miRNA and Asymmetric siRNA

Small RNAs with Large Effects on Bone Metabolism

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Abstract

RNA interference (RNAi) is a post-transcriptional gene silencing process elicited by double-stranded RNA, such as micro-RNA (miRNA) and small interfering RNA (siRNA). They are 18-25 nucleotide long, small non-coding RNAs acting as critical regulators in eukaryotic genome expression. They play an important role in regulating a wide range of biological processes such as cell cycle control, differentiation, aging and apoptosis. However, their role in supporting skeletal development and bone homeostasis is still poorly understood.

Osteoporotic fractures constitute a tremendous and growing problem in our ageing populations, with an annual incidence of approximately 60000 osteoporotic fractures in Sweden. Osteoporosis is referred as the “Silent epidemic” because bone loss is gradual and a basically symptomless development until a fracture occurs.

Results presented in this thesis provide a novel insight into crucial roles of miRNAs in regulating bone homeostasis. The initial aim for the thesis was to perform global miRNA expression profiling in human bone cells, and to correlate these levels to global mRNA levels. We identified and functionally characterized several miRNAs that were differentially expressed and acted in important bone signaling pathways such as the Wnt and BMP pathways. These miRNAs included hsa-miR-29b, hsa-miR-30c2 and hsa-miR-125b, which we found targeting genes highly relevant to bone metabolism e.g. COL1A1, SPARC, RUNX2, BGLAP and FRZB.

Thereafter, the effect on the microRNAome upon external stimuli (e.g., Dexamethasone and Parathyroid hormone) was assessed by SOLiD sequencing. We observed a substantial difference in the expression of miRNAs between PTH and DEX treated cells. Understanding the changes in miRNAome in human bone cells under different conditions could provide new insight in bone remodeling, specifically differentiation and functional properties of osteoblasts.

Based on these studies, we furthermore identified Dlx5 as potential common target of miR-203 and miR-320b and these miRNAs negatively regulate BMP-2-induced osteoblast differentiation.

To activate the RNAi pathway, siRNA or miRNA molecules must be conveyed into the cytoplasm of target cells. Since challenges in cellular delivery of these small silencing RNA molecules so far have limited their clinical utility, we developed a new siRNA design that demonstrates a novel carrier-free cellular delivery. This development could potentially have a major impact in RNAi therapeutics.

In conclusion, this thesis provides novel insight of miRNAs that play a major role in the regulation of bone remodeling and differentiation and functional properties of osteoblasts. Our findings may have diagnostic and/or therapeutic implications in disorders of bone metabolism.

Keywords: miRNA, cp-siRNA, RNAi, bone, Osteoblast, Sequencing, Differential expression, Wnt pathway

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Dedicated to my beloved family ♥
To the power of hope, the spirit of knowledge, and the secrets of miRNA.
Cover: miR-29b, miR-30c2, miR-125b, miR-203, miR-320b, two novel miRNAs and siRNAs overlaid on human trabecular bone. Illustrated by Ramu, Vedaraja N.K and Suneel Kadekar.
List of Papers

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


III **Laxman N***, Mallmin H, Nilsson O, Kindmark A. miR-203 and miR-320 regulate Bone Morphogenetic Protein-2-induced osteoblast differentiation by targeting Distal-less Homeobox 5 (*Dlx5*). *Under Review in Bone*


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### Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>BGLAP</td>
<td>Bone gamma-carboxyglutamate (Gla) protein/osteocalcin</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>Basic multicellular unit</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Collagen type I alpha 1</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>Dlx5</td>
<td>Distal-less homeobox 5</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FRZB</td>
<td>Frizzled-Related Protein</td>
</tr>
<tr>
<td>HOB</td>
<td>Primary human osteoblast</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity pathway analysis</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
</tr>
<tr>
<td>Lrp</td>
<td>Low density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MATra</td>
<td>Magnet assisted transfection</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Pri-miRNA</td>
<td>Primary microRNA</td>
</tr>
<tr>
<td>Pre-miRNA</td>
<td>Precursor microRNA</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-immunoprecipitation assay buffer</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich in cysteine/Osteonectin</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Minimum essential medium α</td>
</tr>
</tbody>
</table>
Francis Crick in 1958 proposed the central dogma of molecular biology [1] which stated that RNA is the intermediary of information transfer between DNA and proteins. However, in the last decade this dogma has been challenged by the human genome project, which revealed that only 1.5% of the human genome codes for protein while the remaining 98.5% of the genome has regulatory function, unlike as previously thought as “junk” DNA [2, 3]. Recently, a significant fraction of RNA which does not code for protein was designated-small noncoding RNAs (ncRNA) [4], and plays a regulatory role in modulating gene expression and epigenetics. Regulatory short ncRNAs are characterized by their short length (~20-30 nucleotides) and their coalition with Argonaute protein family, and are classified into three major classes in animals: microRNA (miRNA), small interfering RNAs (siRNA) and Piwi-interacting RNAs (piRNA).

The focus of this thesis are, two classes of ncRNAs: miRNA and siRNA which play a central role in RNA interference (RNAi). We provide novel insight of miRNAs that play a crucial role in regulating bone cell function. Cellular delivery of these small RNA molecules has limited their clinical utility. In this thesis, so far we also aimed to address this by developing a new siRNA design that demonstrates a novel carrier-free cellular delivery also in bone cells.

Micro RNAs (miRNAs) and small interfering RNAs (siRNAs)

The first miRNA, lin-4 was discovered in Caenorhabditis elegans by Ambrose and coworkers in 1993 [5]. Later, RNA interference (RNAi) was discovered in 1998 by Andrew Fire and Craig C. Mello [6], who reported that double-stranded RNA i.e. siRNA can post-transcriptionally silence gene expression. RNAi is one of the powerful tools, which hold promise in therapeutic gene silencing of disease causing genes.

miRNAs are small non-coding ~21 nucleotide long RNA molecules that are involved in the regulation of gene expression. The 3’UTR of the target mRNA has a complementary binding site to miRNAs specific sequence
called seed sequence, which is essential for target site recognition [7, 8]. Recent studies also suggest that miRNA can also target the 5’UTR or the coding sequence of the mRNA [9]. miRNAs exert their control by determining when and how the genes regulate a variety of biological processes including cell growth, death, differentiation and development. It has been estimated that miRNAs target about 60% of human protein coding genes [10].

siRNA are usually derived from long double-stranded RNAs which are of either endogenous [11, 12] or exogenous origin. They are introduced into a cell by transfection or viral infection [13, 14]. They are usually processed into ~21-22 nucleotide long siRNAs by the enzyme Dicer. They regulate gene expression by cleaving and degradation of the target mRNA. Chemically synthesized 22 nucleotide long siRNA designed by specific algorithm with complete complementarity for specific targets can also show a similar function when introduced in to the cells by transfection.

miRNA and siRNA are similar in many aspects e.g. molecular characteristics, biogenesis and target recognition. miRNA and siRNA are processed by the same RNase-III processing enzyme, Dicer. Subsequently they are incorporated in the RISC complex. In spite of their similarities they differ in many aspects, as shown in Table 1.

<table>
<thead>
<tr>
<th>Features</th>
<th>miRNA</th>
<th>siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>~19-24 nucleotides</td>
<td>~21-22 nucleotides</td>
</tr>
<tr>
<td>Configuration</td>
<td>Double stranded</td>
<td>Double stranded</td>
</tr>
<tr>
<td>Occurrence</td>
<td>• Endogenous in origin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Occurs naturally in plants and animals</td>
<td>• Endo and exogenous origin</td>
</tr>
<tr>
<td></td>
<td>• Naturally occurs in plants and animals</td>
<td></td>
</tr>
<tr>
<td>Processing enzymes</td>
<td>Drosha and Pasha processes premiRNA followed by Dicer</td>
<td>Dicer</td>
</tr>
<tr>
<td>Biogenesis</td>
<td>Expressed by genes that code for miRNA</td>
<td>Expressed by genes that code for siRNA</td>
</tr>
<tr>
<td>Mechanism of action</td>
<td>Translational repression followed by mRNA degradation</td>
<td>mRNA cleavage</td>
</tr>
<tr>
<td>Complementarity to target mRNA</td>
<td>Not perfect complementarity</td>
<td>Usually a perfect complementarity</td>
</tr>
<tr>
<td>Target specificity</td>
<td>a single miRNA can target many mRNAs</td>
<td>target specific mRNA</td>
</tr>
<tr>
<td>Function</td>
<td>Regulation of protein-coding genes</td>
<td>Regulation of protein-coding genes and transposons</td>
</tr>
</tbody>
</table>

Table 1. Distinguishing features of miRNAs and siRNAs.
miRNA and siRNA structure and nomenclature

Thousands of different miRNAs have been identified across different species and therefore a nomenclature system has been adopted. The names or identifiers of a miRNA, is for e.g. hsa-mir-30c2. The prefix ‘mir’ refers to pre-miRNA and ‘miR’ refers to mature miRNA followed by a unique identifier number of each miRNA. miRNA are preceded by the species they are observed in e.g. in *Homo sapiens* (hsa-mir-30c2). Lowercase lettered suffix denotes miRNAs with closely related mature sequences (hsa-mir-30c) and distinct precursor sequences and genomic loci that express identical mature sequence are specified with an additional number (hsa-mir-30c2). A ‘3p’ or ‘5p’ suffix denotes miRNA originating from the 3’ or 5’ end of the miRNA [15].

A pre-miRNA secondary structure is usually 60-80 nucleotides long and consists of a hairpin-shaped stem loop structure containing various mismatches and forms irregular internal loops and bulges. A pre-miR is cleaved by Dicer to form a miRNA/miRNA* or guide/passenger strand (*Figure 1A*).

*A*

![Secondary structure of hsa-mir-30c2](image)

*Figure 1. Structure of miRNA and siRNA. (A) Secondary structure of hsa-mir-30c2 and (B) GAPDH siRNA.*

A siRNA is a double stranded structure, with a guide strand that is perfectly complementary to the target mRNA strand and passenger strand. Each strand is usually 19 nucleotides long with a 3’ overhang of 2 nucleotides and consists of a passenger strand and guide strand (*Figure 1B*).
miRNA and siRNA Biogenesis

The sequential process to miRNA biogenesis has several steps: transcription, pri-miRNA processing, transport to the cytoplasm, precursor miRNA (pre-miRNA) processing, strand selection, target binding, and transcript fate [16]. In the nucleus, primary miRNAs (pri-miRNAs) are transcribed in either introns of protein coding genes or intergenic regions by RNA polymerase II. Pri-miRNAs are several kilobases long, have a hairpin structure, and are capped and polyadenylated [17]. Pri-miRNAs are excised by a member of the RNase III family, Drosha (Double stranded RNA specific ribonuclease) and DGCR8 (DiGeorge syndrome chromosomal region 8), into a 60-80 nucleotide precursor miRNA (pre-miRNA). Pre-miRNA is recognized and transported to the cytoplasm by exportin-5, a nuclear export factor [18, 19].

In the cytoplasm, Dicer together with its catalytic partner TAR-binding protein (TRBP) processes the pre-miRNA to a ~20 nucleotide long duplex molecule comprising the mature miRNA guide strand and the passenger strand and delivers them to RISC (RNA-induced silencing complex) [20-22].

The comparisons of miRNAs and siRNAs in the RNAi pathway are illustrated in Figure 2. The siRNA pathway begins when a double stranded RNA is artificially introduced into an animal cell during a gene knockdown experiment. The siRNA is processed by Dicer but not Drosha and incorporated into RISC (RNA-induced silencing complex) [5].

In the RISC, helicase unwinds the two strands of miRNA or siRNA. The RISC complex also consists of one or more Argonaute proteins (Ago1, 2, 3 and 4) [23, 24] which help to anchor and guide the small ncRNAs to target mRNA molecules for inhibiting the protein translation process or silencing the mRNA by cleavage. In human cells, Ago2 have also been implicated in transcriptional silencing. Both the Dicer and Argonaute proteins are involved in the selection of the guide strand of siRNAs and miRNAs whereas the passenger strand is discarded [24-28]. To warrant the correct strand selection by the RISC, a strand selection mechanism exists; the asymmetry rule, where a relative thermodynamic stability at the 5’ ends of the 2 strands determine which strand is recruited into the RISC, the strand which has high A/U content at the 5’ end is relatively unstable and selected as the guide strand and strand which is more stable (high G/C) is the passenger strand [29]. Finally, the target mRNA is identified by RISC complex and then the miRNA and siRNA present in the RISC complex regulates the expression of the target mRNA by the degree of base pairing with the mRNA. miRNA usually binds to the 3’ UTR with incomplete complementarity, but the siRNA binds with complete complementarity with the coding sequence of the mRNA. siRNA causes mRNA cleavage by endonuclease activity of RISC using Ago2 due to full complementarity. Unlike siRNA, the lower degree of complementarity between miRNA and target mRNA inhibits protein synthesis and later induces their degradation [26, 30-32].
Figure 2. Biogenesis of miRNAs and siRNAs. miRNAs are transcribed as long primary miRNA (pri-miRNA). They are processed by Drosha and its cofactor DGCR8 yielding a hairpin precursor miRNA (pre-miRNA) in the nucleus. Exportin5 translocates the pre-miRNA to the cytoplasm whereas the siRNA are artificially introduced into the cell. The Dicer complex processes it into a guide strand-passenger strand duplex. This duplex is incorporated into the RISC complex and the passenger strand is degraded and the mature miRNA later binds to the specific 3'UTR of target mRNA. Depending on the complementarity miRNA represses the translation and siRNA degrade the target mRNA.

miRNAs in osteoblast differentiation and skeletal development

miRNAs play an important role in regulating skeletal development and homeostasis. They mediate their effect by tightly regulating and coordinating the osteoblast differentiation. Several profiling studies have identified over 50 miRNAs designated as “OsteomiRs”, that positively or negatively regulate osteoblast differentiation and bone formation by targeting negative regulators or osteogenic factors [33, 34]. miRNAs are associated with all the steps as a cell progresses through the osteoblast differentiation program from MSCs to pre-osteoblast, mature osteoblast and the completion of bone formation at the terminal mineralization stage.
miRNAs regulate the expression of multiple transcription factors (Dlx5 (distal-less homeodomain-containing family 5), Runx2 (Runt-related transcription factor 2), Osterix (Osx/SP7) and ATF4 (activating transcription factor 4)) and signaling pathways including the BMP, Wnt and TGFB pathways that cooperatively regulate the osteoblastic genes essential for the development of the osteoblast and bone phenotype. Dlx5 a bone-inducing transcription factor plays an essential role in upregulation of the downstream osteogenic master transcription factor Runx2 and Osterix expression [35, 36]. miR-141 and miR-200a expression triggers the translational repression of Dlx5, inhibiting pre-osteoblast differentiation induced by BMP-2 [37]. Osterix (Osx/SP7) is expressed in all developing bones and acts mainly at the terminal differentiation of osteoblasts. Osx is located downstream of Runx2 and the Osx promoter is directly bound by Runx2 [38]. mir-93 attenuates osteoblast mineralization by directly targeting Osx [39] and miR-31 has also been shown to regulate Osx [40]. Osx in turn promotes the expression of Alp and Bglap.

Runx2 is a key bone specific transcription factor that regulates the differentiation of MSCs into the osteogenic lineage. The expression of several osteoblast differentiation genes such as collagen, osteocalcin (BGLAP), osteopontin (SPP1) and bone sialoprotein (IBSP) are regulated by Runx2. An assortment of 11 miRNAs, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, miR-23a, miR-218 and miR-338 target and decrease the expression of Runx2 thereby inhibiting osteoblast differentiation [41, 42]. The expression of Runx2 protein levels are tightly regulated during the late mineralization stage of osteoblast differentiation, by the strikingly increased expression of majority of miRNAs targeting Runx2. miR-26a and miR-135 targets SMAD1 and SMAD5 by modulating osteoblast differentiation [43, 44]. miR-214 directly targets ATF4 (activating transcription factor 4) and plays a role as an important inhibitor of bone formation [34]. Although several miRNAs inhibit osteoblast differentiation, there are many miRNAs that positively regulate osteoblast differentiation. miR-218 and miR-35-5p promote osteoblast differentiation by downregulating the Wnt inhibitors sclerostin (SOST), Dickkopf2 (DKK2), Dickkopf1 (DKK1) and secreted frizzled-related protein (SFRP2), and as a result activate the Wnt pathway [45, 46]. The expression of the histone deacetylase 5 (HDAC5), an enhancer of Runx2 degradation, is repressed by miR-2861 and as a result plays an important role in activating osteoblast differentiation [47]. miR-29b promotes the progression of differentiation by targeting negative regulators of osteoblastogenesis, i.e. by relieving the inhibitory effect of HDAC4, TGFβ3, ACVR2A, CTNNBIP1, and DUSP2 proteins, thereby promoting osteogenesis [48-51].

miRNAs also regulate the extracellular matrix (ECM) secretion by osteoblasts. The miR-29 family suppresses the genes COL1A1, COL4A2, and COL5A3, decreasing the production of collagens type I, IV, and V, and as a
result contributes to positive regulation of osteoblast differentiation [48]. Osteonectin (SPARC), the most abundant non-collagenous matrix protein that contributes to bone mass, is repressed by miR-29a/c [52]. miR-208 represses the expression of osteopontin, by the regulation of Est1, a transactivator of osteopontin [53] (Figure 3).

**Figure 3.** Schematic illustration highlighting the activity of miRNAs and transcription factors in regulating osteoblast lineage. The differentiation of MSCs to lineage specific phenotype is determined by specific miRNAs targeting the transcription factors. Runx2 directs the MSCs to the pre osteoblast lineage, DLX5, Osterix and ATF4 and the corresponding miRNAs that regulate them contributes to the formation of bone, thereby eliminating the potential to differentiate into adipocyte, chondrocyte and myoblast. References: adipocytes [54, 55], myoblasts [56, 57] and chondrocytes [58, 59]
Bone

Biology of bone

Bone is an exceptional structural material; bones support and protect various organs of the body and store minerals. Bone is a complex and porous structure that attains its maximum strength while remaining relatively lightweight. Bone is not a static tissue; it is highly dynamic with a unique capacity to continually be modified, reshaped, remodeled and overhauled without leaving a scar through one’s lifetime. The combined action of the bone cells; osteoprogenitors, osteoblasts, osteocytes and osteoclasts, helps to attain and maintain bone homeostasis.

Bone cells

Bone cells produce the bone tissue and they work together to help maintain the skeletal system by development and maintenance of bone and also in regulation of its metabolic functions.

The different types of bone cells are:

- **Osteoblasts** – its function is to form new bone matrix
- **Osteoclasts** – it functions to resorb and remove mineralized bone
- **Osteocytes** – its function is to monitor and maintain bone
- **Lining cells**- serves as an interface between the bone tissue and the rest of the body and regulate the movement of calcium and phosphate into and out of the bone.

Osteoblasts are derived from mesenchymal osteoprogenitor cells and they are responsible for the synthesis of new bone matrix through a process called osteogenesis [60]. They are mononucleate cells which produce a matrix called osteoid, mainly consisting of type I collagen. Osteoid later mineralizes and becomes mature bone. Ultimately, some osteoblasts become trapped and surrounded by bone matrix and differentiate into the osteocyte phenotype.

Osteoclasts are large multinucleate cells derived from hematopoietic stem cells of the monocyte/macrophage lineage. Osteoclasts resorb bone, they secrete enzymes and acids which degrade bone tissue by removing its mineralized matrix and after which the osteoclasts undergo apoptosis.

Osteocytes are the most abundant of the three types of cells which have differentiated from mesenchymal stem cells through osteoblast differentiation. They lie deeply embedded in the bone matrix where they are encased in osteoid by mineralization when new bone is formed. These cells form highly connected extensive filamentous dendrites that extend to other osteocytes as well as to the lining cells on the bone surface. Mechanical stress on the bone is sensed by these cells which translate signal to modulate osteoblasts and
osteoclasts to act at the site, thus playing an essential role in bone homeostasis [61].

Bone lining cells are inactive osteoblasts that have become flat and extend over the bone surface. They have a role in regulating mineral homeostasis [62]. Lining cells express receptors for hormones that initiate bone remodeling.

Osteoblast differentiation

Osteoblasts originate from mesenchymal stem cells (MSCs). MSCs function as precursors for different cell types like osteoblasts, myoblasts, chondrocytes and adipocytes. Specific transcription factors determine their lineage commitment. Chondrocytic differentiation is regulated by Sox5, 6 and 9, while PPARγ and Myo D regulate differentiation of adipocytes and myoblasts respectively. The essential transcription factors that regulate osteoblast differentiation are Dlx5, Runx2, Osx and ATF4 [34, 38, 63, 64].

The coordinated process of osteoblast differentiation is delineated by four stages; lineage commitment, proliferation, matrix maturation, and finally mineralization. During osteoblast differentiation, the pluripotent mesenchymal stem cells (MSCs) are induced to differentiate into osteoprogenitors by the action of parathyroid hormone (PTH) and growth factors and signaling molecules like Bone Morphogenetic Protein 2 (BMP2), Insulin-like growth factor (IGF) and Fibroblast growth factor (FGF) [65], and then differentiate into pre-osteoblasts. Pre-osteoblasts start production of a non-mineralized extracellular matrix (ECM) called osteoid consisting mainly of type I collagen, which acts as a template for the deposition of minerals. Pre-osteoblasts eventually develop into mature osteoblasts. The ECM production is maintained by mature osteoblasts, which also produce noncollagenous ECM proteins including osteonectin (SPARC), osteocalcin (OC) and osteopontin (OPN) and they also initiate mineralization of the ECM. Some active osteoblasts become encased within the matrix they secrete, thereby becoming osteocytes. The other mature osteoblasts either undergo apoptosis or become bone lining cells. The matrix maturation phase is characterized by maximal expression of alkaline phosphatase (ALP). Finally, at the beginning of matrix mineralization, OC, Bone sialoprotein (BSP), and OPN are expressed, and hydroxyapatite (HA) crystals begin to attach to the ECM, with ALP aiding the mineralization process. Mineralization is a well-orchestrated process when calcium phosphate crystals are laid down within the collagen fibril matrix [66].

Bone formation

Bone formation (osteogenesis) occurs during the development of human body. It begins during prenatal development and continues through child-
hood and adolescence. The formation of bone occurs by two processes: Intramembranous ossification and endochondral ossification. The process of intramembranous ossification occurs when mesenchymal cell aggregate differentiates into osteoblasts and produce e.g. flat bones of the skull, mandible and clavicle. Endochondral ossification causes formation and growth of long bones such as the femur by replacing a cartilaginous model with bone tissue.

Bone remodeling

Bone remodeling is a lifelong process which is responsible for maintaining bone integrity and strength. To maintain homeostasis, new bone continuously replaces mature bone tissue during fracture and micro damage. The Basic Multicellular Unit (BMU) consists of osteoclasts which remove old bone and osteoblasts which synthesize new bone, working in tandem during the remodeling process [67].

In response to mechanical stress or a crack, osteocytes detect strain and release factors that triggers bone resorption. One recently described such factor, sclerostin, is a signaling molecule involved in the regulation of bone remodeling. It is synthesized by osteocytes and is a potent inhibitor of bone formation and BMP-induced osteoblast differentiation. On the surface of osteoblasts, sclerostin binds to Lrp4, Lrp5 or Lrp6 (low density lipoprotein receptor-related proteins). These single transmembrane receptors are known to be involved in Wnt signaling and have a role in regulating bone mass. Activation of Wnt signaling in bone stimulates osteoblastic activity and increases bone formation. Sclerostin binding to Lrp4, Lrp5 or Lrp6 has an inhibitory effect on Wnt signaling, and reduces osteoblastic activity and bone formation. The Wnt pathway is also believed to play a key role in the ability to strengthen bone in response to mechanical stress. Osteocytes responds to stress by producing less sclerostin which results in an increase in osteoblast mediated formation of new bone [68, 69].

Pre-osteoclasts are generated when hematopoietic cells are induced by Receptor activator of nuclear factor kappa-B ligand (RANKL) and M-CSF (Macrophage Colony Stimulating Factor) [70, 71]. RANK-L is a protein released from osteoblasts, and plays a key role in osteoclast formation, function and survival by the interaction of its receptor RANK on their surface of pre-osteoclasts [72]. Multinucleated osteoclasts are formed by the fusion of pre-osteoclasts [73]. Osteoclasts anchor themselves to the bone matrix with integrins, creating a sealing zone Figure 4. [74]. The cell surface where the osteoclast is directly in contact with bone differentiates into a ruffled border to increase the surface area of resorption. Within this zone, osteoclasts first create an acidic environment that dissolves the bone mineral content, and then the enzymes released from the osteoclasts dissolve the collagenous bone matrix to complete the process of resorption. As the entire BMU
moves, new osteoclasts are activated to resorb bone and they later undergo apoptosis.

Figure 4. Illustration of bone remodeling which occurs at BMU (Basic Multicellular Unit) consists of osteoclasts, which remove old bone, and osteoblasts which synthesize new bone; the two cell types work in tandem in the remodeling process.

Pre-osteoblasts are generated from mesenchymal precursors which require a signal from the Wnt-frizzled-Lrp5 (low density lipoprotein receptor-related protein5)-β-cat ein signaling pathway. Growth factors e.g. BMP, FGF (Fibroblast growth factor) and TGF-β also induce differentiation. Interleukins and bone morphogenic proteins (BMPs) play a key role in osteoblast differentiation. Osteoblast differentiation is further controlled by transcription factors Runx2 and Osterix. Estrogen limits the amount of RANKL expression by osteoblast and OPG binds to RANKL thereby reducing osteoclast activity. A team of new osteoblasts move into the resorption space and begins to replace the resorbed bone. Osteoblasts produce an organic matrix, osteoid, consisting primarily of type I collagen and other compounds. This forms the scaffold into which minerals like calcium and phosphate begins to crystallize [65]. Some active osteoblast becomes encased within the matrix they secrete and thereby becoming osteocytes, that have an extensive canalicular network that extends to other osteocytes as well as the lining cells on the bone surface. Other osteoblasts undergo apoptosis or revert back to lining cells when the process is complete. Lining cells, upon exposure to PTH or mechanical stress, still possess the ability to redifferentiate into osteo-
blasts. The dynamic and active process of bone remodeling is maintained through a balance between coupled process of bone resorption by osteoclasts and bone formation by osteoblasts.

Osteoporosis

Osteoporosis literally means “porous bone”. It is a metabolic bone disease caused by an imbalance in the regulation of bone remodeling resulting in a slight deficit of bone tissue for every remodeling cycle. This results in decreased bone density and increased fracture risk, **Figure 5**. Osteoporosis is a global problem and referred as the “Silent epidemic” because bone loss is gradual and a basically symptomless development, until a fracture occurs. There is a 30-50% life time risk for osteoporotic fractures in women, and 15-30% for men worldwide [75]. Risk of osteoporosis and fracture is known to increase with age. Osteoporosis can be classified as primary and secondary, where primary osteoporosis is the most common type and usually occurs in post-menopausal women due to a decrease in estrogen hormone around menopause (post-menopausal osteoporosis). Estrogen deficiency at menopause accelerates the loss of bone mineral density and increases the risk of fracture [76]. Secondary osteoporosis develops due to an underlying medical condition e.g. hyperthyroidism, diabetes or by actions of drugs such as glucocorticoids with negative effects on bone.

![Osteoporotic bone](image1.png) ![Normal bone](image2.png)

*Figure 5. Micro-CT scanned image of osteoporotic and normal bone. The osteoporotic bone showing deteriorated bone trabecular structure.*
The currently recommended treatments for osteoporosis include:

- Calcium and vitamin D supplements – if the patient is deficient, or is started on active osteoporosis treatment
- Bisphosphonates (Antiresorptives - cause apoptosis of osteoclasts and reduces bone turnover)
- Denosumab (Antiresorptive - human monoclonal antibody designed to inhibit RANKL)
- Injectable teriparatide (Recombinant form of PTH which is anabolic and stimulates new bone formation)

RNAi as therapeutic agents

RNA-based therapeutics provides a great potential as a treatment option for human diseases. Small interfering RNA (siRNA) and miRNA have recently been started to be used in therapies and they are being clinically investigated due to their specificity and efficacy. Attempts are underway to use siRNA and miRNA as diagnostic as well as therapeutic molecules.

siRNA therapeutics have been assessed in several diseases and advanced to clinical trials, it also offers the potential to treat diseases caused by RNA dysregulation [77]. However, there are several factors that limit the utility of siRNA in therapeutics. A classic siRNA is double stranded which has a perfectly complementary guide strand that target specific mRNA. The guide and passenger strand is 19 nucleotides long paired region with a 2 nucleotide 3’ overhang. Major hurdles for the use of siRNAs include the delivery of the molecule to target cells, stability of the molecule, off-target effects and stimulation of the immune system. To resolve some of these issues like stability, these siRNAs were chemically modified by adding moieties like 2’-O-Methyl nucleosides into one strand of the siRNA. Another challenging factor is the safe and effective delivery of siRNA to the target tissue. The siRNA need to cross the cell membrane and escape the endosome to be delivered to the cytoplasm [78]. Once in the cytoplasm it is recruited to the RISC.

In spite of these challenges, several siRNA-based therapeutics have successfully advanced to clinical trials and will likely revolutionize the treatment of human diseases. Currently, many of the treatments use intravitreal and intranasal administration. A summary of siRNA currently undergoing clinical trials are presented in Table 2.
miRNA-based therapeutics have been developed by 2 approaches: miRNA antagonists/inhibitors and miRNA mimics. miRNA antagonists, are chemically modified passenger stand of miRNAs that inhibit active miRNA strand and cause a gain-of function in the diseased tissue. Chronic hepatitis C was efficiently treated in animal models by therapeutic inhibition of miR-122 [79]. A LNA-modified oligonucleotide miraviren (or SPC3649) was designed to inhibit this liver specific miRNA which targets Hepatitis C virus (HCV). Miraviren is in Phase IIa clinical trials and when administered subcutaneously showed a promising reduction of HCV RNA levels in patients. 2’-fluoromethoxyethyl-phosphorothioate modified anti-miR33a/b treatment showed a significant decrease in very-low-density lipoprotein (VLDL)-triglycerides and an increase in high-density lipoprotein (HDL), thus making them potential therapeutic targets for the treatment of cardiovascular diseases.

miRNA mimics are double stranded oligonucleotides that act as functional equivalents to endogenous miRNAs that restore loss of function by the re-introduction of miRNAs into diseased tissue which are otherwise normally expressed in healthy tissue, also known as ‘miRNA replacement therapy’. miR-34a mimics containing nanoparticle liposome MRX34 was injected into mice with post-menopausal osteoporosis and observed reduction in bone loss [80]. miR-34a is a osteoclastic suppressor decreasing osteoclastogenesis and bone resorption. Furthermore, systemic administration of miR-34a suppresses bone metastasis.

### Table 2. Summary of current siRNA based treatments undergoing clinical trials. Information from clinicaltrials.gov.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Delivery system</th>
<th>Disease</th>
<th>Phase</th>
<th>Status</th>
<th>Company</th>
<th>Clinical trial Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALN-RSV01</td>
<td>RSV nuleocapsid</td>
<td>Naked siRNA</td>
<td>Respiratory syncytial virus infections</td>
<td>II</td>
<td>Completed</td>
<td>Alnylam pharmaceutical</td>
<td>NCT00658086</td>
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<td>ALN-TTR02</td>
<td>TTR</td>
<td>LNP</td>
<td>Transthreitin-mediated amyloidosis</td>
<td>II</td>
<td>Recruiting</td>
<td>Alnylam pharmaceutical</td>
<td>NCT01617987</td>
</tr>
<tr>
<td>TD101</td>
<td>K6a(N171K mutation)</td>
<td>Naked siRNA</td>
<td>Pachyonychia congenita</td>
<td>II</td>
<td>Completed</td>
<td>Pachyonychia congenita Project</td>
<td>NCT00716014</td>
</tr>
<tr>
<td>QPI-1007</td>
<td>CASP2</td>
<td>Naked siRNA</td>
<td>Optic atrophy,optic neuropathy</td>
<td>II</td>
<td>Completed</td>
<td>Quark Pharmaceuticals</td>
<td>NCT01064505</td>
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<tr>
<td>PF_655</td>
<td>RTP801</td>
<td>Naked siRNA</td>
<td>diabetic retinopathy, diabetic macular oedema</td>
<td>II</td>
<td>Active</td>
<td>Quark Pharmaceuticals</td>
<td>NCT01445899</td>
</tr>
<tr>
<td>Bevasiranib</td>
<td>VEGF</td>
<td>Naked siRNA</td>
<td>Diabetic macular oedema,macular degeneration</td>
<td>II</td>
<td>Completed</td>
<td>Opko health</td>
<td>NCT00306904</td>
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<td>SYL1001</td>
<td>TRPV1</td>
<td>Naked siRNA</td>
<td>Ocular pain, dry-eye syndrome</td>
<td>I,II</td>
<td>Recruiting</td>
<td>Sylentis</td>
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<tr>
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<tr>
<td>SPC3649</td>
<td>Hepatitis C</td>
<td>LNA siRNA</td>
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<td>Ia</td>
<td>Completed</td>
<td>Santaris</td>
<td>NCT00979927</td>
</tr>
</tbody>
</table>

LNP-lipid nanoparticle, LNA-Locked nucleic acid
A single miRNA can have multiple target sites in genes involved in osteoblast differentiation, reciprocally, a single gene can have many evolutionarily conserved sequences that can interact with a cluster of miRNAs. The advantage of miRNA based therapy is that they are cost effective compared to peptide based therapies. Many challenges exist for the use of miRNAs as therapeutic agents including the issue of delivery, off-target effects, safety and avoidance of activating immune response. [81-85].

The encouraging results obtained from these clinical trials could lead the way for the progress of RNAi from a research tool into clinical evaluation.
Aims of this thesis

The General Aim
The overall aim of this thesis was to characterize the miRNAome in human bone cells. This thesis also aims to broaden the knowledge of miRNA regulation in bone cells. In addition, the thesis aims to identify targets for miRNA as well as to characterize phenotypes derived after over- or down regulation of miRNAs in osteoblasts. Lastly, the goal was to initiate the development for potential novel therapeutic approach for skeletal diseases.

Specific aims
The specific aims of the study are elucidated in the papers included:

Paper I: To investigate the possible association between miRNAs and bone metabolism by correlation to mRNA expression in human osteoblasts.

Paper II: To investigate the impact of treatment with PTH and DEX for 2 and 24 hours by RNA sequencing of miRNAs in primary human bone cells.

Paper III: To identify miRNAs that target transcription factor \textit{Dlx5} that is involved in BMP2-induced osteogenesis using primary human osteoblasts.

Paper IV: To constitute the first cell-penetrating, asymmetric siRNA, possessing endosmolytic properties that promote carrier-free transfection to human cancer cells and hard-to-transfect primary cells.
Materials and Methods

Cell culture (Paper I-IV)
The following cell lines were used in the experiments.

Primary human osteoblast (HOB) cells were isolated from human trabecular bone belonging to a cohort of patients with osteoarthritis undergoing total hip or knee replacement, with no other bone-related pathologies reported ([86, 87]). The bone chips obtained from each donor were thoroughly minced and washed with PBS. The minced bone chips were cultured in medium containing α-MEM (Sigma-Aldrich, Haverhill, UK) supplemented with 2 mmol/l L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% fetal bovine serum (Sigma-Aldrich) at 37°C with 5% CO₂ until confluence was reached. The culture medium was changed twice weekly. The study was approved by the local ethics committee (Ethical approval # Ups 03-561).

Keratinocyte and fibroblast cells were isolated from skin tissue. Keratinocytes were grown in Keratinocyte-SFM medium, Gentamicin 50mg/ml medium, BPE (Bovine Pituitary Extract) medium 25µg/ml and EGF Recombinant human protein 0.2ng/ml medium (all from Gibco Life Technologies) at 37 °C with 5% CO₂ until confluence was reached.

MG63 (human osteosarcoma cell line), HCT116 (human colon cancer cell line), were obtained from American Type Culture Collection, ATCC (Manassas, VA, USA). These cells were cultured in medium containing α-MEM (Sigma-Aldrich, Haverhill, UK) supplemented with 2 mmol/l L-glutamine, 100 U/µL penicillin, 100 mg/µL streptomycin and 10% fetal bovine serum (Sigma-Aldrich) at 37°C with 5% CO₂ until confluence was reached. The culture medium was changed twice weekly.

Transfection (MATra) (Paper I-IV)
Cells were seeded at a density of 35,000 cells in 24-well cell culture plates to achieve 60-80% confluence at the day of transfection. To over express and inhibit the function of miRNA, the cells were transfected with either mirVana mimic or mirVana inhibitor and with mirVana miRNA inhibitor negative control and mirVana miRNA mimic negative control at different concentra-
tions which differed between studies using Magnet Assisted Transfection (MATra-si) reagent (IBA GmbH, Göttingen, Germany) according to the manufacturers' protocols. Each transfection was performed in triplicate. Post transfection, cells were incubated for a varying number of hours depending on the study.

For carrier-free transfection experiments, cpRNAs were directly added to different cell types at 50 nM concentrations. Cells were also transfected with negative control siRNA (scrambled sequence) and untreated cells were taken as controls. Each transfection was performed in triplicate. (Paper IV)

**Gene and miRNA expression**

**RNA isolation and cDNA synthesis (Paper I-IV)**

Total RNA was isolated from cells by homogenizing the cell lysates with QIAshredder (Qiagen, Germany), later RNA was extracted from the cell lysates with the RNeasy Mini Kit (Qiagen). Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA) was used to confirm high RNA quality for all samples. The BioAnalyzer reports an RNA Integrity Number (RIN) RIN values between 8.0-9.5 were taken forward for the experiments [88]. The concentrations were determined using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) with OD 260/280 values ranging between 1.95-2.03. Equal amounts of RNA were reverse transcribed to cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, USA). cDNA synthesis was performed in triplicate.

**Real-time RT-PCR (Paper I-IV)**

Quantitative PCR reactions were performed using TaqMan 2x Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and. The amplification was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems).

The 7500 software automatically calculates raw Ct values, baseline and a threshold values. Data of samples with a Ct value equal to or below 35 were further analyzed. Samples were normalized with endogenous controls and differences in cycle number thresholds were calculated using comparative quantitation $2^{-\Delta\Delta CT}$ method (also called the $\Delta\Delta CT$ method) [89].

**miRCURY™ LNA microRNA microarray (Paper I)**

Total RNA from 11 females and 8 males were labeled with Hy3™ and common reference with Hy5™ fluorescent label, using the miRCURY™ LNA Array power labeling kit (Exiqon, Denmark). The miRCURY™ LNA
array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and the image analysis was carried out using the ImaGene 8.0 software (BioDiscovery, Inc, USA). The quantified signals were background corrected using Normexp with offset value 10,[90] and normalized using the global LOESS (LOcally WEighted Scatterplot Smoothing) non-linear regression algorithm (Details provided in Supplementary Methods), where after data was subjected to unsupervised hierarchical clustering [91] using MeV 4.9 release (MultiExperiment Viewer).

Global mRNA expression (Paper I)
mRNA expression profiling of the 95 samples, each with three biological replicates, was performed using the Illumina HumRef-8v2 BeadChips (Illumina Inc.) where 200 ng of total RNA was processed according to the protocol supplied by Illumina as previously reported [87] The biological replicates were always separated and hybridized on different BeadChips in order to avoid that results are confounded by technical effects. The raw data were imported to Bioconductor [92] using the R 2.5.0 package for variance-stabilizing transformation and robust spline normalization to obtain normalized mean expression values. The microarray data have been deposited in the Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo, accession no. GSE15678).

Osteogenic differentiation and Alizarin red S and ALP staining (Paper I, III)
HOBs were seeded at a density of 35,000 cells in a 24-well cell culture plate to achieve 60-80% confluence at the day of transfection. After transfection as described above, at 80% confluence, osteogenic differentiation was induced by replacing the medium with complete medium supplemented with 10 nM dexamethasone, 0.2 mM L-ascorbic acid, and 10 mM β-glycerophosphate. The cells were cultured in differentiation medium for 12 days and the medium was changed every 3 days. Alizarin Red staining was performed to detect matrix mineralization, after the HOBs were fixed in 70% cold ethanol for 1 hour at room temperature, and then stained with 2% Alizarin Red S (Sigma, St. Louis, MO, USA), pH 4.2, for 10 min at room temperature. Each experiment was repeated in triplicate. Calcium deposition was visualized as red color. Alizarin red was quantified by destaining the cultures using 10% cetylpyridinium chloride (CPC) (Sigma, St. Louis, MO, USA) in 10mM sodiumphosphate, pH 7.0, for 15 min at room temperature. The liquid was transferred to a 96-well plate and the concentration of Alizarin Red S
was determined by measuring the absorbance on an ELISA plate reader (Thermo Scientific) at 540nM. ALP activity was performed using ALP staining kit (Sigma) following the manufacturer’s instruction. The mineralized nodules were visualized as dark purple color under the digital inverted microscope (10x and 20x objective) (AMG Evos XL Core, Fisher Scientific, Pittsburgh, PA).

miRNA and Target identification

miRNA Sequencing by SOLiD4 and data analysis (Paper II)

cDNA was synthesized and libraries were amplified by emulsion PCR using SOLiD library reagents. 24 barcoded small RNA libraries for SOLiD™ sequencing were constructed according to the manufacturer’s instructions, at the Uppsala Genome Center, Rudbeck Laboratory, Uppsala. The libraries were sequenced on a SOLiD v4 with 35bp read-length according to the manufacturer’s instructions. miRNA expression was analysed using the smallRNA pipeline included in LifeScope™ v2.5.1 (all LifeTechnologies, Carlsbad, USA) with miRBase20 and hg19 as references and default mapping parameters. miRNA expression is presented as mapped reads per million sequenced reads.

Discovery of novel miRNAs (Paper II)

The web-based tool miRanalyzer [93] was used to discover previously unidentified miRNA sequence using hg19 and miRBase20 as references. Novel miRNAs were identified and chosen from each treatment group. The RNAstructure software [94] was used to predict secondary-structures and free energy, and the DIANA-microT v3.0 software [95, 96] was used to predict gene targets for the candidate novel miRNAs by comparing the seed region complementarity of the mature miRNA sequence to all the known mRNA 3’UTR sequence in the genome. The sequence conservation between species for each of the mature novel-miRNA was determined by the aligning the sequence of these miRNAs in BLAT tool in the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat).

Target prediction and Pathway analysis (Paper I,II,III)

Predicted targets of differentially expressed miRNA in this study were identified using TargetScan, Release 6.2:June 2012 (http://www.targetscan.org/), miRanda algorithm [97, 98] and Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Redwood City, CA). miRNA Target Filter was
used to assess the interaction between miRNAs and their experimentally validated target genes.

Luciferase reporter assay (Paper III)
The psiCHECK-2 vector, a dual-luciferase plasmid, has both the synthetic Firefly Luciferase (Fluc) gene and the synthetic Renilla Luciferase (hRluc) gene incorporated into them, and they each possess their own promoter and poly (A)-addition sites. Luciferase reporter plasmids were constructed by inserting a perfectly complementary (Wild type) 3' UTR fragment of DLX5 between the XhoI–NotI restriction sites in the multiple cloning regions in the hRluc gene in the psiCHECK-2 vector (Promega, Madison, WI, USA) by Generay Company (Shanghai, China). Luciferase assays were conducted in 96 well plates cells, were HOBs were co-transfected with 100ng/well of the wild reporter plasmid or the 3 mutant reporter plasmid and 40 nM of mirVana mimic or inhibitor (Ambion) or with mirVana miRNA inhibitor negative control and mirVana miRNA mimic negative control negative control (NC) using 0.2 μL/well DharmaFECT™ Duo, Dharmacon™. 48 hours post transfection, cells were lysed using passive lysis buffer. Firefly and Renilla luciferase activity were measured consecutively using the Dual-Luciferase® Reporter Assay System (Promega, Cat #E1910) by using Lumat LB 9507 luminometer (Berthold Technologies) per manufacturer’s instructions. Renilla luciferase activity was normalized to that of Firefly luciferase. All experiments were performed in triplicates. The relative luciferase activity was expressed as a ratio to the negative control miRNA.

siRNA design (Paper IV)
Cellular studies with cpRNA were carried out using dT₅-modified siRNA at 3'-end of sense strand, unless otherwise stated. HPLC purified siRNA duplexes were purchased from Sigma-Aldrich, Sweden. The lyophilized duplexes were resuspended in RNase free water at 100 μM stock concentrations.

Protein analysis
Western blot analysis (Paper I-IV)
Cells were washed twice with ice-cold PBS and total cell lysates was prepared using RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 1.0% Igepal CA-630 (NP-40), 0.5% sodiumdeoxycholate, 0.1% SDS, supplemented with 1.0% protease inhibitor cocktail (SIGMA-ALDRICH®).
Lysates were incubated on ice for 30 minutes and centrifuged at 10,000-RPM for 20 minutes to collect supernatant. Protein concentration was quantified by using Coomassie Plus - The Better Bradford Assay™ Reagent (Thermo Scientific). 20 µg of soluble protein was subjected to SDS-PAGE and the separated proteins were transferred to polyvinylidene difluoride membrane (Millipore). The protein bands were probed with specific primary antibodies. Secondary antibodies were used to detect the primary antibodies, followed by the target protein visualization with EMD Millipore Immobilon™ Western Chemiluminescent HRP Substrate (ECL). Images were acquired using LI-COR Odyssey® Fc Dual-Mode Imaging system (LI-COR® Biosciences) and Image Studio Software.

Cell proliferation assay (Paper IV)
Cell proliferation was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) reagent. Cells were transfected with 50 nM or 100 nM concentrations of cpRNA or scrambled siRNAs in presence or absence of MATra-si. The untreated cells were used as control. Each transfection experiment was performed in triplicate. Post-transfection, cells were incubated for 24 hours at 37 °C. The cell viability was evaluated by MTS assay, using CellTiter 96® AQueousOne Solution Cell Proliferation Assay (Promega, USA) according to the manufacturer's protocol. The enzymatic reduction of MTS to formazan was quantified at 490 nm by ELISA plate reader (Thermo Scientific).

Interferon assay (Paper IV)
MG63 cells were transfected with 50 nM or 100 nM concentrations of cpRNA and equivalent scrambled siRNA in presence or absence of MATra-si as mentioned above. Untreated cells served as control. Each transfection experiment was performed in triplicates. After 24 h, interferon inductions were quantified by RT-PCR experiments using primers specific for interferon α, β and γ.

Confocal microscopy (Paper IV)
For confocal imaging experiments, MG63 cells were plated (1×10⁴) in an 8 well chamber slide for 24 h. At 70% confluency, the cells were transfected with 50 nM Cy3-cpRNA or Cy3-siRNA and incubated at 37 °C for 24 h. The cells were subsequently fixed with 4% PFA (paraformaldehyde) dissolved in
PBS and washed with PBS. Then the slides were mounted using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA) to stain the nuclei of cells. Images were acquired with an LSM 700 confocal microscope (Carl Zeiss, Thornwood, NY, USA) using 40X objective. Images were analyzed by Zen 2009 software (Carl Zeiss) and processed with ImageJ software (NIH, Bethesda, MD, USA).

**GFP knockdown in MG-63 cells (Paper IV)**

MG63 cells with constitutively expressing GFP protein were seeded (1×10⁴) in an 8 well chamber slide. After 24 h the cells were transfected with 50 nM of GFP-cpRNA or GFP-siRNA and untreated cells were used as control. The cells were then incubated for 48 h. To evaluate gene knockdown (GFP levels), cells were fixed with 4% PFA (paraformaldehyde) dissolve in PBS and washed with PBS. Slides were mounted using Vectashield mounting medium containing DAPI to stain the nuclei in all the samples. Images were acquired with an LSM 700 confocal microscope (Carl Zeiss, Thornwood, NY, USA) using 40X objective. Images were analyzed by Zen 2009 software (Carl Zeiss) and processed with ImageJ software (NIH, Bethesda, MD, USA).

**Data analysis**

**RT-PCR expression normalization (Paper I-III)**

Assessment of the stability of the miRNA gene expression was performed using softwares geNorm, version 3.5 [99] and Normfinder [100]. The program geNorm is a Visual Basic application tool for Microsoft Excel. The program selects from a panel of candidate reference genes the two most stable genes or a combination of multiple stable genes for normalization [101]. The NormFinder (freely available on the Internet http://www.mdl.dk) is a Microsoft Excel add-in and calculates the stability values of the individual candidate reference genes for normalization [99]. A low stability value indicating a low combined intra- and inter-group variation proves high expression stability. Using this approach, the most stable single gene is calculated.

**Downstream data analysis (Paper I)**

Downstream data analysis was carried out with R statistical computing framework version 2.11.1 (http://www.R-project.org) (The R Foundation for Statistical Computing 2010). The association between paired miRNA and mRNA profiles was tested by Pearson correlation coefficients. False Discovery Rate (FDR) and p-values <0.05 were also computed. The R software
package adjusts p-values generated in multiple hypotheses testing of gene expression data. The software applies multiple testing procedures that control the False Discovery Rate (FDR) criterion introduced by Benjamini and Hochberg [102]. For visualization of miRNA and mRNA correlations, the Statistica v10 software was used (StatSoft Inc, OK, USA).

Bioinformatic analysis of correlations – Ingenuity Pathway Analysis (Paper I, II and III)

The Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) a web-delivered application was used to explore molecular functions and relevant networks and gene targets [103], where the significant correlations were associated and/or overrepresented. Our data set containing gene identifiers and corresponding miRNA vs mRNA correlation values and p-values for correlation was uploaded into the IPA application. Each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base. A correlation p-value cut off of 0.05 was set to designate differentially regulated genes. Molecular networks were algorithmically generated, and right-tailed Fisher’s Exact Test was used to calculate an IPA p-value for association of our correlated genes related to the molecular networks, biological functions and top canonical pathways from IPA, or in the case of transcription regulators, overrepresentation. The p-value associated with a biological process or pathway annotation designates where overrepresented functions or pathways have more Functions/Pathways/Lists Eligible molecules than expected by chance.

Statistical analysis (Paper I-IV)

Correlations between miRNA fold change values and mRNA expression values were determined using the R statistical software 3.11.1 package in Bioconductor (Release 3.0). The statistical significance of differential expression measured by miRNA sequencing was assessed by R package version 3.1.1 using DESeq available in Bioconductor (Release 3.0). DESeq provides a method to identify differentially expressed (DE) miRNAs using the negative binomial distribution with variance. All $P$-values obtained were adjusted for false discovery rate due to multiple testing. The Student's $t$-test was used to determine statistical differences between pairs of groups. Two-way analysis of variance (ANOVA) was used to evaluate the statistical significance for comparisons within groups. $p < 0.05$ (two-sided) was considered as statistically significant. $p$-values obtained were adjusted for false discovery rate due to multiple testing correction. Data were analyzed using Statistica v12 software (StatSoft Inc, OK, USA) and GraphPad Prism software package (version 6.0).
Results and Discussion

Paper I

The aim of this study was to investigate miRNA–mRNA interactions that may be relevant for bone metabolism by assessing correlations and inter-individual variability in miRNA levels as well as global correlations between miRNA and mRNA levels in a large cohort of primary human osteoblasts (HOBs). The study was conducted on HOBs from 104 donors. Global miRNA expression was assessed using LNA arrays in HOBs derived from 11 females and 8 males. 251 miRNAs passed quality control (QC) criteria for the miRNA array. A subset of 24 miRNAs exhibited interindividual variation, and 9 showed differential expression (DE) between cells from males and females. Relative expression levels of 12 of these differentially expressed miRNAs as well as global mRNA levels were determined in HOBs from 95 subjects. Correlations between miRNA and global mRNA levels were analyzed and putative interactions between miRNAs and target genes were assessed. Bioinformatic pathway analyses showed that several miRNAs exhibiting DE act in important bone signaling pathways.

Functional studies by overexpression and knockdown of miRNAs show that, several of the differentially expressed miRNAs i.e. hsa-miR-29b, hsa-miR-30c2 and hsa-miR-125b target genes highly relevant to bone metabolism such as, COL1A1, SPARC, RUNX2, BGLAP and FRZB. These miRNAs play a regulatory role affecting osteoblast differentiation and extracellular matrix production.
Figure 6. **Regulation of osteoblast differentiation by miRNAs.** MiRNAs influence different stages of osteoblast differentiation from mesenchymal stem cell to osteocyte. MiRNAs target important transcription factors and genes and affect regulation during each step of differentiation. MiRNAs may have a positive or negative effect depending on the target and stage of osteoblast differentiation. Runt-related transcription factor 2 (RUNX2); Osteocalcin (BGLAP) and Frizzled-Related Protein (FRZB).

**Paper II**

In this study, we investigated the impact of treatment of PTH and DEX for 2 and 24 hours on global miRNA expression in HOBs by second generation sequencing of small RNA. Clinically, intermittent PTH administration has been demonstrated to be a bone anabolic agent and DEX a catabolic agent. Second generation sequencing was performed using SOLiD4 on barcoded library constructs of small RNA 50bp plus barcode. Sequence reads were aligned to a scaffold consisting of all known miRNA sequences, and number of sequence reads mapping uniquely to each miRNA were counted.

A total of 207 million reads from the small RNA library constructs was obtained, and normalized absolute expression was retrieved for the 379 most abundant miRNAs. The 10 miRNAs that exhibited the differential expression across the four experiments per individual were taken forward for downstream analyses. Results show a significant effect of treatment with PTH vs. treatment with DEX at 2 hours and even more pronounced at 24 hours on miRNA expression. Interestingly, several miRNAs exhibiting significant differences in expression have predicted mRNA targets involved in bone metabolism, e.g. miR-30c2, mir-203 and mir-205 targeting RUNX2. miR-320 expression in bone cells is correlated with β-catenin (CTNNB1) mRNA expression. CTNNB1 and RUNX2 expression was decreased with DEX treatment and increased with PTH treatment. Our analysis also identified 2 putative novel miRNAs in PTH and DEX treated cells at 24 hours.
Our miRNA absolute expression data from second generation sequencing show that PTH and DEX affect miRNA expression in primary human bone cells, and that these miRNAs in turn are correlated to expression levels of mRNAs known to affect bone metabolism.

Figure 7. qPCR validation of differentially expressed miRNAs by RNA sequencing. Detection of miRNA by TaqMan qPCR miRNA assay in cells treated with PTH or DEX for 2 and 24h. Relative quantitation data represent mean±SD normalized to miR-125b using ∆∆Ct method. Statistical significance is indicated as *P<0.05. Dotted lines indicate fold change >1.5 or < 1.5. Effects were generally larger at the 24 hour time point.
Figure 8. **Effect of PTH and DEX on gene expression.** Gene expression in cells treated with PTH or DEX for 2 and 24h was detected by TaqMan qPCR assays. Relative quantitation (Log2) data represent mean±SD normalized to GAPDH using ΔΔCt method. Statistical significance is indicated as *p<0.05, ** p<0.01. Dotted lines indicate fold change >1.5 or < 1.5.

**Paper III**

In Paper I, we have indicated that miRNA plays an important role in regulating osteoblast differentiation, growth and death. In Paper II, we identified miR-203 and miR-320b as important miRNAs modulating osteoblast differentiation. In this study, we identified *Dlx5* as potential common target of miR-203 and miR-320b. We confirmed this by Western blot analysis, qPCR, target prediction algorithm and by luciferase reporter assay. We examined the effect of miR-203 and miR-320b on osteoblast differentiation by modulating the miRNAs activity with pre- and anti-miR-203 and miR-320b. Knockdown of miR-203 and miR-320b increased osteoblast differentiation and stimulated alkaline phosphatase activity and matrix mineralization, whereas over-expression of miR-203 and miR-320b reversed these effects. We show that miR-203 and miR-320b negatively regulate BMP-2-induced osteoblast differentiation by suppressing *Dlx5*, a bone-inducing transcription factor, which in turn suppresses the downstream osteogenic master transcription factor Runx2 and Osx and together they suppress osteoblast differentiation. Taken together, we propose that miR-203 and miR-320b suppresses BMP-induced osteogenic differentiation by suppressing *Dlx5* and its downstream signaling. We summarize that these miRNAs are candidates for biomarkers of osteoblast differentiation and should be considered in the development of therapeutic agents for skeletal disorders.
Figure 9. Time course for the expression of Dlx5, Runx2, Osx and miR-203 and miR-320b. The time course shows an increase in the relative expression levels of DLX5 (A), RUNX2 (B) and OSX (C) at the mRNA level in BMP-2 induced cells. A concomitant decrease in the miR-203 and miR-320b expression levels is seen in the cells treated with BMP-2. Expression levels in cells untreated with BMP-2 were set to 1. All values are represented as mean ± S.D. of three independent experiments. Statistical significance is indicated as *p < 0.05 and **p < 0.001.
Figure 10. Schematic outline highlighting the complex regulation of BMP-2-induced osteoblastogenesis. BMP-2 binding to the BMP-2 Receptor (BMPR) activates the canonical BMP signaling pathway that regulates osteoblastic differentiation. Our present experiments suggest that BMP-2 downregulates the expression of miR-203 and miR-320b, that in turn suppresses the transcription factor DLX5 (Distal-less Homeobox 5). This activates both the transcription factors Runx2 and Osx to promote osteoblast differentiation. This in turn results in the upregulation of genes important for bone formation and calcification e.g. collagen type I (COL1A1), alkaline phosphatase (ALP), Osteopontin (OPN) and osteonectin (ON).
Paper IV

Harnessing RNA interference (RNAi) using short interfering RNA (siRNA) has the potential to knock down expression of any disease-causing gene, greatly expanding the scope of current interventional therapies. Delivering siRNAs into specific cells and tissues is, however, still the major hurdle that has limited its translation from a generic research tool to therapeutic molecules.

To address this, we developed a novel siRNA design- endosmolytic cell penetrating siRNA (cpRNA) comprising natural nucleotides which display ~4-fold higher activity than canonical siRNA. Such RNA design, promoted efficient cellular uptake, carrier-free transfection and successful endosomal escape with ~80% gene knockdown in cancer cells and hard-to-transfect primary human osteoblasts. This was achieved by incorporating pentadeoxy nucleotides having thymine or adenine nucleobase at the 3’-end of the sense strand, which resulted in ~80% gene knockdown at the mRNA level and near quantitative knockdown at the protein level. Unlike viral RNA, cellular uptake of cpRNA does not trigger immune activation. Our discovery of cpRNA could be critical in the field of nucleic acid therapeutics, with the possibility of bringing gene-silencing technology from bench to bedside. This significant breakthrough has the potential to develop RNAi based platform technologies for the treatment of a diversity of global diseases.

Figure 11. Confocal microscopy images of MG63 cells transfected with (A) Cy-3 labeled siRNA and (B) Cy-3 labeled siRNA with dT₅ the overhang. Red: Cy3-labeled siRNA; blue: DAPI-stained nuclei.
Figure 12. Gene silencing of cpRNA. Carrier-free transfection experiments using siRNA dT₂, and cpRNAs (dT₅ and dA₅) in MG63, HOB, HCT116 cells, Fibroblasts and Keratinocytes. The percentage of GAPDH knockdown was determined using RT-PCR experiments (***P<0.0001).
Most Important findings

**Paper I** - We identified hsa-miR-29b, hsa-miR-30c2 and hsa-miR-125b and their target genes as important modulators of bone metabolism. These miRNAs orchestrate the activities of key regulators of osteoblast differentiation and extracellular matrix proteins by their convergent action on target genes (*collagen, osteonectin, Runx2, osteocalcin* and *FRZB*) and pathways to control the skeletal gene expression.

**Paper II** - Our study provides new insights into the action of miRNAs and consequently gene regulation following PTH and DEX induced perturbation of osteoblast differentiation. We observed substantial differences in the expression of miRNAs between PTH and DEX treated human bone cells, suggesting a potential role of a subset miRNAs in the regulation of bone remodeling and osteoblast differentiation by PTH and DEX.

**Paper III** - We found a novel function of miR-203 and miR-320b in negatively regulating BMP-2-induced osteoblast differentiation by suppressing *Dlx5*, which in turn suppresses the downstream osteogenic master transcription factor *Runx2* and *Osx*. Taken together, we propose that miR-203 and miR-320b may be promising therapeutic targets for enhancing bone formation and treatment of osteoporosis.

**Paper IV** - We have resolved one of the major bottlenecks of RNAi technology by developing a novel siRNA design that is capable of performing transfection without any transfection agent with ~3–4 fold higher efficiency than canonical siRNA.
Conclusions

Our studies demonstrate the importance of miRNA operative in the skeletal lineage cells. miRNAs play a distinct and important role in coordinating a wide variety of processes to control skeletal gene expression. The expression profiles and dynamic regulation of the miRNAs need to be studied to enhance our understanding of skeletal gene expression and bone formation. miRNAs play a vital role in maintaining the homeostatic equilibrium essential for bone remodelling. miRNAs orchestrate the activities of key regulators of osteoblast differentiation and extracellular matrix proteins by the convergent action of various miRNAs on target genes and pathways.

Different phases of bone formation are influenced by miRNAs, where cell differentiation, secretion and mineralization of extracellular matrix proteins are regulated by different subset of miRNAs. However some miRNAs can conciliate both activities, increasing the complexity of miRNA regulatory networks. These miRNAs can inhibit positive and negative transcriptional regulators, which in turn can affect their downstream signalling cascades thereby having an effect on the phenotype.

In this thesis, we used an integrated analysis of global mRNA – miRNA correlations, bioinformatics analysis and functional studies to identify novel target genes for miRNAs with the potential to regulate osteoblast differentiation. Our study is unique in demonstrating that expression of miRNAs exhibits interindividual variation in primary bone cells from a large cohort and also in showing differential expression of miRNAs between men and women. Among different miRNAs evaluated, we identified hsa-miR-29b, hsa-miR-30c2 and hsa-miR-125b as key regulators of human osteoblast differentiation and extracellular matrix proteins by their convergent action on target genes and pathways to control skeletal gene expression for e.g. COL1A, SPARC, RUNX2, BGLAP and FRZB.

Similar to PTH, BMP-2 is also known to have a positive effect on osteogenic differentiation. We found that miRNA expression is affected by glucocorticoids. We have investigated the impact of treatment of PTH and DEX for 2 and 24 hours on global miRNA expression in HOBs by second generation sequencing of small RNA. Clinically, PTH has been demonstrated an anabolic agent and DEX a catabolic agent during osteogenesis. Our studies show that DEX suppresses Runx2 and β-catenin expression by upregulating miR-320 expression, inhibiting the Wnt pathway by enhancing expression of Wnt antagonist sFRP-1, suggesting one mechanism by which
glucocorticoids suppress osteoblastic differentiation. We also focused on miRNAs that are modulated by BMP-2, a highly efficient inducer of osteogenesis. We identified that miR-203 and miR-320b target Dlx5, a bone-inducing transcription factor. Our results indicate that these miRNAs functions as a negative regulator of BMP-2-induced osteoblast differentiation by suppressing Dlx5 and the downstream osteogenic transcription factors.

The emerging information on miRNA deregulation in osteoporosis could be harnessed for its treatment. miRNA and siRNA holds great promise as therapeutic agents and one of the bottlenecks of RNAi therapeutics is their appropriate cellular delivery. We have resolved this challenge of siRNA delivery, which could pave the way to transforming this technology from a generic research tool to a potent therapeutic intervention. To the best of our knowledge, cpRNA represents the first example of transfection agent–free cellular delivery of active siRNA using unmodified nucleotides. We are currently evaluating the potential of our technology for microRNA therapeutics.
Future perspectives

Osteoporosis and other bone related diseases pose a significant burden on both the individual and society, and the frequency of osteoporotic fractures is rising. There is a dire need to develop better, more efficient and cost effective treatment for these bone related diseases. Studies show that freely circulating miRNAs in blood potentially could be used as biomarkers for diagnosis of osteoporosis and fracture risk prediction [104]. This indicates that development of clinical miRNA biomarkers could be of importance for these bone related diseases.

Our studies identify miRNAs as being important for osteoblast differentiation and their up regulation or down regulation may have an effect in the development of osteoporosis. Thus these miRNAs could potentially be used as biomarkers for bone metabolism-related disorders. Apart from biomarkers, miRNA can also be used for therapy, but several barriers need to be overcome for them to be used clinically. Current challenges for implementing miRNA therapies include the lack of safe delivery methods with high degree of specificity. Uptake and expression of a miRNA in a vast spectrum of cells with a broad range of genes it regulates demands a delivery system with high specificity and safety. We tried to address this by developing a new siRNA design that demonstrates a novel carrier-free cellular delivery and successful endosomal escape. This could be critical in the field of nucleic acid therapeutics, with the possibility of bringing gene-silencing technology from bench to bedside. This significant breakthrough has the potential to develop RNAi based technologies in the future for the development of therapeutic agents for bone disorders and for accelerating fracture healing.
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My apologies if I missed anyone.

![Signature]

_Navya Laxman_
References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)