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FACTORS AFFECTING GROWTH, DIFFERENTIATION AND APOPTOSIS OF OSTEOBLASTIC AND OSTEOSARCOMA CELLS

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To my family

ABSTRACT

Osteoblasts play a fundamental role in determining bone structure and function. These cells originate from mesenchymal stem cells (MSCs) and through proliferation and differentiation develop into preosteoblasts and then into mature cells. Most of these cells undergo apoptosis before reaching their terminal differentiated stages of either osteocytes or bone lining cells. These processes, i.e. proliferation, differentiation, and apoptosis, are affected by systemic hormones and local factors. In addition, there are exogenous regulators, which can either be natural substances or synthetic compounds.

This thesis describes investigations of the effects of several selected factors on proliferation, differentiation, and apoptosis of osteoblastic cells. The thesis is based on four papers: In the first paper, the effects of Sirt1 regulators, resveratrol (RSV), nicotinamide (NAM), and isonicotinamide (INM), on the commitment of mesenchymal stem cells (MSC) were studied. We found that the Sirt1 activators, RSV and INM, inhibited adipocyte formation and enhanced osteoblast differentiation, while the inhibitor NAM had the opposite effect. In the second paper, osteoblastic cells from different origins, mouse, rat, and human, were treated with $1\alpha,25(OH)_2D_3$ and its analogue, 2-methylene-19-nor-(20S)-$1\alpha,25(OH)_2D_3$ (2MD). Species-dependent effects on cell growth and alkaline phosphatase (ALP) activity were clearly seen. In the third paper, we found that the expression of Interleukin-6 (IL-6) receptor increased during osteoblast differentiation. IL-6 acted as a differentiation accelerator in the early stage and an apoptosis inducer at late mature stage. In the forth paper, the effects of Sirt1 activators, RSV and INM, on proliferation and apoptosis of human osteosarcoma (OS) cells were studied. We found an inhibitory effect of Sirt1 activators on OS cells and showed a synergism between RSV and L-asparaginase (ASNase), which is a selective nutritional restrictor.

In summary, the work presented in this thesis provides new information about the effects of two osteoblast differentiation regulators, $1\alpha,25(OH)_2D_3$ and IL-6. Additionally, certain compounds affecting Sirt1 activity were found to influence osteoblast differentiation; RSV and INM which increase Sirt1 activity also had a profoundly negative effect on growth of OS cells in vitro.

SAMMANFATTNING

Osteoblastaktiviteten är avgörande för benvävnaden struktur och funktion. Dessa celler utvecklas från mesenkymala stamceller (MSC). Genom proliferation och differentiering utvecklar de sig till omogna osteoblaster och vidare till mogna osteoblaster. De flesta av cellerna dör genom programmerad celldöd innan de når sin slutliga utvecklingsfas till lining cells eller osteocyter. Dessa processer, proliferation, differentiering och celldöd påverkas av hormoner och lokala faktorer. Vidare kan de påverkas av olika tillförda naturliga och syntetiska kemiska ämnen.

Denna avhandling baseras på studier av vissa utvalda faktorer som påverkar proliferation, differentiering och celldöd av osteoblaster. Avhandlingen är baserad på fyra artiklar: I den första artikeln beskrivs effekten av ämnen som påverkar Sirt1 hos MSC nämligen resveratrol (RSV), nikotinamid (NAM) och isonikotinamid (INM). Vi fann att att Sirt1 aktivatorerna RSV och INM hämmade bildningen av fettceller och stimulerade osteoblastmognaden, medan NAM hade motsatt effekt. I den andra artikeln studerades behandling med den aktiva vitamin D-metaboliten $1\alpha,25(OH)_2D_3$ och dess analog, 2-methylene-19-nor-(20S)-$1\alpha,25(OH)_2D_3$ (2MD) på osteoblaster från olika arter, som mus, råtta och människa. Artspecifika effekter på celltillväxt och alkalisk fosfatasaktivitet var uppenbara. I den tredje artikeln fann vi att uttrycket av IL-6 receptorn ökade under osteoblastmognaden. IL-6 stimulerade differentieringen i tidigt skede och medförde ökad celldöd bland fullt differentierade osteoblaster. Den fjärde artikeln beskriver hur Sirt1- aktivatorerna RSV och INM påverkar celldelning och celldöd av humana osteosarcom- celler (OS). Vi fann en hämmande effekt av Sirt1 aktivatorerna på OS och visade även på en synergi mellan RSV och L-asparaginas, som är en selektiv näringsbegränsare.

Sammanfattningsvis ger resultaten i denna avhandling ny information om effekterna av vissa regulatorer som påverkar osteoblastmognaden nämligen $1\alpha,25(OH)_2D_3$, 2MD och IL-6. Vidare visar de att ämnen som påverkar Sirt1:s aktivitet har betydelse för utvecklingen av osteoblaster liksom att RSV och INM som ökar Sirt1-aktivitet även har en klart negativ effekt på celltillväxten av OS celler in vitro.

LIST OF PUBLICATIONS

This thesis is based on the work of the following papers. They are referred to by their Roman numerals.

I. Bäckesjö CM, **Li Y**, Lindgren U, and Haldosén LA. Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells. J Bone Miner Res 2006; 21: 993-1002.

II. **Li Y**, Bäckesjö CM, Haldosén LA, and Lindgren U. Species difference exists in the effects of 1α,25(OH)2D3 and its analogue 2-methylene-19-nor-(20S)-1,25-dihydroxyvitamin D3 (2MD) on osteoblastic cells. Accepted Manuscript, The Journal of Steroid Biochemistry and Molecular Biology. DOI: 10.1016/j.jsbmb.2008.09.004

III. **Li Y**, Bäckesjö CM, Haldosén LA, and Lindgren U. IL-6 receptor expression and IL-6 effects change during osteoblast differentiation. Cytokine 2008; 43:165-73.

IV. **Li Y**, Bäckesjö CM, Haldosén LA, and Lindgren U. Resveratrol inhibits proliferation and promotes apoptosis of osteosarcoma cells. Conditionally Accepted Manuscript. The European Journal of Pharmacology

CONTENTS

LIST OF ABBREVIATIONS

AA	L-ascorbic acid
ALP	alkaline phosphatase
ASNase	L-asparaginase
β-GP	β-glycerophosphate
BMP	bone morphogenetic protein
BSP	bone sialoprotein
Cbfa1/Runx2	core binding factor 1
CCD	cleidocranial dysplasia
CD	cluster of differentiation
C/EBPs	CCAAT enhancer-binding proteins
COL-1	type I collagen
CR	calorie restriction
Dex	dexamethasone
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
INM	isonicotinamide
JAK	Janus kinase
MSC	mesenchymal stem cells
NAD	nicotinamide adenine dinucleotide
NAM	nicotinamide
OCN	osteocalcin
OPN	osteopontin
OS	osteosarcoma
Osx	osterix
PPARγ	peroxisome proliferator-activated receptor γ
PTH/PTHrP	parathyroid hormone and its related proteins
RANKL	receptor activator of nuclear factor-κB ligand
RB	retinoblastoma
RXR	retinoic X receptor
RSV	resveratrol
Sirt1	sirtuin-1
sir2	silent information regulator
TNAP	tissue nonspecific alkaline phosphatase
TRACP	tartrate-resistant acid phosphatase
VDR	vitamin D receptor
VDRE	vitamin D-responsive element
2MD	2-methylene-19-nor-(20S)-1α,25$(OH)_2D_3$

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Bone is a specialized tissue that serves important mechanical, protective and metabolic functions in the human body. As in all other connective tissues, the fundamental constituents in bone are the cells and the extracellular matrix. The latter contains type I collagen and a large number of non-collagenous proteins (e.g. osteocalcin, osteonectin, bone sialoprotein and various proteoglycans) which contribute to the unique characteristics of bone to become mineralized through deposition of calcium and phosphate [1]. Calcified bone undergoes constant remodeling. This process is coordinated by the activities of two types of cells, osteoclasts and osteoblasts [2]. The osteoclasts are members of the monocyte/macrophage lineage and are formed by multiple cellular fusions from their mononuclear precursors. They mediate bone resorption through dissolution of bone mineral and degradation of organic matrix by secretion of HCl and different protein enzymes [3]. The osteoblasts originate from mesenchymal stem cells (MSCs), which through proliferation and differentiation develop into preosteoblasts and then into mature osteoblasts, and finally reach the non-proliferative, terminal differentiatied stage either as osteocytes or as bone lining cells [4]. Both preosteoblasts and mature osteoblasts contribute to the production of bone matrix. Additionally, in a local paracrine manner, the osteoblasts produce receptor activator of nuclear factor-κB ligand (RANKL) as well as the decoy receptor osteoprotegerin (OPG), which control the osteoclasts and bone resorption [5]. Thus, osteoblasts play a major role in bone metabolism and understanding the mechanisms controlling their proliferation, differentiation and apoptosis is one of the fundamental areas in bone research.

Many systemic hormones and local factors affect osteoblastogenesis. “New” regulators, either as natural substances or synthetic compounds, are continually being found or developed. This thesis describes new aspects of two known regulators, 1α,25$(OH)_2D_3$ and IL-6, as well as the synthetic 1α,25$(OH)_2D_3$ analogue, 2-methylene-19-nor-(20S)-1α,25$(OH)_2D_3$ (2MD), and the natural phytoalexin resveratrol. In particular, we studied proliferation, differentiation and apoptosis during the development of osteoblastic cells. Furthuremore, the potential use of resveratrol for the treatment of osteosarcoma is discussed.

1.2 THE OSTEOBLAST LINEAGE

1.2.1 The Origin of Osteoblasts

One of the current dogmas of bone biology is that the osteoblasts are differentiated from the multipotent precursors present in bone marrow, namely, MSCs. These cells are defined as a group of clonogenic cells capable of multilineage differentiation and having the capacity for self-renewal [6]. Fredenstein and co-workers were the first to describe the existence of MSCs [7-10]. Through a series of classic studies they found

that the plastic adherent cells from bone marrow could form fibroblast colony forming units, which had the capacity to differentiate towards osteoblasts, chondroblasts, and adipocytes under defined *in vitro* culture conditions [7, 8]. Later on, after seeding in a suitable scaffold and transplantation in host animals these cells could give rise to a wide range of connective tissues, including bone, cartilage, muscle, fibrous and adipose tissues [9, 10]. Beginning from Friedenstein's pioneering studies much effort has been focused on the direct identification of MSC from bone marrow. Dozens of surface markers have been studied and some of them, like STRO-1, CD29, CD73, CD90, CD105, CD106, CD146, CD166 and CD271 are expressed by MSC while the haematopoietic markers, like CD34, CD45 and CD14 are not [11-13]. However, as shown in different studies the expression pattern of these markers are not always consistent and several studies have shown that MSCs could either gain or lose some of the markers without losing their multipotential capacity [14-16]. Therefore these markers can not exclusively be used to identify MSCs [13]. On the other hand, MSC-like cells have also been derived from a number of other adult and fetal tissues, such as circulating blood, cord blood, placenta, amniotic fluid, heart, skeletal muscle, adipose tissue, synovial tissue and pancreas [17]. Although these non-bone marrow MSCs retain the multipotential capacity and express similar patterns of MSC surface markers they do demonstrate differences in their differentiation capacity, even if cultured in exactly the same microenvironment [18-20]. Therefore, the perception is that the MSCs are both phenotypically and functionally heterogenous and today the ability of multilineage differentiation is still the "gold standard" to confirm the "stemness" of bone marrow derived cells. Also, it is important to mention the minimal criteria to define human MSCs outlined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy in 2006 [11]. These are: (1) adherence to plastic in standard culture conditions; (2) expression of CD105, CD73 and CD90 combined with a lack of expression of CD45, CD34 and CD14 or CD11b and CD79 or CD19 and HLA-DR surface molecules; and (3) differentiation into osteoblasts, adipocytes and chondroblasts *in vitro*.

1.2.2 Commitment of Mesenchymal Stem Cells to Osteoprogenitors

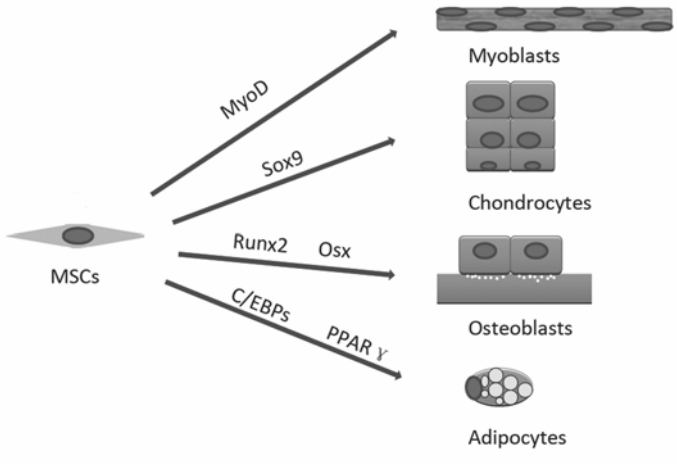

Figure 1 Commitment of MSCs to different cell lineages and the related key transcriptional factors.

From the traditional point of view, the stem cells develop into specialized cell types by a two-step process, determination and differentiation. Determination results in the conversion of multipotent stem cells into lineages of cells with restricted differentiation potential, called committed (or determined) cells. The committed cells proliferate and then differentiate, activating genes that encode the proteins functionally appropriate to that differentiated cell type. In 1984 evidence for the regulatory genes controlling MSCs determination was first shown by Konieczny et al. [21]. With clonal and 2D protein gel analyses they demonstrated that 5-azacytidine converted C3H/10T1/2 cells into three stably determined, but undifferentiated, stem cell lineages, which under permissive culture conditions developed into myofibers, chondrocytes, and adipocytes. They proposed that these three lineages were specified by separate regulatory loci, and 5-azacytidine activated these "determination loci" by random hypomethylation. However, such "determination loci" have not been identified. Following increased understanding of gene regulatory mechanisms, the search for transcription factors which could act as "master switch" has been intensified. Today several of these transcription factors have been identified (for reviews, see [22-24]). Basic helix-loop-helix genes of the *myo*D family are known to specify the commitment of MSCs into myoblasts [25] (figure 1). The C/EBP and PPAR family members play central roles in the regulation of adipocyte differentiation [26-28], Sox9 is required for the commitment to chondrocytes [29], and as described below, Cbfa1/Runx2 and osterix (Osx) are indispensable for osteoblast development [30-32].

The transcription factor core binding factor 1 (Cbfa1/Runx2) gene is one of the three mammalian genes that encode protein homologues to *Drosophila* Runt, which are crucial for proper embryonic development [33]. Its essential role for osteoblastogenesis was discovered by several simultaneous studies in 1997. Through homologuous recombination experiments, two groups, Otto et al. and Komori et al., independently generated Cbfa1-defient mice [30, 31]. Grossly, the homozygous mutants have entirely cartilaginous skeleton and lose all of the intramembranous-formed skeletal elements, such as calvarias and clavicles, and without a rigid chest cage these mice die at birth due to respiratory failure. Histologically, the skeleton of these mice presents a complete lack of bone tissue and osteoblasts. At the molecular level, there is no expression of bone extracellular matrix proteins such as osteopontin (OPN), bone sialoprotein (BSP), and osteocalcin (OCN) [30, 31]. Additionally, Otto et al. noticed that the viable heterozygous Cbfa1 mutants presented hypoplastic clavicles as well as a severe delay of the closure of the fontanels [31]. These features are similar to the classic mutant mouse strain named *Ccd*, which is the phenocopy of a well-characterized human genetic disease, cleidocranial dysplasia (CCD) [34]. Through human genetic studies it was shown that mutations of Cbfa1 causes CCD [35, 36]. Moreover, the expression pattern of Cbfa1 during mouse embryo development has been described. In 12.5 days post-coitum embryos Cbfa1 is expressed by a cell population with both osteoblastic and chondroblastic potential. However, during later embryo development the expression of Cbfa1 is restricted to cells of the osteoblast lineage. Further proof for the essential role of Cbfa1 for osteoblastogenesis was the identification of binding sites for Cbfa1 in the promoters of four osteoblast specific genes, OCN, α 1(1) collagen, BSP and OPN, and that overexpression of Cbfa1 can induce the expression of osteoblastic genes in *in vitro* models [37].

Another major breakthrough in understanding of osteoblastogenesis was the identification of Osx in 2002. Through screening osteoblast-specific cDNAs Nakashima et al. identified this novel zinc finger-containing transcription factor. In Osx null mice no bone was formed through either intramembraneous or endochondral ossification [32]. Furthermore, unlike the Runx2-defient mice, which presented maturational disturbance of chondrocytes [38], absence of a normal bone marrow cavity and lack of osteoclasts/chondroclasts [31], the only deficiency of the Osx mull mice was the absence of osteoblastic mineralization. Additionally, the osteogenic cells in Osx null mice expressed Runx2 at levels comparable to those in wild-type osteoblasts while Osx is not expressed in Runx2-defient embryos; thus Osx is a downstream gene of Runx2 [32]. This evidence indicates that at least two steps are involved in osteoblastogenesis: First, with activation of Runx2, MSCs are committed to osteoprogenitors which have the bipotential capacity to form both endochondral and membranous skeletal elements. Second, with expression of Osx these osteoprogenitors are prevented from choosing the chondrocyte differentiation pathway and start to express the characteristic osteoblast marker genes and differentiate into mature osteoblasts.

Beside these breakthrough findings, dozens of other transcriptions factors and co-regulators have been proposed to be involved in osteoblastogenesis, including β-catenin, ATF4, AP1, homeobox proteins, helix-loop-helix (HLH) proteins. The regulation of the osteoblastic transcription factors by systemic hormones, local factors and mechanical forces has also been extensively investigated (for recent reviews see [22-24, 39, 40]). However, at present a full understanding of the commitment process and the exact roles of regulating factors is not at hand. In several studies the complexity of the determination process has been shown. For examples, Liu et al. and Geoffroy et al. reported that osteopenia and frequent bone fractures are seen in transgenic mice overexpressing Runx2 in bone tissue [41, 42]; Yoshida et al. reported that Runx2 and Runx3 are essential for chondrocyte maturation, and that Runx2 regulates limb growth through induction of Indian hedgehog [43]; Gutierrez et al. and Hata et al. found that the C/EBP family proteins, which are known as the key regulators of adipocyte commitment, can associate with Runx2 and promote osteoblast differentiation [44, 45]. In addition, multiple studies have suggested that a certain degree of plasticity exists within the “committed” MSC lineages. For example, myoblasts can be converted to adipocytes through expression of PPARγ and C/EBPα [46, 47]. Myoblasts can also be converted to mineralizing osteoblasts through expression of Runx2 [48]; bone morphogenetic protein (BMP) can induce osteoblastic trans-differentiation from both preadipocytes and myoblasts [49, 50] and osteoblasts can be trans-differentiated into adipocytes through downregulation of β-catenin under estrogen deficiency [51]. Therefore, the “determination” process of MSC is much more complex than presently understood.

1.2.3 Osteoblast and Adipocyte

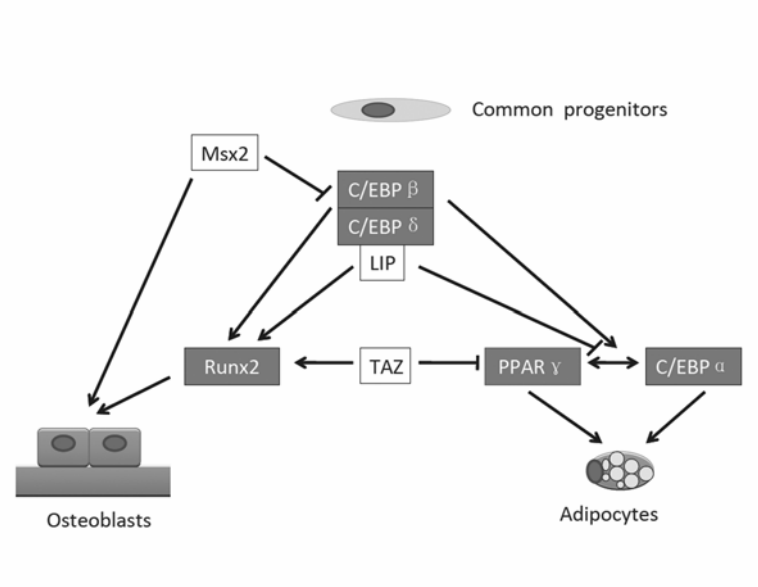

Figure 2 Reciprocal relationship between osteoblast and adipocyte and the putative regulators.

Infiltration of bone marrow by fat is a common feature of osteoporotic bone associated with aging, immobility, or following glucocorticoid treatment [52-55]. Whether the accumulating fat just occupies the space left by the disappearing bone or results from an imbalance in the commitment of MSC between osteoblast and adipocyte is being actively investigated (for reviews, see [56-59]). As mentioned above, the program for adipogenesis is controlled by two key transcriptional factors, the CCAAT enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ). In a proper environment, the committed MSCs (or adipocyte progenitors) express C/EBPβ and C/EBPδ, which in turn induce the expression of C/EBPα and PPARγ. C/EBPα and PPARγ together promote maturation of preadipocytes by activating adipose-specific gene expression and maintaining each others expression at high levels [60]. Accumulating evidence indicates a mutually exclusive relationship between the commitment of MSC into osteoblast and adipocyte. For example, PPARγ $^{-/-}$ embryonic stem cells (ES) fail to differentiate into adipocytes but spontaneously differentiate into osteoblasts, and the PPARγ deficient mice display enhanced bone formation [61, 62]. Consistently, the thiazolidinedione antidiabetic drug rosiglitazone, which is a potent PPARγ agonist elicits significant bone loss while increasing adipogenesis in both mice and humans [63-66]. Moreover, BMP-2-induced increase of Runx2 expression in MSC enhances osteoblast commitment at the expense of adipocyte maturation [67] and in Runx2 deficient MSC an increase in the expression of adipocyte marker genes is seen [68]. These data indicate that a balance between the activity of Runx2 and PPARγ plays a key role in specifying the two alternate cell fates. Recently, a possible regulator of this balance was described. Transcriptional coactivator with PDZ-binding motif (TAZ), a 14-3-3-binding protein, can bind to the Pro-Pro-X-Tyr motif within the activation domains of both Runx2 and PPARγ. While functioning as an endogenous coactivator of Runx2 in MSC, TAZ represses the PPARγ-dependent transcriptional events. Thus, TAZ promotes osteoblastogenesis while simultaneously impairing adipocyte differentiation [69].

However, there are also reports that suggest that Runx2 and PPARγ may not be the major determinants for cell fate determination. For example, the determination process has been suggested to be regulated through the activities of C/EBPs. C/EBPs belong to the leuzine zipper family of transcriptional factors, which form homodimer or heterodimer complexes among family members. C/EBPβ and δ are important for the early stages of adipogenesis [70, 71]. Interestingly, C/EBPβ is also up-regulated during osteoblast differentiation, and through interaction with Runx2, C/EBPβ and δ activate the expression of osteoblast specific genes such as OCN [44]. The liver-enriched inhibitory protein (LIP) is an isoform of C/EBPβ. Due to lack of the transcriptional activation domain it usually functions as a transcriptional repressor [72], Although LIP inhibits adipogenesis in a dominant-negative fashion it was recently shown that LIP functions as a coactivator of Runx2 and stimulates osteoblastic differentiation [45]. Therefore, it could be that the ratio of C/EBPβ-LIF is a critical factor for commitment of MSC to osteoblast or adipocyte lineages. Another potential regulator for MSC commitment is the homeobox gene Msx2, a mammalian homologue of the *Drosophila* muscle segment homeobox. Msx2 is known to be induced by bone morphogenetic proteins (BMPs), which play critical roles in bone formation and osteoblast differentiation [73]. Msx2 deficient mice develop defects in osteogenesis [74]; while transgenic mice overexpressing Msx2 show enhanced growth of calvariae [75]. Consistently, gain or loss of Msx2 function by mutations in humans is often associated with accelerated or delayed skull ossification [76-79]. The expression Msx2 can be induced by BMP2 in Runx2-deficient MSCs. In normal MSCs, besides promoting osteoblast differentiation, Msx2 inhibits the transcriptional activity of C/EBPβ and δ, as well as C/EBPα and PPARγ, thereby suppressing adipocyte differentiation [80]. Thus, Msx2 functions as a determination regulator upstream of C/EBPs and Runx2. In summary, the above findings indicate that the commitment of MSC to osteoblast or adipocyte may be regulated at multiple levels. However, it is clear that more studies are needed in order to better understand the intricate interplay between different regulatory commitment factors.

1.2.4 Differentiation of Osteoblastic cells

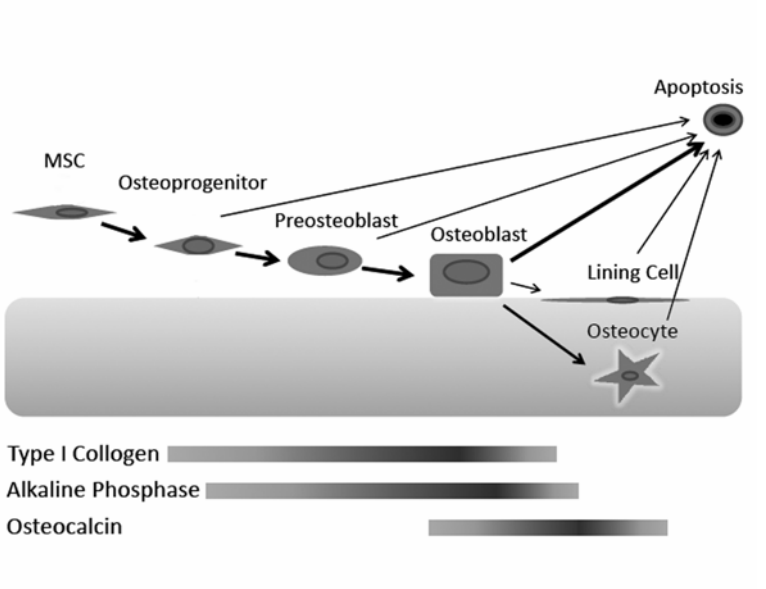

Figure 3 Transitional stages during osteoblast differentiation and the expression of lineage markers Col-1, ALP and osteocalcin.

1.2.4.1 The Transitional Stages during Osteoblast Differentiation

For convenience, the differentiation process of the osteoblast committed MSCs is artificially divided into several stages, namely, osteoprogenitors, preosteoblasts, osteoblasts, osteocytes and bone lining cells. Most of these definitions are originally made by morphological and histochemical criteria, coupled with proliferation analysis.

Osteoblasts: The osteoblasts are defined as post-proliferative, cuboidal, strongly alkaline phosphatase-positive cells lining bone matrix at sites of active matrix production. They have a large nucleus, enlarged Golgi apparatus, and extensive endoplasmic reticulum, which are typical features of cells highly active in protein production [81].

Preosteoblasts: The preosteoblasts are considered the immediate precursor of the osteoblasts and are identified in part by their localization in the adjacent one or two cell layers' distance from the osteoblasts lining bone formation surfaces. Although preosteoblasts resemble osteoblasts histologically and stain for ALP activity, they have not yet acquired many of the other characteristics of fully mature cells, and unlike osteoblasts, preosteoblasts are thought to possess a limited proliferation potential [81].

Osteocytes: The osteocytes are non-proliferative, terminally differentiated cells of the osteoblast lineage. They are non-migratory cells, locked inside small lacunae in the mineralized bone matrix or the newly formed osteoid. Compared with mature osteoblasts, the osteocytes are smaller, have decreased ALP activity and cytoplasmic organelles. However, there is an emerging consensus that osteocytes play an important role in the maintenance of bone structure, i.e. participating in extracellular exchanges

between different components and acting as mechanosensory cells and determining the place and time of bone remodeling [82-84].

Bone lining cells: Another type of the terminally differentiated osteoblast is bone lining cells, which are flat, thin and elongated cells, covering most if not all the nonremodeling bone surfaces. Similar to osteocytes, the bone lining cells are considered postproliferative, inactive in matrix production and may take part in mechanotransduction and protection of the bone matrix from osteoclastic resorption [81, 85]. Another feature of the bone lining cells is their potential to be reactivated to cuboidal, active osteoblasts in response to particular stimuli [86].

Osteoprogenitor cells: In contrast to the other transitional stages, the "osteoprogenitor" is mainly functionally defined. As the name indicates, osteoprogenitors are able to clonally generate cells of osteoblast lineage but lack self-renewal capacity [87]. Some authors have reported that these cells are spindle-shaped and reside in close proximity to the preosteoblast layer with a distance from bone formation surfaces in the periosteal layer of bone [81]. However, accumulating evidence indicates that the "osteoprogenitors" can be gained from different sites, including calvaria, bone marrow, fat, peripheral blood, and accordingly, demonstrate different morphological features [88].

Although the above "compartmentalization" categorizes and describes the characteristics of the osteoblast differentiation process, the boundaries between the adjacent transitional stages are not distinct. For example, the difference between osteoprogenitor and preosteoblast is not clear; and although the "self-renewal capacity" can be used as a conceptual distinction between osteoprogenitor and MSC, it has almost no practical use. In my opinion, this drawback of "compartmentalization" is unavoidable because these different transitional stages actually exist as a developmental continuum rather than as distinct compartments. However, this "compartmentalization" is problematic for *in vitro* studies because the cells change dramatically in morphology from their *in vivo* state. Thus, it is most practical to use molecular markers to evaluate the differentiation process.

1.2.4.2 Phenotypic Markers of Osteoblastic Cells

Besides the change of morphological features and proliferation ability, the differentiation of osteoblasts is associated with temporal modification in the expression of a set of macromolecules. These molecules include membrane-associated enzymes (such as ALP), bone matrix proteins (such as Col-1, OPN, BSP and OCN), and receptors for systemic hormones and local factors (such as parathyroid hormone receptors, estrogen receptors, and different cytokine receptors) [81]. These macromocules not only play important roles in regulating cell proliferation and function but also provide a panel of markers that reflect the transitional stages in osteoblast development. The following phenotypic markers have been frequently used in my studies.

Type I collagen (COL-1): Col-1 is a primary product of osteoblasts during bone matrix formation, constituting approximately 90% of the total organic matrix in mature bone [89]. Together with mineral, collagen governs the mechanical property and functional integrity of the osseous tissue. From a number of *in vitro* studies Col-1 has been proposed to induce osteoblast differentiation and facilitate mineralization [90]. However, as a phenotypic marker, Col-1 is nonspecific; it is synthesized by many cell types and distributed in almost all connective tissues (with the exception of hyaline cartilage). In osteoblast lineage cells, Col-1 is clearly expressed before the preosteoblast stage and it has been documented that up-regulation of Col-1 occurs prior to up-regulation of any of the other matrix molecules and prior to ALP [91].

Tissue nonspecific alkaline phosphatase (TNAP): Although not specific for osteoblast lineage cells, ALP is the most frequently used phenotypic marker to evaluate their activities [92, 93]. The physiologic isoforms of ALP are coded by four gene loci: three loci located on chromosome 2 and each encodes one of the three tissue specific ALPs, expressed in germ-cells, placenta and intestine respectively. The other locus, located in chromosome 1, encodes the bone-liver-kidney isoform, also called the tissue nonspecific alkaline phosphatase (TNAP) [94]. Accordingly, the TNAP is the isoform that we have referred to as osteoblast marker in our studies. In bone, TNAP presents abundantly in the membranes of osteoblasts and matrix vesicles by covalent bounds to glycosylphosphatidylinositol (GPI) [95]. The major role of TNAP in bone is to facilitate mineralization. Clear evidence for this function is seen in the genetic discorder inheritable human hypophosphatasia [96] and in *in vivo* experimental studies of the TNAP gene knockout or mutated mice [97, 98]. *In vitro* studies indicate that ALP facilitates mineralization by generating inorganic phosphate (P_i) from substrates such as β-glycerophosphate (β-GP) [99]; while recent *in vivo* data indicates that mineralization is mainly mediated by ALP-mediated hydrolyzation and elimination of the potent mineralization inhibitor, pyrophosphate (PP_i) [100]. However, TNAP is not only restricted to mature osteoblasts, it can also be detected in subpopulations of osteoprogenitors and preosteoblasts, well before mineralization and prior to the expression of the noncollagenous matrix molecules [87, 101]. Therefore, it has been proposed that TNAP could act as a membrane-bound receptor involved in osteoprogenitor-osteoblast adhesion, migration, and differentiation [102].

Osteocalcin (OCN): OCN is a carboxylated bone protein also known as bone Gla protein. It is the most abundant non-collagenous protein of bone [103] and has a very narrow expression pattern, undetectable in preosteoblasts and expressed only by the mature osteoblasts and osteocytes [104, 105]. Thus, OCN is currently considered the most specific and the latest expressed osteoblast marker. The function of OCN in bone is still unclear. Studies of the in vitro differentiation of rat calvaria osteoblasts showed that OCN is localized intracellularly and in all the extracellular compartments, but concentrated at the mineralization front, supporting a role in regulating mineralization [106]. However, although the OCN-deficient mice develop a phenotype with higher bone mass and bones of improved functional quality, the absence of OCN did not affect bone mineralization or bone resorption [107]. Interestingly, there are recent reports to suggest that OCN can be secreted by osteoblasts as uncarboxylated form and functions as a hormone, which affects glucose metabolism and fat mass through regulation of pancreatic β cells and adipocyte gene expression [108, 109].

1.2.4.3 Apoptosis of Osteoblastic Cells

Apoptosis, or programmed cell death, is recognized as an important component in embryogenesis, organogenesis, and tissue morphogenesis as well as in the maintenance of homeostasis in many adult tissues [110]. In bone, mature osteoblasts have one of three fates upon completion of the synthetic phase of the remodeling cycle: two of which have been previously described, to become osteocytes entrapped within the mineralized matrix or to evolve into bone lining cells that protect the bone matrix from osteoclasts resorption. However, 60-80% of the osteoblasts that originally assemble at the resorption pit cannot be accounted for by either of these two fates, and ample evidence has established that the missing osteoblasts die by apoptosis [111, 112]. In fact, apoptosis is not restricted to mature osteoblasts: a small percentage of apoptotic cells present throughout the *in vitro* culture of rat calvaria-derived preosteoblasts [113]; and apoptotic mesenchymal progenitors are frequently observed in developing long bones, calvariae and sites of fracture healing in animal models [114-117]. In addition, osteocytes are not immortal and undergo apoptosis by aging or under the influence of apoptotic signals such as hormones, pharmaceutical interventions, and mechanical stress [118, 119]. These facts indicate that apoptosis occurs throughout the entire life span of osteoblast lineage cells, and thus its regulation not only determines the rate of bone formation but also helps to maintain a proper functional bone.

As in other tissues, bone cells undergoing apoptosis are recognized by condensation of chromatin, the degradation of DNA into oligonucleosome-sized fragments, and the formation of plasma and nuclear membrane blebs. Eventually the cell breaks apart to form so-called apoptotic bodies [112]. However, except for special locations such as fracture callus and developing calvariae sutures [114, 120, 121], apoptotic osteoblasts with typical features are rarely seen in vivo [116, 122]. This is likely because apoptosis represents only a tiny fraction of the life span of osteoblasts and apoptotic cells vanish rapidly without a trace through effective phagocytic clearance [111]. The only exception is osteocyte apoptosis, which represents cumulative death because the cellular debris is not accessible to phagocytic scavenger cells [123].

The process of apoptosis is highly regulated and can be triggered by both extrinsic and intrinsic signals. The extrinsic signals refer to the cellular binding of proapoptotic factors like Fas ligand which subsequently initiates the apoptosis program. The intrinsic signals usually lead to disruption of the integrity of mitochondria through which the death program is subsequently initiated. The latter represents the most frequent apoptotic mechanism in vertebrates [124]. In the mitochondria pathway, the decision to live or die is determined at the level of the outer mitochondrial membrane. The integrity of this membrane is controlled by the Bcl-2 family proteins, which are made up of pro-apoptotic and anti-apoptotic members. When death signals overwhelm survival signals, the action of anti-apoptotic Bcl-2 proteins are abrogated. This results in the permeabilization of the outer mitochondrial membrane and the release of proteins, such as cytochrome c, from the intermitochondrial membrane space into the cytoplasm [125]. The released cytochrome c activates caspase-9, a protease that normally exists as a latent proenzyme. Once activated, caspase-9 proceeds to activate

other caspases, notably caspase-3, which eventually dismantle the internal components of the cells [126].

A large body of evidence suggests that growth factors, cytokines, hormones, and drugs affect bone development and remodeling, in part by controlling bone cell apoptosis [127]. For example, bone morphogenetic proteins (BMPs) induce apoptosis of mesenchymal osteoblast progenitors in interdigital tissues during the development of hands and feet [128]. TGF-β inhibits apoptosis of cultured osteoblastic cells [111], and conversely, mice lacking Smad-3, which mediates TGF-β signaling, exhibit decreased bone mass associated with increased osteoblast apoptosis [129]. Estrogen deficiency leads to increased bone remodeling and subsequent bone loss due to an excess of resorption over formation, suggesting that estrogen promotes osteoclast apoptosis but prevents osteoblast apoptosis [130]. In addition, the decrease in osteoblast number and bone formation rate in glucocorticoid excess may be explained in part by increased osteoblast apoptosis, which has been reported in studies of murine, rabbit and human bone [131-133]. In summary, apoptosis is an important cellular event during bone development and remodeling and should be considered as critical as the differentiation effects when studying the potential development regulators of osteoblast lineage cells.

1.2.5 Osteosarcoma (OS)

Perturbations of cell differentiation can result in abnormal cellular survival, growth, and behavior [134]. OS might be regarded as a differentiation disease. First, OS is most frequently observed in adolescence, a stage of intensive skeletal growth and locates primarily around the regions with most active new bone formation, such as lower femur and upper tibia [135]. Paget's disease of bone, a benign condition characterized by accelerated bone formation and resorption, is also associated with an increased risk of OS [136]. Together, these observations indicate that tumorogenesis is associated with osteogenesis. Second, up to 70% of OS is of osteoblast lineage with the remaining 30% showing chondroblastic and fibroblastic (~10% each), or anaplastic and small cell phenotype [137]. This kind of lineage distribution represents the mesenchymal origin of the tumor. Third, in osteoblastic OS a certain aspect of the differentiation phenotype is clearly preserved. For example, the tumor cells express lineage specific transcription factors and lay down an aberrant bone matrix [138]. Among these tumor expressed components, the early and nonspecific markers of osteoblastic lineage such as Runx2 and ALP are frequently seen, while the later and more specific markers, such as Osx and OCN, are expressed more ralely or not at all [139, 140], which indicates that the development to the terminal differentiation stage is interrupted. The resistance to mutagens appears to be inversely related to the degree of cell differentiation, as evidenced by the fact that both spontaneous and induced mutation frequencies are much lower in embryonic stem cells than somatic cells [141-143]. Therefore, it is likely that the key tumorogenic events, which result in deregulation of gene expression profiles, occur more frequently in the later stages of osteoblastic differentiation, i.e. from preosteoblast to mature osteoblasts, than the earlier stages such as osteoprogenitors and MSCs.

Several genes have been indicated to be involved in the process of osteoblastic differentiation and transformation resulting in OS. For example, RB-1, which codes for the retinoblastoma protein [144], is mutated in 70% of adolescent OS. Patients carrying germline mutations in RB-1 gene have around 500-fold higher incidence of OS than the general population [145, 146]. The Rb protein can physically interact with Runx2 and participate in the activation of OCN expression [147]. Loss of Rb protein can suppress the terminal differentiation in cultured osteoblast cell lines, and conditional deletion in mouse embryo results in defects in both endochondral and intramembranous ossification [147, 148]. Another studied gene is the tumor suppressor gene p53. Mutations of p53 are observed in 20-60% of sporadic OS [149]. Li-Fraumeni patients, who often carry germ-line mutations in p53, are predisposed to a variety of tumors, 12% of which are bone sarcomas [150, 151]. The role of p53 in osteoblastic differentiation is mainly evidenced by the following facts: p53-deficienct mice display both accelerated osteoblast differentiation and increased bone density [152]; in contrast, hyperactivation of p53, via deletion of the p53-inhibitor Mdm2, suppresses osteoblast differentiation by inhibiting expression of Runx2 [153]. The coupling of transformation and differentiation implies that the tumorogenic events might still be regulated or even reversed under the influence of intrinsic or extrinsic differentiation signals. Indeed, several known differentiation regulators do demonstrate anti-tumor effects as well. For example, transfection of the Osx gene into the mouse OS cells inhibited tumor cell growth in vitro and in vivo and significantly reduced tumor incidence, tumor volume, and lung metastasis following intratibial injection [140]. In addition, the PPARγ agonists, troglitazone and ciglitazone, as well as a RXR ligand all exhibited the ability to inhibit cell proliferation and induce apoptosis in OS cell lines [154]. Thus, differentiation regulators might also be added to the therapeutic arsenal for the treatment of OS.

1.2.6 Factors Affecting Osteoblast Development

The process of initiation, differentiation and apoptosis of osteoblast lineage cells are affected by many systemic hormones and local factors, some of which, such as gonadal steroids (estrogens and androgens), parathyroid hormone and its related proteins (PTH/PTHrP), bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs), have been extensively investigated and reviewed [22-24, 39, 40]. On the other hand, “new” regulators, either as natural substances or as synthetic compounds, are continually being found or developed. The following sections give an introduction of the differentiation regulators that have been investigated in our studies.

1.2.6.1 Sirtuin-1 (Sirt1)

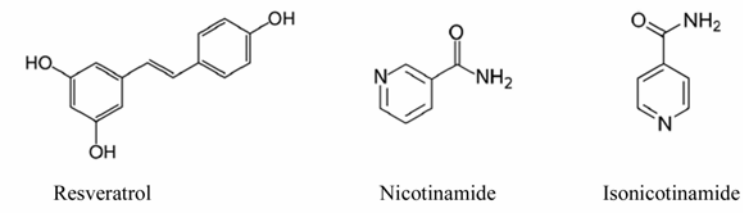

Figure 4 Molecular structures of three Sirt1 regulators

Sirt1: Sirt1 is the mammalian orthologue of the yeast silent information regulator 2 (sir2) [155], which was originally discovered to influence mating-type control in haploid cells by locus-specific transcriptional silencing [156]. The products of sir2 homologues consist of a protein family known as the nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases [157]. These enzymes are distinguished from the other deacetylases mainly for two reasons. First, they are universally conserved proteins, with homologues in all three kingdoms of life [155, 158]. Second, their deacetylation function absolutely requires the coenzyme, NAD^+ [157-160]. The major substrates of sir2 are acetylated lysines such as those present in the N termini of histones [157]. In each reaction cycle, nicotinamide is liberated from NAD^+ and the acetyl group of the substrate is transferred to the ADP-ribose moiety, generating the metabolite O-acetyl-ADP-ribose [161].

The unusual enzymatic activity of sir2 suggests a linkage between protein modification and cell metabolic activity. The metabolic state is known to affect many important aspects in life, with the most striking example being lifespan extension induced by calorie restriction (CR) [162]. The mechanism of this process is largely unknown but studies in yeast indicate that sir2 plays a key role [162]. First, CR does not extend lifespan when sir2 is deleted [163]. Second, the enzymatic activity of sir2 is enhanced during CR [164]. Third, the life extension effect can be induced by a non-specific sir2 activator, resveratrol (as later described) [165]. Fourth, yeast lifespan can be dramatically increased with an extra copy of sir2 gene and decreased with Sir2 dysfunction [166]. In accordance with these findings, studies using *C. Elegans* and *Drosophilia* have demonstrated similar results [167-170].

In mammalian genomes, seven sir2 homologues, Sirtuins 1-7 (Sirt1-7), have been identified [171]. Unlike their homologues in lower organisms the human Sirts appear to have a broader range of substrates and more complicated functions. For example, Sirt2 is mainly found in cytoplasm and mediates tubulin deacetylation [172]. Sirt3 localizes in mitochondria and its function is still unknown [173]. Of these seven Sirts, Sirt1 shares the highest homology to the yeast sir2 and is thus considered to be its orthologue [155]. At the level of chromatin, Sirt1 enzymatic activity preferentially targets histone H1 at Lys26, H3 at Lys9 and Lys4, and histone H4 at Lys16. These modifications are supposed to promote the formation of facultative heterochromatin with resultant silencing [159, 174]. In addition, a wide variety of nonhistone Sirt1 substrates have been identified, such as p53 [175, 176], forkhead transcription factor Foxo3 [177, 178], and transcriptional coactivator PGC-1α [179, 180], stressing the pivotal function that this regulator plays in cellular control and responses. Furthermore, the expression and activity of Sirt1 has been recently reported to be regulated in a circadian manner in cultured cells and in the animal liver, which indicates that Sirt1 could function as a bridge connecting cellular metabolism and the circadian system [181, 182].

There are several reasons to also connect Sirt1 to differentiation of cells. First, the histone deacetylation and gene silencing effects enable Sirt1 to epigenetically modify the mammalian genome. Epigenetic modification is now considered to be a fundamental process in differentiation [183-185]. Second, many transcriptional factors involved in differentiation contain lysine residues, which could be target sites for Sirt1.

Sirt1 binds and deacetylates the p53 protein with specificity for its C-terminal lysine 382 residue. Acetylation of this residue has been shown to be important for the transcriptional activity of p53 [175, 176]. Sirt1 has also been shown to bind and deacetylate Rb resulting in suppressed Rb activity [186]. Third, Sirt1 has been shown to regulate cell differentiation in several cell lineages. Regarding brain cells, activation of Sirt1 by mild oxidation suppresses proliferation of neural progenitor cells and directs their differentiation towards the astroglial lineage at the expense of the neuronal lineage, whereas inhibition of Sirt1 by reducing conditions has the opposite effect [187]. Sirt1 also acts as a redox state sensor and differentiation regulator of muscle cells. Overexpression of Sirt1 retards muscle differentiation via formation of a complex with the acetyltransferase p300/CBP-associated factor (PCAF) and MyoD, whereas in cells with reduced Sirt1 activity, muscle gene expression and differentiation are enhanced [188]. In adipocyte precursors, Sirt1 represses PPARγ activity via docking with two of its corepressors, nuclear receptor corepressor (NcoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT), and hence inhibits adipocyte differentiation. Furthermore, in differentiated adipocytes, up-regulation of Sirt1 triggers lipolysis and loss of fat [189]. Since skeletal defects, such as craniofacial abnormalities and delayed mineralization in digits, has been identified in the Sirt1 deficient mice [190, 191], it is highly likely that Sirt1 affects osteogenesis as well. In our studies the influence of Sirt1 activity on differentiation of MSC to osteoblast has been investigated and is described below.

Resveratrol (RSV): The unusual mechanism of sir2-catalyzed deacetylation permits opportunities for chemical intervention to enhance or inhibit its enzymatic activity. The most studied activator is 3,4,5'-trihydroxystilbene (RSV) [165], a polyphenol found in many plant sources, including nuts, berries, and grape skins (and therefore in red wine). From a botanical point of view, RSV belongs to a class of defense molecules called phytoalexins that protect the plant against infection and environmental harshness [192, 193]. Indeed, RSV is found to be a major ingredient in many herbal medicines that have been used by oriental people for thousands of years [194, 195]. Recent studies attribute this natural, old, and simple molecule a broad range of health benefits, including tumor prevention, cardio and neuro-protection, improvement of metabolic states and lifespan extension [196].

In 2003, Howitz et al. showed that among the 15 sir2 activators RSV demonstrated the most potent deacetylation enhancing effect [165]. Dose-response experiments, using p53-acetylated peptide as substrate, showed that RSV increased the rate of Sirt1-mediated deacetylation. RSV demonstrated no significant effect on the two V_{max} determinations when either substrate or NAD^+ was varied. However, it was shown to lower the Michaelis constants (K_m) for both acetylated substrates and NAD^+, indicating that RSV is an allosteric effector that alters the substrate-binding affinity of Sirt1 [165]. These findings are consistent with the findings that RSV treatment mimics sir2-dependent lifespan extension in various species [163, 165, 170, 197, 198]. However, in mammalian cells several other enzymes or transcription factors could be modulated by RSV. Although it could be that some of these RSV targets are modulated through Sirt1 deacetylation [199], others, such as cyclooxygenase-1 (COX-1), have been found to be affected through direct interaction with RSV [200]. Thus, despite the high potency RSV is a nonspecific Sirt1 activator.

Nicotinamide (NAM): NAM, also known as niacinamide and nicotinic acid amide, is the amide of nicotinic acid (vitamin B_3). NAM has demonstrated anti-inflammatory actions which may be mediated through suppression of antigen induced-lymphocytic transformation and inhibition of 3'-5' cyclic AMP phosphodiesterase [201]. Animal studies have shown that nicotinamide may also work in a way similar to benzodiazepines so as to exert anti-anxiety properties [202]. As well, NAM has been shown to have chemopreventive effects on cancer and type I diabetes through unclear mechanisms [203].

NAM has been described to non-competitively inhibit sir2-catalyzed deacetylation [204, 205]. To date NAM remains among the most potent sir2 inhibitors [206-208] and is the only compound that has been reported to inhibit sir2-dependent lifespan extension [204]. The mechanism of NAM inhibition has been clarified by studies of Sauve et al. [206] and Jackson et al. [207]. Briefly, the enzymatic activity of sir2 can be considered as a combination of two reactions, base-exchange and deacetylation. These two reactions are competitive processes emerging from a common intermediate, ADP-ribosyl-enzyme-acetyl peptide. Unlike deacetylation the base-exchange process is reversible, and by this reason NAM can also be considered as a base-exchange substrate. Therefore, an increase of NAM concentration will favor the base-exchange process, regenerating NAD^+ from the ADP-ribosyl-enzyme-acetyl peptide intermediate, and sacrifice the other process, deacetylation.

Several additional findings contribute to the physiological importance to NAM-mediated sir2 inhibition. First, the inhibitory effect of NAM is phylum-dependent. For bacterial and yeast enzymes, approximately 79 and 35%, respectively, of the maximal sir2 deacetylation rates remained following millimolar NAM treatment [206]. However, the IC50 of human Sirt1 is less than 50 μM [204], and for the mouse enzyme, over 95% inhibition occurred at the presence of 160 μM NAM [206]. Consistent with these findings, studies of Sirt1-deficient mice failed to find a global defect in gene silencing [190, 209], while in yeast continuous sir2-catalyzed deacetylation is required for the maintenance of the heterochromatin [204]. Since levels of NAM in mammalian tissues are found to lie in the range of 10-400 μM [210-213] and NAM appears to be concentrated mostly in the nucleus, with a limit concentration of 150 μM [214], it is highly likely that the deacetylation activity of Sirt1 is largely blocked by NAM in mammalian cells. Furthermore, studies in yeast revealed that NAM produced from NAD^+ cleavage is converted to nicotinic acid, a vitamin B_3 form with no inhibitory effects on sir2, through deamination catalyzed by Pnc1, a nicotinamidase [215]. Interestingly, the expression and enzymatic activity of Pnc1 increased dramatically in response to glucose restriction, amino acid restriction, salt and heat stresses, which are known stimuli to extend yeast lifespan. In addition, overexpression of Pnc1 extends yeast lifespan and suppresses the inhibitory effects of exogenously added NAM on sir2-mediated gene silencing and lifespan extension. Neither of the two phenomena can be observed in sir2-deficient strains [216, 217]. Similar to sir2, the nicotinamidase is a ubiquitous protein and the purified enzyme has been extracted from microbial, plant, insect, and mammalian sources [218-221]. Although the physiological function of the mammalian Pnc1 has not been clarified, the fact that CR prolongs lifespan in mammals strongly indicates that the NAM inhibition of Sirt1 could be reversed under nutritional

stresses. Second, the mechanism of NAM-mediated sir2 inhibition suggests that NAM analogues capable of inhibiting base-exchange but not deacetylation would cause in vivo activation of sir2. Indeed, one such sir2 activator, isonicotinamide (INM), has been identified [214]. INM (pyridine-4-carboxamide) is a chemical based on the NAM structure, but in which the amide is in the 4-position and not the 3-position. INM antagonizes sir2 inhibition by endogenous NAM in yeast cells and causes an increase in sir2 deacetylation activity. Furthermore, INM substantially increases transcriptional silencing at sir2-regulated loci in both wt strains and strains lacking the Pnc1 nicotinamidase [214]. Finally, it needs to emphasize that the mechanism used by NAM and its analogues for regulating sir2 deacetylation is different from that used by the polyphenolic compounds such as RSV. As described previously, polyphenols are proposed to increase sir2 deacetylation by changes in the Michaelis constant for both substrate and NAD^+. In contrast, NAM and INM regulation of sir2 deacetylation activity is achieved without affecting substrate or NAD^+ binding by altering the proportion of intermediate-enzyme complexes proceeding toward the deacetylated products. This difference suggests that a combination of the two mechanistically distinct pathways may synergistically enhance deacetylase activity of sir2 in vivo.

1.2.6.2 1α,25(OH)$_2$D$_3$

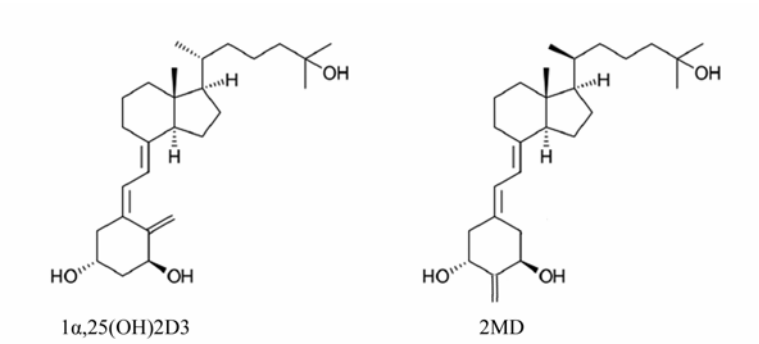

Figure 5 Molecular structures of 1α,25(OH)$_2$D$_3$ and 2MD

1α,25(OH)$_2$D$_3$: The physiologically active form of vitamin D, 1α,25(OH)$_2$D$_3$, is a seco-steroid hormone. Its effects are mediated primarily via the vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily. When bound by its ligands, the VDR dimerizes with the retinoic X receptor (RXR) and binds to promoter regions of responsive genes to either activate or repress transcription [222, 223]. It is generally agreed that 1α,25(OH)$_2$D$_3$ affects bone formation mainly by indirect mechanisms on calcium homeostasis [223]. Though direct effects on osteoblasts have been reported, the findings have sometimes been contradictory. In vitro experiments show that 1α,25(OH)$_2$D$_3$ increases the production of OCN and ALP in rat and human osteoblasts [224, 225], while in mouse osteoblasts 1α,25(OH)$_2$D$_3$ down-regulated expression of Phex, a mature osteoblast marker, and block in vitro mineralization [226, 227]. Controversial results have also been reported from in vivo studies. The rat is the most commonly used animal for vitamin D studies. Similar to humans, rats respond to the treatment of 1α,25(OH)$_2$D$_3$ with increase in intestinal calcium absorption and bone calcium mobilization [228]. Rat experiments suggest that 1α,25(OH)$_2$D$_3$ prevents experimental osteoporosis in vivo [229-234]. VDR-knockout mice show typical

features of vitamin D-dependent rickets type II, such as failure to thrive after weaning, appearance of alopoecia, hypocalcaemia and infertility, as well as severely impaired bone formation [235]. However, studies from VDR knockout mice show that the rickets caused by interruption of $1\alpha,25(OH)_2D_3$ signaling can be totally prevented by high calcium diet alone [236]. Furthermore, transplantation of bone from VDR knockout mice to wild-type mice revealed a significant increase in bone volume and density compared with control (wild-type bone transplanted to wild-type mouse), which indicated that $1\alpha,25(OH)_2D_3$ suppress bone formation [237]. Undesirable effects of $1\alpha,25(OH)_2D_3$ have also been reported in other species, such as in rabbits, in which $1\alpha,25(OH)_2D_3$ prevented fracture healing and aggravated immobilization or prednisolone-induced osteoporosis [238]. The reason for these paradoxes is presumed to be the difference in the regulation of calcium metabolism [238]. Still, a clear understanding of this issue is not at hand. However, reported data indicate that the direct effect of $1\alpha,25(OH)_2D_3$ on osteoblastic cells is species-dependent.

2-methylene-19-nor-(20S)-1α,25(OH)$_2$D$_3$ (2MD): As a synthetic $1\alpha,25(OH)_2D_3$ analogue, 2-methylene-19-nor-(20S)-$1\alpha,25(OH)_2D_3$ (2MD) differs structurally from $1\alpha,25(OH)_2D_3$ by the absence of the 19-methylene carbon and the addition of a methylene group in the 2-position between the 3β-hydroxyl and 1α-hydroxyl. In addition, the C-20 configuration is changed to 20S compared with a 20R configuration in naturally occurring vitamin D compounds [239]. In rat studies, 2MD stimulated bone formation without triggering noticeable hypercalcemia and hypercalciuria. Additionally, although 2MD was about 30-100 fold more potent than $1\alpha,25(OH)_2D_3$ in bone calcium mobilization it was no more effective in promoting intestinal calcium absorption [240]. Further studies in ovariectomized rats showed that low doses of 2MD increased total bone mass and promoted the synthesis of both trabecular and cortical bone with high quality, whereas much higher doses of $1\alpha,25(OH)_2D_3$ only prevented bone loss over the same period [241]. Such bone anabolic effects imply that 2MD increases bone formation through a direct action on bone cells.

1.2.6.3 Interleukin-6 (IL-6)

Interleukin-6 (IL-6): IL-6 is a multifunctional cytokine that can activate target genes involved in proliferation, differentiation, survival and apoptosis in a variety of cells [242]. Its effects occur after binding to its transmembrane receptor (IL-6R or alpha receptor) and the signal transducer gp130 (beta receptor). This heterodimeric receptor-ligand interaction activates receptor-bound Janus kinase (JAK). JAK phosphorylates the tyrosine residues of the cytoplasmic tail of gp130, which leads to recruitment of SH2-domain containing proteins, such as members of the signal transducers and activators of transcription (STAT). STAT phosphorylation causes the formation of STAT-protein dimers that migrate to the nucleus and initiate gene transcription. In bone IL-6 can be produced by a variety of cells, such as osteoblasts, mononuclear phagocytes, endothelial cells, chondrocytes, fibroblasts and lymphocytes [243, 244]. Over-production of IL-6 is seen in postmenopausal osteoporosis, Paget's disease, rheumatoid arthritis and other diseases with accelerated bone turnover [245-247]. Additionally, transgenic mice with IL-6 overexpression are associated with severe skeletal defects including osteopenia, defective ossification, and growth plate

abnormalities [248]. Regarding IL-6 deficient mice, although the evident phenotype is normal [249] microstructure abnormalities are found in the cortical bones and delayed fracture healing is reported [250, 251]. Furthermore, IL-6 deficient mice are protected from bone loss by estrogen depletion, in which case high level of IL-6 are found in the wild types [250]. These findings indicate that IL-6 is an important regulator of bone remodelling and an adequate amount of this cytokine is crucial for bone homeostasis. However, although extensive studies have been done to understand its mechanism of action, the direct effects of IL-6 on osteoblasts are still unclear. For example, many studies indicate IL-6 as a differentiation inducer [252-254] whereas effects such as inhibition of osteocalcin expression [255] and bone nodule formation [256] also have been reported. Additionally, although high levels of IL-6 are usually found where osteoblast apoptosis rates are high [118, 131, 257] several in vitro studies demonstrate that IL-6 is an apoptosis inhibitor [258].

IL-6 receptors (IL-6R): The action of IL-6 is mediated through two kinds of receptors: the cell surface receptor, surface IL-6R (also known as alpha receptor, CD 126 or gp80), and the signal transducer gp130 (beta receptor or CD130). In contrast to gp130, which is ubiquitously expressed on different cell types and shared by several interleukins, the surface IL-6R is expressed in a more restricted pattern [259]. Additionally, the cell surface IL-6R can be functionally replaced by a soluble counterpart, generated by either shedding or alternative mRNA splicing [260]. It is largely unknown which form of IL-6R functions as the major mediator in bone. Many in vitro studies suggest that osteoblasts lack surface IL-6R and are inert to IL-6 unless soluble receptors are added [252, 261-263]. However, there are also studies indicating that surface IL-6R is expressed abundantly in osteoblasts in vivo [247, 264, 265].

2 PRESENT INVESTIGATION

2.1 AIMS OF STUDIES

The aim of the work presented in this thesis was to study the effects of selected factors on growth, differentiation and apoptosis of osteoblast lineage cells.

The specific aims were:

1. To study the effects of Sirt1 regulators on the commitment of MSCs to osteoblast or adipocyte lineage.

2. To study the direct effects of $1\alpha,25(OH)_2D_3$ and its synthetic analogue, 2MD, on osteoblastic cells.

3. To study the effects of IL-6 and specifically the role of IL-6R on osteoblast differentiation.

4. To study the effects of Sirt1 regulators on proliferation and apoptosis of OS cells.

2.2 COMMENTS ON METHODOLOGY

In the presented studies, cell proliferation was evaluated by cell counting and WST-1 analysis. The differentiation stages of MSCs and osteoprogenitors were evaluated by several methods, including quantitative realtime PCR analysis for mRNA expression of osteoblast and adipocyte markers, staining for fat vesicles in adipocytes by oil red O, and staining for ALP expression and bone nodule formation in osteoblast cultures by using an ALP staining kit or with the Alizarin red and Von kossa methods. Cell apoptosis was measured by Annexin V staining followed by flow cytometry analysis or by measurement of caspase-3 activity. These are commonly used methods in cellular research and have been described in details in the attached papers. The following comments on methodology are based on the osteoblast differentiation model used in the presented studies. This model is composed of three fundamental components: cells, bone inducing medium (BIM), and differentiation regulators. The osteoprogenitors used include primary cultured bone marrow stromal cells, C3H/10T1/2 cells and MC3T3-E1 cells. The bone inducing medium is made from normal culture medium supplemented with L-ascorbic acid (AA), β-glycerophosphate (β-GP), and dexamethasone (Dex).

2.2.1 Isolation, Expansion and Differentiation of Bone Marrow Mesenchymal Stem Cells (BM-MSCs)

There is no standard protocol for isolation and expansion of BM-MSCs. In our studies, cell isolation is based on plastic adherence and neither magnetic cell sorting nor a

special culture medium was used (see paper I, II, and IV). However, as shown in many studies, the cells harvested by this method were unavoidably contaminated by hematopoeitic cells and macrophages [266-268]. Accordingly, although the adherent cells tended to be homogeneous as culture prolonged, around 20-30% $CD11b^+$ and $CD45^+$ cells remained when tested on passage 3 and the macrophages could not be removed effectively even after passage 10, when the cells already started to show phenotype changes [269, 270]. In our pilot studies, rat BM-MSCs of passage 4 were cultured for 4 days and then stained with tartrate-resistant acid phosphatase (TRACP), a marker of the activated macrophages. We found that $TRACP^+$ cells were frequently seen in cultures using normal medium; however, in cultures with BIM no $TRACP^+$ cells were found, and instead, most of the cells were stained for ALP. This finding indicates that the BIM has a potential role for purification of the BM-MSCs and that the heterogeneity does not interfere with the differentiation of BM-MSC following osteoblast lineage.

Several studies have been performed in order to find suitable protocols for isolation, expansion and differentiation of BM-MSC. Schrepfer et al. evaluated the effect of different media on proliferation of mouse BM-MSCs. They claimed that cells cultured with DMEM/F12 showed the highest proliferation rates and that supplementation with human growth factor-1 (40 ng/ml) most effectively accelerated cell proliferation. However, without magnetic cell sorting, the cells remained heterogenous after 10 passages [269]. Harting et al. studied the immunophenotype of *in vitro* cultured rat BM-MSCs. They made use of a special media, namely, multipotent adult progenitor cell (MAPC) media, which contains 60% low-glucose DMEM, 40% MCDB-201, 1 x insulin-transferrin-selenium, 1 x linoleic acid bovine serum albumin, 10^{-9} M dexamethasone, 10^{-4} M ascorbic acid 3-phosphate, 100 U penicillin, 1000 U streptomycin, 2% fetal bovine serum, 10 ng/mL human platelet-derived growth factor, 10 ng/mL mouse epidermal growth factor and 1000 U/mL mouse leukemia inhibitory factor. With culturing in the MAPC media the $CD11b^+$ and $CD45^+$ cells declined to less than 2% by passage 4 and cells expressing CD29, CD49 and CD90 increased to 99%, which indicated that the MAPC media was effective for the isolation of a nearly homogenous population of BM-MSCs [270]. Tokalov et al. studied the effects of age on the presence and differentiation capacity of rat BM-MSCs. They found that the number of MSCs in bone marrow significantly reduced with aging. However, no age-related difference regarding the phenotype and differentiation potential was seen [271]. The effects of plating density and culture time on rat BM-MSC characteristics were studied by Neuhuber et al. They found that the optimal cell growth appeared at a plating density of 200 cells/cm^2; however, it was the time in culture, instead of plating density, that affected the differentiation capacity [272]. The above studies provide valuable information regarding culture media, donor age and plating density for studies of murine BM-MSCs, which imply that modifications could be done in order to more effectively isolate and expand BM-MSCs in our future studies.

2.2.2 C3H/10T1/2 cells

The murine multipotent mesenchymal cell line C3H10T1/2 (clone 8) was obtained from American Type Culture Collection. The cells were cultured in BME medium

supplemented with 10% FBS, 1 x L-glutamine, and 100 μg/ml gentamicin. This cell line was developed at the McArdle Laboratory for Cancer Research in 1972 from a line of C3H mouse embryo cells [273]. The cells are very sensitive to post-confluence inhibition of cell division, do not produce tumors in syngeneic mice and have no background of spontaneous transformation [274]. On the other hand, the C3H10T1/2 (clone 8) cells are highly susceptible to transformation by chemical agents, and thus was originally used in studies on chemical oncogenesis in *vitro* [275]. In 1977, Constantinides et al. reported the formation of functional striated muscle cells after the culture of C3H10T1/2/Cl8 cells treated with 5-azacytidine [276]. Two years later, following similar experimental procedure, two other mesenchymal derivatives, i.e. biochemically differentiated adipocytes and chondrocytes capable of the biosynthesis of cartilage-specific proteins, were identified by a cloning method [277]. Thus, the C3H/10T1/2/Cl8 cell line became a frequently used model to study the commitment of multipotent stem cells to cells of restricted lineages. The differentiation of C3H/10T1/2/Cl8 cells towards osteoblastic lineage was firstly suggested by Karagiri et al. in 1990 who found that the recombinant human bone morphogenetic protein-2 (rhBMP2) and retinoic acid induced ALP activity in C3H/10T1/2/Cl8 cells [278]. Since then, this cell line has been used to investigate a range of different factors that may regulate the commitment of multipotent stem cells to osteoprogenitors, including BMPs [279, 280], sonic hedgehog [281, 282], parathyroid hormone [283], Cbfa1 [284], notch [285], β-catenin [286], and Osx [287]. In pilot studies, we found that the majority of C3H/10T1/2/Cl8 cells expressed ALP after 7 days of culture in bone inducing medium. However, after 14 days of culture 10-20% of the cells showed an adipocyte phenotype, e.g. showed cellular lipid droplets. Thus, we considered the C3H/10T1/2/Cl8 cell line a suitable model to investigate the factors directing BM-MSCs to osteoblast or adipocyte lineage. However, two factors should be taken into account when considering the results from C3H/10T1/2/Cl8 cells. First, the C3H/10T1/2/Cl8 cells are not normal mouse cells. The modal number of chromosomes has been shown to be 81, which is hypertetraploid for mice (2N is 40). A small chromosome has been found in around 85% of cells [273]. Second, the cells originate from mouse embryo tissues. Thus, whether they properly represent the adult BM-MSCs is not firmly established. Although we found that these cells were stained positively for ALP and expressed several osteoblast markers, as measured by real-time RT-PCR, they did not form bone nodules even after growing up to 40 days in bone inducing medium.

2.2.3 MC3T3-E1 cells

MC3T3-E1 cells are derived from calvaria of newborn mice and thus they are often used as a preosteoblast model. The cells we used belong to subclone 14, which has been shown to exhibit high levels of osteoblast differentiation capacity. These cells express mRNAs for the osteoblast markers, including Runx2, BSP, OCN, and PTH/ PTHrP receptor, and when cultured in BIM the cells differentiate along the osteoblast lineage and produce a mineralized matrix in less than 2 weeks [288]. We found that unlike cells from primary cultures, the growth and differentiation of MC3T3-E1 cells did not stop even when the serum concentration was reduced to as low as 1%. This property is particularly useful for the study of an individual cytokine because the serum-reduced medium can minimize a possible interference from the investigated

cytokine or other cytokines and growth factors in the fetal calf serum, which is at least 10% in common osteoblast cultures.

2.2.4 Bone Inducing Medium (BIM)

Despite the wide use of BIM for *in vitro* osteoblast differentiation from preosteoblasts and MSCs, the exact mechanisms of action for three important components are relatively unclear.

L-ascorbic acid (AA): AA, also known as vitamin C, is an essential cofactor for the hydroxylation of proline and lysine residues in collagen, the most abundant protein in bone [289]. AA is essential for the formation of bone and other connective tissues and is necessary for the in vivo and in vitro differentiation of osteoblasts [290, 291]. In vivo autoradiographic studies demonstrated that radiolabled vitamin C, when injected systemically, accumulates at sites of active bone formation [292]. Later studies found that osteoblasts contain a specific Na^+-dependent AA transport system that is essential for the intracellular accumulation of vitamin C [290, 293]. In primary cultures of osteoblast-like cells and MC3T3-E1 cells, AA stimulates procollagen hydroxylation, processing, and fibril assembly followed by a dramatic induction of specific genes associated with the osteoblast phenotype, including OCN, ALP, and BSP [294-296]. The mechanism for AA promoting osteoblast differentiation has been suggested to be related to collagen matrix formation [295-298]. However, recent studies using micro-array assays found that preosteoblast cells respond to AA with up or down regulation of genes involved in a broad range of activities, including cell growth, metabolism, morphogenesis, communication, and survival [299].

β-glycerophosphate (β-GP): β-GP is used in BIM mainly for supplementation of phosphate, which has been found to be a limiting factor in the initiation of mineralization in vitro [300]. In *in vitro* osteoblast cultures, β-GP is cleaved by ALP produced by osteoblasts resulting in release of inorganic phosphate, which is later incorporated into the newly formed bone nodules [301]. However, the nodules formed in this manner are nonapatitic, and similar mineral particles could form in a cell-free system in the presence of ALP and β-GP and deposit into a collagen matrix [301]. Thus, instead of promoting osteoblast differentiation, β-GP seems only to facilitate the expression of one phenotype of osteoblast, ALP activity. However, although β-GP alone does not regulate osteoblast activity, a combination of β-GP and AA has been reported to increase the production of metalloproteinases, which could facilitate mineralization [302].

Dexamethasone (Dex): Dex is a synthetic glucocorticoid that exerts a powerful promoting effect on the *in vitro* differentiation of osteoprogenitors. For primary cultured bone marrow stromal cells this compound significantly increases the expression of osteoblastic markers, such as ALP, OPN, and OCN, and is required for initiation of mineralization [303-305]. The mechanism for these effects of Dex is not clear. Due to the fact that Dex also promotes adipocyte formation in MSC cultures [306, 307] and adipocyte and osteoblast share a common progenitor, it could be

assumed that Dex has regulatory functions at the early stage of cell development or stimulates MSC into differentiation.

2.3 RESULTS AND DISCUSSION

2.3.1 Paper I: **Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells**

This paper describes an investigation of Sirt1 regulators, RSV, NAM and INM on the commitment of MSCs to osteoblast or adipocyte lineages. Briefly, the mouse C3H10T1/2 cells and rat bone marrow stromal cells were cultured in BIM. We found that in both models a variable amount of cells demonstrated adipocyte phenotype as identified by oil red O staining and flow cytometry analysis after staining with Nile red. Western blot analysis with anti-Sirt1 antibody showed that Sirt1 protein was expressed in these cells both at pre- and post-differentiation stages. With C3H10T1/2 cells, we found that without addition of Sirt1 regulators around 13% of the cells were adipocytes after 2 weeks of culture in BIM. The amount of adipocytes increased significantly with the addition of 10 mM NAM to BIM (21%) as well as with the addition of 0.75 μM PPARγ agonist, troglitazone (34%). In contrast, addition of 50 μM Sirt1 activator RSV totally blocked the adipocyte formation and 25 mM INM attenuated adipocyte formation to 2%. As expected, a comparable inhibition effect could be seen with 1 nM $1\alpha,25(OH)_2D_3$, a known downregulator of PPARγ expression [308]. We next analyzed the adipocyte and osteoblast markers using quantitative real-time PCR. We found that cells treated with troglitazone and NAM displayed a general downregulated expression of the osteoblast markers, ALP, Col-1, OCN, Runx2, IL-6, OPG, and RANKL, while an upregulated expression was seen for the adipocyte markers, ap2 and PPARγ. For the Sirt1 activators, although both compounds dramatically decreased the adipocyte markers ap2 and PPARγ, they demonstrated different expression patterns of osteoblastic markers: RSV increased the expression of OPG, IL-6, ALP, and RANKL, while INM increased OPG and OCN. To substantiate the findings with C3H10T1/2 cells, we also studied the effects of Sirt1-activating and -inhibiting compounds on rat primary bone marrow stromal cells. We found osteoblast markers ALP and Col-1 decreased in NAM-exposed cells. With INM, osteoblast markers ALP, Runx2, and OCN increased. RSV increased expression of Runx2 and OCN. We also analyzed the effects of these compounds on mineralization in cultures of rat primary cells. Mineralization was detected by von Kossa and alizarin red staining when cells were cultured in BIM. This was prevented with the addition of NAM and troglitazone to medium. Mineralization in INM-exposed cells resembled mineralization in bone-inducing medium, whereas RSV increased mineralization.

To our knowledge, this was the first time the effect of Sirt1 activity and RSV, INM, and NAM on differentiation of MSCs had been studied. It is likely that the demonstrated decrease in adipocyte differentiation was through inhibition of PPARγ. The increase in osteoblast differentiation could either be caused by inhibition of PPARγ that results in earlier initiation of the osteoblast differentiation program or that Sirt1, in addition to inhibition of PPARγ, also stimulates mechanism(s) regulating osteoblast

differentiation. As mentioned in Section 1.2.5 and 1.2.6.1, one possible mechanism could be deacetylation of p53, a potential inhibitor for osteogenesis and osteoblast differentiation. Sirt1 deacetylates the p53 protein and thus decreases its stability and transcriptional activity. However, it is also likely that Sirt1 can deacetylate and regulate other key transcriptional regulators in osteoblast development. This remains to be investigated.

Sirt1 activation results in prolonged lifespan in several lower organisms. Whether Sirt1 regulates aging in mammals has not been directly shown, but it is clear that caloric restriction prolongs lifespan in mammals. In relation to these findings, it is interesting to note that in age-related osteoporosis, adipose cells are increased in bone marrow. It remains to be studied if reduced Sirt1 activity could be one explanation for this. The results from our study further support the idea, as was suggested earlier [61], that inhibition of PPARγ could be considered as a means for treating osteoporosis. One way to do this is by using PPARγ antagonists. The finding that RSV and INM markedly inhibited adipocyte and promoted osteoblast differentiation is interesting and shows that other ways to inhibit PPARγ exist. However, further in vitro and in vivo studies are needed to understand the molecular details of the Sirt1 mechanism of action in MSCs. Results from this study could also contribute to the evolving field of cell-based tissue engineering. By identifying factors that can control osteoblast differentiation in vitro, better protocols for growing and differentiating MSCs to be used for bone reconstruction will hopefully be developed.

2.3.2 Paper II: Species difference exists in the effects of 1α,25(OH)$_2$D$_3$ and its analogue 2-Methylene-19-nor-(20S)-1,25-dihydroxyvitamin D$_3$ (2MD) on osteoblastic cells

In this study, we evaluated the in vitro effects of 1α,25(OH)$_2$D$_3$ and its analogue, 2MD, on osteoblasts from three different species, i.e. bone marrow stromal cells from the Sprague-Dawley (SD) rat, from the C57BL/6 mouse, as well as human osteoblast NHOst cells and human osteosarcoma derived MG-63 cells. We found that in rat cells, both compounds increased cell proliferation, inhibited cell apoptosis and increased alkaline phosphatase (ALP) activity. In mouse cells, however, both compounds initiated cell apoptosis and inhibited ALP activity. In human cells, although cell proliferation was inhibited by both compounds, cell apoptosis was inhibited and ALP activity was enhanced. In each species, 2MD was much more potent than 1α,25(OH)$_2$D$_3$.

First, our results are consistent with in vivo findings in rats and VDR knockout mice. Thus, the different effects of 1α,25(OH)$_2$D$_3$ on bone formation among different species are probably influenced by its direct effects on osteoblasts. The transcriptional and posttranscriptional control of gene expression is mediated by 1α,25(OH)$_2$D$_3$ at multiple levels. Therefore, the molecular mechanisms contributing to the species differences are likely to be complex. A nucleotide sequence variation between rat and mouse osteoblasts has been found in the distal half motif of osteocalcin vitamin D-responsive element (VDRE) and contributed significantly to the different response to

$1\alpha,25(OH)_2D_3$ [309]. Our results indicate that such nucleotide sequence variations in VDRE probably exist for many other genes which control cell growth and activity and thus determine the different response of the osteoblasts from different species to VDR ligands.

Second, our results in part explain the potent bone anabolic effects of 2MD. In rat cells 2MD demonstrated a much stronger anti-apoptotic effect and a higher potency in increasing ALP activity than $1\alpha,25(OH)_2D_3$. ALP plays an important role in facilitating bone mineralization through mechanisms such as breakdown of the major mineralization inhibitor, inorganic pyrophosphate [310]. The combined effects of inhibiting apoptosis and enhancing ALP activity in osteoblasts may result in a larger amount of osteoblasts which can form more organic bone matrix in a microenvironment favorable of bone mineralization. These specific effects on osteoblasts, together with its moderate effects in enhancing intestinal calcium absorption, could be the explanation for the dramatic bone anabolic effects of 2MD in rat. However, despite the higher potency of 2MD compared with $1\alpha,25(OH)_2D_3$ we did not find any qualitative difference between the two VDR ligands on osteoblasts. It is possible that the effects of 2MD on osteoblasts could be elicited by a higher dose of $1\alpha,25(OH)_2D_3$ but in vivo this would entail a high risk of hypercalcemia. However, in human osteoblastic cells, although 2MD dramatically increased ALP activity the cell number was actually reduced. Thus, more human-based experiments are needed in order to confirm the potential of 2MD as an agent used for treating osteoporosis.

2.3.3 Paper III: **IL-6 receptor expression and IL-6 effects change during osteoblast differentiation**

In this study, we found that IL-6, without soluble receptor, decreased MC3T3 cell proliferation only when the cells were cultured in medium containing ascorbic acid (AA) and β-glycerophosphate (β-GP). With this culture condition it has been shown that fully differentiated osteoblasts, i.e. cells that form mineralized noduli, can be obtained [311, 312]. This finding led us to study the expression pattern of IL-6R during osteoblasts differention and to further study the effects of IL-6 on differentiation and apoptosis of osteoblasts at different maturing stages. We detected IL-6R expression in early differentiated cells and also found that IL-6R expression was increased in osteoblasts during the *in vitro* culture period in bone inducing medium containing AA and β-GP or dexamethasone. Interestingly, we also found that IL-6 enhanced differentiation at early culture stage and later that it increased apoptosis.

The results of this study have several implications. First, from many in vitro studies it has been suggested that IL-6R is either only weakly expressed or absent from stromal/osteoblastic cells [252, 261-263], and accordingly the combination of IL-6 and soluble IL-6R is extensively used when studying IL-6 effects on bone cells [111]. However, as found in our study, with culture conditions known to favor osteoblast differentiation this is unnecessary. Furthermore, with this culture condition it is likely that for osteoblasts proper intracellular signaling mechanisms are evoked. Thus, data using soluble IL-6R on immature osteoblasts may not properly identify the physiological role of this cytokine. Second, based on our results, IL-6 strongly

increased osteoblast apoptosis in both MC3T3 and rat bone marrow stromal cells, and this pro-apoptotic effect was restricted to the late differentiation stage. As discussed in section 1.2.4.3 and 1.2.6.3, apoptosis has a substantial role in regulating the amount of functional osteoblasts and bone formation, and increase of apoptosis has been observed in several osteoporosis conditions associated with high levels of IL-6.

2.3.4 Paper IV: **Resveratrol inhibits proliferation and promotes apoptosis of osteosarcoma cells**

In paper I, we found that Sirt1 could affect the differentiation of osteoblast lineage cells. With this in mind we found it interesting to investigate if Sirt1 regulators could influence OS cells. This hypothesis also follows the assumption, as discussed in section 1.2.5, i.e. that differentiation regulators might be effective therapeutic agents in the treatment of OS. Another reason for us to investigate Sirt1 as a potential target in OS treatment is that the activity of Sirt1 can be affected by small molecules such as RSV and NAM, which have been shown to be relative safe when used at large doses in clinical settings or animal experiments.

In paper IV, we evaluated the effect of the Sirt1 activators, RSV and INM, on growth and apoptosis in four OS cell lines, HOS, Saos-2, U-2 OS and MG-63 and also in one normal human osteoblast cell line, NHOst. We found that Sirt1 was expressed at roughly similar levels in these cell lines as analyzed by Western blot using a Sirt1 antibody. Measurement of cell proliferation indicated that RSV dose dependently inhibited cell growth in all four OS cell lines, an effect already seen at the lowest tested concentration, 5 μM. Furthermore, apoptosis analysis through flow cytometry showed that RSV, at all tested concentrations (5-100 μM), elicited significant apoptosis in all four osteosarcoma cell lines. However, for the normal human osteoblast cell line NHOst, it was only at a concentration of 100 μM that RSV induced significant apoptosis. Similar to RSV, INM also dose-dependently increased apoptosis in all four OS cell lines. As discussed in section 1.2.6.1, the limitation of Sirt1 deacetylation could be relieved under nutritional stresses through induction of Pnc1 and deprivation of NAM. Therefore, we found it interesting to investigate if nutritional stress could enhance the effect of RSV on OS cells. In comparison with normal tissue, tumor cells seem to be deficient of asparagine synthetase and have to rely on external sources of L-asparagine to keep up with their rapid growth [313, 314]. We first tested different concentrations of L-asparaginase (ASNase) on the four OS cell lines and, as a comparison, the lymphoblastic cell line Jurkat. No changes for Sirt1 protein expression was noticed before and after treatment; however, these cells did show a variable pattern of responses to ASNase, i.e. the lymphoblastic leukemia cells Jurkat, HOS and Saos-2 cells underwent apoptosis in a dose-dependent fashion, while U-2 OS and MG-63 cells showed minimal or no response. This differential response to ASNase could be due to different metabolic adaptation after ASNase exposure, partly explained by different expression of glutamine synthetase in the different tumor cell lines [315]. When OS cells were exposed to a combination of ASNase and RSV an apparent synergistic effect on apoptosis was found.

Our results indicate the Sirt1 activators, RSV and INM, could complement the therapeutic arsenal used in the treatment of osteosarcoma. However, whether the anti-tumor effects of these small molecules are mediated through Sirt1 needs further confirmation. Several other mechanisms have been proposed for RSV mediated tumor inhibition. The most established mechanism involves p53. It has been reported that RSV induces apoptosis only in cells expressing wild-type p53, but not in p53-deficient cells [316] and that ERKs, p38 kinase, and JNKs are mediators of RSV-induced p53 activation and apoptosis [317, 318]. The osteosarcoma cell lines used in our study have different p53 status. The wild-type p53 has only been detected in U-2 OS cells [319], whereas it has been shown to be mutated in HOS and Saos-2 and totally deleted in MG-63 cells [320, 321]. However, U-2 OS cells were not more sensitive to RSV compared with the OS cells with a p53 mutation, indicating p53 is not the target for RSV in the four OS cell lines used in this study.

The relationship between Sirt1 activity and tumor initiation and progression and growth of established tumors is at present not fully understood [322]. On one hand, Sirt1 has been found to be highly expressed in several types of tumors [323] and may be correlated with the development of chemotherapy resistance [324]. On the other hand, Sirt1 is an important mediator of CR-associated tumor prevention [325, 326]. Effects such as suppressing intestinal tumorigenesis and colon cancer growth has recently been reported [327]. Although the fact that both Sirt1 activators in our study induced apoptosis is a strong indication that activation of Sirt1 initiated apoptosis in OS cells, more studies, in vitro and in vivo, are needed to confirm this and to clarify the underling mechanism(s). However, the results of this study suggest that the use of Sirt1 activators presents an interesting and potential therapeutic strategy for the treatment of OS.

3 CONCLUDING REMARKS

The aim of this thesis was to study osteoblast lineage cells and the effects of selected factors on proliferation, differentiation and apoptosis. Based on the results, the following conclusions can be drawn:

1. The commitment of MSCs to osteoblast or adipocyte lineage is affected by Sirt1 regulators. Sirt1 activitors, RSV and INM, promote osteoblastogenisis and block adipocyte formation, while the inhibitor, NAM, exerts opposite effects.

2. The direct effects of $1\alpha,25(OH)_2D_3$ and 2MD on osteoblastic cells are species-dependent. In the studied species, 2MD was more potent than $1\alpha,25(OH)_2D_3$.

3. IL-6R expression increases during differentiation of osteoblastic cells. IL-6 accelerates differentiation of preosteoblasts but induces apoptosis of mature cells.

4. The Sirt1 activators, RSV and INM, inhibit proliferation and induce apoptosis of OS cells. The inhibitory effect of RSV on OS cells can be enhanced by selective nutrition restriction using ASNase.

4 FUTURE PERSPECTIVES

The number and functions of osteoblasts are regulated by a number of systemic hormones and local factors. Our findings provide new aspects on two putative regulators, $1\alpha,25(OH)_2D_3$ and IL-6. Additionally, a role for Sirt1 in regulating osteoblast differentiation and in the treatment of osteosarcoma is proposed.

The mechanism for the species-dependent effect of $1\alpha,25(OH)_2D_3$ on osteoblasts demand further exploration. In addition, since $1\alpha,25(OH)_2D_3$ is a pluripotent systemic hormone with target cells in several organs and exerting a broad range of physiological effects, it is of interest to investigate if similar species-dependent effects exist in other target cells.

Our study of IL-6 demonstrates that, besides the earlier known function to activate osteoclastic bone resorption, this cytokine affects both differentiation and apopotosis of osteoblasts. The association of IL-6 and bone diseases, such as rheumatoid arthritis and postmenopausal osteoporosis, has initiated development of IL-6 antagonist for future use in treatment of these diseases. However, further studies are needed in order to fully understand the role IL-6 plays in bone physiology.

The findings about Sirt1 are exciting. Sirt1 has been found to be related to cellular stress, metabolic state and aging. Sirt1 affecting MSC commitment indicates a potential role of this protein in age-related osteoporosis, which is usually associated with an increase of bone marrow fat. Additionally, effects of Sirt1 on tumor cells suggest the potential application of its activators in the treatment of osteosarcoma. Furthermore, the mechanism(s) underling these interesting effects are needed to be clarified, for example, Sirt1 activity is mostly inhibited by NAM, which can be degraded by nicotinamidase. Studies about the expression and activity of nicotinamidase during aging and metabolic disturbance should be performed.

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