HHS Public Access

Author manuscript

Connect Tissue Res. Author manuscript; available in PMC 2017 February 10.

Published in final edited form as:

Connect Tissue Res. 2017 January ; 58(1): 116–141. doi:10.1080/03008207.2016.1194406.

Emerging roles for long noncoding RNAs in skeletal biology and disease

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Abstract

Normal skeletal development requires tight coordination of transcriptional networks, signaling pathways, and biomechanical cues, and many of these pathways are dysregulated in pathological conditions affecting cartilage and bone. Recently, a significant role has been identified for long noncoding RNAs (lncRNAs) in developing and maintaining cellular phenotypes, and improvements in sequencing technologies have led to the identification of thousands of lncRNAs across diverse cell types, including the cells within cartilage and bone. It is clear that lncRNAs play critical roles in regulating gene expression. For example, they can function as epigenetic regulators in the nucleus via chromatin modulation to control gene transcription, or in the cytoplasm, where they can function as scaffolds for protein-binding partners or modulate the activity of other coding and noncoding RNAs. In this review, we discuss the growing list of lncRNAs involved in normal development and/or homeostasis of the skeletal system, the potential mechanisms by which these lncRNAs might function, and recent improvements in the methodologies available to study lncRNA functions *in vitro* and *in vivo*. Finally, we address the likely utility of lncRNAs as biomarkers and therapeutic targets for diseases of the skeletal system, including osteoarthritis, osteoporosis, and in cancers of the skeletal system.

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Keywords

Bone; cartilage; chondrocyte; long noncoding (Inc) RNAs; osteoarthritis; osteoblast; osteosarcoma

Introduction

The human genome project commenced in 1990, and its first complete assembly was announced in 2003. From this endeavor, it was found that while 70–90% of the human genome is actively transcribed, there is only 1–2% of the genome that contains protein-coding information (1,2). The remaining noncoding RNA (ncRNA) transcripts were found to lack conserved open reading frames (ORFs) and were predicted to have no biological functions in the cell. As such, these ncRNAs were usually initially referred to as "junk RNA."

However, the past two decades of research on ncRNAs has overwhelmingly shown that these ncRNAs are in fact not "junk," but rather play crucial and multifunctional roles in the regulation of cell phenotype. For example, classes of short ncRNAs [e.g., microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs)] have been shown to function as gene silencing agents (3–13). More recently, much interest has focused on another class of ncRNAs, known as long noncoding RNAs (IncRNAs). IncRNAs are defined as any ncRNA containing 200 or more nucleotides. The majority of IncRNAs are transcribed by RNA polymerase II (RNA Pol II), and they are often spliced (and even alternatively spliced) during RNA processing. From the most recent release of GENCODE, it is predicted that there are currently 15,941 human IncRNAs and 8793 mouse IncRNAs (14). In this article, we review the current literature on IncRNAs and their role in skeletal development and maintenance. A summary of the IncRNAs discussed here can be found in Table 1.

IncRNAs: nomenclature and categorization

It is encouraged that IncRNAs should be named based on their functions (15), although at this point, many of their specific functions remain to be determined. For example, *XIST* is named for "X (Inactive)-Specific Transcript" (16,17), while *DANCR* stands for "Differentiation Antagonizing Non-Protein Coding RNA" (18–20). However, when IncRNAs are initially identified without a known function, they are named based on the associated genomic context or assigned an arbitrary name based on the GENCODE categorization, which divides IncRNAs into the following sub-categories (21,22):

1. Antisense: IncRNA whose sequence overlaps the exonic sequence of a protein-coding gene on the opposite strand (e.g., *HIF1a-AS1*)
2. lincRNA: IncRNA located within the intergenic regions of protein-coding genes (e.g., *LINC00152*)
3. Sense-overlapping: IncRNA whose intronic sequences encode a protein-coding or noncod-ing gene without overlap of exonic sequences (e.g., *SOX2-OT*)
4. Sense intronic: IncRNA residing within introns of a coding gene (e.g., *SPRY-IT1*)

5. Processed transcript: lncRNA that does not contain an ORF and does not fit in any of the above categories

lncRNAs: mechanisms of action

lncRNAs function in diverse ways by virtue of their primary sequence as well as their secondary and tertiary structures. In addition to interacting via base pair complementarity with DNA and RNA partners, they also fold into unique conformations that allow them to interact with DNA, RNA, and proteins. Importantly, these functions are not mutually exclusive, and the unique ability of lncRNAs to utilize these features simultaneously allows them to have far-reaching effects on cellular processes. While a more extensive discussion of the molecular mechanisms of lncRNAs can be found elsewhere (23), in this review we aim to highlight the general mechanisms by which lncRNAs execute their functions and to discuss the growing list of lncRNAs known to be involved in skeletal biology.

lncRNAs in the nucleus

lncRNAs appear to play a number of roles within the nucleus. For example, immunoprecipitation of various chromatin-modifying complexes reveals interactions with many nuclear lncRNAs, indicating a likely function for lncRNAs in epigenetic regulation (24,25). In a variety of vertebrate and invertebrate systems, lncRNAs have been identified that function as scaffolds for repressive chromatin modifiers such as: (i) the Polycomb repressive complexes 1 and 2 (PRC1, PRC2), which methylate his-tone 3 at lysine 27 (26), (ii) the repressive histone demethylase complex LSD1 (27), which demethylates histone 3 at lysine 4, and (iii) the heterochromatin-inducing EHMT2/G9a histone methyltransferase, which methylates histone H3 at lysine 9 (28). In contrast, others have been shown to interact with activating chromatin modifiers such as lncRNA-binding WDR5 subunit of the MLL (mixed-lineage leukemia) complex (29), which methylates histone 3 at lysine 4 (29–32). In some cases, these lncRNA-protein interactions are dictated by the structure of the lncRNA, as in the case of the stem-loop structures in some Polycomb-interacting lncRNAs (26,33). Notably, the presence of discrete structural “modules” along the length of the lncRNA can influence the binding of different protein complexes to the lncRNA, further diversifying its function [reviewed in (34)]. While it is not completely clear how lncRNAs navigate to their targets within the nucleus, it is apparent that lncRNAs can interact with transcription factors themselves (35), which could promote lncRNA targeting to discrete loci. In addition, a number of models have been proposed to describe how lncRNAs identify and interact with their targets, including sequenced-based or structure-based recognition of DNA loci (24,36).

A second layer of complexity in epigenetic regulation by lncRNAs is influenced by their ability to regulate nearby loci *in cis* (32), loci far from its site of transcription *in trans* (31), or a combination of both. Interestingly, knockdown of long intergenic ncRNAs (lincRNAs) demonstrated that the majority of their regulation occurred *in trans* (37), while lncRNAs near protein-coding genes often regulate these nearby loci *in cis*, as in the case of enhancer-associated RNAs [reviewed in (38)]. Notably, a number of lncRNAs are capable of regulating nearby and distant loci and may also bind chromatin-modifying complexes with

opposing functions, suggesting that the regulatory capacity of a given lncRNA can be locus specific (39).

In addition to the well-established functions in regulating expression of discrete loci, lncRNAs also play important roles in nuclear organization and the establishment of chromosome territories. A special example of this occurs during X chromosome inactivation (XCI) in female mammalian cells, where one copy of the X chromosome is silenced to ensure proper gene dosage. Here, the *Xist* lncRNA interacts with the Polycomb repressive complex PRC2 to induce the formation of heterochromatin across the inactive X chromosome (26). Interestingly, *Xist* itself is regulated by other lncRNAs including *Tsix* and *Jpx*. *Tsix* is transcribed antisense to *Xist* and represses *Xist* expression on the active X chromosome via DNA methylation, while *Jpx* induces *Xist* transcription by sequestering a transcriptional repressor CTCF (40–42). A second well-documented example of lncRNA functioning in nuclear organization is the lncRNA *NEAT1*, which functions as an essential scaffold for the organization of RNA processing and editing "factories" called para-speckles (43). Knockdown of *NEAT1* resulted in ablation of paraspeckles and alterations in export of mRNAs containing inverted repeats (44), suggesting an important role for this lncRNA in regulating this process.

lncRNAs in the cytoplasm

A number of lncRNAs have been demonstrated to be primarily localized within the cytoplasm or to shuttle between the nucleus and cytoplasm. These RNAs also function in diverse ways, interacting with a variety of protein-binding partners. lncRNAs have been shown to regulate protein levels (45) and function by base-pairing with the mRNA to upregulate protein translation, while some regulate mRNA degradation, recruiting proteins via the double-stranded RNA structures formed by extensive base-pairing between lncRNA and mRNA (46). Others regulate trafficking of proteins into the nucleus via their associations with the nuclear pore complex (47).

Furthermore, a growing body of evidence suggests that cytoplasmic lncRNAs can function as decoys for other ncRNAs such as miRNAs, and have been termed competing endogenous RNAs (ceRNAs) [reviewed in (48)]. While many ceRNAs function as a "sponge" to sequester miRNAs, others bind to mRNA targets and prevent miRNA-mediated downregulation (49). Interestingly, artificial ceRNAs have been utilized for more detailed characterization of miRNAs in mammalian biology [reviewed (50)].

lncRNAs regulating stem cell maintenance or differentiation

lncRNAs have also been shown to play a crucial role in maintaining pluripotency (35,37). One study (37) performed a systematic loss of function experiment in which 147 lincRNAs were knocked down in mouse embryonic stem cells (ESCs). While the differentiation capacity of these cells after lincRNA knockdown was not assessed, they observed that 93% of the lincRNAs targeted likely played an important role in maintenance of pluripotency, as their depletion resulted in gene expression profiles that were highly similar to those obtained when key pluripotency markers such as *Nanog* or *Oct4* were depleted.

A subsequent study demonstrated that lncRNAs are not only important in maintaining pluripotency, but may also act as key regulators in promoting and preserving cell identity after differentiation (35). Using a microarray chip to quantify expression of 6671 lncRNA transcripts, Ng *et al.* identified 934 differentially expressed ncRNAs as human ESCs were differentiated into neuron progenitors *in vitro*. Their functional studies focused on two classes of lncRNAs: those that were much more abundant in ESCs than in neural progenitors (NPCs) and likely played a role in maintenance of pluripotency and those that were much more abundant in NPCs than in ESCs and likely played a role in neuronal differentiation. As expected, knockdown of several lncRNAs more abundant in ESCs led to spontaneous differentiation into a variety of cell types, while knockdown of several neurogenic lncRNAs resulted in inhibition of neuronal differentiation. Functional characterization of some of these neuronal lncRNAs (e.g., *RMST, LINC01109*, and *CACNA2D1*) showed that they were localized to the nucleus and interacted with either the Polycomb repressive complex PRC2 or the neuronal transcription factor REST. In contrast, the neurogenic lncRNA *MIR100HG* was localized to the cytoplasm. This lncRNA also serves as a precursor to the miRNAs miR-125b and let-7 within its introns, which are known to function in neural development (51,52). Knockdown of *MIR100HG* substantially reduced the expression of these miRNAs, suggesting this lncRNA may also function as a reservoir for miRNAs important for differentiation.

Although the majority of lncRNA studies in stem cell differentiation have been carried out *in vitro*, comprehensive libraries of lncRNA expression during different stages of murine neurogenesis *in vivo* have also been described (53). In the study from Ramos *et al.*, this library was developed by integrating multiple RNA-sequencing approaches (RNA-seq and RNA CaptureSeq (54)) with assays of chromatin state using chromatin immunoprecipitation and sequencing (ChIP-seq), which provides not only a comprehensive list of expressed transcripts and splice isoforms, but also provides some indication of their regulation *in vivo*. Interestingly, comparisons of murine ESCs and micro-dissected neural stem cells (NSCs) demonstrated that many lncRNAs exhibit substantial changes in chromatin state during differentiation that correspond with transitioning from a "poised" state to an active state. For example, *lnc-pou3f2*, which is encoded upstream of the well-established neurogenic transcription factor POU3F2, is bivalently marked with both activating (H3K4me3) and repressive (H3K27me3) histone modifications in ESCs, but transitions to a fully active state marked by only H3K4me3 in NSCs. However, a substantial number of lncRNAs remain bivalently marked in NSCs, suggesting that these lncRNAs may function later during neural lineage specification. Functional validation of several lncRNAs confirmed their important roles in NSC self-renewal or differentiation, demonstrating that well-constructed lncRNA expression libraries are extremely valuable resources for investigating the roles of lncRNAs in development and disease *in vivo* across various cell types and tissues.

Other studies have demonstrated the function of lncRNAs in the definition of other cell/tissue fates, including cardiac development. The lncRNA *Fendrr* was identified by differential expression in posterior mesoderm compared to other early somite-stage mouse tissues, and *in vivo* studies in which the transcript was prematurely terminated clearly demonstrated that loss of *Fendrr* was embryonic lethal (39). Defects in both cardiac development and in development of the ventral body wall suggested defects in

differentiation of the lateral mesoderm, which was verified using gene expression analysis and chromatin profiling. Similarly, Klattenhoff and colleagues again utilized RNA-seq to identify lncRNAs that were most abundant in the murine heart compared to other tissues and identified the candidate *Braveheart (Bvht)*, a lncRNA which has no obvious homologs in human or rat tissues (55). *In vitro* studies of *Bvht* in ESC differentiation showed that this lncRNA is not required for ESC maintenance or global differentiation but plays an important role in cardiac differentiation via interactions with the Polycomb repressive complex, PRC2. Notably, these studies were possible due to the natural propensity for ESC-derived embryoid bodies to generate cardiac tissue and well-established cardiomyocyte differentiation assays, highlighting the importance of developing well-defined *in vitro* differentiation assays for other cell types of interest.

LncRNA *H19* in genomic imprinting

The highly coordinated process of genomic imprinting, in which one of two parental alleles is silenced, is a process involving lncRNAs that is of special importance to musculoskeletal biology and cancer development. One human disease associated with defective imprinting is Beckwith–Wiedemann syndrome, which results in an "overgrowth" phenotype and is caused by genetic or epigenetic changes within imprinting control regions (ICRs). These regions are regulatory loci defined by differential DNA methylation and are responsible for regulating a network of imprinted genes, including the lncRNA *H19* and insulin-like growth factor, *IGF2*; however, mutations or epigenetic changes at many other loci involved in the imprinted gene network, such as the lncRNAs *Kcnq1ot1* and *Airn*, can also confer similar phenotypes. As a result of the clear phenotypes seen with errors in imprinting and its conservation between mouse and human, the imprinted gene network involving *H19* and *IGF2* and the regulation of these loci is one of the best studied loci for genomic imprinting.

In normal circumstances, *IGF2* is expressed from the paternal allele, and the downstream lncRNA *H19* is expressed from the maternal allele. Generation of a series of genetic knockout (KO) models in mice (reviewed in (56)) revealed that genomic imprinting elements within the *H19-IGF2* locus are essential for normal imprinting and thus properly regulated *H19* and *IGF2* expression. In order for normal imprinting to occur, the zinc finger protein CTCF binds to ICRs comprised of differentially methylated regions (DMRs) and the matrix attachment region (MAR) on the maternal allele to shield the *IGF2* promoter from its enhancer. As a result, this enhancer is accessible to *H19* and promotes *H19* expression. Meanwhile on the paternal allele, methylation of the ICR blocks CTCF binding, allowing contact between the *IGF2* promoter and enhancer to promote *IGF2* expression. Simultaneously, the paternally inherited *H19* promoter is also methylated, rendering it inaccessible by both enhancers and transcription factors. In Beckwith–Wiedemann syndrome, loss or epigenetic dysfunction of an ICR typically results in either overexpression of *IGF2* or loss of the imprinted gene *CDKN1C*, which regulates cell proliferation; these phenotypes are thought to contribute to the overgrowth phenotypes observed in patients with this syndrome.

The proper regulation of *H19* and *IGF2* is critical for normal development and tissue homeostasis. During fetal development, *H19* and *IGF2* are highly expressed in endodermal

and mesodermal tissues. More importantly, their expression is tightly coordinated with identical spatial and temporal patterns (57,58), and *IGF2* utilizes fetal-specific promoters for its expression during development (59). Interestingly, in a KO mouse model in which the entire *H19* sequence was deleted, normal imprinting patterns were observed on both parental alleles (60), suggesting that *H19* does not function *in cis* to regulate *IGF2* gene expression. However, other evidence proposes that *H19* may regulate *IGF2* expression post-transcriptionally by binding to insulinlike growth factor II mRNA-binding proteins (IMPs) (61,62). *H19* can also be processed into miR-675, whose targets such as *IGFR1* and *Rb* (Retinoblastoma) are involved in cell growth and regulation (63–65).

Given that *H19* is highly abundant in the mesoderm during embryogenesis (66,67), it could be hypothesized that this IncRNA may play a role in regulating skeletal development. In the remaining sections of this review, we will focus on recent studies describing the expression and potential function of IncRNAs, including *H19*, in chondrogenesis and osteogenesis, as well as in cartilage and bone homeostasis and disease.

Cartilage Development

Cartilage is an important connective tissue that can be classified into three groups: hyaline (e.g., joint articular cartilage), elastic (e.g., ear), and fibrocartilage (e.g., meniscus and intervertebral discs in the spine). These cartilage types differ in extracellular matrix (ECM) composition and cell (chondrocyte) phenotype, thereby providing each tissue type with specific mechanical properties to carry out their function in the body.

Hyaline cartilage development is a complex process that begins with the condensation of mesenchymal stem cells (MSCs) that are derived from the mesoderm germ layer (68). In response to various signaling pathways, chondro-progenitor cells are formed. These cells proliferate and differentiate into chondrocytes that are responsible for generating the ECM which, in hyaline cartilage, consists primarily of type II collagen and the large aggregating proteoglycan, aggrecan. Two types of hyaline cartilage exist in the limb: articular cartilage and growth plate (epiphyseal) cartilage. Some controversy remains as to whether articular and growth plate cartilages are derived from common precursor cells or if the outer region of articular cartilage (i.e., the superficial zone) is derived from distinct precursor cells during synovial joint development (69,70). In any case, we know that chondrocytes of articular cartilage remain as permanent, differentiated chondrocytes that synthesize an ECM with appropriate mechanical properties to permit transmission of loads within the synovial joint during development and aging. Chondrocytes of the growth plate, however, are distinctly localized in columns where they continue to proliferate, thereby lengthening the limb during development. These cells then terminally differentiate to form large hypertrophic chondrocytes. The production of hypertrophic chondrocytes is essential to permit long bone formation via the process of endochondral ossification whereby the cartilage template is essentially replaced by bone (71). It was thought that the majority of hypertrophic chondrocytes become apoptotic during endochondral ossification, but recent studies have shown that these cells also contribute toward the osteoblast pool or that there are osteoprogenitor cells in hypertrophic cartilage that can differentiate toward bone-producing osteoblasts (72–75).

There is an abundance of knowledge with respect to the transcription factors and the signaling pathways/ mediators that regulate chondrocyte differentiation and ECM synthesis. In addition to protein-coding genes, many studies have been published on noncoding miRNAs and how their expression changes during chondrocyte differentiation (76–78) as well as how they may function in controlling chondrogenesis (79–82). In keeping with the epigenetic theme, studies are now emerging to investigate the expression and potential function of lncRNAs in cartilage development and disease.

lncRNAs regulating chondrocyte differentiation

A common method to study chondrogenesis *in vitro* involves three-dimensional culture of human mesenchymal stem/stromal cells (MSCs) isolated from bone marrow (83,84) or other sources such as adipose, synovium, and umbilical cord (85,86). A recent study utilized human bone marrow-derived MSCs (HBMSCs) to determine lncRNA expression signatures in cells 2 weeks following *in vitro* chondrogenesis compared to HBMSCs at day 0 (control) of the differentiation assay (87). Following microarray analysis (lncRNA + mRNA Human Gene Expression Microarray V4.0; CapitalBio Corp, Beijing, China), over 3000 lncRNAs were found to be significantly differentially expressed (±twofold) between control and chondro-induced cells. Among these, three lncRNAs were identified as upregulated following chondrogenesis (*ZBED3-AS1, CTA-941F9.9*, and *ENST00000433576.1*) and one was identified as downregulated (*LINC00707*; it should be noted that this lncRNA was reported as two individual genes in this report). Moreover, it is not known if these lncRNAs showed the most dramatic fold changes in this work since the entire array data was not reported. Another caveat with this study involves the relatively small increase in type II collagen expression (~twofold increase) after 2 weeks of chondrogenesis. Normally, *COL2A1* levels are increased by several orders of magnitude during such MSC chondrogenesis assays (88,89). Also, no *COL2A1* expression data was shown from their array study. It will be interesting to determine lncRNA expression by RNA-seq analysis in HBMSC-induced cells that produce higher levels of *COL2A1* during chondrogenesis and investigate if the same lncRNAs found in this study by Wang *et al.* are also found to be differentially expressed.

Another recent study identified that the transcription factor, SOX4, can regulate the proliferation and chondrogenic differentiation of human synovium-derived MSCs (SMSCs) and that activation of the lncRNA, *DANCR*, is involved in this process (90). Here, SMSCs were extracted from the synovium of osteoarthritis (OA) patients and aggregate cultures were utilized for *in vitro* chondrogenesis. Apparently, the differentiation medium in these assays did not include TGF-β1/3, and so it is surprising that efficient chondrogenic differentiation was achieved. In any case, SOX4 overexpression was found to increase the expression of chondrocyte markers over 2 weeks in culture. To attempt to decipher SOX4 function in this scenario, promoter analysis of the lncRNA *DANCR* revealed a SOX4-binding site. Luciferase and ChIP assays showed that SOX4 can interact with its binding site on the *DANCR* promoter. Inhibition of *DANCR* by RNAi apparently suppressed the effects of SOX4 in promoting chondrogenesis, thereby suggesting that SOX4 functions upstream of *DANCR. DANCR* (Differentiation Antagonizing Non-protein Coding NA) was first described as a lncRNA (previously named *ANCR*), which functions in suppression of

progenitor cell differentiation (18). Two recent studies have shown that *DANCR* can increase stem cell-like features in hepatocellular carcinoma cells (91) and that it can suppress odontoblast-like differentiation of human dental pulp cells by inhibiting the Wnt/β-catenin pathway (92). Given this "anti-differentiation" function, it is interesting that *DANCR* activation via SOX4 in SMSCs resulted in increased proliferation and differentiation toward the chondrocyte lineage in this study. Further research is required to thoroughly decipher the role of *DANCR* in chondrogenesis.

With respect to human skeletal pathology, a lncRNA was recently found to be associated with brachydactyly type E (BDE), a condition characterized by shortening of metacarpals and metatarsals. In this study (93), two families with autosomal dominant BDE were identified with translocations in chromosome 12 that resulted in genomic disruption of an important cis-regulatory element named *CISTR-ACT*, which also produces the lncRNA *DA125942*. Notably, the *CISTR-ACT* locus interacted *in cis* with the *PTHLH* locus, which encodes the chondrogenic regulator parathyroid hormone-like hormone (94), and *in trans* with the *SOX9* locus, which encodes the master chondrogenic transcription factor SOX9 (95). Importantly, knockdown of *DA125942* in a chondrogenic cell line resulted in downregulation of *PTHLH* and *SOX9*, as well as a plethora of other genes important in skeletal patterning (*HOXB* cluster, *ETV4*) or chondrogenesis (*HIF1A*), suggesting a potentially important role for this lncRNA in regulating chondrogenic differentiation.

Studies on lncRNAs associated with *HOX* genes have provided insights into their functions in regulating skeletal development. *HOX* genes are a conserved family of developmental transcription factors that elicit specific developmental programs along the head-to-tail axis of animals (96). For example, lncRNA genes in the 5′ *HOXA* and *HOXD* clusters are known to regulate limb and spine growth and patterning (97–100). *HOTAIR* (HOX Transcript Antisense RNA) was the first vertebrate lncRNA described to regulate HOX function (31). Specifically, *HOTAIR* is expressed from the *HOXC* locus and functions to recruit EZH2 and SUZ12, part of the Polycomb repressive complex PRC2, to the *HOXD* cluster, thereby establishing a silent chromatin state using H3K27 methylation and resulting in repression of several 5′ *HOXD* genes (Figure 1). Targeted deletion of *Hotair* in mice resulted in patterning malformations in the lumbosacral junction and in the metacarpal and carpal bones in the limbs (101). Interestingly, cyclic stretch has been shown to decrease *HOTAIR* levels in human aortic interstitial cells (AVICs), and reducing *HOTAIR* levels via siRNA in AVICs results in increased expression of calcification genes (102). These findings suggest that *HOTAIR* is mechanoresponsive and may thus may play a potential role in mechanically regulated calcification, although additional work is needed to test this hypothesis in other cell types.

In contrast, *HOTTIP* (HOXA Transcript at the Distal Tip) is an enhancer lncRNA that regulates the expression of 5′ *HOXA* genes to control the growth and elongation of skeletal elements of the limb (32). *HOTTIP* functions by regulating chromosome looping to recruit the WDR5/ MLL histone methyltransferase complex to the 5′ HOXA genes, conferring an active chromatin state via H3K4 methylation (32,103) (Figure 2B). While *HOTAIR* has been shown to act *in trans* (located on the HOXC locus and regulates HOXD genes) (Figure 1), *HOTTIP* was noted to act *in cis* (Figure 2B) and its proximity was crucial for HOXA gene

expression, as expression of *HOXA* genes was not changed when *HOTTIP* was ectopically expressed via lentivirus.

An elegant study published recently by Stadler's group has identified another lncRNA located within the HOXA locus, named *lncRNA-HIT*, which appears to function as a critical epigenetic regulator of chondrogenesis (104). *LncRNA-HIT* (HOXA Transcript Induced by TGF-β) was initially characterized as a TGF-P-responsive lncRNA during epithelial-to-mesenchymal transition in mammary epithelia (105). *LncRNA-HIT* was previously mapped as a single exon in the mouse genome between *Hoxa11* and *Hoxa13* (106). Carlson *et al.* found that *LncRNA-HIT* was expressed in the developing mouse limb and hypothesized that it may play a role in chondrogenesis. RNA FISH analysis showed that *LncRNA-HIT* is localized to the nucleus of limb mesenchymal cells, while mass spectroscopy and immunoprecipitation experiments revealed that it associates with p100/CBP complexes (Figure 2A). Knockdown of *lncRNA-HIT* in micromass cultures of murine limb mesenchymal cells was found to inhibit chondrogenesis (i.e., cartilage nodule formation) in these cultures. Further experiments in this study strongly suggest that, mechanistically, *lncRNA-HIT* functions as an enhancer lncRNA via association with p100/CBP complexes to maintain H3K27ac at specific chromatin,sites thereby resulting in the activation of 5′ *HoxA* genes. In addition to affecting the expression of *HoxA* genes, *lncRNA-HIT* siRNA experiments also revealed decreased expression of a number of other genes, including *Bmpr1b*. This finding may also explain why knockdown of *lncRNA-HIT* reduces cartilage formation of murine limb mesenchyme *in vitro* since loss of *Bmpr1b* is known to negatively affect chondrogenesis in mice and humans (107–110). Overall, this study is the first to provide detailed mechanistic insights into the role of a specific lncRNA in chondrogenesis and reveals another level of complexity toward our understanding of how chondro-progenitor commitment and cartilage tissue development is controlled.

lncRNAs in cartilage homeostasis and osteoarthritis

OA is a painful and debilitating disease of the synovial joints that is characterized by progressive degenerative changes in the articular cartilage and other joint tissues such as the synovium and subchondral bone. Under normal circumstances, mature articular cartilage is maintained in a healthy balance of anabolic and catabolic processes by the activity of the chondrocytes. While many factors can contribute to the onset and progression of OA, disruption of cartilage homeostasis can occur if a joint is subjected to altered loading caused by malalignment or trauma (e.g., meniscal or ligament injury, articular cartilage fracture) (111–116). Obesity can also affect joint homeostasis due to increased loads as well as chronic inflammation (113,117–119). As a result, catabolism often predominates causing joint abnormalities including articular cartilage degradation, subchondral bone sclerosis, osteophyte formation, and synovitis which, collectively, can be defined as OA (120). OA is a challenging disease to treat, given that articular cartilage has little or no intrinsic regenerative capacity. Many of the cellular and molecular changes that occur in OA are known, such as alterations in growth factor and cytokine signaling, inflammation, oxidative stress, chemokine signaling, and metabolism (120–122). However, no effective therapies have yet been discovered to ameliorate or stop OA progression. It is now apparent that epigenetic modifications (e.g., DNA methylation, histone modifications) may confer

susceptibility to OA (123–132) which could open up new avenues for alternative therapeutic approaches.

To date, a small number of studies have been published on IncRNA expression and potential function in mature chondrocytes within the context of OA. The IncRNA *H19* was first reported by Dudek *et al.* to be highly expressed in mature primary articular chondrocytes and to be regulated by SOX9 (133). This study also showed that *H19* knockdown in primary chondrocytes reduced *COL2A1* levels; however, *H19* also serves as the precursor for the miRNA miR-675, and they demonstrated that the effects of *H19* knockdown could be rescued by overexpression of miR-675. Thus, they concluded that miR-675 is an important functional component of *H19* to regulate the chondrocyte phenotype.

In addition, Steck *et al.* reported that *H19* expression, as well as *IGF2*, was elevated in human OA cartilage compared to healthy, control tissue; however, *H19* and *IGF2* levels did not correlate significantly in cartilage (134). This study also showed that *H19* levels are increased in chondrocytes under hypoxic conditions, while levels decreased in response to proinflammatory cytokines. From these findings, it was speculated that *H19* may play a role in regulating chondrocyte metabolism in response to stress as well as induce chondrocyte anabolism. As will be discussed more later, *H19* was recently shown to induce osteoblast differentiation via TGF-β1/Smad3/HDAC or β-catenin/Wnt signaling pathways (135,136). It remains to be discovered if *H19* functions in a similar manner to induce chondrogenesis. Finally, a recent report revealed a new function for *H19* in regulating DNMT3B-mediated DNA methylation (137). This is interesting from the standpoint that changes in DNA methylation patterns have been detected in human OA cartilage and that *H19* levels have been reported to be higher in OA (134,138).

A study by Kim *et al.* showed that *HOTTIP* expression was increased (assessed by microarray analysis) in chondrocytes from OA patients compared to non-OA or normal control cells (139). They also detected a decrease in *HOXA13* expression as a result of increased *HOTTIP* levels. However, it has been reported (and mentioned earlier in this review) that *HOTTIP* functions to facilitate H3K4 methylation and, therefore, activation of distal HOX genes. It is, therefore, curious that an increase in *HOTTIP* would lead to a reduced *HOXA13* expression in this study. Further mechanistic studies are needed to clarify the functional role of *HOTTIP* in regulating cartilage homeostasis.

Other microarray-based studies have been published reporting IncRNA expression changes in OA chondrocytes compared to healthy control cells (138,140,141). One of these studies reported 121 differentially expressed IncRNAs, 73 of which were significantly upregulated including *HOTAIR, GAS5, PMS2L2, RP11-445H22.4, H19*, and *CTD-2574D22.4* (138). Of these IncRNAs, *HOTAIR* and *GAS5* were also found to be upregulated in OA chondrocytes in the Kim *et al.* study mentioned above (139). The study by Liu *et al.* (141) identified 152 differentially expressed IncRNAs and focused specifically on one IncRNA (*IncRNA-CIR*; Cartilage Injury Related) which was upregulated over tenfold in OA chondrocytes, as confirmed by qPCR, and was also shown to increase in chondrocytes following treatment with IL-1β and TNF-a. Attempts to decipher the function of *IncRNA-CIR* were done by knockdown (siRNA) and overexpression approaches; it was concluded from these

experiments that *IncRNA-CIR* can induce chondrocyte catabolic events via increased expression of MMP13 and ADAMTS-5. The study by Fu *et al.* (140) provided expression data of differentially expressed IncRNAs followed by target predictions and pathway analyses of those IncRNAs found to be most highly up- or downregulated in OA. They utilized the same microarray (Human IncRNA Array v2.0; 8×60K; Arraystar) that was used by Liu *et al.* (141) and reported many more differentially expressed IncRNAs (4714 IncRNAs) based on the fact that they included IncRNAs whose levels changed by twofold or more (as opposed to the Liu *et al.* study that reported IncRNAs with an eightfold or more change in expression).

Another recent report also showed increased expression of *GAS5* (Growth Arrest-Specific 5) in OA cartilage compared to healthy control tissue (142). RNA FISH analysis showed nuclear localization of *GAS5* in healthy chondrocytes, while localization in OA chondrocytes also revealed cytoplasmic distribution. Overexpression of *GAS5* in chondrocytes *in vitro* resulted in increased metalloproteinase expression, decreased expression of markers associated with autophagy, and decreased expression of miR-21. Manipulation of miR-21 levels in murine articular cartilage *in vivo* via lentivirus showed some degree of chondro-protection following OA-induced surgery by destabilization of the medial meniscus (DMM) (143). The overall conclusion from these findings is that *GAS5* overexpression appears to contribute to a catabolic phenotype via regulation of miR-21 in chondrocytes. Another study was published recently reporting decreased expression of the IncRNA *MEG3* (Maternally Expressed Gene 3) in OA and that its expression levels were inversely associated with *VEGF* levels (144). However, no functional data was included in this study to be able to make any conclusions on *MEG3* regulation of VEGF in chondrocytes.

Finally, a study recently accepted for publication by Pearson *et al.* aimed to identify IncRNAs associated with the inflammatory response in human primary hip OA chondrocytes (145). These cells were treated for 4 h with IL-1β and differentially expressed IncRNAs between treated and untreated cells were identified following RNA-seq analysis (sequencing data is publically available via the GEO data repository: GSE74220). Of the 983 IncRNAs identified, 125 were found to be differentially expressed following IL-1β treatment including *PACER* (p5-Associated COX2-Extragenic RNA) and two novel chondrocyte inflammation-associated lincRNAs (*CILinc01* and *CILinc02*). The increase in expression of these three lincRNAs was confirmed by additional *in vitro* assays using IL-1β-induced primary knee or hip chondrocytes followed by qPCR analysis. Interestingly, expression of these lincRNAs was found to be lower in OA cartilage compared to healthy control tissue. This finding may be due to the fact that the inflammatory status (and hence IncRNA expression) of chondrocytes embedded within late-stage OA cartilage tissue may not correlate with that in cultured chondrocytes isolated from cartilage following treatment with IL-1β. In any case, this report has provided in-depth information on a number of inflammatory-regulated IncRNAs that may be worth pursuing in greater detail with respect to determining their biological functions in regulating chondrocyte homeostasis.

Bone development

Unlike cartilage, bone is a rigid tissue made up mainly of collagen fibers, calcium carbonate, and hydroxyapatite. Three cell types reside within the bone tissue: osteoblasts that synthesize bone ECM, osteoclasts that resorb bone, and osteocytes that are the mechano-sensing cells embedded deep within the bone (146–148). During embryonic development, bone is formed from cells of three sources: the somites, the lateral plate mesoderm, and the cranial neural crest. Formation of bone can occur via two processes: endochondral ossification or intramembraneous ossification.

The vertebral, limb, and rib skeletons are formed by endochondral ossification that begins with the formation of a cartilage intermediate template (71). Under the control of multiple signaling pathways (e.g., BMP superfamily growth factors, FGFs, Wnts, Hedgehog), mesodermal stem cells become committed toward the chondrocyte lineage and undergo a process known as mesenchymal condensation (146). Here, the influence of cell adhesion molecules such as N-cadherin and N-CAM are noted to be highly critical in maintaining cell–cell contacts (149–151). While the master chondrocyte transcription factor Sox9 is one of the earliest markers expressed in the chondrocyte lineage, this transcription factor requires the co-activators L-Sox5 and Sox6 to fully induce the expression of other chondrogenic genes such as *Col2a1* (type II collagen) and *Acan* (aggrecan). The cells later enlarge and become hypertrophic chondrocytes that express elevated levels of *Runx2, Mmp13, Alp, Col10a1*, and *Vegf*. This alteration in gene expression permits the generation of an ECM that can be mineralized and vascularized (152–154). At this stage, the hypertrophic chondrocytes can become apoptotic, although as mentioned earlier in this review, recent evidence suggests that these cells, or progenitor cells within hypertrophic cartilage, can also contribute to the osteoblast pool (72–75). Following vascularization of hypertrophic cartilage, pre-osteoblasts and pre-osteoclasts infiltrate this primary center of ossification. Mature osteoblasts then function to generate new bone matrix and, at the same time, a bone marrow cavity is created. Mature osteoclasts function to resorb the trabecular bone within the ossification center. Overall, there is a critical balance between osteoblasts and osteoclasts to maintain bone homeostasis.

In contrast, intramembraneous ossification occurs when the stem cells in mesenchymal tissue differentiate directly toward the osteoblast lineage without the requirement of a cartilage template. This can be seen perhaps most clearly by the construction of our facial skeleton. Under the influence of BMPs, the cranial neural crest cells differentiate to become pre-osteoblasts which express elevated levels of *Runx2*. These cells subsequently mature into osteoblasts that can produce an osteoid matrix rich in collagen, proteoglycan, and calcium (155). There are many signaling pathways involved in intramembraneous ossification. For example, BMP, IHH (Indian Hedgehog), and PTHrP (parathyroid hormone-related protein) signaling pathways function in the commitment of cranial neural crest cells toward an osteoblast identity (155). In addition, FGF and SHH (Sonic Hedgehog) are also involved in defining the shape and size of the facial midline (156–162).

lncRNAs regulating osteoblast differentiation

To date, studies on the role of lncRNAs in bone biology have been limited. However, one report showed that homozygous mice lacking the lncRNA, *Hotair*, resulted in lumbosacral transformation and fusion of the metacarpal and carpal bones (101). This skeletal phenotype was similar to transgenic mice with ectopic expression of HoxD resulting in elevation and anteriorization of *Hoxd10* and *Hoxd11* expression. It has been suggested earlier that *Hotair* acts *in trans* and regulates the expression of *HoxD* genes by recruiting the Polycomb repressive complex PRC2 to the *HoxD* locus (31). RNA-seq data of WT, *Hotair* +/−, and *Hotair*−/− confirms the earlier hypothesis: *Hotair* deletion leads to demethylation of H3K27 and methylation of H3K4 methylation at targeted genes, resulting in derepression of *HoxD* genes. Interestingly, several imprinted loci were also identified as *Hotair* targets, including *Dlk1-Meg3* and *Igf2-H19*. Since *Hotair* and its well-known related gene *Hottip* regulate the *Hox* loci, and *Hox* genes are involved in body patterning and limb development, it was not surprising that mutation of this lncRNA led to such striking skeletal patterning defects. It will be interesting to investigate whether *Hottip* is also involved in the regulation of osteogenesis.

Recent studies have demonstrated that *H19* is upregulated during osteogenesis (135,136). Different mechanisms, which may not be exclusive of one another, have been proposed to describe *H19* function in this context. Huang and colleagues demonstrate that *H19* can function to promote osteogenesis. They showed that H19 is processed into miR-675, which targets *TGFB1* for degradation (136) (Figure 3A). Since TGFβ signaling has been previously shown to impede osteogenesis by inducing heterochromatin formation at several promoters such as *RUNX2* and *OCN* (163), a drop in TGF-β1 would lead to derepression of these osteogenic markers and subsequently promote osteogenesis. Additionally, this study also showed that H19/miR-675 downregulated levels of HDAC4/5 which also resulted in increased expression of osteoblast genes, although it was not clear whether this effect is direct or indirect. Interestingly, a specific function for *H19* outside of miR-675 biogenesis was not described by Huang *et al.* In contrast, the proposed mechanism described by Liang and colleagues demonstrated that *H19* itself functions as a ceRNA, binding to miR-141 and miR-22 and sequestering them away from their mRNA targets (Figure 3B). Notably, these miRNAs were previously established to target and degrade *CTNNB1* (β-catenin) (164), and *H19* may relieve their repressive effect on the Wnt-signaling pathway by competitively binding to these miRNAs, thereby inducing osteoblast differentiation. As *H19* had previously been shown to be enriched during chondrogenesis (133), it may be the case that *H19* suppresses the stemness of MSCs in general instead of promoting specific differentiation programs in chondrogenesis and/or osteogenesis, although further evidence on the molecular mechanism of *H19* in MSCs is required to prove this hypothesis.

There have been two reports of the lncRNA *DANCR* in osteogenesis. In one study, it was proposed that *DANCR* regulates expression of the osteogenic regulator *RUNX2* by recruiting the Polycomb repressive complex component EZH2 to the *RUNX2* promoter, thereby resulting in suppression of osteogenesis (165). While RNA pulldowns showed a direct association of *DANCR* and EZH2, there was no evidence that *DANCR* was directly binding or recruiting EZH2 to directly bind to *RUNX2* promoter, which will be of interest

for future investigation. A similar study manipulated *DANCR* expression in human periodontal ligament stem cells, and also showed that downregulation of *DANCR* was critical for osteogenesis (166). Here, *DANCR* knockdown upregulated β-catenin/Wnt signaling pathway in these cells, which has been well-documented to promote osteoblastogenesis. However, this study only suggests a correlation between *DANCR* and the osteogenic markers, and no direct interactions between the lncRNA and the genomic elements regulating osteogenic genes were investigated in the study. In addition, since *DANCR* was first proposed to function as an anti-differentiation player (18–20), it might be the case that reducing *DANCR* expression evokes a global effect on the epigenetic state of the cells. Instead of directly affecting their osteogenic capability, *DANCR* may be acting to make the cells more competent for differentiation into specific cell types given the appropriate medium conditions.

Additionally, a set of lncRNA microarray expression data was also generated from a mouse MSC cell line that was stimulated with BMP2 (167). The study identified 116 lncRNAs that were differentially expressed: 59 upregulated and 57 downregulated. However, several issues were noted in this study: (i) the cut-off values used for differential expression were arbitrary and changed between time points, (ii) the experimental methods were not well-described, and (iii) data showing appropriate osteogenic induction were relatively weak. Furthermore, no functional characterization was performed to test the requirements for these lncRNAs in osteogenesis. Therefore, additional validation of differentially expressed lncRNAs in this system is still needed.

The expression of the lncRNA *HIF1A-AS1* in HBMSCs was also characterized, and the authors imply a potential role for this lncRNA in osteogenesis which is dependent on the histone deacetylase *SIRT1*, an important positive regulator of osteoblastogenesis and bone mass (168,169). Here, overexpression of *SIRT1* downregulated *HIF1α-AS1* expression, while knockdown and pharmacological inhibition of SIRT1 upregulated *HIF1a-AS1* expression. Interestingly, *HIF1a-AS1* knockdown decreased the expression of *HOXD10*, suggesting a potential role for this lncRNA in HOX gene regulation. However, the authors describe a pro-osteogenic role for TGF-β, which has been well documented to function in an anti-osteogenic capacity. In addition, the regulation of *HIF1a-AS1* during *in vitro* osteogenic assays was not described, and it was not clear whether these experiments were performed under osteogenic conditions; so further work is needed to determine the function of this lncRNA during osteogenesis.

Last, the function of the lncRNA *MEG3* was shown to play an important role in osteogenesis, especially in the context of multiple myeloma, a hematological cancer that also affects bone mass by inhibition of osteoblastogenesis of MSCs (170). Here, it was demonstrated that *MEG3* expression increases during osteogenic differentiation of MSCs from normal patients, but its expression is reduced overall and is unchanged during osteogenesis. In normal cells, *MEG3* knockdown reduced osteogenic differentiation and functioned by reducing transcription of *BMP4*, and ectopic *MEG3* overexpression or treatment with exogenous BMP-4 rescued the osteogenic defect of MSCs from multiple myeloma patients. Mechanistically, it was shown that *MEG3* regulates *BMP4* transcription by disrupting the interaction between the SOX2 transcription factor and the *BMP4* promoter.

Interestingly, overexpression of *MEG3* in MSCs from multiple myeloma patients also improved chondrogenic differentiation, suggesting a potential role for this lncRNA in chondrocyte differentiation as well.

lncRNAs in osteosarcoma

Osteosarcoma is a type of bone cancer that occurs at a rate of 5% in pediatric malignancies (171,172). In humans, osteoblastic osteosarcoma is characterized by highly mineralized tissues that are abundant in osteoids with multinucleated cells (173,174). From recent improvements in surgical, therapeutic, and radiation care, the survival rate of osteosarcoma patients has increased from less than 20–70% over the past 40 years (175–179). However, elucidation of the molecular basis of disease development and progression is required to establish better treatment options. Recently, several lncRNAs have been identified that show differential expression in osteosarcoma tissue.

One lncRNA that is dysregulated in osteosarcoma is *MALAT-1* (Metastasis-Associated Lung Adenocarcinoma Transcript 1), a 6.8kb transcript that is conserved among 33 mammalian species (180). Unlike most lncRNA, *MALAT-1* is not poly-adenylated at its 3′ end but instead folds into a triple helix (Figure 4), a unique structure that protects the RNA from degradation by nucleases (181). *MALAT-1* is expressed in many types of cancers, where it often promotes proliferation [reviewed in (182)]. Therefore, it is not surprising that *MALAT-1* was also observed to be upregulated in osteosarcoma (183,184). These independent studies established that knockdown of *MALAT-1* in osteosarcoma cells not only decreased proliferation but also induced apoptosis, and knockdown reduced osteosarcoma growth and metastasis *in vivo*. In addition, a third study utilizing the MG63 osteosarcoma cell line demonstrated that *MALAT-1* was downregulated by high-dose estrogen, and this downregulation reduced proliferation, migration, and invasion (185). *MALAT-1* appeared to sequester the splicing complex factor SFPQ from its splicing co-regulator PTBP2 (Figure 4), and downregulation of *MALAT-1* resulted in a higher accumulation of the SFPQ/PTBP2 complex. Interestingly, similar results have been observed in colon cancer cells, in which sequestration of SFPQ by *MALAT-1* results in PTBP2-mediated increases in cellular proliferation and migration (186).

Many lncRNAs have been identified using similar methods, and *in vitro* studies show that they may play important roles in osteosarcoma. Much like *MALAT-1*, the lncRNA *SNHG12* (Small Nucleolar RNA Host Gene 12) is upregulated in osteosarcoma patients (187,188). Both studies demonstrated that expression of *SNHG12* correlated with expression of *AMOT* (angiomotin), a gene with unknown functions in osteosarcoma. While modulation of *SNHG12* substantially altered expression of *AMOT*, the mechanism of regulation is not clear, and the function of this lncRNA and AMOThas not been clearly established (187). Similarly, the lncRNA *HULC* (Highly Upregulated in Liver Cancer) is upregulated in osteosarcoma tissues (189), and knockdown of *HULC* in osteosarcoma cell lines *in vitro* suppressed proliferation, migration, and invasion. In contrast, the lncRNA *TUSC7* (Tumor Suppressor Candidate 7), also known as *LOC285194*, is substantially downregulated in osteosarcoma, and low expression of this lncRNA correlates with poor survival in osteosarcoma patients (190,191). Interestingly, expression changes in this lncRNA seemed

to correlate with copy number alterations rather than an epigenetic silencing of the genes. In contrast to *MALAT-1*, knockdown of *TUSC7* or *LOC285194* led to increased proliferation and/or decreased apoptosis, and *in vivo* xenograft studies showed that *TUSC7* silencing promoted tumor growth. Last, the promoter-upstream transcript *HIF2PUT* has been proposed to regulate expression of *HIF2A* in osteosarcoma (192). While the expression of *HIF2PUT* and *HIF2A* in osteosarcoma appeared to be relatively weakly correlated, knockdown of *HIF2PUT* increased cell proliferation and invasion *in vitro* in an osteosarcoma cell line, suggesting that this class of IncRNAs may also function in osteosarcoma.

In addition, IncRNAs that function in chondrocyte or osteoblast differentiation and homeostasis have also been found to be dysregulated in osteosarcoma, including *DANCR, PACER, HOTAIR, HOTTIP*, and *H19*. Much like *MALAT-1*, knockdown of *DANCR* in osteosarcoma cell lines reduced cell proliferation and resulted in cell cycle arrest (193). Similarly, the IncRNAs *PACER, HOTAIR*, and *HOTTIP* were shown to be upregulated in osteosarcoma, and knockdown of these IncRNAs inhibited proliferation and/or invasion of osteosarcoma cells (194–196). Mechanistic investigation of *PACER* function demonstrated that it upregulates the expression of the oncogene *COX2* in an NF-κB-dependent manner. A role for *H19* in osteosarcoma has been proposed by Chan and colleagues, who developed a model for spontaneous development of osteoblastic osteosarcoma in which *p53*-heterozygous mice containing an osteoblast-specific inactivation of *Patch1* results in partial upregulation of Hedgehog signaling (173). In this model, the Hippo pathway transcriptional coactivator yes-associated protein 1 (Yap1) was upregulated by Hedgehog signaling, and in turn upregulates *H19*. Interestingly, loss of imprinting at the *H19-IGF2* has previously been observed in osteosarcoma (197), suggesting a potential function for *H19* and genomic imprinting in this setting.

Finally, other clinical investigations have revealed correlations between IncRNA expression levels and osteosarcoma patient survival. Decreased expression of *MEG3* (198) and upregulation of *TUG1* (Taurine-Upregulated Gene 1) (199) correlate strongly with poor prognosis in osteosarcoma, while the IncRNA *ODRUL* (Osteosarcoma Doxorubicin-Resistance Upregulated LncRNA) correlates strongly with poor response to doxorubicin chemotherapy (200). These preliminary data suggested that we may eventually be able to utilize IncRNAs as predictive biomarkers.

Characterization of IncRNAs

The field of IncRNA biology is relatively young by scientific standards, and significant investments are still being made to identify new IncRNAs across different tissues and among species. Many techniques that have been utilized to study well-characterized IncRNAs for some time will continue to be used to interrogate the functions of these newly identified IncRNAs. However, advances in other areas such as next-generation sequencing have improved our ability to characterize IncRNAs and have drastically improved assay throughput. Various approaches being utilized to determine IncRNA localization and function will be discussed in this section.

Identification of novel lncRNAs

While protein-coding genes can be predicted computationally from genomic sequences by virtue of their coding capacity and high levels of conservation, the identification of new lncRNAs cannot be performed in this manner, as noncoding transcripts lack these features. Notably, although comparisons of known lncRNAs demonstrated some degree of positive selection and conservation of secondary structure (201–203), predictive algorithms have a high false-positive rate for identification of new lncRNAs (204, 205). Furthermore, some well-characterized lncRNAs such as *HOTAIR* show highly divergent sequences and gene structures across species and are missed by these predictions, yet the function appears to be conserved (101).

As a result of this and significant advances in sequencing-based technologies and analysis pipelines, RNA sequencing (RNA-seq) experiments remain the gold standard for identification of lncRNAs and have been implemented to identify novel lncRNAs across a variety of tissues and species. While sufficient depth of sequencing (100–200 million reads) is a critical parameter for high-quality studies seeking to exhaustively identify novel low-abundance lncRNAs in a given tissue (206), lower-coverage experiments such as those utilized by Iyer and colleagues (207) can be pooled together to facilitate preliminary identification of novel transcripts. Identification of novel lncRNAs from RNA-seq datasets typically relies on analytical pipelines such as TopHat, which aligns reads that may contain splice junctions to the genome, and Cufflinks, which assembles transcripts *de novo* using these alignments (208,209).

In addition to these first-pass strategies for identification of novel lncRNAs, additional methods are typically used to define the ends of the transcript and to validate the presence of additional splice isoforms. In many cases, these methods have been updated such that high-throughput sequencing-based methods may be used. While isoforms for a single gene can be identified from cDNA by PCR, detailed interrogation of many loci of interest can be accomplished utilizing protocols such as Capture-Seq, which enrich for sequences of interest using hybridization-based methods and significantly reduce the required depth of sequencing for identification of novel transcripts (54). In addition, identification of the 5′ end of the transcript, which is often underrepresented in sequencing libraries, using rapid amplification of cDNA ends (RACE) can be parallelized using methods like Deep-RACE (210).

Identifying clues to lncRNA function

Perhaps the most telling indicator of lncRNA function is its subcellular localization, as this provides insight into its potential binding partners and signals its capacity for re-wiring gene regulation at the transcriptional or post-transcriptional levels. A prominent method for lncRNA localization is single-molecule RNA *in situ* hybridization. When combined with fluorescence microscopy, these techniques can provide information relating to not only whether the lncRNA is nuclear or cytoplasmic, but whether it is present near putative mRNA or genomic targets within the cell (211). Furthermore, this methodology can also allow one to estimate the range of expression among single cells, and as a result, to more rigorously quantify the lncRNA of interest (212). Other methods relying on cellular fractionation can

yield additional complementary information regarding lncRNA function. While the functions of ribosome-associated lncRNAs are not yet clear, ribosome profiling and ribosome fractionation experiments identified a number of ribosome-associated lncRNAs (213), and nuclear fractionation studies revealed a large number of lncRNAs associated to actively transcribed chromatin (214).

Identification of the DNA, RNA, and/or proteins that interact with a given lncRNA generates a second tier of information regarding its function (215). Unbiased approaches to identify these partners often rely on hybridization-based methods to isolate the lncRNA and its binding partners from the cell. Subsequently, next-generation sequencing can be used to identify interactions between other RNA molecules using RNA antisense purification (RAP-seq; (216)), chromatin domains using chromatin isolation by RNA purification (ChIRP; (217)) or capture hybridization analysis of RNA targets (CHART; (218)), or proteins using a wide range of RNA pulldown techniques coupled to mass spectrometry [RAP-MS; (219); Csy4 nuclease coupled by MS; (220)]. Notably, more direct interrogation of the functions of nuclear lncRNAs in regulation of individual loci is now possible with the development of the CRISPR-Display technique (221). This technique uses locus-specific guide RNAs to target a Cas9–lncRNA complex to the desired genomic location, which not only facilitates the detailed characterization of the lncRNA on the locus of interest, but also allows the identification of specific features within the lncRNA that are required for its function.

A third powerful predictor of lncRNA function is the secondary and tertiary structure of the transcript. For example, stem-loop structures are frequently present in ncRNAs that interact with the Polycomb complex protein PRC2, a key epigenetic regulator (33). As mentioned previously, a variety of methods are available to predict RNA secondary structure (202,203,222). However, more rigorous experimental analysis of RNA secondary and tertiary structure is frequently performed using chemical and enzymatic probes. Much like the previously described methods, these methods have been recently adapted to be compatible with next-generation sequencing technologies, yielding techniques like Structure-seq, SHAPE-seq, and FragSeq (223–225).

Finally, while RNA-seq is a powerful technique for identifying new lncRNAs, it also proves useful in predicting potential functions of lncRNAs *in vivo* through the identification of co-regulated gene networks. By identifying mRNAs that are regulated similarly to the lncRNAs of interest in response to developmental or environmental stimuli, a network of genes affecting specific pathways can be developed and tested using these techniques (226).

Validation of lncRNA function through reverse genetics

Probing the functions of lncRNAs using reverse genetics *in vivo* is complex, but a number of techniques can be used easily *in vitro* and *in vivo*. Antisense knockdown of lncRNAs has become commonplace, and techniques exist for robust knockdown of both nuclear and cytoplasmic lncRNAs (227,228). Furthermore, *in vivo* electroporation of short hairpin RNAs (shRNAs) to knockdown lncRNAs have also been used to target the *Miat* lncRNA, suggesting that this may be a relatively useful tool for characterizing lncRNA function. However, strategies relying on RNA interference may be limited by delivery of the siRNA or

shRNA, low knockdown efficiency, and off-target effects, and the importance of including well-designed experimental controls can complicate the design of *in vivo* studies.

The utility of KO animal models for protein-coding genes has been essential for thorough investigations of their functions, but the design of similar models for lncRNAs is much more challenging. Exon-deletion strategies and the use of CRISPR-Cas technologies to introduce frameshifts in protein-coding genes have been very successful (229), but these are likely much less effective for lncRNAs, as there is no ORF to be altered and a lncRNA may retain its function even if significant regions are deleted (21,230–232). Furthermore, as the entire genomic region encoding the lncRNA needs to be removed in order to generate a true null mutant, it is especially important to ensure that regulatory regions that may be encoded within the locus are not perturbed.

Despite these challenges, a number of KO animal models have been developed for lncRNAs and have been reviewed elsewhere (233). In addition, a number of lncRNAs have been modeled successfully by a variety of genetic manipulations including gene disruptions [*Xist*; (234)], targeted promoter deletions [*Kcnq1ot1*; (235)], premature termination strategies [*Kcnq1ot1*; (28)], and the previously mentioned *in vivo* knockdown strategies [*Miat*; (236)]. Thus, as with other genetic models in cell culture and *in vivo*, recent advances in CRISPR-Cas technologies, especially those improving efficiency of targeted knock-in strategies, will likely improve the speed at which lncRNA targeting studies can be accomplished (237).

Conclusions and future studies

Although the half-lives of lncRNAs are, in general, less than those of mRNA (238), they are being recognized as potentially important biomarkers for a number of diseases, particularly cancers (239). For example, one of the most specific biomarkers for human prostate cancer identified to date is the lncRNA, *PCA3* (prostate cancer antigen 3) (240,241). Circulating lncRNAs have been detected in whole blood, plasma, serum, and urine and have also recently been proposed as biomarkers for diseases such as gastric cancer and heart failure (242,243). LncRNAs have also been found in extracellular vesicles (i.e., exosomes), which likely increases their half-life *in vivo*, and thus their potential as a biomarker (244–246). In this regard, lncRNAs could potentially serve as a biomarker of OA or repair processes in the joint, although to date, little is known regarding the relationship between lncRNAs and OA severity. Similarly, future studies will inform us of whether or not lncRNAs could be used as predictive or prognostic biomarkers of bone diseases such as osteoporosis.

Given the size and cellular location of lncRNAs, challenges exist to attempt to knockdown or overexpress these ncRNAs as a means to treat disease. However, we can employ the knowledge acquired from manipulating protein-coding gene expression. In this regard, there are various ways to alter the expression of lncRNAs, including the use of viral or non-viral vectors, RNA-based or genome-editing approaches. While there have been approximately 1800 approved gene therapies in clinical trials from 1989 to 2012 (247), viral-based vectors face major limitations, most notably insertional mutagenesis and immunogenicity. For this reason, the use of non-viral vectors is an attractive alternative. While typical delivery systems (such as lipofectamine or polyethyleneimine) are associated with low efficiency and

cytotoxicity (248,249), recent advances have presented the field with new improvements, such as chitosan for non-viral gene delivery that is both efficient and non-toxic (250). In addition, other methods to alter gene expression have also been explored. In the field of RNA therapeutics, it was demonstrated that higher potency and lower dose administration can be achieved with engineered antisense oligonucleotides, such as locked nucleic acid (LNA)-based gapmers (251), multimers, or multi-targeting oligonucleotides (252). Finally, lncRNA expression can potentially be controlled with modified CRISPR–Cas9 complexes. CRISPR (Clustered Regularly Interspaced Palindromic Repeats) and its associated nuclease Cas9 is a powerful and breakthrough genome editing tool. One of the many uses of CRISPR-Cas9 is knocking out protein-coding genes, mainly by introducing frame-shift mutations. However, the system is posed with difficulty when it comes to knocking out lncRNAs due to their lack of an ORF. Nevertheless, this can be achieved by establishing dual cuts to remove the entire gene fragment (237). Alternatively, modified CRISPR-dCas9 systems can also be exploited to enhance or reduce lncRNA expression. A derivative of Cas9, dCas9 is engineered to be nuclease deficient, and can be fused with various effectors to achieve desired transcriptional control. This includes, but not limited to, dCas9-KRAB (253,254) for gene repression and dCas9-VP48 (255), dCas9-VP64 (256) (257), dCas9-p300 (258), dCas9-VP64/p65 (259) for gene activation.

Clearly, lncRNAs play significant roles in regulating cellular fate and function, and in many cases, their dysregulation is associated with disease. With respect to chondrogenesis and osteogenesis, research on how lncRNAs regulate differentiation of stem/progenitor cells toward the chondrocyte or osteoblast lineage is still in its infancy. We predict that research interests in this area will significantly increase over the coming years and provide us with important knowledge on new players involved in the regulation of cartilage and bone development. Similarly, nothing is known yet on how lncRNAs could potentially control homeostasis of mature cartilage or bone; such discoveries will have a profound impact on understanding skeletal diseases such as OA and osteoporosis, and in determining new therapeutic strategies to treat these conditions. Given the size and cellular location of lncRNAs, challenges exist to attempt to knockdown or overexpress these ncRNAs as a means to treat disease. However, through advancing technologies, it is anticipated that novel, effective approaches to modulate lncRNA expression or function *in vivo* will soon be discovered.

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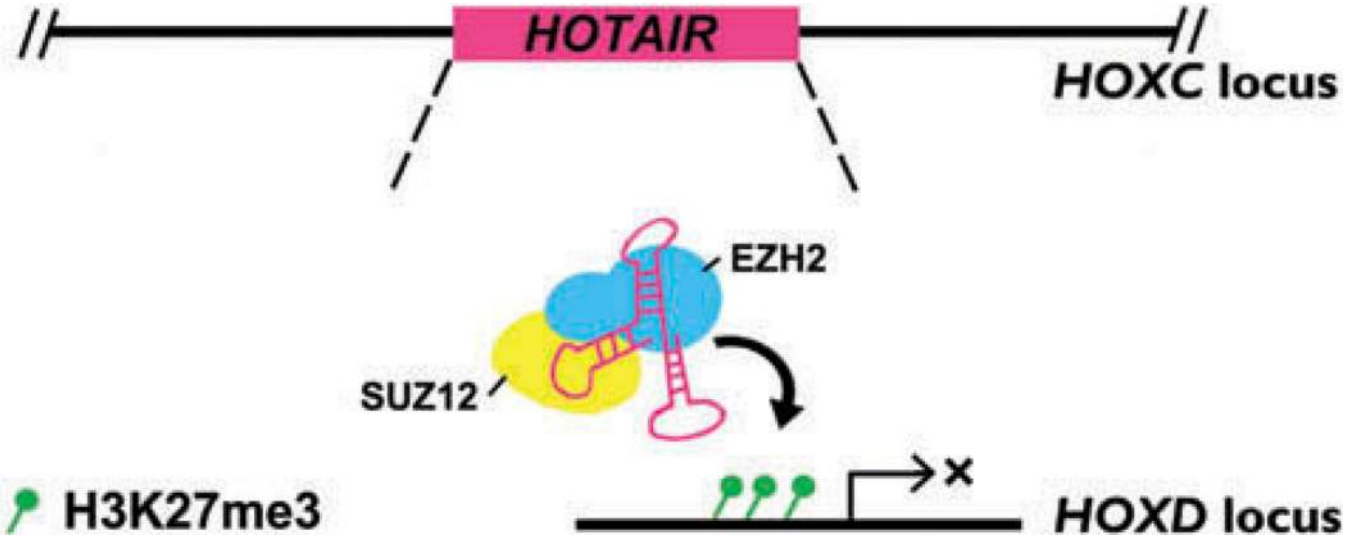

Figure 1.

HOTAIR function in regulating HOXD gene expression. Expressed from the HOXC locus, HOTAIR binds to SUZ12 and EZH2, part of the PRC2 complex. HOTAIR acts *in trans* by recruiting the PRC2 complex to the HOXD locus and silences the locus by establishing H3K27me3 marks (30).

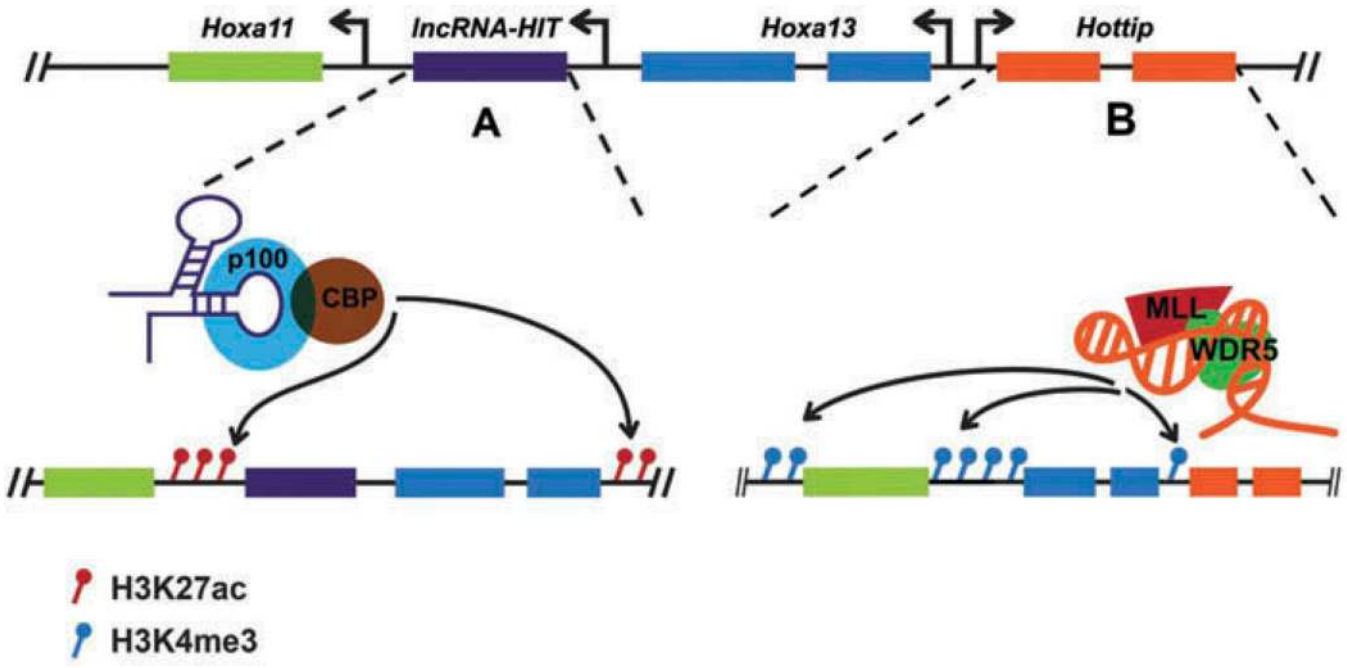

Figure 2.

IncRNA-HIT and Hottip function in regulating Hoxa gene expression. (A) IncRNA-HIT binds to the p100/CBP complex and maintains H3K27ac marks at HoxA genes, resulting in gene activation (103). (B) Hottip acts *in cis* by recruiting the WDR5/MLL histone methyltransferase complex to the Hoxa genes, resulting in gene activation by establishing H3K4me3 marks (31).

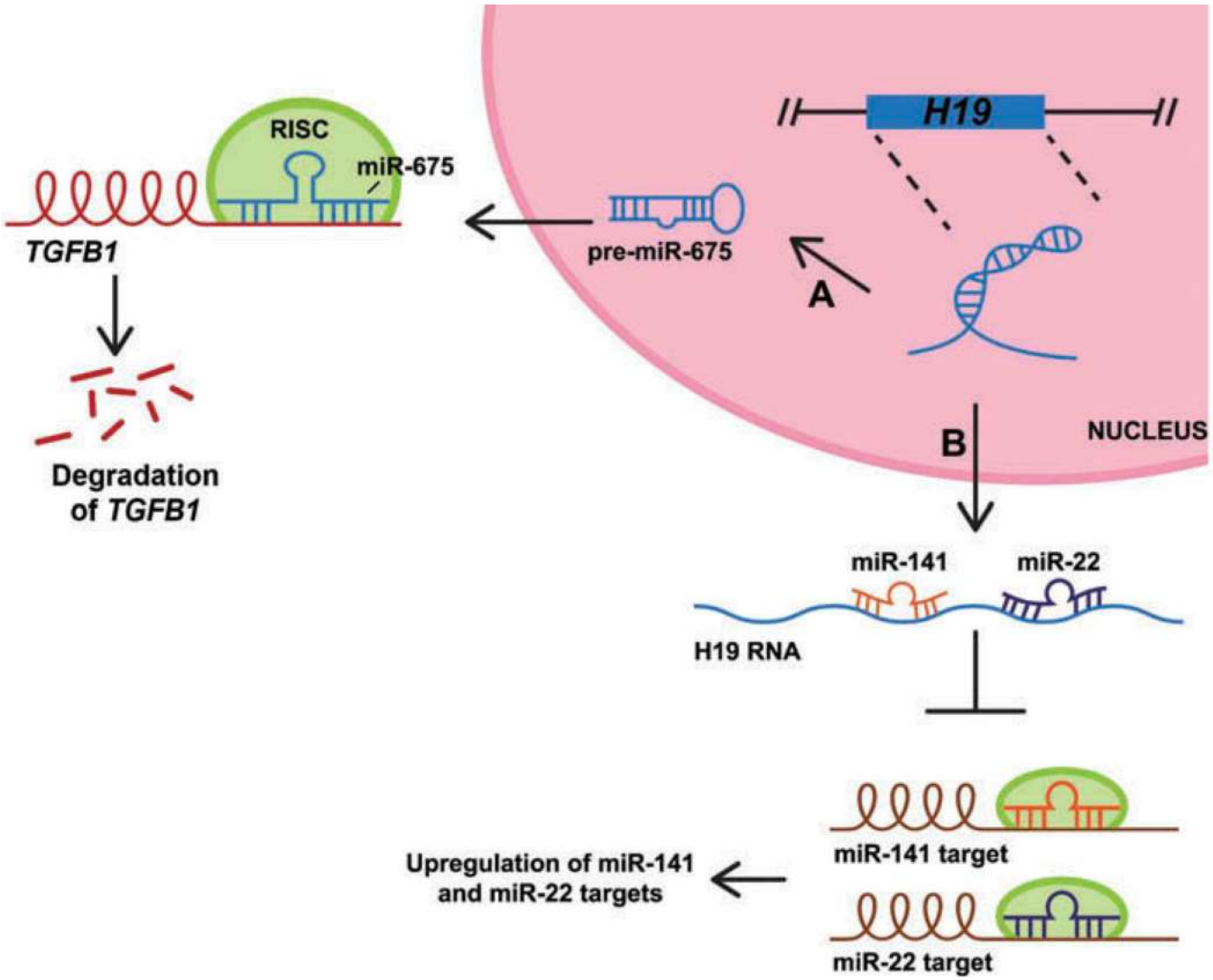

Figure 3.

Different mechanisms of H19 in osteogenesis. (A) H19 is processed into miR-675, which targets TGFB1 for degradation (135). (B) H19 acts as a sponge to sequester miR-141 and miR-22 away from their targets, thus leading to an upregulation of targeted mRNAs that may be required for osteoblast differentiation (134).

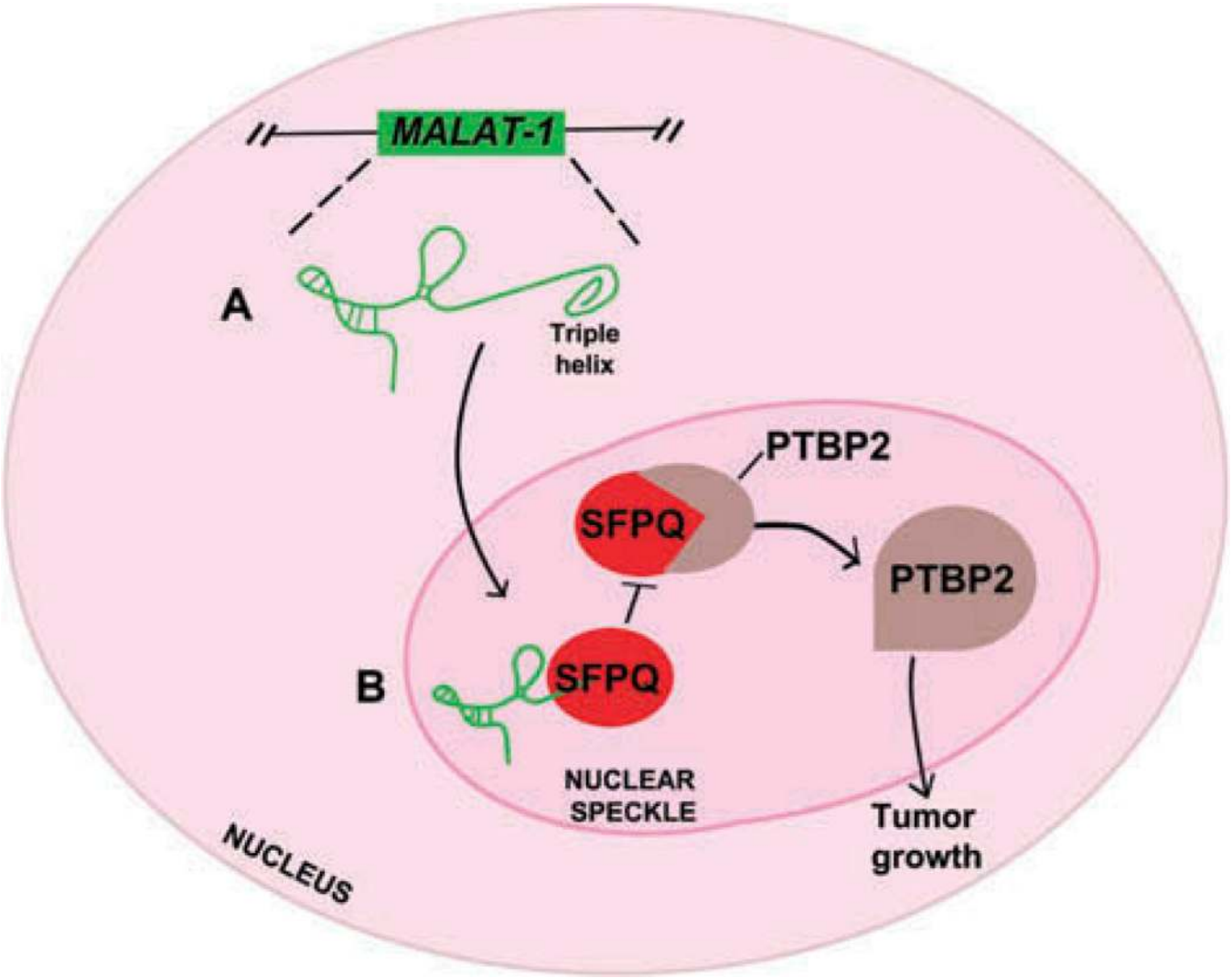

Figure 4.

MALAT-1 function in osteosarcoma. (A) MALAT-1 lncRNA is not polyadenylated, but instead contains a triple helical domain at its 3′ end that protects it from degradation (180). (B) MALAT-1 binds to SFPQ in nuclear speckles. This sequestration prevents the formation of the SFPQ/PTBP2 complex. Subsequently, unbound PTBP2 can induce tumor growth by increasing cellular proliferation and migration (185).

Table 1

Summary of identified IncRNAs involved in skeletal biology and disease.

Gene name	Gene symbol	ENSEMBL Gene ID	Expression pattern	Proposed function	Reference
Chondrogenesis associated transcript	*DA125942* Synonym: *CISTR*	ENSG00000260492	Genetic mutation results in brachydactyly type E (BDE)	Interacts with PTHLH *in trans* and with SOX9 *in cis*	(93)
Differentiation antagonizing non-protein coding RNA	*DANCR* Synonym: *ANCR*	ENSG00000226950	Upregulated during chondrocyte differentiation	May promote cell proliferation and differentiation toward the chondrocyte lineage	(90)
			Downregulated during osteogenesis	Recruits EZH2 to the RUNX2 promoter and suppresses RUNX2 expression	(165)
				Inhibits osteogenesis via suppression of Wnt/βcatenin pathway.	(166)
			Upregulated in osteosarcoma		(193)
F0XC2 antisense RNA 1 Synonym: Osteosarcoma doxorubicin-resistance upregulated IncRNA	*FOXC2-AS1* Synonym: *ODRUL*	ENSG00000260944	Expression correlates with poor response to doxorubicin chemotherapy		(200)
Growth arrest-specific 5	*GAS5*	ENSG00000234741	Primarily expressed in the nucleus of healthy chondrocytes, and in the cytoplasm of OA chondrocytes	Induces catabolic effect by regulating miR-21	(142)
			Elevated in OA chondrocytes		(138, 139)
H19, imprinted maternally expressed transcript (*Mus musculus*)	*H19*	ENSMUSG00000000031	*H19* is upregulated by overexpression of *Hh* and *Yap1*, Overexpression of *Yap1* and *H19* is responsible for the pathogenesis of osteoblastic osteosarcoma		(173)
H19, imprinted maternally expressed transcript (*Homo sapiens*)	*H19*	ENSG00000130600	Highly expressed in mature chondrocytes	Functions downstream of SOX9. *H19* is processed into miR-675 that subsequently upregulates *COL2A1*.	(133)
			Elevated in OA chondrocytes		(134, 138, 139)
			Upregulated in osteogenesis	*H19* is processed into miR-675, which targets *TGFB1* for degradation.	(136)
				H19 acts as a ceRNA, binding to miR-141 and miR-22, thus preventing their binding to target mRNAs.	(135)
HIF1A antisense RNA	*HIF1A-AS1*	ENSG00000258777		Knockdown of *HIF1a-AS1* decreases *HOXD10*	(168)

Gene name	Gene symbol	ENSEMBL Gene ID	Expression pattern	Proposed function	Reference
				expression	
Highly upregulated in liver cancer conserved region	*HULC*	ENSG00000276019	Upregulated in osteosarcoma		(189)
HOX transcript antisense RNA (*Mus musculus*)	*Hotair*	ENSMUSG00000086903	Hotair knockout mice exhibit patterning malformations in the limbs and lumbosacral junction	Recruits EZH2 and SUZ12 to the HoxD cluster and silences the locus.	(101)
HOX transcript antisense RNA (*Homo sapiens*)	*HOTAIR*	ENSG00000228630	Elevated in OA chondrocytes	Recruits EZH2 and SUZ12 to the HoxD cluster and silences the locus.	(138, 139)
			Upregulated in osteosarcoma		(195)
			Decreased in AVICs by cyclic stretch, leading to calcification	β-catenin is a stretch responsive signaling pathway that represses *HOTAIR*	(102)
HOXA transcript at the distal tip	*HOTTIP*	ENSG00000243766	Elevated in OA chondrocytes		(139)
			Upregulated in osteosarcoma		(194)
HOXA transcript induced by TGFβ	*lncRNA-HIT* Synonym: *9530018H14RIK*	ENSMUSG00000102373	Expressed in the developing mouse limb	Binds to p100/CBP complexes to maintain active chromatin mark H3K27ac at the *HoxA* locus	(104)
KCNK15 antisense RNA 1	*KCNK15-AS1* Synonym: *RP11-445H22.4*	ENSG00000244558	Elevated in OA chondrocytes		(138)
Long intergenic non-protein coding RNA 707	*LINC00707*	ENSG00000238266	Downregulated during chondrogenesis		(87)
Long intergenic non-protein coding RNA 1589	UNC01589 Synonym: *CTA-941F9.9*	ENSG00000238120	Upregulated during chondrogenesis		(87)
Maternally expressed gene 3	*MEG3*	ENSG00000214548	Downregulated in osteoarthritis chondrocytes		(144)
			Increased during osteogenic differentiation	Upregulates *BMP4* by disrupting the interaction between the SOX2 and BMP4 promoter.	(170)
			Decreased expression of *MEG3* is correlated with poor prognosis in osteosarcoma		(198)
Metastasis-associated lung adenocarcinoma transcript 1	*MALAT1*	ENSG00000278217	Upregulated in osteosarcoma		(183, 184)
			Downregulated in MG63 osteosarcoma cell line by high-dose estrogen	Sequesters SFPQ and disrupts the SFPQ/PTBP2 complex, resulting in PTBP2-mediated increases in cellular proliferation and migration.	(180, 185)

Gene name	Gene symbol	ENSEMBL Gene ID	Expression pattern	Proposed function	Reference
Not assigned	*CTD2574D22.4*	ENSG00000260114	Elevated in OA chondrocytes		(138)
Not assigned	*RP11–815M8.1*	ENSG00000238042	Upregulated during chondrogenesis		(87)
PMS1 homolog 2, mismatch repair system component pseudogene 2	*PMS2L2*	ENSG00000278416	Elevated in OA chondrocytes		(138)
PTGS2 antisense NFKB1 complex-mediated expression regulator RNA Synonym: PS-associated COX2 extragenic RNA	*PACERR* Synonym:	ENSG00000273129	Increased following 1L1-β treatment of primary OA chondrocytes		(260)
	PACER, PTGS2-AS1		Upregulated in osteosarcoma	Upregulates oncogene COX2 in an NF-KB dependent manner	(196)
Small nucleolar RNA host gene 12	*SNHG12*	ENSG00000197989	Upregulated in osteosarcoma		(187, 188)
Taurine upregulated 1	*TUG1*	ENSG00000253352	Increased expression is correlated with poor prognosis in osteosarcoma		(199)
Tumor-suppressor candidate 7	*TUSC7* Synonym: *LSAMP-AS3 LOC285194*	ENSG00000243197	Downregulated in osteosarcoma		(190, 191)
Vimentin 2, pseudogene Synonym: Cartilage injury-related IncRNA	*VIM2P* Synonym: *IncRNA-CIR RP11-162L10.1*	ENSG00000220548	Downregulated in OA chondrocytes		(141)
ZBED3 antisense RNA 1	*ZBED3-AS1*	ENSG00000250802	Upregulated during chondrogenesis		(87)