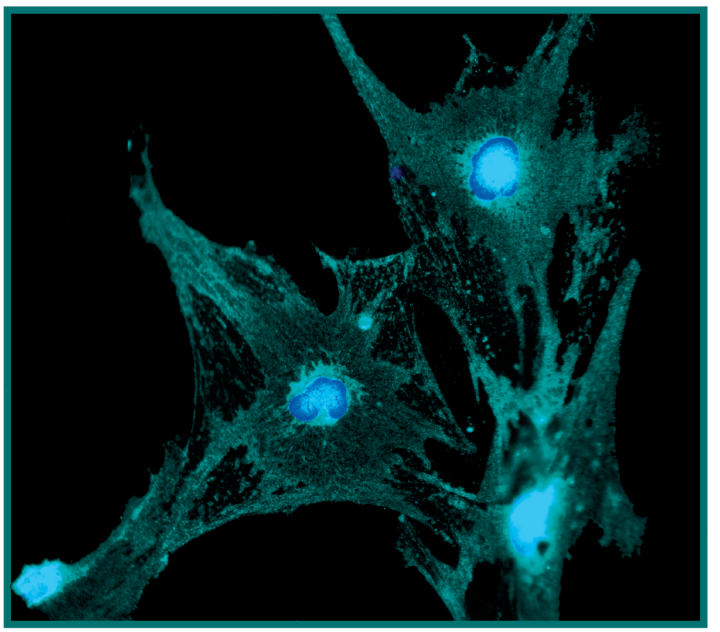
The Osteogenic Potential of Human Mesenchymal Stem Cells

- Novel markers and key factors for differentiation



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Cover illustration: It's art, it's cell culture.

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Curie

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ABSTRACT

Mesenchymal stem cells are multipotent stem cells with ability to differentiate into cells of the connective tissue lineage, such as adipocytes, osteoblasts and chondrocytes, both *in vitr*o and *in vivo*. The main objective of the present thesis was to study different aspects of the osteogenic potential of MSCs. By examining markers of differentiation, exploring approaches for enhanced osteogenesis through the use of small molecule substances, and studying the interactions between MSCs and inflammatory cells/signals, we aimed to gain new insights into factors and mechanisms involved in regulation of the osteogenic differentiation process.

Through both a virtual ligand-based screening method combined with several *in vitro* screening steps, and a chemical inhibition of the PPAR-γ transcription factor, it was demonstrated that osteogenic differentiation of MSCs can be modulated by the use of a small molecule substance. Furthermore, a link between PPAR-γ, leptin and osteogenic differentiation was revealed.

The surface markers CD10 and CD92, and intracellular protein CRYaB were demonstrated as suitable markers for monitoring and evaluating the differentiation of MSCs. CD10 and CD92 were shown to be markers of both osteogenic and adipogenic differentiation, whereas CRYaB was revealed as a marker specific for the osteogenic lineage.

Activated human monocytes communicate pro-osteogenic signals to MSCs, independent of direct cell-cell contact. Furthermore, membrane vesicles isolated from gram-positive bacterial strains *Staphylococcus aureus* and *Staphylococcus epidermidis* also promote osteogenic differentiation of MSCs as well as modulate their secretion of signals related to inflammation and immune-modulation.

In conclusion, the present thesis presents new findings regarding the phenotype of MSCs characteristic for osteogenic differentiation. Furthermore, through the results presented here insight is gained into several key factors, both of synthetic and biological origin, important in this process. This knowledge is valuable for future strategies with the aim of enhancing osteogenic regeneration.

Keywords: Mesenchymal stem cells, mesenchymal stromal cells, osteogenic differentiation, adipogenic differentiation, bone regeneration, inflammation, monocytes, infection, bacterial membrane vesicles, compromised bone healing, cell surface proteins, CD-markers, osseointegration, regenerative medicine.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Mesenkymala stamceller är en typ av adulta stamceller som finns i bland annat benmärg. Dessa celler kan, till skillnad från vanliga cellerna i kroppens vävnader, mogna till celltyper som återfinns i bindväv såsom fettceller, benceller och broskceller. Denna mognadsprocess benämns differentiering och cellerna kan även kallas adipocyter, osteoblaster och kondrocyter. Syftet med denna avhandling var att studera olika aspekter av de mesenkymala stamcellernas potential att mogna till osteoblaster, så kallad osteogen differentiering. Vi har undersökt detta närmare i tre separata, men ändå relaterade, forskningsprojekt där många faktorer i mesenkymala stamcellers osteogena mognad täckts in. Två olika strategier för förbättrad osteogen differentiering genom användandet av små läkemedels-liknande substanser har prövats. Genom att även utforska i fall vissa proteiner uttrycks specifikt under denna mognadsprocess, har möjligheten att använda sådana eventuella proteiner för att identifiera mesenkymala stamcellernas mognadsgrad undersökts. Slutligen har samspelet mellan mesenkymala stamcellers och inflammatoriska cellers respektive signaler studerats.

I dessa forskningsprojekt har vi bland annat kunnat visa att en strategi som kombinerar en databassökning, efter nya kemiska föreningar, med en utvärdering av kandidatsubstanser i ett odlingssystem med mesenkymala stamceller kan ha potential som läkemedelsutvecklingsstrategi. Vi sökte efter kemiska föreningar som liknar en ligand, med tidigare påvisad effekt på mesenkymala celler, och utvärderade sedan deras förmåga att förhöja den osteogena differentieringen av celler. Därefter har kemisk blockering av fettdifferentiering visats ha en mycket positiv effekt på de mesenkymala stamcellernas bendifferentiering. I denna avhandling är det första gången denna typ av kemisk hämning av fettdifferentiering har länkats till ökad osteogen differentiering och uttrycket av proteinet leptin.

De proteiner som identifierades som specifika för differentiering av mesenkymala stamceller var CD10, CD92 och CRYaB. Medan CRYaB endast uttrycktes under osteogen differentiering, och därför är en mycket bra markör för denna process, uttrycktes CD10 och CD92 även under fettdifferentiering. De senare kan därför istället användas som markörer för allmän bindvävsdifferentiering av mesenkymala stamceller.

Sambandet mellan inflammation/infektion och nybildning av benvävnad är ofullständigt utredd. Forskningen som presenteras här visar att aktiverade monocyter, en typ av inflammatoriska celler som ingår kroppens försvar mot främmande ämnen och organismer, kommunicerar signaler som påverkar den

osteogena differentieringen av mesenkymala stamceller på ett positivt sätt. Slutligen så har forskningen i avhandlingen visat att även membranvesiklar, membranomslutna informationspaket i nanostorlek som skickas ut av celler, isolerade från två vanliga bakteriestammar *Staphylococcus aureus* och *Staphylococcus epidermidis* kan främja osteogen differentiering av de mesenkymala cellerna. Innehållet i dessa membranvesiklar kan även, på ett fortfarande okänt sätt, modulera de mesenkymala stamcellernas utsöndring av signaler som kan påverka andra celler i deras närmaste omgivning.

Sammanfattningsvis presenterar denna avhandling ny, tillämpbar kunskap om den fenotypen som är karakteristisk för mesenkymala stamceller under differentiering. Dessutom ger de resultat som presenteras här insikt i flera faktorer, både av syntetiska och biologiska ursprung, som är viktiga i denna process. Denna kunskap kan användas som ett verktyg i strävan efter förbättrad regeneration av benvävnad.

LIST OF PAPERS AND MANUSCRIPTS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Virtual ligand-based screening reveals purmorphamine analogs with the capacity to induce the osteogenic differentiation of human mesenchymal stem cells.**
Granéli C, Karlsson C, Lindahl A, Thomsen P.
Cells Tissues Organs 2013;197(2):89–102.

II. **The effects of PPAR-γ inhibition on gene expression and the progression of induced osteogenic differentiation of human mesenchymal stem cells.**
Granéli C, Karlsson C, Brisby H, Lindahl A, Thomsen P.
Connective Tissue Research 2014; Accepted for publication.

III. **Novel markers for osteogenic and adipogenic differentiation of human bone marrow stromal cells identified using a quantitative proteomics approach.**
Granéli C, Thorfve A, Rüetschi U, Brisby H, Thomsen P, Lindahl A, Karlsson C.
Stem Cell Research 2014;12(1):153–165.

IV. **The stimulation of an osteogenic response by classical monocyte activation.**
Omar O, Granéli C, Ekström K, Karlsson C, Johansson A, Lausmaa J, Larsson-Wexell C, Thomsen P.
Biomaterials 2011;32(32):8190-8204.

V. **The effects of bacterial cell-wall components and bacterial membrane vesicles on the osteogenic differentiation and secretory profiles of human mesenchymal stem cells**.
Granéli C, Wang X, Vazirisani F, Trobos M, Brisby H, Lindahl A, Omar O, Ekström K, Thomsen P.
In manuscript.

TABLE OF CONTENTS

ABBREVIATIONS

ACAT2	Acetyl-CoA acetyltransferase 2
ADAM19	ADAM metallopeptidase domain 19
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1
ALP	Alkaline phosphatase
AM	Adipogenic medium
AMAC-1	Alternative macrophage activation-associated CC chemokine 1 (CCL18)
ASC	Ascorbic acid
AT-MSC	Adipose tissue MSC
β-GPH	Beta-Glycerophosphate
BA	Bone area
BIC	Bone-implant contact
BM-MSC	Bone marrow MSC
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BMSC	Bone marrow stromal cell
BSP	Bone sialoprotein
C/EBP	CCAAT-enhancer-binding proteins
C10orf10	Chromosome 10 open reading frame 10
CB-MSC	Cord blood MSC
CCND1	Cyclin-D1
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming units
ChM	Chondrogenic medium
CM	Conditioned medium
COL	Collagen
CRYaB	Crystallin alpha B
DEPP	Decidual protein induced by progesterone
DEX	Dexamethasone
DLX5	Distal-less homeobox 5
DMEM	Dulbecco's modified eagle medium
DMEM-HG	DMEM High glucose
DMEM-LG	DMEM Low glucose

DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinases
ESC	Embryonic stem cell
FABP4	Fatty acid binding protein 4 (aP2)
FACS	Flow assisted cell sorting
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FC	Fold change
FDR	False discovery rate
FGF	Fibroblast growth factor
FSC	Forward scatter
FT-ICR	Fourier transform ion cyclotron resonance
FZD	Frizzled
G-CSF	Granulocyte colony-stimulating factor
GDF5	Growth/differentiation factor 5
GF	Growth factor
GLI	Glioma-associated oncogene homolog 1
GLUT4	Glucose transporter type 4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene ontology
HA	Hydroxyapatite
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
Hh	Hedgehog
HSC	Hematopoietic stem cell
IBSP	Integrin-binding sialoprotein
IGF	Insulin growth factor
IHh	Indian hedgehog
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
IFN-γ	Interferon gamma
iPSCs	Induced pluripotent stem cells
JNK	c-Jun N-terminal kinases

L-Glut	L-Glutamine
LDH	Lactate dehydrogenase
LEF	Lymphoid enhancer-binding factor
LEP	Leptin
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP	Low-density lipoprotein receptor-related protein
LTA	Lipoteichoic acid
LTQ	Linear ion trap
M-CSF	Macrophage colony-stimulating factor
MA	Machined
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein 1 (CCL2)
MIP-1	Macrophage inflammatory protein 1
MMP13	Matrix metallopeptidase 13
MO	Monocyte
mRNA	Messenger RNA
MS	Mass spectrometry
MSC	Mesenchymal stem cell
MSC	Multipotent stromal cell
MSC	Mesenchymal stromal cell
MSX2	Msh homeobox 2
MV	Membrane vesicles
MyD88	Myeloid differentiation primary response gene 88
OCN	Osteocalcin
OM	Osteogenic medium
ON	Osteonectin
OPG	Osteoprotegrin
OPN	Osteopontin
OSX	Osterix
OX	Anodically oxidized
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDK4	Pyruvate dehydrogenase lipoamide kinase 4

PE/ST	Penicillin-Streptomycin
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PS	Polystyrene
PTCH	Patched
PTH	Parathyroid hormone
qPCR	Quantitative PCR
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor κ B
RANK-L	RANK ligand
RCAN2	Regulator of calcineurin 2
RIA	Radio-immuno assay
RNA	Ribonucleic acid
RUNX2	Runt-related transcription factor 2
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SDF-1	Stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SHh	Sonic hedgehog
SILAC	Stable isotope labeling in cell culture
SMO	Smoothened
SOX	Sex Determining Region Y-Box
SSC	Side scatter
sTNF-αR1	Soluble TNF-alpha Receptor 1
TCF	T-Cell factor
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAP	Tartrate-resistant acid phosphatase
UC-MSC	Umbilical cord MSC
VEGF	Vascular endothelial growth factor
WJ-MSC	Wharton's jelly MSC
Wnt	Wingless-related integration site

1 INTRODUCTION

There is something exciting going on in your bones! From before we are born until the end of our lives it is a never-ending process. Some cells add tissue whilst other cells remove it and this is how it should be. It is called homeostasis. But sometimes things happen that disturb this balance, for example a fractured bone that cannot heal or diseases that affects us due to age or a "typo" in our genetic code. This can result in, amongst many things, reduced function and mobility for a patient and large costs for our health care systems. For these reasons, or just because it is interesting, we are looking into ways to study and begin to solve issues concerning the regeneration of bone tissue using a unique cell that is hiding amongst millions of other cells in your bone marrow.

1.1 Bone

The skeletal system has many functions important for the human body. It provides the framework that supports the body, protects many of the vital organs and allows for body movements. In addition to these features, which are mostly based on the rigidness of the skeleton, it is also involved in more dynamic processes important for the human survival. The skeleton as an organ system is crucial in endocrine signaling that regulates energy metabolism, and is the site of hematopoiesis. The adult human skeleton consists of over 200 individual bones, with many differences in size, structure and composition[1]. Common for all these bones is that they are not constituted by a homogenous material. Generally, bone has an outer layer of compact bone, also known as cortical bone, surrounding a more porous center, the trabecular bone. Bone marrow is found inside the highly vascularized trabecular bone, and also in larger cavities of long bones. The main component of bone is a mineralized extracellular matrix (ECM) composed of an inorganic and an organic phase. The inorganic constituent of this ECM is hydroxyapatite (HA), which is a mineral formed by calcium and phosphate. The organic phase is composed of collagen fibers, mainly type I collagen, as well as noncollagenous proteins such as fibronectin, osteocalcin (OCN) and ostenectin (ON), and glycosaminoglycans[2].

1.1.1 Bone cells

There are several different cell types associated with bone. Osteoblasts are derived from mesenchymal stem cells (MSCs) and are the bone-forming cells responsible for deposition of ECM and its mineralization[3]. Osteoblasts can mature into osteocytes when entrapped in bone ECM[4]. Osteoclasts are large multinucleated cells formed by fusion of macrophages and are thereby of the hematopoietic lineage[5]. These cells are responsible for bone degradation or resorption. In addition to these cell types, the bone marrow and its stroma,

comprises many other cell types such as white blood cells, fibroblasts and adipocytes[6].

1.1.2 Bone formation

In the growing fetus the bone tissue of the skeleton is formed by two processes: endochondral and intramembranous ossification. These processes are also involved in fracture healing in the adult human[7]. During endochondral ossification, cartilage tissue formed by MSCs, which have differentiated into chondrocytes, is subsequently mineralized and transformed into bone by osteoblasts (Figure 1). The formation of long bones during fetal development starts with a cartilage template and the periosteum is then formed around this cartilage structure. In the center of the long bone chondrocytes undergo terminal differentiation, become hypertrophic and the ECM becomes mineralized. This site in the diaphysis develops to the primary center of ossification. It is vascularized and new bone forming cells arrive at the site, thereby creating a trabecular bone tissue. Two secondary ossification centers are formed in the epiphyses of the bone and eventually the mineralized areas fuse together. The outer cortical bone is formed by ECM deposition and mineralization by osteoblasts beneath the periosteum[8]. A similar endochondral ossification process takes place during fracture healing with the cartilage callus, formed after the hematoma, serving as the cartilage template[9].

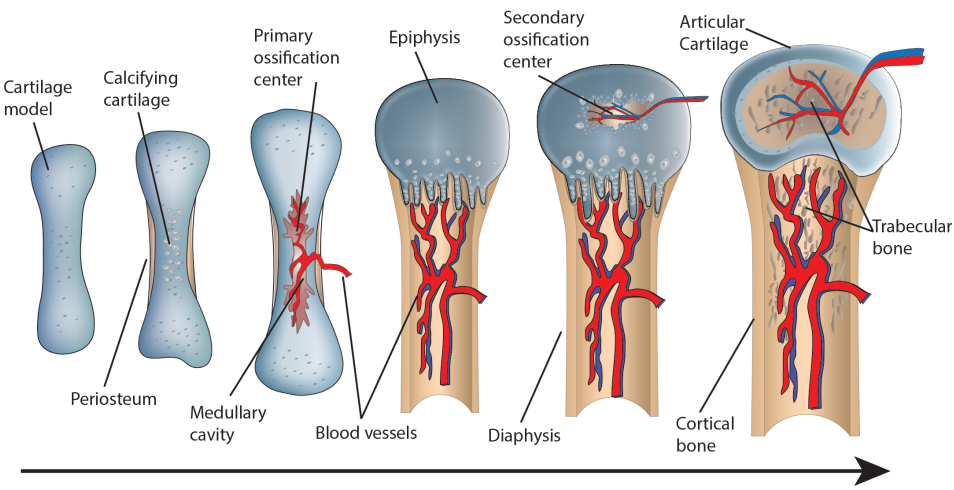

Figure 1. Endochondral bone formation

The progression of endochondral bone formation during embryonic development, from a hyaline cartilage model to a long bone, with trabecular and cortical bone elements.

Intramembranous ossification occurs during the formation of flat bones. In contrast to endochondral ossification, this process starts in the connective tissue matrix and not with a cartilage template. During intramembranous ossification osteoblast progenitors cluster and form a nodule or ossification center. The cells line the nodule and produce an immature unmineralized bone matrix, called the osteoid, towards the nodule-center. As the matrix is mineralized, osteoblasts become trapped and are then terminally differentiated into osteocytes[8]. Intramembranous ossification is the main route whereby implants become osseointegrated[10].

1.1.3 Bone structure

The two types of bone, trabecular and cortical, are schematically illustrated in Figure 2. Cortical bone is denser and stiff compared to trabecular bone and is based on a system of subunits called osteons. Each osteon is formed around a Haversian canal containing blood vessels and nerves. The osteon consists of layers of compact bone, lamella, concentrically organized around the Haversian canal. Osteocytes trapped in between the lamella, in individual lacuna, are in contact with each other through cytoplasmic protrusions running though canals called canaliculi. The canaliculus constitute an important part of the mechano-sensing system whereby osteocytes and osteoblasts communicate[11]. Trabecular bone is composed by an irregular interconnected network of fine tissue spicules or trabeculae. Each such trabecula consists of osteocyte-lined lamellae but unlike the osteon this structure lack the Haversian canal and has a more irregular structure[12].

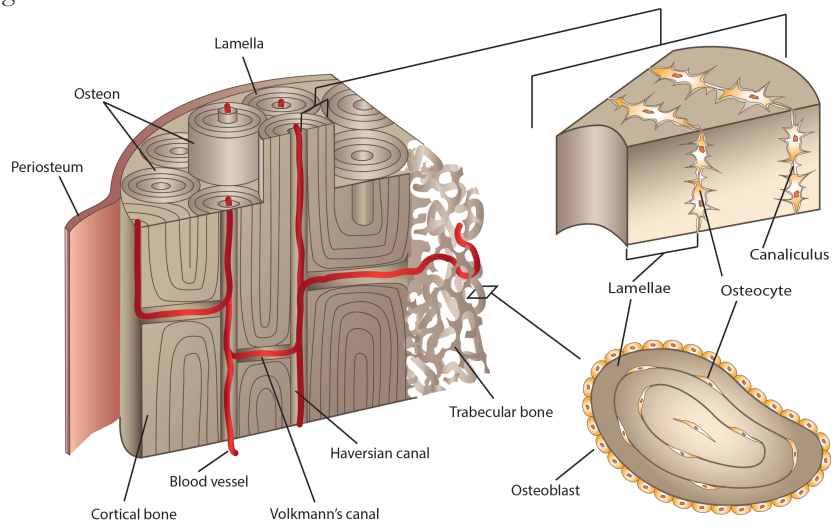

Figure 2. Bone structure

The architecture of cortical and trabecular bone, from osteocytes between bone lamellae to osteons of the cortical bone.

In addition to this division of bone types, bone ECM can be categorized into two types based on the pattern in which collagen fibers are deposited. Woven bone is characterized by a random organization of the collagen fibers, which results in a bone tissue with limited mechanical strength. This type of ECM is firstly produced by osteoblasts and subsequently replaced by the second type of tissue, lamellar bone. In contrast to woven bone matrix, the collagen fibers in lamellar ECM have a high degree of parallel alignment, forming collagen sheets and resulting in a bone tissue with high mechanical strength[12].

1.1.4 Bone remodeling

In adult bone there are four surfaces at which tissue can be added or removed: the periosteal, the endosteal, the intracortical (Haversian canal) and the trabecular surfaces. The process in which bone at distinct sites is resorbed by osteoclasts and re-formed by osteoblasts is called bone remodeling. This is a continuously on-going, physiological process with the purpose of maintaining normal bone mass and repairing micro-damages in the bone.

Osteoclastic progenitors migrate from bone marrow or peripheral circulation and fuse into multinucleated immature osteoclasts in response to macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κ B ligand (RANK-L) and the expression of the osteoclast-specific enzyme tartrate-resistant acid phosphatase (TRAP) is induced. RANK-L is expressed on the cell membrane of osteoblasts or MSCs and the RANK receptor on osteoclast. Continuous presence of RANK-L and physical contact between the two cell types are required for further differentiation of the osteoclast precursor into a mature bone-resorbing osteoclast (osteoclastogenesis)[5,13]. Osteoclasts bind to bone matrix via integrins and bone is resorbed in the space created between the ruffled membrane of the cell and the bone surface. Hydrogen ions are pumped into this compartment, creating an acidic environment that solubilizes the HA and the organic part of the ECM is subsequently broken down by enzymatic degradation. This resorptive process ultimately creates pits in the bone called Howship's lacunae[14].

The process of bone resorption by osteoclast is induced and dependent on RANK-L, a signal produced by osteoblasts. New bone formation by osteoblast, following bone resorption, is in a similar manner dependent on signals released from the ECM during the osteolytic process[15]. In response to transforming growth factor beta (TGF-β) and insulin-like growth factor 1 (IGF-1), as well as other signals, osteoblasts begin to form new ECM and build up bone tissue in previously resorbed area.

In addition to biological signals, mechanical stimulus is essential for bone remodeling. Loading has a profound effect on this process and its absence causes a rapid loss of bone mass[14]. Wolff's law, suggested to be replaced by the term bone functional adaptation, is the theory describing how bone is adapted in response to the mechanical loading it is subjected to. This will for example result in orientation of collagen fibers and directed bone growth to maximize the strength of the bone at points of high mechanical stress[16].

Local regulation of bone metabolism

Osteoblasts possess an important regulatory function in bone remodeling, since they are able to control the rate of osteoclastogenesis by either promoting it through up-regulation of RANK-L or inhibiting it via production of osteoprotegerin (OPG). OPG is a soluble decoy receptor for RANK-L and by inhibiting RANK/RANK-L interaction it may suppress osteoclastogenesis[17]. In addition to factors produced by osteoblasts to regulate osteoclastogenesis, a number of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and IL-1 are involved in modulating the bone remodeling process. These cytokines, produced by several cell types including osteoblasts and osteoclasts, stimulate the production of M-CSF and RANK-L[18]. The OPG/RANK/RANK-L triad is an important regulatory network for bone homeostasis. Dysregulation and imbalance of the expression of these molecules have been implicated in several disease processes[19].

Systemic regulation of bone metabolism

There is also a systemic regulation of bone cell function in which mainly four hormones, parathyroid hormone (PTH), calcitonin, vitamin D3 and estrogen, modulate bone remodeling through paracrine signaling. PTH is one of the most important regulators of calcium homeostasis and it is involved in regulation of both bone formation, through its effect on osteoblast differentiation and survival, and bone resorption indirectly through stimulating osteoblast expression of M-CSF and RANK-L[18]. Furthermore, PTH stimulates the production of calcitriol, an active form of Vitamin D3 that also in an indirect manner promotes bone resorption[15]. In contrast, the hormone calcitonin inhibits bone resorption by affecting the integrity of the ruffled border of osteoclasts, which leads to a decreased ECM breakdown[20]. Estrogens affect both osteoblasts and osteoclasts and thereby have a crucial role in bone biology. Osteoblasts increase their anabolic activities and M-CSF and RANK-L expression in response to estrogen whereas activation of estrogen receptors on osteoclasts and osteoclast progenitor cells decreases differentiation, inhibiting their bone-resorbing activity and increasing apoptosis[18].

1.1.5 Compromised bone situations

There are several diseases that can affect the skeletal system of which some are connected to abnormalities in the bone remodeling and bone formation processes. One such disease, which with an aging population represents an increasing burden on the healthcare system, is osteoporosis and it is commonly divided into three types. Primary type 1 osteoporosis is most common in women after menopause and connected to decreasing levels of estrogen. Primary type 2 osteoporosis, also known as senile osteoporosis, affects both genders after the age of 75, although more common in women. Secondary osteoporosis is the result of for example other diseases or prolonged use of pharmacological agents affecting bone quality.

The clinical definition and diagnosis of osteoporosis is the occurrence of a low-energy fracture, commonly to a vertebra, the wrist or hip as a result of lowered bone mass or bone mineral density (BMD). This is a result of a deterioration of the microstructures in the bone tissue due to increased bone resorption. In type 1 osteoporosis reduced level of estrogen results in both increased bone formation and resorption[21]. However, the increase in osteoclastogenesis, through the loss of this hormone, out-weighs the anabolic effects[22]. The expression of RANK-L is up-regulated in MSCs isolated from postmenopausal women, which would result in increased numbers and activity of osteoclasts[23]. Furthermore, increased levels of pro-inflammatory cytokines, as a result of estrogen deficiency, have been demonstrated to negatively affect bone mass in this type of osteoporosis[24]. Estrogen deficiency also affects the bone status in type 2 osteoporosis in both genders. However, there are also other mechanisms that affect the BMD in these patients. Increased levels of PTH as well as decreased levels of vitamin D and IGF have been suggested to be reasons for the increased bone resorption and decreased bone formation seen in this group of patients[25].

In similarity with osteoporosis, the bone remodeling is also altered in Paget's disease. However, in contrast to osteoporosis, which affects the whole skeleton, Paget's disease is usually limited to a few bones. Many patients with Paget's disease are asymptomatic whilst others suffer from bone pain, bone deformities and secondary arthritis. The bone is compromised by increased osteoclast activity and bone resorption, which in the case of Paget's disease induces an increase in osteoblast activity and new bone formation. However, the resulting trabecular bone is of lower quality with an unorganized ECM structure characteristic for woven bone. Viral infections as well as both hereditary and non-hereditary mutations have been suggested as causes for Paget's disease[22].

Several other diseases may also affect the human skeleton. For example, patients with diabetes are more prone to osteomyelitis (bacterial infection of the bone)[26]. These patients, as well as those diagnosed with for example rheumatoid arthritis and inflammatory bowel disease are also more likely to get osteoporosis. In these cases the secondary osteoporosis is potentially due to, amongst other factors, elevated levels of inflammatory cytokines compared to healthy individuals[27,28]. Furthermore, children and adolescents with early onset of type 1 diabetes and hyperglycemia have a decreased bone mineral density, reduced plasma osteocalcin and increased OPG expression in peripheral blood leukocytes, indicating a risk for impaired growth[29].

1.2 Bone injury and regeneration

1.2.1 Bone healing

Healing of a bone injury such as a fracture is normally divided into four phases; early inflammatory, cartilage callus, primary bone formation and secondary bone formation or bone remodeling phases. Although these phases are overlapping, the processes ongoing in each individual phase have distinct characteristic features. After the initial trauma there is bleeding and subsequent blood coagulation. The repair process is initiated by inflammatory cells and macrophages and their release of inflammatory cytokines like IL-1, IL-6 and TNF-α, which peaks only 24 hours post-fracture[30,31]. As the platelets trapped in the hematoma become degranulated, platelet derived growth factor (PDGF) and TGF-β are released, which are recruiting signals for MSCs. Over the next couple of days MSCs will be recruited, proliferate and stimulated to differentiate into chondrocytes by TGF-β, and into osteoblasts by bone morphogenetic proteins (BMPs) released from the affected bone matrix[32]. This will generate a cartilage callus at the fracture site. A crucial step in the repair process is the vascularization of this callus, which is initiated early by the expression of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and angiopoietin 1[7]. As the healing process continues there will be a shift from cells of the chondrogenic lineage to cells of the osteoblast lineage and a first cycle of ECM resorption will take place. During this primary bone formation phase, bone is formed through endochondral ossification by newly recruited MSCs. Towards the end of the process there will be a decrease in pro-osteogenic signals like BMPs and a secondary increase of pro-inflammatory cytokines[30]. The osteoblasts will up-regulate their expression of M-CSF and RANK-L[31] which, in combination, will stimulate the recruitment, differentiation and activity of osteoclasts and result in active remodeling of the newly formed bone tissue, characteristic for the last phase of the repair process.

There are many instances in which bone does not heal properly after a fracture. For example, in diabetic patients increased levels of TNF-α and other pro-inflammatory cytokines may increase the osteoclastogenesis at an early time point resulting in excessive removal of the cartilage tissue, which may subsequently lead to altered bone formation and impaired fracture healing[33]. Furthermore, not only do patients with osteoporosis suffer increased risk of fractures, the healing process is also altered in this group. In a rat model it has been demonstrated that osteoporosis leads to less callus formation and it has been suggested that the repair process is delayed[34].

The reader interested in the details of fracture healing is referred to the excellent reviews by Dimitriou *et al.*[32] and AI-Aql *et al.*[33]

1.2.2 Bone-anchored implants

There are several types of bone-anchored implants in clinical use. For example, internal fixation of fractures by pins and screws, other orthopedic implants such as hip prosthesis, and dental implants. The repair process that takes place in the bone tissue after the insertion of such an orthopedic or dental implant have similarities with that of fracture repair. However, bone formation around an implant will predominantly be an intramembranous ossification process. Additional differences in the sequence of events composing the repair process, compared to normal fracture healing, may vary due to the implant material, topography and stability. During the initial blood clot formation adsorbing proteins cover the implant surface. The response of the blood cells, such as erythrocytes, platelets and inflammatory cells such as granulocytes and monocytes, which arrive at the implantation site, will be affected by the implant surface and protein-profile they encounter[35]. The recruited inflammatory cells will secrete growth factors (GFs) and cytokines such as IL-1, IL-6, TNF-α and PDGF and the fibrin matrix initially formed will act as a scaffold for the subsequent migration and tissue-formation of MSCs and osteoblastic progenitor cells. The newly arrived tissue forming cells will in turn produce GFs such as BMPs and TGF-β, further stimulating the bone formation.

The recruited osteoblastic cells produce a woven bone either as solitary islands in the ECM or at the surface of existing bone, which gradually advances towards the implant surface[36,37], a process referred to as appositional bone formation or distance osteogenesis. In addition, during osseointegration of an implant, woven bone has been found in direct contact with the implant surface. This newly formed bone is thought to be formed by a process called contact osteogenesis in which MSCs and osteoblasts migrate to the implant surface and produce an ECM that is subsequently mineralized[38,39]. For details on the cellular and molecular processes during osseointegration, see Palmquist and co-workers[40].

1.3 Inflammation

Inflammation is an adaptive response to harmful stimuli, for example tissue injury and infection. Inflammation serves to contain, neutralize, dilute, or wall off the injurious agent or process. Generally, the acute inflammatory reaction, provoked by such stimuli, has a distinct endpoint characterized by resolution and repair of the damaged tissue. However, in some instances a pathological dysregulation of the inflammatory process leads to a prolonged, chronic inflammation, instead characterized by for example permanent tissue damage, fibrosis and/or scaring[41].

1.3.1 Inflammatory cells and signals

A local inflammatory response is initiated when tissue residing macrophages and mast cells becomes activated, resulting in a release of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 as well as leukocyte-recruiting chemokines such as monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 alpha and beta (MIP-1 α/β) and IL-8[42]. Mast cells release histamines, which act on vascular endothelial cells resulting in increased permeability of blood vessels and a gradient of chemokines selective recruit and induce migration of leukocytes, firstly neutrophils and subsequently monocytes, into the affected tissue[43]. Neutrophils become activated at the site of inflammation, either by direct contact with pathogens or through pro-inflammatory cytokines secreted by cells in the affected tissue. These cells attempt to kill the invading agents by releasing the toxic contents of their granules, incidentally also causing damage to host cells and surrounding tissue[43,44].

Recruited monocytes/macrophages have versatile roles in the inflammatory process. Depending on which signals that are present in the affected tissue and their maturation-state, monocytes and macrophages can regulate the progression of inflammation by a pro-inflammatory or an anti-inflammatory and repair oriented response[45].

In response to stimuli such as bacterial cell wall component lipopolysaccharide (LPS) and interferon-gamma (IFN-γ), monocytes will produce pro-inflammatory cytokines with the aim of amplifying the cell-mediated immune response and recruiting more cells to the site[45]. If this acute inflammatory response fails to eliminate the pathogen, the inflammatory process persists and acquires more chronic characteristics, which include continuous low-grade tissue destruction, neovascularization and fibrosis[46]. The repair monocyte/macrophage phenotype (also known as alternative activated monocytes) is induced by IL-4 and/or IL-13 stimulation and characterized by increased expression of the

mannose receptor, MHC class II, alternative macrophage activation-associated CC chemokine-1 (AMAC-1) and MCP-1. This subset of monocytes also produce anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1RA) and tissue formation-stimulatory GFs such TGF-β[47].

1.3.2 Infection and inflammation in bone repair

The treatment regime for open fractures includes surgical irrigation and debridement as well as antibiotics to manage any possible infection. However, although this method is relatively effective, open fractures is one of the ways in which a bacterial infection can reach the bone and cause osteomyelitis. Other causes include hematogenous spread from other infected organs or following the placement of an internal fixation device or other type of implant. Two of the most common bacterial strains in osteomyelitis and biomaterial associated infections are gram-positive *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*)[26].

In normal bone repair the inflammatory phase is transient, self-limiting and likely to be necessary for the subsequent regeneration and tissue healing[48]. However, in the case of an infection, the inflammatory response will be persistent until clearing of invading microorganism is achieved, and if the microbial challenge cannot be eliminated, the infection can become chronic and result in tissue degradation and bone loss. The increased risk of an infection and severe inflammatory response in cases where a biomaterial has been implanted is due to the possibility of colonization and biofilm formation on the implant surface[49]. Bone-anchored implants are particularly associated with chronic osteomyelitis since antibiotic treatment often is ineffective in these cases as a result of the biofilm formed by the pathogen at the implant surface[50].

Also inflammatory diseases such as rheumatoid arthritis (RA), diabetes mellitus and inflammatory bowel disease can affect bone quality, resulting in secondary osteoporosis[51]. The mechanism behind this catabolic process is, at least partly mediated by the high prevalence of pro-inflammatory signals. This will lead to an imbalance between the activities of bone forming osteoblasts and bone resorbing osteoclasts, including RANK/RANK-L interactions and result in decreased bone mass[52,53].

Although this influence of abnormal inflammatory conditions on the bone remodeling process is well characterized, far less is known about the effects of such conditions on fracture healing and bone repair. However, during fracture healing in diabetic mice increased levels of TNF-α were shown to increase chondrocyte apoptosis as well as lead to premature loss of cartilage matrix and enhanced osteoclastogenesis[54,55]. Also the healing around an implant inserted in

bone can be negatively affected by inflammation. Peri-implantitis is defined as a destructive inflammatory reaction around an osseointegrated implant with a subsequent loss of supporting bone[56] and it has been suggested to be caused by infection and possibly biofilm-formation on the implant surface. However, what induces this degenerative process around an already osseointegrated implant remains unclear[57].

Patients suffering from a disease with pathological inflammatory processes, such as RA, Crohn's disease or diabetes, which entails compromised bone quality, have been suggested as high-risk groups in the aspect of dental implant failure. However, possibly due to the relatively unaffected bone of the jaw, in a recent large systematic literature review neither of these conditions were found to be associated with higher risk of treatment failure or complications[58].

1.4 Mesenchymal stem cells

In 1966 Friedenstein and co-authors demonstrated that bone marrow stroma could generate bone, fat cells and cartilage following heterotopic transplantation[59]. This finding suggested a connective tissue lineage progenitor cell residing in bone marrow stroma. From this, the concept of the MSC developed in the 1990's as a precursor cell, easily isolated by plastic adherence, with multipotency and self-renewal capacity[3,60,61]. Since then the multipotency of MSCs has been narrowed down to trilineage potential, i.e. osteoblast, adipocyte and chondrocyte.

The classification of MSCs as a stem cell population is much debated and disputed in the literature. Stem cells are defined by functional assays to meet the two criteria of multipotency and self-renewal. The embryonic stem cell (ESC) is for example defined by its pluripotency i.e. potential to differentiate into cells from all three germ layers, endoderm, ectoderm and mesoderm, as well as by its unlimited proliferative capacity. In a similar way, the strict definition of MSCs is a cell type that can generate fully differentiated tissues within its lineage *in vivo*, which proves its multipotency, and can reconstitute itself *in vivo* and give rise to cells identical in phenotype and potency, which proves self renewal[62]. In that sense it has been demonstrated that only a subset of the MSC-population generated by conventional isolation methods can actually be classified as multipotent stem cells[63]. Therefore multipotent mesenchymal stromal cells, mesenchymal stromal cells (both also abbreviated MSCs) and bone marrow stromal cells (BMSC) are terms that have been suggested as more appropriate for this *in vitro*-expanded heterogeneous cell population than mesenchymal stem cells. The name mesenchymal stem cell is a term that perhaps should be more stringently used and reserved for the proposed *in vivo* precursors or stem cells of the mesenchymal lineage[64,65]. However, the name MSCs remains prevalent and

is nevertheless used to denote a stromal precursor population with trilineage potential throughout the literature and also in this thesis.

The International Society for Cellular Therapy has suggested a set of minimal criteria for the definition of multipotent mesenchymal stromal cells. The MSCs must be plastic-adherent, express several specific surface antigens: CD105, CD73 and CD90, and lack the expression of other antigens CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. In addition, the cells must be able to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* [66]. Although these markers are an excellent guideline and tool in the defining process of MSCs there are also several other markers used to identify MSC populations such as CD29, CD44, CD146, and CD166[67].

1.4.1 MSCs *in vivo* – the niche

The distinct niches in bone marrow that support survival and control proliferation and differentiation of hematopoietic stem cells (HSCs) are well described. They are formed by stromal precursors or their progeny but the exact identity or maturity of these lining cells remains unclear. Furthermore, the question whether these are dual stem cell niches in which both HSCs and MSCs reside is still debated.

One niche has been described at the endosteal surface of the trabecular bone, where the lining cells are of the osteoblastic lineage albeit heterogeneous in their degree of maturity, that spans from bone-synthesizing osteoblasts to MSCs[68]. A second perivascular niche is found at the site of the bone-marrow sinusoids, where stromal progenitor cells or MSCs have been found in close proximity to the endothelial cells of blood vessels[64]. The cells of mesenchymal lineage in these niches express proteins regulating the fate of HSCs such as angiopoietin, stromal derived factor 1 (SDF1)[69,70] and osteopontin (OPN)[71]. Subsets of them have been demonstrated to be multipotent MSCs and suggested to express both CD146[63] and nestin[72].

Interestingly, Baksh and co-authors presented a wider concept of an ubiquitous MSC-niche as they questioned the logic behind MSCs isolated from other tissues when the general concept is an MSC-niche co-localized with the established HSC niche in the bone marrow[73].

1.4.2 MSC sources

Since MSCs were originally isolated from bone marrow (BM-MSCs), this tissue has served as the foundation in this area of research. However, MSCs or MSC-like cells also referred to as MSCs, have also been found in adipose tissue, connective tissue of the umbilical cord and in cord blood. Although the MSC-

populations isolated from these different sources in many aspects are similar to one another, they display variations in both potential and phenotype.

Isolation of MSCs from the umbilical cord (UC-MSCs) and cord blood (CB-MSCs) is an appealing alternative to bone marrow with a harvest technique that is not painful or invasive in any way. Furthermore, it does not afflict any adverse effect to the donor such as donor site morbidity and is in abundant supply at delivery clinics worldwide. To date, MSCs have been isolated from several compartments of the umbilical cord. However the most common sources are the perivascular cells[74] and the cells found in connective tissue in the intervascular zone also known as the Wharton's jelly[75]. It has not been clearly demonstrated whether MSCs isolated from the different sites of the umbilical cord are different populations of cells. One advantage with the UC-MSCs is that they have a higher proliferative capacity than their bone marrow counterpart[76]. Wharton's jelly MSCs (WJ-MSCs) have a surface marker expression profile similar to that of BM-MSCs as they do not only express the surface markers that defines the MSC population, CD73, CD90 and CD105, but also in similarity with BM-MSCs express, CD13, CD29, and CD44[77]. CB-MSCs have, in similarity to WJ-MSCs, a higher proliferative capacity compared to MSCs from other sources and express most of the required MSC markers with the exception of CD105[78,79]. Another source of MSCs is adipose tissue and these cells can be isolated by enzymatic digestion and centrifugation of lipoaspirates. Also adipose tissue MSCs (AT-MSCs) are similar to the MSC-population isolated from bone marrow in terms of surface marker expression and proliferation[78,80].

When it comes to trilineage multipotency there are differences in potential between MSCs isolated from different sources. AT-MSCs are more prone to adipogenic differentiation compared to the other types whereas WJ-MSCs and CB-MSCs have been demonstrated to have higher osteogenic potential than BM-MSCs[78,81,82]. AT-MSCs have an inferior potential for both osteogenesis and chondrogenesis compared with the BM-MSCs[83], and CB-MSCs have a reduced adipogenic potential compared to not only AT-MSCs but also BM-MSCs[78,80].

1.4.3 MSC differentiation

One of the MSC criteria presented by Dominici and colleagues is the trilineage differentiation potential, which means the capacity of these cells to differentiate into chondrocytes, adipocytes and osteoblasts *in vitro*[66]. These differentiation processes and the signaling pathways involved have been extensively studied, primarily in well-established *in vitro* systems with culture expanded MSCs. It is therefore important to be restrictive with applying this knowledge obtained *in vitro* to the native MSCs cells found *in vivo*. However, some of the major factors involved in maturation of MSCs into different cells of connective tissue lineage

have also been characterized *in vivo* and can thereby be used to describe the differentiation processes in more general terms. Furthermore, several proteins are regulators of more than one of these differentiation pathways and crosstalk and cross-regulation between the different lineages is a major element. Therefore, a brief overview of both the chondrogenic and adipogenic differentiation pathways will be presented here, although the focus of MSC-differentiation will be on the osteogenic lineage (Figure 3).

Chondrogenic differentiation

The master switch of chondrogenesis is the transcription factor sex determining region Y-box 9 (SOX9) and its continuous expression is required throughout the chondrogenic differentiation[84]. SOX9, together with other SOX transcription factors for example SOX5 and SOX6, will induce the expression of proteins essential for the chondrocyte phenotype such as collagen type II alpha 1 (COL2A1) and aggrecan[85]. The expression of these transcription factors is induced by members of the TGF-β superfamily. Several TGF-β isoforms (in particular TGF-β1) and BMPs (mainly BMP2, BMP4 and BMP14 also known as growth differentiation factor 5 (GDF5)) are potent inducers of chondrogenic differentiation[86]. These proteins form a complex with two types of trans-membrane receptors that leads to receptor phosphorylation and activation of a SMAD-signaling cascade[87] inducing transcription of chondrogenic genes. When fully differentiated chondrocytes become hypertrophic there is an increase in runt-related transcription factor 2 (RUNX2) expression, decrease in SOX9 expression and a phenotypic shift to a mineralizing and collagen type X, alpha I (COL10A1) expressing cell type.

The Wnt signaling pathway is involved in the regulation of endochondral differentiation during embryonic development and has also been implicated in chondrogenic differentiation of MSCs. In embryonic chondrocytes canonical Wnt activation leads to reduced chondrocyte differentiation, decreased SOX9 and COL2A1 expression as well as increased expression of markers of hypertrophic chondrocytes RUNX2 and COL10A1 [88]. *In vivo*, over-expression of a Wnt-activator resulted in enhanced ossification and reduced chondrocyte formation[89]. Furthermore, the same study also demonstrated that canonical Wnt signaling inhibition led to enhanced chondrogenic differentiation of mouse MSCs. The negative regulation of chondrogenesis by canonical Wnt signaling has also been demonstrated in human MSCs in which canonical Wnt signaling inhibition increased early chondrogenesis and up-regulation of COL2A1 and SOX9[90].

Adipogenic differentiation

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is the main transcription factor controlling the adipogenic differentiation of MSCs and its effect on adipogenesis is thoroughly demonstrated both *in vitro* and *in vivo*[91]. It belongs to a family of nuclear hormone receptors and it is therefore believed that PPAR-γ promotes adipogenic differentiation both through ligand dependent activation and increased expression of the transcription factor itself. This induces an up-regulation of a majority of proteins that characterizes the adipocyte phenotype including fatty acid synthase (FAS), glucose transporter type 4 (GLUT4), lipoprotein lipase (LPL) and fatty acid binding protein 4 (FABP4), and ultimately results in intracellular lipid accumulation[92]. The upstream mechanism that induce expression of PPAR-γ and its downstream targets include signal transduction due to insulin and IGF-1 binding to their respective receptor[93]. However, also several BMPs have been indicated as stimulators and regulators of adipogenesis[94].

In addition to PPAR-γ there are three proteins of the CCAAT-enhancer-binding protein (C/EBP) family, which play a central role in regulation of adipogenic differentiation. Of these three, C/EBPα is the most potent inducer of differentiation. As PPAR-γ is up-regulated due to external signals it induces an increased expression of C/EBPα, which in turn gives rise to a positive feedback loop, further increasing the expression of PPAR-γ[95].

Osteogenic differentiation

In similarity to the key transcription factors SOX9 and PPAR-γ regulating MSC differentiation into the chondrogenic and adipogenic lineages, respectively, RUNX2 is known to be the master switch of osteogenesis. Its crucial and essential role is demonstrated by a cartilaginous skeleton and complete absence of ossification in RUNX2 knockout mice[96]. It has been hypothesized that RUNX2 acts early to commit MSCs to the osteochondral lineages, and that in later differentiation stages expression of this transcription factor induces the production of bone related proteins such as collagen type I, alkaline phosphatase (ALP), OCN and bone sialoprotein (BSP). These proteins are all vital for the osteogenic phenotype. OCN and BSP are two of the most abundant non-collagenous proteins in bone and BSP serves as a nucleating site for HA crystal formation[97]. ALP is a key enzyme in the process of matrix mineralization and together these proteins represent both early and late markers of osteogenic differentiation[98].

Another important transcription factor involved in regulating osteogenic differentiation is osterix (OSX). Although, OSX is vital in promoting the earlier stages of osteogenesis it is not enough to achieve a fully differentiated osteoblast

and it appears to act downstream of RUNX2[99,100]. Several other transcription factors affect the osteogenic differentiation process, for example distal-less homeobox 5 (DLX5) and msh homeobox 2 (MSX2). Overexpression of DLX5 can accelerate osteoblast differentiation *in vitro* whereas MSX2 overexpression actually inhibited osteogenic differentiation and ECM mineralization[101,102]. However, *in vivo* MSX2 is thought to promote osteogenesis by stimulating proliferation in osteogenic progenitor cells[92].

BMPs, the main inducers of osteogenic differentiation, are members of the TGF-β superfamily. There are several BMPs in this group of proteins although the most potent inducers of osteogenesis, both *in vitro* and *in vivo*, are BMP-2, BMP-6, BMP-7 and BMP-9. Signaling of the BMP-pathway is initiated by the binding of one of the BMP-proteins to the heterodimer receptor complex (BMPR). This leads to the phosphorylation of the receptor-SMADs 1, 5 or 8 and subsequent complex formation together with SMAD4, a complex that is then translocated to the nucleus where expression of key osteogenic genes is induced[103]. Receptor SMADs 2 and 3 are specific for TGF-β signaling and the induction of chondrogenic genes and SMAD 3 is thought to inhibit RUNX2 expression[104]. In addition to the SMAD signaling cascade, BMP2 induces the expression of ALP and OCN through a MAP kinase (MAPK) signaling cascade involving ERK, JNK and p38, probably converging with other signaling pathways at the regulation of the RUNX2 expression[105].

The importance of Wnt signaling in bone was discovered when Osteoporosis–pseudoglioma, a disease characterized by low bone mass, was shown to be caused by an inherited loss of function mutation in the low-density lipoprotein receptor-related protein 5 (LRP5) gene[106]. Furthermore, a mutation to this gene resulting in increased Wnt signaling, generates a high bone mass phenotype whereas LRP5 knockout mice develop a low bone mass phenotype[107,108]. Through several *in vitro* experiments it has been demonstrated that the Wnt/β-catenin pathway i.e. the canonical Wnt pathway is not only involved in embryonic skeletal development but also affects the osteogenic differentiation process[89,109]. This pathway is active when a Wnt ligand binds to LRP5/6-frizzled (FZD) receptor complex, which leads to the binding of a β-catenin destruction complex to the receptor. As a result, the degradation of β-catenin is inhibited and this protein accumulates in the cytoplasm and subsequently in the nucleus. This accumulation leads to expression of downstream target genes through the activation of a transcription factor complex consisting of lymphoid enhancer-binding factor and T-cell factor (LEF/TCF). TCF1 enhances RUNX2 expression and RUNX2 promoter activity and thereby the expression of genes related to the osteogenic phenotype such as OCN[110].

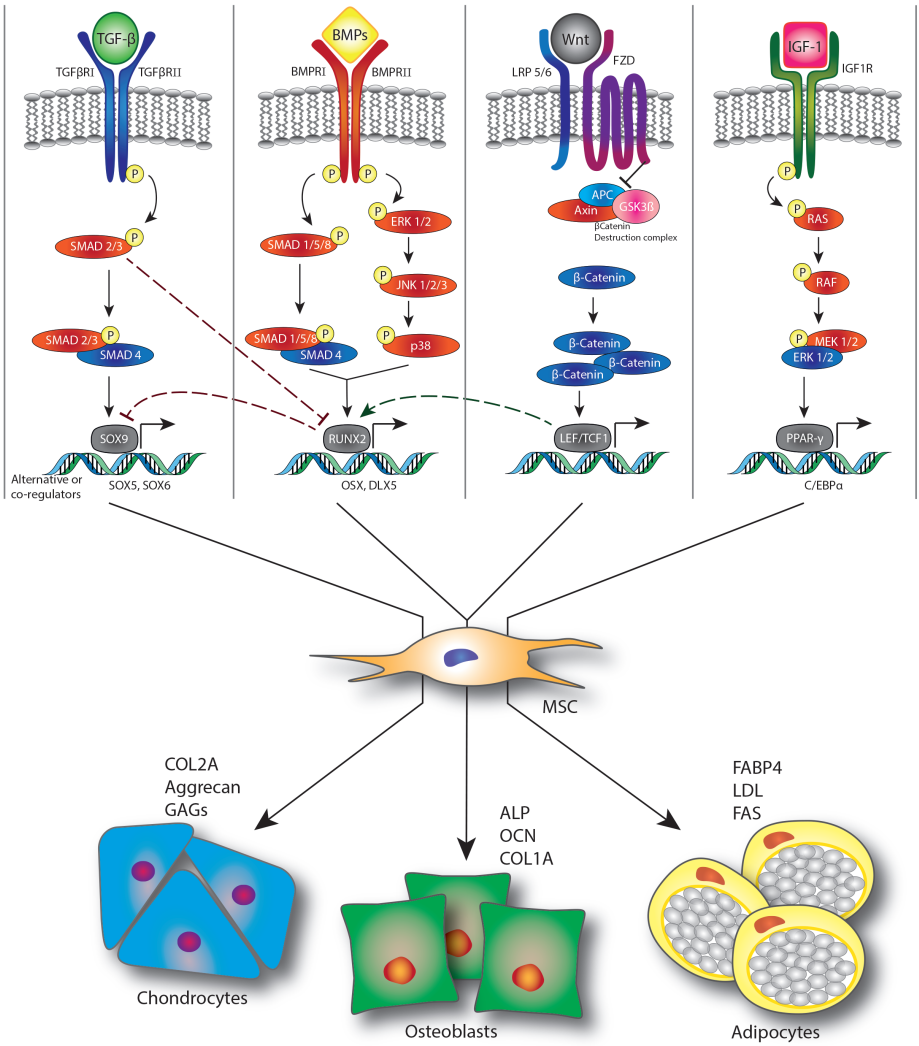

Figure 3. Signaling pathways in MSC differentiation

The main signaling pathways involved in chondrogenic, osteogenic and adipogenic differentiation of MSCs, including inducing growth factors, their receptors, signaling cascades and transcription factors, as well as the resulting phenotypical markers characteristic for each cell type.

However, how Wnt signaling is involved in the regulation of osteogenic differentiation seems to be a matter of timing[111]. Reports with conflicting data regarding Wnt-pathway activation and osteogenesis have been published of which some indicates an inhibitory effect of Wnt signaling activating ligands on the osteogenic process[112].

Hedgehog (Hh) signaling is activated during many processes involved in embryonic development and activation of this signaling pathway can increase the osteogenic differentiation of MSCs, both *in vitro* and *in vivo*[113,114]. The Hh pathway is activated when one of three different Hh ligands, sonic Hh (SHh), indian Hh (IHh) or desert Hh, binds to a cell surface complex consisting of two proteins, patched (PTCH) and smoothened (SMO). This binding results in conformational changes of the receptor complex and subsequent activation of transcription factor glioma-associated oncogene homologs (GLI) 1, 2 or 3[103]. It has been suggested that Hh signaling regulates MSC-commitment into the osteogenic lineage by affecting RUNX2 expression and also that Hh signaling acts upstream of the canonical Wnt pathway to promote osteoblast maturation[115-117].

1.4.4 MSC and inflammatory stimuli

The inflammatory environment that arises from the immune response towards an infection, or in inflammatory diseases such as rheumatoid arthritis is detrimental for bone and exerts negative effects on bone forming cells and their progenitors, the MSCs. At the same time it has been demonstrated that MSCs act on both the adaptive and innate immune systems by for example suppressing the expression of pro-inflammatory cytokines[118]. These immunomodulatory properties of MSCs are, although not fully understood, relatively well established and make these cells an interesting tool in cell-based therapies for diseases of autoimmunity and inflammation. However, the connection between the immunomodulatory properties of MSCs and their regenerative capacity is only recently starting to be elucidated.

One possible connection between these two traits is the toll like receptors (TLRs). TLRs are expressed on cells of the innate immune system and are capable of recognizing pathogen associated molecule patterns (PAMPs)[119]. Apart from the recognition of molecules related to invading pathogens, these receptors can also detect host specific molecules important for "self-recognition" and have been shown to be involved in the pathogenesis of autoimmune and chronic inflammatory diseases[120]. The different TLRs and their respective ligands can be found in Table 1, adapted from Akira *et al*[121].

Table 1. Toll-like receptors (TLRs) and their ligands

Receptor	Ligand	Origin of ligand
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
TLR2	Lipoproteins / Lipopeptides	Bacteria and mycobacteria
	Glycolipids	Bacteria and mycobacteria
	Porins	Gram-negative bacteria
	Peptidoglycans	Gram-positive bacteria
	Lipoteichoic acid (LTA)	Gram-positive bacteria
	Phenol-soluble modulin	MRSA *S. epidermidis*
	Zymosan	Fungi
	Possible ligand:	
	Heat-shock protein 70	*Host*
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide (LPS)	Gram-negative bacteria
	Taxol	Plants
	Possible ligands:	
	Heat-shock protein 70	*Host*
	Type III repeat EDA of fibronectin	*Host*
	Oligosaccharides of hyaluronic acid	*Host*
	Fragments of heparan sulphate	*Host*
	Fibrinogen	*Host*
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	Mycoplasma
	Lipoteichoic acid (LTA)	Gram-positive bacteria
	Zymosan	Fungi
TLR7	Single-stranded RNA	Viruses
TLR8	Single-stranded RNA	Viruses

Interestingly, several TLRs are expressed on MSCs and they have been implicated as key proteins in immunomodulation[122]. Furthermore, although there are studies with conflicting results, recent reports have suggested that activation of different TLRs on MSCs can have an impact on their differentiation *in vitro*[123-125]. Furthermore, Waterman *et al.* have proposed a new hypothesis suggesting that MSCs can be polarized by TLR activation into either a pro-inflammatory or immunosuppressive and regenerative phenotype, much in parallel to the classification applied to monocyte/macrophages[126].

2 AIMS OF THE THESIS

The main objective of this thesis has been to explore different approaches to enhance the osteogenic capacity of MSCs and thereby gain new insight into the factors and mechanisms that are involved in the regulation of this process.

2.1 Specific aims of the included studies

- To investigate the potential to enhance osteogenic differentiation of MSCs by the use of small molecule substances (addressed in paper I and II) and to explore the possibilities of a drug development approach involving virtual ligand-based screening in combination with an *in vitro* functionality assessment (addressed in paper I).

- To further elucidate how adipogenesis and osteogenesis are related in cells of the connective tissue lineage by investigating the effects of PPAR-γ inhibition on osteogenic differentiation of MSCs *in vitro* (addressed in paper II).

- To develop methods for monitoring the progression of osteogenic differentiation of MSCs by identifying new markers, preferably easy accessible surface markers, for this process (addressed in paper III).

- To study how signals from inflammatory cells and immune-triggering molecules affect osteogenic differentiation and cell-cell communication of MSCs (addressed in paper IV and V).

3 MATERIALS AND METHODS

3.1 Mesenchymal stem cells

3.1.1 Isolation and expansion

The MSCs used in this thesis were isolated from bone marrow biopsies obtained from patients undergoing surgical spinal fusion at Sahlgrenska University Hospital. Bone marrow was aspirated from the iliac crest using heparin-coated syringes to prevent coagulation and transferred to vials containing a heparin-PBS solution (ethical approval number 532-04, T936-13). After removal of the lipid content by centrifugation, a mononuclear cell population was isolated from the biopsies by density gradient centrifugation.

The mononuclear cell fraction, containing the MSCs, was seeded in tissue culture flasks at a density of approximately 250,000 cells/cm^2 in DMEM-LG supplemented with 2 mM L-glutamine (L-Glut), 1x PE/ST (0.1 units/ml penicillin, 100 μg/ml streptomycin) and 10% fetal bovine serum (FBS). After 24 hours the culture flasks were rinsed and unattached cells discarded. The adherent cell population was expanded in culture medium consisting of DMEM-LG supplemented with 2 mM L-Glut, 1x PE/ST, 10 ng/mL human recombinant FGF-β and 10% FBS, and through expansion MSCs were enriched in the cell population due to their proliferative capacity. During expansion, the cells were passaged at 80% confluency using 0.05% trypsin with EDTA and reseeded at a density of approximately 8,000 cells/cm^2. Throughout the cell culture experiments the culture medium was prepared fresh every week, changed every three to four days and the culture flasks/well-plates were kept in an incubator at 37°C in 5%CO_2. Before the cells were used in experiments their phenotype and population purity were analyzed by flow cytometry.

3.1.2 Mesenchymal stem cell differentiation

Induction of osteogenic differentiation

For the induction of osteogenic differentiation, MSCs were seeded in flasks or wells at a density of 5,000 cells/cm^2 in osteogenic medium (OM). The OM consisted of DMEM-LG supplemented with 2mM L-Glut, 1x PE/ST, 10% FBS, 45 mM ascorbic acid (ASC), 20 mM β-glycerophosphate (β-GPH) and 1 μM dexamethasone (DEX). These additives are known to induce osteogenic differentiation of MSCs and although they are used at varying concentrations throughout the literature it is the standard procedure for inducing differentiation towards this lineage[127]. In paper I the cells were additionally stimulated to

differentiate down the osteogenic lineage by 25 ng/ml human recombinant BMP-2.

Induction of chondrogenic differentiation

For the induction of chondrogenic differentiation, MSCs were seeded at a density of 20,000 cells/cm^2 in monolayer culture or in pellet mass culture (200,000 cells/pellet) with chondrogenic medium (ChM). The ChM consisted of DMEM-HG supplemented with 1x PE/ST, 5.0 μg/mL linoleic acid, 1x insulin, transferrin and selenium, 1.0 mg/mL human serum albumin, 10 ng/ml human recombinant TGF-β_1, 0.1 μM DEX and 14 μg/mL ASC. The cells for the pellet mass culture were placed in a conical polypropylene tube and centrifuged at 500 g for 5 minutes, after which the pellet at the bottom of the tube was gently loosened.

Induction of adipogenic differentiation

For the induction of adipogenic differentiation, MSCs were seeded at a density of 5,000 cells/cm^2 in adipogenic medium (AM). The AM consisted of DMEM-LG supplemented with 2 mM L-Glut, 1x PE/ST, 20% FBS, 5.0 μg/mL insulin, 1,0 μM DEX, 0.5 mM isobutylmethylxathine and 60 μM indomethacin.

3.2 Monocytes

3.2.1 Isolation and culture

Human mononuclear cells were isolated from buffy coat obtained from healthy donors by density gradient centrifugation. The resulting mononuclear cell fraction, consisting mainly of human monocytes (MO), was collected and washed. The concentration of the viable cells was determined by the NucleoCounter system and monocyte purity was determined by flow cytometry. The isolated cells were re-suspended at concentration of 500,000 cell/ml in DMEM-LG supplemented with 1% FBS and 1x PE/ST.

3.3 Cell stimuli

In paper I MSCs were stimulated during osteogenic differentiation by different purmorphamine analogs. In paper II the cells were treated with GW9662, a potent PPAR-γ inhibitor during induced osteogenic differentiation. In paper IV MOs were activated by either LPS or by recombinant human IL-4 and in paper V the MSCs were stimulated by either LPS, lipoteichoic acid (LTA) or bacterial membrane vesicles (MVs) isolated from *S. aureus* or *S. epidermidis*.

3.3.1 Bacterial membrane vesicles

In paper V MVs were isolated from bacterial cultures (10^9 CFU/ml) of *S. aureus* (strain ATCC 25923) and *S. epidermidis* (strain ATCC 35984) obtained from the Culture Collection University of Gothenburg. One colony from each strain was grown in 100 ml Tryptic Soy Broth at 37°C for 22 hours with gentle shaking. The bacterial cells were subsequently pelleted by centrifugation and the remaining supernatant was filtered sequentially through a 0.45 and 0.22 μm pore-size vacuum filters to remove the remaining bacterial cells. A sample from the supernatant was cultivated on Columbia horse blood agar plates (Clinical Microbiology Lab, Sahlgrenska University Hospital, Gothenburg, Sweden), to confirm that it was free of bacteria. The bacterial MVs were collected from the supernatant by sequential ultracentrifugation and filtration steps. The resulting MV-pellet was washed in PBS and collected by ultracentrifugation and total protein content determined by a BCA kit.

Nanoparticle tracking analysis

In paper V the sizes of isolated MVs were determined using the NanoSight LM10/LM14 instrument. MVs were diluted in PBS and injected into the LM14 module. Three videos were captured, of three different injections and then subjected to nanoparticle tracking analysis using the Nanosight particle tracking software 2.3. This provided the nanoparticle concentrations and size distribution profiles.

3.4 Titanium surfaces

In paper IV two types of titanium surfaces were used. Machined (MA) titanium and anodically oxidized (OX) titanium.

3.4.1 Discs

For the *in vitro* experiment in paper IV, monocytes were cultured on MA or OX titanium discs, with polystyrene (PS) as a control surface. The discs were prepared according to the following procedure: Discs with a diameter of 12 mm and a thickness of 1 mm were machined from commercially pure titanium (Grade 2). The discs were cleaned by ultrasonication and in successive baths of heptane, acetone and ethanol for 10 minutes in each bath. The OX discs were supplied with a thick oxide layer by spark anodization in a sulphuric and phosphoric acid electrolyte. Prior to *in vitro* experiments all discs were soaked in 70% ethanol and exposed to ultraviolet light for 24 hours for sterilization.

3.4.2 Implants and implant preparation

The implants used in the animal study in paper IV were commercially available dental implants. The MA surface was represented by Branemark system

Original™ screws and the OX surface was represented by Branemark system TiUnite™ screws, all produced by Nobel Biocare.

LPS was dissolved in HBSS and the solution was diluted to a concentration of 10 mg/ml. The implants were placed in individual glass vials and LPS-solution was added to each vial with an implant and left for 1 hour in room temperature and thereafter an additional 24 hours at 8 °C. The implants were removed from the LPS-solution and sterile balanced salt solution was gently dripped on each implant. The implants were dried for 2–3 days at room temperature.

3.5 Animal surgery

3.5.1 Pigs

The titanium implants were inserted into the femurs of two female pigs, weighing 75 kg each. Implantation was performed in the femoral diaphysis under general anesthesia using Ketalar intramuscularly (Ketamin 50 mg/ml), Stresnils (Azaperon, 40 mg/ml) and Hypnodil (Metomidate hydrochloride, 1 g). The bone was exposed by a 10 cm long incision, through the skin and muscles, from the distal part of the femur and proximally. The muscles were divided and the bone exposed after elevation of the periosteum. The screws were implanted into the femoral diaphysis. Four implants were inserted in each femur in the following order, proximal to distal; MA, OX, MA+LPS and OX+LPS. Both animals served as their own control and were operated twice, with implantation into one femur performed 4 weeks after the other, resulting in observation times of 2 and 6 weeks. Animals were sacrificed with an overdose of Stresnil and Hypnodil and the implants and the surrounding tissue retrieved.

3.6 Gene expression analysis

Transcription of mRNA from genes encoded in the DNA is the first step in the process of producing functional proteins from the information stored in the genome. Changes in the number of copies of a specific gene that is produced can occur due to many reasons for example in response to internal and external stimuli. Determination of the relative expression level of genes of interest is used in all papers of this thesis to analyze the on-going processes in the cell.

3.6.1 RNA isolation

In all papers total RNA was isolated from the samples using an RNeasy mini or micro kit, with on-column DNase digestion to reduce genomic DNA contamination, according to the manufacturers protocol for animal cells.

3.6.2 Microarray analysis

For the microarray analysis in paper II biotinylated and amplified sense-strand cDNA was generated from total RNA, representing the expressed genome, using the Ambion WT Expression Kit. Subsequently, the cDNA was hybridized onto Human Gene 1.0 ST GeneChip® arrays for 16 hours in at 45°C and rotated at 60 rpm. The arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G. The microarray analysis was performed by technicians at Uppsala Array Platform at Uppsala University.

Microarray data analysis

The raw data was normalized and analyzed in the gene expression and functional profiling analysis suite Babelomics 4.3. The normalization was performed using the robust multi-array average (RMA) method. Subsequently, the background was reduced by removing transcripts for which the intensity was less then 5 in all samples. The mean intensities for the inhibitor treated group and the control group were calculated and genes differently expressed between the two groups, with a fold change (FC) > 1.5, were selected. Differently expressed genes were analyzed in DAVID Bioinformatics Resources 6.7 where the enrichment of genes annotated in different gene ontology (GO) classifications, based on biological processes, was analyzed.

3.6.3 Reverse-transcriptase quantitative PCR

In all paper mRNA was transcribed to cDNA using the High capacity reverse transcription kit including random hexamer primers. The qPCR reactions were performed using cDNA equivalent to 2.5 ng RNA and the TaqMan Universal PCR master mixture with 1× assay-on-demand mixes of primers and TaqMan MGB probes. All samples were analyzed in methodological duplicates and the qPCR was performed using the 7900HT real time PCR System. The relative gene expression was evaluated by the $2^{-\Delta\Delta_{Ct}}$ method either manually or in GenEx Enterprise 5.2.3.13.

3.7 Protein expression analysis

The expression of functional proteins is what ultimately determines the phenotype and functions of a cell. This expression can for example be analyzed by antibody-based techniques such as ELISA and flow cytometry in which an epitope on the proteins of interest is recognized by a specific antibody. The antibody used can either be labeled for direct detection or linked to an enzyme allowing detection and quantification based on the conversion of a substrate leading to a colorimetric shift.

3.7.1 Flow cytometry

For flow cytometry analysis cells were resuspended in FACS buffer consisting of PBS with 5% FBS, 1% BSA and 2 mM EDTA and stained with antibodies or the appropriate isotype control. The flow cytometry analysis was carried out on the BD FACS ARIA flow cytometer, using Comp Beads plus to calculate the compensation and FlowJo software for analysis. Cells were acquired and gated by forward scatter (FSC) and side scatter (SSC) to exclude debris and cell aggregates. To calculate the percentage of cells positive for each of the selected markers, a maximum of 0.5% false positive gate was set using the isotype control. For all experiment in this thesis the MSC-phenotype was verified by their CD105, CD166, CD45 and CD34 expression. In paper IV the monocyte phenotype verification was performed using markers CD45, CD14, CD3 and CD19 and in paper III the MSC expression of surface markers CD10, CD49e, CD59, CD92, CD105, CD140b, CD146, CD147 and CD166 was investigated.

3.7.2 RIA and ELISA

In paper II the leptin secretion by the MSCs to the cell culture medium was analyzed by radioimmunoassay (RIA) according to the manufacturer's instruction. In paper III, the levels of crystalline-αB (CRYaB) were analyzed using a sandwiched ELISA according to the manufacturer's instructions. For this analysis the MSCs were lysed using RIPA lysis buffer with protease inhibitor added and CRYaB content in whole cell extract determined. Generally, for a sandwiched ELISA the plate is coated with a capture antibody over night, after which the cell lysate is added to the wells. After incubation the plate is washed multiple times and the detection antibody, usually conjugated to a SA-HRP, subsequently added. Finally, the substrate for the HRP is added and the absorbance measured at 450 nm. In paper IV the secretion of TNF-α, AMAC-1, TGF-β_1, BMP-2, PDGF-BB, sTNF-αR1, MCP-1 and SDF-1 by MO was determined by ready-made Quantikine ELISA assays from R&D systems according to manufacturers protocol.

3.7.3 Cytokine multiplex ELISA

In paper V the presence of 17 different cytokines and chemokines in culture medium from MSCs was determined after 72 hours and 1 week using the magnetic bead-based multiplex assay Bio-Plex Pro Human Cytokine 17-plex kit from Bio-Rad Laboratories. The analysis was performed according to the manufacturers protocol and the outcome analyzed on the Bio-Plex 100 system. The expression of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β and TNF-α was analyzed using this assay.

3.7.4 SILAC and quantitative mass spectrometry

In paper III the stable isotope labeling in cell culture (SILAC) method was used in order to identify proteins that were differentially expressed in MSCs undergoing osteogenic differentiation with the aim of finding new markers for this process. MSCs were cultured in the presence of either "heavy" arginine ($^{13}C_6$-arginine) and lysine ($^{13}C_6$-lysine) isotopes during differentiation or normal, "light" arginine and lysine ($^{12}C_6$-arginine, $^{12}C_6$-lysine) during expansion. The mass difference between identical peptides due to the incorporation of these amino acids into the newly synthesized proteins can be detected by mass spectrometry (MS)-analysis and the difference in peak intensities in the mass spectrum of one such peptide reflects the difference in protein abundance between the two culture conditions.

After two weeks of culture, in heavy or light condition, the MSCs were lysed and membrane proteins isolated by sub-cellular fractionation through serial centrifugation. The protein concentrations of the resulting supernatants were determined and the undifferentiated and differentiated samples were mixed at a 1:1 protein ratio and pelleted by ultracentrifugation. The sample was separated on a SDS-PAGE gel and the protein-containing lanes were excised and divided. The gel pieces were treated with 10 mM DTT to reduce disulfide bonds and the resulting cysteine residues were modified using 55 mM iodoacetamide. In-gel trypsinization was followed by peptide extraction and the extracts were then evaporated to remove remaining solvents. Before MS-analysis the peptides were dissolved in 0.1% formic acid.

Online peptide separation was performed on a 75 μm fused silica column and mass analyses were performed with a hybrid linear ion trap/Fourier transform ion cyclotron resonance (LTQ/FT-ICR) mass spectrometer. The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey MS spectra (from m/z 350 to 1500) were acquired in the FT-ICR and the three most intense ions in each full scan were fragmented and analyzed in LTQ.

The raw MS-data files were analyzed using MaxQuant software (version 1.0.14.11). MS/MS spectra were searched against the International Protein Index (IPI)-human database version 3.62 using the Mascot search engine and a false discovery rate (FDR) of 0.01 was used. Ratios of heavy versus light peptides were calculated using MaxQuant software and the data subsequently transferred into the ProteinCenter software where candidate markers were selected based on the criteria of an expression ratio above two in at least three peptides per protein.

3.8 Colorimetric assays

The following assays were performed by technicians at the accredited C-Laboratory, Sahlgrenska University Hospital, Gothenburg Sweden.

3.8.1 ALP activity

In paper I, II and V the ALP activity was determined as a measurement of osteogenic differentiation. For this analysis, samples in cell culture well-plates were rinsed with DMEM-LG and the cells were then lysed. The ALP activity in cell lysates was subsequently measured using p-nitrophenylphosphate as substrate. The quantity of p-nitrophenylphosphate, which determined by absorbance measurements at 405 nm, was considered directly proportional to the ALP activity.

3.8.2 LDH activity

Lactate dehydrogenase (LDH) is a cytosolic enzyme that when the cell-membrane integrity is compromised, either from apoptosis or necrosis, is released into the culture medium and can therefore be measured as an indicator of cellular toxicity. In papers I, II, IV and V in this thesis the LDH activity in the cell culture medium was analyzed using a Cytotoxicity Detection Kit. Culture medium was collected and incubated with the substrate mixture from the kit. The LDH activity was subsequently determined in a coupled enzymatic reaction, during which nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH. The rate at which NADH increases is proportional to the activity of LDH and can be measured spectrophotometrically at 340 nm.

3.8.3 ECM mineralization

In order to determine the degree of mineralization of the ECM cell culture samples in well-plates were rinsed with DMEM-LG and fixed in Histofix™. After rinsing the wells with distilled H_2O the samples were demineralized by incubation in 0.6M HCl for 24 hours after which the supernatants were collected for calcium and phosphate concentration measurements.

Calcium and phosphate measurements

The calcium concentration in the dissolved ECM was measured using the ortho-cresolphthalein complexone (OCPC) method. Under alkaline conditions, this reagent forms a complex with calcium that can be detected at 600 nm and the absorbance is directly proportional to the calcium concentration. The phosphate concentration was in a similar way determined by colorimetry of phospho-vanado-molybdic acid. This reagent forms, under acidic conditions, a complex that can be detected at 340 nm and the absorbance is directly proportional to the phosphate concentration.

3.9 Histochemical staining

Four different histochemical stainings were used for monolayer cell cultures and pellet mass cultures in this thesis. After fixation in Histofix™ osteogenically differentiated cultures were stained using Alizarin Red for the identification of calcium deposition in the ECM (2.0% Alizarin red in ddH_2O) or von Kossa for the identification of phosphate depositions (0.1 M $AgNO_3$ in ddH_2O in dark, followed by bright light exposure).

Chondrogenically differentiated cultures were stained using Alcian Blue (0.5% Alcian Blue in 0.5% acetic acid) or Safranin-O (0.01% Fast Green FCF in ddH_2O followed by 0.1% Safranin-O in ddH_2O) for the identification of proteoglycans in the ECM. Adipogenically differentiated cells were stained using Oil Red O solution (0.5% in isopropanol), which stains triglycerides in lipid vacuoles.

3.10 Histological techniques

3.10.1 Section preparation

In paper IV implants together with the surrounding tissue were removed *en bloc* by sawing, immersed in formaldehyde for fixation and then embedded in polyacrylate resin. After polymerization, the embedded implants were divided in to two blocks longitudinally by sawing[128]. From one block, sections were cut by sawing from which ground sections (10-15 um thick) were prepared by stepwise grinding with increasing grain-size number. Finally, sections were stained for histological evaluation using 1% toluidine blue.

3.10.2 Histomorphometry

Histomorphometric measurements were used to determine the degree of integration between the implant and the surrounding bone tissue in paper IV. Two separate measurements were used, the contact between the implant surface and the surrounding bone tissue (bone-to-implant contact; BIC) and the relative proportions of bone tissue within the threads (bone area; BA). The three best consecutive threads of each implant were evaluated.

3.11 Microscopy

Throughout the experiments included in this thesis the MSC morphology was continuously evaluated by light microscopy using an inverted light microscope. In paper IV the histological evaluation of *in vivo* samples was performed using a Nikon Eclipse E600 light microscope.

3.11.1 Electron microscopy

In paper IV human MOs cultured on titanium discs were analyzed by scanning electron microscopy (SEM). Samples were fixed in Karnowsky solution (pH 7.4). The specimens were then post-fixated in 1% osmium tetroxide and dehydrated in increasing concentrations of ethanol and dried in hexamethyldizilasane. Specimens were mounted on stubs, on carbon coated adhesive tape, and sputter-coated with about 10 nm of palladium.

In paper V MVs isolated from *S. aureus* and *S. epidermidis* were analyzed by transmission electron microscopy (TEM). MVs resuspended in PBS were loaded onto formvar carbon coated grids. Samples were then washed, fixed in 2% paraformaldehyde, washed in PBS and post-fixated in 2.5% glutaraldehyde. After washing the specimens were contrasted with 2% uranyl acetate and dried. The specimens were then examined using TEM (Tecnai G2 20) operating at 200 kV in bright field mode.

3.12 Statistical analyses

In paper I, II and IV statistically significant differences among groups were evaluated using Kruskal-Wallis test followed by a Mann-Whitney test to determine significant differences between individual groups. In paper III the gene expression results was analyzed by one-way ANOVA and other data evaluated by an independent t-test for normally distributed data. In paper V all data was analyzed using the Wilcoxon test for paired non-parametric data. A statistical significant difference was defined a $p < 0.05$. All statistical analyses, apart from those carried out in Babelomics 4.3 and David Bioinformatics 6.7, were performed in SPSSStatistics17.0

3.13 Ethical approval

3.13.1 Biopsies

Bone marrow was obtained from donors undergoing surgical spinal fusion at the Sahlgrenska University Hospital (Gothenburg, Sweden) under ethical approval 532-04 (with addendum T936-13) from the Regional Ethical Review Board, University of Gothenburg. Written consent was collected from bone marrow donors. The consent form was part of the ethical application and was approved by the committee

3.13.2 Animal study

The animal experiment was approved by the University of Gothenburg Local Ethical Committee for Laboratory Animals (Dnr 242-97).

4 SUMMARY OF THE RESULTS

4.1 Paper I

In this first study a virtual ligand based screening for chemical compounds similar to purmorphamine was performed. This search generated a list of 1,069 compounds, from which 11 substances were chosen for further testing. In an initial *in vitro* screening, MSCs were treated with one of the 11 compounds in two concentrations, 2.0 or 5.0 μM, and the LDH- and ALP activities were measured after two weeks of osteogenic culture. After five weeks the ECM mineralization was quantified by calcium and phosphate measurements.

The LDH activity was elevated in culture medium from MSCs treated with the higher concentration of two of the substances, whereas the LDH activity in medium from the MSCs treated with all other substances were within the normal range, indicating no toxic effects at the used concentrations. The initial screening revealed five substances with increased levels of ALP activity and/or ECM mineralization and these were chosen for further *in vitro* investigation.

In a subsequent concentration optimization step, these five analogs were tested in concentrations ranging from 0.5 to 10.0 μM and their ability to affect osteogenic differentiation of MSCs compared against purmorphamine. Cells treated with three substances displayed elevated levels of ALP activity and/or ECM mineralization and the osteogenic differentiation related to these three compounds were evaluated by a more extensive osteogenic panel.

Only one substance generated a significant increase in the MSC gene expression of RUNX2 compared to the control. This compound, as well as the original substance purmorphamine, also generated an increase in the gene expression of OCN. SHh-pathway activation was evaluated by target gene GLI1 expression and only purmorphamine led to strongly up-regulated expression of GLI1. Wnt-pathway activation was evaluated by target gene CCND1 expression and purmorphamine treatment as well as one additional substance resulted in an increase in the expression of this Wnt reporter gene. A correlation analysis revealed a significant positive correlation between the ALP activity and the gene expression of both OCN and CCND1. Furthermore, when the MSCs were stimulated with BMP-2 in combination with the different analogs, a strong positive correlation was seen between the ALP activity and the calcium and phosphate content of the ECM.

4.2 Paper II

In paper II the effects of PPAR-γ inhibition on osteogenic differentiation of MSCs *in vitro* were investigated. The cells were treated with PPAR-γ antagonist GW9662 during induced osteogenic differentiation and the progression of the differentiation was evaluated after two and five weeks. Inhibition of PPAR-γ resulted in elevated levels of ALP activity after two weeks, in donor two. In donor one the treatment resulted in increased ALP activity with increased inhibitor concentration, however not significantly higher than the control. When investigated by qPCR the gene expression of ALP was found to be increased in two of three donors. The matrix mineralization was quantified by measuring the calcium and phosphate content of the ECM produced by the MSCs after five weeks of osteogenic differentiation. The calcium content of the ECM was significantly increased by PPAR-γ inhibition in all three donors. The phosphate content of the ECM was significantly increased in two out of three donors.

After two weeks of osteogenic differentiation, the gene expression of the MSCs treated with the inhibitor was analyzed by microarray and compared to untreated control. After normalization, background reduction and removal of duplicates a list of 217 transcripts with a difference in expression between the control and inhibitor treated groups of a fold change (FC) > 1.5 was revealed.

Among the up-regulated genes several interesting genes connected to osteogenesis and adipogenesis were detected; Pyruvate dehydrogenase lipoamide kinase 4 (PDK4), leptin (LEP), chromosome 10 open reading frame 10 (C10orf10) that encodes decidual protein induced by progesterone (DEPP), ADAM metallopeptidase with thrombospondin type 1 (ADAMTS1) and regulator of calcineurin 2 (RCAN2). Also several genes involved in these two differentiation processes were found to be down-regulated, for example matrix metallopeptidase 13 (MMP13) that encodes collagenase 3, bone sialoprotein or integrin-binding sialoprotein (IBSP), ADAM metallopeptidase domain 19 (ADAM19) and acetyl-CoA acetyltransferase 2 (ACAT2).

To verify the difference in expression of the LEP gene, qPCR analysis was performed. The qPCR analysis validated the previous finding and demonstrated that inhibition of PPAR-γ during osteogenic differentiation generated a significant up-regulated expression of LEP. A decreased expression of RUNX2 in two out of three donors and a large reduction in OSX expression following treatment with the inhibitor in all donors was also demonstrated by qPCR analysis. Further investigation of the protein expression of leptin demonstrated a translation of increased LEP gene expression into increased secretion of functional protein.

4.3 Paper III

In this study the SILAC method was used to identify potential new markers for osteogenic differentiation of MSCs. Firstly, the MSC-phenotype and lineage multipotency was demonstrated. The cells were positive for CD105 and CD166, and lacked expression of CD34 and CD45. Trilineage capacity was demonstrated through osteogenic potential by positive alizarin staining of calcium deposits, adipogenic potential by positive oil-red O staining of triglycerides, and chondrogenic potential by positive alcian blue and safranin-o staining of proteoglycans, as well as the expression of lineage specific genes.

Membrane proteins from isotope labeled osteogenically differentiated and undifferentiated cells were analyzed by MS, which revealed 52 quantified proteins with an expression ratio above two in differentiated cells compared to undifferentiated cells. Among these proteins, 11 membrane proteins and two intracellular proteins were of interest, due to their large differences in expression between the two conditions. CD10 and CD92 showed the largest relative expression change between the differentiated and undifferentiated state, with a ratio of 9.4 and 15.9 respectively. Also, two non-membrane proteins with increased expression levels in differentiated MSCs compared with undifferentiated cells were identified. One of these was CRYaB, which was up-regulated almost 8 times during osteogenic differentiation.

For verification of the MS results, the protein expression in MSCs from three individual donors of 8 differentially expressed CD markers was analyzed by flow cytometry. The CD markers CD10 and CD92 were again significantly higher expressed in the osteogenically differentiated cells. The difference in expression of CD10 was continuously large over time, whereas a declining trend could be seen for CD92. The difference in expression of the intracellular protein, CRYaB, between osteogenically differentiated and undifferentiated MSCs, was verified using a sandwich ELISA. This demonstrated that the expression of CRYaB was significantly up-regulated in all three donors during osteogenic differentiation of the MSCs as compared to the undifferentiated cells.

To evaluate the lineage specificity of the selected markers, the expression during adipogenic and chondrogenic differentiation of CD10, CD92 and CRYaB was investigated. The results showed that the expression of CD10 and CD92 was up-regulated also during adipogenic differentiation of MSCs but not significantly elevated during chondrogenic differentiation. CRYaB was not up-regulated during chondrogenic and adipogenic differentiation of MSCs.

4.4 Paper IV

In paper IV the effects of signals communicated from activated human MO on the osteogenic response of MSCs *in vitro* were investigated. MOs were stimulated by either LPS, to induce classical activation or by IL-4 to induce alternative activation, and the MO-conditioned medium (CM) subsequently transferred to MSCs. The effects of different titanium culture substrates on the signals communicated from MOs to MSCs were also evaluated.

Classically activated MOs secreted high levels of TNF-α whereas alternatively activated MOs responded with increased production of AMAC-1. The IL-4 stimulated MOs also up-regulated PDGF-BB secretion. When cultured on the MA and OX titanium surfaces without any simulation the MOs secreted low to moderate levels of TNF-α and MCP-1. LPS stimulation by MOs on titanium surfaces increased the secretion of both these factors irrespective of surface type.

SEM revealed surface-dependent variations in MO-morphology. Cells on MA surfaces were rounded showing prominent cytoplasmic ruffling whereas MOs on the OX surfaces displayed a flattened morphology with some cytoplasmic protrusions. The addition of LPS resulted in further changes in morphology with increased ruffling and massive amounts of filopodia evident on cell cultured on the MA surface.

In response to CM from classically activated MOs, MSCs up-regulated their expression of RUNX2 and BMP2 whereas CM from alternatively activated MOs did not affect the osteogenic gene expression in MSCs. CM from unactivated MOs cultured on OX titanium on induced an up-regulation of BMP2 expression in MSCs after 24 hours in contrast to unactivated MOs cultured on MA titanium. In general, the effect of LPS-stimulation on the osteogenic gene expression was markedly stronger compared to that of the surface chemistry and topography of MA and OX titanium.

The effect of LPS on bone regeneration was further investigated in an *in vivo* pilot experiment. Titanium implants, with and without LPS incubation were inserted in pig cortical bone and the bone formation was evaluated two and six weeks after implantation. LPS incubation resulted in an initial decreased integration of the implant into the bone. At this time large areas of inflammatory infiltrates with active bone resorption were detected in the bone surrounding the LPS incubated implants of both surface types. After six weeks, the healing response and the amount of bone around both LPS incubated and control implants appeared fairly similar and there were no differences in measurements reflecting implant integration.

4.5 Paper V

In the final study of this thesis, MSCs were stimulated by LPS, LTA or MVs isolated from gram-positive strains *S. aureus* and *S. epidermidis* and the effects on their osteogenic differentiation and secretory profiles investigated. The MVs isolated in this study were characterized by NanoSight Nanoparticle Tracking analysis and TEM. This demonstrated that the *S. aureus* and *S. epidermidis* MVs are spherical membrane-enclosed vesicles similar to each other in size, with a mean size of 100-150 nm.

All stimuli used in this study promoted osteogenic differentiation of MSCs. This was demonstrated by significantly increased levels of ALP activity and ECM mineralization. The results from qPCR analysis of the gene expression of several osteogenic markers were along the same line. An increased RUNX2 expression was shown after LPS or LTA stimulation whereas the expression of BMP2 increased in response to all treatments.

In paper V, MSCs displayed a pronounced up-regulation of TLR2 expression in response to treatment with LPS, and a moderate up-regulation in response to MV-stimulation. The expression of TLR3 and TLR4 was not affected to the same extent by any of the stimuli. However, LPS treatment did increase the TLR3 expression and LTA decrease the TLR4 expression in these cells. qPCR analysis also revealed an up-regulation of several genes involved in TLR-signaling in response to LPS stimulation, indicating that this TLR-ligand results in a NF-κB activation through TLR/myeloid differentiation primary response gene 88 (MyD88) signaling. MVs from *S. aureus* resulted in a similar up-regulation, indicating TLR-activation also by PAMPs present in/on these MVs.

Five cytokines assayed in this study were not detected by multiplex ELISA in response to any stimuli, i.e. IL-1β, IL-5, IL-10, IL-13 and GM-CSF. In response to stimulation with LPS during osteogenic differentiation, MSCs significantly increased the production of a majority of the cytokines analyzed, both compared to unstimulated cells and the other stimuli. Compared to other cytokines relatively high levels of IL-6, IL-8 and MCP-1 were detected, irrespective of stimuli. Interestingly the levels of IL-8 and MCP-1 secreted by LPS stimulated MSCs decreased significantly from 72 hours to one week. Apart from LPS, also bacterial MVs had distinct effects on cytokine secretion. The levels of IL-17 and TNF-α were elevated in cultures treated with *S. epidermidis* MVs compared to unstimulated cultures whereas *S. aureus* MV-stimulation resulted in increased IL-4 secretion. Over time, a majority of the stimuli, including the osteogenic medium alone, resulted in an increase in IL-2, IL-4, IL-17 and TNF-α.

5 DISCUSSION

Early reports regarding MSCs communicated hopes of a bright future with many potential applications for these cells in the field of medicine. The cells were suggested to be possible to use in tissue engineering and gene therapy as well as a diagnostic and prognostic tool[129,130]. Although some of these applications have become a reality, or are currently on the verge of making the transition into clinical settings, many have not. It is interesting to note that it was not in the most popular context of tissue engineering that MSCs first made this transition, but it has been in the field of immune modulation that they have, to this date, had the most clinical success[69]. However, there are still applications of MSCs emerging and room for improvement of elements of existing approaches by better understanding and use of their potential. It is also of importance, that the biological context of these cells is not forgotten. The MSCs have a crucial role in many *in vivo* processes and there is still much knowledge to be gained in how the MSCs respond to the numerous signals present in their environment in the human body. This thesis investigated several factors that not only could be of importance in future translational use of MSCs, but possibly also affect the cells and their function *in vivo*.

5.1 Methodological considerations

With this thesis, as with any scientific contribution, the results of an experiment are never more reliable than the methods used to retrieve them. Therefore, critical assessment of scientific methods is at the core of experimental research.

All of the *in vitro* studies included in this thesis have been based on human samples. For the isolation of MSCs, bone marrow biopsies were retrieved from patients undergoing other forms of surgical treatment. This type of adult human stem cell is highly valuable in basic research experiments. However, due to the ethical aspects and the possible patient discomfort associated with the biopsy-procedure, of which both are necessary to consider, the number of biopsies available for research purposes is limited. The main advantage of working with primary human cells, in contrary to cell lines, is the high biological relevance of such cells. Furthermore, the variations associated with using material from different individuals does, to some extent, reflect the heterogeneity of the human population, whereas cell lines due to their homogeneity can be considered a poor representation of the population.

Biopsies from several individuals, commonly referred to as biological replicates, should be discriminated from technical replicates, which is the repetition of an experiment several times using the same biological sample. Occasionally, especially in cases with immortalized cell lines, the use of repeated experiments

performed at different time points can be seen in method descriptions in the scientific literature. These should also be considered technical replicates since no biological variation has been introduced. However, technical replicates are also of importance, as they will account for methodological variations and instabilities.

Two of the more advanced methods used in this thesis were quantitative proteome analysis by SILAC and mass spectrometry, and quantitative global genome expression analysis by microarray. Although they are powerful and validated methods, these multiple step procedures have some limitations and drawbacks of which the researcher should be aware. Common limitations for some microarrays are skewed comparisons between samples, due to limitations in signal detection range (i.e. fold change compression), and difficulty to detect genes with a low expression. Mass spectrometry analysis of a SILAC sample, or any complex protein sample, suffer from a similar limitation. Within the problem of "under-sampling", common for this type of analysis, lies the missed identification/quantification of low abundance proteins. One drawback that these two methods have in common is the database searches used to analyze and process the results. The problem here lies within the use of only previously described, annotated, genes and proteins to compare and describe the findings.

5.2 MSCs as a scientific tool

5.2.1 *In vitro* screening using MSCs

Traditional drug discovery uses high throughput screening in cell-based systems of chemical libraries to identify candidate substances that bind molecular targets of interest e.g. cell surface receptors. Many of these systems are based on immortalized cell lines such as the HeLa or HEK293 cell lines. Such cell lines have some advantages over primary cells, for example unlimited proliferative capacity and their homogeneity within the cell population. However, as discussed above there are disadvantages and such cells often have little phenotypic resemblance to the target cell type and behave differently from human primary cells. Another problem with such cell lines, both in research and drug discovery, is cell line cross contamination. It has been suggested that somewhere around 20% of immortalized cell lines used are misidentified[131]. One possible solution to such issues could be to use human adult stem cells, ESCs or induced pluripotent stem cells (iPSCs) in drug screening setups[132]. This could potentially reduce the risk of false positive results at early time points in the drug development process and thereby reduce the time and cost for generating candidate drugs. In reality it is perhaps only MSCs, isolated from a suitable source, that would be a feasible option for a drug screening system using adult

stem cells. This is due to their relatively easy isolation protocol and the fact that this cell type can be produced in the quantities needed. MSCs or an osteogenically differentiated counterpart have been suggested by Rissanen and co-author as an *in vitro* screening step in the search for new drugs for the treatment of osteoporosis[133]. In similarity to this suggested strategy, we performed a screening study in paper I where we combined a virtual ligand-based screening method with several *in vitro* screening steps, using MSCs. The three resulting candidate substances identified had similar or better capacity to enhance osteogenic differentiation of MSCs compared to the original small-molecule drug. This suggests that an MSC-based screening system could be a possible strategy in drug development.

5.2.2 MSCs as an *in vitro* model system

The most widespread use of MSCs, as well as many other types of stem cells, is as a tool to study biological mechanisms in a controlled *in vitro* model. These systems are used as a simplification of the complex situation *in vivo*. In paper II we were interested in how the cross-communication between MSCs undergoing adipogenic and osteogenic differentiation affects the differentiation process. Since this is an intricate system involving many different factors, an *in vitro* model was set up in which we inhibited PPAR-γ, the main adipogenic regulator, during the induction of osteogenic differentiation. In paper II it was demonstrated, that such an inhibition results in a significant increase in mineralization of the ECM, as well as increased activity or gene expression of ALP, the key enzyme involved in matrix mineralization.

Furthermore, microarray analysis revealed several genes differentially regulated in response to PPAR-γ inhibition during osteogenic differentiation. Amongst them a 2-fold up-regulation of LEP was considered to be of key interest and the gene and protein expression was further verified.

Leptin is a key factor in the communication between fat and bone *in vivo* and it is has been demonstrated to have both systemic and local modes of action[134]. The systemic, endocrine communication of leptin is controlled via the hypothalamus and uses the sympathetic nervous system for reaching its target tissue. Ducy *et al.* demonstrated that leptin secreted from fat tissue acts via the hypothalamus, affecting bone formation[135]. Further studies have since demonstrated that leptin receptors in the brain activates β-adrenergic receptors (ADRB2) on osteoblasts in the bone tissue, increasing the expression of osteoclast differentiation factor RANK-L and thereby resulting in a net bone loss[136,137]. However, in a recent report Turner *et al.* question this prevailing hypothesis of catabolic systemic effects of leptin and suggest that it is instead

peripherally in the tissue that leptin primarily exerts its effects, and that these are in contrast anabolic[138]. Their findings are corroborated by previous reports in which peripheral administration of leptin, in leptin deficient mice, were shown to have a positive effect on both BMD as well as result in increased bone marrow adipocyte apoptosis[139,140]. The effects of leptin on bone formation and osteogenic differentiation have since been further elucidated using *in vitro* models. Leptin stimulation of MSCs in culture results in an increased mineralization, OCN expression and ALP activity as well as a reduced adipogenic differentiation[141-143]. However, in similarity with the divergent data for leptin reported from *in vivo* models, it is still not established that leptin has a positive effect on osteogenesis *in vitro*. In the study by Scheller and co-workers, MSCs isolated from mice were not affected in terms of osteogenic differentiation by recombinant leptin[144].

The connection between PPAR-γ and leptin is not fully understood. Hollenberg and co-authors demonstrated that the LEP promotor contains a PPAR-γ response element and that LEP expression is negatively regulated by this transcription factor in adipocytes[145]. Furthermore, the leptin stimulatory effects on tumor growth in a mouse breast cancer model was reduced by PPAR-γ activation and restored by inhibition of this transcription factor[146]. It is therefore likely that also in MSCs an inhibition of PPAR-γ would increase the opportunity for positive regulatory elements to bind the promotor and induce LEP transcription. However, this was demonstrated for the first time in paper II, which also adds substantial evidence that leptin has an important role of the cross-regulatory network between adipogenic and osteogenic differentiation of MSCs. Furthermore, in a recent study the leptin receptor was found to be significantly up-regulated and osteogenic tissue formation promoted, in a calvarial defect two weeks after it was treated with GW9662 stimulated MSCs[147].

In paper II an *in vitro* model was used to study the relationship between osteogenic and adipogenic differentiation of MSCs and revealed a possible connection between down-regulated adipogenesis, up-regulated osteogenesis and leptin.

5.3 Osteogenic differentiation of MSCs

5.3.1 Pro-osteogenic strategies

As the knowledge of the different signaling pathways involved in osteogenic differentiation increases, the number of possible targets for modulating this process expands, and ranges from transcription factors to cell-surface receptors.

In general, there are two pivotal points in most cell differentiation processes, the initial commitment and the execution of the final differentiation. In the context of osteogenic differentiation RUNX2 is considered to be the master switch that needs to be turned on for osteogenic differentiation to commence[103]. However, it has also been demonstrated that this transcription factor is highly involved in the embryonic chondrogenic development indicating that this switch is applicable for early osteochondral commitment. In contrast, the role of OSX is central in the final differentiation step in osteogenesis and it has been suggested to be independent of RUNX2 expression[99]. It is therefore possible that OSX is a more suitable and specific target than RUNX2, when the intention is to modulate osteogenic differentiation of MSCs.

As previously discussed, PPAR-γ can modulate the differentiation process of MSCs in multiple lineages. In paper II we demonstrated that inhibiting PPAR-γ during induced osteogenesis of MSCs actually progressed the osteogenic differentiation. However, the two transcription factors RUNX2 and OSX were simultaneously down-regulated. The mechanism via which this effect is mediated remains unclear. However, there are other effector proteins involved in osteogenesis, such as MSX2, which cannot only induce osteogenic differentiation in a RUNX2-independent manner but also interfere with PPAR-γ/DNA-binding and thus suppress adipogenic differentiation[148].

In paper I the target was instead the SHh signaling pathway. Purmorphamine activates this pathway, which has been clearly demonstrated by us, and others, through the up-regulation of PTCH/SMO downstream targets and results in an increased osteogenic differentiation of MSCs. However, in other studies it has been reported that such a SHh pathway activation leads to a suppression of osteogenic differentiation of MSCs and mineralization by osteoblast[149]. It has been speculated on the temporal aspect of SHh activation during osteoblast commitment and MSC differentiation. A recent report showed that during the induction of osteogenic differentiation of MSCs, SHh related genes were only up-regulated during the first week of differentiation[150] and it is possible that the divergent data regarding purmorphamine and its osteogenic effect is a matter of timing. Furthermore, in line with our findings in paper I, BMP-2 and purmorphamine have been shown to act reciprocally during induction of both adipogenesis and osteogenesis[151,152]. These data further highlight the complexity of administering the proper cues at a time point i.e. when the cells will be susceptible for the specific signal, when trying to maximize osteogenic differentiation of MSCs *in vitro*.

5.3.2 Chemical *vs.* biological stimuli

All of the signaling pathways discussed above are possible targets for the modulation of MSC differentiation *in vitro* as well as *in vivo*, and there are numerous others. One of the most common ways of augmenting osteogenic differentiation is the use of recombinant BMPs. These proteins are amongst the few recombinant proteins, for this application, that are in clinical use. For example, recombinant BMP-2 and BMP-7 are used in spinal fusions, fracture treatment and jaw augmentations[153]. One of the benefits with a human recombinant protein is the specificity. Generally, a growth factor will only bind to and activate its specific receptor, which is of course a great advantage when attempting to modulate biological processes. However, there have been several reports of adverse events such as heterotopic ossification, hematoma and dysphonia in connection with the administration of a high doses of these potent proteins[154-157].

In paper I and II, we demonstrate that it is possible to use an alternative approach when aiming at enhancing osteogenic differentiation of MSCs. The application of a small molecule substance for addressing bone regeneration is promising but under development. Several substances including simvastatins, bisphosphonates and purmorphamine, targeting SMAD, RANK-L and Hedgehog signaling, respectively, have shown promising pre-clinical results[158]. Furthermore, the large costs it entails to safely produce and purify recombinant growth factors are incomparable to the low production cost of a small molecule drug alternative. The major concern regarding small molecule therapeutics is their nonspecific side effects, which needs to be addressed before such strategy becomes a clinical reality[159].

5.3.3 Markers of differentiation

In the field of tissue engineering, the possibility to monitor cells in culture or cell-based constructs in a bioreactor by minimally invasive techniques is essential. A construct engineered for osteogenic applications needs to have and meet quality control requirements guaranteeing that the cells are of the osteogenic lineage and producing an osteogenic tissue[160]. Also in other fields of research the identification of cells undergoing this differentiation process could be of importance. In study III, we identified two new surface markers for MSCs undergoing osteogenic and adipogenic differentiation, CD10 and CD92.

CD10 is a metallo-endopeptidase which is capable of cleaving several proteins participating in osteogenesis, such as calcitonin, osteostatin and osteogenic growth peptide and thereby controlling their biological activity[161]. Furthermore the activity of CD10 has been demonstrated to have an impact on body mass

and fat accumulation and it has been identified as a regulator in the development of obesity[162]. As previously discussed, there is a close relationship between adipogenic and osteogenic lineage differentiation and the similarities in CD10 expression does therefore not come as a surprise. However, based on the findings in paper III CD10 is suggested, for the first time, as a potential marker for osteogenically and adipogenically differentiating MSCs. CD92 is a transporter protein and its main function is to transport choline, a component of phosphatidylcholine, across the cell membrane[163]. Phosphatidylcholine is found at sites of both intramembranous and endochondral bone formation and has been shown to affect ALP activity[164]. It is a possible hypothesis that the strong up-regulation of CD92 in osteogenically differentiated cells is related to the increased synthesis of PC during osteogenic differentiation.

In addition to the membrane associated proteins CD10 and CD92, another potential marker was identified in paper III. The intracellular protein CRYaB, a small heat shock protein, has been shown to be significantly regulated in association with bone metabolism, and also found in a gene microarray study of MSCs during differentiation into osteoblasts[165,166]. Finding reliable markers for the differentiating MSC, which are unique for each of the three osteogenic, chondrogenic and adipogenic lineages, is challenging. This is due to the fact that these differentiation processes activate many of the same signaling pathways, for example both the TGF-β and PDGF pathways[167]. The novel osteogenic marker CRYaB, found in paper III, could therefore be a significant contribution in the task of identifying lineage specific cells early during the differentiation process.

5.4 Inflammation and regeneration

As described earlier the effect of pro-inflammatory molecules on bone and bone forming cells has traditionally been described as catabolic. However, the response of MSCs or osteoblastic progenitor cells to inflammatory signals such as cytokines, with respect to survival and regeneration, is only recently starting to be elucidated. In paper IV we found that MSCs up-regulated their gene expression of BMP2 and RUNX2 in response to signal secreted from LPS-activated MO and in paper V it was demonstrated that MSCs exposed to inflammatory agents derived from microorganisms, responded with increased osteogenic differentiation.

During the inflammatory process signals are secreted that affect both immune cells and regenerative cells. MSCs have been shown to up-regulate their ALP activity as well as gene expression of OCN and RUNX2 in response to unknown signals secreted by activated T cells[168]. Further investigation by Rifas and co-authors revealed that MSCs up-regulate their secretion of BMP2 and

ALP activity in response to a cytokine and GF cocktail. The cocktail used was representative of the secretory profile of activated T cells and consisted of TNF-α, TGF-β, IFN-γ, and IL-17[169]. Taken together with the results from paper IV, it is plausible that cytokines considered as inflammatory, secreted by monocytes and T cells, also have an important role during the maturation of MSCs and tissue repair. In addition, three out of four proteins in the cocktail used by Rifas *et al.* were, in paper V, shown to be secreted by MSCs in response to inflammatory stimuli, demonstrating that there is autocrine signaling of these cytokines by MSCs potentially resulting in increased tissue formation.

In paper IV, the medium conditioned by LPS activated MO was shown to contain cytokines and GFs such as TNF-α, TGF-β and MCP-1. Exposure of MSCs to TNF-α increases their proliferation and ECM mineralization as well as BMP2 and ALP expression[170,171]. However, it has been demonstrated that TNF-α inhibits RUNX2 expression[172] suggesting that the mechanism regulating the osteogenic process in response to TNF-α is RUNX2-independent and that the increased RUNX2 gene expression demonstrated in paper IV is due to some other soluble factor secreted by the MO. Using a similar experimental set-up as in paper IV, an alternative/additional explanation to the communication of soluble osteogenic signals from monocytes to MSCs was recently suggested: a release of exosomes by monocytes upon stimulation by LPS resulted in the uptake of exosomes (containing RNA, including RNA in the size of microRNA) and the promotion of osteogenic differentiation in recipient MSCs[173].

MCP-1, a factor commonly associated with inflammatory cell-recruitment, has also been shown to induce migration of MSCs[174]. The expression of MCP-1 *in vivo* was shown to be differentially regulated in the bone surrounding implants with either a MA or OX titanium surface[175]. The *in vitro* results in paper IV are in agreement with these findings showing a role of the implant surface properties for the secretion of TNF-α and MCP-1 from MOs. Both cytokines were produced to a greater extent by MOs cultured on titanium compared to PS and levels of MCP-1 were higher in medium from MOs cultured on MA titanium as compared to OX. It is likely that these effects of surface chemistry and topography on cytokine secretion could be factors affecting the osseointegration of implants possibly through the recruitment of different cell types affecting the tissue healing. However, as evident from the results of both *in vitro* and *in vivo* experiments in paper IV, the effect on cell differentiation and tissue response by inflammatory agents by far out-weighs the effects of different surfaces.

In paper IV, the positive control medium used, which contained 10 ng/ml LPS, did not elicit any effect on osteogenic gene expression of MSCs, in contrast to

the response of these cells to the soluble signals secreted by the LPS-activated MO. However, in paper V a strong pro-osteogenic response and altered secretory profiles could be seen when MSCs were stimulated with LPS. In previous studies the varying effects of LPS on osteogenic differentiation of MSCs have been reported[123,176,177]. These discrepancies may, at least partly, be explained by the LPS dose. In paper IV 10 ng/ml was used whereas 1μg/ml was used to stimulate MSCs in paper V. Based on previous literature a possible threshold can be seen at around 0.1-1.0 μg /ml for provoking a response by MSCs *in vitro*[123,176,178]. Although there is a large difference in the dose that triggers a response *in vitro* between the two cell types it is difficult to know how this relates to the *in vivo* situation and what local concentrations can be detected during a pathological process *in vivo*.

5.4.1 The effects of MVs on MSCs *in vitro*

In case of a traumatic bone event, such as an open fracture, or insertion of orthopedic and dental implants and prostheses, there is a risk of bacterial contamination. Common invading pathogens are gram-positive bacterial strains *S. aureus* and *S. epidermidis*, triggering a host-defense response through for example TLR-activation on host cells, with a subsequent inflammation in the affected tissue. Gram-negative bacterial cell wall component LPS is a known TLR-ligand and TLR signaling has been suggested to regulate the observed pro-osteogenic effect[179]. However, whether it is a direct effect mediated via effector proteins of the TLR-signaling pathway or indirect, for example paracrine secretion due to TLR-activation, remains unclear and to be established.

In addition to having proteins present on the surface, of which many can be recognized by TLRs on host-immune cells, gram-positive bacteria have been shown to secrete proteins and MVs into their extracellular surroundings[180,181]. The secretome and MVs contains lipids, membrane-associated proteins, genetic materials, and other factors associated with virulence[180-182]. It is not unlikely that the function of these extracellular "communications" is for the transfer information, cell-cell signaling, elimination of competing organisms and delivery of virulence factors to host cells.

In paper V it was demonstrated that MVs isolated from *S. aureus* and *S. epidermidis* induce a similar pro-osteogenic response in MSCs as LPS. In a recent study, heat in-activated whole cell lysate of *S. aureus* induced proliferation of MSCs, whereas in the same study, in concurrence with the results presented in paper V, LPS promoted both proliferation and osteogenic differentiation[183]. These results suggest that the factors responsible for the augmented osteogenic differentiation demonstrated in paper V are specific for the MVs, and that they

are not expressed or active on the bacterial cell. However, the identity of these factors or proteins, and the mechanism through which they affect osteogenesis, remains unknown. Possibly the MVs, in similarity with LPS, activate TLR-signaling in MSCs through one or several TLRs. However, the connection between TLR-signaling and tissue repair, both in the context of normal homeostasis and as a result of host response against infections, needs to be further investigated.

MVs isolated from *S. aureus*, but not *S. epidermidis*, induced an increase in IL-4 secretion in MSCs compared to unstimulated cells. IL-4 is one of the key factors that induce alternative activation and a reparative phenotype of monocytes and macrophages. It has also been demonstrated that addition of recombinant IL-4 to macrophage cultures infected with gram-positive *Streptococci intermedius*, resulted in reduced amounts of pro-inflammatory IL-1 secreted by macrophages and that IL-4 increases expression of anti-inflammatory mediator IL-1RA[184,185]. Taken together with the results from paper V, these observations suggest that MSCs in response to *S. aureus* MVs may secrete signals, which modulate the inflammatory host response.

S. epidermidis is considered to be less pathogenic compared to *S. aureus*. It is therefore interesting that *S. epidermidis* MVs, but not MVs isolated from *S. aureus*, induced increased secretion of both TNF-α and IL-17 in MSCs compared to unstimulated cells. TNF-α and IL-17 are pro-inflammatory cytokines with their primary function being to amplify inflammation. Further, IL-17 has been shown to synergize with TNF-α, thereby increasing the inflammatory response[186]. Albeit, this cytokine has also been demonstrated to induce proliferation of MSCs and decrease their ALP activity[187,188]. This may indicate that the pro-osteogenic effects seen with *S. epidermidis* MVs in paper V are a result of increased MSC-proliferation under osteogenic conditions.

The up-regulated production of IL-6 in response to all the inflammatory stimuli used in paper V may also be a contributing factor for the increase in osteogenic differentiation of the MSCs. IL-6 in combination with its soluble receptor, IL-6R have previously been demonstrated to increased mineralization and proliferation of MSC-like cells[189,190]. Furthermore, out results are supported by a previous study in which peptidoglycans isolated from the membrane of *S. aureus* induced increased IL-6 expression in MSCs[124].

It is interesting to note that the induction of osteogenic differentiation in MSCs (without the addition of stimuli) induced secretion of relatively high levels of IL-6, IL-8 and MCP-1 by the MSCs. The present findings are supported by previous observations of high, although declining, levels of IL-8 and MCP-1

after 10 to 20 days of induced osteogenic differentiation[191]. Although the levels of IL-2, IL-4, IL-17, IL-8 and TNF-α were much lower, by only the addition of osteogenic factors, there was an increased secretion of these cytokines over time. Taken together, the data indicates that the induction of osteogenic differentiation in MSCs results in a transient secretion of several pro-inflammatory cytokines with possible autocrine and/or paracrine effects.

6 CONCLUSIONS

In papers I and II we show that the osteogenic differentiation of MSCs can be modulated by the use of a small molecule substance. Furthermore, in paper I the potential of virtual ligand-based screening in combination with an *in vitro* functionality assay as a drug development approach was demonstrated. In this proof-of-concept study we identified several purmorphamine analogs with similar or increased osteo-conductive properties.

In paper II we demonstrated that PPAR-γ inhibition during induction of osteogenesis increases the osteogenic differentiation of MSCs *in vitro*. The inhibition increased the ALP expression or activity and the ECM mineralization. Microarray analysis revealed the regulation of leptin, amongst several interesting genes connected to both osteogenesis and adipogenesis, as a result of this treatment. The link between PPAR-γ inhibition, leptin and osteogenic differentiation demonstrated in this study reveals new knowledge regarding the web of interactions and the intricate relationships between the osteogenic and adipogenic signaling in MSCs.

The results in paper III revealed significant differences in the proteome between differentiated MSCs and undifferentiated MSCs. The surface markers, CD10 and CD92, were significantly higher expressed in both osteogenically and adipogenically differentiated MSCs, and could therefore be used as markers for cells undergoing adipogenesis or osteogenesis. In addition, the results in paper III also revealed that intracellular protein CRYaB is specifically up-regulated in osteogenically differentiated MSCs. This protein could therefore be a suitable candidate for monitoring and evaluating the progression of the osteogenic differentiation process in MSCs.

In paper IV we showed that activated human mononuclear cells communicate pro-osteogenic signals to MSCs in absence of direct cell-cell contact. These unknown signals resulted in the up-regulation of BMP2 and RUNX2 expression in MSCs. The *in vitro* model used in this study also revealed that the effects of culture substrate surface properties, e.g. representing different implant materials, on the release of pro-osteogenic signals by monocytes are relatively low and transient in comparison with the profound and prolonged effects of classical activation, via LPS, of the monocytes.

Lastly, the results in paper V show that pro-inflammatory and immune-triggering molecules can promote the osteogenic differentiation of MSCs. In this study it is for the first time demonstrated that MVs released from the two gram-positive bacterial strains *S. aureus* and *S. epidermidis* can promote osteogenic

differentiation of MSCs and modulate the secretion of signals related to inflammation and immune modulation *in vitro*. Taken together, these results indicate that the secretory response of the MSCs may have a contributory effect on the recruitment of inflammatory and immune cells, and thereby enforcing the host defense toward the inflicting microbes.

In conclusion, this thesis presents new knowledge regarding the phenotype that is characteristic for MSCs undergoing osteogenic differentiation. Furthermore, new knowledge has also been gained regarding several factors of importance in this process. The results can potentially be translated into useful tools for basic as well as applied research in the field of osteogenic regeneration.

7 FUTURE PERSPECTIVES

The MSCs have gone from being considered a great promise for future medicine in the eighties and nineties, to somewhat of a frowned upon cell type in the beginning of the new century. They were overshadowed by the novelty and potential of ESCs and also later the iPSCs, and in addition there has been much controversy regarding definition and nomenclature. However, in the last five or ten years, through the discovery of new traits and applications, they have made an interesting comeback. We are now starting to see new potentials and learn more about this cell type in a broader context than merely in the initially dominating perspective of tissue engineering. This thesis has covered many of these aspects although with one pervading focus: the differentiation of MSCs towards the osteogenic lineage. The finding of three novel markers of MSC-differentiation, through the work of this thesis, represents a highly applicable tool in further research regarding MSCs. However, their biological functions during the differentiation process remains to be elucidated.

As the world's population grows larger, and more importantly as the demographic profile of it becomes dominated by elderly, we will face huge challenges to meet the demand of health care and to maintain good quality of life. One small but very significant piece in this puzzle has been discovered and developed here in Gothenburg. It is the osseointegrated implant, which restores the functions primarily of teeth, but also to some extent of limbs, in a remarkable way. However, will it work just as well with an implant in the bone of a 100-year old person? We do not know. Local stimulation of new bone tissue formation by MSCs *in vivo*, for example by an easy to produce and cost efficient small molecule drug, might be necessary to successfully osseointegrate dental and orthopedic implants in this patient-group. In this thesis we have presented new insights regarding the modulation of MSC-differentiation that will be valuable in the development of such an approach.

With implants, fracture healing or other types of tissue repair, our bodies are able to heal in an extraordinary way. However, infection and dys-regulated inflammation are examples of situations in which the body's response can instead of promoting repair potentially result in tissue degradation. MSCs have proven to be a helpful tool in relieving the detrimental effects of chronic inflammation and suppressing unwanted immunological or autoimmune reactions. In this thesis we have studied how inflammatory cells and signals affect MSCs with regards to regeneration. From this work it is clear that if we can learn to harness these signals through further research on their mechanistical function, we could increase the potential of the MSCs yet again and take new steps towards successful bone regeneration also in compromised bone situations.

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