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Functional Analyses of Candidate Genes for Osteoporosis: *RUNX2* and *LRP5* interplay during differentiation of the hFOB human osteoblast cell line

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Functional Analyses of Candidate Genes for Osteoporosis: *RUNX2* and *LRP5* interplay during differentiation of the hFOB human osteoblast cell line

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THIS WORK IS DEDICATED TO MY FAMILY

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INTRODUCTION

1. Introduction

1. 1 Osteoporosis

Osteoporosis is a general skeletal disease. It is characterized by a low bone mass and a microarchitectural deterioration of bone tissue, which increase bone fragility and susceptibility to fracture. The risk of osteoporosis is greater in women than in men; also, the risk varies between countries [1, 2].

The term ‘osteoporosis’ first entered medical terminology in the nineteenth century in France and Germany, as a term for the porosity of the histological appearances of aged human bone. The World Health Organization (WHO) in 1994 defined osteoporosis as BMD (Bone Mineral Density) measurements in women that fall more than 2.5 standard deviations below the mean BMD value of young women [3]. Osteoporotic fracture is the major health consequence of osteoporosis that can occur at any skeletal site. The primary sites of osteoporotic fracture are the spine, hip (proximal femur), humerus and distal forearm [4].

Osteoporosis is a major public health problem due to its association with fragility fractures. It is estimated that 10 million Americans who are over 50 years old have osteoporosis; and another 34 million are in the risk zone also Almost 25% of European women aged over 50 years are affected [3, 5, 6]. Osteoporosis can be classified as primary type 1, primary type 2, primary type 3, and secondary (Figure 1).

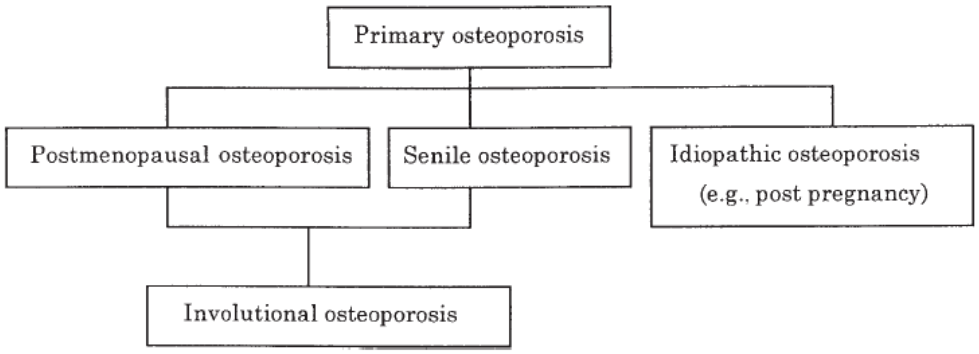


Figure 1. Classification of primary osteoporosis [7].

Primary type 2 is also known as senile osteoporosis, and primary type 3 is referred to as idiopathic osteoporosis.

Secondary osteoporosis occurs in all ages, and is caused by other factors which affect BMD. Examples of these factors include endocrinological, nutritional, drug induced, immobilization and congenital [7].

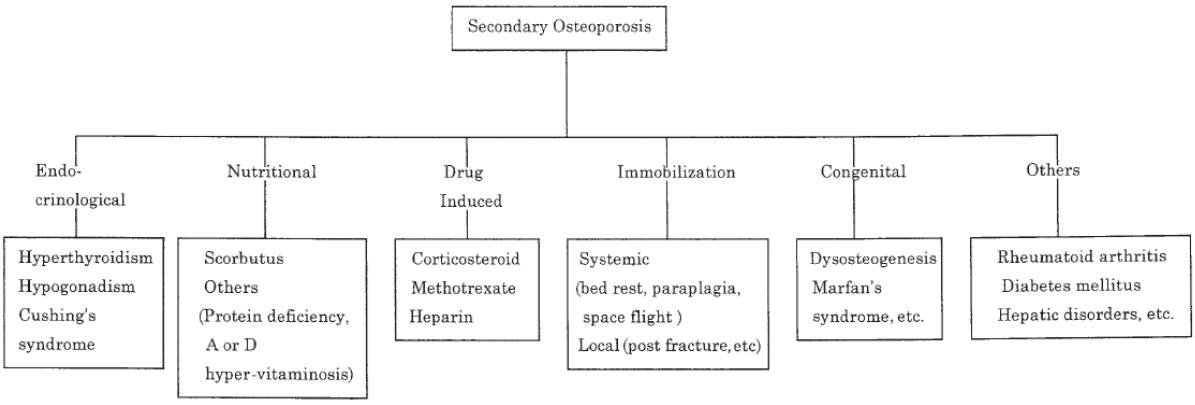


Figure 2. Classification of secondary osteoporosis [7].

The gold standard method for diagnosing osteopenia or osteoporosis is BMD measurement by dual-energy x-ray absorptiometry (DXA) of the hip (femoral neck or total hip). Table 1 [8].

Table 1. Definitions* of osteopenia and osteoporosis in white women by World Health Organization [8].

Normal	Hip BMD > 1.0 SD below the young adult female reference mean** (T score above -1.0)
Osteopenia	Hip BMD between 1.0 and 2.5 SDs below the young adult female reference mean** (T score between -1.0 and - 2.5)
Osteoporosis	Hip BMD > 2.5 SDs below the young adult female reference mean** (T score at or below -2.5)
Severe osteoporosis or established osteoporosis	Hip BMD > 2.5 SDs below the young adult female reference mean** in the presence of 1 or more fragility fractures

*Based on hip bone mineral density (BMD) measurements assessed with dual-energy x-ray absorptiometry.
** The young adult female reference mean is determined with use of the mean hip BMD from National Health and Nutrition Examination Survey reference database of women aged 20-29 years.

Bone mineral density is reported as the mineral weight in grams per cm^2 for the tested bone. It is noted that BMD also decreases with increasing age; see e.g. Figure 3 [9].

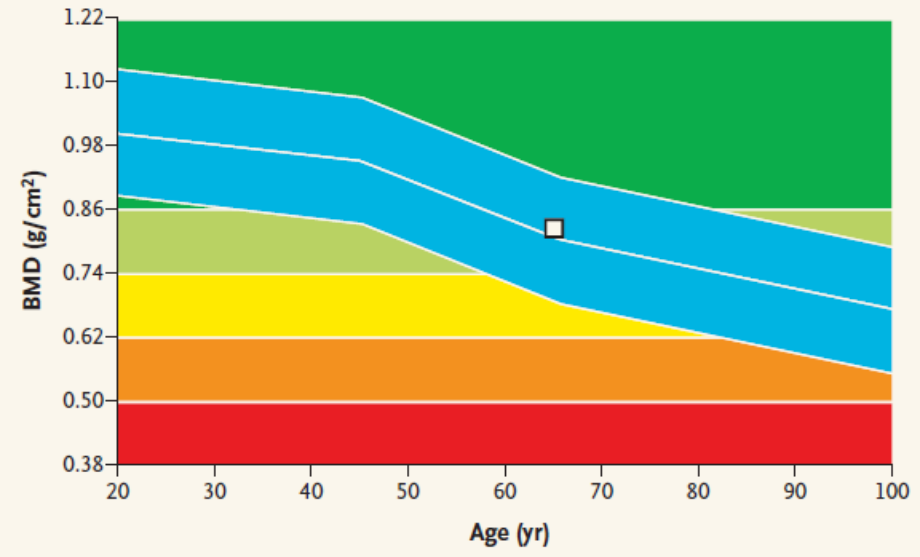


Figure 3. Diagnosis of osteoporosis on the basis of BMD of the hip [9].The Color stripes indicate the degree of concern related to bone density; red denotes hogh concern and green low concern.

1.2 Bone biology

Bone denotes a family of materials, which are made of mineralized collagen fibrils. Bone materials have highly complex structures with different functions such as locomotion, mechanical support and protection of different limbs, support of hematopoiesis in the bone marrow and repair of fractures and other bony defects [10]. About 10% of the bone mass in adults is replaced each year and complete renewal of the mass occurs every 10 year. Bone formation and bone resorption are ongoing processes, which are normally in balance; imbalance can have serious consequences [11]; this will be elaborated in the coming sections.

1.2.1 Bone modeling

Bone modeling refers to either the formation of bone by osteoblasts (Formation modeling) or resorption of bone by osteoclasts (Resorptive modeling) on a bone surface. The main function of bone modeling is to increase the bone mass and maintain or change the bone shape. The process of modeling occurs in two stages, activation and formation or resorption. Activation is the process of recruitment of precursor cells that differentiate into mature osteoblasts or osteoclasts, which will be discussed in chapter 1.2.2. Once the appropriate cells are activated, the processes of

formation or resorption take place until sufficient bone mass is produced or removed. Bone modeling is most prominent during human growth and development, and the adult skeleton does also undergo modeling [12].

1.2.2 Bone remodelling

Bone undergoes a process denoted as remodelling, which involves the breaking- down (resorption) and build-up (formation) of bone. These processes occur in micro-scale throughout the skeleton and will take over several weeks. Cells that are involved in bone remodelling can be divided in 3 different types, osteoblast, osteoclast and osteocytes [13, 14]. There is a connection between bone resorption and bone formation; imbalances in these two processes can lead to massive distresses in skeletal structure and function, and potentially to morbidity and shortening of the lifespan [14, 15]. Activities of these 3 cell lines are balanced by a complex signal system, which involves three major components. The components are receptor activator of nuclear factor kappa B (NF- κB) (RANK), RANK ligand (RANKL) and Osteoprotegrin (OPG) known as RANK/ RANKL/ OPG system. Expectedly, modification of this RANK/ RANKL/ OPG signaling pathway has major effects on bone remodeling [16].

1.2.2.1 Osteoblast and bone formation

Osteoblasts develop from bone marrow derived from multipotent mesenchymal stem cells (MSCs). Osteoblasts differentiate then into proliferating preosteoblasts, mature osteoblasts, and eventually into osteocytes (Figure 4) [15, 17].

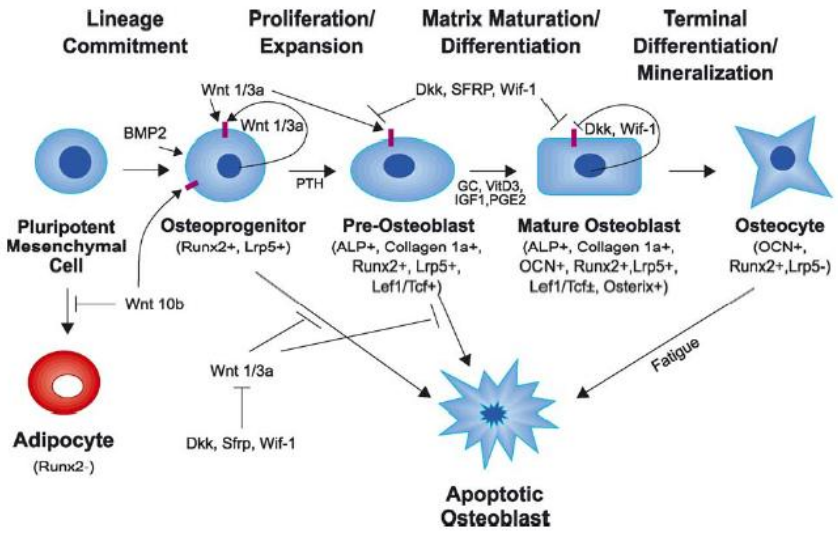


Figure 4. Mesenchymal stem cell differentiation toward osteoblastic lineage [18].

RUNX2 is the earliest osteoblastic marker. It is necessary for the progenitor cell differentiation along the osteoblast lineage, which is turn regulates expression of the genes encoding of Osteocalcin, RANKL and Sclerostin. Another transcription factor that is essential for the osteoblast differentiation is Osterix [19-21]. A large number of paracrine, autocrine, and endocrine factors affect the osteoblast development and maturation. Examples include bone morphogenetic proteins (BMPs), growth factors like FGF and IGF, angiogenic factors like endothelin-1, hormones like PTH and prostaglandin agonists. The action of PTH and BMPs (Figure 4) is closely associated with the activation of Wnt signaling pathways. Wnts are secreted glycoproteins, which are active in all cells of the osteoblastic lineage, and crucial for the development and renewal bone tissue. The activation of canonical Wnt-signaling promotes osteoblast differentiation from mesenchymal progenitors, which leads to improved bone strength; while suppression of it causes bone loss. Canonical Wnt signaling in osteoblast differentiation is modulated by Runx2 and osterix [17, 22-24].

The fully differentiated osteoblast is characterized by the co-expression of alkaline phosphatase and type I collagen; both are important for synthesis of bone matrix and the subsequent mineralization. At the end of their lifespan osteoblasts transform into either osteocytes or into lining cells, which become embedded in the mineralized matrix and cover all surfaces of bone [18, 25-27].

1.2.2.2 Osteoclast and bone resorption

The osteoclast is a tissue-specific macrophage polykaryon, which is differentiated from monocyte/macrophage precursors cells, at or near the bone surface (Figure 5).

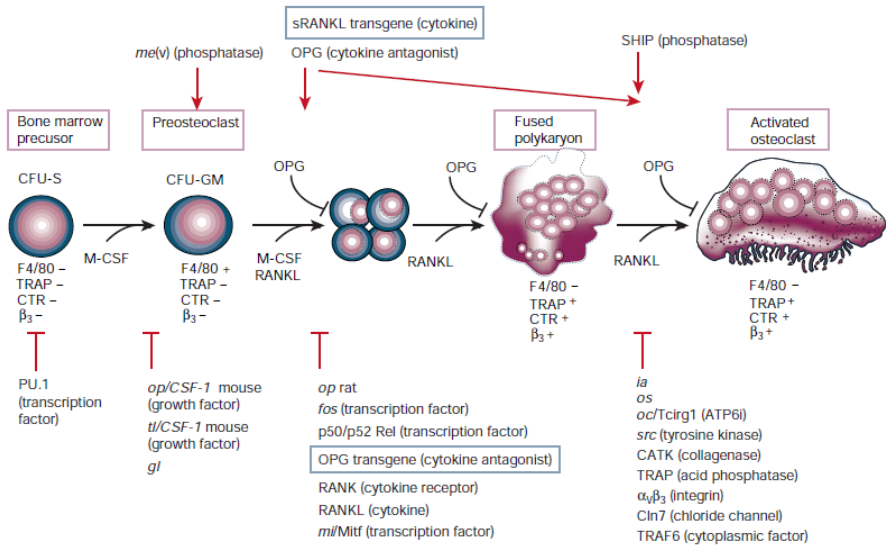


Figure 5. Osteoclastogenesis [14].

The close contact between stromal and bone marrow cell types allows for the production of the two stromal derived factors, TNF-related cytokine RANKL and the polypeptide growth factor CSF-1; which are necessary and sufficient for proliferation and maturation of pre-osteoclast .

The osteoclast cell body must be polarized in order to start bone resorption. In response to the activation of RANK by RANKL, the osteoclast cell undergoes internal structural changes that prepare it to resorb bone. Degradation products (collagen fragments and solubilized calcium and phosphate) are processed within the osteoclast and released into the circulation.

The survival of the mature osteoclast and its participation in successive rounds of bone resorption is regulated in part by hormones and cytokines [28, 29]. At least 24 genes or loci have been shown to positively and negatively regulate osteoclastogenesis and osteoclast activation (Figure 5) [14].

1.2.2.3 Osteocytes

Osteocytes represent terminally differentiated osteoblasts as mentioned in section 1.2.2.1, and function within syncytial networks to support bone structure and metabolism. Osteocytes lie within lacunae of the mineralized bone.

Osteocytes do not normally express alkaline phosphatase but do express osteocalcin and several other bone matrix proteins. Osteocytes are active during osteolysis and may function as phagocytic cells as they contain lysosomes [30]. Osteocytes respond to mechanical load, and they detect the related bone microdamage due to the skeletal loading and fatigue. Translation of microdamage into targeted remodelling occurs by mechanosensory signals from the osteocyte network into biochemical signals of resorption or formation in osteoclast, and osteoblast activity on trabecular surfaces (Figure 6) [15, 31, 32].

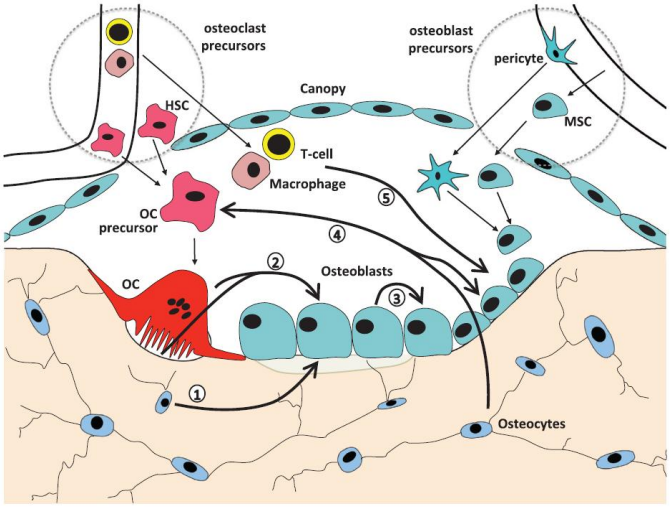


Figure 6. Intercellular communication pathways [33].
(1) Stimulatory and inhibitory signals from osteocytes to osteoblasts.
(2) Stimulatory and inhibitory signals from osteoclasts to osteoblasts.
(3) Signaling within the osteoblast.
(4) Stimulatory and inhibitory signals between the osteocyte and osteoclast lineages.
(5) Marrow cell signals to osteoblasts.

It is believed that osteocytes initiate and direct the subsequent remodelling process that repairs damaged bone [13, 34].

1.3 Genetics of osteoporosis

Osteoporosis is a complex disease in which the phenotype is caused by the effects of multiple quantitative trait *loci*. The effects of these genes on bone mass are in turn altered by interactions of the genes with several environmental factors [35, 36]. Studies of female twins, including studies of postmenopausal twins, have shown heritability of BMD to be 57% to 92% [37-40]. Intergenerational family studies have also identified substantial heritability of BMD (44% to 67%) [41-43]. Bone fracture is also heritable to some extent, but heritability is known to decrease with age. For example, a study of Swedish twins showed that the heritability of hip fracture was about 68% in those under the age of 65, but the heritability dropped rapidly with age to reach a value of close to zero by the age of 80 [44] . Segregation analysis in families has shown that determination of BMD and other osteoporosis-related phenotypes is polygenic. The effects are not caused by a few genes having a large effect; rather there are many genes each with a relatively small effect. There is evidence suggesting that genes with larger effects may be involved in at least some populations [41, 45].

1.4 Approaches for the identification of osteoporosis susceptibility genes and variants

Several approaches have been used to identify the genes responsible for osteoporosis, including linkage analysis, association studies [candidate gene and genome-wide (GWAS)] and animal studies.

1.4.1 Linkage analysis

Linkage analysis is a classical approach for gene discovery in an inherited disease. Linkage analysis tracks alleles (as they are transmitted between generations of a family) to determine whether a trait and genetic *loci* co-segregate within a family [46]. Linkage analysis is very powerful for the identification of causal genes in Mendelian disorders (i.e. diseases caused by mutation in a single gene).

Severe osteoporosis, bone fragility, or abnormally high bone mass may be inherited as the result of mutations in single genes, as summarized in Table 2.

Table 2. Monogenic bone diseases associated with abnormal bone mass [35, 47, 48].

Disease	Phenotype	Genes
Osteogenesis imperfecta	Low BMD, fractures	*COL1A1,COL1A2, CRTAP, LEPRE1, SERPINEF1, IFTTM5*
Osteopetrosis	High bone mass, fractures, bone marrow failure, blindness, osteoarthritis	*CLCN7, TCIRG1, CATK, OSTM1, RANKL, RANK, PLEKHM1, SNX10, TNFRSF11A, TNFSF11*
High bone mass syndrome	High bone mass, torus palatinus	*LRP5, LRP4*
Sclerosteosis	High bone mass, bone overgrowth, nerve compression syndromes	*SOST*
van Buchem disease		
Aromatase deficiency	Osteoporosis	*CYP17*
Estrogen receptor deficiency	Osteoporosis, tall stature	*ESR1*
Osteoporosis pseudoglyoma	Early-onset osteoporosis; ocular pseudoglioma or vitreoretinopathy	*LRP5*

Following linkage studies, one of the genes that was discovered to be a key regulator of bone mass in the osteoporosis– pseudoglioma syndrome (OPPS) and high bone mass syndrome, is lipoprotein receptor-related protein 5 (*LRP5*) [49-51].

1.4.2 Association study

1.4.2.1 Candidate gene study

Candidate gene association studies have been widely used in the field of osteoporosis by identifying allele frequency differences which might be associated with the disease. Association studies are easy to perform and are capable of detecting small effects of alleles if sufficiently large sample-sized are used. Otherwise, false positive results may be easily obtained. Replication in a second cohort is a necessary step. But, if insufficient care has been paid to matching cases and controls then

erroneous results will raise due to population stratification. However, these problems can partly be overcome by a careful study-design, and with statistical corrections for confounding factors and population stratification.

Approximately 150 candidate genes have been investigated for their relationship with BMD or fractures in humans. Very few genes have been examined in large-scale studies such as collagen type 1 α 1 (*COLA1*), estrogen receptor 1 (*ESR1*), vitamin D receptor (*VDR*) and lipoprotein receptor related protein 5 and 6 (*LRP5 and LRP6*). A brief summary of the investigations have been listed below.

For example, Ralston et al. [52] investigated the role of COLIA1 Sp1 alleles as an indicator of BMD and fracture. These authors found that the COLIA1 Sp1 polymorphism is associated with reduced BMD and could influence the incident vertebral fractures in women, independent of BMD. Ioannidis et al. [53] investigated whether 3 common ESR1 polymorphism are associated with BMD and fractures. The authors observed that none of the 3 polymorphisms had any statistically significant effect on BMD; furthermore, effects on fractures were independent of BMD. Uitterlinden et al. [54] studied the relation between VDR polymorphisms, BMD, and fractures. Comparisons of BMD at the lumbar spine and femoral neck showed insignificant differences. Also, it was shown that FokI, BsmI, ApaI, and TaqI VDR polymorphisms are not associated with BMD or with fractures. However, the results indicated that the Cdx2 polymorphism may be associated with risk for vertebral fractures. van Emeurs [55] studied the association of 2 common variants of LRP5 (Val667Met, Ala1330Val) and1variant of LRP6(Ile1062Val) with BMD and fracture risk. It was found that Common LRP5 variants are consistently associated with BMD and fracture risk across different white populations. Also the LRP6 Ile1062Val polymorphism is not associated with any osteoporosis phenotype.

1.4.2.2 Genome wide study

Advances in genome-wide SNP identification and in genotyping technologies have made it possible to perform association studies on a genome-wide basis, by

analyzing large numbers of SNPs spread at regular intervals across the genome, rather than focusing on a specific candidate gene. GWAS have been successfully applied to the study of many complex diseases, including osteoporosis [56-58].

The first GWAS reported for BMD in children, identified the *SP7* locus (which encodes the transcription factor osterix) as being associated with BMD [59].

Meta-analysis is the statistical analysis of a large collection of results from individual studies for the purpose of integrating the findings. The first large-scale meta-analysis for BMD by Rivadeneira et al. identified 13 novel regions that were genome-wide-significant for BMD [60]. The meta-analysis (Figure 7) identified 467 SNPs from 20 genomic loci exceeding the GWS threshold of association with the BMD traits.

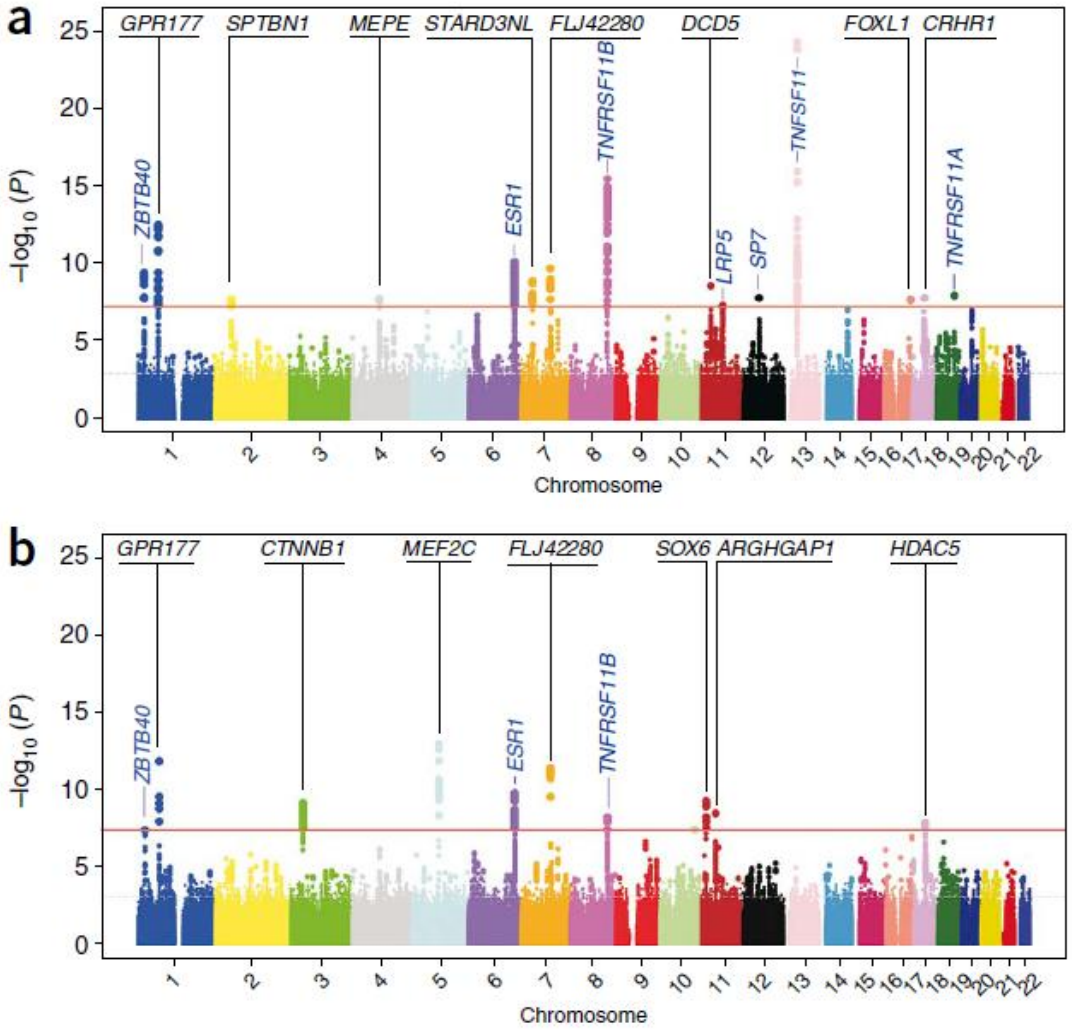


Figure 7. Manhattan plots. (a,b). The plots display newly discovered and previously reported (known) loci associated at genome-wide significant level (GWS) with lumbar spine BMD (a) and femoral neck BMD (b) for all 2,543,686 HapMap CEU-imputed SNPs analyzed using fixed-effects. The 13 new GWS loci are in underlined type. Previously reported GWS loci are in blue type [60].

The Meta-analysis study doubled the number of loci that are associated with BMD at a genome-wide-significant level. A second Osteoporosis (GEFOS) meta-analysis by Estrada et al., in which all subjects were of European or East Asian ancestry, identified 32 novel loci for BMD [61]. This study also covered osteoporotic fracture as a phenotype. The obtained results pointed to the highly polygenic nature of BMD variation, and the critical role of several biological pathways that influence osteoporosis and fracture susceptibility. In addition to the Wnt factors, which are known to be associated with BMD (CTNNB1, SOST, LRP4, LRP5, WLS, WNT4 and MEF2C), several of the newly discovered loci implied additional Wnt signaling factors (including WNT5B, WNT16, DKK1, PTHLH, SFRP4 and AXIN1). Furthermore, another clearly delineated pathway was discovered to be involved in the mesenchymal stem cell differentiation, which included the newly identified RUNX2, SOX4 and SOX9 BMD-associated loci (along with the previously known SP7).
Another bone-relevant pathway is endochondral ossification, which involves essential processes during fetal development of the mammalian skeleton; in which several of the identified BMD-associated loci are implicated, among others SPP1, MEF2C, RUNX2, SOX6, PTHLH, SP7 and SOX9 [55, 57].

1.4.3 Cell and animal studies

1.4.3.1 Cell lines

Cell lines provide information about the processes of skeletal development, bone formation and bone resorption. The information can be used to formulate new forms of treatment for common bone diseases such as osteoporosis. Osteoblast systems offer unique advantages by increasing our understanding of bone development, differentiation, gene expression and responses to hormones and local factors. These systems include primary cultures of bone cells and organ cultures, osteosarcoma cell lines, non-transformed cell lines and experimentally immortalized cell lines.

Primary cell cultures, especially the calvaria derived ones, undergo changes which mimic osteoblastic differentiation in vivo, thus providing insights into the stage-wise regulation of gene expression in osteoblastic cells. The limitation of the mentioned cell cultures are their inherent instability and the possibility of abnormal behavior associated with many of the clonal cell lines [62].

The most widely used osteosarcoma cell lines are the UMR 106 and the ROS 17/2. The cell lines are derived from a transplantable rat ^{32}P-induced malignant osteogenic sarcoma and spontaneous tumor in an ACI rat, respectively. Other osteoblastic cells derived from human osteosarcomas include the Saos2 (our present research focuses on this cell line), U2OS, OHS- 4, TE-85, KPDXM, TPXM and CAL7 [63-70].

The UMR 201and MC3T3-E1 cell lines are clonal non-transformed cell lines derived from neonatal rat and newborn mouse calvaria, respectively. UMR 201 has a limited life span and phenotypic features of pre-osteoblasts [71-73]. Other cell lines, which belong to clonal non-transformed osteoblastic cells, are CRP 4/7, CRP 7/4, CRP 7/7, CRP 10/3 and CRP 10/30; isolated from neonatal calvarial bone cells in the presence of TGFβ and EGF [74].

RCT-1 and RCT-3 are immortalized cell lines, derived from embryonic rat calvarial cells, and transfected with a recombinant retrovirus containing the cDNA for SV40 large T antigen. RCT-1 cells, after induction by retinoic acid, express osteoblastic traits [ALP, pro- α1(I) collagen, PTH-responsive adenylate cyclase]; and RCT-3 cells express osteoblastic markers except for osteocalcin [75, 76]. Other immortalized cell lines, which are used to study certain stages of osteoblast differentiation, are adult human osteoblast-like (hOB) cells [77] and the human fetal osteoblast cell line hFOB. hFOB is derived from biopsies obtained from a spontaneous miscarriage. This immortalized cell line has minimal chromosome abnormalities and has the ability to form bone in vivo without developing transformation. Furthermore, it has been shown that the cells have the ability to spontaneously produce mineralized nodules and to form extracellular matrix in *in vitro* cultures. These cells are known to express many osteoblastic markers including high alkaline phosphatase activity, 1,25 dihydroxyvitamin D3- inducible osteocalcin expression, and parathyroid hormone

(PTH)-inducible cAMP production. Furthermore, hFOB 1.19 is conditionally immortalized with a gene coding for a temperature-sensitive mutant, tsA58, of SV40 large T antigen [78, 79].

Several immortalized cell lines with phenotypic characteristics of osteocyte-like cell lines such as MLO-Y4 (murine long bone osteocyte Y4), MLOA5, MLO-A2, MLO-D1 and MLO-D6 have been established by Bonewald et al [80].

Unlike osteoblasts, osteoclasts are difficult to study *in vitro*, because they are relatively scarce, terminally differentiated, adherent to mineralized surfaces and fragile. However, different methods have been developed to isolate these cells in vitro or to induce their formation in bone marrow cultures. Mature, multinucleated functional osteoclasts are obtained either directly from bone as the primary source, or secondarily generated in vitro from hematopoietic progenitors or peripheral blood mononuclear cells (PBMNC). RAW264.7 is a mouse osteoclast-like myeloma cell line capable of differentiating into osteoclasts when treated with RANKL. Other main characteristics of osteoclasts are: tartrate resistant, acid phosphatase (TRAP) staining, multinuclearity, formation of actin ring structure and a polar cell body during resorption, and contraction in response to calcitonin. Osteoclasts express a number of molecular markers, such as calcitonin receptor, RANK (receptor of RANKL, receptor activator of NFκB ligand), c-fms (receptor of M-CSF, macrophage-colony stimulating factor), cathepsin K, c-src, fosL1 and the vitronectin receptor (integrin αvβ3) [81-85].

1.4.3.2 Animal models

Animal models have aided our understanding of the pathophysiology and treatment of osteoporosis. They are used for multiple purposes including investigation of signaling pathways that regulate bone growth, turnover, and repair, characterization of the cellular, biomechanical, biochemical- and molecular mechanisms, study of prevention and reversal of bone loss, and investigation of bone repair following an osteoporotic fracture.

Animals that are particularly important in osteoporosis research are chicken, mouse, rat, rabbit, dog, pig, sheep, and nonhuman primate species. The usefulness of each animal model is summarized in Table 3. Each species has advantages and shortcoming; and no laboratory animal can be used to model all of the risk factors that are associated with osteoporosis [86, 87].

Table 3. Summary of in vivo animal models for osteoporosis [86]**.**

Attribute	Human	Avian	Mouse	Rat	Dog	Pig	Sheep	Primate
Growth and adult phases?	Yes	Ok	Ok	Ok	Yes	Yes	Yes	Yes
Menstrual/estrus cyclicity	28 Days	Daily	Inducible	4–5 Days	205 Days	21 Days	21 Days seasonal	21–28 Days
Natural menopause	Yes	No	Yes*	Yes*	No	?	?	Yes
Bone loss after estrogen depletion	Yes	?	Probably	Yes	Not consistent	Weak	Weak	Yes
Response to estrogen	Turnover ßz4	Formation ßz3	Formation ßz3	Turnover ßz4	Not consistent	?	?	Turnover ßz4
Development of osteoporotic fractures	Yes	No	No	No +	No +	?	?	No +
Cancellous remodeling	Yes	No	No	Some	Yes	Yes	Yes	Yes
Haversian remodeling	Present (study site difficult)	No	No	Low levels; INducible	Yes	Yes	Yes	Yes
Time frame compression	No	?	Yes	Yes	No	Some	Some	Some
Convenience	OK*	Yes	Yes	Yes	Weak	Poor	Poor	Depends
Drug dose range like humans?	Yes	?	No	No (1/100)	Close	?	?	Yes
Cost effectiveness	Yes*	No	Yes	Yes	Weak	?	?	?

"Ok" denotes possible model for the study of Osteoporosis. "Yes" denotes definite model for the study of Osteoporosis. "*" refers to convenience dependent on the attribute of the animal.

1.4.3.2.1 Rats

Rats are commonly used laboratory animal for the study of osteoporosis. Rats are studied because they are inexpensive to purchase and maintain, grow rapidly, have a relatively short life span, and have a well-characterized skeleton. There are striking similarities as well as fundamental differences between rats and humans in bone

growth and remodelling that affect the use of the rat as an animal model for human osteoporosis [87].

The ovariectomized (OVX) skeletally mature rat is proven to have a predictive value as a preclinical model for therapies to prevent and treat postmenopausal osteoporosis. This rat is an interesting model for postmenopausal osteoporosis because acute ovarian hormone deficiency leads to elevated cancellous bone turnover [88].

The growing rat can potentially be used as a model for evaluating the effects of genetics, gender, endocrine, and environmental factors on peak bone mass. The rat is also used for the investigation of early-onset juvenile and adolescent onset osteoporosis [87, 89, 90]. Growing and adult orchiectomized rats are useful for investing factors related to the actions of sex steroids on growth and maintenance of peak bone mass in males [87].

The rat has also extensively been used as a model for disuse osteoporosis and understanding the etiology and severity of alcohol-induced bone loss [91-96]. The other purposes of using rat models are investigation of senile osteoporosis, glucocorticoid-induced osteoporosis; inflammation-induced osteoporosis and primary hyperparathyroidism; which is thought to contribute to age-related increases in bone turnover and bone loss [97-101].

1.4.3.2.2 Mice

The mice are currently used as a model for the study of osteoporosis. The advantages and disadvantages of the small size of the rat are even more pronounced in the mouse. The two rodents have similar growth characteristics. However, they differ radically in their physiologies that offer unique advantages as laboratory animal models for osteoporosis research. The mouse is an exquisite laboratory animal model for studying the genetic risk factors of osteoporosis and age-related bone loss. It has also successfully replicated the skeletal phenotypes related to several genetic disorders in humans [49, 102-105]. There are numerous well-characterized mouse strains with differences in bone mass and there is a long and growing list of transgenic mice with altered bone metabolism that are obtained by manipulation of specific gene

expression. Similarly, many factors that 'regulate' bone mass and turnover have been identified in gene knockouts, knockins, and loss and gain of function mutations in mice; some of which will be discussed in chapter 1.5 and 1.6.

The use of mouse as a preclinical model for postmenopausal osteoporosis is unproven and cannot be recommended for research in this area of research [106, 107].

Mice have also been used with mixed results for the investigation of disuse osteoporosis [108-110], senile osteoporosis [105, 111, 112], in studies of glucocorticoid-induced bone loss [113-116], and of the bone anabolic response to intermittent PTH [117-121].

1.4.3.2.3 Dogs and Primates

Dogs are appropriate for studies of intracortical bone remodelling because, similar to humans, they have well-developed bone remodelling, and also have major advantages as a model for highly localized bone fragility and generalized disuse [122]. The large size and relatively long life span also discourages the use of the dog model because of the increased cost of its maintenance. An additional consideration in the studies is the reduced availability of molecular probes specific to dogs compared to rats and mice [87].

Several species of monkeys have been used as models for osteoporosis. The physiology of monkey is more similar to human physiology than other commonly used animal models for osteoporosis. The most compelling evidence for generalized age-related osteopenia in an animal model is in monkeys. Unfortunately, extensive bone loss has not been reported in monkeys. The use of monkeys as a model for osteoporosis is greatly limited by their expense, long life span, limited availability, and ethical concerns [123].

1.5 RUNX2

RUNX2, also known as CBFA1 or AML3, is a major transcription factor in controlling osteoblast commitment and differentiation. It belongs to the Runt family of transcription factors (Table 4).

Table 4. Genes of the *Runx* family [124].

Runx family	**Main function**	**Related diseases in humans**
Runx1/Cbfa2/Pebp2αB	Hematopoietic stem cell differentiation	Acute myeloid leukemia
Runx2/Cbfa1/Pebp2αA	Osteoblast differentiation, chondrocyte maturation	Cleidocranial dysplasia
Runx3/Cbfa3/Pebp2αC	Growth regulation of gastric epithelial cells	Gastric cancer
Cofactor of Runx2		
Cbfb/Pebp2β	Essential factor for Runx1	Acute myeloid leukemia

Runx2 is initially detected during embryogenesis on day 9.5 in the notochord, at day 10.5 in the mesoderm and by day 11.5 strongly expressed in mesenchymal cells at the onset of skeletal development. It is also present in osteoblasts throughout their differentiation (Figure 8) [124, 125].

Runx2 is necessary for the regulation of skeletal genes, hypertrophic chondrocytes as well as endochondral and intramembraneous bone formation and skeletal development [20, 125, 126].

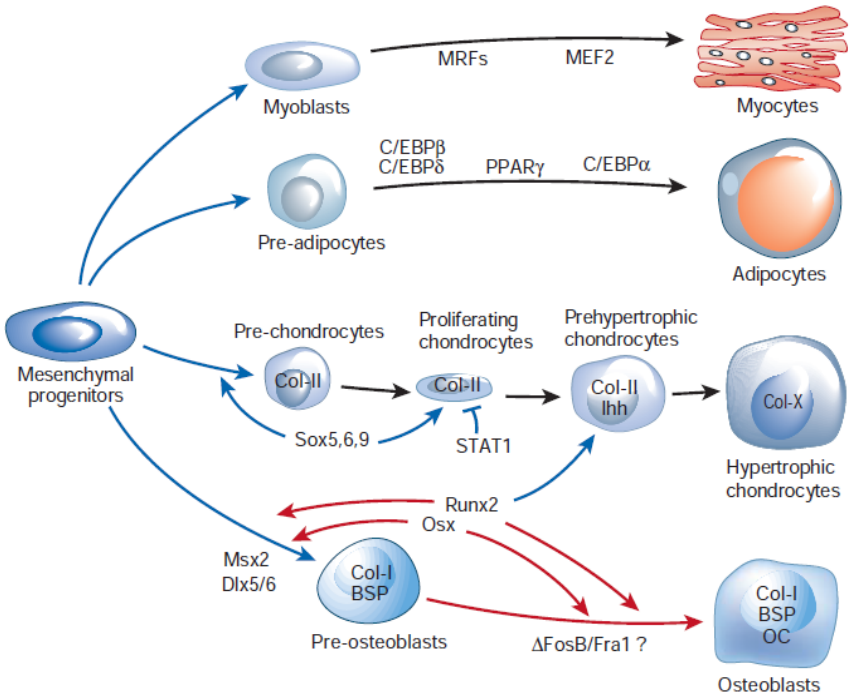


Figure 8. Transcriptional control of osteoblastic, chondrocytic, adipocytic and myocytic differentiation. [127].

Mutations in the *RUNX2* locus in human cause cleidocranial dysplasia (CCD), which is an autosomal-dominant condition accompanied with hypoplasia/aplasia of clavicles, patent fontanelles, supernumerary teeth, short stature, and other changes in skeletal patterning and growth (Figure 9).

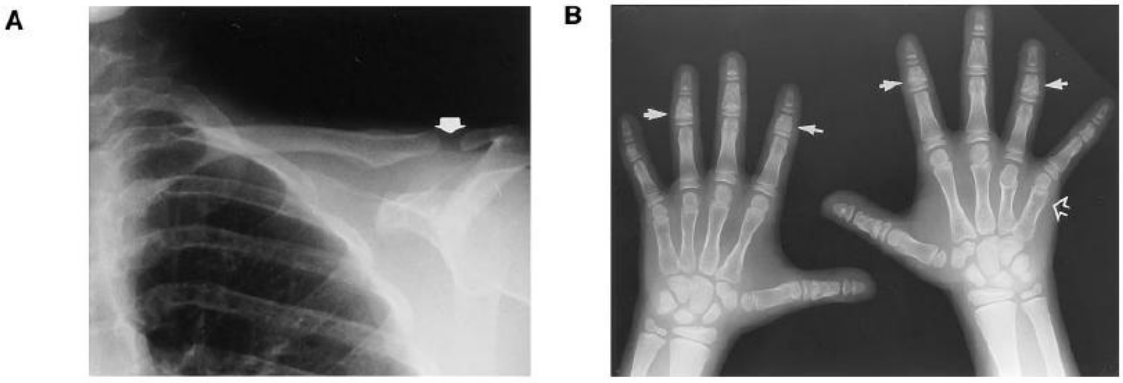


Figure 9. Radiographs of clavicle and hands of affected Individuals in family CCD [126].

A. Left shoulder region with distal gap in clavicle (arrow).

B. Affected child with prominent brachydactyly: distal phalanges are hypoplastic and middle phalanges have cone-shaped epiphyses (arrow).

Skeletons from homozygous Runx2 -/- (knock out) mice showed complete lack of functional osteoblasts and were devoid of mineralized bone (Lack of Ossification) ; Figure 10a-b [125].

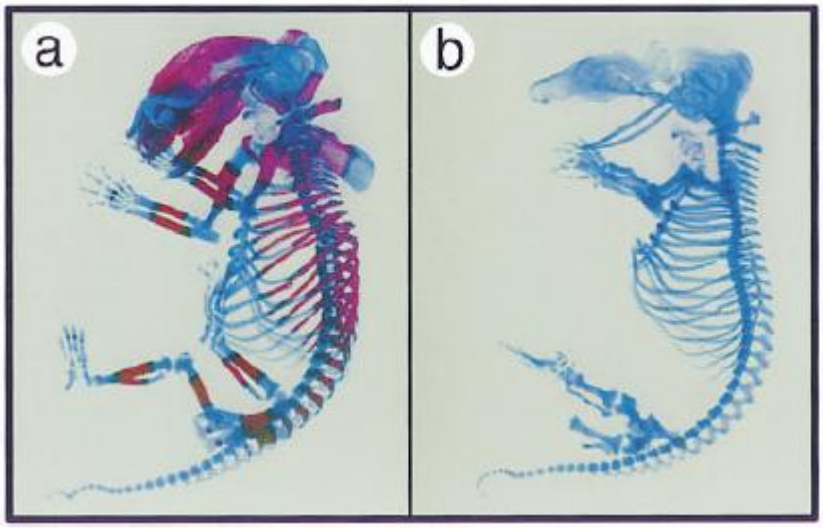


Figure 10. Lack of ossification in *Cbfa1* mutant mice day 17.5 wild type (a) and homozygous mutant (b) embryos were stained with Alcian blue/Alizarin red. Membranous and endochondral ossification are absent in *mutant* mice. Bone is stained red and cartilage blue [125].

Runx2 activates and regulates osteogenesis as the targeted gene of many signaling pathways, including transforming growth factor-beta 1 (TGF-β1), BMP, Wingless type (Wnt), Hedgehog (HH), and (Nel)-like protein type 1 (NELL-1). The DNA-binding sites of Runx2 in major bone matrix protein genes, including Col1a1; Col1a2; Spp1; Ibsp/BSP; Bglap2; Fn1/fibronectin; Mmp13, and Tnfrsf11b/Opg, have been identified, and Runx2 induced the expression of these genes or activated their promoters in vitro [128-130].

1.6 LRP5

The LRP5 (low density lipoprotein receptor-related protein 5) gene on chromosome 11q13 encodes a single pass trans-membrane member of the low-density lipoprotein receptor family. LRP5 consists of five conserved motifs, which are characteristic of the LDL-receptor family: (i) LDL-receptor repeats that are required for ligand binding, (ii) four EGFreceptor-like cysteine-rich repeats with associated

YWTD (Tyr-Trp-Thr-Asp) spacer domains, (iii) a putative signal peptide for protein export, (iv) a single membrane-spanning segment and (v) a cytoplasmic tail with NPXY (Asn-Pro-X-Tyr) motifs for receptor internalization. LRP5 is highly conserved among species. There is a 95% identity between human and mouse LRP5 proteins, and 40% identity with the drosophila homolog, arrow [131, 132].

LRP5 is widely expressed in adult and embryonic tissues, including bone, macrophages and fat (and to a lesser extent in brain, heart, liver, skin and pancreas). In bone, it is expressed by osteoblasts of the endosteal and trabecular bone surfaces but not by osteoclasts [133, 134].

Genetic studies show that LRP5 has a major influence on the entire spectrum of BMD from osteoporosis to normal BMD to high-bone-mass phenotypes. Loss-of-function mutations in the *LRP5* gene cause osteoporosis pseudoglioma syndrome (OPPG), (MIM 259770). Osteoporosis pseudoglioma syndrome is an autosomal-recessive condition of juvenile onset, which is characterized by blindness due to aberrant vitreo-retinal vascular growth and osteoporosis resulting in fractures and deformation. Gain-of-function mutations in the *LRP5* gene cause high bone mass phenotype, (MIM 601884) an autosomal dominant condition of increased BMD [46, 49, 134].

LRP5 has been shown to affect the normal population variation of BMD and diseases marked by abnormal bone turnover. (Table 5) [46].

Table 5. Examples of coding variants described for *LRP5*.

Disease	Amino Acid	Population	Ref
FEVR	(p.S1450fs*1502)	British family	[135]
FEVR	(p.K1374fs*1549)	US family	[136]
FEVR	(p.R1270fs*1438	Indian patient	[136]
FEVR	p.T173M	British female	[136]
FEVR	p.Y1168H	British female	[136]
FEVR	p.C1361G	Australian male	[136]
FEVR	p.R570Q	European family	[135]
FEVR	p.R752G	European family	[135]
FEVR	p.E1367K	European family	[135]
OPPG	p.W10*	OPPG families	[49]
OPPG	p.R428*	OPPG families	[49]
OPPG	p.D490fs (c.1467delG)	OPPG families	[49, 137]
OPPG	p.R570W	OPPG families	[49]
OPPG	p.D718*	OPPG families	[49]
OPPG	p.Q853*	OPPG families	[49, 137]
OPPG	p.E1270fs (c.3804delA)	OPPG families	[49, 137]
Osteopetrosis	p.D111Y	Argentinean family	[138]
Osteopetrosis	p.G171R	Belgian family	[138]
Endosteal hyperostosis	p.A214T	US family	[138]
Osteosclerosis	p.A214T	English family	[138]
Endosteal hyperostosis	p.A242T	US and Sardinian families	[138]
Osteopetrosis	p.A242T	French family	[138]
Osteopetrosis	p.T253I	Two Danish families	[138]
Areal and lumbar BMD, height	p.V667M	European Caucasians	[139]

* FEVR, familial exudative vitreoretinopathy; OPPG, osteoporosis pseudoglioma syndrome.

LRP5 acts as a co-receptor for Wnt. Wnt/β-catenin signaling plays an important role in the development and maintenance of many organs and tissues, including bone. In the absence of a Wnt ligand, the transcriptional co-activator β-catenin is continuously degraded in the cytoplasm by a protein complex that includes scaffolding protein Axin, tumor suppressor APC (adenomatous polyposis coli) and GSK3 (glycogen synthase kinase 3) and CK1a (casein kinase 1a). The Axin complex mediates CK1a and GSK3 phosphorylation of β-catenin, to provide a binding site for E3 ubiquitin ligase, resulting in β-catenin ubiquitination and subsequent degradation. Wnt signaling can be blocked by interactions of Wnt with inhibitory factors including WIF-1 and sFRP or the interaction of LRP5/6 with the Dkk/Kremen complex or sclerostin (*SOST* gene product). This process is inhibited when a Wnt ligand brings together two types of receptors, the Frizzled (Fz or FZD) serpentine receptors and the low-density lipoprotein receptor-related protein 5 or 6 (LRP5 or LRP6). The intracellular regions of FZD and LRP5 or LRP6 recruit the cytoplasmic proteins Dishevelled (DVL) and Axin, respectively. Recruitment of Axin to the membrane by the Fz- LRP5/6 complex inhibits β-catenin phosphorylation and allows β-catenin levels to accumulate, which results in β-catenin entering the nucleus and interacting with TCF/LEF (T cell factor/lymphoid enhancer factor) transcription factors to activate Wnt target gene transcription [18, 140, 141]. The process has been described in Figure 11.

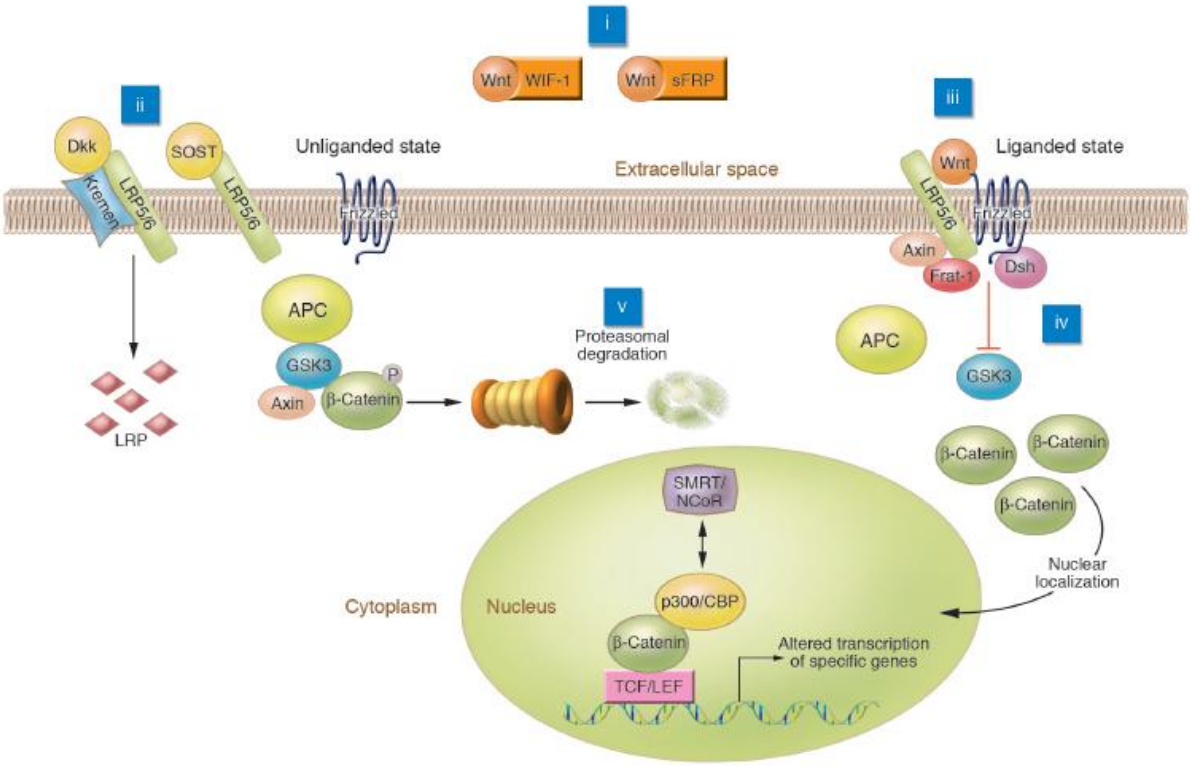


Figure 11. Elements of Wnt/β-catenin signaling [141].

LRP5 has a central role in human bone mass regulation. Loss of function mutations in *LRP5* result in osteoporosis-pseudoglioma (OPPG) primarily characterized by low bone mass [49]. On the other hand *LRP5* gain-of-function missense mutations, which are clustered in the first β-propeller, cause high bone mass (HBM) [50, 51], likely as a result of disruption of binding and inhibition of LRP5 by its antagonists DKK1 and Sclerostin/SOST [142, 143].

Wnt/ β -catenin signaling regulates osteogenesis through multiple mechanisms. Wnts repress alternative mesenchymal differentiation pathways such as adipocyte and chondrocyte differentiation, and promote osteoblast differentiation, proliferation, and mineralization activity, while blocking osteoblast apoptosis (Figure 12) [141].

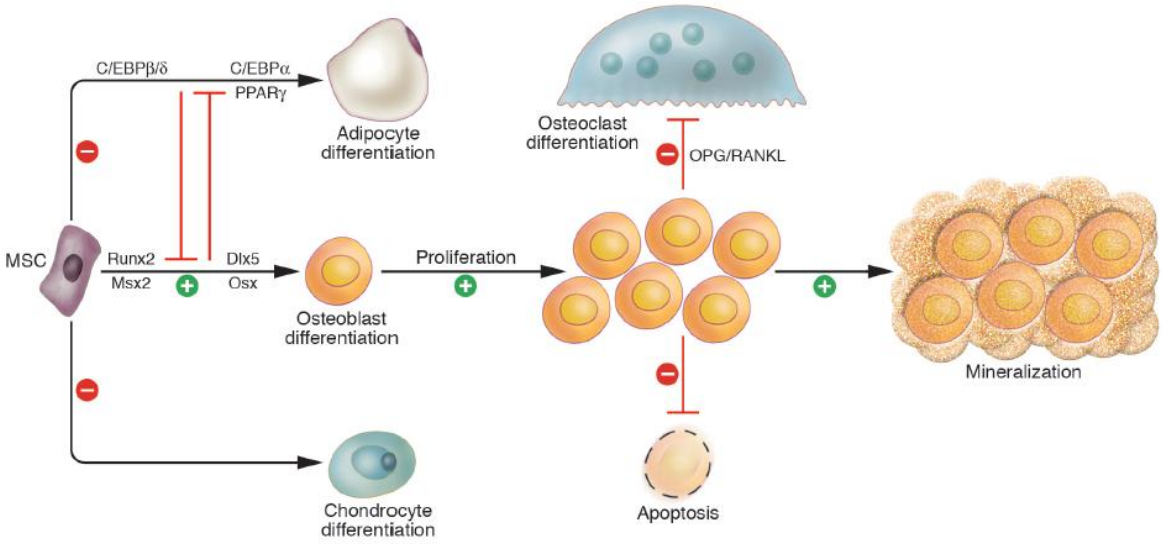


Figure 12. Wnt/β-catenin signaling regulates osteogenesis. Wnts repress alternative mesenchymal differentiation pathways such as adipocyte and chondrocyte differentiation and promote osteoblast differentiation, proliferation, and mineralization activity while blocking osteoblast apoptosis. By increasing the ratio of osteoprotegerin (OPG) to RANKL, β-catenin represses osteoclastogenesis. Green plus signs indicate positive effects of Wnt; red minus signs indicate inhibitory effects of Wnt. Dlx5, distal-less homeobox 5; MSC, mesenchymal stem cell; Msx2, msh homeobox homolog 2; Osx, osterix; Runx2, runt-related transcription factor 2. [141].

1.7 Connection of Runx2 and Wnt pathway

Functional RUNX2 binding sites have been described in the promoters of genes of some Wnt signaling pathway elements, such as *SOST* and *AXIN2* genes [144, 145]. Khaler and colleagues described Lef1, a final effector of Wnt signaling, as a *RUNX2* transcriptional regulator [146]. Gaur and colleagues and Reinhold and Naski also reported cooperation between LEF/TCF and RUNX2 factors in *RUNX2*'s own promoter and the FGF18 promoter, respectively [147, 148]. McCarthy and Centrella presented evidence for bidirectional crosstalk between the Wnt pathway and RUNX2 in osteoblasts [149]. Lumbar spine BMD-associated SNP, rs312009, is located in the LRP5 5' region. Gel-shift experiments have identified a RUNX2 binding site in this region. Four more RUNX2 binding sites were identified and characterized in the 3.3-kb region upstream of LRP5 by using in silico prediction and alignment tools. Luciferase experiments revealed the involvement of each of them in the RUNX2 response. These showed that *RUNX2* and *LRP5* are directly connected [150, 151]. Even though there is no doubt regarding direct connection between RUNX2 and

LRP5, there is lack of knowledge concerning the Runx2 protein-*LRP5* gene physical interaction in a live cell in different stages of osteoblast differentiation. Hence, it is proposed that the lack of knowledge should be addressed. This is the topic of the present PhD thesis.

OBJECTIVES

2. Objectives

1. To set up an osteoblast differentiation protocol using the hFOB (human Fetal Osteoblast) cell line.

2. To characterize in detail 5 stages of osteoblast differentiation (days 0, 3, 7, 14 and 21 days) at the level of mRNA for several key genes, and at the mRNA and protein levels for *RUNX2* and *LRP5*.

3. To investigate the binding of RUNX2 to the 5 sites of the *LRP5* promoter in an *in vivo* setting by means of chromatin immunoprecipitation (ChIP) experiments.

   In particular, to test these putative bindings in Saos2 and in the hFOB cell line at different stages of osteoblast differentiation.

MATERIALS AND METHODS

3. Materials and methods

3.1. Cell culture

The human osteosarcoma cell lines Saos2 and human fetal osteoblastic hFOB 1.19 (hereafter, hFOB) were obtained from the American Type Culture Collection (ATCC no. HTB-85 and ATCC no. CRL-11372, respectively; Manassas, VA, USA). The hFOB 1.19 line was established by transfection of limb tissue with the temperature sensitive expression vector pUCSVtsA58, which bears a temperature-sensitive SV40 T antigen, and the neomycin resistance expression vector pSV2-neo. Cells grown at 33.5°C exhibit rapid cell division and present an undifferentiated phenotype, whereas at 39.5°C little or no cell division occurs and the cells have the ability to differentiate into mature osteoblasts expressing the normal osteoblast phenotype. Saos2 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), and 1% antibiotic (penicillin and streptomycin; Invitrogen, Carlsbad, CA, USA). hFOB were cultured in 1:1 mixture of Dulbecco's Modified Eagle's medium/Ham's F12 medium without phenol red supplemented with 10% heat-inactivated fetal calf serum (FCS), and 0.3 mg/ml antibiotic (Geneticin; Invitrogen, Carlsbad, CA, USA). hFOB cells were grown at the permissive temperature of 335°C to have confluent plate. The temperature was then shifted to 39. 5°C to promote osteoblast differentiation. During the differentiation process media was changed every 48 hours.

3.2. MTT assay

The assay was performed in accordance with the method of Van de Loosdrecht et al. [152]. Cells were cultured in 6 well plates as indicated above. At the appropriate time points, cells were washed with 2 ml of PBS and 200 µl MTT (5mg /ml Thiazolyl Blue Tetrazolium Blue,Sigma Diagnostics, St. Louis, USA) was added to each well. Cells were then incubated for 3 hours at the same temperature. After formation of the formazan crystals the culture medium supernatant was aspirated from the wells without disturbing the formazan precipitate. The crystals were thereafter dissolved in 1600 µl dimethyl sulphoxide (DMSO 100%, Sigma) per well, by shaking for 5 min at

150 rpm. The absorbance was measured at 560 nm using a GloMax® 96 Microplate spectrophotometer (Turner Biosystem, USA).

3.3. Alizarin red assay

A process similar to that proposed by Gregory et al [153] was employed for this assay. Cells were cultured in 6 well plates, and thereafter washed 3 times with PBS. The cells were then fixed with 3.7% (v/v) formaldehyde (Sigma) at room temperature for 20 minutes. Cells were then washed 3 times with Milli-Q water during 4 minutes prior to the addition of 40 mM Alizarin red S (ARS, pH: 4.2, Sigma) per well. The plates were then incubated at room temperature for 20 minutes by gently shaking the plates at 50 rpm. After aspiration of the unincorporated dye, the wells were washed 4 times with Milli-Q water during 4 minutes. The plates were then left tilted for 2 minutes to facilitate the removal of the excess water. Stained cells were visualized by phase microscopy. Pictures were taken with a Leica DML microscope in combination with a Leica DFC300FX camera and Leica application software (Leica microsystem).

3.4. Alkaline phosphatase assay

Cells were cultured in 6 well plates, and then washed with PBS, twice. An ALP assay kit (Sigma Diagnostics, St. Louis, USA) was used for staining and histochemical determination of the osteoblast-like cells. The cells were fixed in 3.7% (v/v) formaldehyde (Sigma) at room temperature for 4 minutes, upon which they were gently rinsed with an alkaline-dye mixture. Incubation of the cells in an alkaline-dye mixture for 15 minutes was thereafter followed. Stained (blue) cells were visualized by phase microscopy using an inverted microscope.

3.5. RNA extraction

Saos2 and hFOB cells were cultured in 100 mm dishes. RNA extractions were performed at the chosen time points using the high pure RNA isolation kit (Roche, Germany) and RNA were stored at -80ºC until use. The RNA quantity and quality

were assessed by spectrophotometry (Nanodrop ND-1000 Spectrophotometer; Nanodrop Technologies Inc., Wilmington, DE, USA). Two µg of total RNA was reverse-transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions and kept at -20°C until use.

3.6. Protein extraction:

Cells were cultured in 100 mm plates, and harvested and protein was extracted using CellLytic TM NuCLEAR^{TM} extraction kit (Sigma). Both cytoplasmic and nuclear fractions were obtained for the analysis of LRP5 and RUNX2, respectively. Total protein was quantified by the DC protein assay kit (Bio-Rad), and assessed in a GloMax® 96 Microplate (Turner Biosystem, USA). Samples were stored at -80°C until further use.

3.7. Western blot

Thirty micrograms of protein per lane were resolved by SDS-PAGE (12.5% polyacrylamide), transferred onto nitrocellulose membranes, and analyzed by standard immune staining using the following primary antibodies: RUNX2 at 1:5000 dilution (R6282; Sigma, St Louis, Mo, USA) on nuclear fraction, LRP5 at 1:100 dilution (ab38311; Abcam, Cambridge, CB4 0FL, UK) on cytoplasmic fraction, and GAPDH at 1:800 dilution (SC 365062; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for the loading control, and a horseradish peroxidase-conjugated secondary antibody (GARPO and SAMPO; Sigma). Immuno-reactive bands were detected by incubating the membrane in the following solution; 10 ml of 100 mM Tris-HCL (pH 9.0), 50 µl of 45 mM ρ-coumaric acid, 50 µl of Luminol and 10 µl of 30% H_2O_2.

3.8. Expression analysis at the mRNA level

One hundred ng of the resulting cDNA was used for subsequent quantitative real time PCR in a Light Cycler® 480 (Roche, Basel, Switzerland) with Light Cycler® 480 Probes Master (ref# 04887301001, Roche, Basel, Switzerland) under standard conditions. Amplification was performed using the primers and the UPL probes

(Universal Probe library, Roche Diagnostics) indicated in Table 6. GAPDH mRNA was used for normalization.

Table6. Sequences of the primers and probes used in expression analysis.

Gene name	Forward primer	Reverse primer	Taqman probe (UPL library number)
RUNX2	cagtgacaccatgtcagcaa	gctcacgtcgctcattttg	# 41, cttcagcc
LRP5	agaacatcaagcgagccaag	atgtcgatgctgaggtcgt	# 27, caggcagc
OC	tgagagccctcacactcctc	acctttgctggactctgcac	# 81, ggccctgg
ALP	gtgcccgtggtcaattct	gacggacccgtcactctc	# 77, ggtggtgg
SOST	agctggagaacaacaagacca	gctgtactcggacacgtcttt	# 77, ggtggtgg
GAPDH	gctctctgctcctcctgttc	acgaccaaatccgttgactc	# 60, ggccctgg

3.9. ChIP

3.9.1. Cross-linking and cell lysis

Cells were grown in 150 mm culture dishes containing 20 ml of growth media. When the cells reached about 80% confluency they were used for fixation. This was done for Saos2 and hFOB during differentiation day 0, day 7 and day 21. Cells were cross-linked for 10 minutes at room temperature by adding 550 µl of 37% (v/v) formaldehyde (Sigma F8775) to 20 ml of growth media. Cross-linking was terminated by incubation of cells with glycin solution for 5 minutes. They were then re-suspended in lysis buffer, followed by re-suspension in nuclear lysis buffer by using EZ-Magna ChIP (Millipore, Darmstadt, Germany), following the manufacturer instruction.

3.9.2. Sonication

Sonication was done by Bioruptor^{R} Sonication system (Diagenode sa. Belgium). It was found that the period of sonication (while samples were maintained on ice at 4 °C) should be approximately 3 minutes to achieve the optimal average chromatin fragment size of 500 bp.

The DNA size range and efficiency of DNA fragmentation were evaluated by electrophoresis on a 2% agarose gel.

3.9.3. Immunoprecipitation

Chromatin immune precipitation was done by using the EZ-Magna ChIP kit (Millipore, Darmstadt, Germany), following the manufacturer instruction. One percent of the shared chromatin was saved at 4°C to be used as input. The remaining sample was used at the time of protein/ DNA complexes elution and reverse cross-link of protein/ DNA complexes to obtain free DNA. Fully re-suspended protein A/G magnetic beads along with the indicated antibody (see below) were added to the shared chromatin obtained from the last step. In particular, the following antibodies were added to each tube:

- For a positive control of the ChIP procedure, anti-RNA polymerase (was included in the kit) 1.0 µg of antibody per tube.
- For the negative control of the experiment, normal mouse IgG (was included in the kit), 1.0 µg of antibody per tube.
- For the experiment, anti-RUNX2 (SC-10758x; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), 10 µg of antibody per tube.

For Saos2 and hFOB day 0, day 7 and day 21, all three tubes were set up in triplicate. Overnight incubation was done to achieve optimal binding of the antibody.

3.9.4. Elution and DNA extraction:

The day after the immunopercepitation step, cross-linking was reversed and DNA was purified following the manufacturer instruction, using EZ-Magna ChIP (Millipore, Darmstadt, Germany) to obtain free DNA.

3.9.5. Amplification

Ten µl of each ChIP DNA was used for subsequent amplification in a Light Cycler® 480 (Roche, Basel, Switzerland) with Light Cycler®480 SYBR Green I Master Mix (ref# 04887352001, Roche, Basel, Switzerland) under standard

conditions. Amplifications were performed for Saos2 and hFOB at day 0, day 7, and day 21 for all 5 RUNX2 binding sites on the *LRP5* promoter, for the RUNX2 binding sites on the *CDKN1A* and *SERPINE1* genes and for the *GAPDH* promoter.

Primers for all RUNX2 binding sites on *LRP5* promoter and RUNX2 binding sites on *CDKN1A* and *SERPINE1* genes (which have been described by Deen et al[154]) are given in Table 7.

Table7. Sequences of the primers used in ChIP amplification.

Gene name	**Forward primer**	**Reverse primer**
BS1	tttttctgtatcccttcccaaa	cctcttggactcaagtggatg
BS2	tcctggagcttccatgcta	ttagcctcgtggctcactct
BS3	agcagagaacagcaagcaca	ttccttagccactcccttcc
BS4	aagggagtggctaaggaagc	ccccttccacctgatctcat
BS5	ggtgcattctcgattcctct	ggaaactggcttagggaagg
CDKN1A	gtaaatccttgcctgccaga	gagccacaaatctggctttt
SERPINE1	tggattctcccaactgaacc	gtgtggtctggatgttgtgg

RESULTS

4. Results

4.1. hFOB osteoblast differentiation in culture

4.1.1 Cell viability decreases progressively from day 3

Cell viability was evaluated by the MTT assay. The MTT colorimetric assay is based on the ability of living cells to reduce 3- [4,5- dimethylthiazol-2-yl]-2,5 diphenyltetrazolium (MTT) into formazan. The MTT assay is based on the assumption that only viable cells reduce tetrazolium salts into colored formazan, which can be quantified spectrophotometrically. hFOB cells exhibited rapid cell division at the permissive temperature of 33.5ºC, which was considered as day 0. Thereafter, when the temperature was switched to the restrictive temperature of 39.5ºC, cell division slowed down, differentiation increased and a more mature osteoblast phenotype was produced. Cells were analyzed at days 3, 7, 14 and 21 at this temperature. The number of living cells increased at day 3 and then decrease continuously up to day 21, reaching an absorbance of 20% of that at day 0 in the MTT assay (Figure 13). The Saos2 cell line showed an OD value close to that of hFOB at day 0.

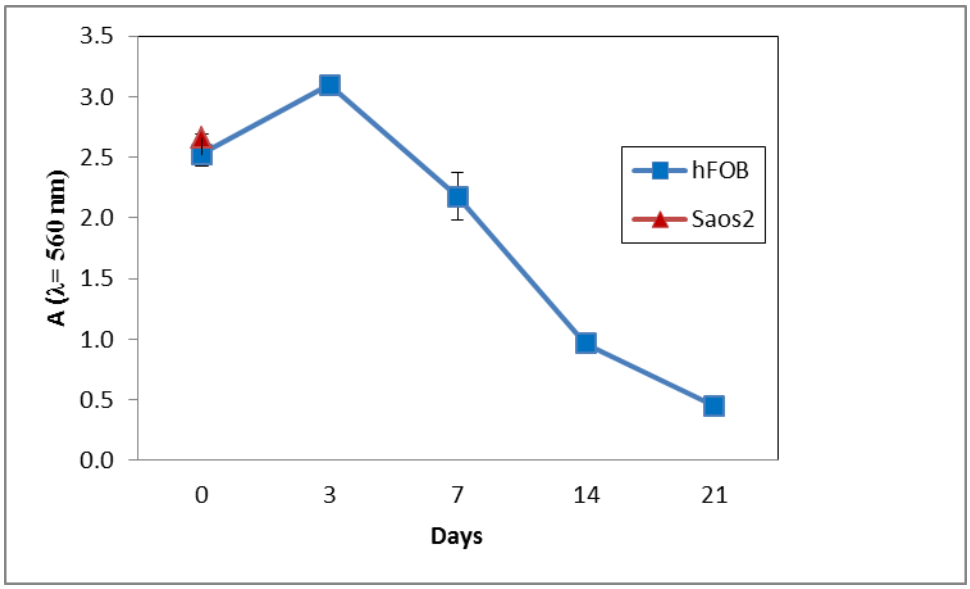


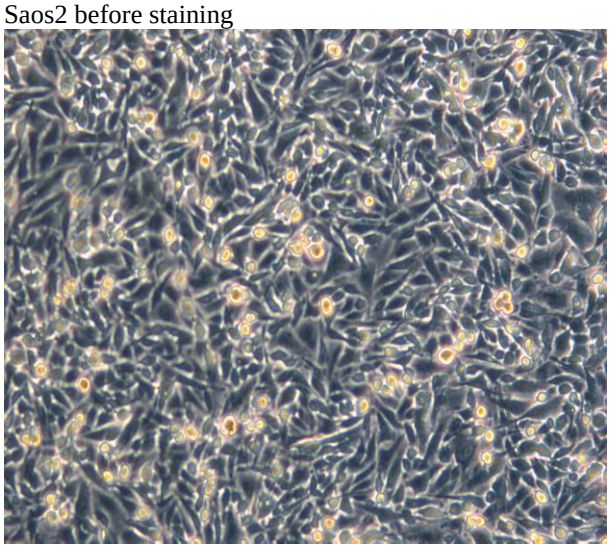
Figure 13. Decrease of cell viability (in terms of formazan absorbance after MTT treatment) during hFOB differentiation from day 0, day 3, day 7, day 14 and day 21. The results are representative of two experiments performed in triplicate.

4.1.2 Extracellular calcium salt deposition increases

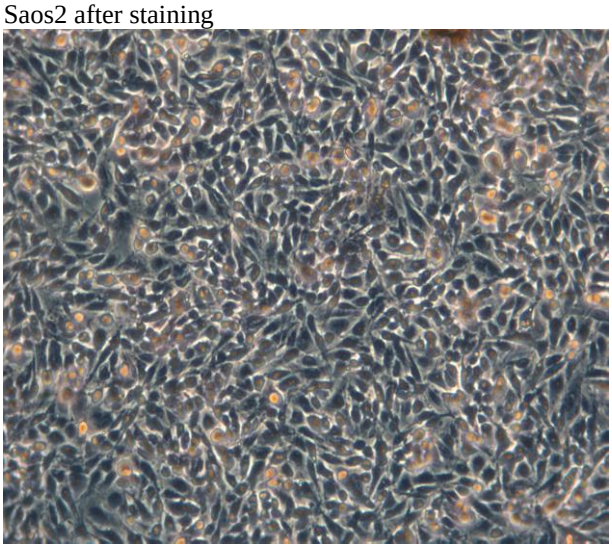
Alizarin Red-S (ARS) is a dye which binds selectively to calcium salts, widely used for calcium mineral histochemistry. ARS staining was used to detect mineralization.

Cells displayed increasing alizarin red staining (Fig. 14) from day 0 (pink) to day 21 (dark red), indicative of deposition of mineralized nodules, which is characteristic of late stages of osteoblast differentiation in culture. Saos2 showed a pale pink staining, similar to hFOB at day 0. Therefore, hFOB cells appear to be relatively undifferentiated cells, programmed to be differentiated upon shift to restrictive temperature. Some hFOB cells displayed large, flat, triangular shapes and formed sheet-like filopodia, which increased by day and are clearly observed at day 21 (Fig. 14).

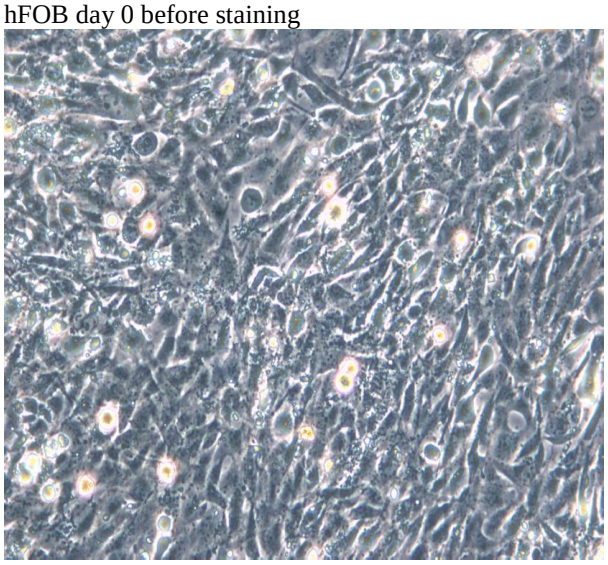
Saos2 before staining



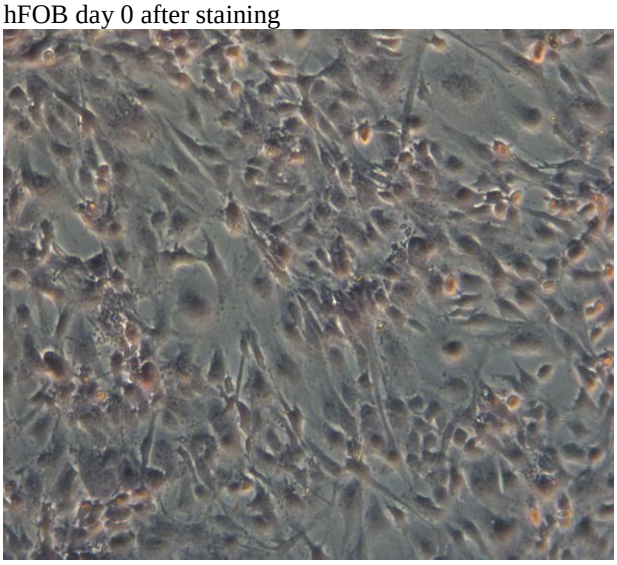
Saos2 after staining



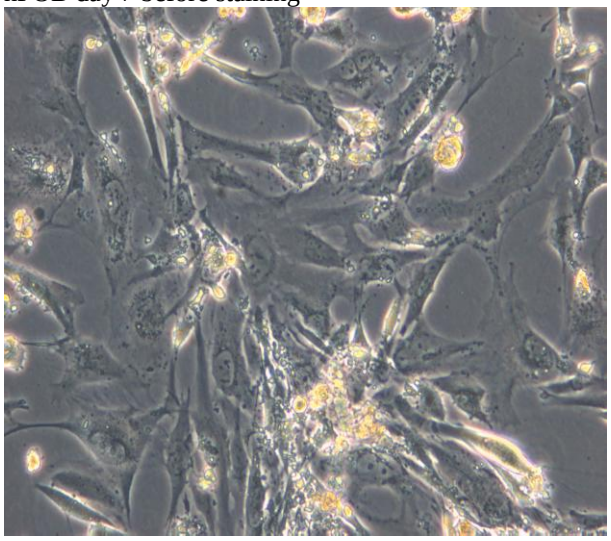
hFOB day 0 before staining



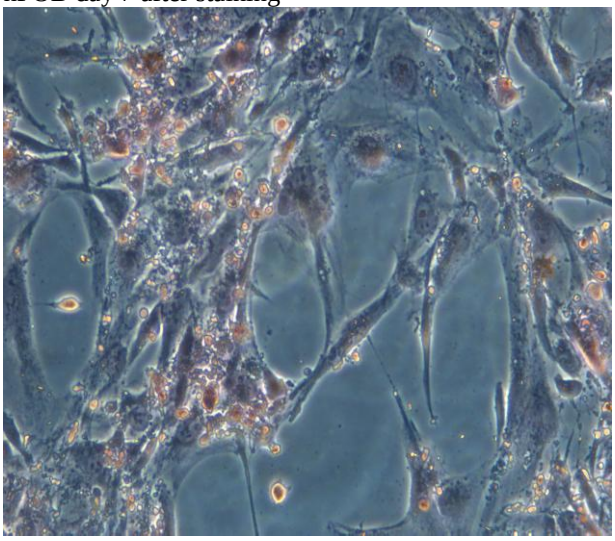
hFOB day 0 after staining



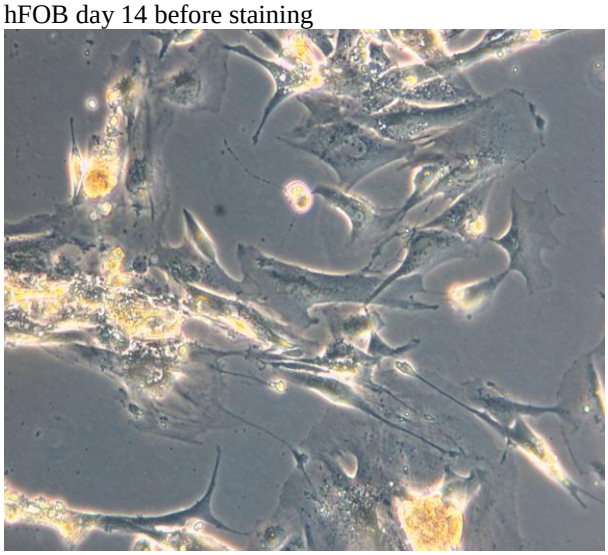
hFOB day 7 before staining



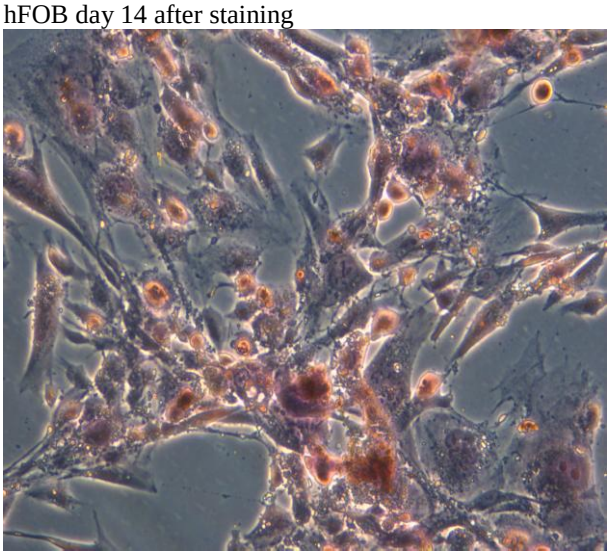
hFOB day 7 after staining



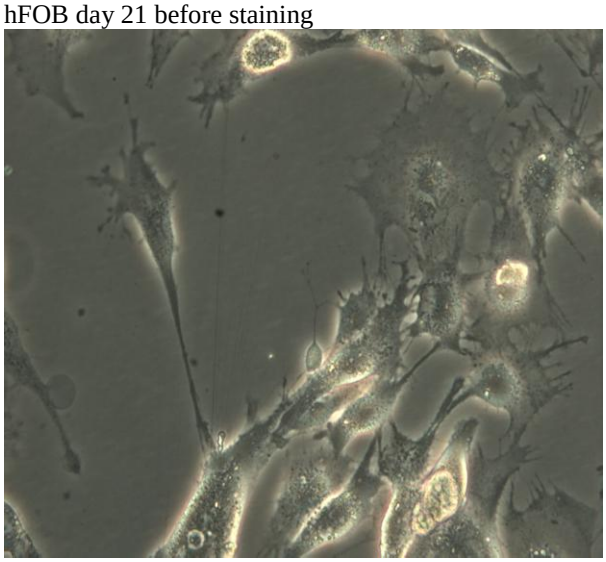
hFOB day 14 before staining



hFOB day 14 after staining



hFOB day 21 before staining



hFOB day 21 after staining

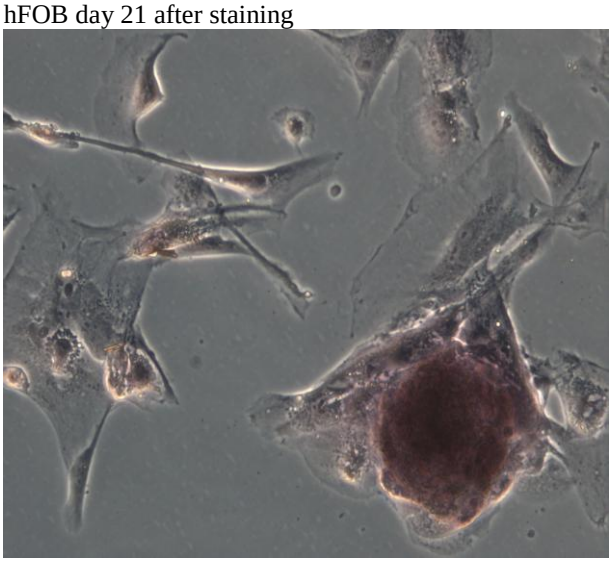
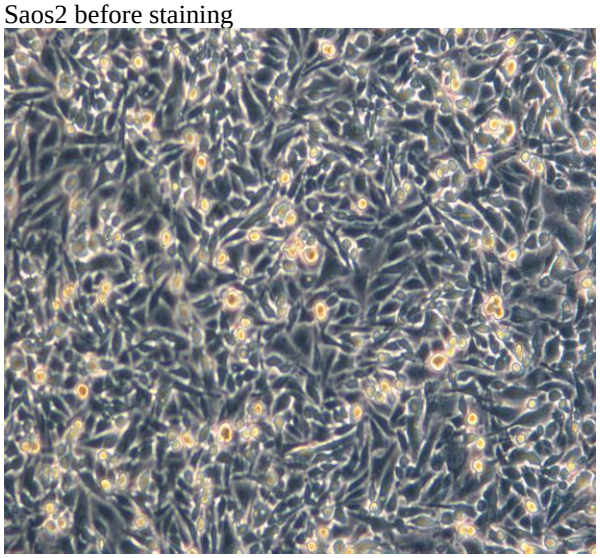


Figure 14. Mineralization of hFOB cells at day 0, day 7, day 14, day 21 and Saos2 cells was visualized by alizarin red staining. hFOB cells demonstrated calcium deposition in the extracellular matrix. Results shown are representative of two experiments in duplicate.

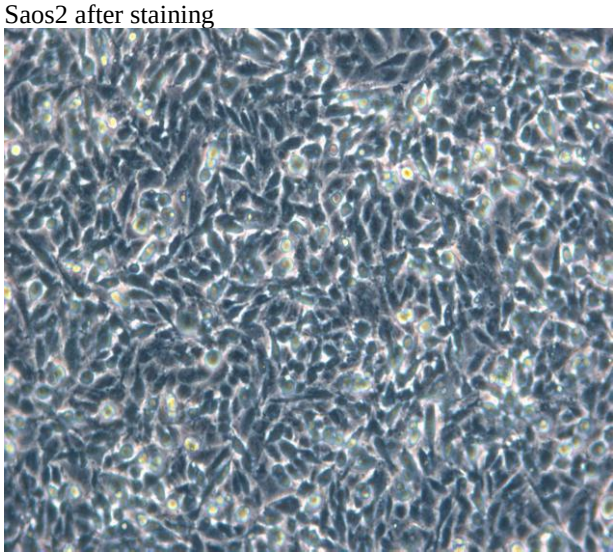
4.1.3 Alkaline phosphatase activity increases with differentiation

The cells were screened for alkaline phosphatase activity by blue membrane substrate based staining technique. The alkaline phosphatase (AP) activity was higher at the restrictive temperature, starting at day 7, compared to that at the permissive temperature (day 0) (Fig. 15). Saos2 cells, which were used as control, showed relatively high levels of alkaline phosphatase activity (Fig. 15).

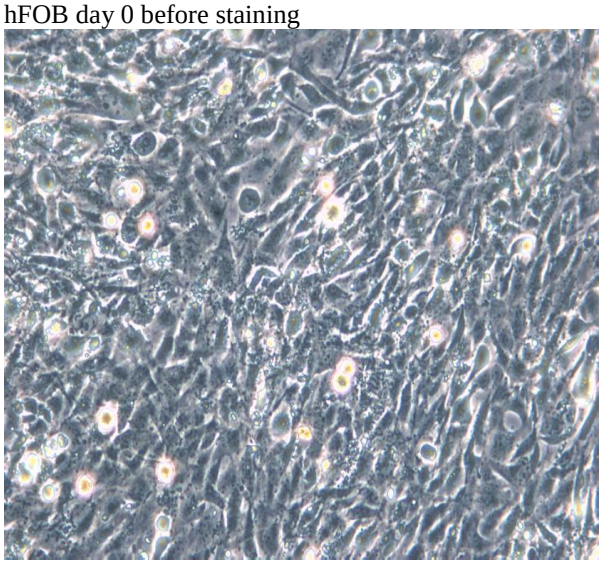
Saos2 before staining



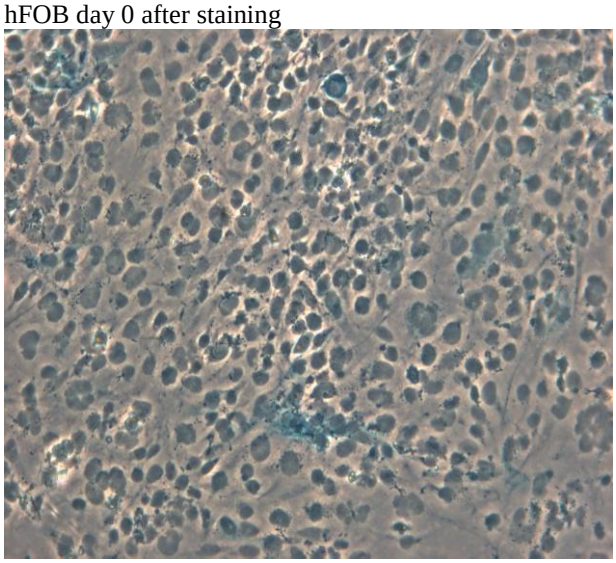
Saos2 after staining



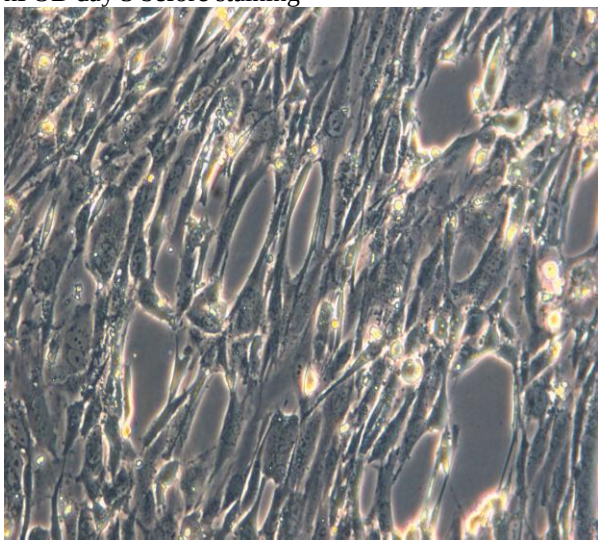
hFOB day 0 before staining



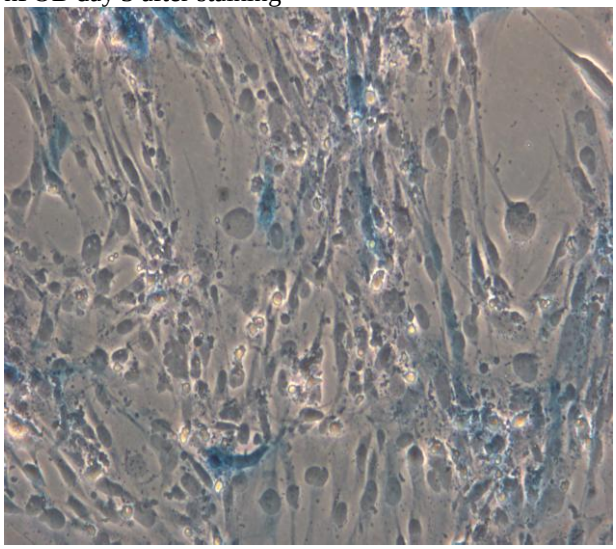
hFOB day 0 after staining



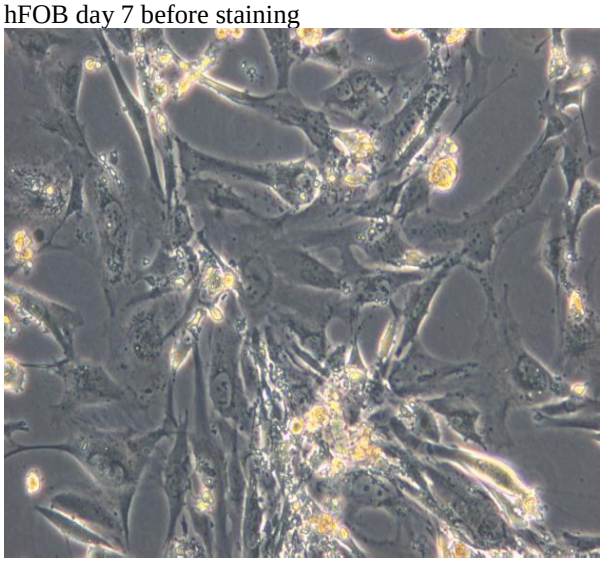
hFOB day 3 before staining



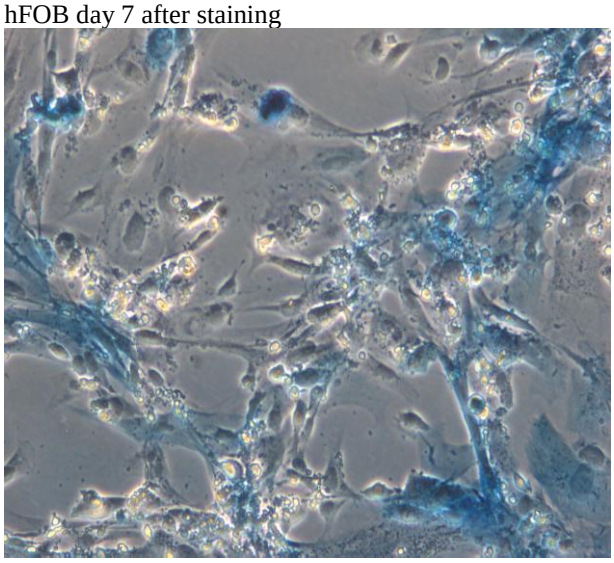
hFOB day 3 after staining



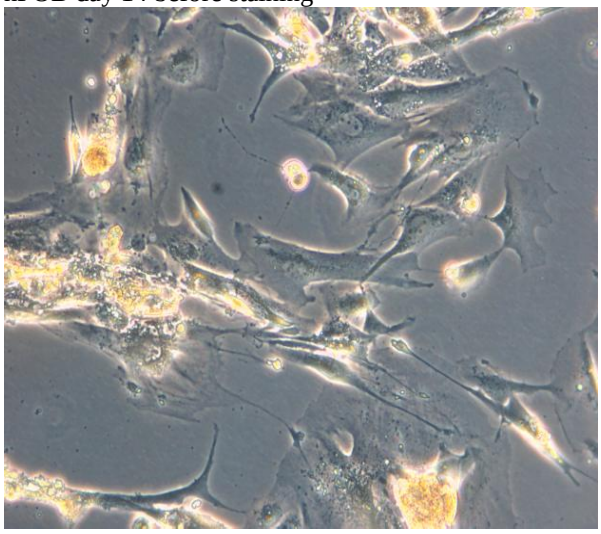
hFOB day 7 before staining



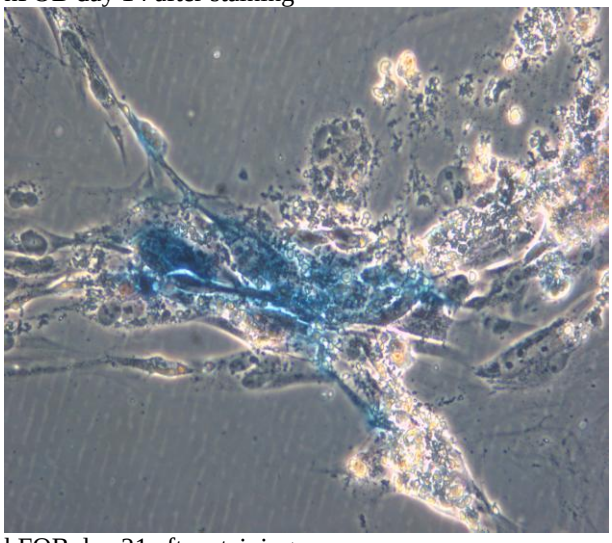
hFOB day 7 after staining



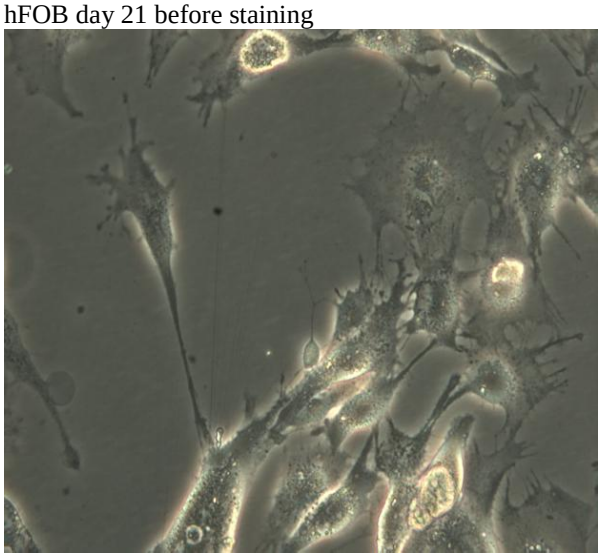
hFOB day 14 before staining



hFOB day 14 after staining



hFOB day 21 before staining



hFOB day 21 after staining

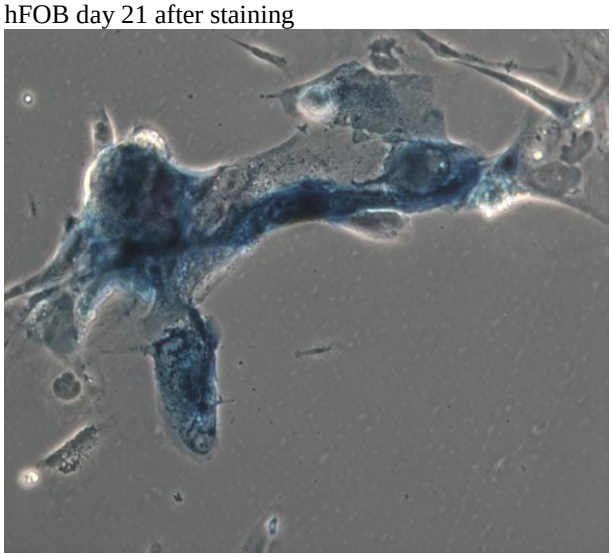


Figure 15. Alkaline phosphatase activity in Saos2 and hFOB cells. The cells were incubated either at 33.5°C or 39.5°C. Dark-staining indicates high AP activity. The results shown are representative of two experiments performed in duplicate.

4.2. Transcriptional and translational profile

4.2.1 The transcriptional profile of hFOB cells shows high levels of *LRP5* and low levels of *RUNX2* when compared to Saos2

RUNX2 and *LRP5* transcriptional profiles were obtained at 5 stages of the osteoblast differentiation along with several other key genes (Osteocalcin, Alkaline Phosphatase and *SOST*).

RNA of each stage was extracted to test the expression of these genes by quantitative PCR and *GAPDH* mRNA was used for normalization. For each gene and stage of hFOB differentiation results were expressed relative to the corresponding expression in Saos2 (Figure 16).

LRP5 mRNA levels in hFOB cells were above those of Saos2 at all time- points with a pick at day 3. *OCN* levels were above the levels in Saos2 cells only at day 3, and below those levels in all other time-points. *RUNX2*, *OCN* and *ALP* mRNA levels in hFOB cells were below those of Saos2 cells in all cases.

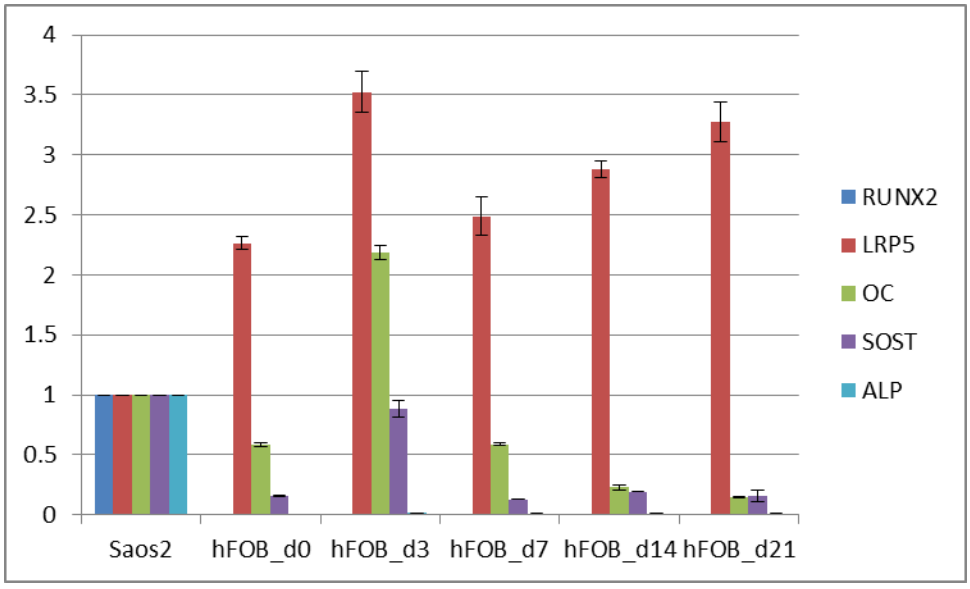


Figure 16. Real-time PCR quantification of *RUNX2*, *LRP5*, *OC*, *SOST* and *ALP* in hFOB during differentiation compared to Saos2. *GAPDH* was used as the internal normalization gene. The results are representative of two experiments in triplicate.

The results were also analyzed by relating the expression levels of the four stages of differentiation (days 3, 7, 14 and 21) to the expression in hFOB at day 0 (Figure 17).

A steady increase was observed for *RUNX2*, reaching a maximum of 4-fold at day 14. All the other genes analyzed showed a peak at day 3, more than 5-fold for *SOST* and above 4-fold for *ALP*. *OCN* and *SOST* showed a clear decrease after day 3, while *ALP* showed a slow decrease, and *LRP5* maintained relatively similar mRNA levels (Fig. 17).

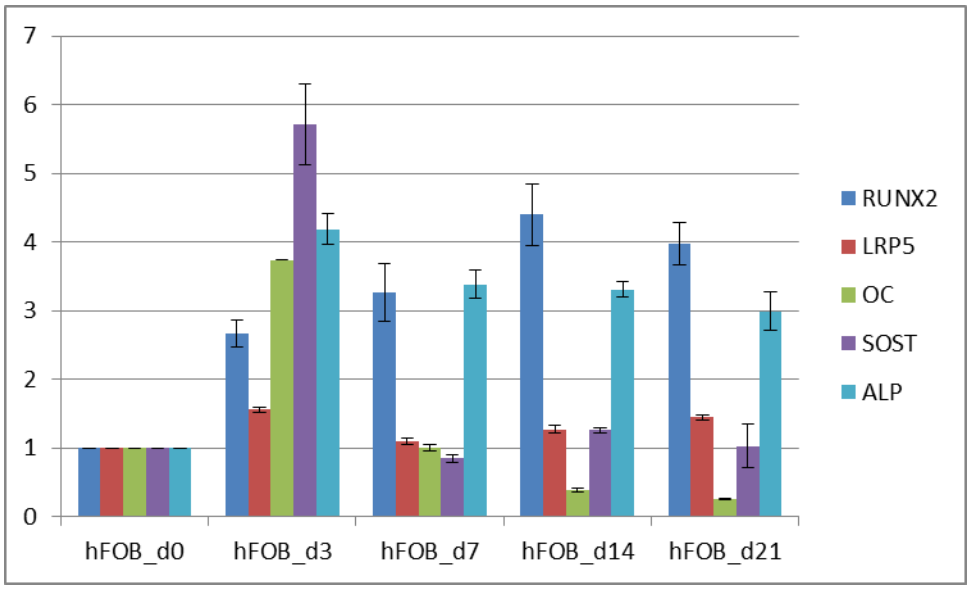


Figure 17. Real-time PCR quantification of *RUNX2*, *LRP5*, *OC*, *SOST* and *ALP* in hFOB during differentiation compared to undifferentiated hFOB. *GAPDH* was used as the internal normalization gene. Results shown are representative of two experiments in triplicate.

4.2.2 RUNX2 and LRP5 proteins were expressed at low levels and decreased along differentiation

The RUNX2 and LRP5 protein levels in Saos2 and 5 stages of osteoblast differentiation were also monitored by western blot (Figure 18). It was found that the level of RUNX2 protein in Saos2 is higher compared to hFOB at all stages of differentiation (days 0-21). Both RUNX2 and LRP5 proteins were detected in Saos2 cells and in hFOB at day 0, 3 and 7. Low levels of LRP5 were also observed at day 14. Overall, the protein levels of both RUNX2 and LRP5 decrease during differentiation of hFOB cell lines.

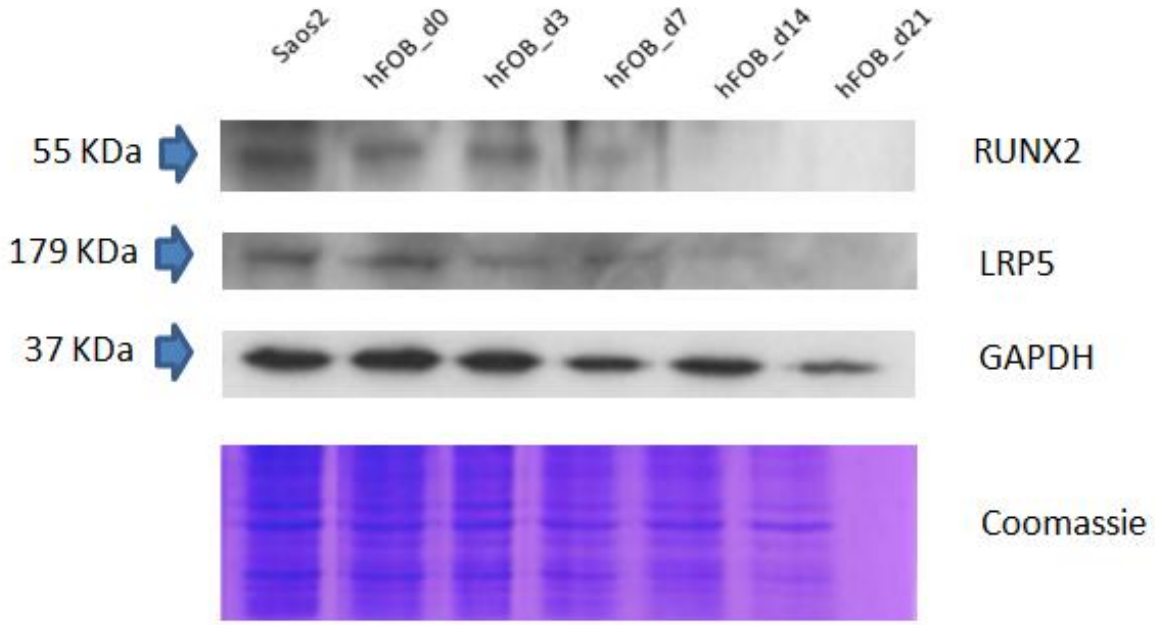


Figure 18. RUNX2 and LRP5 protein levels in Saos2 and hFOB cells at several time-points along differentiation. Molecular weights of the proteins are shown at the left. Thirty µg of nuclear (RUNX2) or cytoplasmic (LRP5 and GAPDH) protein extracts was loaded in each lane. Comassie blue staining of cytoplasmic extracts is shown below for reference. Results shown are representative of two experiments in duplicate.

4.3. In vivo *interaction of RUNX2 to 5 elements on the human LRP5 upstream region*

Chromatin immunoprecipitation (ChIP) was performed to assess *in vivo* binding of RUNX2 to the five BSs in the *LRP5* upstream region, in Saos2 and in hFOB cells on days 0, 7 and 21. The *CDKN1A* and *SERPINE1* promoters, known to be bound by RUNX2 in Saos2 cells [21], were used as positive controls. Data is expressed as fold enrichment of DNA immunoprecipitated with anti-RUNX2 antibody, relative to DNA immunoprecipitated with IgG. An immunoprecipitation reaction with antibody against RNA POL II and quantitation of the enrichment of the GAPDH promoter was performed as a positive control of the ChIP experiment. As expected, RNA POL II strongly associated with the GAPDH transcription start site (TSS) in chromatin of both Saos2 and hFOB (undifferentiated or differentiated) cells (Figure 19). Fold enrichment was above 10^3 in Saos2 and hFOB up to day 7, and dropped at day 21.

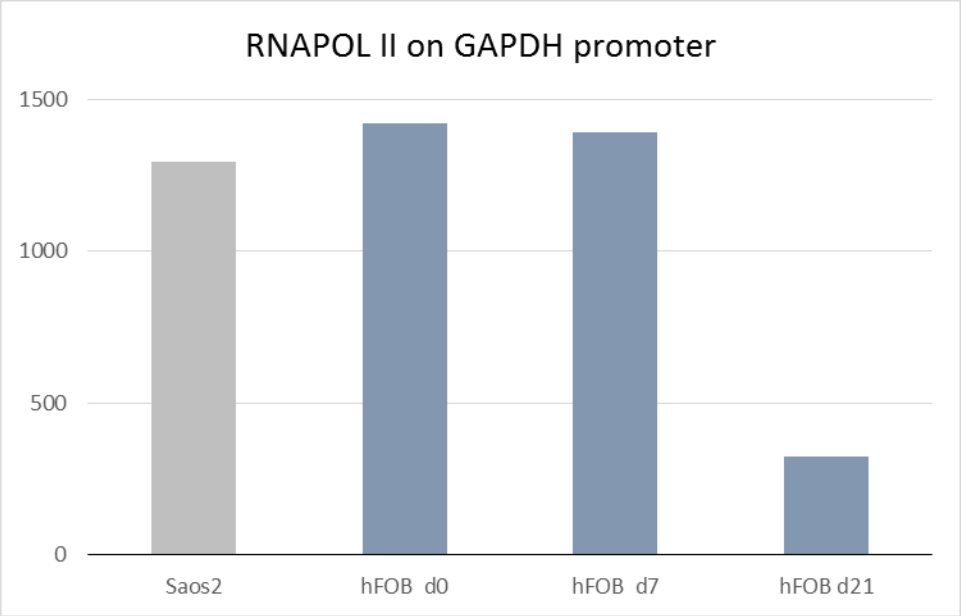


Figure 19. RNAPOLII ChIP followed by qPCR analysis of GAPDH promoter in Saos2 and hFOB days 0, 7 and 21.

4.3.1 RUNX2 binds the *LRP5* promoter region in Saos2 cells

Occupancy of *CDKN1A* and *SERPINE1* promoters by RUNX2 was confirmed in chromatin of Saos2 cells (1.5 and > 2.5 fold enrichment, respectively). In these cells, RUNX2 was observed to bind to all the *LRP5* promoter binding sites with the exception of BS1 (Figure 20).

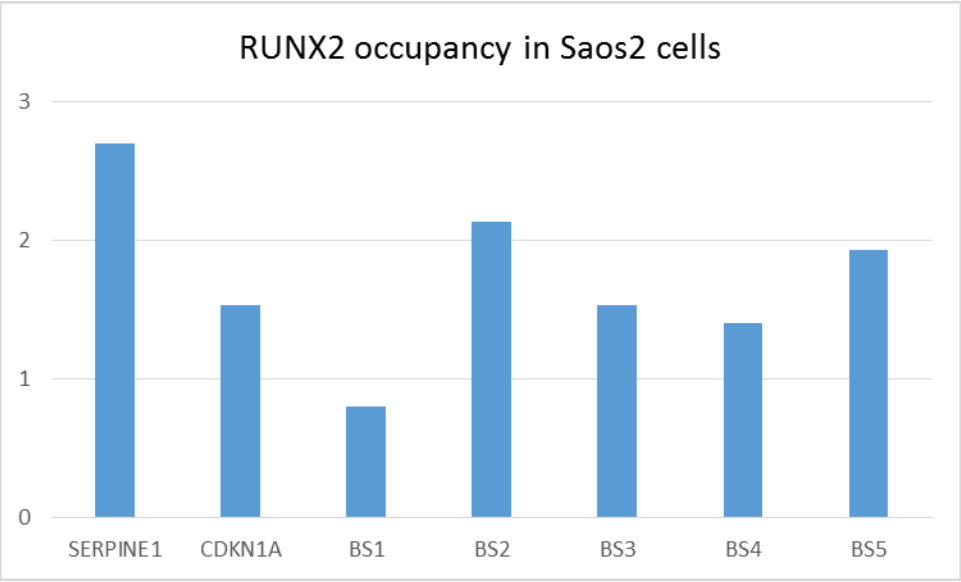


Figure 20. RUNX2 ChIP followed by qPCR analysis of the 5 RUNX2 binding sites of *LRP5* promoter in chromatin from Saos2 cells. *SERPINE1* and *CDKN1A* promoters were used as controls of RUNX2 binding.

4.3.2 RUNX2 binds the *LRP5* promoter region in hFOB cells

hFOB chromatin was used to test the binding of the RUNX2 transcription factor firstly to the promoters of the *CDKN1A* and *SERPINE1* genes. As shown in Figure 21, binding of RUNX2 was observed at both promoters, in day 7 of hFOB differentiation. Binding was also observed at *CDKN1A* in day 0. RUNX2 binding to these promoters in hFOB cells is described here for the first time.

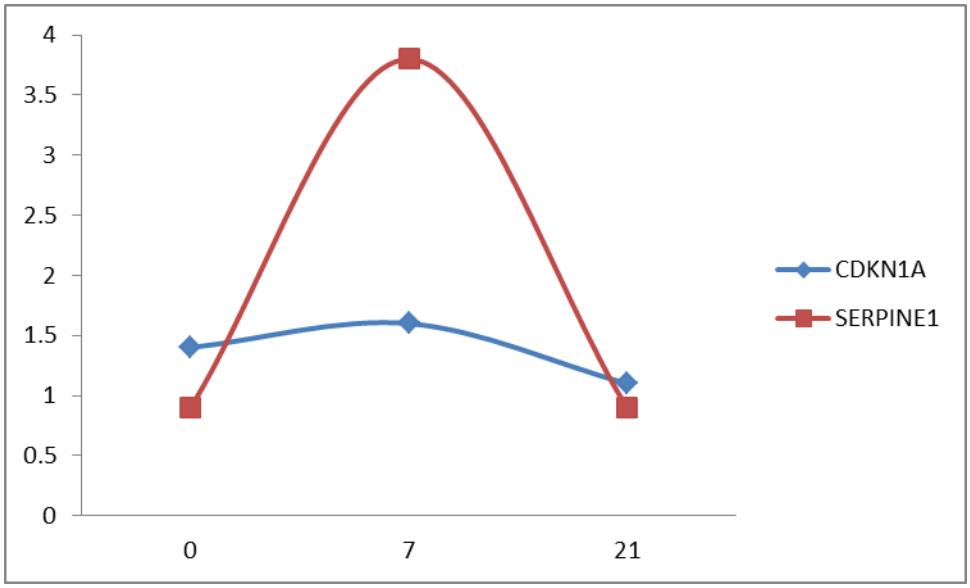


Figure 21. Runx2 ChIP followed by qPCR analysis of the Runx motifs on the *CDKN1A* and *SERPINE 1* promoters in hFOB cell line at day 0, day 7 and day 21.

Subsequently, binding of RUNX2 to the 5 RUNX2 elements in the LRP5 promoter was assessed in chromatin from undifferentiated (day 0) and differentiated (days 7 and 21) hFOB cells (Figure 22). Again, binding at all five sites was observed at day 7 (above 3-fold enrichment in all cases), while it was negligible at days 0 and 21.

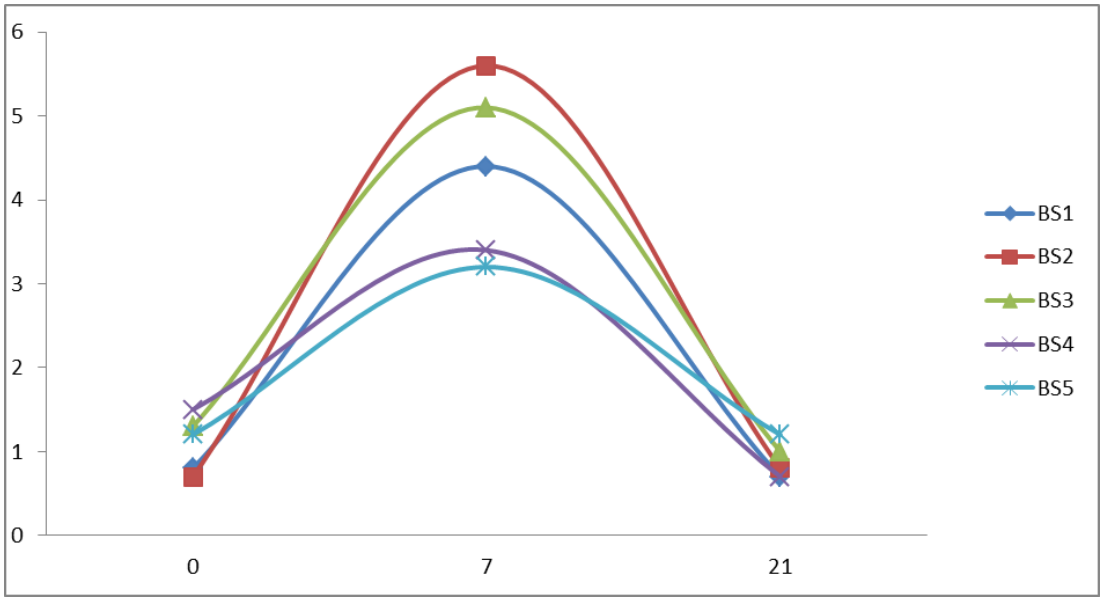


Figure 22. Runx2 ChIP followed by qPCR analysis of the 5 Runx motifs on the *LRP5* promoter in hFOB cell line at day 0, day 7 and day 21.

DISCUSSION

5. Discussion

The osteoporosis was studied by the combination of different methods of analysis, which led to several interesting observations, which are highlighted and discussed in the following sections.

5.1. Choice of cell culture

Different types of osteoblastic cells include primary cultures (derived from normal human and rodent bone tissue), as well as osteosarcoma cell lines (derived from human and rodent tumors) widely used for the understanding of osteoblast biology as was mentioned in introduction section. Each of these model systems has limitations with regard to their application in the study of human osteoblast biology. Osteoblastic cultures derived from rodent species may exhibit species-specific phenotypic characteristics which differ from those of human osteoblastic cultures. Primary cultures derived from normal human bone have an osteoblastic phenotype but proliferate at a very slow rate and become senescent after a relatively short time in culture [79, 155].

Saos2 is a line of osteosarcoma cells that possesses several osteoblastic features and, as malignant cells, they express differentiated features of the tissue of origin along with cellular immortality [66]. Both of these features were the reason behind the choice of this cell line as a control for the hFOB cell line during differentiation. It is interesting to point out that osteosarcoma cell lines have an inherent unreliability with regard to their phenotype (similar to untransformed cells) and the exact nature of the genetic transforming event.

hFOB was chosen for the study of osteoblast differentiation because of the unique characteristics of this cell line. This cell line is a clonal immortalized human fetal cell line with capability to osteoblastic differentiation. hFOB was cultured at 33.5°C until the cells got confluent; this point was set as day 0. Thereafter, the temperature was switched to 39.5°C for the differentiation study at different time points: day 3, day 7, day14 and day 21. As seen in the present work, at day 21 hFOB cells are very quiescent and difficult to work with.

5.2. MTT assay and cell viability during the differentiation study

hFOB cells have little or no cell division at their restrictive temperature, 39.5°C. It is noted that at this point cell division slows down, the differentiation increases, and a more mature osteoblast phenotype is produced.

The recording of the proliferating rate during differentiation steps is a necessity; this can be achieved by several methods of staining or radioactive. Radioactive methods need large number of cells and are expensive. On the other hand, staining methods require washing steps, which increase the processing time and number of sample variations. The MTT assay was used to measure viability of cells during the differentiation steps. This assay has the ability to detect living cells but not dead cells; hence, the method can be used as a rapid, simple and inexpensive method to measure cell proliferation [152, 156-158]. The MTT assay on Soas2 cells showed an OD close to that of the hFOB cell line at day 0 (Figure 13), which is considered as the undifferentiated step of hFOB cells. However, hFOB cells showed a linear relationship between OD (at 560 nm) and days of culture, during differentiation (day 3-21). This correlation with the decrease in number of viable cells is related to differentiation.

Hausser et al. [159] propagated Saos2 cells for up to 100 passages. These authors observed that cells from later passages exhibited higher level of MTT than in earlier passages, which indicate a higher proliferation rate. Wenna Liang [160] cultured hFOB cells at 37˚C in a humidified incubator with 5% CO_2; the hFOB 1.19 cell viability was assessed by MTT colorimetric assay. They showed that the cell viability increased by increasing the concentration of icariin. Hence, the MTT assay study of this thesis constitutes a novel work. As indicated above, the results clearly showed a strong loss of viability, especially at day 21.

5.3. Alizarin red assay and Alp activity based staining

Alizarin Red S (ARS) staining is an established method for the evaluation of calcium-rich deposits by cells in culture. It is an accurate method for the mineralization in monolayer cultures [153]. Many osteoblastic cell lines have been

shown to form mineralized nodules during the processes of cell differentiation and matrix mineralization.

It was observed that hFOB cells at the time of post-confluent cultures (day 0) at 33.5°C formed mineralized nodules gradually (Figure 14). Nodule formation was also observed and became extensive when cells were cultured at 39.5°C, at day 3, day 7, day 14 and day 21 (Figure 14). To visualize calcium deposit within the nodules, the cells were stained by the ARS procedure and examined under light microscopy. Nodules with mineralized matrix were stained dark. Saos2 cells have low level of mineralization at this step (Figure 14). The results agree with those of Hausser et al [159] who studied Saos2 during differentiation with alizarin red staining. These authors studied over 100 passages and observed that Saos2 cells exhibit higher level of mineralization when the number of passages was increased and the minimum level was seen at passage number 9.

Mineralized nodules formation is a characteristic of the late stages of osteoblast differentiation in culture. Our results reveal that hFOB cells appear to be relatively undifferentiated cells, programmed to differentiate. Harris et al [79] used the von Kossa procedure for evaluation of mineralized nodules on the hFOB cell line. Both Von Kossa and ARS staining allow simultaneous evaluation of mineral distribution by phase contrast microscopy. However, ARS staining detects calcium, and Von Kossa detects phosphate and carbonate anions. The von Kossa method can also give a positive staining result to dystrophic mineralization with unknown origin. However, this should be taken with caution since calcium-binding proteins and proteoglycans are also detected by ARS staining [153, 161, 162].

Changes of alkaline phosphatase activity by blue membrane substrate based staining were also determined during the differentiation study to verify the ARS staining results. The results showed (Figure 15) that alkaline phosphate activity based staining started at day 0 and increased during differentiation from day 3 to day 21. Saos2 also showed alkaline phosphatase activity as a test control.

It is interesting to compare these findings with those of Harris et al. [79], who analyzed the hFOB cells at 33.5°C in post-confluency at day 0, day 2, day 4, day 8,

day 10, and also at 39.5°C. It is noted that the studies of Harris et al. contrast the investigations of this thesis, which were conducted at 39.5°C, at days 0, 3, 7, 14 and 21; also, an investigation was conducted at 33.5°C in confluency. However, despite the discrepancies in the protocols of the different studies, the results of this thesis and those of Harris et al. indicate that the levels of alkaline phosphatase activity in hFOB cells cultured at 39.5°C are higher than those of cells cultivated at 33.5°C. But, Harris et al. observed that the alkaline phosphatase activity increased dramatically at either temperature (33.5°C or 39.5°C).

The results of alkaline phosphatase activity based-staining displayed differentiated phenotype in post-confluent hFOB cells at restrictive temperatures. An increase in mineralization at restrictive temperature was shown, which can be explained by the osteoblast differentiation. These authors propagated Saos2 cell line over 100 passages and showed that higher passage cells exhibited lower specific alkaline phosphatase activity and higher mineralization. [159].

5.4. Expression analysis of key genes during osteoblast differentiation

Alkaline phosphatase (ALP) is a glycosylated membrane bound enzyme that catalyzes the hydrolysis of phosphor monoester bonds. The role of the enzyme is to provide inorganic phosphate for mineralization, and to play a role in the degradation of pyrophosphate. Pyrophosphate is a natural inhibitor of mineralization. It is thought that alkaline phosphatase is a primary factor in mineralization. ALP is present early in osteoblast development; therefore, it has been suggested to be a progression factor in osteoblast differentiation [163].

The ALP mRNA levels in Saos2, undifferentiated hFOB and also during hFOB differentiation at days 3, 7, 14 and 21 were monitored. Saos2 showed high levels of ALP mRNA compared to the low levels observed in hFOB undifferentiated cells (Figure 16). During differentiation, it was shown that there was a rapid increase in ALP mRNA level at day 3; it raised 4.4 folds, as an early marker of differentiation. Then, from day 7 to day 21 a slight decrease was observed (Figure 17). This data agree with the result of Harris et al. [79] who found that the activity was 2 to 3 times

higher in hFOB cell cultures at 39.5°C compared with cells at 33.5 °C. The authors showed that the amount of ALP specific staining was much higher in confluent cells than in sub-confluent cells. As shown in Figure 17, during the differentiation, the number of cells decreases. Therefore, such a small decrease in the level of ALP could be due to the changes from a confluent to a sub-confluent step.

This pattern of expression of ALP (Figure 17) was also similar to that observed in chicken calvaria-derived osteoblasts (COB) and rat calvaria osteoblasts (ROB) [164, 165]. In these types of cells alkaline phosphatase levels rose 5-6-fold and then remained constant.

Prince et al. [166] studied ALP levels in 04-T8, 03- CE6 (CE6) and 03-CE10 (CE10) cell lines. All cell types were immortalized human cell lines and were derived from trabecular bone of a 14 year-old girl and from cortical explant culture from a 77 year-old woman, respectively [167, 168]. The authors showed that the basal level of ALP was low at 34°C. However, the ALP levels were found to increase at 39°C in 04-T8 cell compared to 34°C. The results of this thesis (Figure 17) showed that the level of mRNA increased at 39.5°C compared to 33.5°C. The ALP mRNA level increased at day 7 in 04-T8, slightly decreased at day 14 and then increased again at day 21. However, a sudden decrease was seen from day 21 to day 35 of differentiation (not shown). In the CE6 cell line the ALP mRNA levels increased from day 4 to day 14 and then decreased to day 21 and, finally, an increase was observed from day 21 to day 35. In CE10, ALP mRNA levels increased from day 4 to day 14 and then decreased to day 28 without any changes until day 35. The maximum amount of mRNA level was seen at day 21, day 35 and day 14 in 04-T8, CE6 and CE10 cell lines, respectively. The results in Figure 17 showed that the maximum level of ALP mRNA occurs at day 3. The maximum expression of ALP levels in 04-T8, CE6 and CE10 was seen at different time points. Hence, the maximum level of ALP in immortalized cell line can be at early, mid-stage or late stage of differentiation [166].

Osteocalcin (OC) is one of the genes expressed to maximal levels at the onset of mineralization, along with other bone genes. These genes code for bone-synthesized proteins, which are known to associate with the mineralized matrix in vivo [164, 169].

Osteocalcin expression is restricted to the bone tissue, where it is incorporated into the bone extracellular matrix and into the circulation [170]. RUNX2 is one of the transcription factors important for the regulation of its expression [124].

Due to this fact, in this thesis it was decided to study the expression of this gene along with the other key genes of osteoblast differentiation. We monitored the expression of this gene in Saos2 as control and in hFOB during differentiation. The results (Figure 16) showed that the level of osteocalcin was two times higher in Saos2 compared to hFOB. In hFOB cells, OC mRNA levels initially increased up to day 3, and then steadily decreased to day 21 (Figure 17). It is interesting to compare these observations with those in the literature.

In mice, osteocalcin expression is first detectable in the embryo at day E15.5 concomitant with mineralization [171]. OC mRNA levels steadily increase in various osteoblast cell models (including MC3T3-E1, Saos2 cultures) as the osteoblast phenotype progresses. It is noted that OC has been shown to peak late [66, 172]. The results obtained in the present study (Figure 17) are similar to those obtained by Gerstenfeld et al. [164] who studied osteocalcin level in chicken osteoblasts at days 6, 12, 18, 24 and 30. Their results showed a temporal increase from day 6 to day 18, and then a decrease to day 30. Aronow et al. [165] studied osteocalcin expression in ROB (Rat Osteoblast) cells. These authors showed that the expression of OC occurs subsequent to initiation of ALP activity, and accompanies the formation of mineralization. These authors further showed a temporal expression of osteocalcin in ROB cells, which displayed maximum level in primary culture, and then a decrease from day 7 to 14 and to 21 in subcultures. However, Prince et al. [166] showed that in immortalized human cell lines, 04-T8, CE6 and CE10, the osteocalcin was not detected at 34°C during the growth period but appeared at 39°C, at a low level. It is noted that these authors checked the level of osteocalcin by radio immunoassay technique at protein secreted level, and during 24 hours in the differentiation step [166].

RUNX2 plays a crucial role in osteoblast development. RUNX2 binding sequences, known as OSE2 (osteoblast specific element 2), were identified in the osteocalcin

gene promoter, and their ability to enhance its expression was reported [173, 174]. The results obtained in this thesis (Figure 17) showed that from day 7 to day 21, at the time in which the OC mRNA decreased, RUNX2 mRNA levels increased. This finding is similar to that reported in MC3T3-E1 cultures, where the levels of OC mRNA and RUNX2 mRNA go in the opposite direction. In MC3T3-E1 cells during the differentiation, RUNX2 mRNA level decreases and OC mRNA level increases [172].

RUNX2 expression is tightly controlled at the transcriptional level. Rat OC promoter contains three recognition sites for RUNX2 interactions and it was shown that RUNX2 regulated tissue-specific expression of the OC promoter [175-178]. Hence, in this study it was decided to measure RUNX2 protein levels. The results (Figure 18) showed that there was a relationship between the amount of protein and the down-regulation of osteocalcin expression. The amount of RUNX2 protein was higher at days 0 and 3 compared to day 7, which correlates with osteocalcin expression. However, the amount of RUNX2 protein is null at day 14 and day 21. The decrease and low level of osteocalcin expression in hFOB during differentiation can be explained by the lack of RUNX2 to promote osteocalcin expression.

There are other transcription factors that regulate osteocalcin expression. For example, ATF4 (CREB2; cAMP response elements binding protein 2) is one of the important transcription factor that controls osteoblast. ATF4 regulates transcriptional activity of osteocalcin by interaction with RUNX2. Xiao et al. [179] showed that ATF4 alone did not stimulate osteocalcin mRNA in mouse and the stimulation is RUNX2 dependent. The activity of ATF4 protein is regulated at the post- transcriptional level, because it is degraded after its ubiquitination in osteoblast [180]. Therefore, this expression pattern of osteocalcin during hFOB differentiation could be due to the effect of other factors.

The *SOST* gene has a key role in the regulation of bone mass; it is another endogenous inhibitor of the Wnt pathway. The canonical Wnt pathway fundamentally regulates osteoblast differentiation and bone formation. The product of the *SOST* gene is sclerostin. It has been found that sclerostin antagonize canonical Wnt signals by

binding to the Wnt co-receptors LRP5 and LRP6 [141, 181-183]. There is still much to be learnt about effects of sclerostin on human osteoblast function. Sutherland at al. [184] reported that *SOST* expression increased in mineralized culture of human mesenchymal stem cells.

Sevetson et al. [144] identified a RUNX2 binding site on the *SOST* promoter. The authors showed a connection between *SOST* expression and RUNX2 as a key regulator of osteoblast growth and function. Mutations in the *SOST* gene cause the high-bone-mass disease sclerosteosis in humans and its down regulation can cause Van Buchem disease [183, 185]. This information prompted us to study *SOST* expression during hFOB differentiation.

The study focused on measuring the mRNA level of the *SOST* gene. The results (Figure 16) showed that *SOST* levels in Saos2 were higher than in hFOB at day 0. During differentiation, the levels of *SOST* mRNA increased 6.1 times on day 3 and then decreased on day 7, and remained mostly constant at the same level as day 0 (Figure 17).

Li et al. [181] showed that the expression of *SOST* during osteoblast differentiation of MC3T3 cells happens at late stages of osteoblast differentiation, coinciding with that of osteocalcin.

In the present study it was observed (Figure 17) that the expression of sclerostin had a similar time course than that of osteocalcin, and only increased at day 3 during differentiation of hFOB cells. The same time course of expression of both genes was seen in mouse osteoblastic MC3T3 cells, but in these cells it happened at the late stages of differentiation.

RUNX2 (CBFA1, AML3) has been identified as the major transcription factor controlling osteoblast commitment and differentiation. It belongs to the Runt family of transcription factors and it is the earliest marker of osteoblast differentiation. Its expression by mesenchymal cells at the onset of skeletal development is high and present in osteoblasts throughout their differentiation in vivo and in vitro. RUNX2 deficient mice lack osteoblasts, and also completely lack bone formation. This demonstrates that RUNX2 is essential for osteoblast differentiation [20, 125, 186].

Previous studies showed that persistent expression of the protein in mature osteoblasts has deleterious consequences [129, 187, 188]. RUNX2 is prominently involved in activation of bone genes in pluripotent cells. It is required for commitment to the osteogenic lineage and supports final progression to the mature osteocyte and expression of obligatory genes for mineralization [128, 189, 190].

In the present study RUNX2 mRNA was monitored in Saos2 and throughout different time points of hFOB differentiation. RUNX2 mRNA levels were high in Saos2 compared to the low levels observed in hFOB at 33.5°C (Figure 16). In hFOB there was a steady increase between days 0 to day 21, which indicates that RUNX2 has an impact during the post-proliferative maturation and mineralization. This showed that RUNX2 is positively regulated at the mRNA level during osteoblast differentiation in hFOB cells.

An opposite pattern with steady decline in RUNX2 mRNA level from day 1 to day 14 has been shown in the MC3T3-E1 model [172]. However, northern blot analysis of RUNX2 transcript during osteoblast differentiation in human osteoblast cell lines 04-T8, CE6 and CE10 by Prince et al. [166] showed that the RUNX2 mRNA expression levels remained constant during differentiation time points at day 4, 7, 15, 22, 29 and day 36.

Yu et al. [191] showed that in SK11 cells (a clonal progenitor cell line derived from human embryonic stem cells), RUNX2 expression was essentially unchanged throughout the time course of day 5 to 9 of the experiment. Ducy et al. [174] showed that Runx2 mRNA is a good marker for early commitment of mesenchymal stem cells to the osteoblast lineage. The present work suggests that it can be used to measure progression of the osteoblast phenotype in committed cells.

It can be hypothesized that the pattern of RUNX2 expression in human osteoblasts is due to the immortalization characterization or to species differences. This study showed that RUNX2 mRNA is expressed at similar levels throughout stages of post proliferative and mineralization, while osteocalcin and *SOST* are being temporally expressed (Figure 17). These findings support that RUNX2 expression is required for the onset of osteogenesis and osteoblast differentiation [20, 173, 174].

The Wnt signaling pathway is required for osteoblast proliferation as well as for bone matrix deposition by differentiated osteoblasts. *LRP5* (encoding the Wnt co-receptor) is expressed in osteoblasts at early and late stages of differentiation, and is required for optimal Wnt signaling in osteoblasts [134]. Previous studies have shown that loss of function mutations in the human *LRP5* gene cause the osteoporosis pseudoglioma syndrome (OPPG). On the other hand, gain of function mutations in the same gene result in a high bone mass (HBM) phenotype [49-51, 138]. Kato et al. [134] showed that LRP5 is required for osteoblast proliferation and matrix deposition by differentiated osteoblasts. The authors found that in mice, in absence of LRP5, RUNX2 had normal expression which suggests a role for RUNX2-independent pathways in the control of osteoblast proliferation and function. Until recently, RUNX2 and LRP5 had not been shown to be directly connected. However, the studies of our group revealed the presence of five RUNX2 binding site at 2.9 kb from the transcription start site of the *LRP5* promoter [150].

LRP5 is modulated by RUNX2. Inhibition of RUNX2 by siRNAs in U-2 OS cells lead to a decrease of the endogenous *LRP5* mRNA levels. However, in Saos2 cells this effect has not been seen, maybe due to the high levels of RUNX2 present in this cell line [150]. In the present study LRP5 expression levels were measured, and it was found that *LRP5* mRNA levels in hFOB were above those of Saos2 and that the gene was expressed during differentiation with small variations in levels. While *RUNX2* mRNA levels increased during differentiation, this increase was not accompanied by an increase of *LRP5* (Figures 16 and 17).

Kruppel-like factors (KLFs), which belong to a subclass of the zinc-finger family of transcriptional regulators, are critical regulators of growth and differentiation in a broad range of mammalian cell types [192, 193]. Sp1 (zinc-finger protein), which belongs to the Sp family, was identified as the major protein forming complex with oligonucleotides containing Sp1 and KLF15 binding motifs. LRP5 promoter contains Sp1-binding and KLF15-binding sites, which play critical roles in the regulation of the basal transcription of the human LRP5 gene [194]. The characterization of the human

LRP5 basal promoter represents a starting point to the further unravelling of the underlying molecular mechanisms that regulate human LRP5 expression.

5.5. Western blotting and protein analysis

It has been reported that there is a regulation of RUNX2 at the translation level [195]. Therefore, in this study it was decided to measure the total RUNX2 protein levels during hFOB osteoblast differentiation. The results (Figure 18) showed that the level of RUNX2 protein in Saos2 was much higher than in hFOB at day 0. In hFOB, the levels of both RUNX2 protein and RUNX2 mRNA were low at days 0 and 3. But, the protein level decreased at day 7, 14 and 21, which is opposite of the RUNX2 mRNA expression level during the same time interval. The results further showed that the protein levels of LRP5 (the same as RUNX2) decreased during the differentiation study.

Pregizer et al. [172] analyzed the protein level at day 1, 4, 8, 11 and 14 of differentiation in MC3T3-E1 cells. The authors showed that on day 1 the level of RUNX2 mRNA was maximum and that the amount of its protein level was minimum. This is in agreement with the studies of this report (Figure 18). At day 14 and day 21 in immortalized hFOB cells, an opposite pattern in the RUNX2 expression and protein level was observed. However, Yu et al. [191] showed that loss of RUNX2 during differentiation is attributable to post-translational mechanism. These authors showed that the mRNA level did not decrease, and that the protein level decreased due to the loss of its stability in SK11, ST2 and MC3T3 cells. However, Yu et al. showed that the stability loss did not happen on the same day for each cell line during differentiation.

The ubiquitin ligase Smurf1 interacts with the PY motif in the C terminal end of the RUNX2 protein and promotes its degradation by the proteasome. Schnurri-3 is the other protein that promotes RUNX2 protein degradation. This protein controls RUNX2 protein levels by recruiting the E3 ubiquitin ligase WWP1 to RUNX2 [196, 197]. The dissociation between the presence of RUNX2 mRNA on day 14 and 21 and

absence of the protein level at these days reflects multiple levels of control for gene regulation [172, 191, 195].

5.6. Chromatin immunoprecipitation (ChIP) assay

RUNX2 has DNA-binding sites in all the major osteoblast genes, including *COL1A1*, and the osteopontin, bone sialoprotein, and osteocalcin genes, which serve to regulate their expression. RUNX2 induces the expression of these genes by activating their promoters [174, 198-202]. In the present study, RUNX2 occupancy of the 5 different RUNX2 biding sites on the LRP5 promoter [150] was monitored via ChIP assay, on days 0, 7 and 21 of hFOB osteoblastic differentiation and in Saos2 cells. For a control of the ChIP experiment and a control of RUNX2 binding to specific DNA sites, binding of RNA POL II to the GAPDH promoter was measured along with binding of RUNX2 on the *CDKN1A* and *SERPINE1* promoters. The protocol used in this thesis was the same as in the contribution of Van Der Deen et al. [154]. As expected, RNA POL II associated to the transcription start site of *GAPDH* in chromatin of both differentiated and undifferentiated hFOB cells (Figure 19). The level of enrichment was higher in the undifferentiated state and on day 7 of differentiation than in day 21. In the Saos2 cell line the degree of RNA POLII enrichment on *GAPDH* was close to the value observed in hFOB undifferentiated cells.

Also, as expected, the results in the Saos2 cell line showed (Figure 20) that *CDKN1A* and *SERPINE1* promoters exhibited RUNX2 binding. The enrichment of RUNX2 on the *SERPINE1* promoter was higher than that on the *CDKN1A* promoter, which is in agreement with the results of Van Der Deen et al. [154] in this cell line.

Results showed that all but one (site 1) of the RUNX2 binding sites on the *LRP5* promoter were enriched when Saos2 chromatin was immunoprecipitated with an anti-RUNX2 antibody (Figure 20). This observation is in agreement with the results of Agueda et al. [150], in the sense that siRNA (against RUNX2) treatment did not change the expression of *LRP5* in Saos2 due to the excess level of RUNX2 in this cell type.

Enrichment of *CDKN1A* or *SERPINE1* promoters by immunoprecipitation with the RUNX2 antibody along hFOB differentiation (days 0, 7 and 21) followed different curves (Figure 21). While RUNX2 bound the promoter of *CDKN1A* at both differentiated (day 7) and undifferentiated forms of hFOB cells, enrichment for *SERPINE1* happened only in the on day 7 of differentiation. In the hFOB cell line (Figure 22), on day 0, RUNX2 binding to the 5 *LRP5* promoter binding sites 3, was negligible. In contrast, on day 7, the occupancy of RUNX2 on all binding sites increased dramatically, in spite of the decrease of the RUNX2 protein level. However, the occupancy on binding sites 1, 2 and 3 was stronger than on binding sites 4 and 5. Finally, on day 21, no enrichment could be detected.

RUNX2 occupancy on the osteocalcin promoter in MC3T3-E1 was tested by Pregizer et al. [172]. The authors showed a maximum occupancy in the first week, between day 4 and 11, and then a decrease of the occupancy level in the second week at day 14. This result is similar to the results of the present study, on the occupancy of RUNX2 on the *LRP5* promoter as a function of time.

On day 0, when both RUNX2 mRNA and protein were detected, it was observed that the level of enrichment was low. This may be due to eukaryotic cell-specific post-translational modifications, such as phosphorylation or glycosylation, which might be necessary before RUNX2 can interact with the *LRP5* promoter.

RUNX2 requires several partner proteins to play its transcriptional activator or repressor role. Histone deacetylases (HDACs), transducin-like enhancer of split (TLE) proteins, mSin3a, and Yes associated protein (YAP), are examples of co-repressors which bind to RUNX2 and regulate its transcriptional regulation capacities. These proteins do not bind the DNA directly and they act by preventing RUNX2 from binding DNA and altering chromatin structure [179, 203]. A putative high level of one or several of these co-repressors might explain the lack of RUNX2 binding to th *LRP5* promoter at day 0.

On day 14 and day 21, when the RUNX2 expression was at the maximum level, the protein was not observed by western blot and the occupancy of the RUNX2 on the *LRP5* promoter was absent, which can be explained by the translational block of

RUNX2. With the translational block of the RUNX2 in this step, there would be no RUNX2 protein to occupy the *LRP5* promoter. Consistent with its protein levels, the occupancy of the RUNX2 on all 5 binding sites increased on day 7 and was absent at all binding sites at day 21 (Figure 22). At these time points *LRP5* has its steady expression which means that RUNX2 is not the sole regulator of *LRP5* transcription. Hence, other factors can also be involved in its transcription level [194].

5.7. Proposal for further studies in the field of Osteoporosis

This work managed to unlock several features of osteoporosis; however, the field is still an uncharted territory. The following suggestions are proposed as possible research topics in the field.

RUNX2 has binding sites on the *OC* gene, which play an important role on osteoblast differentiation. Therefore, the study of RUNX2 binding on the *OC* promoter during hFOB differentiation along with its binding on the *LRP5* promoter will reveal the importance of RUNX2 in the osteoblast differentiation.

It would also be interesting to study the effect of drugs such as icariin on the hFOB differentiation. It has been shown that this drug significantly increases the viability, ALP activity and the number of calcified nodules in the hFOB cell line [160]. Also, it has been shown that the drug increases the expression of *RUNX2* along with the level of the RUNX2 protein. Therefore, it would be of interest to study the effect of icariin during differentiation of hFOB, and evaluate the interplay between the key genes in osteoblast differentiation and RUNX2 interaction on *LRP5* promoter.

Human bone marrow contains mesenchymal progenitors that can be differentiated to osteogenic, chondrogenic and adipogenic lineage cells [204]. Therefore, the study of human mesenchymal stem cells is important to understand how the expression level of *RUNX2* and other key genes of osteoblast differentiation regulate mesenchymal progenitors to osteogenic lineage cells. This could open new strategies for developing new therapeutic drugs for osteoporosis.

During the differentiation process from mesenchymal progenitors, osteoblast differentiation is regulated by different types of hormones and cytokines, among

which BMPs are the most relevant. BMP-7 induces the expression of *RUNX2* before induction of osteocalcin mRNA [174]. It has also been shown that the level of RUNX2 mRNA increases with BMP-2 in immortalized human bone marrow stromal cell (hMC), C2CL2 cells and 2T3 cells [189, 190, 205]. Hence, studies should be conducted to investigate the effects of BMP-7 and BMP-2 on the hFOB cell line.

It has been shown that sclerostin binds to LRP5, and that responsiveness to Wnt and proliferation is reduced in the absence of LRP5 in mouse primary osteoblast [134]. The present study revealed that LRP5 decreased during differentiation. Studies should also be conducted on sclerostin protein along with the LRP5 protein, and its effect on Wnt signaling in hFOB during differentiation. Furthermore, since RUNX2 has binding site on the *SOST* gene, studies on RUNX2 binding on the *SOST* promoter by ChIP should also be considered.

Osteoporosis is a severe disease that profoundly affects the quality of life of millions of people. This thesis is part of the endeavors that have been initiated by the research community, and supported by governmental bodies, to unlock the origins of the ailment. It is the hope of this author that the reported studies may improve our knowledge of the pathological process of osteoporosis, and lead to the better treatment of the disease.

CONCLUSIONS

6. Conclusions

- An osteoblast differentiation model based on the hFOB was set up, which displayed characteristic osteoblastic markers of mineralization and alkaline phosphatase activity. The low viability observed at day 21 makes this time point of poor utility for further studies.

- It was shown that *LRP5* mRNA was expressed in hFOB cells at higher levels than in Saos2 and that *RUNX2* mRNA was also expressed, but at lower levels. Increasing from day 0 to day14.

- RUNX2 and LRP5 proteins were detected in hFOB cells at day 0, albeit at low levels, faded away along differentiation and were undetectable at day 21.
  No correlation was observed between mRNA and protein levels for RUNX2 and LRP5, suggesting the existence of post-transcriptional regulatory mechanisms.

- ChIP experiments clearly showed, for the first time, the *in vivo* binding of RUNX2 to RUNX2-bidning sites in the *LRP5* promoter in chromatin of both Saos2 and day-7 hFOB cells.

- No good correlation was observed between RUNX2 binding and *LRP5* expression, which leaves the functional effect of RUNX2 binding to the *LRP5* promoter as an unsolved question.

- RUNX2 binding to the *SERPINE1* promoter was observed in chromatin of day-7 hFOB cells for the first time.

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