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Non-coding RNAs: Epigenetic regulators of bone development and homeostasis

Mohammad Q. Hassan[a,*], **Coralee E. Tye**[b], **Gary S. Stein**[b], and **Jane B. Lian**[b]

[a]Department of Oral & Maxillofacial Surgery, School of Dentistry, The University of Alabama at Birmingham, Birmingham, AL, USA

[b]Department of Biochemistry and University of Vermont Cancer Center, University of Vermont College of Medicine, Burlington, VT, USA

Abstract

Non-coding RNAs (ncRNAs) have evolved in eukaryotes as epigenetic regulators of gene expression. The most abundant regulatory ncRNAs are the 20–24 nt small microRNAs (miRNAs) and long non-coding RNAs (lncRNAs, <200 nt). Each class of ncRNAs operates through distinct mechanisms, but their pathways to regulating gene expression are interrelated in ways that are just being recognized. While the importance of lncRNAs in epigenetic control of transcription, developmental processes and human traits is emerging, the identity of lncRNAs in skeletal biology is scarcely known. However, since the first profiling studies of miRNA at stages during osteoblast and osteoclast differentiation, over 1100 publications related to bone biology and pathologies can be found, as well as many recent comprehensive reviews summarizing miRNA in skeletal cells. Delineating the activities and targets of specific miRNAs regulating differentiation of osteogenic and resorptive bone cells, coupled with in vivo gain-and loss-of-function studies, discovered unique mechanisms that support bone development and bone homeostasis in adults. We present here “guiding principles” for addressing biological control of bone tissue formation by ncRNAs. This review emphasizes recent advances in understanding regulation of the process of miRNA biogenesis that impact on osteogenic lineage commitment, transcription factors and signaling pathways. Also discussed are the approaches to be pursued for an understanding of the role of lncRNAs in bone and the challenges in addressing their multiple and complex functions. Based on new knowledge of epigenetic control of gene expression to be gained for ncRNA regulation of the skeleton, new directions for translating the miRNAs and lncRNAs into therapeutic targets for skeletal disorders are possible.

Keywords

MicroRNA; miRNA biogenesis; LncRNAs; Osteoblasts

[*]Corresponding author at: School of Dentistry, The University of Alabama at Birmingham, Department of Oral & Maxillofacial Surgery, 1720 2nd Avenue South, Birmingham, AL 35294-0007, USA.

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1. Introduction

Normal bone functions as an organ through balanced activities among different cell types, and also communicates with many other organs and biological networks that impact on bone turnover and maintaining tissue homeostasis [1]. Complex regulatory controls and transcriptional activities are required to support gene expression in response to hormone-, growth factor- and cytokine-mediated signaling cascades in bone forming and resorbing lineage cells. Only 1–2% of the human genome is transcribed into protein with a vast majority represented by non-coding RNAs (ncRNAs), implicating their significant role in contributing to regulation of gene expression [2]. This decade has witnessed an explosion of information for understanding epigenetic control of gene expression by the different classes of ncRNAs. Pursuit of epigenetic mechanisms operative in bone tissue is equally important as understanding gene-centric regulation of the skeleton.

Among the well-known classes of regulatory ncRNAs with epigenetic functions are the long non-coding RNAs ranging in size from >200 nt to 100 kb and different types of small RNAs which include small interfering siRNAs (18–30 nt), piwiRNAs (24–30) nt and the 20–24 nt microRNAs (miRNAs). The number of miRNAs exceeds over 2500 in humans and nearly 2000 are found in mouse databases (http://www.mirbase.org). The number of long non-coding RNAs is in the range of 16,000–32,000. Large databases have been compiled for miRNAs and their targets, and both long interfering non-coding RNAs (lincRNA) and long non-coding (lncRNA) are also annotated in databases. However, all the ncRNA databases are based on sequence data and structure and function, but a fair percentage of miRNA and most lncRNAs have not been validated or studied for their activity and many lncRNAs are not even annotated.

Detailed in Section 2 are the biogenesis, maturation and function of miRNAs which are regulated at multiple levels with stringent sequential control during processing from precursor to metastasis [3,4]. The mechanism by which miRNAs induce messenger RNA silencing is by functioning as a reader of the bases of its 5′ ‘miRNA seed’ sequences that are complementary with each mRNA-binding site. These sites are typically present in the target mRNA 3′ untranslated region. Dysregulation of miRNA biogenesis is associated with human diseases including developmental disorders and cancer [5–7].

LncRNAs, which are not translated into proteins, have a broad expanse of biological functions regulating chromatin states to support activation or repression of transcription. They affect gene expression by translational and transcriptional mechanisms that are distinct from the classical binding of transcription factors to their cognate DNA regulatory elements [8]. LncRNAs present technical challenges in characterizing their complex functional roles in the skeleton. However, newer technologies make such studies feasible and are discussed in Section 4.

Prompted by the recently identified regulation of miRNA processing enzymes and the interplay between miRNAs with mRNAs and lncRNAs for regulation of the genome, this review presents: a) recent advances in the biosynthesis, processing, function and stability of miRNAs; b) the emerging concepts of miRNA regulation of the skeleton which are based on

the abundance of well characterized miRNAs that have conveyed an increased understanding of epigenetic regulation of the bone genome; and c) new directions for discovery of lncRNA activities in regulating bone tissue formation.

2. Biogenesis and regulation of miRNAs: complexity for diversity

2.1. Background

MicroRNAs (miRNAs/miRs) represent a leading class of small ncRNAs, and are implicated in diverse biologic and pathologic processes. Before beginning the journey of biogenesis, a word about the well-organized naming of miRNAs is relevant. While the first mammalian miRNAs were named by the gene where they were located, a numerical system was adopted as miRNA sequences were identified. However, to date there remain inconsistencies and variability in miRNA nomenclature. Those miRNAs that have nearly identical "seed" sequences (8 nt, with nt positions 2 to 7 being 99% conserved) of the 20–24 nt mature miRNA, are considered a miRNA family and are, for example, the miR-29a,b,c, let-7a–h, and miR-30a–e families. Based on binding specificity of the mature miRNA, the two isoforms can exhibit similar or diverse functions. miRNA isoforms of miR-23 (23a and 23b) can target the same miRNA as SATB2, in addition to other different targets [9]. The miR-24-1 is an oncomiR involved in the regulation of MEN1 [10] and miR-24-2 is part of a cluster regulating osteoblast differentiation [9]. Sometimes a mature miRNA is transcribed from multiple genomic loci, in which case, a numeric suffix is added after the letter or at the end of the miRNA name (for example, miR-125b-1 and miR-125b-2). In addition, each precursor miRNA generates two mature miRNAs: one from the 5′ region of the stem and one from the 3′ region of the stem (termed as miR-27a-5p and miR-27a-3p). Because each locus produces two mature strands, the strand with functional activity and abundancy is termed "guide" strand, while the other is a "passenger" strand which is designated as miRNA*. The mature miRNAs are targeted to 3′ UTR of mRNAs matching a seed sequence. This small sequence can occur in just a few messenger RNAs or several hundred transcribed messages. Nearly two-thirds of 21,000 human protein-coding genes have high, moderate and poor miRNA-binding affinity [11], but across species, most mammalian mRNAs are conserved targets of microRNAs. Together these properties of miRNA control of gene expression provide a refined layer of regulation that refrain a broad spectrum of biological processes. However, there is recognition that while the identification of a miRNA with characterization of a single target may have a significant biological effect, but the miR should be examined in a broader context of its effects on other targets and a biological pathway in the cell context being studied.

To note, the siRNAs are related to miRNAs, but their biosynthesis and mechanism of action differ from miRNAs. The siRNAs are processed from double stranded or stem loop structures, only by Dicer in the cytoplasm. Both miRNAs and siRNAs are loaded into the *R*NA-*I*nduced *S*ilencing *C*omplex (RISC) to silence target miRNAs. However, siRNAs are 100% complementary to mRNA *coding* sequences, and therefore the siRNAs target only one mRNA. This results in direct degradation of messenger RNA, with a readout of decreased gene expression, while miRNAs can either control translation repression of protein or mRNA degradation.

MicroRNAs are derived from genomic sequences where the majority of miRNAs are located in the intronic sequences and are defined by their stem loop structure that is dependent on the underlying chromatin structure of the RNA [3,4]. For those miRNAs that do not rely on expression by a host gene, their own promoters have been characterized and found to be regulated by numerous transcription factors. A recent review [12] has listed the miRNAs that are co-regulated with transcription factors that represent specific cell phenotype, developmental processes and growth control; for example, p53, MYC, ZEB 1 and 2 and MYO D1 [13,14]. RUNX2 and RUNX1, essential transcription factors for bone development and hematopoiesis respectively, directly bind and control transcription of the miR-23a–27a–24-2 cluster [9,15]. Runx1 mutations causing various leukemias contribute to deregulation of the cluster [15]. The targets of this cluster regulating osteogenesis is are discussed in Section 3. These studies are a clear demonstration of tissue specific transcription factors regulating miRNAs that support differentiation programs.

2.2. miRNA processing: the essential enzymes

Present knowledge of miRNA biogenesis, maturation and function has revealed that many components of miRNA processing are tightly regulating cellular levels of miRNAs. Studies have indicated the importance of each of the many enzymes and factors involved in miRNA biogenesis, processing, maturation, and silencing miRNAs (Fig. 1). We describe the enzymes and their co-regulator roles in miRNA biogenesis for potential understanding of complex skeletal disorders that could be associated with deregulated processing. Two endoribonucleases of the RNaseIII family process microRNAs: Drosha in the nucleus and Dicer in the cytoplasm are considered the essential regulators of miRNA biogenesis [3,6,16,17]. Drosha and DGCR8 (DiGeorge syndrome critical region gene 8) in the nucleus remove the primary miRNA and process it to a pre-miR, while Dicer and co-regulator Ago2 (an Argonaute protein) in the cytoplasm continue processing the mature miRNA duplex. These enzymes are crucial for life [17–21]. Drosha deletion causes early embryonic lethality by E7.5 in mice. Likewise, knockout of *Dgcr8*, a subunit of the Drosha complex, results in arrest early in embryonic development [17–19]. *Dicer* knockout also results in early embryonic lethality [20] and mice null for Ago2 are embryonic lethal and show numerous developmental defects by E 9.5 [21,22].

Conditional deletion of the enzymes in specific cell types has identified their crucial importance in organ development. Related to the skeleton, conditional deletion of either Dicer, Dgcr8, or Drosha in cells comprising bone and cartilage tissues, demonstrated the requirement of miRNAs for the differentiation of osteoblasts, osteoclasts and chondrocytes. Osteoclast differentiation is clearly dependent on both Drosha and Dicer with both mouse models having defective bone resorption [23–25]. Dicer excision in chondrocytes (by using Col2a-Cre) resulted in viable mice, but with compromised endochondral bone formation and early post-natal death [26]. However, with an inducible Cre driver expressed in articular chondrocytes (by PRG4-Cre) mice had normal life span, but developed an osteoarthritis phenotype, and interestingly in males only [27]. The significance of this finding is further supported by the observation that lubricin (PRG4), which is regarded as chondroprotective, is highly responsive to mechanical compression [28]. Striking phenotypes were also found by Dicer deletion in mesenchymal progenitors and implicate a subset of miRNAs for

induction of bone formation and fetal survival, while excision in mature osteoblasts (by osteocalcin-Cre) increased bone mass [29]. In another study, deletion of Dicer in mesenchymal osteoprogenitors was found to compromise hematopoiesis to the point of inducing a leukemia phenotype, underscoring crucial communication between mesenchymal and hematopoietic cells by miRNAs [30]. The depletion of miRNAs in either cell severely altered the phenotypes of both cells. The conditional deletion of Dicer in mesenchyme by Wnt1-Cre or epithelium by shh-Cre revealed very different defects in tooth development by loss of Dicer. Extra incisors formed due to compromised epithelium, but no defects in molars were observed; however deficient miRNA processing in mesenchymal tissue resulted in an arrest in tooth development [31]. Together these in vivo phenotypes resulting from deficiencies in mature miRNAs show a spectrum of activities of the principle miRNA processing enzymes; and, that the consequences of miRNA deficiencies in different bone cell populations have revealed the importance of miRNAs essential for normal bone homeostasis throughout life. The many findings implicate subsets of miRNAs for fetal survival, induction of bone formation and regulation of bone turnover, and the importance of epigenetic control of bone mass by miRNAs. In future studies, perhaps miRNAs may be discovered that represent a heritable epigenetic signature that determines bone size and quality.

The mature miRNA sequences that are embedded in the stem region of the stem-loop structure of the primary-miRNA (pri-miR) are the target of Drosha. Following transcription of the miRNA, Drosha and cofactor DGCR8 (DiGeorge syndrome critical region 8) with RNA helicase p68 form the “microprocessor complex”. This critical regulator of miRNA biogenesis crops the pri-miR to yield an 80–100 nucleotide precursor miRNA (pre-miRNA). Receptor activated SMAD proteins (R-SMADs) and the tumor suppressor gene p53 associate with the complex to regulate the processing [14,32] as shown in Fig. 1A. Ribonucleoproteins including hnRNPA1 [33], the splicing regulatory factor KSRP [34] and Lin-28 Homolog (LIN28) bind selectively to the terminal loop of the pri-miRNA. The miR-18a or let-7 regulates stability of Drosha-mediated processing [35]. AGO2 and TRBP phosphorylation results in the dissociation of these processing co-factors from Dicer to attenuate pre-miRNA processing (Fig. 1B). Furthermore, post-translational modifications (e.g., phosphorylations and acetylation of Drosha and DGCR8 protein) can modulate the processing activity of the complex. Immediately after Drosha processing, the nuclear cargo exportin 5 (EXP5) forms a “delivery” complex with GTP-binding protein RAN-GTP to export pre-miRNA into the cytoplasm for further maturation of the pre-miRNA by the Dicer enzyme [36].

The formation of a Dicer enzyme complex is required for generating the functional miRNA (Fig. 1C). The mammalian TAR RNA-binding protein (TRBP) first associates with Dicer to mediate pre-miRNA processing by [37]. Analogous to regulation of Drosha, KSRP and LIN28 in the cytoplasm bind with pre-miRNA and to yield mature miRNAs [34,38,39]. The Dicer generated 22–24 nt duplex RNA from the pre-miRNA is then loaded on the Ago2 “leading complex” to assemble a pre-*R*NA *I*nduced *S*ilencing *C*omplex (pre-RISC). Heat shock proteins, HSC70 and HSP90 facilitate a conformational change of AGO proteins to remove the passenger strand which results in an active RISC complex with the guide strand (as shown in Fig. 1C) [40]. The miRNA in the RISC complex recognizes mRNA targets by

base pairing typically with the 3′ UTR of an mRNA and mediates repression of translation followed by degradation of mRNA (Fig. 1D) [41].

Argonaute-mediated protein repression is regulated at many levels (Fig. 1C), by hydroxylation, phosphorylation and ADP-ribosylation of Argonaute proteins which modulates miRNA binding and stability to repress their targets [42–44]. Additionally ubiquination (proteasome-mediated degradation) or autophagy of AGO proteins has also been suggested to contribute to stability of the RISC complex engaged in protein repression [45,46]. At the final stage of miRNA biogenesis is consideration of the degradation of miRNAs. The 3′ (Fig. 1E) ends of miRNAs are subject to modifications that include adenylation and methylation which stabilizes miRNAs at the 3′ end (Fig. 1E) [47,48]. In contrast, uridylation of 3′ end makes the miRNA highly susceptible to trimming and tailing for degradation of the miRNA and also the RISC complex [4] (illustrated in Fig. 1E).

We have focused on canonical miRNA biogenesis, however non-canonical pathways to miRNAs can occur where Drosha and Dicer function independent of each other, to regulate messenger RNAs [49]. Mouse models where different phenotypes were observed by separate deletion of the two enzymes provided evidence of non-canonical pathways for biogenesis and different functions of the enzymes. An example is the finding that Drosha, and not Dicer, regulated the proliferation of hMSCs through a miRNA independent mechanism, potentially by regulating ribosomal RNA processing [50]. Also, Argo proteins are mainly in the cytoplasm (canonical pathway) but are also found in the nucleus where they may have transcriptional functions independent of miRNA processing [51].

To summarize, the complexity of miRNA biogenesis is required for stringent control of miRNA cellular levels that is still not completely understood. The multiple enzymes are themselves the target of miRNA regulation and other epigenetic modulators, as well as transcriptional control. There is no doubt that the process of miRNA biogenesis may be far more regulated by mechanisms yet to be uncovered in specific cell types. For example, control of Dicer/Ago processing of miRNAs is known to be regulated by physiologic hypoxic conditions [52,53] and cellular stresses [54]. There are likely other physiological cues from hypoxia [55] or hormones e.g., glucocorticoids, vitamin D, and estrogen [56–58], that can contribute to regulating processing (in addition to BMP TGFβ SMADs and p53 indicated earlier). The findings of repeated sequences in Drosha and Dicer associated with a fragile X syndrome [59], SNPs located in Drosha in breast cancer [56], and the recent discovery of SNPs in Drosha and Dgcr8 discovered in Wilms tumor, highlight the consequences of compromising activity of the miRNA processing enzymes in human diseases [60,61].

3. MicroRNAs in bone: orchestrating cellular activities

Osteoblasts originate from the pluripotent mesenchymal stem cell (MSCs) and osteoclasts are derived from hematopoietic lineage cells with miRNAs regulating their phenotype differentiation. Many excellent reviews have been published in just the past few years which summarized miRNAs identified in different bone cell populations that are either up or downregulated during their differentiation and we cite the most recent reviews as of this

writing [62–65]. In this section, we will refer to only a few miRNAs, as examples to illustrate the different modes of miRNA control over bone activities. Transcriptional control in response to developmental, hormonal and growth factor signals is the key elements for induction of a cell phenotype for tissue formation; and, partnering these mechanisms are the changes in miRNA expression in response to the biological signals.

3.1. miRNAs: stemness and lineage allocation

It is now clear that from the hundreds of studies of miRNA in cell populations resident in bone that the miRNAs have key roles in maintaining pluripotency of stem cells for tissue renewal, as well as regulating induction of a specific cell phenotype and providing phenotype stability to the tissue histocyte. The “guiding principle” of commitment to a phenotype regulated by miRNAs is clearly demonstrated by progression of osteoblast differentiation originating in the MSCs. A key feature of studies in rodent or human bone marrow-derived stem cells (BMSCs) or fat tissue-derived MSCs is that miRNAs are downregulated by osteogenic media or when induced into osteogenesis by BMP2. Nearly all miRNA profiling studies show similar miRNAs in undifferentiated MSCs that preserve stemness. These miRNAs support options for lineage allocation by inhibiting cellular protein levels of essential tissue-specific transcriptional regulators (e.g., Runx2, Sox 9, PPARg, C/EBPa, MyoD) until they receive a stimulus by developmental, hormonal factors or other physiological signals required for phenotype differentiation. It has been demonstrated in several osteoblast cell models that BMP2 commits a cell to osteogenesis by down-regulating miRNAs that target activators of bone formation, among which are, Smad co-receptors [66–68], Wnt receptors [69], Runx2 [67], ATF 4 [70,71], Osterix/Sp7 [72,73] and other bone related transcription factors [74]. Thus BMP2, by decreasing cellular levels of miRNAs that target required factors for bone formation, releases these osteogenic regulators from suppression.

Lineage allocation of MSCs is only partly understood. A fundamental mechanism of commitment to a specific cell phenotype is the requirement of an essential transcription factors (TFs) for phenotype development, e.g., C/EBPa and PPARg for adipogenesis and Runx2 and Osx/Sp7 for osteogenesis. While “master” transcriptional regulators are the essential players of lineage commitment, miRNAs support lineage direction by one miRNA having opposing effects in the same cell by promoting one phenotype, and inhibiting another. This control of the “bone” genome is best illustrated by Runx2, which to date, is known to be down-regulated by at least 19 miRNAs that target the 3′ UTR. Many of these miRNAs are expressed in non-osseous mesenchymal lineage cells to assure their phenotype stability. For example, miR-133 promotes myogenesis, but strongly inhibits Runx2 when myogenesis is induced. Likewise, miR-30 which downregulates Runx2 [67,75,76] and recently reported to promote myogenesis [77]. Another study found that miR-30e in BMSCs, by targeting/inhibiting IGF2 [78] will block osteogenesis and increase adipogenesis in MSC or promote differentiation of smooth muscle cells. The in vivo consequence of this dual action was shown in Apo−/− mice that exhibited cardiovascular calcification along with increase Runx2, OPN and IGF2 and the phenotype could be rescued by expressing miR-30e which suppresses the Smad1–Runx2 axis [79]. miR-30e also targets LRP6, a crucial co-receptor for Wnt signaling required for osteogenesis [69]. MicroRNAs are now considered

key regulators of the adipogenic–osteogenic phenotype switch, which has recently been reviewed [80,81]. MicroRNAs reported to drive adipogenesis, but inhibit osteogenesis include miR-204/21 by targeting Runx2 [82] and miR-320 [83], while miRNAs promoting osteogenesis will block fat cell differentiation, for example, miR-27a [84,85] and 27-b [86] by inhibiting PPARγ and let7 [87] and miR122 by targeting HDAC6, an inhibitor of Runx2 [88]. The many studies documenting phenotypic switches in MSCs reinforce the concept that miRNAs represent a level of epigenetic regulation to assure that transcriptional drivers of differentiation will not be competing against other master transcription factors. Such miRNAs are clinically relevant for therapeutic intervention in musculoskeletal and metabolic bone disorders to correct imbalances in cell populations.

3.2. MiRNAs: signaling pathways and networks

A basic tenet of miRNA biology is the complexity that occurs because: a) one miRNA can target many mRNAs with distinct effects on individual genes in a single cell; and b) one gene can be targeted by many different miRNAs, as described above for transcriptional control of lineages. In bone tissue, the biological advantage of these properties is also highlighted by the ability of miRNAs to: 1) dynamically control progression of osteoblast differentiation through stages of maturation; and 2) orchestrate the activities of osteogenic signaling pathways through feed forward and feedback circuits. This is illustrated by miR-218 repression of several inhibitors of the Wnt signaling. The miR-218 functions as a continuous driver of differentiation through a positive feedback loop (Fig. 2A). However, this feature is deregulated in metastatic breast cancer cells and miR-218 becomes a pathologic driver of metastatic bone disease by sustaining Wnt activity. The tight control over Wnt activities by miRNAs is evidenced by multiple miRNAs expressed in osteoblasts that target inhibitors of Wnt signaling to support osteoblastogenesis and mineralization; for example, miR-27 decreases sFRP1 [89], miR-346 inhibits GSK3B in MSCs [90], and miR-335-5p blocks Dkk1 [91].

The multitasking of a single miRNA to target different genes during osteoblast differentiation is illustrated by several studies examining the miR-29 family, another pro-differentiation miRNA. miR-29b inhibits SFRP1 and collagen only in mature osteoblasts [92,93], miR-29a inhibits DKK1 but also osteonectin [94,95], and is reported as an enhancer of mineral deposition [92] and to protect against glucocorticoid-induced bone loss [96]. Of interest all three miR-29 family members were identified in osteoclasts. By inhibiting the miRNA, osteoclast differentiation was decreased [97]. Thus, miR-29 that is promoting osteoclast differentiation may be having a greater role in bone tissue “fine tuning” bone turnover. Cluster miRNAs which are regulated through their own promoter are also important for coordinating intracellular signaling pathways and can be appreciated for their diverse effects in bone cells. The cluster miR-17 and -20a-support periosteal bone formation and can inhibit apoptosis of osteoblasts [66,98–100]. The regulated expression of three miRNA clusters by Runx2 is described in Fig. 2. The miRNA-218 is upregulated during osteoblast differentiation, reaching peak levels at the mineralization stage [93]. Although miR-218 targets and decreases Runx2 in a reporter assay, it does not inhibit osteoblast differentiation [75]. Rather it drives differentiation to the mineralization stage and osteocyte formation by activating Wnt signaling through downregulation of multiple inhibitors of the

Wnt pathway during stages of osteoblast differentiation. However, it is also highly expressed in breast cancer metastatic cells compared to non-metastatic cells where increased Wnt signaling promotes the expression of genes related to homing and adhesion of tumor cells to bone (osteomimicry) [101] (Fig. 2A). The miR-23a–27b-24-1 cluster forms a regulatory network that inhibits three essential transcriptional regulators which promote bone formation, SatB2, Runx2 and Hoxa10 (Fig. 2B). Thus negative regulation of transcription of this cluster by Runx2 relieves repression of osteogenesis due to these miRNAs. However, each miRNA in the cluster feeds back to target Hoxa10 and Runx2, thus maintaining a physiological balance of transcriptional control when cells reach the final stage of mineralization. In an opposite fashion, Runx2 activates two miRNAs, miR-3960 and miR-2861, each targeting an inhibitor of Runx2, although it is not clear if these miRNAs are clustered (Fig. 2C). Here repression of the targets results in increased Runx2 cellular protein levels. The miR-2681 was first associated with primary osteoporosis in two related adolescents due to a homozygous mutation in pre-miR-2861 that blocked its expression and resulted in high levels of HDAC5, a potent inhibitor of Runx2 activity [102]. The mechanism along with miR-3960 which targets Hoxa2, also a negative regulator of Runx2, was later characterized [103].

Together studies characterizing multiple functions of a single miRNAs in skeletal cells demonstrate mechanisms by which they: a) orchestrate levels of different components of signaling pathways for progression of differentiation; b) are involved in intricate feed-forward and feed-back signaling; and c) selectively use miRNA isoforms at different stages to promote to attenuate or inhibit differentiation and regulate extracellular matrix proteins essential for the regulation of mineral density and bone mass. Both the diversity and complexity of miRNAs that can target multiple components of a signaling pathway are necessary to achieve specificity in miRNA actions. This is understandable for the regulation of factors with broad expression in many cell types. For consideration of miRNAs as a druggable target, a specific miRNA may be enriched in a cell population with functions related to the cell phenotype.

4. Long non-coding RNAs: uncovering their secrets of epigenetic control

Long non-coding RNAs (lncRNAs) are a family of transcripts with greater than 200 nucleotides that do not encode proteins. Initially thought to be transcriptional noise (referred to as “the dark matter” of the genome), lncRNAs have emerged in the last few years as novel regulators of nuclear architecture and gene expression during development. They are involved in numerous cellular processes including X-chromosome inactivation, imprinting, and the regulation of cell cycle, differentiation, transcription and translation [8,104–109]. LncRNAs appear to be involved in numerous diseases and are being investigated as targets of novel therapies [110–115].

More than 16,000 lncRNAs have been identified in the human genome, with more transcripts being identified with each updated GENCODE annotation [116]. Expression of lncRNAs is largely tissue, cell and developmental stage-specific, although many are ubiquitously and constitutively expressed. LncRNAs are regulated by transcription factors and they display chromatin signatures ($H3K4me^3$ and $H3K36me^3$) similar to mRNA

[117,118]. Expression of lncRNAs is generally lower than mRNA, and while many are nuclear, others are cytoplasmic. LncRNAs tend to have a low degree of homology, however they demonstrate higher conservation in their promoters and exons, and genomic positioning is often maintained across species, suggesting that lncRNAs have conserved function [119,120].

4.1. Identification and characterization of lncRNAs: technology meets the challenge

With the advances seen in microarray technology and next-generation sequencing over the last decade, a large number of groups are now are identifying and cataloging hundreds or thousands of lncRNAs expressed during the differentiation of various cell types [105,121] and they are comparing the expression of lncRNAs between normal and diseased tissues [115,122–124]. Generally, a select one or two molecules are then investigated for possible function and mechanism using in vitro cell models. With tens of thousands of lncRNAs to understand, this is a daunting process. However, it is one of great importance, as it has been demonstrated that lncRNAs are required for life and normal development. Sauvageau et al. investigated the knockout of 18 lncRNAs and found that deletion of three lncRNAs resulted in peri- and post-natal lethal phenotypes and two others had growth defects [125]. Examining for a phenotype in more depth (e.g., at the organ level by histology, of the remaining 13 knockout animals) may reveal roles for those lncRNAs in processes required for normal development.

Only a proportionally small number of lncRNAs have been fully characterized. Unlike protein-coding genes, very little can be ascertained from lncRNA sequence and to date there are no defined motifs to identify potential function. Therefore, while interest and investigation into lncRNAs have developed tremendously, there is a long way to go into understanding the roles of lncRNAs in normal development and disease. LncRNAs function through a diverse number of mechanisms to regulate gene expression. They can interact with both DNA and/or RNA via base-pairing and bind proteins via structural motifs. One of the first ways lncRNA function was inferred was through “guilt-by-association” [117]. Using an informatics approach, lncRNAs and protein-coding genes that are tightly expressed are presumed to be co-regulated. Using this approach, associated pathways were correctly predicted for several lncRNAs. This guilt-by-association method has successfully been utilized in several other studies as well [125–127]. Of course, careful in vitro and in vivo functional analyses are required to verify these predictions.

Generally, lncRNA function is initially investigated in vitro in cell culture. LncRNA expression may be altered by standard siRNA or shRNA techniques, however, when using these approaches, one must consider the subcellular location of the expressed lncRNA. siRNA may work well with cytoplasmic lncRNAs, but nuclear lncRNAs will not be easily targeted by standard siRNA methodology. More recently, researchers have begun using used antisense oligonucleotides (ASOs) to target nuclear lncRNAs for RnaseH digestion. This is an exciting strategy as ASOs could be used in vivo in mouse studies and may be translatable into a viable therapeutic strategy to target lncRNAs in disease [128]. With the discovery of CRISPR (clustered regularly interspaced short palindromic repeats) methodology, investigators are now deleting entire lncRNA sequences from the genome [129]. When

performing these large genomic deletions, one must keep in mind that key regulatory regions may also be deleted and importantly, this would not be a suitable technique to study lncRNAs that overlap other genes. Therefore when planning to perform a loss-of-function experiment to examine lncRNA function, one must consider not only its genomic location (i.e., overlapping, antisense, intergenic) but also its subcellular location. RNA fluorescent in situ hybridization (FISH) is an ideal starting place in determining whether a lncRNA is nuclear and/or cytoplasmic [130,131].

In vivo analysis is the ultimate goal in determining lncRNA function. This generally entails loss-of-function studies in mouse models. Unfortunately, targeting strategies often adopted with protein-coding genes such as insertion of stop codons, exon replacement or insertion, truncation and mutation are not applicable to lncRNAs [132]. LncRNAs are not translated, and we are generally unaware of their functional domains, therefore the entire transcript needs to be prevented from being transcribed. Conventional and conditional knockout methods have been used with lncRNAs in mice, as have lacZ reporter knock-ins to replace lncRNA genes. An alternative strategy involves deletion of the lncRNA promoter, provided that this promoter is not in close proximity to another transcribed gene. Integration of a premature polyadenylation cassette into the first exon of the lncRNA is another possibility. More details on these methods and considerations, as well as information regarding lncRNA knockout mice generated to date can be found in [132].

With the ability to generate CRISPR knockout mice in a fraction of the time of standard knockout technologies (as short as one month), and the ability to target greater than five genes simultaneously [133], researchers are now using this technique to study lncRNAs in vivo [134]. The CRISPR technique has successfully been used with other species as well, including rat, rabbits and goats, thereby increasing the ability to study lncRNA function in a variety of animal models [135–137]. Regardless of the manner knockout animals are generated, it is important to perform a genetic rescue with re-expression of the deleted lncRNA in knockout animals to confirm any deleterious effect, is in fact due to the lncRNA and not other regulatory elements that may have also been deleted [138].

Purified chromatin contains twice as much RNA as DNA [139], and lncRNA function is often associated with histone modification and chromatin remodeling. LncRNAs associated with chromatin-modifying complexes can be identified through RNA immunoprecipitation techniques (RIP-Seq) whereby an antibody to a chromatin modifying enzyme or protein component of the modification complex is used to pull-down associated RNAs followed by RNA-Seq [140]. A similar method involves crosslinking and immunoprecipitation followed by high-throughput sequencing (Clip-Seq) [141]. Alternatively, one can use the lncRNA as bait to identify protein, DNA and RNA-binding partners via chromatin isolation by RNA purification (ChIRP) or capture hybridization analysis of RNA targets (CHART) [142,143].

An additional consideration when studying lncRNAs is to evaluate their structure. The study of lncRNA structure is very much in its infancy, however this can have tremendous implications in helping our understanding of mechanisms of lncRNA function and provide critical information for lncRNA-based therapeutics [144,145]. At this time it is still unclear if lncRNA mechanism involves higher order structure, if their structure is compact or

extended and if individual lncRNAs contain multiple, functionally active domains. It has been proposed however, that RNA secondary structures might be preserved throughout evolution and this might explain the lack of sequence conservation [146].

4.2. LncRNAs and bone: a crystal ball or Pandora's box?

LncRNAs are believed to be critical to bone formation, not only due to inference from other tissues, but it has been demonstrated that the targeted disruption of the lncRNA Hotair results in malformation of metacarpal–carpal bones and homeotic transformation of the spine [85]. Hotair is expressed in the posterior trunk and distal limb buds, and in mesenchymal cells in embryonic forelimbs [85,147]. Hotair binds to PRC2, which is involved in the methylation of H3K27, and also the Lsd1 complex, which demethylates H3K4. It has been suggested that Hotair enforces a silent chromatin state and is involved in repressing the expression of the HoxD genes.

Very little else is understood about lncRNAs expressed during osteogenesis or how they may function. The lncRNA DANCR was characterized during differentiation of hFOB1.19 cells, and it is believed that DANCR may function in mesenchymal stem cell (MSCs) as a molecular switch regulating cell commitment [148]. A small array study identified 116 lncRNAs differentially expressed during early BMP-2 induced differentiation of C3H10T1/2 MSCs [149]. A more complete RNA-Seq study profiling lncRNAs during osteogenesis will likely produce a much larger list of expressed lncRNAs. The next challenge for understanding biological control of developmental programming, homeostasis of organ systems and disease induction and progression, is the functional characterization of tissue specific lncRNAs.

5. Summary remarks and future directions

The multifaceted components of the different classes of non-coding RNAs that contribute to epigenetic control of the skeleton are leading to exploration of novel mechanisms that would explain human traits and diseases far more than DNA mutations, particularly regulated by the long non-coding RNAs. There are many gaps to fill in our existing information for miRNAs. While much of the miRNA literature has identified regulated or dysregulated cellular levels and characterized a target(s) to individual miRNAs, it is now apparent that miRNAs must be considered in a broader context of their range of activities in order to consider their potential for therapeutic intervention. In vivo studies of exogenously expressed miRNAs or using a knockdown strategy are emerging for the skeleton and demonstrating the effectiveness of miRNAs to effect changes in bone in animal models; for example in metastatic bone disease [150,151]. But for human studies, a challenge would be specific delivery to cell of interest and characterization of off-target effects in vivo. We know very little of the miRNAs that are found in the circulation related to bone tissue metabolism and turnover. Studies measuring circulating miRNAs as potential "biomarkers" are emerging for cancer and other diseases. This would be a future direction for skeletal disorders, as miRNAs reflect changes in activity of cells which may occur as a warning of disease progression, a response to therapy and possibly inform an early intervention strategy.

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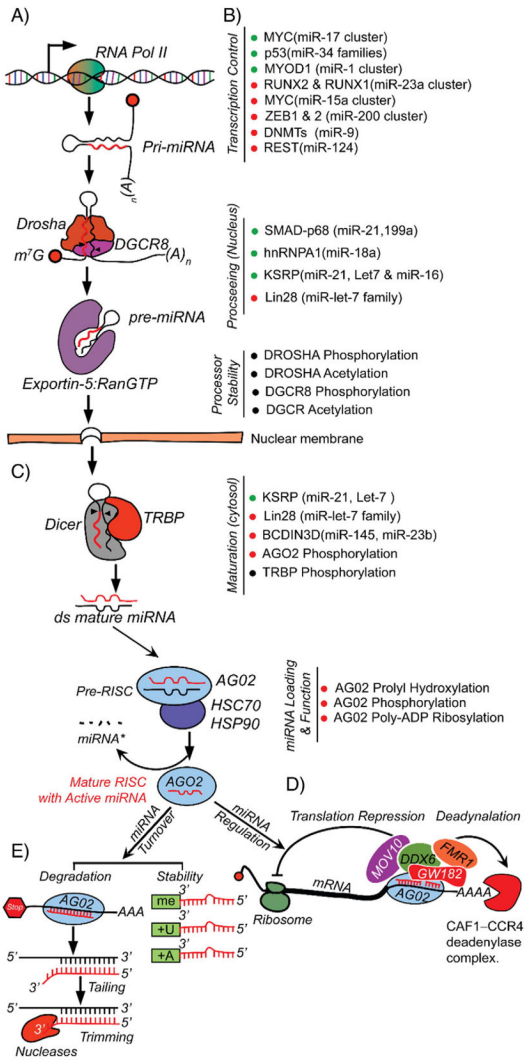

Fig. 1.

MicroRNA biogenesis, maturation, function and decay. Nuclear events: (A) schematic model of microRNA (miRNA) transcription to synthesize primary miRNA (pri-miRNA) by RNA polymerase II (Pol II), and processing of pri-miRNA by the Drosha–DGCR8 microprocessor complex to generate precursor miRNA (pre-miRNA) in the nucleus. Exportin 5 (EXP5) RAN•GTP complex exports pre-miRNA from nucleus to cytoplasm; (B) examples of miRNA transcriptional control and processing: tumor suppressor p53, growth factor MYC and myoblast specific transcription factor MYOD1 transactivate miR-34, miR-17 and miR-1 clusters, respectively. Osteoblast specific factor Runx2, leukemic factor Runx1, MYC, and zinc finger transcription factors ZEB1 and ZEB2 transcriptionally suppress the miR-23a cluster, miR-15a cluster, and miR-200 cluster. DNA methyltransferases (DNMTs) and RE1-Silencing Transcription Factor (REST) epigenetically regulate miR-9 and miR-124, respectively, at the level of transcription. Numerous RNA-binding proteins, including p68, KH-type splicing regulatory protein (KSRP), heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and LIN28, regulate the processing of primary miRNAs including miR-21, 199a, miR-21, let-7, miR-16, miR-18a, and let7. The

phosphorylation and acetylation of Drosha and DGCR8 proteins control the processing activity of these proteins; (C) cytoplasmic events: illustration of pre-miRNA maturation and formation of pre-and mature RNA Induced Silencing Complex (RISC). Dicer associates with TRBP (TAR RNA-binding protein) and processes pre-miRNA to generate 22–24 nt mature duplex miRNA that subsequently loaded onto AGO2 to form pre-RISC complex. Heat shock protein 90 (HSP90) and heat shock cognate 70 (HSC70) form a complex that hydrolyses ATP to load the RNA duplex on to the RISC. The miRNA* (passenger strand) is further degraded and the mature miRNA ‘guide’ strand remains in the RISC complex. Post-translational modifications AGO proteins, including prolyl hydroxylation, poly-ADP ribosylation and phosphorylation influence its efficiency and ability to control the processing of Dicer, RISC formation and miRNA activity; (D) RNA helicases, including MOV10 [152], DDX6 [153], translational repressor FMR1 [154], GW182 and AGO2 [4] are present in the active RISC and mediate miRNA-dependent repression of translation of complementary mRNAs by Argonaute proteins. Succeeding translation repression cognate mRNA is degraded by CAF1–CCR4 deadenylase complex [155]; (E) target guided miRNA degradation by tailing and trimming mechanism [156].

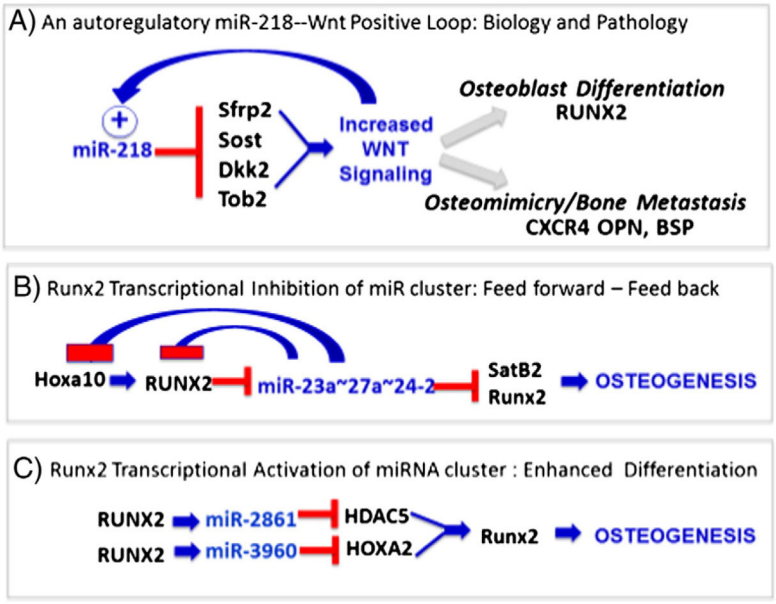

Fig. 2.

miRNA circuitry: pathways supporting bone formation. (A) Positive regulation of miR-218 for normal osteogenesis and in promoting metastasis. Wnt signaling is activated by miR-281 which increases Runx2 and multiple genes that promote matrix formation and mineralization during osteoblast differentiation. However high levels of miR-218 are associated with cancer and their osteomimetic properties promote metastasis to bone. (B) Negative regulation of the cluster MiR-23a by Runx2 transcriptional down-regulation of the cluster at a Runx site in its promoter. This action relieves the inhibition of both Runx2 and Satb2 which form a complex that drives differentiation. Restraints are placed on the feed forward path to bone formation by miR-23a targeting Runx2 and miR-27a by targeting Hoxa10 an activator of Runx2 in osteoprogenitors. (C) Positive regulation by Runx2 of two miRNAs, miR-3960 and miR-2861, targets an inhibitor of Runx2, HDAC5 and Hoxa2, respectively. The transcriptional activation of these miRNAs downregulates the inhibitors and generates a feed forward circuit for osteogenesis.