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MicroRNAs AS NOVEL REGULATORS OF SKELETAL HOMEOSTASIS

by

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To my family

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MicroRNAs as novel regulators of skeletal homeostasis
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Annales Universitatis Turkuensis, Medica-Odontologica, 2013

ABSTRACT

The human skeleton is composed of bone and cartilage. The differentiation of bone and cartilage cells from their bone marrow progenitors is regulated by an intrinsic network of intracellular and extracellular signaling molecules. In addition, cells coordinate their differentiation and function through reciprocal cell-to-cell interactions. MicroRNAs (miRNAs) are small, single-stranded RNA molecules that inhibit protein translation by binding to messenger RNAs (mRNAs). Recent evidence demonstrates the involvement of miRNAs in multiple biological processes. However, their role in skeletal development and bone remodeling is still poorly understood.

The aim of this thesis was to elucidate miRNA-mediated gene regulation in bone and cartilage cells, namely in osteoblasts, osteoclasts, chondrocytes and bone marrow adipocytes. Comparison of miRNA expression during osteogenic and chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (MSCs) revealed several miRNAs with substantial difference between bone and cartilage cells. These miRNAs were predicted to target genes essentially involved in MSC differentiation. Three miRNAs, miR-96, miR-124 and miR-199a, showed marked upregulation upon osteogenic, chondrogenic or adipogenic differentiation. Based on functional studies, these miRNAs regulate gene expression in MSCs and may thereby play a role in the commitment and/or differentiation of MSCs. Characterization of miRNA expression during osteoclastogenesis of mouse bone marrow cells revealed a unique expression pattern for several miRNAs. Potential targets of the differentially expressed miRNAs included many molecules essentially involved in osteoclast differentiation.

These results provide novel insights into the expression and function of miRNAs during the differentiation of bone and cartilage cells. This information may be useful for the development of novel stem cell-based treatments for skeletal defects and diseases.

Keywords: microRNA, bone, cartilage, mesenchymal stem cell, osteoblast, osteoclast, chondrocyte, adipocyte

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TIIVISTELMÄ

Ihmisen tukiranka koostuu luusta ja rustosta. Se muodostuu sikiöaikana tapahtumasarjassa, jossa mesenkyymisolut erilaistuvat rusto- tai luusoluiksi ja alkavat tuottaa ympäröivään kudokseen soluväliainetta. Luu- ja rustosolut erilaistuvat luuytimen mesenkymaalisista ja hematopoieettisista kantasoluista tarkasti säädeltyjen solunulkoisten ja -sisäisten sekä solujenvälisten viestinvälitysreittien ohjaamina. mikroRNA(miRNA)-molekyylit ovat pieniä yksijuosteisia RNA-molekyylejä, jotka estävät proteiinien tuottoa sitoutumalla lähetti-RNA:n sekvenssiin. Viimeaikaisten tutkimusten perusteella miRNA-molekyylit säätelevät monia elimistön prosesseja, mutta niiden merkitys luuston kehityksessä ja uudismuodostuksessa tunnetaan puutteellisesti.

Tämän väitöskirjatyön tavoitteena oli selvittää miRNA-molekyylien roolia luuytimen mesenkymaalisten kantasolujen erilaistumisessa luu-, rusto- tai rasvasoluiksi. Pyrimme myös selvittämään miRNA-molekyylien merkitystä luun hajotuksessa tutkimalla niiden ilmentymistä osteoklasteissa. Kun kymmenien miRNA-molekyylien ilmentymistä verrattiin luu- ja rustosoluissa, havaittiin että joidenkin miRNA-molekyylien ilmentymisessä on moninkertainen ero luu- ja rustosolujen välillä. Näiden miRNA-molekyylien kohdegeenien joukosta löytyi useita luun ja ruston muodostumiselle tärkeitä geenejä. Ihmisen kantasoluilla tehdyssä tutkimuksessa erottui kolme miRNA-molekyyliä, joiden ilmentyminen lisääntyi luu-, rasva- tai rustosolujen erilaistumisen aikana. Näiden miRNA-molekyylien osoitettiin säätelevän mesenkymaalisten kantasolujen erilaistumiseen vaikuttavien geenien ilmentymistä. Myös osteoklasteilla havaittiin omaleimainen miRNA-profiili, mikä luo pohjaa uusien miRNA-säätelyreittien tunnistamiseen osteoklasteissa.

Tutkimuksen tulokset antavat uutta tietoa miRNA-välitteisestä geenisäätelystä luu- ja rustosolujen erilaistumisen aikana. Näiden mekanismien parempi tuntemus on edellytyksenä uusien kantasoluihin perustuvien hoitomuotojen kehittämiselle luuston sairauksiin ja vaurioihin.

Avainsanat: mikroRNA, luu, rusto, mesenkymaalinen kantasolu, osteoblasti, osteoklasti, rustosolu, rasvasolu

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ABBREVIATIONS

ACAN	aggrecan
Adamts-5	a disintegrin and metalloproteinase with thrombospondin motifs 5
Ago	Argonaute
ALK2	activin A receptor, type I
ALP	alkaline phosphatase
AP1	activator protein 1
APC	adenomatous polyposis coli
ATF4	activating transcription factor 4
BAMBI	BMP and activin membrane-bound inhibitor homolog
BAT	brown adipose tissue
BMC	bone marrow cell
BMD	bone mineral density
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BRC	bone remodeling compartment
C/EBP	CCAAT/enhancer binding protein
CAII	carbonic anhydrase II
Cat K	cathepsin K
CHOP	C/EBP-homologous protein
ClC-7	chloride channel protein 7
COMP	cartilage oligomeric matrix protein
COX-2	cytochrome c oxidase subunit II
CRIM1	cysteine-rich motor neuron 1
DAP12	DNAX-activating protein 12
dC(T)	delta cycle threshold
DC-STAMP	dendrocyte expressed seven transmembrane protein
DDIT3	DNA-damage-inducible transcript 3
DGCR8	DiGeorge syndrome critical region gene 8
Dkk	Dickkopf
DLX	distal-less homeobox
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
ECM	extracellular matrix
EID-1	EP300 interacting inhibitor of differentiation 1
ERK1/2	extracellular signal-regulated protein kinase 1/2
FABP4	fatty acid binding protein 4
FACIT	fibril-associated collagens with interrupted triple helices
FAK	focal adhesion kinase
FBS	fetal bovine serum
FcRγ	Fc receptor common γ chain
FGF	fibroblast growth factor

FGFR	fibroblast growth factor receptor
GAG	glycosaminoglycan
GDF	growth and differentiation factor
Gla	γ-carboxyglutamic acid
GLUT4	glucose transporter type 4
GPCR	G protein-coupled receptor
GSK	glycogen synthase kinase
hADMSC/ hADSC/hASC	human adipose (tissue)-derived mesenchymal stem cell
HBM	high bone mass
HCV	hepatitis C virus
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGMA2	high mobility group AT-hook 2
hMADSC	human multipotent adipose tissue-derived stem cell
HOXA2	homeobox A2
hPBMC	human peripheral blood mononuclear cells
HSC	hematopoietic stem cell
Ihh	indian hedgehog
IL	interleukin
iNOS	inducible nitric oxide synthase
IPA	Ingenuity Pathway Analysis
IRAK1	interleukin-1 receptor-associated kinase 1
ISCT	International Society of Cellular Therapy
ITAM	immunoreceptor tyrosine-based activation motif
Ldbr/DBR1	debranching enzyme (*S. Cerevisiae*) homolog 1
LDL	low density lipoprotein
LEF	lymphoid-enhancer-binding factor
LNA	locked nucleic acid
LRP	low-density lipoprotein receptor-related protein
M-CSF	macrophage colony-stimulating factor
miR/miRNA	micro ribonucleic acid
MITF	microphthalmia-associated transcription factor
MMP13	marix metallopeptidase 13
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem (stromal) cell
MSX	msh homeobox
NFATc1	nuclear factor of activated T cell-1
NFI-A	nuclear factor I/A
NF-κB	nuclear factor kappa B
OA	osteoarthritis
OC-STAMP	osteoclast stimulatory transmembrane protein
OP	osteoporosis
OPG	osteoprogeterin
OSCAR	osteoclast-associated receptor

OSX	osterix
PDCD4	programmed cell death 4
PFA	paraformaldehyde
PKA	protein kinase A
PPARγ	peroxisome proliferator-activated receptor gamma
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related protein
PTK2	protein tyrosine kinase 2
PTN	pleiotrophin
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor κ B
RANKL	ligand for receptor activator of nuclear factor κ B
RBL2/p130	retinoblastoma-like 2 (p130)
RGD	Arg-Gly-Asp
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RT-PCR	real-time polymerase chain reaction
RUNX2	runt family transcription factor 2
SATB2	special AT-rich sequence-binding protein 2
sFRP	secreted frizzled-related protein
Shh	sonic hedgehog
siRNA	small interfering ribonucleic acid
SMAD	mothers against decapentaplegic homolog
SOCS1	suppressor of cytokine signaling 1
SOST	sclerostin
SOX	sex-determining region Y-type high motility group box
TCF	T-cell-specific transcription factor
TF	transcription factor
TFBS	transcription factor binding site
Tgfbr	transforming growth factor beta receptor
TGF-β	transforming growth factor β
TNF	tumor necrosis factor
TRACP	tartrate-resistant acid phosphatase
TRAF	tumor necrosis factor receptor-associated factor
TREM2	triggering receptor expressed in myeloid cells 2
TRPS1	Tricho-Rhino-Phalangeal Syndrome I
UTR	untranslated region
V-ATPase	vacuolar-type proton adenosine triphosphate
VEGFA	vascular endothelial growth factor A
WAT	white adipose tissue
XPO5	exportin-5
YAT	yellow adipose tissue

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-III. In addition, unpublished results are included. The original publications have been reproduced with the permission of the copyright holders.

I **Suomi S**[*], Taipaleenmäki H[*], Seppänen A, Ripatti T, Väänänen K, Hentunen T, Säämänen A-M and Laitala-Leinonen T (2008). MicroRNAs regulate osteogenesis and chondrogenesis of mouse bone marrow stromal cells. *Gene Regul Syst Bio*. 2:177-91. [*]Equal contribution

II **Laine SK**, Alm JJ, Virtanen SP, Aro HT and Laitala-Leinonen TK (2012). MicroRNAs miR-96, miR-124 and miR-199a regulate gene expression in human bone marrow-derived mesenchymal stem cells. *J Cell Biochem.* 113(8):2687-95.

III **Laine SK**, Taipaleenmäki H, Hentunen T, Säämänen A-M and Laitala-Leinonen T. Characterization of microRNA expression during osteoclastogenesis. Submitted manuscript.

1 INTRODUCTION

Skeletal development and the dynamic balance between bone resorption and formation are coordinated by the activities of three specific cell types: osteoblasts and osteoclasts in bone, and chondrocytes in cartilage. Osteoclasts derive from the monocyte-macrophage lineage of the hematopoietic stem cells (HSCs), whereas osteoblasts and chondrocytes originate from the non-hematopoietic compartment of bone marrow termed mesenchymal stem/stromal cells (MSCs). However, the commitment and differentiation of osteoblasts, osteoclasts and chondrocytes from their progenitors appear to be closely related through wide-ranging molecular signaling pathways and cellular interactions. A detailed understanding of the mechanisms behind lineage commitment and differentiation of bone and cartilage cells is of great importance in the development of novel therapeutics for skeletal diseases.

MicroRNAs (miRNAs) comprise a group of small (19-25 nucleotides) non-coding RNAs that regulate gene expression by binding to their target messenger RNAs (mRNAs) resulting in translational repression or mRNA degradation. More than 17 000 mature miRNA sequences have been identified in over 140 species including various plants, green algae, viruses and an increasing number of animals (Kozomara and Griffiths-Jones, 2011). With more than half of human protein-coding genes predicted to encounter miRNA regulation (Friedman et al., 2009), it may be difficult to find a biological process that is not influenced by miRNAs. Besides their importance in normal physiology, miRNAs have been implicated in the pathogenesis of several human diseases, including cancer, neurodegenerative disorders, as well as viral and metabolic disease (Mendell and Olson, 2012a). However, their role in the regulation of bone formation and homeostasis has become only recently appreciated.

This thesis project aimed to characterize the role of miRNAs in the differentiation of bone and cartilage cells. For this purpose, the expression of selected miRNAs was analyzed during osteogenic and chondrogenic differentiation of mouse bone marrow cells. In addition, the function of specific miRNAs was studied in human MSCs upon osteogenic, chondrogenic or adipogenic differentiation. To evaluate the role of miRNAs in osteoclasts, the expression of selected miRNAs was monitored during early and late stages of osteoclastogenesis.

Synthetic miRNA mimics and inhibitors offer promising tools for the development of clinical treatments for various diseases (van Rooij et al., 2012). The information of miRNA expression and function during the differentiation of bone and cartilage cells may prove useful for the development of new therapeutics or enhanced *in vitro* culture techniques required for stem cell-based therapies in the future regenerative medicine.

2 REVIEW OF THE LITERATURE

2.1 The skeleton

The adult skeleton, mainly composed of bone and cartilage tissues, is multifunctional by providing mechanical support, protecting vital internal organs and, by providing sites of muscle attachment, permitting locomotion. In addition, bones maintain mineral homeostasis and acid-base balance, serve as a reservoir of growth factors and cytokines, and provide a protected environment for hematopoiesis. Furthermore, recent discoveries have shown that bone cells produce endocrine-like factors that control phosphate and glucose homeostasis, thus suggesting that bone acts as an endocrine organ.

2.1.1 Development and growth

Bones develop through two distinct mechanisms: intramembranous and endochondral ossification. Intramembranous ossification, which predominates in cranial bones and parts of the mandible and clavicle, takes place in mesenchymal condensations through differentiation of embryonic mesenchymal progenitors into bone-forming osteoblasts. The ossification center increases in size by appositional growth as more osteoblasts are formed from the mesenchymal cells of the surrounding periosteum. The collagen-proteoglycan-rich matrix produced by osteoblasts becomes calcified, resulting in woven bone with irregular orientation of type I collagen fibrils. The woven bone is later remodeled and replaced by mature lamellar bone.

In contrast, the majority of the skeleton is developed through endochondral ossification which is characterized by the formation of a cartilaginous anlage and subsequent replacement of the cartilage with bone. The cartilaginous anlage is formed as embryonic mesenchymal cells condense and differentiate into chondroblasts, which secrete various components of the extracellular matrix (ECM), including type II collagen and the proteoglycan aggrecan. Cartilage increases in size through two different mechanisms. Appositional growth occurs at the external parts of the anlage in the perichondrium, where mesenchymal cells continue to differentiate into chondroblasts, and secrete the cartilaginous matrix. In contrast, interstitial growth occurs within the cartilaginous anlage through chondrocyte division and enlargement. As the endochondral bone formation proceeds, chondroblast mature into chondrocytes, which, in turn, become hypertrophic (enlarged) and change their genetic program to synthesize type X collagen. Consequently, capillaries invade the perichondrium, and mesenchymal cells therein differentiate into osteoblasts, which start to produce bone matrix. This results in formation of a bone collar underneath the periosteal layer, which is now referred to as the periosteum. Matrix mineralization is followed by the

vascular invasion and the migration of osteoblast precursor cells into the cartilaginous model, triggering the formation of the primary ossification center inside the bone. The primary spongiosa formed on the cartilaginous remnants is later remodeled by osteoclasts, and woven bone as well as cartilage remnants are replaced with lamellar bone. In late fetal life and early childhood, secondary ossification centers appear in the epiphyses of long bones by a mechanism very similar to that seen in the formation of the primary center. The diaphysis will be separated from the epiphyses by cartilaginous growth plate that will ensure the longitudinal growth of the skeleton. In humans, growth plates close by fusion of epiphyses and metaphyses when growth ceases at puberty. However, in small rodents, such as the mouse and rat, growth plates are active throughout life. (Karaplis, 2008; Kronenberg, 2003; Mackie et al., 2011)

Bone development and skeletal growth are controlled by a complex network of signaling pathways comprising of transcription factors, growth factors, hormones, vitamins, and other signaling molecules. These factors contribute to skeletal development mainly through modulation of bone and cartilage cell proliferation and/or differentiation and are discussed in chapters 2.1.4.1-2.1.4.4.

2.1.2 Macroscopic structure

Bone

Macroscopically, bones can be divided into long bones, such as tibia and femur, and flat bones, such as skull bones, scapula, and mandible. Furthermore, two types of bone can be distinguished according to the pattern of extracellular matrix: cortical bone and trabecular bone (Figure 1). Cortical bone comprises about 80% of the skeleton and is predominantly located at the external part of the bone and in the diaphysis of long bones. It is structured as parallel osteons composed of dense layers (lamellae) of calcified tissue with Haversian canals in the middle. Trabecular bone is predominantly located in the inner parts of small bones and in the epiphyses of long bones. In addition, it makes up the majority of the vertebral bodies. It is composed of a network of thin calcified trabeculae. Cortical bone provides the skeleton with mechanical and structural support whereas trabecular bone is responsible for the metabolic functions of bone.

The spaces between the trabeculae are occupied by bone marrow consisting of highly branched vascular sinuses and a number of different cell types, including HSCs, MSCs and their progeny. The main function of bone marrow is to produce and maintain an appropriate amount of blood cells through a process called hematopoiesis. In addition, bone marrow plays a central role in the immune system, being the site of B-cell maturation.

The outer surface of cortical bone, except at joints where bone is lined by articular cartilage, is surrounded by a layer of connective tissue called periosteum which is attached to bone by strong collagenous fibers called Sharpey's fibres. In addition, periosteum provides an attachment for muscles and tendons.

Periosteum consists of fibroblasts and osteochondroprecursor cells embedded in fibrous extracellular matrix. These progenitors and their differentiation into osteoblasts or chondrocytes are responsible for appositional bone growth and fracture healing. The inner surface of cortical and trabecular bone is covered by a membranous structure called endosteum, which contains blood vessels, osteoblasts and osteoclasts as well as their progenitor cells. Bone resorption typically exceeds bone formation on the endosteal surface with aging, leading to enlarged marrow space. (Clarke, 2008)

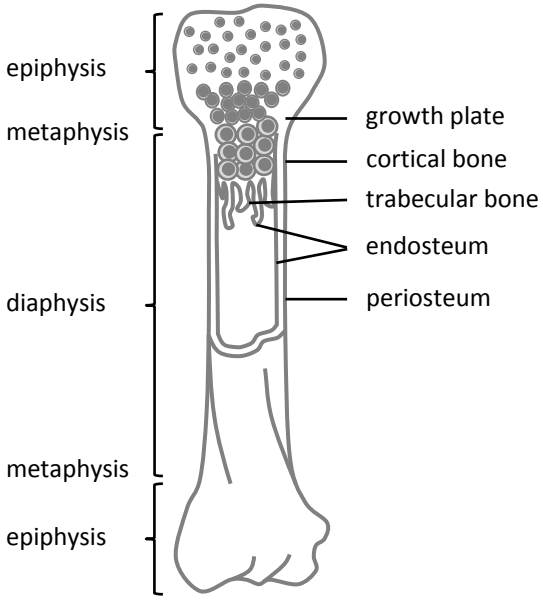

Figure 1. A schematic presentation of the structure of a long bone.

Cartilage

Cartilage is a semi-rigid form of connective tissue found in many areas of the vertebrate body, including joints, rib cage, ears, nose, bronchial tubes and intervertebral discs. It is characterized by the lack of vasculature, nerves and lymphatic vessels, which explains its slow growth and inefficient regenerative capacity. Based on the content of fibrous proteins, three types of cartilage can be identified: hyaline cartilage, elastic cartilage and fibrocartilage. Hyaline cartilage, consisting of type II collagen, is the most abundant type of cartilage found as supportive tissues in the nose, ears, trachea, larynx, and smaller respiratory tubes. Hyaline cartilage covers the articular surfaces of bones in synovial joints and is therefore called articular cartilage. During fetal development, hyaline cartilage forms the temporary skeleton that is later replaced by bone through endochondral ossification. Elastic cartilage, containing elastic fibers in addition to type II collagen, is found in the epiglottis, the auricle, walls of the external auditory canal and eustachian tubes where it provides flexibility. In contrast to

hyaline cartilage, elastic cartilage does not become calcified. Fibrocartilage contains both type II and type I collagen and is present in intervertebral discs, pubic symphysis and tendon attachments to bone providing these tissues flexibility and toughness.

Cartilage, except articular cartilage in synovial joints, is surrounded by a dense layer of connective tissue called perichondrium. Perichondrial cells secrete a number of different paracrine factors that control the proliferation and differentiation of chondrocytes. During endochondral bone formation, mesenchymal cells in the perichondrium differentiate into osteoblast and give raise to the bone collar and the primary spongiosa (Kronenberg, 2007).

The growth plate

The growth plate is organized to a continuum of four zones, designated as resting, proliferating, prehypertrophic and hypertrophic zones. In the resting zone, chondrocytes are nearly spherical and appear to be randomly arranged, surrounded by abundant ECM rich in type II collagen and proteoglycans. Parts of this zone are mitotically inactive, while others provide a stem cell source for chondrogenic differentiation. Eventually, cells in this zone either remain as permanent chondrocytes or turn into discoid and arrange into rather regular columns forming the proliferating zone. In the proliferating zone, chondrocytes divide to flattened daughter cells and produce large amounts of ECM components, resulting in bone growth. In prehypertrophic zone, chondrocytes stop dividing, enlarge and lose their characteristic shape. Here, the elongation of bone is mainly due to chondrocyte enlargement. Eventually, chondrocytes continue to their terminal differentiation stage in the hypertrophic zone. Hypertrophic chondrocytes continue to enlarge and secrete matrix rich in type X collagen. The terminal differentiation phase is eventually followed by apoptosis of chondrocytes. The molecular mechanisms controlling the differentiation and proliferation of chondrocytes in the growth plate are discussed in chapter 2.1.4.2. (Karaplis, 2008)

2.1.3 Extracellular matrix

Bone and cartilage are characterized by the relatively large amount of ECM compared to the number of cells. Bone matrix is composed of inorganic and organic components. The inorganic matrix, which constitutes about 70% of bone volume, consists of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], and gives bone its characteristic mechanical rigidity and load-bearing strength. The organic matrix is made of collagen fibers and non-collagenous proteins and is responsible for the elasticity and flexibility of bone. Cartilage has two main extracellular components: fibrous proteins, which are responsible for mechanical stability, and proteoglycans, which resist deformation by compressive forces. As with bone, cartilage matrix contains also a number of ECM proteins that are neither collagens nor proteoglycans.

The cartilage ECM comprises several compartments, characterized by distinct morphology and biochemical composition. The ECM immediately adjacent to the chondrocyte cell membrane is referred to as the pericellular matrix. It is characterized by a high content of large proteoglycan aggregates and by the relative absence of organized fibrillar collagens. The pericellular matrix is surrounded by the territorial matrix that encapsulates individual cells or groups of cells. These compartments, termed as lacunae, consist of basket-like network of crosslinked fibrillar collagen providing cells with mechanical support. Chondrocytes are connected to their territorial matrix through numerous cytoplasmic processes and specific matrix molecules. The largest compartment of ECM, situated most distantly from the cell membrane, is the interterritorial matrix comprising most of the collagen fibrils and proteoglycans. (Thonar et al., 1999)

2.1.3.1 Collagens

Collagens comprise a large family of multimeric extracellular matrix molecules that among various other functions provide cells with structural integrity (Gordon and Hahn, 2010). They are composed of three α chains that are coiled into left-hand triple helical domains due to the repeating peptide triplets of Gly-X-Y. The organic matrix in bone is mainly composed of type I collagen, with trace amounts of types III and V and non-fibrillar FACIT (fibril-associated collagens with interrupted triple helices) collagens restricted to certain stages of bone formation (Robins and Brady, 2008). Type I collagen contains two α1(I) chains and one α2(I) chain, and in its Gly-X-Y repeats, X is often proline and Y is frequently hydroxyproline or hydroxylysine (Bou-Gharios and de Crombrugghe, 2008). In adult cartilage, the most abundant (~90% of the collagens) component among the fibrous proteins is type II collagen (Thonar et al., 1999). It is a homotrimer of α1(II) chains encoded by the COL2A1 gene. In cartilaginous ECM, type II collagen associates with type IX and XI collagens, forming co-polymeric bundles that are organized into a fibrous network (Mendler et al., 1989). In addition, small amounts of other collagens, including types VI, XII, XIV and XXVII, are present in the cartilaginous ECM. Type X collagen is produced exclusively by prehyperthophic and hypertrophic chondrocytes (Gordon and Hahn, 2010).

2.1.3.2 Non-collagenous proteins

Non-collagenous proteins in bone matrix may be divided into three categories: proteoglycans, glycosylated proteins, and γ-carboxylated proteins. In cartilage, the non-collagenous part of matrix is primarily composed of proteoglycans but also small amounts of non-collagenous and non-proteoglycan proteins are present.

Proteoglycans are macromolecules that contain glycosaminoglycans (GAG) covalently attached to a central core protein. Two small leucine-rich proteoglycans, decorin and biglycan, are highly enriched in bone matrix. They are assumed to play roles in modulation of collagen fibrillogenesis and regulation of growth factor

availability and activity (Heinegård, 2009; Hildebrand et al., 1994). Decorin and biglycan bind to type I collagen and transforming growth factor β (TGF-β), a multifunctional cytokine involved in inflammation, apoptosis, cell proliferation, and differentiation. Knock-out mice deficient in *biglycan* and *decorin* show abnormal formation of collagen fibrils and reduced bone mass (Ameye and Young, 2002). The most abundant proteoglycan in cartilage is aggrecan which is assembled to large aggregates by interactions with the polysaccharide hyaluronan (Heinegård, 2009). The GAG chains, which comprise approximately 90% of the total mass of the aggrecan macromolecule, are keratan sulphate and chondroitin sulphate. The main function of aggrecan is to provide the high osmotic environment and thereby the hydrated gel structure that provides the cartilage with load-bearing properties. Mutation in the human *aggrecan* gene (*ACAN*) results in spondyloepiphyseal dysplasia associated with premature osteoarthritis (OA) (Gleghorn et al., 2005), and mice with disrupted *Acan* as a result of mutation exhibit cartilage matrix deficiency (Watanabe et al., 1994). The glycosaminoglycan hyaluronan, besides its function with aggrecan, provides lubrication to joints and skin. Other proteoglycans in cartilage include decorin, biglycan, fibromodulin and perlecan.

Glycosylated matrix proteins may be divided into two groups based on their capacity to mediate cell attachment. This feature is attributed by an amino acid sequence Arg-Gly-Asp (RGD), which allows ECM proteins to bind to the integrin class of cell surface receptors. The cell-attachment proteins in bone include various members such as fibronectin, vitronectin, osteopontin, bone sialoprotein, fibrillins, and thrombospondins. The non-RGD containing glycoproteins in bone include osteonectin, alkaline phosphatase and tetranectin. Except vitronectin, the glycosylated proteins present in bone have been also identified in cartilaginous ECM. (Robey, 2008)

Vitamin K-dependent proteins or γ-carboxylated proteins are characterized by the presence of γ-carboxyglutamic acid (gla) residues that provide these proteins their calcium-binding properties. Gla proteins identified in bone and cartilage include osteocalcin (bone gla protein), matrix gla protein (MGP), and protein S (Robey, 2008). In addition, bone and cartilage ECM contain several other non-collagenous and non-proteoglycan proteins. These include structural proteins, such as cartilage oligomeric matrix protein (COMP), fibronectin, chondroadherin, and dentin matrix protein 1, as well as various regulatory molecules, such as TGF-β, bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs).

2.1.4 Cellular compartment

The skeleton comprises several functionally different cell types: osteoblasts, osteocytes and osteoclasts in bone and chondrocytes in cartilage. In addition, bone marrow contains a heterogeneous population of HSCs, MSCs and their progeny. Osteoblasts and chondrocytes originate from nonhematopoietic compartment of bone marrow stem cells that have been variously termed mesenchymal stromal cells, skeletal stem cells, and most frequently, mesenchymal stem cells or MSCs, among other variations. In addition to osteoblast and chondrocyte lineages, MSCs

are capable of differentiating into at least adipocytes, fibroblasts, and myoblasts. The origin and *in vivo* identity of MSCs is under intensive investigation. Perivascular cells, termed as pericytes, have been suggested to represent the *in vivo* counterparts of isolated, *ex vivo* expanded cells (Crisan et al., 2008). Recently, an alternative source of MSCs was presented as the mislocalized bone in the human disease Fibroplasia Ossificans Progressiva, carrying heterozygous activating mutations in ALK2 (activin A receptor, type I), was demonstrated to originate from vascular endothelium (Medici et al., 2010). Endothelial cells from these patients, as well as TGF-β2 or BMP4-stimulated wild-type endothelial cells were described to undergo endothelial-to-mesenchymal transition and to give rise to osteoblasts and chondrocytes.

Osteoclasts are derived from the monocyte/macrophage lineage of the HSCs that are recognized for their ability to produce blood cells. The commitment and further differentiation of mesenchymal and hematopoietic stem cells into osteoblast, chondrocyte, adipocyte and osteoclast lineages is controlled by multiple lineage-specific transcription factors and evolutionarily conserved signaling pathways. Although these factors are summarized in the following chapters in cell type-specific manner, most of them initiate and maintain a particular pathway at the expense of others, thereby playing a role in other lineages as well. In addition to tightly regulated intercellular signals, stem cells are controlled by their surrounding microenvironment. In bone marrow, HSCs reside in specific areas near the endosteal surface of bone and blood vessels, known as HSC niches (Schofield, 1978). The cellular and molecular composition of bone marrow niches is under intensive investigation. Based on recent findings, at least specific subtypes of MSCs associate with HSCs within the niches and thereby contribute to the control of stem cell quiescence, self-renewal and differentiation (Méndez-Ferrer et al., 2010; Omatsu et al., 2010; Sacchetti et al., 2007).

2.1.4.1 Osteoblasts

Osteoblasts are polarized bone-forming cells located on the surface of bone matrix. They are characterized by a round nucleus, a large circular Golgi complex, a well-developed endoplasmic reticulum, and a cuboidal morphology. In addition, osteoblasts are distinguished by the production of type I collagen, alkaline phosphatase and osteocalcin. Besides matrix synthesis, osteoblasts are known to regulate the mineralization process, which will be discussed in chapter 2.1.5. Bone-lining cells of osteoblast lineage are observed as single layers of flattened cells on quiescent bone surfaces undergoing neither bone formation nor resorption (Everts et al., 2002). Although identified already years ago, very little is known about the differentiation or function of these cells. Another subtype of osteoblastic cells is constituted by osteocytes located within the bone matrix. They are thought to originate as osteoblasts become trapped in the synthesized matrix and are thereby considered as the terminal differentiation stage of osteoblast lineage. Osteocytes are connected with each other via long cytoplasmic processes which pass through the bone matrix. Through this network of thin

canaliculi, osteocytes are suggested to account for the biomechanical regulation of bone mass and structure (Klein-Nulend and Bonewald, 2008).

Differentiation of osteoblasts

Differentiation of bone marrow mesenchymal stem cells into osteoprogenitors, preosteoblasts and finally into mature osteoblasts involves multiple transcription factors and signaling pathways. **Runx2** (runt family transcription factor 2, formerly called Cbfa1) is recognized as the master regulator and, at the same time, the earliest marker of osteogenesis (Figure 2). Deletion of *Runx2* in mice results in complete lack of bone tissue and death just after birth (Komori et al., 1997; Otto et al., 1997). Due to the maturational arrest of osteoblasts, the skeleton in these mice comprises only chondrocytes and cartilage with no evidence of vascular or mesenchymal invasion. Besides its function in osteogenesis, Runx2 regulates chondrocyte hypertrophy thereby acting as a molecular switch between osteogenesis and chondrogenesis. Runx2 expression increases in cells of the osteoblast lineage during development, but decreases and disappears in the prehypertrophic chondrocytes (Takeda et al., 2001). The activation of osteogenesis by Runx2 is mediated through several target genes, including *Osteocalcin*, *Osteopontin* and *Bone sialoprotein* (Ducy et al., 1997). A number of different molecules have been identified to interact with and modify Runx2 activity during skeletogenesis, such as transcription factors Twist1 and Twist2 (Bialek et al., 2004), and the signal transducers of TGF-β superfamily receptors, SMADs (mothers against decapentaplegic homolog) (Ito and Zhang, 2001; Phimphilai et al., 2006).

Osterix (Osx/Sp7) is another transcription factor crucial for osteoblast development, both during embryonic development and in postnatal life (Figure 2). It acts downstream of Runx2 and in its absence no bone formation occurs leading to perinatal lethality (Nakashima et al., 2002). Osx is specifically expressed in osteoblasts of all skeletal elements. Its transcription is known to be regulated positively by Runx2 (Nishio et al., 2006) and Nfatc1 (nuclear factor of activated T cells, cytoplasmic 1) (Koga et al., 2005; Winslow et al., 2006), and negatively by the tumor suppressor p53 (Wang et al., 2006). However, other mechanisms that regulate Osx expression as well as the detailed information on its function remain poorly understood. Other transcription factors with important roles in osteoblast differentiation include activating transcription factor 4 (**ATF4**), helix-loop-helix proteins, various members of the activator protein 1 (**AP1**) subfamily, and homeobox transcription factors **Msx1**, **Msx2**, **Dlx5** and **Dlx6**.

Wnt/β-catenin signaling has emerged to play an important role in osteoblast differentiation and skeletal development. Wnts comprise a family of 19 secreted proteins that activate intracellular signaling pathways by binding to a membrane receptor complex composed of Frizzled (FZD), G protein-coupled receptors (GPCRs) and low-density lipoprotein (LDL) receptor-related proteins (LRPs). Wnts can function through canonical and noncanonical pathways, of which the canonical Wnt/β-catenin pathway is better characterized. In a canonical pathway, the binding of a Wnt ligand to LRP5 or LRP6 leads to inhibition of a

protein complex consisting of axin, glycogen synthase kinase (GSK)-3β and adenomatous polyposis coli (APC) protein. This leads to the stabilization of β-catenin, since the axin/GSK-3β/APC complex normally promotes the proteolytic degradation of the β-catenin. The activation of Wnt target gene expression occurs as β-catenin translocates to the nucleus and activates lymphoid-enhancer-binding factor (LEF)/T-cell-specific transcription factors (TCFs). In the absence of Wnt signal, cytosolic β-catenin becomes phosphorylated, leading to ubiquitination and degradation of β-catenin by the 26S proteasome complex. (Johnson, 2008)

Wnt/β-catenin signaling has been shown to regulate osteogenesis through multiple mechanisms. Activation of Wnt/β-catenin signaling appears to be required for osteoblast lineage commitment as conditional deletion of *β-catenin* gene in mesenchymal progenitors results in increased chondrocyte formation at the expense of osteoblast differentiation both *in vitro* and *in vivo* (Day et al., 2005; Hill et al., 2005). On the other hand, Wnt seems to favor osteogenesis at the expense of adipogenesis by inhibiting the adipogenic transcription factors CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) in bipotential mesenchymal precursors (Bennett et al., 2005; Kang et al., 2007). The positive effect of Wnt/ β-catenin on osteogenesis has been shown to involve a direct stimulation of *Runx2* gene expression (Gaur et al., 2005). Wnt/β-catenin signaling also promotes the proliferation and mineralization activity of mature osteoblasts and reduces osteoblast apoptosis, as evidenced by *Lrp5* and *Lrp6* knockout mice (Kato et al., 2002; Kokubu et al., 2004). Mice lacking *Lrp5*, similarly as patients with inactivating *LRP5* mutations, exhibit low bone mass due to impaired bone formation (Gong et al., 2001; Kato et al., 2002). In contrast, a single point mutation G171V in *LRP5* is associated with increased bone mass in at least two human kindreds (Boyden et al., 2002; Little et al., 2002). The same mutation in HBM (high bone mass) transgenic mice displays a similar phenotype, characterized by high bone mass and enhanced bone strength (Babij et al., 2003). Currently, a large number of additional *LRP5* mutations associated with increased or decreased bone mass have been reported, indicating the importance of Wnt/β-catenin signaling in the control of bone mass (Johnson, 2008). The phenotype of *Lrp6*-deficient mice with delayed ossification at birth and a low bone mass phenotype in adults corresponds to those observed in mice carrying mutations in different Wnt genes (Kokubu et al., 2004). However, the data concerning the polymorphism of human *LRP6* is limited to a single point mutation I1062V, which is associated with higher fracture risk in men (van Meurs et al., 2006). With respect to osteoclasts, Wnt/β-catenin signaling can suppress osteoclastogenesis through activation of osteoprotegerin expression in osteoblasts (Glass et al., 2005).

The Wnt/β-catenin signaling pathway is controlled by a number of regulatory proteins which, as a consequence, contribute to the regulation of osteoblast differentiation and function. For instance, the members of the Dickkopf (Dkk) family of proteins, together with their receptors Kremen1/2, inhibit Wnt/β-catenin signaling through internalization of Lrp5/6 from the cell surface (Mao et al., 2002). As a consequence, the Dkk/Kremen complex prevents the binding of Wnt proteins to the Lrp5/6 co-receptor. Ectopic expression of ***Dkk1*** results in

impaired osteoblast differentiation and maturation *in vitro* while mice deficient in a single *Dkk1* allele exhibit a high-bone-mass phenotype (Morvan et al., 2006). **Dkk2** appears to play a role in late stages of osteoblast differentiation and mineralization of bone matrix. Mice lacking *Dkk2* are osteopenic and osteoblasts from these mice exhibit impaired mineralization in response to osteogenic induction (Li et al., 2005). Sclerostin (**Sost**) is a Wnt inhibitor produced by osteocytes (Poole et al., 2005). It is thought to function as a "brake", holding the Wnt signaling pathway in bone in the "off" position when needed. Mice overexpressing human *SOST* exhibit low bone mass and decreased bone strength (Winkler et al., 2003) while *Sost*-deficient mice develop a high-bone-mass phenotype (Li et al., 2008a). Secreted frizzled related proteins (**sFRPs)** are competitive inhibitors of Wnt/β-catenin pathway, having the capacity to bind to soluble Wnts and Frizzled receptors (Moon et al., 1997). Mice lacking *sFRP-1* have increased bone mass phenotype due to impaired osteoblast and osteocyte apoptosis in addition to increased osteoblast proliferation and differentiation (Bodine et al., 2004). Also chondrogenic differentiation and endochondral bone formation are affected in response to *sFRP-1* deletion (Gaur et al., 2006).

Osteoblast differentiation is also influenced by a combination of secreted molecules including **TGF-β**, **BMPs**, and **FGFs**. Besides their function in osteogenesis, these factors regulate chondrocyte proliferation and differentiation during endochondral bone formation and will be discussed in chapter 2.1.4.2.

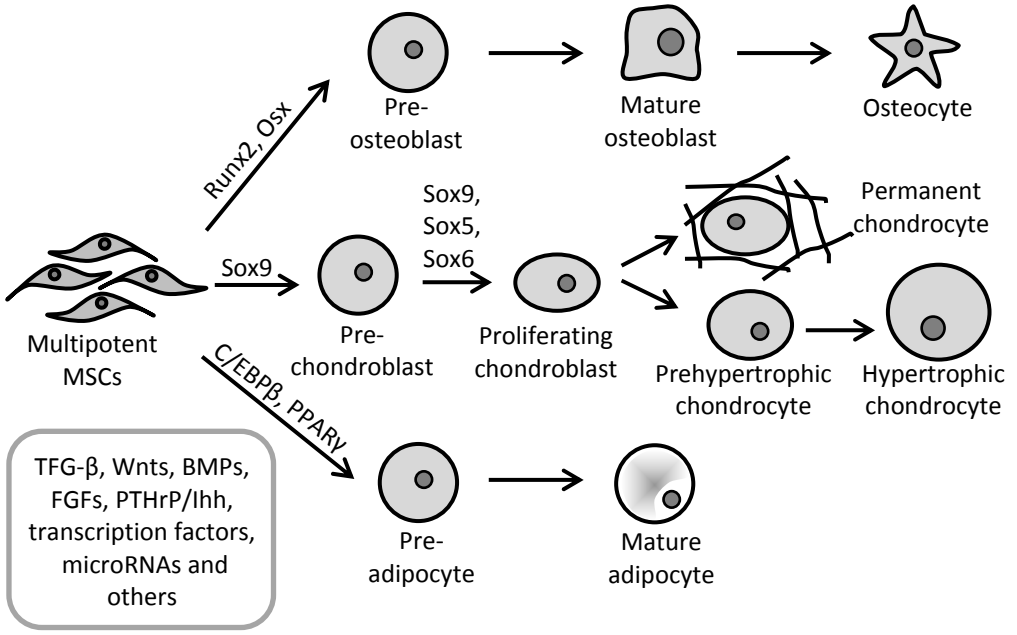

Figure 2. The differentiation of multipotent MSCs into osteoblasts, chondrocytes, and adipocytes involves key lineage-associated transcription factors (above arrows) and a number of other regulatory molecules (the box). Intrinsic signals from the surrounding ECM, cells nearby and the cell itself coordinate the proliferation and differentiation of precursor cells to certain lineages during development and tissue remodeling.

2.1.4.2 Chondrocytes

Chondrocytes reside embedded in spaces called lacunae within the cartilaginous matrix they produce. Since there is no vasculature in cartilage, chondrocytes live in hypoxic environment and receive their nutrients through diffusion via the ECM. Chondrocytes also lack cell-cell contacts and therefore communicate though the ECM. Inactive chondrocytes are characterized as small, spherical-shaped cells whereas upon activation they increase in size and display prominent nucleus, extensive rough endoplasmic reticulum and Golgi apparatus. Chondrocytes originate from osteochondroprogenitors through several stages distinguished by morphological criteria and the expression of specific ECM markers and differentiation factors. In brief, chondrogenesis includes condensation of the precartilaginous mesenchyme, commitment to the chondrogenic lineage, and differentiation into type II collagen and aggrecan depositing chondroblasts, and finally into mature chondrocytes. As chondrocytes turn into hypertrophy, they increase in size and begin to secrete type X collagen. After hypertrophy, chondrocytes undergo programmed cell death or apoptosis. Although most prominent in epiphyseal growth plate, chondrocytes exhibiting differential developmental stages may be identified also in other cartilaginous compartments such as articular cartilage and fracture callus.

Differentiation of chondrocytes

The transcription factor **Sox9** (sex-determining region Y-type high motility group box 9) is one of the earliest molecules to be expressed during endochondral bone formation and is the key transcription factor for chondrogenesis (Figure 2) (Akiyama and Lefebvre, 2011). Its expression starts at mesenchymal osteochondroprogenitor stage and remains high until cells reach hypertrophy in the growth plate (Zhao et al., 1997). Loss-of-function experiments in the mouse have demonstrated that Sox9, along with two other members of the Sox family, **Sox5** and **Sox6**, are both necessary and sufficient factors for chondrogenesis (Akiyama et al., 2002; Bi et al., 1999; Lefebvre et al., 2001). These transcription factors initiate chondrogenesis and maintain the chondrocyte phenotype through regulation of cartilage-specific genes including *Col2a1* (Lefebvre et al., 1997), *Col11a2* (Bridgewater et al., 1998), *Aggrecan (Acan)* (Han and Lefebvre, 2008), and others. Downregulation of Sox9 in the hypertrophic chondrocytes is critical for vascular invasion, bone marrow formation and bone growth (Hattori et al., 2010). In addition Sox9 appear to prevent osteoblastic transformation by reducing β-catenin and Runx2 activity in prehyperthrophic chondrocytes (Dy et al., 2012).

A number of *in vitro* as well as *in vivo* studies indicate that the regulation of chondrocyte proliferation and differentiation in the epiphyseal growth plate is mediated by a negative feedback loop consisting of parathyroid hormone-related protein (**PTHrP**) and Indian hedgehog (**Ihh**) (Kronenberg, 2006). PTHrP is expressed in perichondrial cells and chondrocytes at the distal zone of the growth

plate. It acts on its receptors on proliferating chondrocytes to maintain them proliferating and, thereby, to delay Ihh production (Karaplis et al., 1994; Lanske et al., 1996; Weir et al., 1996). Ihh is synthesized by prehypertrophic chondrocytes in response to decreased PTHrP level. Ihh increases the proliferation of adjacent chondrocytes, accelerates the differentiation of proliferative chondrocytes, and stimulates the production of PTHrP. Furthermore, it acts on perichondrial cells to convert them into osteoblasts.

Mesenchymal precursors also express **Runx2** and **β-catenin**, which are crucial factors for osteoblast differentiation. They are highly expressed in mesenchymal cells committed to chondrocytic lineage but become downregulated at the early stages of chondrogenic differentiation, in response to increased expression of Sox9 (Akiyama et al., 2004; Dy et al., 2012; Lengner et al., 2005; Yamashita et al., 2009). Runx2 and β-catenin are needed again in differentiated chondrocytes to induce chondrocyte hypertrophy (Day et al., 2005; Hill et al., 2005; Zheng et al., 2003). Their importance is supported by disturbed chondrocyte maturation in response to *Runx2* (Enomoto et al., 2004; Inada et al., 1999) or *β-catenin* (Ryu et al., 2002) inactivation. Runx2 induces the expression of *type X collagen* (Inada et al., 1999; Takeda et al., 2001), *vascular endothelial growth factor A* (*Vegfa)* (Zelzer et al., 2001) and *matrix metallopeptidase 13* (*Mmp13)* (Jiménez et al., 1999), of which the two latter proteins are needed for the invasion of blood vessels during endochondral bone formation. β-catenin promotes chondrocyte hypertrophy at least in part through activation of *Runx2* and subsequent induction of *Col10a1* expression (Dong et al., 2006).

Sonic hedgehog (**Shh**) and **TGF-β** are among the earliest regulatory factors present during chondrogenic differentiation. Shh is a secreted signaling molecule described to be essential for the vertebrate formation (Chiang et al., 1996). Based on *in vitro* observations, Shh is required for BMP-induced somatic chondrogenesis (Murtaugh et al., 1999). Subsequent studies have demonstrated that Shh and BMP signals work in sequence to establish a positive regulatory loop between Sox9 and Nkx3.2, and that increased Sox9 expression will then account for the initiation of chondrogenesis (Murtaugh et al., 2001; Zeng et al., 2002). Related to this, the transcription factor Nkx3.2 was recently shown to promote chondrogenesis also through a Sox9-independent mechanism by directly activating *Col2a1* expression (Kawato et al., 2012). TGF-β belongs to the TFG-β superfamily consisting of multiple signaling molecules, such as TFG-βs, BMPs, growth and differentiation factors (GDFs), activins and inhibins. The three isoforms TGF-β1, TGF-β2 and TGF-β3 are expressed in the mouse embryonic perichondrium and osteochondrium, although each in a distinct cell type reflecting their differential effects on chondrogenesis (Millan et al., 1991). TGF-βs signal through heteromeric type I and II receptor serine/threonine kinases. Conditional inactivation of TGF-β type II receptor (*Tgfbr2*) in the limb mesenchyme results in short limbs and fusion of the joints of the phalanges (Seo and Serra, 2007). Correspondingly, mice lacking type I receptor (*Tgfbr1*) in skeletal progenitor cells have short and wide long bones, reduced bone collars, and trabecular bones (Matsunobu et al., 2009). *In vitro* experiments with *Tgfbr1*-depleted mouse calvarial cells suggest that TGF-

β signaling promotes osteoprogenitor proliferation, early differentiation, and commitment to the osteoblastic lineage (Matsunobu et al., 2009). With respect to chondrocytes, TGF-β signaling appears to promote chondrogenesis in early differentiation stages (Leonard et al., 1991), while inhibiting terminal chondrocyte differentiation (Serra et al., 1997).

Chondrogenesis is also regulated by **FGF** and **BMP** signaling pathways. FGFs constitute a family of 22 structurally related polypeptides with essential functions during embryonic development and postnatal homeostasis (Itoh and Ornitz, 2011). Secreted FGFs signal through four different cell surface FGF receptors (FGFR1-4) while intracrine FGFs act independent of FGFRs. Many of the 22 FGF genes and all FGF receptors are expressed during different stages of endochondral bone formation. There, FGF signaling pathways inhibit chondrocyte proliferation, decrease Ihh production thereby suppressing Ihh/PTHrP signaling, and accelerate the terminal differentiation of hypertrophic chondrocytes (Hurley et al., 2008). BMPs are members of the TGF-β superfamily representing the largest subset within the family (Rosen et al., 2008). As TGF-β, BMPs bind and signal through serine/threonine kinase receptors and subsequently activate various SMAD proteins. The importance of BMP signaling during mesenchymal condensation is supported by enlarged cartilage in mice lacking the BMP antagonist *Noggin* (Brunet et al., 1998). During later stages of bone development, BMPs appear to have opposing effects on chondrogenesis to that of FGFs (Minina et al., 2002; Yoon et al., 2006). However, the molecular mechanisms of BMP/FGF antagonism remain unclear.

2.1.4.3 Bone marrow adipocytes

In addition to osteoblasts and chondrocytes, bone marrow-derived MSCs are able to differentiate into adipocytes. Although not directly involved in skeletal development, adipocytes contribute to bone formation and metabolism through their reciprocal origin and close relationship with osteoblasts (Gimble et al., 2006). In addition, an increasing number of studies suggest a close interaction between bone and adipose tissue with relation to the regulation of energy metabolism (Rosen et al., 2012). Furthermore, bone marrow adipocytes have been described to contribute to osteoclast differentiation through their production of RANKL (ligand for receptor activator of nuclear factor kappa B) (Goto et al., 2011a; Goto et al., 2011b; Kelly et al., 1998).

The central feature of adipocytes is the accumulation of lipid droplets which eventually fuse to form a single large vacuole. Adipocytes are also characterized by a spherical morphology, a compressed nucleus displaced to one side of the cell, and a thin layer of cytoplasm around the periphery. There are two main types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), distinguished by their appearance under microscopy. In addition, brown and white adipocytes differ by their gene expression profile and developmental pattern (Seale et al., 2008; Tseng et al., 2008). While several studies support the existence of a common adipogenic precursor, there is also evidence of a close

developmental relationship between brown adipocytes and skeletal muscle cells (Majka et al., 2011).

WAT is the predominant type of adipose tissue distributed throughout the body. In addition to its central role in energy metabolism, white adipose tissue contributes to the regulation of temperature and functions as a cushion against mechanical stress. BAT is a specialized type of adipose tissue mostly found in newborn mammals. Recent studies using positron emission topography have revealed significant amounts of active BAT also in human adults (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). Besides its role in adaptive thermogenesis, BAT appears to function in protecting against obesity, insulin resistance and diabetes (Cederberg et al., 2001; Enerbäck et al., 1997). Adipocytes in the bone marrow represent a third category of fat tissue, the yellow adipose tissue (YAT). In contrast to WAT and BAT, the metabolic function of YAT is largely unknown. It is also unclear whether YAT constitutes a unique population of cells or a mixture of brown and white adipocytes. Although it resembles WAT by its origin, recent studies have identified BAT-like features in YAT, which are attenuated with aging and diabetes (Krings et al., 2012).

Differentiation of adipocytes

Adipogenic differentiation is characterized by two main phases (Rosen and MacDougald, 2006). In the first step, known as determination, MSCs lose their ability to differentiate into other mesenchymal lineages and commit to become pre-adipocytes. In the second step, defined as terminal differentiation phase, pre-adipocytes acquire the morphology of mature adipocytes and the machinery that is necessary for lipid synthesis, transport and mobilization as well as insulin sensitivity. Mature adipocytes may be identified by their expression of fatty acid binding protein 4 (FABP4), glucose transporter type 4 (GLUT4), leptin and adiponectin. (Cristancho and Lazar, 2011)

The transcription factor peroxisome proliferator activated receptor γ (**PPARγ/PPARG**) is a member of the nuclear hormone receptor superfamily of ligand-responsive transcription factors. It is considered the master regulator of adipogenesis since its expression is both essential and sufficient for adipogenic differentiation (Figure 2). PPARγ has two isoforms (γ1 and γ2) that are formed by alternative splicing. PPARγ2 is the major form expressed in fat; however, the relative contribution of these two isoforms to adipogenesis remains poorly understood. Targeted disruption of *Pparg* in mice results in embryonic lethality (Barak et al., 1999) and mice with selective deletion of *Pparg* in adipose tissue show impaired adipogenesis among their multiple other phenotypes (Jones et al., 2005). Despite the extensive efforts, no factor thus far has been discovered to induce adipogenesis in the absence of PPARγ (Kawai et al., 2010). On the other hand, forced expression of PPARγ is sufficient to induce adipocyte differentiation in many cell types although not normally destined for this lineage (Tontonoz et al., 1994). The critical role of PPARγ in adipogenesis is further illustrated by the association of PPARγ with essentially all other signaling pathways and regulatory

factors involved in adipogenesis (Kawai et al., 2010; Rosen and MacDougald, 2006). Several endogenous ligands have been identified as PPARγ agonists, including 15-deoxy-$\Delta^{12,14}$-prosteglandin J_2 (Forman et al., 1995; Kliewer et al., 1995), components of the oxidized low-density lipoprotein (Nagy et al., 1998), and nitrolinoleic acid (Schopfer et al., 2005), although their contribution to *in vivo* adipogenesis remains unclear. PPARγ can be also activated by exogenous ligands such as derivatives of long-chain polyunsaturated fatty acids (Kliewer et al., 1997) and thiazolidinediones that are clinically of interest due to their insulin sensitizing actions (Lehmann et al., 1995).

In addition, several members of the **C/EBP family** play important roles during adipogenesis. Among the members that are expressed in adipocytes, C/EBPα, C/EBPβ, and C/EBPδ promote adipogenesis, while C/EBPγ and CHOP/DDIT3 (C/EBP-homologous protein or DNA-damage-inducible transcript 3) appear to inhibit adipogenesis. During early adipogenesis, C/EBPβ and C/EBPδ activate the expression of PPARγ leading to induction of C/EBPα. C/EBPα functions in synergy with PPARγ to induce the expression of adipogenic genes and thereby to stimulate adipogenic differentiation (Tontonoz et al., 1994). The inhibitory effects of C/EBPγ and CHOP/DDIT3 on adipogenesis may be explained by their heterodimerization with, and thereby inactivation of C/EBPβ (Darlington et al., 1998)

The differentiation along the osteoblast and adipocyte lineages display an inverse relationship, as evidenced by a large number of *in vitro* and *in vivo* studies (Gimble et al., 2006). In culture conditions, many growth factors and hormones that promote adipogenesis have a negative effect on osteogenesis and *vice versa*. For instance, while the ligands for glucocorticoid receptor and PPARγ typically stimulate adipogenesis, their administration to MSCs is accompanied by reduced osteogenesis (Lecka-Czernik et al., 1999; Weinstein et al., 1998). In addition, Wnt signaling plays an important role in the differentiation of MSC along the osteoblast and adipocyte pathways, as already discussed with respect to osteoblasts. Wnt signaling genes are upregulated during osteoblast differentiation and downregulated during adipocyte differentiation (Taipaleenmäki et al., 2011). Furthermore, the Wnt inhibitor **sFRP-1** is induced during adipogenesis and at least part of the inhibitory effects of Wnt signaling on osteoblast differentiation is suggested to be mediated through sFRP-1. The reciprocal relationship between osteoblastogenesis and adipogenesis is also supported by several *in vivo* observations of increased bone marrow adiposity associated with decreased bone mineral density (Di Iorgi et al., 2010; Martin and Zissimos, 1991; Wren et al., 2011).

2.1.4.4 Osteoclasts

Osteoclasts are large multinucleated cells specialized in bone resorption. They are required during skeletal development to shape the forming bones and throughout life in the process of bone remodeling. Due to their special function, osteoclasts are provided with several unique features including the capacity to polarize on

bone, the formation of specific membrane domains upon matrix degradation, an exceptionally high number of mitochondria and Golgi complexes, and the cytoplasm filled with lysosomes. In addition, osteoclasts are characterized by the formation of actin ring upon attachment to bone (Lakkakorpi et al., 1989) and by the expression of a number of osteoclast-specific genes such as tartrate resistant acid phosphatase (TRACP), cathepsin K (cat K), calcitonin receptor, and $\alpha_v\beta_3$ integrin. Osteoclasts resorb bone via the ruffled border membrane domain facing the bone surface. The resorption lacuna becomes acidic by protons secreted from osteoclasts. The low pH ensures rapid dissolution of hydroxyapatite and the residual organic matrix is digested by lysosomal enzymes derived from osteoclasts. The digested matrix is internalized, transported across the cell, and finally released to the surroundings through the functional secretory domain of the basal membrane. (Väänänen and Zhao, 2008)

Differentiation of osteoclasts

Osteoclasts are derived from the monocyte/macrophage lineage of the hematopoietic progenitor cells. During osteoclastogenesis, osteoclast precursors proliferate and differentiate into mononuclear pre-osteoclasts, which are then fused into multinucleated polykaryons and consequently activated into mature osteoclasts (Figure 3). Osteoclast differentiation and function involves factors derived from stromal cells/osteoblasts, first evidenced by murine co-cultures of bone marrow or spleen cells and bone marrow stromal cells/osteoblasts (Takahashi et al., 1988; Udagawa et al., 1990). Later studies have shown that production of the tumor necrosis factor (TNF)-related cytokine **RANKL** (Yasuda et al., 1998) and the polypeptide growth factor **M-CSF** (for macrophage colony-stimulating factor) (Lacey et al., 1998), and subsequent activation of **RANK** (receptor activator of nuclear factor κ B) on the plasma membrane of hematopoietic precursor cells (Hsu et al., 1999; Nakagawa et al., 1998) are required for osteoclastogenesis. Mice lacking *Rankl* (Kong et al., 1999), *Rank* (Dougall et al., 1999) or *M-csf* (Yoshida et al., 1990) fail to produce osteoclasts and their osteoblasts are not able to support osteoclastogenesis. A similar phenotype is observed in mice lacking ***c-Fms***, the receptor for M-CSF (Dai et al., 2002). Stromal cells/osteoblasts also produce a soluble factor osteoprogeterin (**OPG**), which is structurally similar to RANK and functions as a decoy receptor for RANKL thus inhibiting osteoclastogenesis (Simonet et al., 1997). *Opg*-deficient adult mice show decreased bone mineral density (BMD), marked thinning of parietal bones of the skull, and a high incidence of fractures (Bucay et al., 1998; Mizuno et al., 1998). Essentially, the amount of active osteoclasts and the rate of bone resorption depend on the balance between the expression of RANKL and OPG (Takahashi et al., 2009).

In addition, several other factors have been shown to affect osteoclast differentiation. Transcription factors **PU.1** (Tondravi et al., 1997) and **MITF** (microphthalmia-associated transcription factor) (Hodgkinson et al., 1993) are essential in the commitment of HSCs into the myeloid lineage and for the early

differentiation of macrophage/osteoclast precursors whereas nuclear factor NF-kappa-B (**NF-κB)** (Franzoso et al., 1997; Iotsova et al., 1997) and proto-onkogene **c-Fos** (Grigoriadis et al., 1994) are needed for osteoclastogenesis after lineage separation from macrophages. Mice lacking either of these factors are osteopetrotic due to a block in osteoclast differentiation. TNF-receptor-associated factor (TRAF) family of proteins act as adaptor molecules in various intracellular signaling pathways. In osteoclasts, **TRAF6** is needed for the RANK-mediated activation of NF-κB and mitogen-activated kinase (MAPK) pathways, and mice deficient in *Traf6* develop severe osteopetrosis (Lomaga et al., 1999; Naito et al., 1999). However, TNFα-induced osteoclastogenesis appears to be independent of RANK/TRAF6-mediated signaling, as hematopoietic precursors from mice lacking either *Rankl*, *Rank* or *Traf6* are able to differentiate into osteoclasts *in vitro* (Kim et al., 2005). The transcription factor **NFATc1** functions in downstream of TRAF6 and NF-κB and, in cooperation with other transcription factors, activates the induction of osteoclast-specific genes (Asagiri et al., 2005; Takayanagi et al., 2002). The *in vivo* significance of NFATc1 in osteolastogenesis remains unclear as the deletion of *Nfatc1* in mice results in embryonic lethality. However, embryonic stem cells from *Nfatc1*-deficient mice fail to differentiate into osteoclasts in response to RANKL (Takayanagi et al., 2002). Moreover, ectopic expression of *NFATc1* in precursor cells induces osteoclastogenesis without RANKL signaling, suggesting that NFATc1 functions downstream of RANKL (Takayanagi et al., 2002). NFATc1 expression and thereby osteoclastogenesis are modulated by the immunoreceptor tyrosine-based activation motif (**ITAM**)-dependent pathway that cooperate with RANK to stimulate calcium signaling. Osteoclast-associated receptor (**OSCAR**) and triggering receptor expressed in myeloid cells (**TREM2**) are immunoglobulin-like receptors that pair with ITAM-harboring adaptor molecules Fc receptor common γ chain (**FcRγ**) and DNAX-activating protein 12 (**DAP12**), respectively. The crucial role of the ITAM pathway for osteoclastogenesis is evidenced by suppressed NFATc1 expression associated with impaired osteoclast differentiation in mice lacking *FcRγ* or *DAP12* (Koga et al., 2004). Additionally, mice deficient in both *Fcrγ* and *Dap12* exhibit severe osteopetrosis due to the differentiation blockade of osteoclasts (Mócsai et al., 2004). The importance of ITAM-pathway is further underscored by the human Nasu-Hakola disease, where mutations in the *TREM2* or *DAP12* genes leads to formation of lytic lesions of the bone and an early onset fronto-temporal dementia (Paloneva et al., 2000; Paloneva et al., 2002).

Two transmembrane proteins, **DC-STAMP** (dendrocyte expressed seven transmembrane protein) and **OC-STAMP** (osteoclast stimulatory transmembrane protein), are essential for the cell-cell fusion of osteoclasts (Miyamoto et al., 2012; Yagi et al., 2005). Deletion of these genes results in complete lack of osteoclast cell fusion. In addition, several molecules play essential roles in the bone-resorbing activity of multinucleated osteoclasts. For instance, the integrin $\boldsymbol{\alpha_v\beta_3}$, which is a receptor for vitronectin, and the tyrosine-protein kinase **c-Src** are involved in osteoclast polarization and movement, whereas **CAII** (carbonic anhydrase II), **V-ATPase** (vacuolar-type proton adenosine triphosphate), and **ClC-7** (chloride

channel protein 7) play a role in the acidification of the resorption lacuna, and **cat K** participates in the degradation of organic bone matrix. The importance of c-Src, (Lowe et al., 1993; Soriano et al., 1991), ClC-7 (Kornak et al., 2001) and cat K (Saftig et al., 1998) is evidenced by the fact that osteoclasts in mice lacking either of these molecules exhibit no or very little bone-resorbing activity. Mice deficient in the vitronectin receptor subunit β_3 have increased bone mass due to osteoclast dysfunction (McHugh et al., 2000). With respect to V-ATPase, the a3 subunit of the proton pump appears to be of marked importance as mice deficient in this particular subunit develop severe osteopetrosis (Li et al., 1999). Respectively, mice lacking *CaII* exhibit decreased bone size, renal acidosis and osteopetrosis-like changes in trabecular bone (Lewis et al., 1988; Margolis et al., 2008). Defects in *CAII* in humans are associated with nonfunctional osteoclasts and osteopetrosis (Sly and Hu, 1995), a dysfunction of osteoclasts characterized by brittle bones.

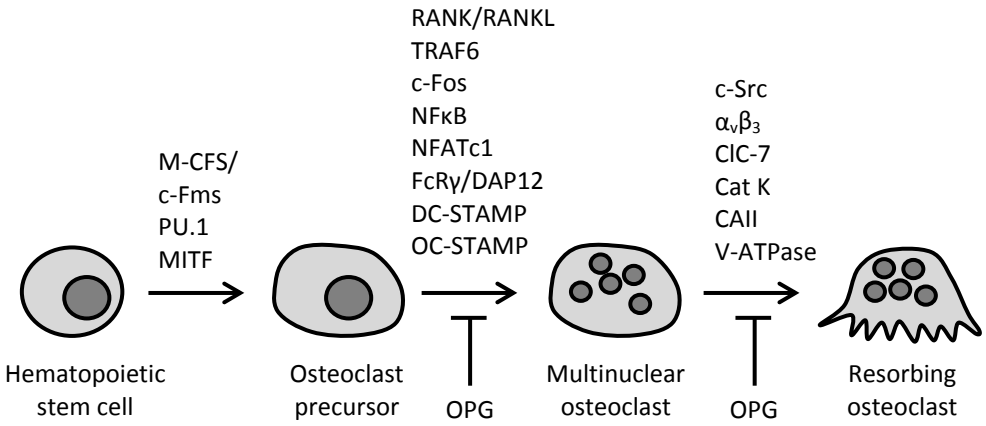

Figure 3. Key molecules involved in osteoclast differentiation and function.

2.1.5 Bone remodeling

Bone modeling (construction) is the process by which bone obtains its size and shape during development and growth, involving uncoupled bone formation by osteoblasts and bone resorption by osteoclasts at distinct anatomic locations. Bone remodeling (reconstruction), in turn, is a lifelong process involving the coupled sequential events of osteoclastic bone resorption and osteoblastic bone formation within discrete anatomic structures. These structures, referred to as bone remodeling compartments (BRCs) are generated by a canopy of flattened osteoblast-like cells, thus separating osteoblasts and osteoclasts from the bone marrow cavity (Andersen et al., 2009; Hauge et al., 2001). Bone remodeling serves to adjust bone architecture to maintain the structural integrity of the skeleton and it assists in repairing microdamages. It also plays an important role in mineral metabolism by incorporating or mobilizing calcium and other components from the bone matrix when needed. On average, 2-10% of the adult human skeleton is

remodeled annually, most of the turnover taking place at the endosteal surface of trabecular bone. Remodeling also takes place in endosteal surface and Haversian channels of the cortical bone, although at substantially lower rate. The bone remodeling cycle consists of four sequential events: bone resorption, reversal phase, bone formation and mineralization. While the resorptive step of remodeling cycle has been estimated to last up to 14 days, the subsequent reversal phase and bone formation phases are more prolonged, requiring 4-5 weeks or several months, respectively (Hadjidakis and Androulakis, 2006).

Although the molecular mechanisms initiating the remodeling cycle remain unclear, several pieces of evidence demonstrate that the need for bone remodeling is signaled via osteocytes. Viable osteocytes have been shown to inhibit osteoclastogenesis, and at least part of the signaling appears to be mediated through TGF-β (Heino et al., 2002). Induction of osteocyte apoptosis in response to damage of intercellular processes releases the inhibition, resulting in activation of osteoclastogenesis *in vitro* (Gu et al., 2005; Kurata et al., 2006) and *in vivo* (Clark et al., 2005). It has been suggested that osteocytes transmit the need for bone remodeling and activate osteoclastogenesis via their processes connected to flattened osteoblasts lining the bone surface (Seeman, 2008). Based on recent observations, osteocytes may also stimulate bone remodeling through RANKL expression (Nakashima et al., 2011; Xiong et al., 2011). These studies suggest that osteocytes trigger osteoclast recruitment to bone resorption sites by inducing the RANKL expression by osteoblastic cells in the local micro-environment.

Bone resorption itself is a multistep process, initiated by the attachment of osteoclasts to the target matrix through specific membrane domains called sealing zones (Väänänen and Horton, 1995). This is followed by the polarization of osteoclast and subsequent formation of the ruffled border between the sealing zones. The process of bone resorption has been described above (see 2.1.4.4). Bone resorption and formation is intermediated by a reversal phase, during which the resorption lacuna is cleaned and the resorbed surface is prepared for subsequent bone formation. The exact molecular events taking place during this phase remain poorly understood but it is suggested to involve the activity of mononuclear cells, most likely bone lining cells (Everts et al., 2002) and/or osteoblast-like cells (Mulari et al., 2004). Osteoclastic resorption is followed by bone formation mediated by the osteoblasts. In the adult skeleton, bone formation is restricted to sites with prior resorption and these processes are therefore coupled both in time and space. It has been suggested that products from the osteoclasts themselves and products from the resorbed matrix, such as type I collagen, osteocalcin and TGF-β, recruit osteoblast precursors to the resorption area and induce osteoblastogenesis (Mundy et al., 1982; Seeman, 2008). Mature osteoblasts lay down osteoid, filling the resorbed area partly or completely and forming the lamellae that will later undergo mineralization. Most osteoblast die after bone formation while others differentiate into lining cells or become entrapped in the osteoid to become osteocytes.

Extracellular mineralization of bone matrix occurs in two stages. During primary mineralization, mineral content is rapidly increased to about 70% of its

final density due to nucleation and crystal multiplication. This is followed by a slow secondary mineralization during which crystals grow in size. The crystals are formed at discrete sites of bone matrix collagen fibrils serving as templates for the initiation and propagation of mineralization. Interfibrillar non-collagenous proteins function as co-factors permitting the crystallite deposition while others inhibit crystal proliferation and/or growth. As bone matures, the mineral crystals become larger and more perfect with fewer impurities. Since the size and distribution of mineral crystals in bone matrix influence its mechanical properties, an optimal composition of crystals is vital for each anatomic site. Following mineralization, bone surface is covered with flattened lining cells and a prolonged resting period begins until new remodeling cycle is initiated. (Bonucci, 2012)

2.2 MicroRNAs

MicroRNAs (miRNAs) are a class of short non-coding RNA molecules that regulate gene expression at post-transcriptional level by binding to specific sequences within a target mRNA (Ambros, 2004; Bartel, 2004). First descriptions of small regulatory RNAs came up in two parallel studies characterizing the genes that control the *Ceanorhabditis elegans* (*C. elegans*) larval development (Lee et al., 1993; Wightman et al., 1993). In these studies, lin-4 RNA was identified as a potential posttranscriptional regulator for LIN-14 expression, and an antisense RNA-RNA interaction via Watson-Crick base pairing was suggested as a mechanism for this action. Only 7 years later, when the second miRNA let-7 was discovered (Reinhart et al., 2000) and subsequently identified in various vertebrate species including humans (Pasquinelli et al., 2000), the existence of small regulatory RNAs was truly appreciated. Encouraged by these findings, extensive cloning efforts were carried out resulting in the identification of numerous novel miRNAs, many of them being evolutionarily conserved across multiple species (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Research on this field has been very intensive and currently more than 17 000 mature miRNA sequences have been identified in over 140 species including diverse plants, green algae, viruses and a variety of animals (Kozomara and Griffiths-Jones, 2011).

2.2.1 Biogenesis

The canonical miRNA biogenesis pathway is closely related to the small interfering RNA (siRNA) pathway, first described by the Nobel laureates Andrew Z. Fire and Craig C. Mello (Fire et al., 1998). The intersection between miRNA and siRNA pathways became evident as targeted inactivation of *Dicer*, an enzyme that acts in the RNA interference pathway, led to accumulation of lin-4 and let-7 RNAs (Grishok et al., 2001; Hutvágner et al., 2001). However, while siRNAs are exogenous of origin, miRNAs are encoded in the genome and are found on all human chromosomes.

Within the genome, miRNA precursor sequences are located in introns of protein-coding or non-coding genes, in exons of non-coding genes, or in intergenic regions and may contain sequences for multiple different miRNAs that are co-transcribed as clusters. Like mRNA transcripts, miRNAs are transcribed by RNA polymerase II or III into long primary transcripts (pri-miRNAs) that are capped and polyadenylated (Cai et al., 2004; Lee et al., 2004).

In canonical miRNA biogenesis (Figure 4), a pri-miRNA is processed by the Microprocessor complex comprised of the RNase III enzyme Drosha (Han et al., 2004; Han et al., 2006; Lee et al., 2003) and the double-stranded RNA (dsRNA)-binding protein DiGeorge syndrome critical region 8 (DGCR8)(also called Pasha) (Denli et al., 2004; Gregory et al., 2004) to produce a 60-110 nt long intermediate precursor-miRNA (pre-miRNA). Pre-miRNA is then exported from the nucleus to the cytoplasm by Exportin-5 (XPO5) (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). In the cytoplasm, the pre-miRNA is further processed by the RNase III enzyme Dicer into a 20-25 nt mature miRNA duplex which is finally unwound by an helicase into a single-stranded miRNA (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). The functional mRNA targeting complex is formed as the miRNA is incorporated into an RNA-induced silencing complex (RISC) constituted by the members of Argonaute (Ago) family proteins (Khvorova et al., 2003; Schwarz et al., 2003). Association of miRNA with its target mRNA results in translational repression or mRNA degradation, which has been thought depend on sequence complementarity between the miRNA and the target mRNA (Giraldez et al., 2006; Yekta et al., 2004). However, recent studies examining the kinetics of miRNA-mediated gene regulation suggest that most targets undergo both regulatory effects, i.e. translational repression followed by removal of the stabilizing poly(A) tail and mRNA decay (Bazzini et al., 2012; Djuranovic et al., 2012).

Recent evidence has demonstrated that the biogenesis of miRNAs is essentially more diverse than was previously thought. An increasing amount of miRNAs has been shown to be processed through mechanisms independent of certain canonical miRNA biogenesis components. The term ‘mirtron’ was initially adopted to define a group of miRNAs that were found to be produced by intronic splicing, a mechanism independent of the Microprocessor components Drosha and DGCR8 (Okamura et al., 2007; Ruby et al., 2007). Where pre-miRNAs in canonical pathways are cleaved by the Microprosessor, the mirtron pre-miRNA is spliced and subsequently linearized by Ldbr/DBR1 (debranching enzyme (*S. Cerevisiae*) homolog 1), allowing the intron to form a structure that is exported to the cytoplasm and further processed by the canonical biogenesis components XPO5 and Dicer, respectively. Although originally identified in *Drosophila melanogaster* and *C. elegans*, the existence and function of mirtrons have now been verified in mammals, including humans (Sibley et al., 2012). However, it was recently demonstrated that a subset of the predicted mirtrons in fact mature through a non-canonical pathway independent of splicing. These miRNAs, termed ‘simtrons’, require Drosha but not it’s binding partner DGCR8 for their maturation (Havens et al., 2012). In contrast to mirtrons and many other small non-coding

RNAs processed through a non-canonical pathway, simtrons are also independent of the endonuclease Dicer. Earlier, a bypass of Dicer cleavage had been described only for miR-451 which is processed through a Dicer-independent biogenesis pathway that requires Ago2 slicer catalytic activity (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). Overall, these studies show the complexity of small RNA processing and demonstrate the diversity of mechanisms that can take place when miRNAs are produced from an RNA transcript.

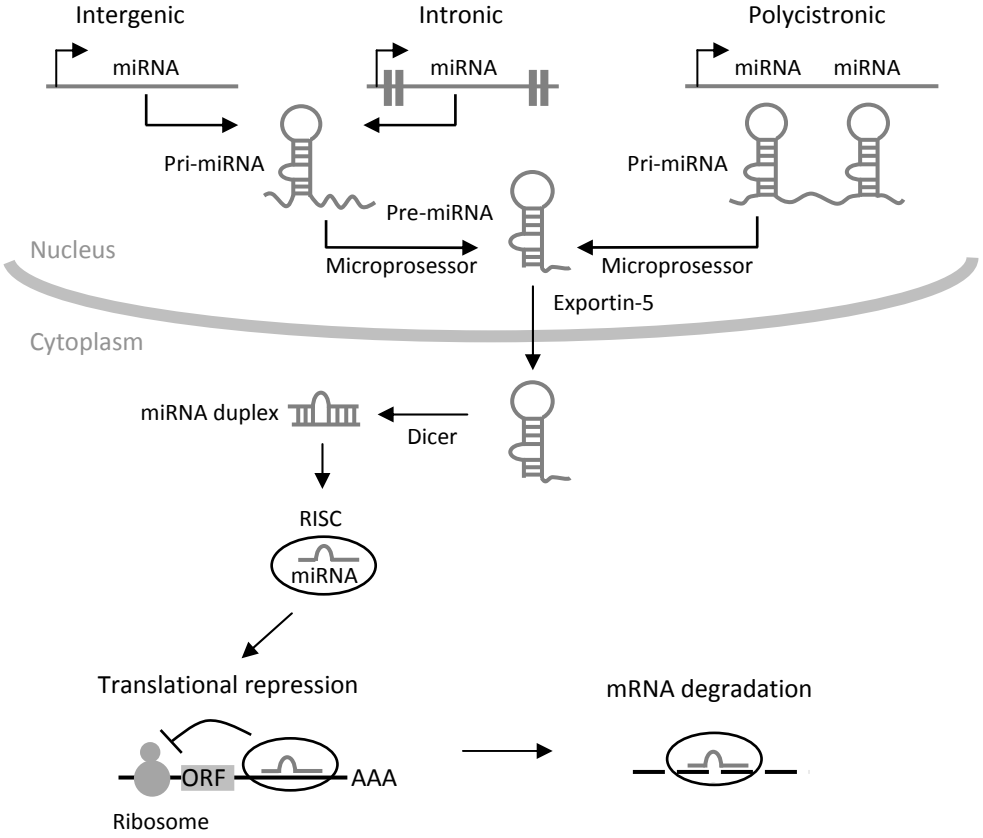

Figure 4. **MicroRNA biogenesis.** In animals, miRNAs are transcribed into primary miRNA (pri-miRNA) transcripts as individual miRNA genes, from introns of protein-coding genes, or from polycistronic transcripts. The pri-miRNAs are processed by Drosha-containing Microprocessor complexes and the resulting hairpin precursor miRNAs (pre-miRNAs) are exported from the nucleus by Exportin-5. In the cytoplasm, the Dicer complex removes the loop region from pre-miRNAs, resulting in a miRNA:miRNA* duplex. The mature miRNA is assembled into the RISC and subsequently bound to the target mRNA. Based on current understanding, miRNAs first block translation of their target mRNA and then mediate its decay.

2.2.2 Function

The main function of miRNAs is to inhibit protein synthesis through translational repression or mRNA degradation. The relative contribution of these two mechanisms remained unclear until recently, when mRNA destabilization was found to account for over 80 % of the decreased protein production (Guo et al., 2010). Although more uncommon, some miRNAs have been shown to function as translational activators (Vasudevan et al., 2007). In fact, the same miRNA may oscillate between translational repression and activation in coordination with the cell cycle status. While most animal miRNAs bind to the 3'untranslated region (UTR) of their target mRNAs, there are also reports of miRNA binding sites within the 5'UTRs and coding regions of mRNAs (Duursma et al., 2008; Lytle et al., 2007). In 2007, Lytle et al. were the first to demonstrate that miRNAs can associate with practically any position of the target mRNA (Lytle et al., 2007). A subsequent computational screen for highly conserved sequences shows an enrichment of miRNA target sequences within the coding region of *Dicer* (Forman et al., 2008). *In vitro* validation of the results demonstrates a direct binding of let-7b to the *Dicer* coding region revealing an autoregulatory negative feedback loop between let-7c and *Dicer*.

The target recognition by miRNAs is mediated by different degrees of base pairing. In plants, miRNAs usually pair nearly perfectly with target sites whereas animal miRNAs typically display partial pairing (Pasquinelli, 2012). The most commonly observed pairing pattern comprises perfect binding in 5' region of miRNAs centered on nucleotides 2-7, which is called the seed region (Lewis et al., 2005). In fact, the seed pairing appears to be rather essential for miRNA-mediated regulation as nucleotide substitutions at seed region are most crucial to miRNA activity (Doench and Sharp, 2004). There are also several validated miRNA-target pairs that do not follow the seed pairing pattern, illustrating the diversity of animal miRNA targeting.

Many different algorithms exist for the prediction of miRNA targets. With some modifications, computational predictions rely on the complementarity between the miRNA seed sequence and the potential binding site in the 3' UTR of a target mRNA as well as the conservation of the binding site among multiple species (Bartel, 2009). In general, hundreds of targets are predicted for each miRNA, causing significant challenge for target validation. Based on current understanding, miRNAs function by fine-tuning the expression of numerous targets, and the subtle changes observed in target levels are likely to be overridden by the additive effect of multiple transcripts. Nevertheless, in order to understand the complex networks behind miRNA-mediated gene regulation, target validation *in vitro* and/or *in vivo* is inevitable. These validations typically involve miRNA gain- and loss-of-function studies accomplished by genetic deletion techniques or the use of synthetic miRNA precursors and/or inhibitors. In addition, novel methods based on the immunoprecipitation of miRNA-protein complexes have proven useful (König et al., 2011).

2.2.3 Clinical applications

Aberrant miRNA expression, and thereby dysregulation of their target genes, has been implicated in human malignancies and a variety of other pathologies. As with protein-coding genes, the deregulation of miRNAs may result from deletion, amplification, mutation, or dysregulation of transcription factors controlling specific miRNAs. In addition, miRNAs may be influenced by epigenetic mechanisms such as DNA methylation and histone modifications. The pioneer work by Calin et al. showed that miR-15a/miR-16-1 cluster is downregulated or deleted in more than 60% of chronic lymphocytic leukemia patients (Calin et al., 2002). The same group also demonstrated that a significant percentage of miRNA genes are located in cancer-related or fragile genomic sites, further supporting the association between miRNA expression and disease (Calin et al., 2004). In subsequent studies over the past decade, an altered miRNA expression pattern has been described in almost all types of cancer and a large number of other human disorders, including cardiovascular disease, neurological disorders, autoimmune disease and skeletal disease (Mendell and Olson, 2012b; van Rooij et al., 2012).

2.2.3.1 miRNAs as molecular biomarkers

Recent studies have shown significant levels of miRNAs in serum and other body fluids, raising the possibility that miRNA expression profiling could provide a noninvasive method for disease diagnosis and prognosis as well as for prediction of therapeutic responses. Indeed, extracellular miRNAs exhibit several features that support their utility as clinical biomarkers: high stability, conservation among species, tissue and disease-specific expression pattern, and easy assessment by various methods, such as microarrays and real-time polymerase chain reaction (RT-PCR) (Creemers et al., 2012; Etheridge et al., 2011). While the mechanisms by which miRNAs are exported remain unclear, the cause of unusual stability of secreted miRNAs has started to be revealed. Extracellular miRNAs are secreted in microparticles (exosomes, microvesicles, and apoptotic bodies) (Valadi et al., 2007; Zernecke et al., 2009) or associate with Ago2 proteins (Arroyo et al., 2011; Turchinovich et al., 2011) or lipoprotein complexes (Vickers et al., 2011) and are thereby protected from degrading ribonucleases. Although the biological function of secreted miRNAs remains inconclusive, their utility as diagnostic or prognostic biomarkers has been demonstrated in multiple diseases, including different types of cancers and cardiovascular diseases, as reviewed by (Etheridge et al., 2011).

2.2.3.2 miRNAs as therapeutics

miRNA gain- and loss-of-function studies in normal tissues often result in minor, if any, phenotype suggesting that other miRNAs are able to compensate for the loss of a specific miRNA. However, under pathologic conditions such as cancer, delivery of synthetic anti-miRNAs selectively affects tumor cell growth and survival without any effect on normal cells, suggesting an increased sensitivity to miRNA regulation under stress (Mendell and Olson, 2012b). Therefore, inhibition or delivery of miRNAs may provide novel mechanisms to modulate disease processes while avoiding toxic effects in normal tissues. This potential has prompted significant efforts to develop miRNA-targeted therapeutics; for review, see (Laitala-Leinonen, 2010).

The most widely used approach to alter miRNA regulation is the anti-sense strategy, which exploits chemically modified oligonucleotides containing antisense sequences against mature miRNAs. A variety of chemical modifications, such as 2'-0-methyl and locked nucleic acid (LNA) modifications, are described to enhance their stability, cellular uptake and efficacy (Elmén et al., 2008; Krützfeldt et al., 2005). The most promising results this far have been obtained with LNA-modified anti-miR Miravirsen (Santaris Pharma) directed against miR-122 as a treatment for hepatitis C virus (HCV) infection. Binding of miR-122 to viral 5' non-coding region is essential for HCV replication, and inhibition of this miRNA with anti-miRs suppresses viral replication in rodents and primates (Jopling et al., 2005; Lanford et al., 2010). The Phase II trial with human patients shows dose-dependent and long-lasting suppression of HCV, without evidence of toxicity (Reesink et al., 2012). Another promising method of miRNA inhibition comprises the use of "microRNA sponges" (Ebert et al., 2007). These inhibitors contain multiple tandem binding sites for a specific miRNA and when introduced to cells, they sequester endogenous miRNAs thereby preventing their interaction with targets.

Compared to miRNA inhibitors, the development of miRNA mimics appears to be more problematic. The main challenge is to generate and effectively deliver a double-stranded RNA molecule in which one strand, the "guide", is identical to the mature miRNA while the complementary strand, or "passenger", accounts for the stability and cellular uptake. Further complications arise from the tendency of passenger strand to function as an anti-miR. However, special delivery methods, such as lipid complexes, nanoparticles and viral vectors, appear to provide potent means for miRNA mimicry in diseases associated with decreased miRNA expression (Laitala-Leinonen, 2010).

2.3 miRNA regulation in the skeleton

MicroRNAs have emerged as essential regulators of skeletal development and homeostasis. They mediate their effects by controlling the cellular protein levels of key transcription factors and signaling molecules involved in bone and cartilage cell differentiation and function. Intensive research on the field has revealed that miRNAs contribute to all steps of differentiation, from stem cells to the fully differentiated cells and mature tissues. Particularly, many miRNAs are involved in MSC lineage fate determination and same miRNAs may play a role, parallel or inverse, across multiple differentiation pathways. For simplicity, these miRNAs are discussed below separately for each cell type.

2.3.1 miRNAs in skeletal development

miRNA regulation is crucial already at the stem cell stage and mice lacking the miRNA processing enzyme *Dicer* do not survive beyond the embryonic day 7.5 (Bernstein et al., 2003). miRNAs are also essential for skeletal morphogenesis during early stages of limb development, as conditional deletion of *Dicer* in limb mesoderm leads to reduced limb size due to massive apoptosis (Harfe et al., 2005). Furthermore, genetic ablation of *Dicer* in bone and cartilage cells results in significant defects, designating the importance of miRNAs in bone formation and bone remodeling. The absence of miRNAs in chondrocytes leads to reduced growth and premature death of mice. Histological analysis of growth plates shows suppressed chondrocyte proliferation and accelerated hyperthrophic differentiation (Kobayashi et al., 2008). With respect to osteoblasts, miRNAs appear to control both early and late steps of bone formation (Gaur et al., 2010). Conditional disruption of *Dicer* in osteoprogenitors leads to embryonic lethality with fetal pubs at E14.5 showing deformed cartilage skeleton and no evidence of bone formation. Inactivation of *Dicer* in mature osteoblasts generates a viable mouse with a transient perinatal phenotype of delayed bone mineralization. Interestingly, at the age of 2-10 months, these mice show increased bone mass with no evidence of impaired osteoclast activity. In contrast, conditional ablation of *Dicer* in osteoclasts results in mild osteopetrosis caused by impaired osteoclast differentiation and function (Mizoguchi et al., 2010; Sugatani and Hruska, 2009).

2.3.2 Regulation of osteogenesis by miRNAs

The differentiation of MSCs to osteoprogenitors, pre-osteoblasts and finally to mature osteoblasts involves a coordinated action of multiple transcription factors and signaling molecules. The regulatory pathways constituted by these factors are post-transcriptionally controlled by a repertoire of miRNAs, as demonstrated by an increasing number of studies ranging from undifferentiated MSCs to mature osteoblasts (Table 1). Although Table 1 appears heavy at first sight, a deeper look

reveals that certain ECM proteins, transcription factors and components of the signaling pathways are enriched among the target genes. In the following section, instead of addressing all currently known osteoblast-associated miRNAs, selected examples of miRNAs associated with transcriptional control of osteogenesis as well as miRNAs related to BMP and Wnt signaling are discussed.

Posttranscriptional regulation of Runx2 and Osx

The commitment and differentiation of MSCs towards osteogenic lineage is driven by the transcription factors Runx2 and Osx. Particularly many miRNAs participate in the regulation of Runx2 expression, illustrating the high redundancy of miRNA regulation associated with vital signaling pathways. For instance, **miR-204** and its homolog **miR-211** are highly expressed in murine MSCs and ST2 mesenchymal progenitor cells and their levels are increased upon adipogenic differentiation, along with decreased Runx2 level (Huang et al., 2010). Overexpression of miR-204 leads to suppressed osteoblastogenesis and increased adipogenesis. An inverse outcome results from miR-204 inhibition, suggesting that miR-204/211 directs mesenchymal progenitor cells towards adipogenic lineage at the expense of osteogenesis. Another Runx2 targeting miRNA, **miR-335**, is abundant in undifferentiated hMSCs whereas its expression is downregulated during osteogenesis and adipogenesis (Tomé et al., 2011). In contrast to miR-204, overexpression of miR-335 inhibits both osteogenic and adipogenic differentiation of hMSCs. Taking into account that miR-335 also suppresses the proliferative and migratory capacities of hMSCs, it may be associated with a common regulatory pathway controlling MSC proliferation, differentiation and migration. A more comprehensive view of the miRNA-mediated regulation of Runx2 signaling was obtained recently when a group of 11 miRNAs was shown to attenuate Runx2 expression during osteoblast and chondrocyte differentiation (Zhang et al., 2011d). These miRNAs include **miR-23a, miR-30c**, **miR-34c**, **miR-133a**, **miR-135a**, **miR-137, miR-204**, **miR-205**, **miR-217**, **miR-218**, **and miR-338**. Seven of these also target Tricho-Rhino-Phalangeal Syndrome I (TRPS1), a transcription factor known to regulate the differentiation, proliferation and apoptosis of chondrocytes within the growth plate (Zhang et al., 2012b). With one exception, these miRNAs effectively suppress osteoblast and chondrocyte differentiation in pre-committed progenitor cells (Zhang et al., 2011d). In contrast, upregulation of Runx2-targeting miRNAs may be required for adipogenic differentiation as their transfection into C3H10T1/2 mesenchymal cells induces the expression of adipocyte marker genes (Zhang et al., 2012b).

Besides being a direct target for several miRNAs, Runx2 contributes to miRNA-mediated regulation of osteogenesis by controlling the expression of osteoblast-related miRNAs. During osteoblast differentiation, induction of Runx2 suppresses the expression of three miRNAs in a cluster (**miR-23a~27a~24-2**) via direct binding to the Runx binding element found in the promoter region of the cluster (Hassan et al., 2010). These miRNAs inhibit the expression of the DNA-binding protein SATB2 (for special AT-rich sequence-binding protein 2), which is

known to synergize with Runx2 to facilitate bone formation, thereby suppressing osteoblastogenesis in mouse primary osteoblasts. In contrast, another miRNA cluster consisting of **miR-3960** and **miR-2861** is upregulated by Runx2 through direct binding to the promoter of the miR-3960/miR-2861 cluster (Hu et al., 2011). These miRNAs promote osteoblastogenesis by suppressing the expression of homeobox protein Hoxa2, a known repressor of Runx2 expression, and histone deacetylase 5 (Hdac5), an enhancer of Runx2 degradation, respectively (Hu et al., 2011; Li et al., 2009a).

Two miRNAs, **miR-93** and **miR-637**, have been identified as direct regulators of Osx. miR-93 is downregulated during mineralization of mouse calvarial osteoblasts (Yang et al., 2012). Forced expression of miR-93 in osteoblasts leads to impaired mineralization, explained at least in part by the reduced Osx expression. miR-637 seems to control the balance between osteogenesis and adipogenesis by regulating OSX protein level in hMSCs (Zhang et al., 2011b). Upregulation of miR-637 inhibits osteoblast differentiation *in vitro* and promotes adipogenesis *in vitro* and *in vivo*. Besides direct regulation by miRNAs, Runx2 and Osx are influenced indirectly by miRNAs targeting their upstream signaling pathways. For instance, **miR-138** attenuates RUNX2 and OSX expression in hMSCs by suppressing the protein tyrosine kinase 2 (PTK2) (Eskildsen et al., 2011). Upregulation of miR-138 results in decreased phosphorylation of focal adhesion kinase (FAK) and its downstream target ERK1/2 (for extracellular signal-regulated protein kinase 1/2). Inhibition of the cascade leads to decreased phosphorylation of RUNX2 and reduced expression of OSX. miR-138 is highly expressed in undifferentiated cells and becomes downregulated upon osteogenic, chondrogenic and adipogenic differentiation. Since ectopic expression of miR-138 inhibits at least osteogenesis and adipogenesis, it may have a role in maintaining MSCs in a proliferative state (Eskildsen et al., 2011; Yang et al., 2011c).

miRNA-mediated regulation of BMP and Wnt signaling

As discussed above, BMP and Wnt signaling are potent inducers of osteoblast differentiation. The components of BMP and Wnt signaling pathways are controlled by a number of miRNAs, which either promote or suppress their activity. For instance, **miR-100** inhibits BMP signaling, and thereby osteogenic differentiation of human adipose derived mesenchymal stem cells (hASCs), by targeting the BMP receptor 2 (BMPR2) (Zeng et al., 2012). Besides their negative effect on Runx2 expression, the members of the **miR-30 family** as well **as miR-135a** interfere with BMP signaling by targeting the signal transducers Smad1 and Smad5, respectively. Smad1 is also targeted by **miR-26a**, and ectopic expression of miR-26a in human adipose tissue-derived stem cells (hADSCs) results in suppressed osteogenesis (Luzi et al., 2008). In contrast, **miR-20a** potentiates BMP signaling, and thereby osteoblast differentiation, by targeting BAMBI (for BMP and activin membrane-bound inhibitor homolog) and CRIM1 (for cysteine-rich motor neuron 1), which are antagonists of the BMP pathway (Zhang et al., 2011c).

On the other hand, a number of studies demonstrate an altered miRNA expression profile in osteoblasts in response to the activation of BMP signaling, indicating a reciprocal interaction between miRNAs and BMP signaling (Huang et al., 2010; Li et al., 2008b; Zhang et al., 2011c).

Several miRNAs, including, **miR-27**, **miR-29a**, **miR-142-3p**, **miR-218**, and **miR-335-5p**, have been described to activate canonical Wnt signaling in osteoblasts. miR-27 and miR-142-3p potentiate osteoblastogenesis by targeting APC protein, resulting in accumulation of β-catenin and thus increased Wnt activity (Hu et al., 2012; Wang and Xu, 2010). miR-29a and miR-218 have been shown to promote osteogenesis through a positive feedback loop with Wnt (Hassan et al., 2012b; Kapinas et al., 2010). miR-29a and miR-218 become upregulated upon activation of Wnt signaling, while, in turn, they promote Wnt signaling by targeting negative regulators of Wnt/β-catenin pathway. Targets of miR-29a include Dkk1, Kremen2 and sFRP-2. In addition, miR-29 has been shown to regulate osteonectin in osteoblasts (Kapinas et al., 2009). Validated targets of miR-218 include Dkk2, Kremen2, sFRP-2, and Sost. miR-335-5p activates Wnt signaling in mature osteoblasts by suppressing the expression of DKK1 protein (Zhang et al., 2011a). *In situ* hybridization in mouse embryos shows abundant expression of miR-335-5p in osteoblasts and hypertrophic chondrocytes, suggesting a potential role for miR-335-5p during endochondral bone formation. Together, these studies illustrate that BMP and Wnt signaling pathways are controlled by miRNAs at multiple levels.

Table 1. Regulatory functions of miRNAs in osteoblasts

miRNA(s)	Target gene(s)	Function	References(s)
miR-20a	*PPARG, BAMBI, CRIM1*	Promotes osteoblast differentiation by targeting negative regulators of BMP signaling in hMSCs	(Zhang et al., 2011c)
miR-21	*SPRY2*	Promotes osteogenic and adipogenic differentiation of hMSCs	(Mei et al., 2012)
miR-22	*HDAC6*	Promotes osteoblast differentiation of hADMSCs	(Huang et al., 2012)
miR-23a/27a/24-2	*Runx2, Satb2*	Inhibit osteogenic differentiation of MC3T3-E1 cells; Suppressed by Runx2 in rOBs	(Hassan et al., 2010)
miR-26a	*SMAD1*	Modulates late osteoblast differentiation of hADSCs	(Luzi et al., 2008)
miR-27	*APC*	Promotes osteoblast differentiation by activating Wnt signaling in hFOB cells	(Wang and Xu, 2010)
miR-29a	*Osteonectin, DKK1, KREMEN2, SFRP-2*	Promotes osteoblast differentiation in hFOB or MC3T3-E1 cells	(Kapinas et al., 2010; Kapinas et al., 2009)
miR-29b	*Hdac4, Tgfb3, Acvr2a, Ctnnbip1, Dusp2, Col1a1, Col5a3, Col4a2*	Promotes osteoblast differentiation and attenuates collagen synthesis of MC3T3-E1 cells	(Li et al., 2009b)
miR-30 family	*Runx2, Smad1*	miRNAs inhibit BMP2-induced osteoblast differentiation in MC3T3-E1 cells and mMSCs	(Wu et al., 2012)
miR-34b/c	*Satb2*	Control bone mass accrual *in vivo*; Inhibit osteoblast proliferation and differentiation	(Wei et al., 2012)
miR-34c	*Notch1, Notch2, Jag1*	Transgenic mice overexpressing miR-34c in osteoblasts exhibit age-related OP *in vivo*	(Bae et al., 2012)
miR-93	*Sp7/Osx*	Inhibits osteoblast mineralization in mOBs	(Yang et al., 2012)
miR-100	*BMPR2*	Inhibits osteoblast differentiation in hACSs	(Zeng et al., 2012)
miR-125b	-	Inhibits osteoblast differentiation in ST2 cells and human vascular smooth muscle cells	(Goettsch et al., 2011; Mizuno et al., 2008)
miR-133a	*Runx2*	Inhibits BMP2-induced osteoblast differentiation of C2C12 cells	(Li et al., 2008b)
miR-135a	*Smad5*	Inhibits BMP2-induced osteoblast differentiation of C2C12 cells	(Li et al., 2008b)
miR-135b	-	Inhibits osteogenic differentiation and mineralization of UUSCs	(Schaap-Oziemlak et al., 2010)
miR-138	*PTK2*	Inhibits osteoblast differentiation in hMSCs	(Eskildsen et al., 2011)

miRNA(s)	Target gene(s)	Function	References(s)
miR-141, miR-200a	*Dlx5*	Inhibit BMP2-induced osteoblast differentiation of MC3T3-E1 cells	(Itoh et al., 2009)
miR-142-3p	*APC*	Promotes osteoblast differentiation by activating Wnt signaling in hFOB cells	(Hu et al., 2012)
miR-182	*Foxo1*	Inhibits osteoblast differentiation and promotes osteoblast apoptosis (C3H10T1/2, MC3T3-E1, mOB)	(Kim et al., 2012b)
miR-196a	*HOXC8*	Promotes osteogenic differentiation of hASCs	(Kim et al., 2009a)
miR-199a, miR-346	*LIF*	Regulate LIF expression during hMSC differentiation	(Oskowitz et al., 2008)
miR-204/ miR-211	*RUNX2*	Inhibit osteogenesis and promote adipogenesis of hMSCs and ST2 cells	(Huang et al., 2010)
miR-206	*Connexin 43*	Inhibits osteoblast differentiation *in vitro* (C2C12 cells) and *in vivo*	(Inose et al., 2009)
miR-208	*Ets1*	Inhibits BMP2-induced osteoblast differentiation in mOBs and MC3T3-E1 cells	(Itoh et al., 2010)
miR-210	*Acvr1b*	Promotes osteoblast differentiation by inhibiting the TGF-β/activin signaling pathway in ST2 cells	(Mizuno et al., 2009)
miR-218	*Sost, Dkk2, Sfrp-2*	Promotes commitment and osteogenic differentiation of mMSCs	(Hassan et al., 2012a)
miR-335	*Runx2*	Inhibits MSC proliferation, and migration, as well as osteogenic and adipogenic differentiation	(Tomé et al., 2011)
miR-335-5p	*Dkk1*	Promotes osteogenic differentiation (mOBs and several mouse cell lines)	(Zhang et al., 2011a)
miR-370	*Bmp2, Ets1*	Inhibits BMP2-induced pre-osteoblast differentiation in mOBs and MC3T3-E1 cells	(Itoh et al., 2012)
miR-378	*Galnt7*	Inhibits nephronectin-mediated osteoblast differentiation in MC3T3-E1 cells	(Kahai et al., 2009)
miR-637	*SP7/Osx*	Inhibits osteogenesis and promotes adipogenesis of hMSCs	(Zhang et al., 2011b)
miR-2861/ miR-3960	*Hdac5, Hoxa2*	Promote osteoblast differentiation in ST2 cells; Upregulated by Runx2	(Hu et al., 2011; Li et al., 2009a)
A group of 11 miRNAs	*Runx2*	Inhibit osteogenic differentiation of MC3T3-E1 cells	(Zhang et al., 2011d)

C2C12, a mouse myoblast cell line; C3H10T1/2, a mouse mesenchymal cell line; hFOB, human fetal osteoblasts; MC3T3-E1, a mouse osteoblast precursor cell line; m/rOB, primary mouse/rat osteoblasts; ST2, a mouse stromal cell line; UUSCs, unrestricted somatic stem cells

2.3.3 Regulation of chondrogenesis by miRNAs

An increasing number of miRNAs have been associated with various steps of chondrogenesis (Table 2) and, as an indication of their importance for skeletal homeostasis, aberrant miRNAs expression has been implicated in the pathogenesis of OA (Table 5). **miR-140** is the most comprehensively studied miRNA with positive effect on chondrogenesis. It is specifically expressed in cartilage tissues during zebrafish (Wienholds et al., 2005) and mouse (Tuddenham et al., 2006) embryonic development. Mice lacking miR-140 are dwarfs as a consequence of impaired chondrocyte proliferation and show age-related OA-like changes (Miyaki et al., 2010). In contrast, transgenic mice overexpressing miR-140 in chondrocytes are protected from antigen-induced arthritis. In line with this, reduced miR-140 levels have been reported in human OA cartilage (Iliopoulos et al., 2008; Miyaki et al., 2009). The positive effect of miR-140 to chondrocyte proliferation is mediated through several targets, including Hdac4, Adamts-5 (a disintegrin and metalloproteinase with thrombospondin motifs 5), and transcription factor Sp1 (specificity protein 1) (Miyaki et al., 2010; Tuddenham et al., 2006; Yang et al., 2011b). Other miRNAs with known positive effects on chondrocyte proliferation include **miR-21**, **miR-337**, **and miR-365**. miR-21 promotes proliferation and increases the expression of Col2a1 and aggrecan in rat chondrocytes embedded in atelocollagen gel (Kongcharoensombat et al., 2010). miR-337 promotes chondrocyte proliferation during early phases of endochondral bone formation through regulating TGFBR2 expression (Zhong et al., 2012). It is highly expressed in neonatal rats whereas the expression level becomes significantly decreased during the maturation phases of endochondral ossification. miR-365 promotes chondrocyte proliferation and differentiation through targeting HDAC4 in primary chicken chondrocytes (Guan et al., 2011). Its expression is induced upon mechanical loading, illustrating the variety of mechanisms that can induce miRNA regulation. Also **miR-23b** is considered a positive regulator of chondrogenesis, as it increases aggrecan expression in hMSCs by suppressing protein kinase A (PKA) expression (Ham et al., 2012).

Among the other chondrocyte-associated miRNAs, **miR-145** controls the early chondrogenesis via suppressing Sox9, thereby inhibiting chondrocyte differentiation in mouse MSCs (Yang et al., 2011a) and human articular chondrocytes (Martinez-Sanchez et al., 2012). This mechanism appears to be specific to chondrogenesis, as no changes are observed in cell proliferation or adipogenic differentiation upon miR-145 overexpression (Yang et al., 2011a). Similarly, **miR-199a*** (also known as miR-199a-3p) inhibits the early stages of chondrogenic differentiation of C3H10T1/2 cells by targeting Smad1, a downstream molecule of BMP2 signaling (Lin et al., 2009a). Besides their functions during osteogenesis, **miR-29a** and **miR-29b** appear to inhibit chondrocyte differentiation in mouse MSCs and mesenchymal cell lines by suppressing Col2a1 expression (Yan et al., 2011). Based on gain- and loss-of-function studies, expression of these miRNAs is controlled by Sox9. **miR-34a** and

miR-221 are induced in chicken mesenchymal cells upon inhibition of chondrogenesis with a JNK inhibitor. miR-34a inhibits cell migration and condensation of chick limb mesenchymal cells through suppressing EPH receptor A5 (Kim et al., 2011a). Furthermore, it modulates the reorganization of the actin cytoskeleton by targeting RAC1 thereby impairing the RhoA/Rac1 cross-talk in chicken chondrogenic progenitors (Kim et al., 2012a). The negative effect of miR-221 on chondrogenesis is mediated through downregulation of MDM2 leading to accumulation of Slug protein (Kim et al., 2010a). **miR-1** and **miR-18a** are repressed during late stages of chondrogenic differentiation and ectopic expression of these miRNAs results in impaired chondrogenesis in human chondrocytic HCS-2/8 cells (Ohgawara et al., 2009; Sumiyoshi et al., 2010). While miR-18a is thought to function through downregulation of the connective tissue growth factor CTGF/CCN2, the mechanism of miR-1 in chondrocytes remains unidentified.

Table 2. Regulatory functions of miRNAs in chondrocytes

miRNA(s)	Target gene(s)	Function	References(s)
miR-1	-	Inhibits chondrogenic differentiation of HCS-2/8 cells	(Sumiyoshi et al., 2010)
miR-18a	*CTGF/CCN2*	Inhibits chondrogenic differentiation of HCS-2/8 cells	(Ohgawara et al., 2009)
miR-21	-	Promotes proliferation and matrix synthesis of rat chondrocytes embedded in atelocollagen gel	(Kongcharoensombat et al., 2010)
miR-23b	*PKA*	Increases aggrecan expression in hMSCs	(Ham et al., 2012)
miR-29a/b	*Col2a1*	Inhibit chondrogenesis in C3H10T1/2 and ATDC5 cell lines; Suppressed by Sox9	(Yan et al., 2011)
miR-34a	*RAC1, EPHA5*	Inhibits precartilage condensation and chondrogenic differentiation of chick limb mesenchymal cells	(Kim et al., 2011a; Kim et al., 2012a)
miR-140	*Adamts-5, Bmp2, Cxcl12, Dnpep, Hdac4, Smad3, Sp1*	Involved in endochondral bone development and cartilage homeostasis; Promotes chondrocyte proliferation; Expression is induced by Sox9; Reduced expression in human OA tissues	(Miyaki et al., 2009; Miyaki et al., 2010; Nakamura et al., 2012; Nicolas et al., 2008, 2011; Pais et al., 2010; Yang et al., 2011b)
miR-142-3p	*ADAM9*	Regulates TGF-β3-mediated region-dependent chondrogenesis in chicken limb	(Kim et al., 2011b)
miR-145	*SOX9*	Inhibits chondrogenesis in human chondrocytes and mMSCs	(Martinez-Sanchez et al., 2012; Yang et al., 2011a)

miRNA(s)	Target gene(s)	Function	References(s)
miR-194	*SOX5*	Inhibits chondrogenic differentiation of hASCs	(Xu et al., 2012)
miR-199a*	*Smad1, Cox2*	BMP2 responsive miRNA that inhibits early chondrocyte differentiation of C3H10T1/2 cells	(Akhtar and Haqqi, 2012; Lin et al., 2009a)
miR-221	*MDM2*	Inhibits chondrogenic differentiation of chick limb mesenchymal cells by preventing the degradation of the Slug protein	(Kim et al., 2010a)
miR-337	*Tgfbr2*	Promotes chondrocyte proliferation at early phases of endochondral bone formation	(Zhong et al., 2012)
miR-365	*HDAC4*	Promotes chondrocyte proliferation and differentiation; Expression is stimulated upon mechanical loading	(Guan et al., 2011)
miR-449a	*LEF-1*	Suppresses chondrogenesis of human osteosarcoma cell lines and hMSCs	(Paik et al., 2012)
miR-488	-	Inhibits cell migration during chondrogenic differentiation of chick limb mesenchymal cells	(Song et al., 2011)

ATDC5, a chondrogenic cell line; C3H10T1/2, a mouse mesenchymal cell line; HCS-2/8, a human chondrosarcoma cell line

2.3.4 Regulation of adipogenesis by miRNAs

A large number miRNAs have validated functions related to adipogenic differentiation and function (Table 3). Based on *in vitro* studies with human adipose tissue-derived stem cells and murine adipogenic 3T3-L1 cells, miRNAs mediate their actions through several targets involved in MSC commitment and subsequent differentiation of pre-adipocytes into mature adipocytes. In addition, miRNAs appear to play a role in the regulation of mature adipocyte function. **miR-143** is the first miRNA associated with adipogenesis (Esau et al., 2004). It is upregulated in differentiating human adipocytes and 3T3-L1 cells, and inhibition of miR-143 blocks adipogenesis (Esau et al., 2004; Yi et al., 2011). The positive effect of miR-143 on adipogenesis is mediated at least in part through suppression of the extracellular-signal-regulated kinase 5 (ERK5) and pleiotrophin (PTN), which are both important regulators of cell proliferation and differentiation.

Owing to the close relationship between osteogenesis and adipogenesis, the same miRNAs may play a role, parallel or inverse, in both differentiation pathways. For example, **miR-22** regulates the balance between adipogenic and

osteogenic differentiation by suppressing HDAC6 expression in human adipose tissue-derived mesenchymal stem cells (hADMSCs). While endogenous miR-22 is decreased during adipogenic differentiation of hADMSCs, increased expression is observed upon osteogenic induction. As forced expression of miR-22 results in repressed adipogenesis and enhanced osteogenesis, miR-22 is considered a negative regulator of adipocyte differentiation. In contrast, **miR-30 family** members seem to favor adipogenesis at the expense of osteogenesis by targeting the transcription factor Runx2. These miRNAs are downregulated upon BMP2-induced osteoblastogenesis of MC3T3-E1 cells (Wu et al., 2012) but become repressed during adipogenic differentiation of hASCs (Zaragosi et al., 2011). At least miR-30a and miR-30d mediate their actions in hASCs by targeting RUNX2 directly. **miR-138** provides an example of reciprocal miRNA regulation as it appears to inhibit both adipogenic and osteogenic differentiation of hMSCs, although through different mechanisms. While miR-138 suppresses osteogenesis at least partially by indirect repression of RUNX2 through PTK2 (Eskildsen et al., 2011), its inhibitory role during adipogenesis is explained by the repression of EID-1 (EP300 interacting inhibitor of differentiation 1) (Yang et al., 2011c). EID-1, in turn, is suggested to promote adipogenesis through suppression of TGF-β.

PPARγ, the key transcription factor for adipogenesis is targeted by at least mir-27 and miR-130 family members **miR-27a/b** and **miR-130a/b** (Karbiener et al., 2009; Kim et al., 2010b; Lee et al., 2011; Lin et al., 2009b). mir-27a is highly expressed in mouse adipose tissue and is inversely correlated with PPARγ expression during adipogenic differentiation of 3T3-L1 cells (Kim et al., 2010b). Correspondingly, miR-27b level is reduced during adipogenic differentiation of hMADSCs (human multipotent adipose tissue-derived stem cells) (Karbiener et al., 2009). Forced expression of miR-27a or miR-27b in 3T3-L1 or hMADSCs, respectively, leads to suppressed adipogenesis in response to repressed PPARγ expression. A similar mechanism has been suggested for miR-130a and miR-130b, which are both downregulated during adipogenic differentiation of human preadipocytes and 3T3-L1 cells (Lee et al., 2011). While overexpression of miR-130a or miR-130b in human preadipocytes suppresses adipogenesis, miR-130 inhibition results in increased expression of adipocyte marker genes and enhanced lipid accumulation.

Let-7 and the miRNA cluster miR-17-92 have been associated with the regulation of clonal expansion in 3T3-L1 cells (Sun et al., 2009; Wang et al., 2008). 3T3-L1 cells are murine fibroblast-like cells that are extensively used as a model to study adipogenesis *in* vitro. Adipogenic induction of these cells initiates a complex sequence of events including clonal expansion, cell cycle exit, and finally terminal differentiation (Rosen et al., 2000). Although the *in vivo* relevance of clonal expansion is unclear, strong evidence exists showing that some mitosis-related proteins contribute to adipogenesis. **Let-7** is upregulated during adipogenic differentiation of murine 3T3-L1 and 3T3-F442A cells as well as in mature adipocytes isolated from mouse adipose tissue (Sun et al., 2009). Ectopic expression of let-7a causes cell cycle delay and repression of several cell

cycle-associated genes. These effects are suggested to result from suppression of Hmga2 (high mobility group AT-hook 2), a transcription factor regulating growth and proliferation and a validated target of let-7 in other contexts. The **miR-17-92** cluster is composed of 3 polycistronic miRNA genes and encodes a total of 15 miRNAs. It is associated with several cancers, where it promotes proliferation thus acting as an oncogene (Concepcion et al., 2012). In addition to malignant tissue, miR-17-92 is upregulated during the clonal expansion phase of 3T3-L1 cells (Wang et al., 2008). Transfection of miR-17-92 into 3T3-L1 accelerates hormone-induced adipogenesis and increases triglyceride accumulation, at least in part by targeting the tumor suppressor RBL2/p130.

With respect to adipocyte function, **miR-103** and **miR-107** have been predicted to target multiple genes involved in acetyl-CoA and lipid metabolism (Wilfred et al., 2007). Based on recent *in vivo* data, these miRNAs are upregulated in obese mice, and silencing of miR-103/107 leads to improved glucose homeostasis and insulin sensitivity. Caveolin-1 was identified as a direct target for miR-103/107 providing an intriguing target for the treatment of type 2 diabetes and obesity (Trajkovski et al., 2011).

Table 3. Regulatory functions of miRNAs in adipocytes

miRNA(s)	Target gene(s)	Function	References(s)
let-7	*Hmga2*	Inhibits clonal expansion and terminal differentiation of 3T3-L1 cells	(Sun et al., 2009)
miR-15a	*Dlk1*	Inhibits cell size and proliferation of 3T3-L1 cells	(Andersen et al., 2010)
miR-17-92	*Rb2/p130*	Accelerates hormone-induced adipogenesis of 3T3-L1 cells	(Wang et al., 2008)
miR-21	*TGFBR2*	Promotes adipogenic differentiation of hASCs	(Kim et al., 2009b)
miR-22	*HDAC6*	Inhibits adipocyte differentiation of hADMSCs	(Huang et al., 2012)
miR-27a/b	*PPARγ*	Inhibit adipogenesis of 3T3-L1 cells and hMADSCs	(Karbiener et al., 2009; Kim et al., 2010b; Lin et al., 2009b)
miR-30a/d	*RUNX2*	Promote adipogenesis in hASCs	(Zaragosi et al., 2011)
miR-30c	*PAI-1, ALK-2*	Promotes adipogenesis in hMADSCs by co-repressing two unconnected pathways	(Karbiener et al., 2011)
miR-103, miR107	*Cav1*	Negative regulators of glucose homeostasis and insulin sensitivity, upregulated in obese mice	(Trajkovski et al., 2011)
miR-130a/b	*PPARγ*	Inhibit adipogenesis in human preadipocytes	(Lee et al., 2011)
miR-138	*EID-1*	Inhibits adipogenic differentiation of hADMSCs	(Yang et al., 2011c)
miR-143	*ERK5, Ptn*	Promotes adipogenesis in primary human adipocytes and 3T3-L1 cells	(Esau et al., 2004; Yi et al., 2011)
miR-210	*Tcf7l2*	Possible positive effect on adipogenesis through suppression of Wnt signaling	(Qin et al., 2010)
miRNA-155, -221, -222	-	Inhibit adipogenesis in hMSCs	(Skarn et al., 2011)
miR-193b-365 cluster	*Runx1t1, Cdon, Igfbp5*	Enriched in BAT, promote brown adipocyte differentiation of C2C12 cells and human myoblasts	(Sun et al., 2011)
miR-375	-	Promotes adipogenic differentiation of 3T3-L1 cells	(Ling et al., 2011)
miR-448	*Klf5*	Inhibits adipogenic differentiation of 3T3-L1 cells	(Kinoshita et al., 2010)

C2C12, a mouse myoblast cell line; 3T3-L1, mouse adipogenic cell line

2.3.5 Regulation of osteoclast differentiation by miRNAs

Despite the obvious phenotype in mice with conditional deletion of *Dicer* in osteoclast lineage cells, only a few miRNAs have been identified to contribute to osteoclastogenesis (Table 4). A global analysis of miRNA expression shows 38 miRNAs with increased expression and 33 miRNAs with decreased expression during RANKL-induced osteoclastogenesis of mouse BMCs (bone marrow cells) (Sugatani et al., 2011), suggesting that osteoclast differentiation may be influenced by the activity of many more miRNAs than currently understood.

miR-223 is the first miRNA associated with osteoclast differentiation and is highly expressed in mononuclear osteoclast precursor cells (Sugatani and Hruska, 2007). Controversial data exist concerning the effect of miR-223 on osteoclastogenesis. Although initially described as a negative regulator of osteoclastogenesis, miR-223 has been subsequently shown to stimulate osteoclast differentiation through a positive feedforward mechanism consisting of the transcription factor PU.1, miRNA-223, nuclear factor I/A (NFI-A), and M-CSF receptor (M-CSFR) (Sugatani and Hruska, 2009). In this cascade, PU.1 induces miR-223 expression leading to downregulation of NFI-A (nuclear factor I/A), a negative regulator of M-CSFR, and thereby to activation of M-CSF. However, in a recent study related to rheumatoid arthritis (RA), miR-223 was described to impair *in vitro* osteoclastogenesis through an unclear mechanism (Shibuya et al., 2012). **miR-155** is a negative regulator of osteoclastogenesis controlling the cell-fate decision between macrophage and osteoclast lineages (Mann et al., 2010). miR-155 level is rapidly upregulated at the onset of macrophage differentiation while ectopic expression of miR-155 in RAW264.7 mononuclear precursor cells leads to impaired osteoclast formation. The effect of miR-155 appears to be mediated through downregulation of MITF and SOCS1 (suppressor of cytokine signaling 1), both essential regulators of osteoclastogenesis (Zhang et al., 2012a).

miR-21 is among the most robustly stimulated miRNAs identified in the global expression profiling during osteoclastogenesis and is suggested to promote osteoclast differentiation by targeting programmed cell death 4 (PDCD4) (Sugatani et al., 2011). Decreased PDCD4 expression removes a repression from c-Fos, which is an essential transcription factor for osteoclastogenesis. On the other hand, the transcription factor c-Fos upregulates miR-21, constituting a feedforward mechanism to drive miR-21 expression and thereby to promote differentiation during osteoclastogenesis. In addition, **miR-146a**, previously identified as a negative regulator of immune and inflammatory responses, seems to play a role in osteoclastogenesis. miR-146 is abundantly expressed in human peripheral blood mononuclear cells (hPBMCs) and elevated levels are observed in patients with RA (Nakasa et al., 2008; Pauley et al., 2008; Stanczyk et al., 2008). Being induced by NF-κβ, miR-146a controls Toll-like receptor and cytokine signaling in human monocytes by targeting IRAK1 (interleukin-1 receptor-associated kinase 1) and TRAF6 (Taganov et al., 2006). With respect to osteoclasts, ectopic expression of miR-146a in hPBMCs results in decreased expression of osteoclast-related transcription factors and reduced number of

TRACP-positive multinucleated cells (Nakasa et al., 2011). The inhibition of osteoclast formation by miR-146a may result from downregulation of TRAF6, which is an essential transcription factor for osteoclastogenesis.

Table 4. Regulatory functions of miRNAs in osteoclasts

miRNA	Target gene(s)	Function	References(s)
miR-21	*Pdcd4*	Promotes osteoclast differentiation though a positive feedback loop of c-Fos/miR-21/Pdcd4	(Sugatani et al., 2011)
miR-146a	*TRAF6*	Inhibits hPBMC osteoclast differentiation and suppresses bone destruction in mice with collagen-induced arthritis	(Nakasa et al., 2011; Taganov et al., 2006)
miR-155	*Mitf, Socs1*	Inhibits osteoclast differentiation of RAW264.7 cells; Essentially involved in the pathogenesis of collagen-induced arthritis in mice	(Mann et al., 2010; Zhang et al., 2012a)
miR-223	*Nfia*	Promotes osteoclast differentiation by upregulating M-CSFR level through suppression of NFI-A in mOC precursors and RAW264.7 cells	(Sugatani and Hruska, 2009)

hPBMC, human peripheral blood mononuclear cells

2.4 Involvement of miRNAs in skeletal disease

Over the past few years, an increasing number of miRNAs have been associated with skeletal diseases, namely osteoporosis (OP) and OA (Table 5). OP is a bone loss disease characterized by reduced BMD and consequently, increased risk of fractures. OA is the result of chronic destruction of articular cartilage and subchondral bone, causing joint stiffness, pain and loss of movement in the joint.

Although comprehensive analysis of miRNA expression and function upon OP is lacking, two specific miRNAs, namely **miR-34c** and **miR-2861**, have been described to contribute to pathogenesis of OP. miR-34c is a negative regulator of osteogenesis and becomes upregulated during BMP2-induced osteoblast differentiation (Bae et al., 2012; Wei et al., 2012). Osteoblast-specific overexpression of miR-34c in mice results in an age-dependent bone loss due to impaired osteoblast proliferation and differentiation and enhanced osteoclastogenesis (Bae et al., 2012). These effects are mediated at least in part through repression of Notch signaling, as Notch1, Notch2, and Jag1, all important components of the Notch signaling pathway, are direct targets for miR-34c. The phenotype of transgenic mice expressing miR-34c in osteoblasts resembles that of mice lacking Notch signaling in osteoblasts (Engin et al., 2008). Suppressed Notch signaling in committed osteoblasts results in downregulation of OPG, and thereby, enhanced osteoclastogenesis. In a very similar experimental setup, miR-34c was

shown to inhibit osteoblast proliferation and differentiation *in vivo* by suppressing Cyclin D1 and Satb2, respectively (Wei et al., 2012), indicating that miR-34c contributes to bone homeostasis through multiple targets. miR-2861 is a rather recently identified miRNA that is predominantly expressed in bone and becomes upregulated during BMP2-induced osteoblast formation (Li et al., 2009a). Besides its important function as a positive regulator of Runx2 signaling, miR-2861 has been associated with OP in humans. Two related adolescents with juvenile OP have been described to carry a homozygous mutation in pre-miR-2861, leading to loss of mature miR-2861 in their bones (Li et al., 2009a). Although this variant appears to be rare, it serves as a significant example of how dysregulated miRNA expression may lead to development or progression of a skeletal disorder.

In addition, polymorphism in miRNA target site may predispose to OP, as suggested in the association study on predicted polymorphisms in miRNA target sites and OP (Lei et al., 2011). These analyses identify three polymorphisms in the FGF2 miRNA binding sites that associate with femoral neck BMD. Furthermore, repressed FGF2 gene expression is observed in subjects with high BMD compared to subjects with low BMD, suggesting an association between impaired miRNA binding and OP.

The expression and function of miRNAs in OA is under intensive investigation and a few specific miRNAs have been identified with relevance to OA (Table 5). Based on miRNA profiling in human bone and cartilage, a number of miRNAs display an altered expression pattern upon OA in comparison to normal tissues (Iliopoulos et al., 2008; Jones et al., 2009). Through a combination of computational pathway analyses and gain- and loss-of-function studies, the most differentially expressed miRNAs have been identified to play a role in inflammatory responses by modulating the expression of TNF-α and MMP13 (Jones et al., 2009). Essentially, three miRNAs, **miR-9**, **miR-22** and **miR-27b,** appear to contribute to MMP-13 expression in OA cartilage. While miR-22 promotes MMP-13 expression indirectly via downregulation of BMP7 and PPARα (Iliopoulos et al., 2008), miR-27b is a direct regulator of MMP-13 thereby suppressing its expression (Akhtar et al., 2010). miR-9 appears to repress MMP-13 level in human chondrocytes, although the detailed mechanisms remain unknown (Jones et al., 2009). As discussed above, **miR-140** is essential for normal cartilage development and homeostasis. Targeted deletion of miR-140 in mice results in abnormal growth and premature OA phenotype, whereas transgenic mice overexpressing miR-140 are protected from antigen-induced OA. Accordingly, several studies demonstrate reduced miR-140 levels in human OA cartilage (Iliopoulos et al., 2008; Miyaki et al., 2009; Tardif et al., 2009).

miR-146a is a negative regulator of immune responses and is thus suggested to play a role in the inflammatory process of OA progression. miR-146a is highly expressed in low-grade OA cartilage, making it an attractive candidate to be used as a biomarker for early diagnosis of OA. miR-146 expression decreases along OA progression, showing a negative correlation with MMP13 level (Yamasaki et al., 2009). Based on a recent study with rat primary chondrocytes,

miR-146a drives its action by suppressing Smad4 expression resulting in reduced cellular responsiveness to TGF-β and accelerated chondrocyte apoptosis (Li et al., 2012). Besides its function in chondrocytes, miR-146a is associated with the OA-associated analgesia by modulating the expression of pain-related molecules, e.g. TNF-α, COX-2 (for cytochrome c oxidase subunit II), inducible nitric oxide synthase (iNOS), and interleukins IL-6 and IL-8, in human glial cells (Li et al., 2011). **miR-199a*** is downregulated in human chondrocytes in response to IL-1β and may play a role in OA-associated analgesia by targeting the inflammation and pain-related enzyme COX-2 (Akhtar and Haqqi, 2012). **miR-455-3p** is expressed during mouse limb development and is upregulated in human OA cartilage compared to normal tissue (Swingler et al., 2012). It is suggested to interfere with TGF-β pathway and thereby to contribute to OA pathogenesis via indirect suppression of Smad2/3 signaling.

In addition, miRNAs hold promise to serve as diagnostic markers of OP and OA. Profiling of miR-16, miR-132, miR-146a, miR-155 and miR-223 in human plasma appears to be sufficient to differentiate OA or RA patients from healthy controls (Murata et al., 2010). Furthermore, synovial fluid concentrations of miR-16, miR-146a miR-155 and miR-223 of RA were significantly higher than those of OA, thus enabling the discrimination between RA and OA. Analysis of miR-133a expression in human circulating monocytes may provide a novel strategy to evaluate OP in postmenopausal women (Wang et al., 2012). Its expression is high in postmenopausal Caucasian women with low BMD (spine or hip Z-score <-0.84) in comparison to women with high BMD (spine or hip Z-score >0.84). Based on computer-based target predictions, miR-133a may regulate genes associated with osteoclast function, but this has not been experimentally validated.

Table 5. Involvement of miRNAs in skeletal diseases

miRNA	Disease	Description	Reference(s)
miR-9	OA	Upregulated in OA cartilage, modulates TNF-α and MMP13 expression	(Jones et al., 2009)
miR-22	OA	Increased expression in human OA cartilage, regulates PPARα and BMP7 expression in human normal and OA chondrocytes	(Iliopoulos et al., 2008)
miR-27b	OA	Decreased expression in IL-1β-stimulated human OA chondrocytes, direct regulator of MMP13	(Akhtar et al., 2010)
miR-34c	OP	Mice overexpressing miR-34c display aged-related OP	(Bae et al., 2012)
miR-140	OA	Downregulated in OA cartilage and suppressed by IL-1β	(Miyaki et al., 2009; Miyaki et al., 2010)
miR-146a	OA	Upregulated in OA cartilage, induced by IL-1β, a direct regulator of Smad4	(Li et al., 2012; Li et al., 2011; Yamasaki et al., 2009)
miR-199a*	OA	Downregulated in OA cartilage, suppressed by IL-1β, a direct regulator of COX-2	(Akhtar and Haqqi, 2012)
miR-455-3p	OA	Strongly expressed in OA cartilage, suppresses Smad2/3 pathway by targeting components of the TGF-β signaling pathway	(Swingler et al., 2012)
miR-2861	OP	Mutations of pre-miR-2861 are associated with primary OP in humans	(Li et al., 2009a)

CIA, collagen-induced arthritis; OA, osteoarthritis; OP, osteoporosis

3 AIMS OF THE STUDY

When the present study was initiated, there was an increasing interest in the role of microRNAs in various cellular processes. Although their essential functions had been pointed out in many other tissues, information related to their expression and function in skeletal tissue was limited. This thesis project aimed to broaden the knowledge of microRNA regulation in bone and cartilage cells, and thereby to identify novel pathways for potential therapeutic approaches of skeletal diseases. The following specific aims were set:

1. To study the expression of miRNAs during osteogenic and chondrogenic differentiation of mouse MSCs.

2. To identify and characterize miRNAs involved in the differentiation of human MSCs towards osteogenic, chondrogenic and adipogenic lineages.

3. To characterize the regulatory networks comprised of transcription factors, miRNAs under study and target mRNAs.

4. To identify miRNAs involved in osteoclast differentiation by studying the expression profile of selected miRNAs during osteoclastogenesis.

4 MATERIALS AND METHODS

Detailed description of the materials and methods is available in the original publications (I-III) as referred below.

4.1 Isolation of cells

4.1.1 Mouse mesenchymal stem cells (I)

Bone marrow cells (BMCs) were isolated from the tibiae and femora of 8–12 week-old male C57BL×DBA mice. To remove rapidly adhering fibroblast-like cells, BMCs were incubated on plastic for 2 h at 37 °C. Non-adherent cells were collected, re-plated and cultured for 48 hours. Adherent cells were collected, expanded and enriched for MSCs by selective culture conditions. Thereafter, cells were trypsinized, immunophenotypically characterized and subjected to osteogenic or chondrogenic differentiation.

4.1.2 Human mesenchymal stem cells (II)

Human bone marrow MSCs (hMSCs) were obtained through collaboration with Professor Hannu Aro (University of Turku and Turku University Hospital, Finland) and were collected with the approval of the local Ethical Committee. These cells have been shown to be multipotent and express surface markers characteristic for MSCs (Alm et al., 2010). hMSCs (passage 2) were recovered from liquid nitrogen, cultured until near confluence, and thereafter subjected to osteogenic, chondrogenic or adipogenic differentiation.

4.1.3 Mouse bone marrow cells (III)

Mouse bone marrow cells (mBMCs) were isolated as described in chapter 4.1.1 and after 2 h of adherence, non-adherent cells were subjected to osteoclast differentiation.

4.2 Differentiation

4.2.1 Osteogenic differentiation (I, II)

For osteogenic differentiation, MSCs were cultured in osteoinductive medium for 3-4 weeks with medium changes every 3-4 days. Dexamethasone was present only for the first week. For mouse MSCs (mMSCs), a subculture method was used (Qu et al., 1998). The initial concentration of cells and the composition of culture media are summarized in Table 6.

Table 6. Culture conditions for osteogenic differentiation

Parameter/Ingredient	mMSC	hMSC	Supplier
Cell density (cells/cm^2)	10 000	2 500	-
Culture medium	α-MEM	α-MEM	Gibco Invitrogen
FBS	12 %	10 %	Gibco Invitrogen
Penicillin	100 U/ml	100 U/ml	Gibco Invitrogen
Streptomycin	100 µg/ml	100 µg/ml	Gibco Invitrogen
Na-β-glycerophosphate	10 mM	10 mM	Fluka BioChemika
Ascorbic acid 2-phosphate	50 µg/ml	250 µM	Sigma-Adrich
Dexamethasone	10 nM	100 nM	Sigma-Aldrich

MEM, minimum essential medium; FBS, fetal bovine serum

4.2.2 Chondrogenic differentiation (I, II)

For chondrogenic induction, MSCs were cultured as pellets (Johnstone et al., 1998) for 21 days, with medium changes every 3-4 days. The initial amount of cells and the composition of culture media are presented in Table 7.

Table 7. Culture conditions for chondrogenic differentiation

Parameter/Ingredient	mMSC	hMSC	Supplier
Cells/pellet	200 000	200 000	-
Culture medium	high-glucose DMEM	high-glucose DMEM	Gibco Invitrogen
Penicillin	100 U/ml	100 U/ml	Gibco Invitrogen
Streptomycin	100 µg/ml	100 µg/ml	Gibco Invitrogen
TGF-β3	10 ng/ml	10 ng/ml	R&D Systems
Dexamethasone	100 nM	100 nM	Sigma-Aldrich
L-proline	40 µg/ml	-	Sigma-Aldrich
Sodium pyruvate	100 µg/ml	-	Sigma-Aldrich
Ascorbic acid 2-phosphate	50 µg/ml	100 nM	Sigma-Aldrich
ITS Premix	50 mg/ml	50 mg/ml	BD Biosciences

DMEM, Dulbecco's Modified Eagle Medium; TFG, transforming growth factor; ITS, insulin, transferrin and selenous acid

4.2.3 Adipogenic differentiation (II)

For adipogenic differentiation, hMSCs were cultured for 4 weeks in α-MEM supplemented with 10% FBS, 10 µg/ml insulin (Sigma-Aldrich), 100 µM indomethacin (Sigma-Aldrich), 500 µM isobutylmethylxanthine (Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco Invitrogen), with medium changes every 3-4 days.

4.2.4 Osteoclast differentiation (III)

For osteoclast differentiation, mBMCs were cultured for 3 or 7 days on bovine cortical bone slices (10^6 cells/slice) following a previously described method (Hentunen et al., 1995). The culture medium consisted of α-MEM supplemented with 10% FBS, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Gibco Invitrogen), 10 ng/ml M-CSF (R & D Systems, UK), 20 ng/ml RANKL (Peprotech, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco Invitrogen).

4.3 Evaluation of differentiation

At selected time points, cells subjected to osteogenic, chondrogenic, or adipogenic stimuli as well as cells cultured under osteoclast differentiation were fixed in 3-4% paraformaldehyde (PFA) and evaluated through histologic methods. Osteogenic differentiation was evaluated based on alkaline phosphatase (ALP) activity or matrix mineralization. Chondrogenic cell pellets were dehydrated,

embedded in paraffin, and thereafter analyzed by toluidine blue staining and immunohistochemical staining for type II collagen. The formation of lipid droplets in cells subjected to adipogenic differentiation was demonstrated by Oil Red O staining. With respect to osteoclast differentiation, the formation of multinuclear (>3 nuclei/cell) cells was demonstrated through nuclear staining. In addition, osteoclast cultures were evaluated by their TRACP activity and through formation of resorption pits. Histological methods and reagents used to evaluate the outcome of differentiation are summarized in Table 8.

Table 8. Reagents and histological methods

Reagent	Application	Supplier/Reference	Used in
2% silver nitrate	Mineralization	(von Kossa, 1901)	I
AlexaFluor 488 Phalloidin	Actin	Molecular Probes	III
CD34 Mouse MAb	ICC for CD34	Vision Biosystems	I
CD45 Mouse MAb	ICC for CD45	Dako	I
Hoechst 33258	Nuclei	Molecular Probes	III
Leukocyte acid phosphatase kit	TRACP activity	Sigma-Aldrich	III
Leukocyte Alkaline Phosphatase Kit	ALP activity	Sigma-Aldrich	I, II
Mouse MAb 6B3	IHC for type II collagen	Chemicon, (Salminen et al., 2001)	I
Oil Red O	Lipids	Sigma-Aldrich	II
Sca-1 Rat MAb	ICC for Sca-1	BD Biosciences	I
1% Toluidine blue	Proteoglycans	Sigma-Aldrich	I, II
WGA-lectin	Resorption pits	Sigma-Aldrich, USA	III

ALP, alkaline phosphatase; ICC, immunocytochemistry; IHC, immunohistochemistry; MAb, monoclonal antibody; TRACP, tartrate-resistant acid phosphatase; WGA, wheat germ agglutinin

4.4 RNA analyses

Reagents used for RNA isolation as well as the analysis of mRNA or miRNA expression are listed in Table 9.

Table 9. Reagents and applications used for RNA analyses

Reagent	Application	Supplier	Used in
DNase I	DNase treatment	Ambion	I, III
DyNAzyme II DNA Polymerase	RT-PCR	Finnzymes	I
mirVana miRNA Isolation Kit	RNA/miRNA isolation	Applied Biosystems	I, II, III
mirVana qRT-PCR miRNA Detection Kit	miRNA expression analysis	Ambion	I, III
M-MLV reverse transcriptase	RT-PCR	Promega	I
RQ1 DNase	DNase treatment	Promega	II
Taq DNA Polymerase	qRT-PCR	ABgene	I, III
TaqMan Gene Expression Assays	qRT-PCR	Applied Biosystems	II
TaqMan MicroRNA Assays	qRT-PCR	Applied Biosystems	II

M-MLV, Moloney Murine Leukemia Virus; RT-PCR, real time polymerase chain reaction; qRT-PCR, quantitative real time polymerase chain reaction

4.5 Functional assays (II)

Functional characterization of miR-96, miR-124 or miR-199a was performed by transfecting Pre-miR miRNA precursors (Ambion, USA), miRCURY LNA anti-microRNAs (Exiqon, Denmark) or their negative controls into hMSCs with a concentration of 100 nM. Transfected cells were seeded at 10 000 cells/cm^2, cultured for 4 days in α-MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin, and thereafter subjected to RNA analyses.

4.6 Bioinformatics and statistical analyses (I-III)

For miRNA target prediction, TargetScan (www.targetscan.org), PicTar (http://pictar.mdc-berlin.de/) and miRBase, former miRanda, (www.microrna.org) were used. Conreal and TRANSFAC professional 9.4. were used to predict transcription factor binding sites (TFBS) in the regulatory regions 500 bp upstream and 100 bp downstream from the starting locus of selected pre-miRNAs. Pathway analyses were carried out using the Ingenuity Pathway Analysis 5.0 (www.ingenuity.com) software. SAS 16.1 was used for statistical analyses.

5 RESULTS AND DISCUSSION

5.1 Characterization and differentiation of MSCs (I, II)

Research on MSC field has been challenged by biological variability as conflicting results are observed in studies reproduced by multiple laboratories. There are several explanations for this inconsistency, one of the most significant being the difficulty of defining the true MSCs. While the surface marker profiles of hematopoietic cells at their various lineages and differentiation stages are well characterized, there is currently no consensus on markers that can separate multipotential MSCs from their more committed progeny. Accordingly, varying sets of surface antigens are used to define MSCs. With an attempt to reduce this diversity, the International Society of Cellular Therapy (ISCT) has suggested the minimal criteria to define hMSCs (Dominici et al., 2006). Based on ISCT criteria, hMSCs should express CD105, CD73, and CD90, but be negative for CD11b or CD14, CD19 or CD79a, CD34, CD45, and HLA-DR.

In order to study miRNA expression and function during osteogenesis, chondrogenesis and adipogenesis, MSCs from mouse and human BM were used. The surface marker patterns of these cells have been characterized by flow cytometry and/or immunohistochemistry, and the detailed results are reported elsewhere (Taipaleenmäki et al., 2008; Alm et al., 2010). In brief, *in vitro* expanded hMSCs expressed CD105, CD73, and CD90, and were negative for the hematopoietic markers CD14, CD34 and CD45 (Table 10). Enrichment of mMSCs is more problematic, and cultures are prone to be contaminated by hematopoietic progenitors (Phinney et al., 1999). In the present study, mMSCs were enriched through serial passaging in a medium that has been demonstrated to inhibit the growth of hematopoietic cells (Eaves and Eaves, 1988). As a result, a homogenous population of cells negative for CD34 and CD45 was obtained, indicative of the successful removal of hematopoietic cells. Culture expanded mMSCs uniformly expressed stem cell antigen-1 (Sca-1), which is a commonly used positive marker for mMSCs (Baddoo et al., 2003; Jiang et al., 2002; Meirelles and Nardi, 2003; Peister et al., 2004).

MSCs from different species are known to display varying surface marker expression patterns (reviewed by Boxall and Jones, 2012). For instance, while cultured hMSCs are constantly described as CD105, CD90, and CD73 positive, some papers report variable expression of these markers in murine MSCs (Peister et al., 2004; Pelekanos et al., 2012). Although not related to this study, varying expression patterns are observed even in MSCs isolated from different mouse strains (Peister et al., 2004). It is important to note, however, that MSCs undergo phenotypic rearrangements during *ex vivo* manipulations (Jones et al., 2002). Therefore, the *in vitro* expression pattern does not necessarily correlate with the *in vivo* situation, and variation may be observed even with increasing passages.

Table 10. The expression of surface markers

Marker	mMSC	hMSC	Reference
CD14	n.a.	-	(Dominici et al., 2006)
CD34	-	-	(Baddoo et al., 2003; Dominici et al., 2006)
CD45	-	-	(Baddoo et al., 2003; Dominici et al., 2006)
CD73	n.a.	+	(Dominici et al., 2006)
CD90	n.a.	+	(Dominici et al., 2006)
CD105	n.a.	+	(Dominici et al., 2006)
Sca-1	+	n.a.	(Baddoo et al., 2003)

n.a., data not available

MSCs are also characterized by their ability to differentiate into osteogenic, chondrogenic and adipogenic lineages (Dominici et al., 2006). By using classical differentiation protocols, MSCs in this study proved their potential for trilineage differentiation, as evidenced by histological methods, and by the expression of genes associated with osteogenesis, chondrogenesis and adipogenesis (I: Figure 2, II:Figure 1, II: Figure 2). The adipogenic differentiation of mMSCs has been described in another context (Taipaleenmäki et al., 2008). We and others (Arrigoni et al., 2009; Martínez-Lorenzo et al., 2009) have noticed that MSCs from various species diverge in their differentiation capacity. Comparison of mouse and human cultures revealed a marked variance in their differentiation capacity. During 3 weeks of osteogenic induction, mMSCs turned into ALP positive cells and von Kossa staining demonstrated the mineralized matrix. Despite the longer culture period for hMSCs, their differentiation capacity remained weaker with no signs of mineralization based on von Kossa staining (data not shown). A similar observation was made in respect to chondrogenic differentiation of mouse and human MSCs, mouse cells being more readily differentiated. For instance, type II collagen was markedly expressed following chondrogenic differentiation of mMSCs (I: Figure 2), whereas it remained undetectable in hMSC-derived chondrogenic pellets (data not shown). Prolongation of the culture period by 1-2 weeks could have resulted in more pronounced differentiation. Nevertheless, this material is too limited to make any conclusions of the differentiation capacity between mouse and human MSCs. The difficulties observed with hMSCs may result from the variation between different individuals, since cells were collected from four donors only.

Another important cause of variation is related to the use of multiple tissue sources. Cells exhibiting MSC-like characteristics have been found from various sites other than the bone marrow, including adipose tissue (Zuk et al., 2002), amniotic fluid (De Coppi et al., 2007), peripheral blood (Kuznetsov et al., 2001), umbilical cord (Rosada et al., 2003) and fetal tissues (In 't Anker et al., 2004). Although these cells share some common properties, their differentiation capacities and gene expression profiles diverge (Wagner et al., 2005). Therefore, results obtained from BM-derived MSCs may not be comparable with those obtained using MSCs isolated from other tissues. Besides the species differences and the tissue of origin, variability may be caused by the differences between

individual donors (Hung et al., 2006; Wagner and Ho, 2007) and even by the variance between different passages of the same donor (Goff et al., 2008; Wagner et al., 2008). In the present study, hMSCs from four individuals displayed rather similar proliferation rate and differentiation capacity, if only assessed through histological staining. However, analysis of gene expression revealed moderate differences, albeit smaller than in the case of miRNA expression (discussed below).

5.2 miRNA expression and function during MSC differentiation

5.2.1 Expression during osteogenic and chondrogenic differentiation of mouse MSCs (I)

When the present study was initiated, there was no previous information on the role of miRNAs in the skeleton. In order to address this issue, miRNA expression was studied during osteo- and chondrogenic differentiation of mouse BM-derived MSCs. The 35 evolutionarily conserved miRNAs were selected based on their reported association with stem cell differentiation, or computationally predicted importance for osteogenesis or chondrogenesis. As around 300 human miRNAs had been identified so far (miRBase, October 2005), our panel of 35 miRNAs represented approximately 10% of all known miRNAs. Illustrative of the rapid progress in this field, the latest release 19 of the miRNA database (miRBase, August 2012, www.mirbase.org) comprises about 2000 human mature miRNA sequences. Among the 35 miRNAs analyzed by real-time PCR (RT-PCR), miR-24 and miR-199b were the most significantly upregulated ones during osteogenic and chondrogenic differentiation (I: Figure 3). In addition to these, miR-101, miR-124 and miR-199a showed increased expression upon chondrogenesis. In contrast, miR-18 and miR-96 were downregulated upon chondrogenic induction. While most miRNAs had a similar expression pattern during osteogenesis and chondrogenesis, eight miRNAs (miR-18, miR-31, miR-96, miR-124, miR-130a, miR-130b, miR-142-3p, miR-199a) were distinguished by exhibiting at least 2-fold difference in their relative expression level (I: Table 1).

Since the beginning of this study, several microarray and real-time PCR-based expression profilings have been carried out in various experimental models revealing that many miRNAs exhibit a selective expression pattern in different stages of osteoblastogenesis and chondrogenesis. For example, global miRNA profiling before and after osteogenic induction of bone marrow-derived hMSCs reveal 13 miRNAs being differentially expressed between undifferentiated and differentiated cells (Gao et al., 2011). Among them, five miRNAs were upregulated and eight miRNAs were downregulated following osteogenic induction, although the variation between the three independent donors was high. In immortalized hMSC cell line, 33 miRNAs showed significantly altered miRNA expression during

osteoblast differentiation with almost equal number of miRNAs being up- and downregulated (Eskildsen et al., 2011). Global miRNA profiling before and after osteogenic induction of hASCs revealed eight differentially expressed miRNAs, of which half were upregulated and half were downregulated (Zhang et al., 2012c). With respect to chondrogenesis, eight miRNAs were upregulated during TGF-β3-induced chondrogenesis of mMSCs, miR-140* and miR-140 being the most significantly increased miRNAs (Yang et al, 2010). Concurrently, five miRNAs showed decreased expression, comprising two miRNA clusters, miR-143/145 and miR-132/212. In hMSCs, global miRNA profiling during chondrogenic differentiation identified four upregulated miRNAs: miR-26b, miR-28, miR-130b, miR-152, and miR-193b (Han et al, 2010). Global miRNA profiling during chondrogenic differentiation of CD146+ hMSCs revealed 36 differentially expressed miRNAs, out of which 12 were upregulated and 24 downregulated (Sorrentino et al., 2008). In general, results obtained from miRNA profiling studies are variable and even opposite expression patterns have been reported for specific miRNAs. Besides the sources of variability for MSCs (chapter 5.1), the comparison of miRNA profiling studies is hindered by the use of variable *in vitro* models, including different cell lines and primary cells obtained from various tissues and species, as illustrated by Tables 1-3 in the Review of Literature.

5.2.2 Expression during osteogenic, chondrogenic and adipogenic differentiation of human MSCs (II)

MSCs offer a great promise for clinical stem cell therapy of degenerative diseases due to their multiple beneficial properties, such as extensive proliferation and differentiation capacity in culture, immunosuppressive and immunomodulatory properties, as well as potential for homing. However, results obtained from mouse studies may not be directly transferrable to the human system. Therefore, we studied also the selected miRNAs in hMSCs. Expression levels of miR-96, miR-124 and miR-199a were analyzed in five different culture conditions representing undifferentiated MSCs, osteoblasts, chondrocytes, adipocytes and control cells cultured for same time period without induction. Based on RT-PCR analysis, miR-96 expression increased during the 4-week culture period in all four culture conditions (II: Figure 3). The most prominent increase was observed upon osteogenic and adipogenic induction while in control and chondrogenic conditions, upregulation of miR-96 was moderate. miR-124 was exclusively expressed during adipogenic differentiation while it stayed undetectable in the other conditions. miR-199a expression increased during osteogenesis and chondrogenesis, but remained at the baseline level during adipogenic induction.

In general, results were comparable with those obtained from mMSCs. However, the fold changes were to some extent higher in mouse than in human MSCs, which may be explained by their distinct differentiation capacities *in vitro*. We also observed rather significant inter-individual variation of miRNA expression between the four donors. This is an unavoidable, well-known problem

associated with human MSC cultures in general (Hung et al., 2006; Wagner and Ho, 2007). Although less comprehensively addressed with respect to miRNA expression, substantial variability between individual donors, as well as between different passages of the same donor, has been reported (Goff et al., 2008; Lakshmipathy and Hart, 2008; Wagner et al., 2008). To minimize such variation in the current study, hMSCs were collected from young healthy females and all inductions were initiated from the same cell passages.

5.2.3 Computer-based prediction of miRNA function (I)

To gain more insight into the potential cellular functions of the differentially expressed miRNAs, target prediction analyses were carried out. There are several miRNA target prediction tools available online, most of which rely on the complementary base pairing between the miRNA seed region and the 3' UTR of the mRNA (Bartel, 2009). In addition, some algorithms take into account the conservation among species and mRNA thermodynamics. However, most algorithms produce widely divergent predictions with considerable amount of false positives and false negatives that are difficult to assess (Min and Yoon, 2010). In order to reduce the divergence, a combination of three commonly used prediction software, PicTar, TargetScan and miRanda (former miRBase), was used. In the study of miRNA expression in mMSC-derived osteoblasts and chondrocytes, intersection sets of the three target prediction tools were produced and used for subsequent transcription factor and pathway analyses. Although this procedure probably reduced the amount of false positives, many genuine mRNA targets may have been missed. Therefore, in later studies, predictions were performed without the requirement of target identification by multiple programs.

In order to elucidate the regulatory networks constituted by miRNAs and transcription factors (TFs), promoter analysis was performed and conserved binding sites for known TFs were predicted for the upstream regions of the differentially expressed miRNA genes. The biological significance of miRNA target genes as well as the interplay between miRNA target genes and TFs was analyzed using Ingenuity Pathway Analysis (IPA) software. IPA is a web-based software application that associates specific data sets with biological mechanisms, functions, and well-known pathways on the basis of published observations (ww.ingenuity.com). The biological functions related to predicted target genes were evaluated and statistically significant functions with 10 or more target genes were identified for five miRNAs (miR-96, miR-124a, miR-130a, miR-130b, miR-199a) (I: Table 2). Based on association analysis, miR-96 target genes were linked to e.g. connective tissue development and function and lipid metabolism.

Collectively, our *in vitro* and *in silico* data suggested that the downregulated miRNAs target genes important for the differentiation pathway in question. For instance, miR-96 expression was markedly decreased upon chondrogenic differentiation while its level remained unaltered during osteogenic differentiation. Based on target prediction and pathway analyses, miR-96 was suggested to control type II collagen expression via Sox5. On the other hand,

miRNAs upregulated during osteogenesis or chondrogenesis appeared to target genes important for the differentiation of other mesenchymal lineages. As an example, miR-124a was upregulated during chondrogenic differentiation and it was predicted to regulate type I collagen expression via RFX1.

When the interplay between miRNA target genes and TFs was analyzed using IPA, statistically significant p-values and extensive interactions were observed (I: Figure 4 and Table S4). Composite loops were observed for three TFs (PBX1, PPARG and HIF1A); these harbored binding sites in upstream regions of miRNAs while they were also predicted as target genes for the same miRNAs. Indeed, the existence of such regulatory interactions between miRNAs and transcription factors has been proven also *in vitro*. For instance, miR-23a, a member of the Runx2-regulated miRNA cluster associated with osteogenesis, represses Runx2 in the terminally differentiated osteocytes thereby constituting a feedback mechanism to attenuate osteoblast maturation (Hassan et al., 2010). The miR-2861/miR-3960 cluster associated with osteoblast differentiation provides an example of an indirect regulatory loop between miRNAs and transcription factors (Hu et al., 2011). miR-2861 targets the transcriptional corepressor Hdac5, resulting in increased Runx2 protein production. Correspondingly, miR-3960 targets Hoxa2, another suppressor of Runx2 activity. On the other hand, experimental data confirm the binding of Runx2 to the promoter of the miR-3960/miR-2861 cluster. Together, miR-2861 and miR-3960 constitute an amplification mechanism to coordinate Runx2 expression and thereby, to drive osteoblast differentiation. A similar kind of positive regulatory loop consisting of c-Fos, miR-21, and PDCD4 has been identified to promote osteoclastogenesis (Sugatani et al., 2011). In conclusion, these experimental observations as well as our analyses reveal how sophisticated functions miRNAs may bear in terms of controlling the bone formation and homeostasis.

5.2.4 Effects of synthetic miRNAs and miRNA inhibitors on hMSCs (II)

Synthetic miRNA precursors and inhibitors were used to study the functional role of miR-96, miR-124 and miR-199a in hMSCs. Transfection of pre-miRNAs into hMSCs significantly increased the expression of the miRNAs in question (II: Figure 4). Correspondingly, decreased expression levels were observed in response to miRNA inhibition. As the endogenous miR-124 level in hMSCs was undetectable, no difference was observed with anti-miR-124. To evaluate the biological effects of pre-miRNAs and anti-miRNAs, the expression of selected genes related to hMSC differentiation was analyzed by qPCR. Among the studied transcripts, ≥ 2-fold change was observed for *aggrecan (ACAN)*, *FABP4*, and *SOX9* (II: Figure 5). Aggrecan and Sox9 are early markers of chondrogenesis, while Fabp4 is associated with adipogenic differentiation. Aggrecan is the major proteoglycan in the articular cartilage and is responsible for the load-bearing properties of the cartilage through its interaction with hyaluronan (Kiani et al., 2002). It is also needed for chondroskeletal morphogenesis during development. Sox9 is a positive regulator of chondrogenesis and it is required for the MSC commitment

during mesenchymal condensation (Akiyama, 2008). Fabp4 is predominantly expressed by adipocytes and macrophages, and belongs to the family of lipid binding proteins that account for the uptake and transport of long-chain fatty acids (Zimmerman and Veerkamp, 2002).

Transfection of pre-miR-96 resulted in increased *FABP4* expression while other transcripts under study remained unaltered. No changes were observed with anti-miR-96. The most substantial outcome was detected with miR-124 precursor, which increased *FABP4* expression more than 10-fold, suggesting a positive role for miR-124 in adipogenesis. Upregulation of miR-124 in hMSCs also resulted in decreased *ACAN* expression and increased *SOX9* expression, although the changes were less prominent. This divergent behavior of *ACAN* and *SOX9* may derive from their different physiological functions in chondrocytes, as presented above, or from the heterogeneity of the cell population under study. The results obtained from anti-miR-124 transfections cannot be considered physiologically reliable, as endogenous miR-124 expression remained undetectable in hMSCs. Pre-miR-199a appeared to function in a way opposite to pre-miR-96 and pre-miR-124, with induced *ACAN* expression while *FABP4* and *SOX9* were suppressed. As expected, opposing results were observed in response to miR-199a inhibition, where *ACAN* expression was suppressed and *FABP4* as well as *SOX9* expression were increased.

To determine whether miR-96, miR-124, and miR-199a have an effect on hMSC proliferation, cell number was determined 14 days after transfection. Cells transfected with pre-miRNAs showed reduced proliferation whereas transfection with anti-miRNAs had no effect or increased the proliferation slightly (II: Figure 6). The most noticeable effects were observed with pre-miR-124 and pre-miR-199a, which reduced the proliferation of hMSCs by 74% and 50%, respectively, in comparison to the control. The anti-proliferative effect of miR-124 may link to its reported regulatory effect on cyclin-dependent kinase 2 (Nakamachi et al., 2009).

In conclusion, we noticed that overexpression or functional inhibition of miR-96, miR-124 or miR-199a altered the proliferation of hMSCs and modulated the expression of genes related to hMSC differentiation. Based on target prediction studies, potential targets for miR-96 include the chondrogenic transcription factors Sox5 and Sox6. Due to low levels of these transcripts in hMSCs, the potential downregulation by pre-miR-96 could not be confirmed. However, increased expression of *FABP4* was observed, suggesting a potential positive role for miR-96 in adipogenic differentiation. A similar effect, although a much more pronounced one, was detected with pre-miR-124, which increased *FABP4* expression by 11-fold. Previous studies have linked miR-124 to postnatal neurogenesis in human and mouse subventricular zone (Cheng et al., 2009; Åkerblom et al., 2012). However, to our knowledge, there are no previous data concerning the expression and potential function of miR-124 in hMSCs or adipocytes. Based on target predictions and pathway analyses, miR-199a was associated with HIF1A and hypoxia. Indeed, miR-199a has been described to play a role in a hypoxia-triggered pathway in cardiac myocytes (Rane et al., 2009). Hypoxia has been shown to promote chondrogenic differentiation of mouse

mesenchymal cell lines, in part by HIF1A-mediated transactivation of Sox9 (Robins et al., 2005). However, in general, the data concerning the role of hypoxia in chondrogenesis are inconsistent. We observed a 1.8-fold increase in *HIF1A* expression during chondrogenic differentiation of hMSCs, and transfection with anti-miR-199a increased the expression of *HIF1A* by 1.3-fold while pre-miR-199a had no effect (data not shown). Nevertheless, miR-199a appeared to have a negative effect on chondrogenesis, as pre-miR-199a decreased the expression of *SOX9* and inhibition of miR-199a resulted in increased *SOX9* levels. Our results are in good accordance with the previous observations showing that miR-199a* excised from the 3' arm of miR-199a-1, and miR-199a-2 precursor molecules inhibit early chondrogenesis of mouse C3H10T1/2 cells at least in part by suppressing Smad1 (Lin et al., 2009a).

Despite the information obtained from functional studies, the relevance *in vivo* remains unclear. As most mRNAs are targeted by several miRNAs and, on the other hand, one miRNA is expected to target multiple mRNAs, individual miRNA-target pairs most likely do not mimic the *in vivo* signaling network of multiple miRNAs. However, the functional characterization of miRNAs *in vivo* has been challenged by the adaptive responses, as the majority of miRNA knockout mice analyzed thus far lack developmental phenotypes. On the other hand, miRNA regulation may be, especially under stress, a rather sensitive mechanism, and subtle changes in miRNA expression may ultimately lead to significant physiological responses (Mendell and Olson, 2012a). Another complicating factor is related to the issue of species differences; although highly conserved across species, due to distinct cellular environments, miRNAs can have diverse expression profiles, target molecules and functions in different species (Terrile et al., 2011). Therefore, observations collected from various model organisms and cell lines may not always be directly applicable to humans.

5.3 miRNA regulation in osteoclasts (III)

In order to gain more information on the potential miRNA-mediated regulatory networks involved in bone remodeling, miRNA expression was characterized during osteoclast differentiation of mouse bone marrow cells (BMCs). Since inconsistent results exist concerning the capacity of different monocyte subpopulations to generate osteoclasts (Väänänen and Laitala-Leinonen, 2008), and on the other hand, since RANKL and M-CSF seem to be potent enough to direct hematopoietic cells to osteoclasts at different stages of their differentiation pathway, unsorted BMCs were used. Cells were cultured on bovine bone slices for 3 or 7 days, representing two different stages of osteoclastogenesis. At day 3, only few, if any, multinucleated cells were present (III: Figure 1). At day 7, several multinucleated TRACP-positive cells were observed. These cells represented the fully differentiated bone-resorbing osteoclast as visualized by the formation of resorption pits.

Compared to osteogenesis and chondrogenesis, studies related to the role of miRNAs during osteoclastogenesis are few. The only miRNA profiling study published this far addresses global miRNA expression in mouse BM-derived monocyte/macrophage precursor cells treated with RANKL and M-CSF for 24h (Sugatani et al., 2011). In that study, miRNA changes were monitored immediately after induction by RANKL and/or M-CSF, while we sought to compare miRNA expression at two different stages of osteoclastogenesis, namely mononuclear osteoclast precursors and functional bone-resorbing osteoclasts. Since previous information on miRNA expression in osteoclasts was lacking, miRNAs were selected based on their reported expression in hematopoietic tissues or embryonic stem cells. Among the 29 miRNAs analyzed, 15 miRNAs showed considerable expression (III: Figure 2). These miRNAs were evaluated by their delta cycle threshold (dC(T)) values and divided into groups based on their expression profiles (III:Table 1). Potential targets for each miRNA were searched using TargetScan 6.2.

Interestingly, two previously described osteoclast-associated miRNAs included in this study, miR-21 and miR-223, ranked to the highest expression group. miR-223 was abundantly expressed in undifferentiated BMCs whereas its level decreased gradually during osteoclastogenesis. In contrast, miR-21 level increased markedly during early stages of osteoclast induction and thereafter tended to decrease. These results are in agreement with the previous observations in the global miRNA profiling of mouse BM-derived monocyte/macrophage precursors (Sugatani et al., 2011). Also miR-24, miR-27a and miR-142-were differentially expressed between early and late stages of osteoclastogenesis. Based on dC(T)-values, miR-24 expression was low in BMCs, moderate in committed pre-osteoclasts, and high in mature bone-resorbing osteoclasts. miR-27a was moderately expressed in BMCs but became upregulated in response to RANKL and M-CSF. miR-142-3p followed the expression profile of miR-223, being highly expressed in BMCs and decreasing slowly upon osteoclast formation. It is to be noted that miR-24 and miR-27a behaved oppositely in the global miRNA profiling, being downregulated in response to osteoclast induction (Sugatani et al., 2011). This may, at least partly, result from the distinct time frame of the experiments, ranging from 24 hours to 3 or 7 days. Since miRNA expression is highly dynamic, it is not surprising that distinct sets of miRNAs are observed depending on the stage of differentiation.

Target predictions for the differentially expressed miRNAs resulted in several genes associated with osteoclastogenesis, bone resorption or the regulation of osteoblast apoptosis (III: Table 1). However, further studies are needed to reveal whether these miRNA-target pairs are functional *in vitro* and/or *in vivo*.

6 SUMMARY AND CONCLUSIONS

The formation and remodeling of the skeleton are complex processes involving the differentiation and crosstalk of multiple cell types, including chondrocytes, osteoblasts, osteoclasts, and bone marrow adipocytes. The activity of these cells is under the control of several key signaling pathways consisting of various hormones, growth factors and cytokines. MicroRNAs have emerged as novel regulators of skeletal gene expression being essentially involved in the control of bone organogenesis, postnatal bone formation, and regulation of bone mass in the adult skeleton.

Based on the results and discussion presented in this thesis, the following conclusions can be made:

1. MicroRNAs constitute complex networks with transcription factors. Based on promoter analyses and target predictions, transcription factors may act as direct miRNA targets, but also as upstream regulators of miRNA target genes, or of the miRNAs themselves. In this way they constitute loops that strengthen or attenuate specific signaling pathways.

2. MSCs isolated from mouse bone marrow exhibited more efficient differentiation capacity than human bone marrow-derived MSCs. This may arise from the fact that isolation of mouse MSCs results in a rather heterogeneous population of cells, some of which have already committed to specific mesenchymal lineages. Alternatively, the differences may result from the varying requirements for culture conditions. Species differences are likely to affect miRNA expression and function as well, although it is not possible to address such differences here.

3. MicroRNAs are differentially expressed during *in vitro* osteogenesis, chondrogenesis and adipogenesis of bone marrow-derived MSCs. Most noticeable changes were observed in the expression levels of miR-96, miR-124 and miR-199a. These miRNAs regulate the expression of genes known to be involved in MSC differentiation.

4. Osteoclastogenesis of mouse BMCs is associated with altered miRNA expression. The differentially expressed miRNAs are predicted to control genes associated with osteoclast differentiation, function and apoptosis.

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