus fibrosus tissue engineering

Scaffolds for AF tissue engineering have been prepared from natural materials. Collagen, a major component of the AF, has been used in the form of gels or as three-dimensional (3D) porous structures and provides a favourable microenvironment for IVD cells in terms of ECM production and gene expression (Gruber et al., 2004). Collagen has been combined with GAGs, such as chondroitin-6-sulphate and hyaluronan, to mimic the native ECM composition (Saad and Spector, 2004; Schneider et al., 1999; Alini et al., 2003). Scaffolds prepared from natural materials adequately support cells. Moreover, hydrogels prepared from natural materials that can be injected into the IVD offer advantages over structures that require more invasive surgical approaches to be implanted (Guterl et al., 2013). However, gels and other structures prepared from natural materials have been mechanically weak compared to AF tissue.
In order to adequately restore the biomechanical properties of the disc, scaffolds should have mechanical properties that resemble those of the native AF. To obtain scaffolds with appropriate mechanical properties, many studies have been conducted on the use of synthetic biodegradable polymeric materials. Synthetic polymeric biomaterials have proven to be useful in many tissue engineering applications, as they have highly controllable syntheses and predictable properties, flexibility in processing into desired structures, and low immunogenicity (BaoLin and Ma, 2014). Poly(ε-caprolactone) (PCL), poly(lactide) and poly(glycolide) and copolymers prepared from the respective monomers have been studied as scaffold material for AF tissue engineering applications (Guterl et al., 2013; Sharifi et al., 2015). These synthetic polymers degrade by hydrolysis of the ester bonds. The newly formed ECM should gradually take over the function of the degrading scaffold. Currently there is not a single polymer of choice for preparing scaffolds for AF tissue engineering (Sharifi et al., 2015). The desired mechanical properties depend on the location of the defect and the extent of degeneration of the AF. Therefore, Wan et al. (2007) developed biodegradable polymer networks of poly(1,8-octanediol malate) and poly(ε-caprolactone triol malate). The degradation rate and deformability of the network could be tuned by adjusting the post-polymerization time.

Fibrocartilages like the AF are highly ordered structures, densely packed with aligned collagen fibres. The highly organized anisotropic structure of the AF is essential for performing its biomechanical function. Scaffolds for AF tissue engineering should therefore not only facilitate good cell adhesion and proliferation, but the structure of the scaffold should also replicate the structural organization of the native tissue. Thereby the scaffold will successfully rescue the mechanical function of the damaged tissue and enable synthesis of an ECM similar to that of native AF tissue. The structural and mechanical environment is suggested to be a critical determinant of the cell phenotype that also may guide the differentiation of MSCs (See et al., 2011; Nerurkar et al., 2011).

Nerurkar et al. manufactured nanofibrous planar sheets from PCL by electrospinning. These planar sheets were seeded with MSCs and combined into bilayers to generate angle-ply laminate tissues that replicate the native internal structure of the AFs. The nanofibrous assemblies directed cell alignment and ECM deposition, thereby replicating the form and function of native AF tissue (Nerurkar et al., 2008, 2009). The laminates could be assembled into whole disc composites containing an angle-ply AF surrounding a central NP, providing a foundation for eventual IVD replacement (Nerurkar et al., 2010b). However, the slowly-degrading PCL nanofibres were not optimized for tissue repair. Furthermore, the angle-ply laminates would be difficult to apply for the repair of small AF defects that differ from patient to patient. In order to obtain a construct suitable for the repair of AF defects, one should establish tissue regeneration in a 3D scaffold that is tailored for the defect.

Among the different methods to prepare such scaffolds, the additive manufacturing technique stereolithography would be the most appropriate. Stereolithography is known to be a most versatile 3D structure processing method, with the highest accuracy and precision (Melchels et al., 2010). Stereolithography is based on the spa-
tially controlled solidification of a liquid resin by photopolymerization in a layer by layer manner. Stereolithography can be used to create complex scaffold designs from a computer-aided design file. The structure and pore characteristics of the scaffold can be precisely designed using 3D drawing software or described using mathematical equations (Melchels et al., 2010; Skoog et al., 2014). Stereolithographic models have been used in medicine since the 1990s (Klimek et al., 1993) for creating accurate 3D models of various anatomical regions of a patient based on datasets acquired by x-ray computed tomography, MRI, or other scans (Bouyssié et al., 1997). Stereolithography can reproduce the complex internal structure of AF tissue into a tailor-made scaffold based on scans of the AF defect.

3D porous structures can be designed and prepared by stereolithography using resins based on poly(trimethylene carbonate)(PTMC) macromers. PTMC is known for its biocompatibility and biodegradability (Zhang et al., 2006; Vyner et al., 2014) but also for its rubber-like properties. Tough, flexible networks can be prepared, whose elastic moduli, elongations and tensile strength increase with increasing PTMC macromer molecular weight (Schüller-Ravoo et al., 2011). Blanquer et al. (2012) prepared scaffolding structures for AF tissue repair by stereolithography using PTMC resins. PTMC macromers with an intermediate molecular weight of 5,000 g/mol allowed for the formulation of suitable resins with small amounts of diluents and materials with good mechanical properties after photo-cross-linking. The resulting highly elastic and flexible scaffolds matched the mechanical properties of tissues such as AF. Although scaffolds with a great variety of pore architectures could have been prepared, the authors chose a pore structure with a gyroid architecture, because this pore structure was shown to be effective for seeding and subsequent proliferation of cells (Melchels et al., 2009).

None of strategies reported in the literature describe a 3D scaffold designed with an oriented channel-like pore architecture that reproduces the complex structure of AF tissue (Figure 2.4). Such scaffolding structures would allow to precisely reproduce the native orientation of collagen fibres in AF tissues, which is vital for performing its biomechanical function.
2.5 Bone tissue

Bones are made up of bone tissue as well as bone marrow, small blood vessels, epithelium and nerves. Bone tissue forms the rigid part of the bones and is the major structural and supportive connective tissue of the body. Bone tissue consists of bone matrix and the bone cells within it. In mature bone in most higher animals, cells usually represent up to 5% of the volume of bone tissue (Heaney and Whedon, 2017).

2.5.1 Bone matrix

The bone matrix is the intercellular substance of bone, consisting of organic material and minerals. Organic material comprises 50% of the volume and 30% of the dry weight of the bone matrix, with minerals making up the remainder (Heaney and Whedon, 2017). The major minerals in bone matrix are calcium and phosphate. When first deposited, mineral is amorphous, but with maturation it becomes typical of the apatite minerals, the major component being carbonated hydroxyapatite (Legros et al., 1987). Bone mineral gives the bone stiffness and compressive strength. The organic material consists of collagen fibres with small amounts of polysaccharides and glycosaminoglycans. Collagen fibres, predominantly collagen type I, are responsible for tensile strength and elasticity of bone tissue. The combination of hard mineral and flexible collagen makes bone harder and stronger than cartilage without being brittle (Heaney and Whedon, 2017).

2.5.2 Bone cells

Cells in bone originate from two cell lineages. Preosteoblasts, osteoblasts, osteocytes and bone lining cells develop from mesenchymal stem/stromal cells (MSCs). Monocytes, macrophages, pre-osteoclasts and osteoclasts differentiate from haematopoietic stem cells.

Osteoblast lineage

MSCs are maintained in self-renewing undifferentiated compartments of bone. They reside at the boundaries of compartments of differentiating cells. Bone is the richest source of MSCs, reflecting the activity of bone remodelling (Blair et al., 2008). Specific conditions that can induce osteoblast formation have not been defined, although isolated MSCs form osteoblasts in vitro under permissive conditions that include dexamethasone, β-glycerol phosphate, and ascorbic acid (Pittenger et al., 1999). The sequence of cellular differentiation markers and transcription factors that are expressed during osteoblast differentiation has been extensively studied (Blair et al., 2008).

During osteoblast differentiation, the developing progenitor cells express runt-related transcription factor 2 (RUNX2). Other transcription factors are involved in osteoblast
differentiation, of which at least transcription factor Sp7 (also known as osterix) is absolutely required (Karsenty, 2008). Commitment to osteoblast progenitor cells coincides with high level expression of receptors for hormones and cytokines including parathyroid hormone, prostaglandins, interleukin 11, insulin-like growth factor-1 and TGF-β. The osteoblast progenitor cells continue to proliferate but have greatly reduced plasticity. Wingless-type proteins (Wnt’s) and BMPs drive these early events, while the helix-loop-helix proteins Twist and Id maintain proliferation (Blair et al., 2008).

Once the cells have reached the state of osteoblasts, the cells stop proliferating and start synthesizing organic bone matrix, or osteoid. Osteoid consists primarily of cross-linked collagen type I. In addition, non-collagenous proteins are produced including osteocalcin, a small vitamin-K-dependent calcium binding protein that is specific to bone, and alkaline phosphatase, which is important to mineral deposition (Blair et al., 2008). Bone gamma-carboxyglutamate protein is secreted solely by osteoblasts at the late differentiation stage. This stage coincides with the onset of mineralization (Caetano-Lopes et al., 2007).

Following matrix formation, osteoblasts can have various fates. The osteoblast can simply die by programmed cell death. Another fraction of the osteoblasts are incorporated within the newly laid down matrix, and become osteocytes (Dudley and Spiro, 1961). Osteocytes are the most commonly found cell in mature bone tissue, and can live as long as the organism itself (Knothe Tate et al., 2004). The osteocyte body and its processes are contained within spaces, called lacunae. Osteocytes maintain contact with each other and with cells on the bone surface via a network of small channels, called canaliculi (Aarden et al., 1994).

Osteoblasts can also transform into bone lining cells. As the name suggests, bone lining cells line the bone surface. To date, there are no accepted markers of bone lining cells or effective techniques to selectively isolate these cells (Matic et al., 2016). Bone lining cells are positive for intercellular adhesion molecule 1 (ICAM1), whereas osteoblasts are not (Everts et al., 2002). Inflammatory cytokines such as interleukin-1 beta can differentiate osteoblasts towards ICAM1-positive bone lining cells (Tanaka et al., 2000). A reverse process also takes place: bone lining cells are capable of transforming back into bone-forming osteoblasts and represent a major source of osteoblasts during adulthood (Matic et al., 2016).

**Mononuclear phagocytes**

Monocytes are leukocytes that develop in the bone marrow from precursors called monoblasts, bipotent cells differentiated from hematopoietic stem cells. Monocytes enter the bloodstream where they make up about 7% of leukocytes. They are the largest cells in the blood, averaging 15-18 µm in diameter. Monocytes are actively motile and phagocytic, capable of ingesting infectious agents as well as red cells and other large particles (Schwartz and Lockard Conley, 2017). Monocytes circulate for
a few days before they migrate into tissue where they develop into macrophages. Macrophages take different forms with various names depending on the tissue, but all these cells are part of the mononuclear phagocytic system. Mononuclear phagocytes have roles in homeostasis, immune defense, and tissue repair, and they express an extremely diverse transcriptome (Ziegler-Heitbrock et al., 2010; Wells et al., 2006). Macrophages and their precursors are capable of fusion with diverse other cell types in tissue repair and metastasizing tumors, as well as with each other in the immune response and formation of osteoclasts (Ovchinnikov, 2008).

Osteoclasts

Osteoclasts have the unique ability to resorb mineralised bone. Osteoclasts form by the fusion and maturation of mononuclear phagocytic precursors into large multinucleated cells (Takahashi et al., 1988). Peripheral blood monocytes from the human circulation, when cultured under the right conditions, form osteoclast-like cells that are capable of bone resorption (Matsuzaki et al., 1998). Osteoclast differentiation involves factors produced by bone marrow stromal cells and osteoblasts. The most important factors are macrophage colony-stimulating factor 1 (M-CSF) (Lacey et al., 1998) and receptor activator of nuclear factor κ-B ligand (RANKL), also known as tumor necrosis factor ligand superfamily member 11 (Yasuda et al., 1998; Suda et al., 1999).

Osteoclasts reside in pits in the bone surface called Howship’s lacunae. At a site of active bone resorption, osteoclasts form a specialized cell membrane facing the bone surface, the ruffled border. This ruffled border facilitates bone removal by the expression of large amounts of a vacuolar-type H\(^+\)-ATPase at the surface of the mineralized tissue to dissolve bone mineral. In addition, several hydrolytic enzymes are released into the compartment by lysosomes to digest the organic components of the matrix. This permits characterization of osteoclasts by their staining for high expression of tartrate resistant acid phosphatase (TR-AP) and cathepsin K (Väänänen et al., 2000).

2.5.3 Bone remodelling

Throughout life, bone is constantly remodelled in a process where small packets of bone are resorbed by osteoclasts, followed by the recruitment of osteoblast progenitor cells that differentiate and replace the amount of removed bone (Figure 2.5). Bone remodelling takes place throughout the skeleton at sites termed basic multicellular units (BMUs). The resorption activity at a BMU of adult human bone takes approximately 3 weeks and the formation response 3 to 4 months. Each year, 5-10% of the skeleton is replaced by remodelling activities. The entire adult human skeleton is replaced in 10 years. Bone remodelling is part of calcium homeostasis and provides a mechanism to respond to demands of mechanical loading (Sims and Martin, 2014).

Bone resorption and bone formation are highly coupled processes, ensuring that new bone is formed with only temporary bone loss. An imbalance in the regulation of bone remodelling results in many metabolic bone diseases. Defects in removal of minera-
lized bone and cartilage results in osteopetrosis, or high bone mass. Osteopenia, or low bone mass, reduces the strength of the skeleton and limits the mineral available for metabolic functions of the skeleton (Blair et al., 2008). Coupling between bone resorption and bone formation involves many cell types and molecular mechanisms. They include not only factors produced at different stages of osteoblast and osteoclast differentiation but also factors originating from cells of the immune system (Sims and Martin, 2014).

Within cortical bone, the BMU is located near blood vessels in Haversian canals. In trabecular bone the BMU is located on the surface and becomes covered by a canopy of bone lining cells (Parfitt, 2001; Sims and Martin, 2014). The need for a remodelling event is recognized by osteocytes. Osteocytes are able to sense and integrate both mechanical and chemical signals from their environment, and signal through their canaliculi to surface cells (Schaffler et al., 2014). When the need for a remodelling event is recognized at a site, bone lining cells separate from the underlying bone and form a raised canopy over the site to be resorbed (Parfitt, 2001). Bone remodelling takes place within the canopy, and in this compartment intercellular communication occurs among the component bone cells, from endothelial and vascular cells, and perhaps also from immune cells that access the BMU through the blood supply.

Osteoclasts are formed by fusion of osteoclast precursors supplied by marrow and the bloodstream. The supply of osteoclast precursors is regulated by chemotactic and chemorepulsive factors such as sphingosine-1-phosphate (S1P) (Ishii et al., 2010) (Figure 2.5 a). Active vitamin D stimulates bone resorption by reducing the expression of the S1P receptor on osteoclast precursors (Kikuta et al., 2013). The final stage of osteoclast differentiation depends on factors produced by osteoblast lineage cells, in particular M-CSF and RANKL (Yasuda et al., 1998; Suda et al., 1999). RANKL interacts with RANK on the cell membrane. RANKL has a decoy receptor osteoprotegerin (OPG). OPG, secreted by osteoblasts, blocks the RANKL-RANK interaction and inhibits the differentiation of the osteoclast precursor into a mature osteoclast (Matsuzaki et al., 1998) (Figure 2.5 b).

Recently it has become clear that signalling also takes place in the reverse direction: osteoclasts secrete coupling factors to promote and coordinate osteoblast activity and bone formation (Karsdal et al., 2007; Boyce, 2013). Osteoclast-conditioned medium induces mineralization in osteoblast cultures, a bone anabolic effect that is partially resorption dependent (Henriksen et al., 2012). Osteoclasts secrete BMP6 and Wnt10b, well-known pro-osteogenic factors. Osteoclasts also produce S1P, which induces osteoblast precursor recruitment (Pederson et al., 2008). Bone resorption is coupled to bone formation through the release of growth factors from the bone matrix. TGF-β1 released during bone resorption induces the migration of MSCs to previously resorbed sites (Tang et al., 2009) (Figure 2.5 c). On the other hand, semaphorin-4D is an osteoclast-derived inhibitor of osteoblast differentiation and bone formation (Negishi-Koga et al., 2011), just as OPG negatively influences osteoclast formation and function. These molecules act as mediators of bone remodelling.
Figure 2.5: Schematic representation of the bone remodelling process, which involves a number of intercellular communication pathways: (a) Chemotactic and chemorepulsive factors such as S1P regulate the recruitment of osteoclast precursors. (b) Osteoclast maturation depends on osteoblast-derived M-CSF and RANKL, and is inhibited by OPG, that is also secreted by osteoblasts. (c) Osteoclasts secrete pro-osteogenic factors BMP6 and Wnt10b, and induce osteoblast precursor recruitment by producing S1P. In addition, TGF-β1 released during bone resorption directs MSCs to previously resorbed sites. (d) Pro-osteogenic factors produced by macrophages include BMP2, oncostatin-M and Wnt family members. Adapted from Servier Medical Art.
After osteoclasts have resorbed an amount of bone at the BMU, demineralized matrix (mainly collagen) is cleared from the bone surface to prepare the surface for bone formation. Given that mononuclear cells (lymphocytes and monocytes) reside near the BMU, it has been suggested that mononuclear phagocytes are involved in the removal of demineralized matrix (Tran Van et al., 1982). However, it was later discovered that bone lining cells carry out this indispensable step linking bone resorption to formation. The removal of demineralized matrix by bone lining cells depends on matrix metalloproteases (MMPs) (Everts et al., 2002). Osteoblast precursor cells come from MSCs in the marrow or from the blood. Osteoblasts can also develop from pericytes and bone lining cells. They differentiate within the BMU to fully functional bone-forming osteoblasts.

Addition of monocyte/macrophages to osteoblast cultures promotes osteoblast differentiation and bone formation in vitro (Chang et al., 2008). Macrophages can express many pro-anabolic bone molecules, including BMP2 (Muller et al., 2014), oncostatin-M (Guihard et al., 2015) and Wnt family members (Cho et al., 2014) (Figure 2.5 d). Mounting evidence supports the contribution of macrophage function to regulating bone remodelling, as reviewed by Batoon et al. (2017). However, research to date has only scratched the surface of the complexity of these contributions. As macrophages are inherently adaptable cells, they are likely to be a receptive target for therapeutic intervention to promote bone regeneration.

2.5.4 Bone regeneration

Bone remodelling gives the bone considerable capacities to repair in response to injury. During fracture healing, basic steps of fetal bone development, including intramembranous and endochondral ossification, are recapitulated (Ferguson et al., 1999). Unlike in other tissues, the majority of bony injuries (fractures) heal without the formation of scar tissue, and bone is regenerated with completely restored pre-fracture properties (Einhorn, 1998). However, despite the considerable regenerative capacity, 5-10% of all fractures lead to delayed bone union or non-union (Henkel et al., 2013). Cases in which intrinsic regeneration capacity is insufficient or impaired include large traumatic fractures, extensive loss of bone substance after tumour resection or failed arthroplasies (e.g. spinal fusion) (Henkel et al., 2013).

In cases in which the normal potential of bone healing is insufficient or impaired, bone grafts are used to replace the missing bone. Bone graft materials include autologous bone (from the same patient) and allogeneic bone (from a donor). Autologous and allogeneic bone combines all the properties desired in a bone grafting material: it holds viable osteoblasts that form new bone tissue (osteogenic), it provides a scaffold for the ingrowth of cells (osteoconductive) and it promotes the proliferation of stem cells and their differentiation into osteoblasts through BMPs and other growth factors (osteoinductive). However, autologous and allogeneic bone grafts also have major drawbacks such as donor site morbidity, limited availability, immune rejection, and
pathogen transfer (Dimitriou et al., 2011). To overcome these limitations, bioactive materials have been developed, either alone or in combination with stem cells, that promote the migration, proliferation and differentiation of bone cells (Amini et al., 2012).

Apart from a combination of an appropriate stem cell source and biomaterial scaffold, bone tissue engineering requires osteoinductive factors. Of these molecules, BMPs have been the most extensively studied, as they are potent osteoinductive factors. Recombinant human BMPs are used in orthopedic applications such as spinal fusions, treatment of large traumatic fractures and oral surgery. Recombinant human BMP-2 and BMP-7 are approved by the U.S. Food and Drug Administration for some uses (Ong et al., 2010). The clinical application of BMPs however, remains controversial. Many complications related to dosage issues and ectopic effects have been reported causing serious safety concerns among clinicians (Tannoury and An, 2014). The choice of growth factors, estimation of dosage and release mechanisms remain big challenges.

The immune system and the haematopoietic system strongly influence tissue repair and regeneration. Ample evidence suggests that an active control of the immune system is an attractive therapeutic approach to induce tissue regeneration (Julier et al., 2017), including bone regeneration. Monocytes for example, are recruited to bone fracture sites, where they produce several pro-osteogenic factors affecting mesenchymal precursors present at the healing site (Champagne et al., 2002; Pirraco et al., 2013; Dong and Wang, 2013).

2.6 Extracellular vesicles

Cells release diverse types of membrane vesicles called extracellular vesicles (EVs) into the extracellular environment. EVs represent an important mode of intercellular communication by transferring a complex cargo of biologically active molecules (e.g. proteins, nucleic acids) between cells (Ratajczak et al., 2006). Many different sub-populations of EVs have been described. The term EV was proposed to encompass all cell-derived membrane-limited vesicles released into the extracellular space (György et al., 2011). There is currently no consensus about the classification of EVs, because the current purification methods often result in heterogeneous mixtures of EV sub-populations. Moreover, detection and characterization of EVs in a standardized manner have proven difficult (Raposo and Stoorvogel, 2013). Current research focuses primarily on two major subpopulations of EVs, exosomes and microvesicles, whose release may represent a universal and evolutionarily conserved process (György et al., 2011). Microvesicles bud directly from the plasma membrane, whereas exosomes are of endosomal origin (Figure 2.6). Exosomes bud as intraluminal vesicles into multivesicular endosomes (MVEs) and are released by fusion of MVEs with the plasma membrane (Harding et al., 1984). Exosomes are vesicles between 40-100 nm in diameter. Microvesicles are generally larger (up to ~1,000 nm in diameter), but also smaller vesicles (100 nm) may bud from the plasma membrane (Booth et al., 2006).
Figure 2.6: Intercellular communications via extracellular vesicles. Exosomes and microvesicles are the major subpopulations of extracellular vesicles. Exosomes are released by fusion of multivesicular endosomes (MVEs) with the plasma membrane. Microvesicles bud directly from the plasma membrane. EVs carry their cargo of mRNA, miRNA, DNA, proteins and lipids to recipient cells. EVs can interact with recipient cells by ligand-receptor interactions, fusion, and endocytosis. HSPs: heat shock proteins, MFGE: milk-fat globule-EGF-factor VIII, MHC: major histocompatibility complex, TSG101: tumor susceptibility gene 101. Adapted from Yoon et al. (2014) and Servier Medical Art.

EVs can be isolated from biofluids, tissues, or cell conditioned medium. Despite increasing scientific and clinical interest, no standard procedures are available for the isolation, detection and characterization of EVs, because their size is below the reach of conventional detection methods (van der Pol et al., 2010). In studies examining EVs released from in vitro cell cultures, EVs are mostly isolated from the conditioned media by performing differential ultracentrifugation. The serum that is used in the cell culture media is depleted of EVs by a similar ultracentrifugation step. EVs can be efficiently separated from protein aggregates based on the EV’s relatively low buoyant density using sucrose density gradients (Raposo et al., 1996). Recently, commercially available EV isolation kits have been developed. Here, the reagents reduce the solubility by lowering the hydration of EVs and lead to precipitation. Using these kits, EVs can be separated at a low gravitational force and with higher yield than ultracentrifugation. However, these kits give low purity due to co-precipitation of protein aggregates, as the solubility of almost all particles including EVs and proteins decreases equally (Sunkara et al., 2016).

Since there is yet no consensus on EV-specific markers, EV are often characterized using a combination of methods and identified based on their size, presence of pro-
teins and their biological activity. EVs can be visualized using transmission electron microscopy, offering information on size and morphology. In addition, biochemical information can be obtained using immunogold labelling (van der Pol et al., 2010). Nanoparticle tracking analysis allows determination of the size distribution of isolated EVs based on the Brownian motion of vesicles in suspension (Soo et al., 2012). The presence of EVs in a sample is often verified by performing Western blots on proteins known to be enriched on EV membranes. EV marker proteins are proteins known to cluster at endosomes or into microdomains at the plasma membrane. These include membrane transporters and fusion proteins ( annexins and flotillin) and major histocompatibility complex molecules. Adhesion molecules such as tetraspanins (CD9, CD63, CD81, CD82), integrins and milk-fat globule-EGF-Factor VIII and cytosolic proteins such as certain heat shock proteins, Tsg101 and Alix are also enriched in EVs (Witwer et al., 2013) (Figure 2.6). The purity of the EV sample is shown by confirming the absence of proteins that are not enriched in EVs and are associated with compartments other than plasma membrane or endosomes. Examples include calnexin (endoplasmic reticulum) and histones (nucleus) (Lötvall et al., 2014).

Encapsulated in the EV, proteins and miRNAs are protected from degradation. EVs can be internalized by recipient cells and deliver their cargo, inducing a response in that cell. Cells for instance exchange mRNAs and microRNAs, which is functional in the recipient cell (Valadi et al., 2007). Staining of EVs with fluorescent membrane dyes such as PKH67, DiD and carboxyfluorescein succinimidyl ester permits the direct visualization of EV internalization and localization within cell organelles using live-cell confocal microscopy (Tian et al., 2010).

2.6.1 Extracellular vesicles in tissue engineering

EVs are capable of inducing specific cellular responses by transferring complex cargo to recipient cells (Ratajczak et al., 2006). Doing so, EVs affect many biological processes including cell viability and proliferation (Zhou et al., 2013), angiogenesis (Janowska-Wieczorek et al., 2005; van Balkom et al., 2013; Bian et al., 2014), regulation of immune responses (Raposo et al., 1996; Blazquez et al., 2014; Zhang et al., 2014), and ECM remodelling (Mu et al., 2013), which may be of specific interest for tissue engineering and regenerative medicine. As alternatives to cellular therapies, EVs have advantages with regard to safety and regulatory concerns (Lener et al., 2015).

EVs from stem or progenitor cells, and especially from MSCs have promising regenerative effects in tissues such as kidney (Reis et al., 2012; Wang et al., 2014), heart (Lai et al., 2010; Barile et al., 2014), and nerve (Zhang et al., 2015) in vivo. The use of progenitor-derived EVs harnesses a significant component of the paracrine effects of these cells (de Jong et al., 2014).

The therapeutic efficacy and versatility of native EVs is limited by their native cargo and targeting capacity. Engineering of EVs for therapeutic delivery offers the potential to load non-native cargo and the targeting of EVs can be modified to a specific recipient
cell type (Alvarez-Erviti et al., 2011). EVs are potentially versatile drug carriers that could be applied for tissue regeneration (Lai et al., 2013; Tian et al., 2014; Sun et al., 2010).

2.6.2 Extracellular vesicles in bone remodelling

Given the importance of cellular interactions in controlling bone remodelling, EVs have attracted attention as regulators of this process. Similar to the regenerative strategies discussed in the previous section, EVs derived from MSCs have been used as biomimetic tools to direct stem cells into the osteoblast lineage. EVs from both undifferentiated and osteogenically differentiated MSCs had the potential to induce osteogenic differentiation, although the EVs from osteogenically differentiated MSCs induced a more robust calcium deposition and calcium phosphate nucleation (Narayanan et al., 2016).

In another study using MSC-derived EVs, their cargo was found to be highly enriched in miR-196a, which upregulated the expression of ALPL, BGLAP, SPP1 and RUNX2 in osteoblasts (Qin et al., 2016). Furthermore, the MSC-derived EVs accelerated healing of calvarial defects in rats (Qin et al., 2016). Cui et al. (2016) showed that EVs from pre-osteoblast MC3T3-E1 cells promoted osteoblast differentiation in bone marrow-derived stromal cell line ST2. The EVs affected miRNA profiles, activating the Wnt signaling pathway by inhibiting Axin1 expression and increasing catenin-beta-1 expression (Cui et al., 2016). The expression of RUNX2 and ALPL, as well as matrix mineralization, was increased (Cui et al., 2016).

Interactions between osteoblasts and osteoclasts partially take place through transfer of EVs. Deng et al. (2015) demonstrated that EVs from osteoblastic cell line UAMS-32P contain RANKL and can transfer it to RAW264.7 osteoclast precursor cells, leading to stimulation of osteoclast formation. EVs interacted with osteoclast precursors through receptor ligand (RANKL-RANK) interaction; other cells without specific receptor were not recognized. The researchers used imipramine to block the release of EVs by osteoblasts, which inhibited osteoclast formation. Mice administered with imipramine were protected from ovariectomy-induced bone loss (Deng et al., 2017).

Osteoclasts use the RANKL-RANK pathway to regulate their own formation in an EV-dependant manner, as was demonstrated by Huynh et al. (2016). EVs from osteoclast precursors promoted osteoclast formation in whole mouse marrow cultures, while EVs from mature osteoclasts inhibited osteoclast formation. A subset of the mature osteoclast-derived EVs expressed large amounts of RANK on their surface (Huynh et al., 2016). Depletion of the RANK-rich EVs relieved the ability of osteoclast-derived EVs to inhibit osteoclast formation in marrow cultures (Huynh et al., 2016). The RANK-rich EVs may function by competing with RANK+ osteoclast precursors for RANKL, thereby inhibiting osteoclast formation in a similar manner as OPG. RANK-rich EVs may also take advantage of the RANK-RANKL interaction to target RANK+ cells for the delivery of other regulatory molecules.

Mononuclear phagocytes that share precursors with osteoclasts may contribute to
maintenance of bone homeostasis. EVs from these cells most likely have a role in the crosstalk between immunity and tissue healing (Silva et al., 2017). Dendritic cell- and monocyte-derived EVs significantly stimulate osteogenic differentiation of MSCs (Wang et al., 2014). MSCs treated with 10 \( \mu \text{g/ml} \) EVs from dendritic cells expressed \( \text{RUNX2} \) at higher levels than control. After 14 days of EV-stimulation, alkaline phosphatase activity levels were significantly higher than control, although lower than in standard osteogenic differentiation conditions (Wang et al., 2014). Ekström et al. (2013) showed that monocytes activated by lipopolysaccharide release EVs that are internalized by MSCs. After 3 days, monocyte-derived EVs had caused an increase in the expression of \( \text{RUNX2} \), \( \text{BMP2} \) and \( \text{BGLAP} \). Although the expression levels were lower than in MSCs cultured in the complete monocyte-condition medium, the difference with the control was significant for \( \text{RUNX2} \) and \( \text{BMP2} \) (Ekström et al., 2013). The authors showed that the pro-osteogenic factors produced by monocytes are partially delivered to mesenchymal precursors via EVs (Ekström et al., 2013). Recent studies however, show that increased levels of miR-214-3p are associates with reduced bone formation, and that miR-214-3p transfers through osteoclast-derived EVs to inhibit osteoblast activity in vitro and reduce bone formation in vivo (Li et al., 2016; Sun et al., 2016).
Chapter 3

Aims of the study

The aim of the thesis work was to develop novel tissue-engineering approaches for the treatment of degenerated IVDs. These skeletal defects appear to increase the burden on public health and therefore there is a huge need for effective and feasible new treatments.

Specifically, the aims of the thesis work were:

• to compare the use of TGF-β1 and TGF-β3, and their combined use, to differentiate human AT-MSCs towards an AF-like phenotype (I, II).

• to engineer AF tissue in vitro using novel AF-mimetic designed scaffolds seeded with human AT-MSCs and differentiation towards an AF-like phenotype under optimized culture conditions (II).

• to evaluate the potency of EVs from activated primary human monocytes and osteoclasts to induce a lineage-specific response in human AT-MSCs (III).
Chapter 4

Materials and methods

4.1 Biomaterials

4.1.1 Fabrication of designed PTMC scaffolds by stereolithography (II)

Porous 3D structures were designed and prepared by stereolithography (Figure 4.1). Resins made out of PTMC macromers with an intermediate molecular weight of 5,000 g/mol allow for the building of scaffolds with suitable mechanical properties after photo-cross-linking (Blanquer et al., 2012). Details on the polymer synthesis and preparation of the scaffolds by stereolithography can be found from publication II.

PTMC scaffolds with a truncated cone design were built that had a height of 4 mm, a maximum diameter of 4 mm and a minimum diameter of 3 mm. The scaffolds had a porosity of 76% with an average pore channel diameter of 420 µm. The mean compression modulus of the cubic porous PTMC scaffolds designed was 0.35 MPa (SD: 0.10).

4.1.2 Structural and mechanical characterisation of the scaffolds (II)

The mechanical properties of the scaffolds were measured by compression testing in the dry state using a material testing machine (Zwick Z020; Ulm, Germany), equipped with a 500 N load cell at a compression rate of 30% per minute to a maximum of 80% strain. Scanning electron microscopy (Philips XL30 ESEM-FEG; Philips, Amsterdam, the Netherlands) was applied to visualize the porous structures. The specimens were sputter-coated with gold and the apparatus was operated at a voltage of 3 kV.
4.1.3 Preparation of hydroxyapatite coatings (III)

Hydroxyapatite coatings were deposited in 24-well culture plates in a two-step procedure consisting of precalcification and crystal growth as described by (Patnirapong et al., 2009). Details on the coating procedure are described in publication III.

Figure 4.1: Computer-aided design representation of the three-dimensional scaffold. (a, b) the pore architecture of the scaffolds was based on the typical angle-ply organization of collagen bundles from 30° to 45° in the native annulus fibrosus (AF); (c) schematic illustration of organized collagen bundle layers in the native AF; (d) the pore channels follow the orientation from peripheral (left) to central (right) like collagen fibres in native AF tissue.
4.2 Cell culture methods

4.2.1 Culture media (I, II, III)

Table 4.1: The compositions of media used in the thesis work

<table>
<thead>
<tr>
<th>Medium</th>
<th>Publications</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance medium (MM)</td>
<td>I, II</td>
<td>DMEM/F12 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA); 5 or 10 vol% human serum (PAA Laboratories GmbH, Pasching, Austria); 100 units/ml penicillin and 100 µg/ml streptomycins (Lonza Biowhittaker, Verviers, Belgium); 1 vol% L-alanyl-L-glutamine (Glutamax I, Gibco)</td>
</tr>
<tr>
<td>EV-depleted MM</td>
<td>III</td>
<td>DMEM/F12 with GlutaMAX (Gibco), 10 vol% fetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco)</td>
</tr>
<tr>
<td>Chondrogenic medium (CM)</td>
<td>I</td>
<td>DMEM/F12; ITS+1 (BD Biosciences, Franklin Lakes, NJ, USA); 0.3 vol% antibiotics (100 units/ml penicillin; 100 µg/ml streptomycin; Lonza); 1 vol% L-alanyl-L-glutamine (Glutamax I; Gibco); 50 µg/ml L-Ascorbic acid 2-phosphate (Sigma-Aldrich, Munich, Germany); 55 µg/ml sodium pyruvate (Lonza); 23 µg/ml L-proline (Sigma-Aldrich)</td>
</tr>
<tr>
<td>Differentiation medium with</td>
<td>II</td>
<td>CM, 10 ng/ml TGF-β1 produced in E. coli (Santa Cruz, Dallas, TX, USA)</td>
</tr>
<tr>
<td>TGF-β1 (DM1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiation medium with</td>
<td>II</td>
<td>CM, 10 ng/ml TGF-β3 produced in Chinese hamster ovarian cells (Prospec, Rehovot, Israel)</td>
</tr>
<tr>
<td>TGF-β3 (DM3)</td>
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<td></td>
</tr>
<tr>
<td>Differentiation medium with</td>
<td>II</td>
<td>CM, 10 ng/ml TGF-β1, 10 ng/ml TGF-β3</td>
</tr>
<tr>
<td>TGF-β1 and TGF-β3 (DM1+3)</td>
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<tr>
<th>Medium</th>
<th>Publications</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus pulposus culture medium</td>
<td>I, II</td>
<td>NPCM basal medium (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA), 2 vol% FBS (ScienCell), 1 vol% Nucleus Pulposus Cell Growth Supplement (ScienCell), 100 units/ml penicillin and 100 mg/ml streptomycin (ScienCell)</td>
</tr>
<tr>
<td>LPS-activation medium</td>
<td>III</td>
<td>EV-depleted MM, 10 ng/ml lipopolysaccharide (LPS, E. coli O111: B4, Merck Millipore, Billerica, MA, USA)</td>
</tr>
<tr>
<td>(EV-depleted) osteoclast differentiation medium (OCDM)</td>
<td>III</td>
<td>(EV-depleted) MM, 10 ng/ml recombinant human macrophage-colony stimulating factor (M-CSF, R&amp;D systems, Minneapolis, MN, USA), 20 ng/ml receptor activator of nuclear factor κ-B ligand (RANK-L, Peprotech, Rocky Hill, NJ, USA)</td>
</tr>
<tr>
<td>EV-depleted osteogenic differentiation medium (ODM)</td>
<td>III</td>
<td>EV-depleted MM, 50 µM L-Ascorbic acid 2-phosphate (Sigma-Aldrich), 10 µM β-glycerophosphate disodium salt hydrate (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich)</td>
</tr>
</tbody>
</table>
4.2.2 Cell types (I, II, III)

Table 4.2: Cell types used in the thesis work

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Source</th>
<th>Culture media</th>
<th>Publications</th>
</tr>
</thead>
<tbody>
<tr>
<td>adipose tissue-derived mesenchymal stem cell (AT-MSC)</td>
<td>human adipose tissues and liposuction aspirates</td>
<td>(EV-depleted) MM, CM, DM1, DM3, DM1+3, ODM</td>
<td>I, II, III</td>
</tr>
<tr>
<td>annulus fibrosus cell (AFC)</td>
<td>annulus fibrosus of human intervertebral disc</td>
<td>NPCM</td>
<td>I, II</td>
</tr>
<tr>
<td>CD14+ monocyte</td>
<td>human blood buffy coats</td>
<td>LPS-activation medium, (EV-depleted) OCDM</td>
<td>III</td>
</tr>
</tbody>
</table>

1 ScienCell Research Laboratories, Inc. (Carlsbad, CA, USA)
2 Finnish Red Cross Blood Service (Helsinki, Finland)

4.2.3 Isolation and characterisation of human AT-MSCs (I, II, III)

In publications I and II, human AT-MSCs were isolated from adipose tissue samples obtained from 6 female donors (ages 40 - 65) in surgical operations performed at the Tampere University Hospital, Tampere, Finland. In publication III, human AT-MSCs were obtained from water-assisted lipotransfer liposuction aspirates (Peltoniemi et al., 2013) from 7 female donors (ages 32 - 60). The thesis work was carried out under approval of the ethical committee of the Pirkanmaa Hospital District (I, II, R03058) and the Helsinki and Uusimaa Hospital District (III, DNro 217/13/03/02/2015) with informed consent from the donors. Detailed description of the isolation procedure are included in the publications.

AT-MSCs used in publications I and II were expanded in MM supplemented with human serum and were all analysed for surface marker expression at passage 1. In publication III, AT-MSCs were expanded in FBS-supplemented MM and were analysed at higher passages. After primary culture, AT-MSCs were characterized by flow cytometry to confirm the mesenchymal origin of the cells. In publications I and II, analysis was performed on $1 \times 10^4$ events per sample using a FACSAlra flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Monoclonal antibodies against CD14-PE-Cy7, CD19-PE-Cy7, CD45RO-APC, CD73-PE and CD105-APC (BD Biosciences); HLA-DR-PE (Immunotools GmbH, Friesoythe, Germany) and CD11a-APC, CD80-PE, CD86-PE, and CD105-PE (R&D Systems Inc., Minneapolis, MN, USA).
USA) were used. In publication III, analysis was performed on $1 \times 10^5$ events per sample using a BD Accuri C6 flow cytometer. We used monoclonal antibodies conjugated with allophycocyanin against CD14, CD19, CD34, CD45, CD54, CD73, CD90, CD105 and HLA-DR (BD Pharmingen).

### 4.2.4 AT-MSC micromass cultures (I, II)

TGF-β1, TGF-β3, and their combined use were compared with respect to their potency to stimulate differentiation of human-derived AT-MSCs towards an AF phenotype. AT-MSCs at passages 3-4 were plated according to the micromass culture technique described earlier (Tapp et al., 2008) in order to stimulate AF differentiation. A high cell density suspension of $10^5$ cells/ml was added as 3 droplets of 10 µL to the centre of wells in 24-well plates (Nunc, Thermo Fisher Scientific). Cultures were incubated for 3 h before the addition of 700 µl CM, or differentiation media consisting of CM supplemented with either 10 ng/ml TGF-β1 (DM1), 10 ng/ml TGF-β3 (DM3) or 10 ng/ml each of TGF-β1 and TGF-β3 (DM1+3; Table 4.1). Experiments were repeated 3 times with different donors. Technical duplicates of each sample were used in all assays. At 14 days and 21 days of culture, the micromasses were collected for biochemical, histological and reverse transcription quantitative polymerase chain reaction (RT-qPCR) analyses. The DM3 medium was selected as the AF differentiation medium for the scaffold experiments based on the obtained results.

### 4.2.5 Annulus fibrosus cell micromass cultures (I)

Human annulus fibrosus cells (AFCs; ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) were plated as passage 1 at 5000 cells/cm$^2$ in cell culture flasks. AFCs were expanded in 20 ml nucleus pulposus culture medium (NPCM; Table 1) at 37°C and 5% CO$_2$ in a humidified atmosphere. Whole medium was refreshed every 2 or 3 days. When the cells were confluent, AFCs were subcultured by detaching with trypsin solution (Gibco), centrifuging at 1000 rpm for 5 min and replating at 5000 cells/cm$^2$.

After expansion, cells of passage 3-4 were plated as micromasses in 24-well plates (Nunc) as described in the section 4.2.4. Cells were allowed to attach for 3 h in the incubator at 37°C and 5% CO$_2$ in a humidified atmosphere before NPCM was added. Cultures were maintained in the incubator for 3 weeks and whole medium was changed 3 times a week. At 14 days and 21 days of culture of culture, micromasses were collected for biochemical and RT-qPCR analyses.

### 4.2.6 Cell seeding and culture of scaffolds (II)

The scaffolds were pre-treated with CM 24 h before cell seeding. At passages 3 or 4, AT-MSCs were seeded in the scaffolds using direct, micromass or fibrin seeding (Figure 4.2). Human AFCs (ScienCell Research Laboratories, Carlsbad, CA, USA) were used as a reference cell type in order to verify the phenotype of the differentiated AT-MSCs.
towards AF tissue. After expansion, AFCs from passage 4 were seeded into the scaffolds using direct and fibrin gel seeding.

In the direct seeding group, $1.8 \times 10^5$ cells were suspended in 40 $\mu$l of MM (AT-MSCs) or NPCM (AFCs) and pipetted directly into the scaffold at the bottom of 0.5 ml microcentrifuge tubes. In the micromass seeding group, AT-MSCs were suspended in MM at high cell density (10$^7$ cells/ml) as described in section 4.2.4, and two cell suspension droplets of 10 $\mu$l were carefully applied to the lateral sides of the scaffold in 24-well plates (Nunc). In the fibrin seeding group, $1.8 \times 10^5$ cells were suspended in 20 $\mu$l of fibrinogen solution (33.3 mg/ml; Baxter Biosurgery, Vienna, Austria) and then combined with 20 $\mu$l of thrombin solution (1 units/ml, Baxter Biosurgery) immediately before pipetting into the scaffold at the bottom of 0.5 ml microcentrifuge tubes.

The cell-seeded scaffolds were incubated at 37°C and 5% CO$_2$ for 3 h to allow cell attachment (direct and micromass seeding groups) or for 1 h to allow fibrin gelation (fibrin seeding group) before transferring the scaffolds to new wells in 24-well plates with 1 ml of DM3 (AT-MSCs) or NPCM (AFCs). Scaffolds with pure fibrin gel without cells were used as blanks in all the assays to take into account the background caused by the fibrin gel in the assays. In the direct and micromass seeding groups, scaffolds without fibrin and without cells were used as blanks. Experiments were repeated with AT-MSCs from 3 different donors. Technical duplicates of each sample were used in all assays. The cell-seeded scaffolds were collected for biochemical, histological and RT-qPCR analyses after 1, 14 and 21 days of culture.

4.2.7 Isolation of human monocytes (III)

Human buffy coats were obtained from the Finnish Red Cross Blood Service (Helsinki, Finland). CD14+ monocytes were isolated from 40 ml of buffy coat by gradient centrifugation and negative selection using a Monocyte Isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. More details can be found from publication III.

4.2.8 Culture of monocytes and generation of osteoclasts (III)

Monocytes were plated at a density of 1.5 x 10$^5$ cells/cm$^2$ in 24-well plates, either on culture plastic (tissue-culture treated polystyrene or TCPS) or coatings of hydroxyapatite. Monocytes were either activated by culturing in MM supplemented with 10 ng/ml lipopolysaccharide (LPS-activation medium, Table 4.1) or stimulated to generate osteoclasts in osteoclast differentiation medium (OCDM, Table 4.1). The cells were cultured at 37°C and 5% carbon dioxide (CO$_2$) in a humidified atmosphere. The culture medium was refreshed every 2 or 3 days. LPS-activated monocytes were cultured in EV-depleted medium from the start of the culture. The osteoclast differentiation cultures were changed to EV-depleted OCDM once osteoclasts had formed. EV-depleted medium was prepared with FBS depleted of EVs by ultracentrifugation for 19 h at 26,000 rotations per minute in an Optima LE-80K ultracentrifuge equipped
Figure 4.2: Schematic representation of the AF engineering strategy. (a) AT-MSCs were seeded in the scaffolds using direct, micromass or fibrin seeding. (b) After initial cell attachment, the cell-seeded scaffolds were cultured for a maximum of 21 days in a defined medium with TGF-β3 (DM3, section 4.2.6). The cell-seeded scaffolds were collected for analysis after 1, 14 and 21 days of culture. (c) We analysed the efficiency of the seeding methods (section 4.4.2), the deposition of extracellular matrix components (section 4.5), the presence of oriented collagen fibres in the pore channels of the scaffolds (section 4.4.3) and the expression of AF marker genes (section 4.6.3). Based on Servier Medical Art.
with an SW-28 swinging-bucket rotor (Beckman Coulter, Inc., Brea, CA, USA).

At the beginning of the conditioned medium collection, samples were taken by fixing of the cells in 4% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO, USA). To confirm the formation of osteoclasts, the cells were stained for osteoclast-specific marker tartrate resistant acid phosphatase (TR-AP) using the Leukocyte acid phosphatase kit (Sigma-Aldrich) according to the manufacturer’s instructions. During the culture and after the staining, cells were monitored and imaged using a Nikon Eclipse TS100 inverted phase-contrast microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon DS-Fi2 camera.

4.2.9 Culture of human AT-MSCs in presence of monocyte- and osteoclast-derived EVs (III)

Human AT-MSCs at passages 4-6 were seeded in 96-well plates at $2.5 \times 10^3$ cells/cm$^2$. The cells were allowed to attach in MM overnight before the medium was changed to EV-depleted MM supplemented with $3 \times 10^9$ EVs/mL from either LPS-activated monocytes, osteoclasts cultured on TCPS or osteoclasts cultured on coatings of hydroxyapatite. The number of EVs was based on the NTA analysis. Cells cultured in EV-depleted MM and cells cultured in EV-depleted osteogenic differentiation medium (ODM, Table 4.1) were used as controls. The cells were cultured for 18 days at 37°C and 5% CO$_2$ in a humidified atmosphere during which the medium was refreshed every 2-3 days. Each time, EVs were freshly added.

At the end of the culture, the culture medium was removed, the wells were washed in phosphate buffered saline (PBS) and the contents were collected for analysis (Figure 4.3).
Figure 4.3: Schematic representation of the experimental set-up in publication III. (a) Primary human monocytes were activated using lipopolysaccharide or generated into osteoclasts by stimulation with M-CSF and RANK-L. Osteoclasts were generated on either tissue culture polystyrene (TCPS) or activated for mineral resorption by culturing on coatings of hydroxyapatite (section 4.2.8). (b) Extracellular vesicles were isolated from the monocyte- or osteoclast-conditioned medium by precipitation and centrifugation (section 4.3.1). (c) AT-MSCs were cultured in the presence of these EVs for 18 days after which gene expression was analysed using microarrays (sections 4.2.9 and 4.6.2). Based on Servier Medical Art.

4.3 Extracellular vesicles

All relevant data of our EV experiments were submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV170018) (van Deun et al., 2017).

4.3.1 Collection of conditioned medium and isolation of EVs (III)

The conditioned medium was collected from the LPS-activated monocytes at 3 and 5 days of culture. The osteoclasts were differentiated for 7 days, after which the medium was changed to EV-depleted medium. The conditioned medium was collected 3 and 5 days after changing to EV-depleted medium. The conditioned medium was depleted of cell debris by centrifuging for 20 min at 2,500 × G and the supernatant was filtered through a .45 μm sterile filter (Merck Millipore). EVs were isolated from the conditioned medium by precipitation using the miRCURY Exosome Isolation Kit (Exiqon A/S, Vedbaek, Denmark) according to the manufacturer’s instructions.

4.3.2 Characterisation of EVs (III)

EVs were visualized using transmission electron microscopy. Nanoparticle tracking analysis (NTA) was used to quantify and determine the size distribution of nanoparticles in the EV samples. Western blotting was performed for EV-associated proteins CD63 (lysosomal-associated membrane protein 3), CD90 (Thy-1) and Hsp70 (70 kD heat shock protein) as described previously (Puhka et al., 2017). Details on these methods can be found from publication III.
4.3.3 Uptake of EVs by AT-MSCs (III)

The uptake of EVs by AT-MSCs was studied using confocal microscopy and flow cytometry. EVs were labeled with 1 \( \mu \)M DiD lipophilic dye (Thermo Fisher Scientific). DiD-labelled PBS without EVs served as a control. AT-MSCs (donor 7, passages 4 or 7) were cultured for 3 days in EV-depleted MM supplemented with \( 3 \times 10^9 \) DiD labelled EVs/ml or PBS control. The cells were labelled with CellTrace carboxyfluorescein succinimidyl ester dye (Thermo Fisher Scientific) and Hoechst 33342 (Sigma-Aldrich) prior to imaging. After imaging, cells were detached by trypsin treatment and analysed by flow cytometry (BD Accuri C6). More details can be found from publication III.

4.4 Histology

4.4.1 Histological analysis of micromass sections (II)

At 14 and 21 days of differentiation, micromasses were fixed for 1 h in 4% paraformaldehyde in PBS, embedded in paraffin and sectioned at 5 \( \mu \)m thickness (described in detail in publication II). Proteoglycan production in the ECM was observed by toluidine blue staining (0.1% vol in dH2O; Sigma-Aldrich). The stained sections were visualized with Nikon E600 Fluorescence/Histology microscope. AFC micromasses were not analysed histologically, because they were not dense enough for sectioning.

4.4.2 Methylene blue stainings of cell-seeded scaffolds (II)

The cell-seeding techniques were evaluated in terms of efficiency and spatially uniform seeding in the scaffolds. Scaffolds were stained using methylene blue for AT-MSCs from one donor (age 55). After 1, 14 and 21 days of culture, scaffolds were fixed in 3.7% paraformaldehyde solution (Sigma-Aldrich) and stained with methylene blue (Sigma-Aldrich). Scaffolds without cells served as negative control. Cell attachment and distribution in the scaffold was assessed under a stereo-microscope (Nikon SMZ-10A) equipped with a Sony 3CCD camera (Sony Corporation, Tokyo, Japan).

4.4.3 Picrosirius red staining of cell-seeded scaffolds (II)

To evaluate ECM deposition, the cell-seeded scaffolds were fixed in 4% paraformaldehyde and processed for cryosectioning (described in detail in publication II). The production of collagen in the cell-seeded scaffolds was evaluated by Picrosirius red (Polysciences, Inc., Warrington, PA, USA) staining following the suppliers protocol. The microscopic preparations were visualized with a Nikon E600 fluorescence/histology microscope. Polarized light was used to detect oriented collagen fibres.
4.5 Biochemical analyses

4.5.1 Cell lysis of cell-seeded scaffolds and micromass cultures (I, II)

In publications I and II, micromass cultures and cell-seeded scaffolds were stored at -20°C in CM. Subsequently, samples were rinsed in PBS and transferred to microcentrifuge tubes. Cells were digested overnight in 600 µl of 100 µg/ml papain (Sigma-Aldrich) at pH 6.4 and 65°C.

In publication III, AT-MSCs were collected in 0.1% triton-x-100 (Sigma-Aldrich) and frozen at -75°C. In order to lyse the cells completely, the lysate was allowed to melt and again frozen at -75°C.

4.5.2 DNA quantification (I, II, III)

DNA amount in cell culture lysates was quantified using Hoechst 33258 nucleic acid stain (Bio-Rad Laboratories Inc., Hercules, CA, USA) with purified calf thymus DNA as a standard (Bio-Rad).

In publications II, cell proliferation in the scaffolds was evaluated by quantification of DNA content using the CyQuant proliferation assay (Thermo Fisher Scientific) after 1, 14 and 21 days of culture. Standards with known cell numbers were used to calculate the number of AT-MSCs and AFCs.

4.5.3 Colorimetric assays (I, II)

The total amount of sulphated GAGs (sGAGs) in the lysates of the micromass cultures (I, II) and cell-seeded scaffolds (II) and cell was analysed with a sGAG assay kit (Blyscan; Biocolor Ltd, Carrickfergus, UK) according to manufacturers instructions.

The total collagen content of the cell-seeded scaffolds (II) was quantified using a hydroxyproline assay (Sigma-Aldrich). Total collagen content was calculated based on the reported hydroxyproline:collagen weight ratio of 1:8 (Edwards and O’Brien, 1980), assuming that elastin content is negligible.

4.6 Gene expression analyses

4.6.1 RNA isolation and quality control (I, II, III)

In publications I and II, total RNA was isolated from micromass cultures and cell-seeded scaffolds using the NucleoSpin RNA II Total RNA isolation kit (Macherey-Nagel GmbH & Co., KG, Dren, Germany) according to the manufacturers instructions. In publication III, the miRCURY RNA Isolation Kit (Exiqon A/S) was used according to the
manufacturer’s instructions to extract total RNA from the AT-MSCs. Total RNA yield was measured by optical density at 260 nm with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific), and sample purity was assessed from the ratio of A260/A280. In publication III, detection and purity of RNA was also checked with the Agilent 2200 TapeStation (Agilent Technologies, Foster City, CA, USA).

4.6.2 RNA microarray analysis (III)

For the microarray analysis, the RNA was pooled into 3 distinct pools per condition, assembled from 5 donor replicates. Microarray analysis was performed using Clarion D Human Arrays (Affymetrix). Functional enrichment was assessed by enrichment analysis of Gene Ontology (GO) terms for genes differentially expressed in presence of monocyte-derived EVs compared to control (EV-depleted MM). The microarray results were verified by RT-qPCR. More details can be found from publication III.

4.6.3 Reverse transcription quantitative PCR (I, II, III)

Total RNA was converted into cDNA by reverse transcription. In micromass cultures, ACAN expression was analysed by RT-qPCR method. In cell-seeded scaffolds, gene expression levels of ACAN, DCN, COL1A1, COL2A1 and COL5A2 was analysed. The results were normalized to the expression of RPLP0. The primer sequences (Oligomer Oy, Helsinki, Finland) are listed in publication II. Cycle threshold values were obtained from the exponential area of amplification curves and adjusted for the efficiency of the chain reactions according to the method described by Yuan et al. (2008).

In publication III, gene expression of ACAN, ALPL, BMP2, COL15A1, FGL2, ICAM1, ITGA3, MMP1 and RUNX2 was quantified using TaqMan assays (Thermo Fisher Scientific). Assay IDs are listed in publication III. Genes were selected based on their established relevance to osteogenesis, or based on their differential expression in presence of EVs compared to EV-depleted MM in our array data. The results were normalized to the geometrical average of multiple reference genes, RPLP0, TBP and YWHAZ (III), which are stably expressed under several experimental conditions (Ragni et al., 2013). The PCR reactions were conducted in triplicates in a Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific).

4.7 Statistical analysis

Technical duplicates (or triplicates) of each sample were used in all assays. Statistical analyses were performed on single observations from independently performed experiments (cell cultures). Statistical testing was performed in R Statistical Software.

In publications I and II, micromass culture experiments were repeated with AT-MSCs from 3 different donors. The commercially obtained AFCs were derived from one donor. The effects of medium composition on DNA, sGAG content and sGAG/DNA in
AT-MSC micromass cultures were analysed using Friedman rank sum tests from the muStat package. The replicated data (3 ×) was blocked based on AT-MSC donor. AT-MSC micromass differentiated in presence of TGF-β1 and/or TGF-β3 were tested for significantly increased DNA and sGAG content against the control (CM) using a one-sided Wilcoxon rank sum tests. The effects of medium composition on AT-MSC gene expression in micromass cultures was analysed using Friedman rank sum tests with unreplicated blocked data based on AT-MSC donor. Only medium compositions with complete blocks were included in the tests. Pairwise comparisons were not performed because the Friedman tests did not detect significant differences between the mean ranks of the groups.

In publication II, experiments with cell-seeded scaffolds were performed with AT-MSCs from 3 different donors. The commercially obtained AFCs were derived from one donor. Differences were assessed between micromass and fibrin seeding of AT-MSCs with respect to the number of cells, sGAG content and collagen content (total amount and per cell), as well as the sGAG:collagen ratio and gene expression in the scaffolds. Wilcoxon rank sum tests were applied on replicated (3 ×) pairs of cell donors.

The effects of medium composition and EV source on normalized gene expression levels were assessed for AT-MSCs from 6 donors per group. Statistical analyses were performed using Kruskal-Wallis one-way analysis of variance by ranks. Mann-Whitney U post hoc tests were conducted to analyse specific conditions against the control (EV-depleted MM) for significant differences.

The rate of type I errors for multiple testing was controlled by false discovery rate (FDR) procedures.
Chapter 5

Results

5.1 Adipose tissue-derived mesenchymal stem/stromal cell characterization (I, II, III)

Human AT-MSCs demonstrated high expression of CD90 ($\geq 96\%$), CD105 (>87%), and CD73 (>57.6%), moderate expression of CD34 ($\geq 1.5\% > 28.6\%$) and CD54 ($\geq 0.7\% > 80.6\%$). AT-MSCs demonstrated no or low expression of CD3 ($\leq 0.3\%$), CD11a ($\leq 0.6\%$), CD14 ($\leq 1.5\%$), CD19 ($\leq 0.8\%$), CD45RO ($\leq 1.3\%$), CD80 ($\leq 0.8\%$), CD86 ($\leq 1.1\%$), and HLA-DR ($\leq 1.3\%$). The results showed that AT-MSCs expressed most of the specific antigens that define human stem cells of mesenchymal origin according to criteria set by the Mesenchymal and Tissue Stem Cell Committee of the ISCT (Dominici et al., 2006).

5.2 Optimization of annulus fibrosus differentiation medium in micromass cultures

5.2.1 Extracellular matrix production in micromass cultures

Toluidine blue staining was performed for the detection of proteoglycans in AT-MSC micromasses (Publication II, Figure S1 c at the back of this thesis on page y). Toluidine blue stains background and nucleic acids blue by orthochromatic staining. Purple colour denotes high proteoglycan content by metachromatic staining of Toluidine blue. Samples cultured in CM supplemented with TGF-β had more intense staining than samples cultured in CM alone. Staining was the most intense in samples cultured in presence of TGF-β3. Staining was slightly more intense in samples at the 14-day time point than at 21 days. At 14 days of culture, the majority of the proteoglycans was detected at the periphery of micromasses, while at 21 days the proteoglycan distribution was more homogenous. Sections of micromasses cultured in CM were notably
smaller compared to samples cultured in the presence of TGF-$\beta$1 and/or TGF-$\beta$3.

AFC micromasses and AT-MSC micromasses cultured in the various differentiation media were harvested at 14 and 21 days of culture. In order to evaluate ECM production, micromasses were lysed in papain after which DNA and sGAG content were analysed using quantitative biochemical assays (Figure 5.1). Results are shown for AT-MSCs from 3 different donors for each of which the experiments were repeated 3 times.

The DNA content was quantified using an assay based on the nucleic staining of Hoechst 33258 (Figure 5.1 a). Supplementation with TGF-$\beta$ increased the DNA content of AT-MSC micromasses compared to culture in CM. DNA content was significantly higher in samples cultured in DM3 and DM1+3 than in CM at both time points. The DNA content of AFC cultures was slightly higher or at least comparable to AT-MSC micromasses in DM3 and DM1+3. In all conditions, DNA content was similar at 14 and 21 days of culture.

The results for sGAG quantification (Figure 5.1 b) are consistent with the Alcian blue stainings, showing that micromasses cultured in presence of TGF-$\beta$3 (DM3) or a combined use of TGF-$\beta$1 and TGF-$\beta$3 (DM1+3) had higher amount of sGAGs than in other conditions. Without TGF-$\beta$ supplementation (CM), sGAG levels were often below the background levels of the assay. Already at 14 days of culture, a significantly higher amount of sGAGs were detected in micromasses cultured in presence of TGF-$\beta$3 alone (DM3) than in CM. At 21 days, the sGAG content of AT-MSC micromasses cultured in all 3 media compositions with TGF-$\beta$ was significantly higher than in CM. The effect of TGF-$\beta$3 supplementation was however bigger than supplementation with TGF-$\beta$1. At both 14 and 21 days of culture, the sGAG content of AT-MSC micromasses cultured in presence of TGF-$\beta$ was similar to that of AFC cultures.

The amount of sGAGs was normalized with the DNA amount (Figure 5.1 c) showing the sGAG content per cell. The Friedman rank sum test did not detect significant differences in normalized sGAG content among groups of AT-MSC micromass stimulation.

In order to evaluate the effect of TGF-$\beta$ supplementation on AT-MSC gene expression, the mRNA levels of ACAN in micromass cultures were analysed at 21 days by the RT-qPCR method (Publication II, Figure 5). Experiments were repeated with AT-MSCs from 3 different donors. Gene expression levels are expressed as relative to the median level in CM.

Irrespective of culture condition, ACAN expression was higher at 21 days of culture than 14 days (Publication II, Figure S1 d at the back of this thesis on page y). Supplementation with TGF-$\beta$ increased ACAN expression by multiple orders of magnitude compared to CM. TGF-$\beta$3 had a greater effect on ACAN mRNA levels than TGF-$\beta$1. Combining TGF-$\beta$1 and TGF-$\beta$3 did only affect ACAN expression at 21 days of culture than TGF-$\beta$3 alone. At 21 days of culture, ACAN expression levels in AT-MSCs micromasses cultured in DM1+3 were similar to expression levels in AFC cultures.
Figure 5.1: DNA content and sGAG content, as well as the sGAG:DNA ratio in AT-MSC and AFC micromasses at 14 days and 21 days of culture. AT-MSC micromasses were cultured in chondrogenic medium (CM), or CM supplemented with 10 ng/ml TGF-β1 (DM1), CM supplemented with 10 ng/ml TGF-β3 (DM3), or a combination of 10 ng/ml TGF-β1 and 10 ng/ml TGF-β3 (DM1+3). Micromasses prepared with annulus fibrosus cells (AFCs) were cultured in nucleus pulposus culture medium (NPCM). * p > 0.05 compared to CM.
No statistical tests were performed to evaluate differences in gene expression between micromass culture conditions, since it is theoretically impossible to reach a significance level of $\alpha = 0.05$ with this small sample size in a non-parametric test.

5.3 Differentiation of AT-MSCs towards annulus fibrosus in a designed PTMC scaffold prepared by stereolithography

5.3.1 Cell attachment and proliferation in the scaffolds

The effectiveness of the cell-seeding methods and uniformity of cells in the scaffolds was evaluated using methylene blue staining. The results are shown in Publication II, Figure 2. This figure is reproduced and printed in colour at the back of this thesis on page x.

Major differences between the different seeding methods were already visible at day 1 as only fibrin gel seeding allowed homogeneous methylene blue staining, indicating uniform distribution of AT-MSCs in the scaffolds. The fibrin gel itself is lightly stained by methylene blue as the staining shows of a scaffolds with fibrin gel without cells (Reproduction of Publication II, Figure 2 at the back of this thesis on page x). However, in cell-seeded scaffolds with fibrin, darkblue spots indicating cells can be seen. Cells were uniformly embedded in the fibrin gel. With direct seeding of the cells only few areas were stained by the methylene blue, suggesting poor cell attachment in the scaffolds. At day 14, the differences were more pronounced as only fibrin seeding led to homogeneous attachment of AT-MSCs throughout the scaffold. Cross-sections showed that the fibrin gel had facilitated the distribution of the cells throughout the pore channels on the inside of the scaffold. AT-MSCs seeded by micromass seeding on the other hand, grew in clusters near the original seeding sites. In scaffolds seeded by direct seeding, only few cell clusters were visible at the periphery of the scaffolds.

The number of cells in the cell-seeded scaffolds was quantified based on their DNA content (Figure 5.2). Direct seeding was only performed with AT-MSCs from a single donor. Direct seeding in the scaffolds resulted in lower cell-seeding efficiency and proliferation of AT-MSCs compared with micromass seeding and fibrin seeding at all measured time points. Direct seeding of AFCs was slightly more successful than direct seeding of AT-MSCs. The difference between micromass and fibrin seeding was most apparent at 21 days, although differences between the AT-MSC donor replicates were still substantial. Significant differences between micromass seeding and fibrin seeding of AT-MSCs were not detected. Fibrin seeding of AFCs slightly improved cell number compared to direct seeding.

Based on the results from methylene blue stainings and DNA quantification, direct
Figure 5.2: The number of AT-MSCs and AFCs of cell-seeded scaffolds based on DNA content. AT-MSCs were seeded into the scaffolds using direct seeding, micromass seeding and the fibrin seeding methods and cultured for 1, 14 and 21 days before cell lysis in papain. Experiments were repeated with AT-MSCs from a single donor for direct seeding and AT-MSCs from 3 different donors for micromass and fibrin seeding.
seeding was excluded from further analysis.

5.3.2 Evaluation of extracellular matrix production in cell-seeded scaffolds

Sulphated GAG and collagen assays were implemented in order to quantify the ECM production of AT-MSCs seeded by fibrin seeding or micromass seeding methods, as well as fibrin-seeded AFCs (Figure 5.3).

Collagen content was higher in scaffolds seeded with AT-MSCs using the fibrin seeding method than micromass seeding (Figure 5.3 c). The collagen content was significantly higher at 21 days of culture. Collagen content per cell was especially high in scaffolds seeded using fibrin as a AT-MSC-carrier: please notice the log₂ scale used to indicate the difference with micromass-seeded scaffolds (Figure 5.3 d). This indicates that the higher collagen content cannot entirely be attributed to the higher number of AT-MSCs. Also the median sGAG content of fibrin-seeded scaffolds was higher than micromass-seeded scaffolds, especially at 21 days (Figure 5.3 a). The amount of sGAG in the scaffolds however, did not differ significantly between the two seeding methods, due to high variability between donor replicates. This variability of sGAG production/retention becomes apparent when the sGAG content per cell is compared between the seeding methods: a log₂ scale was used to show the large variability between the repeats (Figure 5.3 a). Total sGAG and collagen content were small in scaffolds seeded with AFCs than AT-MSCs.

The collagen:sGAG ratio was used to determine whether the composition of the ECM produced by differentiated AT-MSCs and AFCs in the cell-seeded scaffolds was similar to that of AF tissue as reported previously by Mwale et al. (2004) (Figure 5.3 e). In micromass-seeded scaffolds, the collagen:sGAG ratio had decreased between 14 days to 21 days of culture, caused by the diminished collagen content at the later time point. The collagen:sGAG ratio in micromass-seeded scaffolds was significantly higher than in fibrin-seeded scaffolds at 21 days.

5.3.3 Formation and alignment of collagen fibres in cell-seeded scaffolds

Histological stainings of the scaffolds were in accordance with the quantitative results (Publication II, Figure 4). Picrosirius red staining showed the abundance and homogeneous distribution of collagen. Importantly, polarized light showed the formation and alignment of collagen fibres inside the pore channels in this condition (Publication II, Figure 4 f). This was in great contrast with micromass seeding of AT-MSCs in which only few densely stained structures were visible. No aligned collagen fibres were detected using polarized light in this condition. Staining of scaffolds fibrin-seeded with AFCs did not reach beyond background levels as visualised in scaffolds without cells.
Figure 5.3: Extracellular matrix production of AT-MSCs and AFCs in cell-seeded scaffolds. AT-MSCs were seeded into the scaffolds using direct seeding, micromass seeding and the fibrin seeding methods and cultured for after 1, 14 and 21 days before cell lysis and extraction of ECM content in papain. Experiments were repeated with AT-MSCs from a single donor for direct seeding and AT-MSCs from 3 different donors for micromass and fibrin seeding. (a) Sulphated glycosaminoglycan (sGAG) content, (b) sGAG content per cell, (c) total collagen content, (d) total collagen content per cell and (e) collagen:sGAG ratio. * p < 0.05.
5.3.4 Expression of annulus fibrosus marker genes in cell-seeded scaffolds

The seeding method affected the expression of AF marker genes by AT-MSCs (Figure 5.4). Expression of ACAN and COL1A1 by AT-MSCs was significantly higher in micromass-seeded scaffolds at 21 days of culture. DCN expression on the other hand, was higher in fibrin-seeded scaffolds with AT-MSCs. The difference was only statistically significant at the 14-day time point. Expression of the assessed genes was in general relatively low in AFCs. DCN was the exception, which was expressed at similar levels by AT-MSCs and AFCs.
Figure 5.4: Gene expression levels of aggrecan (ACAN), decorin (DCN), collagen type I alpha 1 chain (COL1A1), collagen type II alpha 1 chain (COL2A1) and collagen type V alpha 2 chain (COL2A1) in AT-MSC- and AFC-seeded scaffolds at 14 days and 21 days of culture. AT-MSC were seeded using micromass or fibrin seeding and cultured in DM3. AFCs, seeded in the scaffolds using fibrin seeding, were cultured in nucleus pulposus culture medium (NPCM). Results are shown relative to fibrin-seeded AFCs at 14 days of culture. p < 0.05 compared with micromass seeding of AT-MSCs.
5.4 Potency of EVs from activated primary human monocytes and osteoclasts to induce a lineage-specific response in human-derived AT-MSCs

5.4.1 Monitoring monocyte cultures and formation of osteoclasts

Cell cultures were monitored using a phase-contrast microscope (Publication III, Figure 1). LPS-activated monocytes were visible as small adherent cells. The conditioned medium from these cells was collected at 3 (b) and 5 days (c) of culture. Part of the LPS-activated monocytes was positive for osteoclast-marker TR-AP (b).

Peripheral blood monocytes were differentiated towards osteoclasts using M-CSF and RANK-L. In presence of M-CSF and RANK-L, monocytes started to fuse and form osteoclasts after 5 days of culture (d). Mature osteoclasts were visible after 7 days (e). Non-active osteoclasts were generated on tissue culture plastic (tissue culture polystyrene, TCPS). Mineral-resorbing osteoclasts were cultured on a hydroxyapatite substrate. The formation of active osteoclasts was confirmed by the positive staining of the cells for osteoclast-marker TR-AP and dissolution of the hydroxyapatite substrate (f-k). Osteoclast-conditioned medium was collected at 10 and 12 days of culture.

5.4.2 Characterization of EVs from LPS-activated monocytes and osteoclasts

EVs were isolated from the conditioned medium. Transmission electron microscopy revealed the secretion of intact EVs of various shapes and sizes in all culture conditions (Publication III, Figure 2 a-f). EVs with high and low electron density were present in each condition.

NTA indicated that the large majority (>80%) of the particles was between 50 and 200 nm in diameter (Publication III, Figure 2 g-i). Particles between 50 and 400 nm in diameter were considered EVs. The sizes of osteoclasts-derived EVs were slightly larger than monocyte-derived EVs (Publication III, Table S3). More EVs were isolated from osteoclast cultures than from monocytes.

Western blotting for the EV-associated proteins CD63 (lysosomal-associated membrane protein 3), CD90 (Thy-1) and Hsp70 (70 kD heat shock protein) revealed that osteoclast-derived EVs were positive for CD63 and CD90, while negative for Hsp70. Monocyte-derived EVs on the other hand, were negative for CD63 and CD90, but positive for Hsp70 (Publication III, Figure 3).

5.4.3 Uptake of EVs by AT-MSCs

Our uptake studies confirm that the biological activity of isolated EVs was intact (Publication III, Figure 5). EVs from all tested cell sources were taken up by AT-
MSCs. The signal was higher in conditions with DiD-labelled EVs compared with AT-MSCs incubated with a DiD-labelled PBS control without EVs (a). The highest fraction of EV-positive AT-MSCs were incubated with EVs derived from osteoclasts cultured on TCPS (OC TCPS-EVs; c). When the AT-MSCs were incubated with monocyte-derived EVs (MC-EVs; b) or EVs from osteoclasts cultured on hydroxyapatite coatings (OC HA-EVs; d), only a fraction of the AT-MSCs was positive for EVs.

The interaction between AT-MSCs and monocyte/osteoclast-derived EVs was confirmed by flow cytometry analysis (e-g). Although the background signal varied largely between experiments, substantial EV-positive AT-MSC fractions could be detected. EV-positive AT-MSC fraction was defined as the level of fluorescence greater than 95% of AT-MSCs incubated with the DiD-labelled control (gray area). After incubation with OC TCPS-EVs, the EV-positive fraction was as high as 97.4% (f). Incubation with OC HA-EVs resulted in an EV-positive fraction of 58.4% (g), while only 31.6% had taken up MC-EVs (e).

5.4.4 Gene expression analysis of AT-MSCs cultured in presence of monocyte- and osteoclast-derived EVs

Genome-wide transcriptome analysis using microarrays was performed on AT-MSCs stimulated with monocyte- and osteoclast-derived EVs, as well as AT-MSCs cultured in ODM. AT-MSCs cultured in EV-depleted MM was the reference condition. 2,972 genes were found to be differentially expressed between the treatments and MM (FDR < 0.05). 1,153 genes were upregulated and 1,595 genes were downregulated in ODM than MM.

Despite that osteoclast-derived EVs were taken up by the AT-MSCs, osteoclast-derived EVs did not have a significant effect on AT-MSC gene expression. We did not detect significant regulation of gene expression by culturing AT-MSCs with EVs from osteoclasts cultured on both TCPS and hydroxyapatite.

Stimulation with MC-EVs resulted in 111 upregulated genes and 149 downregulated genes than EV-depleted MM (Publication III, Table 1). Genes that were differentially expressed in presence of monocyte-derived EVs showed great overlap with differentially expressed genes in established osteogenic induction conditions. Of the 260 genes that were significantly regulated by MC-EVs, 122 were also differentially expressed in ODM. The direction of regulation, however, did not necessarily match. Of the 111 upregulated genes in presence of MC-EVs, 5 were also upregulated in ODM and 19 were downregulated in ODM. Of the 149 downregulated genes in presence of MC-EVs, 71 were also downregulated in ODM and 27 were upregulated in ODM. Osteoclast-derived EVs did not result in differential gene expression than EV-depleted MM.

To characterize the function of the genes differentially regulated by MC-EVs, an enrichment analysis of the genes by their functional annotation was performed (Publication III, Figure 4). According to Gene Ontology (GO) term analysis, biological proces-
ses such as cell chemotaxis (enrichment p-value $6.31 \times 10^{-15}$) and more specifically, positive regulation of leukocyte chemotaxis (enrichment p-value $1.29 \times 10^{-12}$) were enriched for genes upregulated by MC-EVs. Genes significantly upregulated by MC-EVs related to these processes include interleukins (ILs) and chemokine ligands. For example, $IL1B$ (log₂ fold change 2.34; FDR $1.21 \times 10^{-2}$), $IL6$ (log₂ fold change 1.79; FDR $2.91 \times 10^{-2}$) and $IL36B$ (log₂ fold change 1.97; FDR $3.30 \times 10^{-2}$) were upregulated in presence of MC-EVs than EV-depleted MM alone. MC-EVs upregulated the expression of 7 chemokine ligands, such as $CXCL5$ (log₂ fold change 3.96; FDR $4.66 \times 10^{-3}$), $CXCL3$ (log₂ fold change 2.53; FDR $9.04 \times 10^{-3}$) and $CXCL6$ (log₂ fold change 2.38; FDR $2.63 \times 10^{-2}$). Other genes upregulated by MC-EVs involved in many enriched processes were retinoic acid receptor responder (tazarotene induced) 2 ($RARRES2$, log₂ fold change 1.39, FDR $5.43 \times 10^{-4}$) and intercellular adhesion molecule 1 ($ICAM1$, log₂ fold change 1.57, FDR $1.05 \times 10^{-3}$).

Collagen catabolic processes (enrichment p-value $2.94 \times 10^{-6}$) and processes involving extracellular structure organization (enrichment p-value $7.27 \times 10^{-6}$) were also significantly enriched for genes upregulated by MC-EVs. The enrichment of these biological processes was due to the upregulation of many matrix metallopeptidases (MMPs) by MC-EVs. In fact, the 3 most upregulated genes by MC-EVs were $MMP3$, $MMP1$ and $MMP8$ (Publication III, Table 1).

We also performed enrichment analysis of the genes upregulated by MC-EVs according to their molecular functions (Figure Publication III, Figure 4). We found enriched molecular functions such as chemokine activity (enrichment p-value $5.63 \times 10^{-12}$), cytokine receptor binding (enrichment p-value $2.30 \times 10^{-8}$) and cytokine activity (enrichment p-value $1.30 \times 10^{-5}$). These molecular processes support the enriched biological processes of (immune) cell chemotaxis. Metalloendopeptidase activity was also enriched (enrichment p-value $1.21 \times 10^{-4}$), reflecting the upregulation of MMPs by MC-EVs.

In publication III, Figure 6 shows a heat map of the differentially expressed genes. Hierarchical clustering of the samples is indicated by the dendrogram. Samples clustering next to each other have the highest degree of similarity with each other. All three replicates for each condition cluster together, except for samples cultured in presence of osteoclast-derived EVs. Samples cultured in ODM are distinct from all other conditions. Samples cultured in presence of MC-EVs cluster away from samples in EV-depleted MM. Samples cultured in presence of OC TCPS-EVs or OC HA-EVs from donor pool a are more similar to each other than to the other samples in their respective conditions, showing similarity of the two types of osteoclast-derived EVs.

We performed direct quantitation of 9 selected gene transcripts by RT-qPCR to validate the microarray findings (Publication III, Figure S1). The RT-qPCR analysis was performed separately for each of the 6 donor replicates. In every case, a strong correspondence between the microarray and RT-qPCR data was observed.
Chapter 6

Discussion

6.1 Optimisation of annulus fibrosus differentiation medium

Isoforms of TGF-β, and their receptors, play important roles in the differentiation and development of the IVD (Sohn et al., 2010; Jin et al., 2011). Furthermore, degeneration of the IVD correlates with decreased expression of TGF-βs and their receptors (Matsunaga et al., 2003). TGF-βs promote cell proliferation, enhance cell viability and stimulate secretion and accumulation of proteoglycan and collagen type II (Risbud et al., 2006; Gruber et al., 2010a; Hayes and Ralphs, 2011). The importance of TGF-β1 or TGF-β3 to maintain the AFC phenotype in vitro has been reported in several studies (Colombini et al., 2015; Guillaume et al., 2014).

The three isoforms TGF-β1, TGF-β2 and TGF-β3 induce differentiation of MSCs towards a fibrocartilaginous phenotype like that of the cells in the outer AF (Barry et al., 2001; Steck et al., 2005; Tapp et al., 2008; Gruber et al., 2010b). Differentiation occurs through two major intracellular pathways: through the small mothers against decapentaplegic homolog (SMAD) signalling transcription factors; and through mitogen activated protein kinase (Augustyniak et al., 2015). Most investigators have used either TGF-β1 or TGF-β3 (Johnstone et al., 1998; Mackay et al., 1998; Barry et al., 2001; Tuli et al., 2003; Mueller et al., 2010). Of these two commonly used isoforms, TGF-3 induces a more rapid and thorough expression of chondrogenic markers (Barry et al., 2001). TGF-β3 has been used to differentiate bone marrow-derived MSCs (Steck et al., 2005) and AT-MSCs (Tapp et al., 2008; Gruber et al., 2010b) towards an AF-like phenotype. Our study appears to be the first to systematically compare the effects of TGF-β1, TGF-β3 and their combination on AT-MSC proliferation and differentiation with the objective to create AF tissue in vitro. A concentration of 10 ng/ml was used as this concentration has been previously shown to be effective for AT-MSCs (Gruber et al., 2010b), and we used serum-free differentiation medium to allow translation of the results to the clinic.
The micromass technique was developed initially to study the chondrogenesis of embryonic mesoderm cells (Ahrens et al., 1977), although it has since been applied to many other cell types including AFCs (Gruber et al., 2010a; Hayes and Ralphs, 2011). The technique makes use of the fact that mesoderm cells or MSCs undergo chondrogenesis when cultured at high density, in a condensation process directed by secreted factors, cell-cell and cell-matrix interactions (DeLise et al., 2000). The micromass culture technique represents a procedurally simple system to study the development of cartilaginous tissues (Barry et al., 2001). It is in this light a promising culture system to study the differentiation towards a phenotype of AF tissue, which shares features with cartilage. The micromass culture technique has been previously used to differentiate AT-MSCs towards an AF-like phenotype (Tapp et al., 2008; Gruber et al., 2010b).

Most common protocols for TGF-β-mediated chondrogenic differentiation, including the conditions described in this thesis, are most suitable for generation of collagen type I-rich tissues like the outer AF. Steck et al. (2005) showed that TGF-β-induced MSC micromasses adopt a gene expression profile that resembled native AF tissue more closely than native joint cartilage. They therefore concluded that TGF-β-mediated induction of MSC micromasses is a promising strategy to engineer AF tissue. The difficulty with assessing an AF-like phenotype is that a single AF phenotype does not exist. The AF is a fibrocartilous tissue which consists of a mixture of fibrous tissues and cartilaginous tissues, with higher proportions of fibrous tissue towards the outer AF. Nevertheless, sustained secretion of proteoglycans is essential when using cell-based therapies for treating degenerated IVDs, as proteoglycans and their GAG chains distribute forces around the circumference of the AF by increasing its hydration (Cassinelli et al., 2001; Pattappa et al., 2012).

Proteoglycan and sGAG content in AT-MSC micromasses was increased in the presence of TGF-β, especially TGF-β3 (Figure 5.1 b and Publication II, Figure S1 c at the back of this thesis on page y). Other studies also indicate that TGF-β increases ECM deposition in MSC micromasses under chondrogenic induction conditions (Barry et al., 2001; Mauck et al., 2006; Hamid et al., 2012). Tapp et al. (2008) and Gruber et al. (2010b) showed that incorporation of TGF-β3 supports the production of an ECM with high sGAG content up to 14 days of culture. Chondrogenic differentiation of MSCs takes approximately 3 weeks and accumulation of sGAGs takes place until the last phase of chondrogenesis (Barry et al., 2001). We therefore assessed sGAG content up to 21 days of culture and showed that the increase in total sGAG content under TGF-β stimulation was sustained up to this time point.

Apart from an increase in ECM components, we observed that TGF-β also heightened DNA content of AT-MSC micromasses. Without TGF-β supplementation, micromasses were smaller in size and total amount of DNA was diminished (Figure 5.1 a and Publication II, Figure S1 c at the back of this thesis on page y). sGAG content per cell was similar across culture conditions indicating that increased total sGAG content after stimulation with TGF-β was mainly due to increased proliferation (Figure 5.1.

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c). Based on these results, we cannot conclude that increased total sGAG content was a consequence of increased sGAG synthesis per cell under TGF-β stimulation. Normalized gene expression of ACAN (encodes for aggrecan) on the other hand, was increased by supplementation with isoforms of TGF-β (Publication II, Figure S1 d at the back of this thesis on page y). Aggrecan is one of the major structural components of IVD, including AF tissue (Pattappa et al., 2012).

Both TGF-β1 and β3 stimulated cell proliferation, but in comparison with TGF-β1, TGF-β3 supplementation resulted in greater accumulation of sGAGs and proteoglycans. We used TGF-β1 produced in E. coli and TGF-β3 produced in Chinese hamster ovarian cells. We cannot exclude that the differences in effects between the two isoforms is partly due to their different sources. Vanhatupa et al. (2015) compared the effect of human recombinant BMP2 produced in E. coli and Chinese hamster ovarian cells on proliferation and osteogenic and adipogenic differentiation of human AT-MSCs. Their results indicated that mammalian-produced BMP2 had a more pronounced effect on mineralization and lipid formation of AT-MSCs compared with BMP2 produced in E. coli. In our study, combined treatment with TGF-β1 and β3 had the most pronounced effect on proliferation, proteoglycan and sGAG content, and resulted in the highest ACAN expression levels compared to either one of the isoforms alone. Nonetheless, combined TGF-β1 and β3 treatment did not affect matrix synthesis more than the sum of the effects of the two isoforms, suggesting that they do not have a synergistic effect.

As opposed to the AT-MSC micromasses, AFC micromasses did not condense. These reference cells were cultured in NPCM. This serum-containing medium stimulated proliferation, which was higher than the AT-MSCs in serum-free conditions. The serum however, might have favoured the attachment of the AFCs to culture plastic, instead of micromass condensation. AFCs only produced small amounts of sGAGs. When normalized by DNA content, sGAG levels were below those of AT-MSC micromasses in CM. This might reflect the senescent state of AFCs after in vitro expansion, but the composition of NPCM might also not have been optimal for this purpose as it was originally designed for culture of human NP cells (ScienCell). Others have obtained ECM production in AFC micromasses under TGF-β stimulation in conditions with serum (Gruber et al., 2010a), or without serum (Hayes and Ralphs, 2011). Guillaume et al. (2014) showed the importance of TGF-β3 to maintain the AFC phenotype in vitro.

Supplementation with TGF-β3 supported proliferation and accumulation of proteoglycans and sGAGs in the micromasses. As the addition of TGF-β1 to the TGF-β3-supplemented medium did not give any significant benefit, we decided to use DM3 for culturing AT-MSCs in scaffolds. Nevertheless, we did not find evidence for increased sGAG synthesis by AT-MSCs in response to TGF-β1 or TGF-β3. The use of growth factors in clinical applications is controversial because of their short half-lives, high costs and general low effectiveness (Fassett et al., 2009). One should therefore consider to avoid growth factors altogether when an AT-MSC-based solution for restoring AF defects is trans-
lated to the clinic.

Sustained proteoglycan production alone is not enough to obtain a tissue-engineered construct that can be used to repair AF defects. Collagen bundles are the major structural and tension-bearing elements in the AF (Smith and Fazzalari, 2009). As we move from a purely cell-based treatment to combined mechanical and biological repair, we will also focus on the collagen content of the ECM.

### 6.2 Annulus fibrosus tissue engineering using AT-MSCs in a designed PTMC scaffold

Owing to the close relation between collagen fibre organisation and biomechanical function of AF tissue, scaffolds for AF tissue engineering should have a pore architecture that facilitates replication of this structure (Guterl et al., 2013). Only a few studies have reported on the development of a scaffold with a pore architecture that reproduces the organization of collagen fibres in the AF. Nerurkar et al. (2009) reported for on an electrospun membrane that replicates the specific angle-ply displayed by the collagen fibres. However, owing to limitations of the electrospinning approach, these membrane scaffolds are difficult to scale to 3D scaffolds tailored for the patient’s defect. The use of a lamellar silk scaffold has also been reported. However, these approaches cannot be adapted to our purpose, either because of the isotropic random pore structure obtained by the scaffold preparation process used, which will influence the biomechanical performance (Park et al., 2012), or because it does not allow the fabrication of a full-sized 3D scaffold that is required to repair a herniated disc (Bhattcharjee et al., 2012). Therefore, none of these previous strategies reported in the literature describe a scaffold design that allows control of the organization and orientation of the collagen bundles. This limitation may be the major reason explaining the inability to reproduce oriented collagen fibres in previous work. In consequence, the work presented here is the first study describing a 3D scaffold designed with an oriented channel-like pore architecture that reproduces the structure of AF tissue.

The scaffold design was achieved by precisely respecting the multilamellar organization and angle-ply of the native AF collagen (Figure 4.1) and was built with high precision by stereolithography. Furthermore, the truncated cone geometry of the scaffold was designed in order to prevent the risk of scaffold extrusion after implantation in the disc defect. This specific geometry allows the use of the scaffold as a plug and increases the stability of the implanted scaffold inside the defect.

Scaffolds for AF tissue engineering should facilitate good cell adhesion and proliferation, and allow the deposition of an AF-like ECM. We evaluated 3 techniques for seeding AT-MSCs and AFCs in the scaffolds with respect to cell content and proliferation. Although direct seeding is the simplest and most widely used method of cell delivery, it is also the least efficient approach (Villalona et al., 2010). Also according to our results, attachment of cells to the hydrophobic PTMC was poor in the serum-free
conditions. Shorter seeding incubation times would have prevented seeded cells from reaching a stable, mature, morphologic state, which can result in cell loss when culture medium is added or when the constructs are directly implanted. Longer cell seeding incubation times on the other hand allow the cells to obtain a more mature morphology and thus increase efficient cell attachment, but may also lead to unfavourable cellular changes.

One of the limitations of using static cell seeding techniques is the difficulty in achieving a uniform distribution of the cells (Villalona et al., 2010). Micromass seeding was successful for AT-MSCs from one donor with cell number already reaching beyond the initial seeding number \((2 \times 10^5)\) at day 1 of culture. Although the total cell number in our micromass-seeded scaffolds was high in some instances, their distribution of the scaffolds was poor. Moreover, micromasses did reach the centre of the scaffold and condensed on the edge of the scaffold as high cell density droplets. Results from scaffolds seeded with AT-MSC micromasses were hard to reproduce, which makes this approach difficult to translate to clinical therapy as its high operator dependency may refrain regulatory agencies from approval (Villalona et al., 2010).

To increase seeding efficiency and uniform distribution, hydrogels have been applied to trap cells on the scaffold or to facilitate cell attachment to the scaffold (Villalona et al., 2010). In order to establish effective seeding of cells, fibrin gel seeding was tested because fibrin is known to maintain the typical phenotype of AF cells (Colombini et al., 2015) and to promote the production of ECM (Sha'ban et al., 2008). We hypothesized that the use of fibrin would also support the differentiation of AT-MSCs towards an AF phenotype, as fibrin was reported to support chondrogenesis of MSCs (Pelaez et al., 2009; Ho et al., 2010; Ahmed et al., 2011). Fibrin gel facilitated good distribution and penetration of the cells through the pores of the scaffolds. Due to the big pore size and their high interconnectivity in the scaffold, dynamic seeding strategies were not necessary for the cells to penetrate into the centre of the scaffold (Roh et al., 2007).

Fibrin seeding of AT-MSCs resulted in enhanced AF-like ECM formation compared with micromass seeding (Figure 5.3). The higher sGAG content of the fibrin-seeded scaffolds may be explained by a higher retention of synthesized GAGs in the fibrin gel compared with micromass seeding (Ameer et al., 2002). Compared to hydrogels of hyaluronic acid or formulations with collagen, fibrin gel formulations have been shown to retain higher numbers of AT-MSCs and GAG content (Park et al., 2010). Fibrin seeding of AT-MSCs resulted in an abundant production of collagen (Figure 5.3 c), which is the main ECM component of AF tissue (Roughley, 2004). The fibrin seeding method together with the designed scaffold fully support the accumulation of a fibrocartilaginous AF-like matrix. Especially at the end of the culture, collagen content corresponded well with the number of cells in the scaffold (Figure 5.3 d), indicating that collagens were retained equally well by the fibrin gel irrespective of seeding efficiency or cell proliferation. The collagen:sGAG ratio for differentiated fibrin-seeded AT-MSCs corresponded closely with the value reported for native human AF tissue (Mwale et al., 2004). In contrast, the sGAG:collagen ratio for micromass-seeded AT-MSCs and AFC-
seeded scaffolds tends towards the ratio reported for NP tissue.

Not only the production of sGAG and collagen was significantly upregulated, but the collagen was also organized in a specific manner. Picrosirius red staining of collagen showed that the AF-mimetic architecture of the scaffold led to regularly packed and aligned collagen bundles inside the designed pore channels (Publication II, Figure 4), which is essential for the biomechanical function of AF tissue (Nerurkar et al., 2010a). The results do not reveal that all the produced collagen fibres follow the pore orientation. Although the pore characteristics of the scaffold obtained have not yet been optimized, the results obtained in the present study do show that collagen bundles can be created and that several collagen bundles appear to be aligned along the pore. This result is remarkable as it was obtained under static conditions without mechanical stimulation. This is in agreement with a previous study showing that collagen orientation is controlled by large-scale microstructures in a scaffold for vascular tissue engineering (Engelmayr et al., 2006). Similarly, de Mulder et al. (2013) used thermal-induced phase separation to prepare a scaffold for meniscus repair, in which collagen fibres were oriented through the channel-like pore architecture of the scaffold. However, this technique does not allow sufficient control of the pore architecture, which is essential to reach the optimal reproduction of the AF tissue structure and function. In consequence, a 3D scaffold with specific micro-architecture able to mimic with high precision the architecture of native AF will guide the cells and the produced collagen to follow the porous orientation and therefore reproduce the desired structure and function.

Although differentiated AT-MSCs showed abundant production of ECM, sGAG and collagen contents remained less than in native AF tissue. sGAGs and collagen constitute 10-20% and 50-70% of the dry weight of native AF tissue, respectively (Cassinelli et al., 2001). We estimate the dry weight of the cultured cell-scaffold construct to be 8 mg based on a total volume of 40 l and 80% water content. Based on this estimate, sGAGs made up about 0.25% and total collagen content made up approximately 1% of the estimated dry weight at 21 days of culture. Mechanical stimulation and in vivo implantation could stimulate the production of an ECM composition similar to native tissue (Nerurkar et al., 2009).

To verify the obtained AF phenotype, the expression of AF markers genes was evaluated. We assessed the expression of ACAN and DCN. DCN encodes for decorin, a small interstitial proteoglycan in IVD tissue, which is predominantly expressed in the AF (Melrose et al., 2001). COL1A1 and COL2A1 encode for the major collagens present in the ECM of AF tissue: collagen type I and collagen type II (Roughley et al., 2006). However, these collagens are not specific for AF tissue alone. Instead, COL5A2 encoding for collagen type V, is a more specific marker for AF tissue, distinguishing AFCs from NP cells and chondrocytes (Clouet et al., 2009). Interestingly, fibrin-seeded AT-MSCs expressed significantly less of ACAN and COL1A1 at the end of the culture, and COL2A1 expression levels were substantially lower than in scaffolds seeded with AT-MSC micromasses. These results indicate that the higher ECM content in scaf-
folds with fibrin-seeded AT-MSCs is not due to increased production compared with AT-MSC micromasses, but rather due to better retention of the ECM products within the fibrin-PTMC scaffold.

The AFCs cultured in NPCM only produced minor amounts of AF-like ECM, both in the micromass cultures and 3D scaffolds. In the scaffolds, expression of AF marker genes was consistently lower in AFCs than in the differentiated AT-MSCs. The use of fibrin gel as a cell carrier has previously been reported to support the production of a fibrocartilaginous matrix during in vivo implantation of AFCs (Colombini et al., 2015). In the present study however, fibrin seeding did not rescue AF-like ECM formation by AFCs.

6.3 Potency of EVs from activated primary human monocytes and osteoclasts to induce a lineage-specific response in human-derived AT-MSCs

Mononuclear phagocytes interact with AT-MSCs through EV-mediated signalling: EVs from activated monocytes and osteoclasts were taken up by AT-MSCs (Publication III, Figure 5). Incubation with all three cell sources led to a fraction of AT-MSCs that was positive for the DiD-stained EVs. The fraction of EV-positive AT-MSCs varied largely between the various EV sources: only 31.6% of AT-MSCs were positive for monocyte-derived EVs while almost all AT-MSCs were positive for EVs from osteoclasts cultured on TCPS. This data however, was derived from three independently run experiments and the technical variability between the staining procedures and flow cytometry runs was significant: background levels varied between these experiments. Performing the experiment for all three EV types in the same run would produce more reliable quantitative data on the effect of cell source on the uptake of EVs by AT-MSCs.

Our microarray data show that MC-EVs affected the gene expression in AT-MSCs significantly. Monocytes secrete many soluble factors promoting osteogenic differentiation and proliferation of mesenchymal precursors (Pirraco et al., 2013; Champagne et al., 2002). The pro-osteogenic signalling becomes more profound when the monocytes are activated by LPS (Omar et al., 2011). Ekström et al. (2013) demonstrated that EVs from LPS-activated monocytes upregulated the expression of osteogenic marker genes RUNX2 and BMP2. We did not observe significant upregulation of osteogenic marker genes by MC-EVs in the present study, nor did we see these genes significantly upregulated in established osteogenic induction conditions. We can however establish that the signals carried by the EVs act on different pathways than the chemical inducers in ODM. We observed noticeable differences between the gene expression patterns of AT-MSCs in presence of MC-EVs and established osteogenic induction conditions.

Part of the activated monocytes was positive for osteoclast-marker TR-AP (Publica-
tion III, Figure 1 b), supporting previous reports that LPS has the potential to induce osteoclast formation (Liu et al., 2009; Mabilleau et al., 2011). Omar et al. (2011) pointed out that pro-osteogenic signalling of LPS-activated monocytes could be consistent with coupling signals from osteoclasts stimulating bone formation. While, according to our data, MC-EVs affected AT-MSC gene expression, the same number of EVs from osteoclasts did not have a significant effect. This result challenges the suggestion that factors secreted by LPS-activated monocytes and osteoclasts are identical. Although osteoclast-derived EVs might regulate the differentiation of MSCs, the effect of osteoclast-derived EVs on MSC gene expression is less pronounced compared to the effect of the same number of MC-EVs.

Osteoclasts provide signals that promote osteoblast activity and osteogenic differentiation of MSCs (Pederson et al., 2008; Henriksen et al., 2012). Osteoclast-derived EVs on the other hand, can also transfer miR-214-3p to inhibit osteoblast activity (Li et al., 2016; Sun et al., 2016). These seemingly contradicting observations show the complexity of how bone remodelling is regulated and that the effect of secreted soluble factors does not necessarily have to match the effect of EVs derived from the same cells. The effects of osteoclast-derived EVs on MSCs remain to be further elucidated.

MSCs attract immune cells by secreting a broad mixture of chemokines. Chemokine secretion by MSCs may represent an immunomodulatory mechanism in which chemotaxis brings specific immune cell subsets in close proximity to MSCs, which makes them more susceptible for the immunosuppressive actions of MSCs (Hoogduijn et al., 2010; Quaedackers et al., 2009). Our data suggests that this mechanism includes a positive feedback loop, in which activated monocytes in their turn promote the chemotaxis of immune cells by MSCs via EV-mediated signalling. We showed that EVs derived from activated monocytes upregulated the expression of various cytokines involved in the chemotaxis of leukocytes. Monocytes recruited to an injury site will become activated and start producing EVs. The EVs involve MSCs into the tissue response by stimulating chemotaxis of other immune cells, while the MSCs simultaneously modulate the activity of immune cells. Patrikoski et al. (2014) reported on the immunosuppressive potential of AT-MSCs in various culture conditions. The strongest immunosuppression and lowest immunogenicity was observed in culture conditions with higher expression of ICAM1. In the present study, ICAM1 expression was significantly upregulated by MC-EVs. ICAM1 is required for lymphocyte-MSC adhesion, and with the help of chemokines, ICAM1 induces MSC-mediated immunosuppression (Ren et al., 2010).

In tissue repair, new ECM deposition takes place only after the old ECM components are degraded (Silva et al., 2017). The remodelling of the microenvironment involves metalloproteinases, such as MMPs, which may be delivered by EVs. Various cell types secrete EVs that carry metalloproteinases (Shimoda and Khokha, 2013), including immune cells (Dalli et al., 2013). Interestingly, monocytes exposed to pro-inflammatory cues secrete increased levels of MMP-containing EV (Li et al., 2013). The present study shows that EVs from activated immune cells can not only carry MMPs, but can also upregulate their expression in mesenchymal progenitors. In bone remodelling,
MMP-dependent removal of ECM components takes place during the reversal phase that precedes bone formation: collagens protruding from the bone surface are removed by bone lining cells (Everts et al., 2002). Given that mononuclear cells (lymphocytes and monocytes) reside near the bone surface, it has been suggested that mononuclear phagocytes are involved in this process (Tran Van et al., 1982). Our data suggest that monocytes stimulate processes related to the reorganization of the ECM structure, in particular the removal of collagens. The signals secreted by monocytes, combining both soluble factors and EV cargo, may direct MSCs to differentiate towards bone lining cells. Our hypothesis is supported by the fact that expression of ICAM1 was upregulated in presence of MC-EVs compared to EV-depleted MM and ODM. ICAM1 is a marker for bone lining cells, whereas cuboidal bone-forming osteoblasts are negative for ICAM1 (Everts et al., 2002). Moreover, inflammatory cytokines such as interleukin 1 beta, which was overexpressed in presence of MC-EVs, can differentiate osteoblasts towards ICAM1+ bone lining cells (Tanaka et al., 2000).

We cannot exclude that at least part of the observed effects of MC-EVs are caused by contaminations of LPS left in the EV suspensions. Presence of LPS could explain the activation of immune cell functions in the AT-MSCs. We used an EV isolation kit based on the reduction of the solubility by lowering the hydration of EVs. These kits usually give lower purity than e.g. ultracentrifugation, as the solubility of almost all particles decreases equally (Sunkara et al., 2016). Future studies would benefit from more specific EV isolation methods. Additionally, one could specifically inactivate remnants of LPS using acyloxyacyl hydrolase after the isolation of the EVs (Munford and Hunter, 1992). Alternatively, one could add a control condition by following the same isolation procedures with unconditioned LPS-supplemented medium.

6.4 Future perspectives

One important feature of IVD degeneration is a decrease in viable and functional cell numbers, with a substantial proportion of cells existing in a senescent state (Roberts et al., 2006). The application of cell therapy could be beneficial in patients with progressive IVD degeneration at an early stage, bridging the gap between symptomatic care and surgical interventions. Other potential beneficiaries of cell therapy would be patients with IVD disease that arises after surgical intervention. Preclinical data on cell therapy shows the promising prospective for regeneration of early IVD degeneration: cellular injection therapy is safe, although long-term results are unknown (Sakai and Andersson, 2015). Furthermore, questions remain regarding the timing of treatment, optimal cell source, cell pretreatment and cell carrier.

As procedures involving the use of autologous AFCs are technically demanding and AFCs from a degenerating IVD often show signs of senescence, the focus has been directed towards the application of MSCs. As was confirmed in the current study, most common protocols for TGF-β-mediated chondrogenic differentiation are suitable for generation of AF tissue (Steck et al., 2005). Advances in chondrogenic differen-
tiation of AT-MSCs have prospects to be translated into stem cell-based therapies for treating large size cartilage defects (Wu et al., 2013). Therefore, we can expect that AT-MSC-based therapies for regeneration of articular cartilage will soon be followed by similar therapies for the regeneration of AF defects. In vitro differentiation allows for expansion to a great number of MSCs and de novo tissue engineering, or even whole IVD engineering. However, well-controlled preclinical testing is needed to address the long-term efficacy of using AT-MSCs and to assess the adverse effects as well as investigate the concerns about the use of stem cells. Much of the published animal data is experimental and does not provide answers to specific questions about the clinical application of cell therapy (Tam et al., 2016).

Currently, techniques to close the AF using suturing of closure devices focus primarily on restoration of the mechanical integrity of the AF. These techniques offer clear solutions for delivery and fixation. Regenerative therapies on the other hand, target the engineering of healthy and functional AF tissue, but lack strategies for implantation and fixation and thus for clinical application (Bron et al., 2009). Our proposed strategy offers immediate mechanical stability and the truncated cone geometry of the scaffold was designed to increase the stability of the implanted scaffold inside the defect. Moreover, stereolithography offers the possibility of tailoring the scaffold geometry to the defect. The build-up of an AF-like tissue structure shows prospects that the engineered tissue will be able to successfully rescue the biomechanical function of the compromised AF. However, up to this date, there is no in vivo data available that clearly indicate to what extent native composition and structure of the engineered tissue are needed to support long-term AF repair (Guterl et al., 2013). Ultimately, large animal in vivo studies will be necessary to show the importance of the implant design for functional AF repair.

Spinal fusion is an established surgical technique that can be effective for disabling back pain due to a degenerated IVD. Bone grafts or degradable biomaterials are used to stimulate bone growth to increase the chances of a successful fusion (Dimitriou et al., 2011), but non-unions are still frequent (Spivak, 2006). Tissue engineering strategies that involve stem cells and growth factors have not yet proven broadly effective in the clinic. There is a need for novel strategies aimed at inducing bone development in a way closer to the physiological process (Dong and Wang, 2013).

The immune system plays a crucial role in the tissue healing process. Immune-mediated mechanisms may support existing regenerative strategies or could be an alternative to using growth factors and/or stem cells (Julier et al., 2017). The roles of EVs in the crosstalk between immunity and tissue healing are of great interest for the field of regenerative medicine, as EVs could serve as a more biomimetic alternative to growth factors (Silva et al., 2017). Our results show the importance of EV-mediated signalling between monocytes and MSCs for controlling the function of the immune system. MC-EVs form promising prospects for the development of MSC-based immune therapy. Signals secreted by monocytes may involve MSCs into remodelling of the microenvironment, a crucial step in tissue repair. MC-EVs could be used to form
functional bone lining cells in vitro. This in vitro model would help us to understand the exact mechanisms involved in bone remodelling. By promoting the formation of bone lining cells, MC-EVs could facilitate enhanced bone remodelling and may prove useful in the treatment of bone disease or injury. Ultimately, MC-EVs could be incorporated in a tissue engineering construct for the regeneration of bone tissue.
Chapter 7

Conclusions

The overall aim of the thesis work was to develop novel tissue-engineering approaches for the treatment of degenerated IVDs.

We performed a systematic comparison between the key isoforms of TGF-β, type 1 and type 3, and their combination on AT-MSC proliferation and differentiation towards AF tissue. A designed scaffold was prepared by stereolithography from resins based on PTMC with a channel pore architecture that reproduces the multilamellar angle-ply organization of the native AF collagen. We evaluated 3 techniques for seeding AT-MSCs and AFCs in the scaffolds with respect to cell content and proliferation. AT-MSCs were differentiated towards an AF phenotype using the optimized differentiation conditions. The most efficient seeding methods were selected for evaluation of the composition of the ECM and expression of AF marker genes.

We analysed the transcriptome of AT-MSCs after stimulation with EVs from LPS-activated primary human monocytes and (mineral-resorbing) osteoclasts.

The following conclusions can be drawn:

- Neither TGF-β1 nor TGF-β3 increase sGAG synthesis by AT-MSCs. Nevertheless, supplementation with TGF-β3 supports proliferation and thereby accumulation of proteoglycans and sGAGs in the micromasses. TGF-β3 is more effective than TGF-β1 in promoting the formation of fibrocartilaginous tissue in AT-MSC micromass cultures. Combining TGF-β1 and TGF-β3 in the differentiation medium does not have a synergistic effect compared to the effect of TGF-β3 alone.

- Fibrin gel seeding of AT-MSCs in the AF-mimetic scaffolds facilitates good distribution and penetration of the cells through the pores of the scaffolds. Combined with TGF-β-stimulation, fibrin gel seeding of AT-MSCs leads to AF-like ECM formation in the construct including alignment of collagen bundles inside the pore channels. The build-up of an AF-like tissue structure shows prospects that the
engineered tissue will be able to successfully rescue the biomechanical function of a compromised AF.

- The effect of osteoclast-derived EVs on MSC gene expression is less pronounced compared to the same number of EVs from LPS-activated monocytes. EVs from LPS-activated monocytes carry signals that act on different pathways than chemical inducers of osteogenic differentiation. EVs derived from activated monocytes upregulate the expression of various cytokines involved in the chemotaxis of leukocytes. In addition, the EVs stimulate processes related to the reorganization of the ECM structure, in particular the removal of collagens. The signals carried by MC-EVs involve MSCs into remodelling of the microenvironment, a crucial step in tissue repair. However, the results should be taken with care because we did not control for possible contaminations of LPS in the MC-EV suspensions.
References


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