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## Noncoding RNAs Involved in Mammary Gland Development and Tumorigenesis: There's a Long Way to Go

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### Abstract

The mammalian genome encodes thousands of noncoding RNAs. These noncoding transcripts are broadly categorized into short noncoding RNAs, such as micro-RNAs (miRNAs), and long noncoding RNAs (lncRNAs) of greater than 200 nt. While the role of miRNAs in development and cancer biology has been extensively studied, much less is known about the vast majority of noncoding transcripts represented by lncRNAs. lncRNAs are emerging as key regulators of developmental processes and as such, their frequent misregulation in tumorigenesis and disease is not unexpected. The role of lncRNAs in mammary gland development and breast cancer is just beginning to be elucidated. This review will discuss the role of lncRNAs in mammalian and mammary gland development. In addition, we will review the contributions of lncRNAs to the stepwise progression of tumorigenesis, highlighting the role of lncRNAs in breast cancer.

### Keywords

Noncoding RNA; Mammary gland development; Alveologenesis; Epigenetics; Breast cancer

### Introduction

Although coding regions occupy less than 2% of the mammalian genome, a much larger portion of the genome is transcribed [1–5]. The initial reports of this pervasive transcription generated some controversy regarding their biological relevance. However, several large scale analyses have since provided evidence that many noncoding transcripts are highly regulated and functional [6–9].

In the past decade, there has been a profound shift in our understanding of the role of RNA molecules. They were once regarded as mere templates for generating proteins, but

noncoding RNAs are emerging as highly versatile molecules that potentially function at every level of cellular regulation. The expanding catalogue of noncoding RNAs provides evidence that they are becoming as vast and varied as their protein-coding mRNA counterparts. Just as there are ubiquitously expressed housekeeping proteins, there are also housekeeping noncoding RNAs including, ribosomal, transfer, small nuclear and small nucleolar RNAs. On the other hand, regulatory noncoding RNAs generally show restricted patterns of expression during development and are frequently involved in regulating gene expression. Regulatory noncoding RNAs includes short RNAs (<200 nt) such as, small interfering RNAs, Piwi-associated RNAs, and of course, microRNAs. This review will focus on another class of regulatory noncoding RNAs, long noncoding RNAs (>200 nt), which have recently moved to the forefront of noncoding RNA research.

Long noncoding RNAs (lncRNAs), like mRNAs, can be capped, spliced and polyadenylated and fall into a variety of categories based on their genomic context as well as their functions. Some are located sense or antisense to other transcriptional units. Others are bidirectional, being transcribed near another gene from the opposite strand. lncRNAs can also be located within the intron of another transcript. Alternatively, intergenic noncoding RNAs lie between two genes or sets of genes. Functionally, lncRNAs have been shown to affect a wide range of cellular processes, including splicing [10], localization [11], transcription [12], survival [13], cell cycle [14], migration [15], metabolism [16], and organization of cellular compartments [17]. However, for the relatively small fraction of lncRNAs whose functions have been revealed, their predominant role seems to be the regulation of gene expression.

lncRNAs utilize a large arsenal of mechanisms to regulate gene expression. One well-studied mechanism is transcriptional interference, where the act of transcribing a lncRNA interferes with transcription initiation, elongation or termination of another sense or antisense gene [18]. Some lncRNAs act as decoys, containing sequences that mimic transcription factor binding sites [16] or miRNA target sites [19] to titrate these factors away from their primary targets thereby inhibiting their function. Other lncRNAs act as co-activators, binding to transcription factors and enhancing their transcriptional activity [12, 20, 21]. lncRNAs can also affect transcription by binding to transcription factors and shuttling them into the cytoplasm to keep them away from their nuclear targets [11]. Recent evidence also suggests that some lncRNAs may have enhancer-like function [22], activating expression of nearby genes by an unknown mechanism.

Many lncRNAs interact with chromatin-modifying complexes to regulate gene expression. The idea that lncRNAs associate with chromatin-modifying complexes initially came from studies of X chromosome inactivation and genomic imprinting, but more recent global analyses have revealed that this may be a more universal characteristic of lncRNAs than originally anticipated. Almost 40% of large intergenic noncoding RNAs (lincRNAs) interact with chromatin-modifying complexes, such as the Polycomb Repressive Complex 2 (PRC2) and the CoREST/REST repressor complex [23]. At least 24% of lincRNAs interact specifically with PRC2 [23] and a recent study has identified thousands of additional lncRNAs that interact with PRC2 [24], many of which are not intergenic RNAs. In addition, several lncRNAs have been shown to interact with the histone methyltransferase associated with the activating trithorax complex, MLL1 [25, 26], and the H3-K9 methyltransferase, G9a [27, 28]. While the functional relevance of these interactions is not yet well understood, several lncRNAs have also been shown to be required for targeting of these complexes to specific loci, either in *cis* or in *trans*, to regulate gene expression [27, 29–31]. Precisely how lncRNAs interact with chromatin-remodeling complexes and how they target specific genes is still largely unknown.

The predominant role of lncRNAs in regulating gene expression, combined with their strict spatiotemporal expression patterns, suggests that they may have a significant role in regulating key processes during development. Indeed, mechanistic evidence is accumulating for the role of lncRNAs in regulating differentiation and development, often epigenetically [32–34]. In addition, numerous lncRNAs have been identified that are misregulated in cancer [35–37]. However, the mechanisms of action of lncRNAs in tumor initiation and progression are largely unknown. In this review, we will discuss the role of lncRNAs during development and tumorigenesis, emphasizing what is currently known in the field of mammary gland biology.

## LncRNAs and Development

### LncRNAs Regulating Differentiation and Development

LncRNAs were originally discovered in association with distinct epigenetic processes, such as X chromosome inactivation in dosage compensation and genomic imprinting, and were therefore regarded by many as anomalies in the field of gene regulation. In mammals, dosage compensation is the process of inactivation of a single X chromosome in female cells to equalize X-linked gene expression with that of male cells. It involves a complex interplay of several lncRNAs including *Xist*, *Jpx*, *RepA* and *Tsix*. These lncRNAs recruit chromatin-modifying complexes to the targeted X chromosome, leading to histone modifications, heterochromatin formation and silencing of the targeted X chromosome [38, 39]. Genomic imprinting is the process whereby certain genes undergo monoallelic silencing during gametogenesis, and are therefore only expressed from the maternal or paternal allele for the lifetime of the organism. Imprinting, like X-inactivation, is mediated by chromatin-remodeling complexes and has been shown to require lncRNAs, such as *Kcnq1ot1* and *Airn* (formerly known as *Air*), that are transcribed from the imprinted loci and silence genes in cis [40]. X inactivation and genomic imprinting are essential developmental processes whose mechanisms have been, and continue to be, studied intensely. While these processes were initially considered unique, an increasing number of lncRNAs are being discovered that interact with chromatin-modifying complexes, and it is now apparent that the lncRNAs associated with X-inactivation and imprinting serve as paradigms to understand precisely how lncRNAs can function as epigenetic regulators.

LncRNAs also play a role in regulating the delicate balance between stem cell self-renewal and differentiation during embryogenesis. Large-scale analysis of mouse embryonic stem cells (mESCs) has identified hundreds of lncRNAs that are differentially expressed in the pluripotent state as compared to various stages of differentiation [26]. Of these, two novel lncRNAs, *Evx1as* and *Hoxb5/6as*, are transcribed from homeotic loci in differentiated ES cells and interact with the methyltransferase MLL1. In addition, the lncRNA *Mira* is thought to play a role in germ layer specification by interacting with MLL1 to activate the expression of the homeotic genes *Hoxa6* and *Hoxa7* during mESC differentiation [25]. These data suggest likely roles for lncRNAs in epigenetically regulating differentiation genes in early embryonic development. In another recent study, functional analysis was carried out on over 200 lincRNAs that are expressed in mESCs [41]. Knockdown of many of these lincRNAs resulted in drastic changes in gene expression in *trans*. Furthermore, knockdown of dozens of these lincRNAs led to the reduced expression of pluripotency genes as well as the induction of differentiation genes, suggesting that these lincRNAs are indeed functioning in the maintenance of the pluripotent state of mESCs. Finally, these investigators also showed that many of these lincRNAs are themselves regulated by pluripotency-associated transcription factors, such as Oct4, Nanog and Sox2. These same transcription factors were reported previously to regulate the expression of other lncRNAs in mESCs [8, 42]. Together, these studies demonstrate lncRNAs function to regulate cellular homeostasis during embryonic development.

Many lncRNAs are regulated both spatially and temporally. Thus, it is not surprising that lncRNAs are also involved in regulating proliferation and differentiation of specific stem and progenitor populations during organogenesis. For example, the lncRNAs *EGO*, *HOTAIRM1* and *LincRNA-EPS* are induced by, and required for, differentiation of distinct lineages during hematopoiesis [43–45]. In addition, global analysis has revealed a large number of lncRNAs that are expressed in the brain and nervous system [7, 46–48] and some lncRNAs have been shown to be necessary for proper neural development. For instance, loss of *Evf2* in mice leads to reduced numbers of GABAergic interneurons in early development and disrupts formation of GABA-dependent neuronal circuitry in the adult brain [49]. Other lncRNAs, such as *Tug1* and *RNCR* (also known as *MIAT* or *Gomafu*), regulate specification and differentiation of various cell types in the developing retina [50, 51]. lncRNAs also have been found that regulate muscle differentiation by novel mechanisms. For example, *Lnc-MD1* acts as a competing endogenous RNA (ceRNA) that binds miRNAs, which normally repress myogenic transcription factors, and inhibits their function, thereby promoting muscle differentiation [19]. Another recent study identified an lncRNA called *ANCR*, which is downregulated during epidermal differentiation and is required to maintain epidermal progenitors [52]. Since the functions of only a relatively small fraction of lncRNAs currently are known, thousands of lncRNAs still have putative undetermined roles in important developmental processes.

### **LncRNAs Regulating Mammary Gland Development**

The mammary gland begins as the epithelial layer of the skin folds inward to form the mammary bud during embryonic development [53]. At birth, the mammary fat pad contains a simple, branched epithelial structure that quiescently lies beneath the nipple until puberty. During puberty, the mammary epithelial cells of the terminal end buds proliferate and drive ductal elongation toward the ends of the fat pad resulting in a branched tree-like structure that fills the fat pad of the adult mammary gland. The virgin mammary gland then remains mostly quiescent until pregnancy.

In the post-pubertal mammary gland, pregnancies result in dramatic cycles of hormonally-regulated proliferation, differentiation, and apoptosis of mammary epithelial cells making the mammary gland an excellent model to study essential developmental processes such as stem and progenitor cell regulation and cell fate commitment [54–57]. In early to mid-pregnancy, mammary epithelial cells undergo proliferation as they expand to generate lobulo-alveolar structures. During mid to late-pregnancy, proliferation slows as mammary epithelial cells begin to differentiate into alveolar cells. At parturition, alveolar cells undergo terminal differentiation to produce and secrete the high volume of milk required to nurse the young during lactation. Finally, following lactation, most of the differentiated alveolar cells undergo apoptosis in early involution. The mammary gland remodels to a pre-pregnancy-like state and again becomes mostly quiescent until the next pregnancy.

Many coding genes, as well as a few miRNAs (see miRNA reviews in this issue), have well-documented roles in regulating embryonic, post-natal and post-pubertal mammary gland development [58, 59]. However, the role of lncRNAs remains largely unexplored. A few lncRNAs have been mentioned in the literature as having a potential role in mammary development, but without any in depth follow-up. For example, *H19* was shown to be hormonally-regulated and expressed highly in alveolar cells during pregnancy and involution, but the specific role of *H19* in alveolar cells remains unknown [60]. Additionally, the lncRNA *SRA* may also play a role in the post-pubertal mammary gland. Overexpression of human *SRA* in mammary epithelial cells of MMTV-*SRA* transgenic mice results in excessive side branching of the adult virgin transgenic gland and precocious alveolar development during pregnancy [61]. However, *SRA* is barely detectable in the

virgin mouse mammary gland and subsequent loss-of-function studies to support a role for *SRA* in mammary development are lacking.

Recently, a large-scale microarray analysis using RNA from mouse mammary glands at day 15 of pregnancy, day 7 of lactation and day 2 of involution, identified 97 lncRNAs that are differentially expressed during post-pubertal development. However, the functions of most of these lncRNAs are not yet understood [62]. Only two lncRNAs, *PINC* and *Zfas1*, have been studied more extensively in the post-pubertal mammary gland (Table 1) [62–64]. Interestingly, both of these lncRNAs are highly expressed in alveolar cells during pregnancy. Their expression decreases during lactation and increases again during early involution. *PINC* and *Zfas1* also appear to play similar roles in regulating cell cycle and differentiation of mammary epithelial cells. Each will be discussed in more detail below.

**PINC**—Pregnancy-Induced NonCoding RNA (*PINC*) is polyadenylated, alternatively spliced and conserved specifically among mammals [63]. There are two sets of splice forms of mouse *PINC* (mPINC), *mPINC1.0* and *mPINC1.6*. Knockdown of *mPINC1.0* and *mPINC1.6* individually in mammary epithelial HC11 cells leads to increased rates of apoptosis and proliferation, respectively. These data suggest *mPINC* may play a role in regulating survival and cell cycle of mammary epithelial cells.

Recent data suggest that mPINC may play a specific role in regulating alveolar differentiation during post-pubertal mouse development (Shore and Rosen, unpublished data). *mPINC* splice isoform levels are coordinately regulated, declining between late pregnancy and early lactation in the mammary gland when alveolar cells undergo terminal secretory differentiation. Interestingly, *mPINC* levels also decrease upon lactogenic differentiation of HC11 cells following hormone treatment. This reduction of *mPINC* expression may be required for lactation as overexpression of *mPINC* inhibits lactogenic differentiation of HC11 cells, while conversely knockdown of *mPINC* enhances differentiation.

*PINC* and retinoblastoma associated protein 46 (*RbAp46*) were originally identified as genes that are persistently upregulated following pregnancy in the rat mammary gland and thought to potentially mediate the protective effect of an early pregnancy [64]. *PINC* and *RbAp46* are both expressed in alveolar cells during pregnancy and their expression persists in the regressed lobules of the involuted rat mammary gland. *RbAp46* is an important regulator of development and a potential tumor suppressor [65–67]. *RbAp46* also associates with the chromatin-modifying PRC2 complex that has been shown to interact with many lncRNAs [68]. Using RNA immunoprecipitation assays, we found that *mPINC* splice forms interact specifically with *RbAp46* and other PRC2 members in HC11 cells and in mammary epithelial cells isolated from day 16 pregnant mammary gland (Shore and Rosen, unpublished data). These data suggest a potential epigenetic mechanism for the regulation of alveolar progenitor cells by *mPINC* during pregnancy and involution of the mammary gland.

**Zfas1**—The lncRNA *Zfas1* initially was identified by microarray analyses of various stages of post-pubertal mouse mammary gland development [62]. *Zfas1* is transcribed in the antisense direction from the promoter region of the *Znfx1* gene. *Zfas1* and *Znfx1* expression is coordinately regulated in several tissues including, brain, kidney, lung and spleen. However, they show distinct patterns of expression during post-pubertal mammary development, suggesting *Zfas1* may have a distinct function from *Znfx1* in the mammary gland. The primary *Zfas1* transcript also contains snoRNAs in three consecutive introns that are differentially regulated in the post-pubertal mammary gland. However, the expression of these snoRNAs is not precisely coordinated with that of *Zfas1*. While it is likely that

transcription from this complex locus is interconnected, the mechanistic implications of this relationship are currently unknown.

*Zfas1* is highly expressed in alveolar cells of the pregnant mammary gland, but declines 10-fold during lactation. *Zfas1* expression levels also decline upon lactogenic differentiation of HC11 cells. Upon lactogenic hormone treatment, *Zfas1* knockdown leads to enhanced dome formation and increased  $\beta$ -casein levels. Knockdown of *Zfas1* in HC11 cells also results in increased proliferation. Together these data suggest *Zfas1* may be highly expressed in the late pregnant mammary gland to regulate proliferation and repress terminal differentiation of alveolar cells until parturition when *Zfas1* levels drastically decline.

## Perspectives on LncRNAs and Mammary Gland Development

*mPINC* and *Zfas1* are both expressed in alveolar cells of the pregnant and involuting mammary gland and are downregulated during lactation when alveolar cells undergo terminal secretory differentiation. In addition, *mPINC* and *Zfas1* levels decline upon hormonally-induced differentiation of HC11 cells. Furthermore, knockdown studies demonstrate that they both negatively regulate lactogenic differentiation of HC11 cells. Therefore, high expression of *mPINC* and *Zfas1* in the late pregnant gland may be necessary to prevent precocious terminal differentiation and maintain alveolar progenitors until lactation (as illustrated in Fig. 1a). Likewise, increased *mPINC* and *Zfas1* levels during early involution may function to preserve alveolar progenitors in the involuted gland so that they can rapidly differentiate into milk-producing cells in subsequent pregnancies. While the mechanism of action of *Zfas1* is unknown, we propose a model where *mPINC* interacts with PRC2 to target and repress alveolar differentiation genes epigenetically, thereby maintaining alveolar progenitors in the pregnant and involuting mammary gland (see hypothetical model in Fig. 1b).

While only a couple of lncRNAs have documented roles in mammary gland development, it is anticipated that the application of RNA-seq in mammary development and breast cancer, through The Cancer Genome Atlas (TCGA) project, will lead to the discovery of many more lncRNAs. The mammary gland is unique to mammals. It has been postulated that the mammary gland evolved from the innate immune system [69], as it provides immunological protection as well as nutrition to offspring during lactation. Previous studies have identified over a thousand lncRNAs that are highly conserved among mammals and, based on functional genomics approaches, a large portion of these have putative roles in regulating innate immunity [8]. Potentially, many of these lncRNAs have a mammalian-specific function in the mammary gland. In addition, milk protein genes and other mammary gland genes are sometimes organized in large genomic clusters that are epigenetically regulated [70, 71]. With the emerging association of lncRNAs and chromatin-modifying complexes, it would not be surprising to identify lncRNAs that govern the coordinated expression of these gene clusters during mammary development. Finally, current studies only reflect a role for lncRNAs in the post-pubertal mammary gland. However, several lncRNAs are induced by signaling pathways that are known to regulate embryonic and post-natal mammary development, such as those regulated by Notch, Wnt, Shh, and BMP [12, 72–75]. Therefore, it is likely that these pathways induce lncRNAs that contribute to early mammary development. Understanding the function of lncRNAs in regulating developmental processes in the mammary gland should help elucidate the potential roles of aberrantly expressed lncRNAs in the initiation and progression of breast cancer.

## LncRNAs in Breast Cancer

Transcriptional profiling has revealed highly aberrant lncRNA expression in human cancers [76]. In breast tumors, expression profiling suggests fundamental differences in global RNA

regulation between normal and cancer cells with the latter showing a lower percentage of genes expressing antisense transcripts [77]. The expression of highly conserved lncRNAs is altered in human cancers including breast [78], with some recent evidence of their susceptibility to mutation. Using mRNA profiling, breast cancer has reproducibly been divided into a number of clinically relevant subtypes [79, 80]. These subtypes also show differential expression of non coding RNAs including both miRNAs [81] and lncRNAs (Herschkowitz, Perou, and Rosen unpublished results). Likewise, the expression of lncRNAs can be predictive of clinical outcome and they may be useful as biomarkers. One example is *HOTAIR* (discussed below) which has been shown to be an independent predictor of breast cancer survival [15].

While the study of lncRNA function is still in its infancy, a role for a number of these transcripts has recently been established in cancer, in general, as well as specifically in breast cancer. Like protein-coding genes and miRNAs, lncRNAs play key roles in tumorigenesis. They have been shown to play a functional role in a number of fundamental processes associated with cancer including cell cycle regulation, apoptosis, the DNA damage response, and metastasis.

### Oncogenic and Tumor Suppressor LncRNAs

Like protein-coding genes, lncRNAs can function as oncogenes or tumor suppressors. Many lncRNAs shuttle between the nucleus and cytoplasm suggesting they may have dual functions, while others are restricted to the nucleus. In the nucleus, lncRNAs are often part of the nuclear architecture and in some cases are critical for the maintenance of sub-nuclear structures.

**MALAT1/NEAT2**—The nuclear-retained lncRNA *MALAT1*, which is highly expressed in many tumor types, regulates alternative splicing by modulating the phosphorylation of SR splicing factors and influencing their distribution to nuclear speckles [10]. While *MALAT1* expression is restricted to the nucleus, a small tRNA-like 61-bp ncRNA is generated by RNase P dependent cleavage of the primary transcript and exported into the cytoplasm [82]. However, the function of this processed RNA in normal development and cancer is not known.

Recent evidence also suggests that non coding RNAs are involved in the localization of transcriptional units to specific sub-nuclear architectural structures, thus regulating cellular proliferation. Specifically by binding to unmethylated Polycomb 2 protein (Pc2)/Cbx4, *MALAT1* has been shown to relocate growth control genes to interchromatin granules, which are thought to represent sites of active transcription. In contrast, the lncRNA *TUG1* binds to methylated Pc2, relocating genes to the repressive environment of Polycomb bodies [83]. Like *MALAT1*, many lncRNAs can give rise to a number of distinct processed RNAs with separate functions, any and/or all of which may contribute to their role in tumorigenesis.

**H19**—*H19* is an imprinted gene expressed exclusively from the maternal allele that maintains the silencing of *IGF2*. Aberrant expression of *H19* is observed in a number of solid tumors. The majority of breast cancers express high levels of *H19* as compared to normal tissues. *H19* is generally overexpressed in stromal cells, rarely in tumor epithelial cells in tumors and was found to be associated with the presence of ER and PR [84]. This is not surprising given that *H19* has been shown to be hormonally regulated in the mammary gland as discussed earlier. There are data indicating both oncogenic and tumor suppressive roles for *H19* in different cancers [85]. In breast cancer cell lines, *H19* RNA expression is directly regulated by E2F1 and promotes cell cycle progression [86]. This supports a

putative oncogenic function for *H19* in breast tumorigenesis. Additionally, *H19* has been shown to be upregulated by hypoxia through HIF1-alpha and suppressed by p53 [87].

**ANRIL/p15AS**—The *INK4/ARF* (*CDKN2a/b*) locus, located at chromosome 9p21 contains three well-characterized tumor suppressors, *p16<sup>INK4a</sup>*, *p15<sup>INK4b</sup>*, and *p14<sup>ARF</sup>*. The lncRNA *ANRIL* (Antisense Noncoding RNA in the *INK4* Locus) was initially identified by examining the deletion of this region in hereditary neural tumors [88]. *ANRIL* is a polyadenylated lncRNA antisense to *CDKN2A* (*p16<sup>INK4a</sup>*) and *CDKN2B* (*p15<sup>INK4b</sup>*). *ANRIL* has been shown to repress *p16<sup>INK4a</sup>* and *p15<sup>INK4b</sup>*, but not *p14<sup>ARF</sup>*, in *cis* through direct binding to CBX7 [89], a PRC1 complex member, and SUZ12 [31], part of the PRC2 complex. Upon recruitment, these complexes apply repressive histone modifications resulting in heterochromatin formation and gene silencing at this locus. Interestingly, genome-wide association studies (GWAS) have revealed that *ANRIL* is associated with increased susceptibility to several diseases, including breast cancer [90].

**ncRNACCND1**—A low-copy number lncRNA, *ncRNACCND1*, transcribed 2 kb upstream of *CCND1* is induced by ionizing radiation and regulates transcription of *CCND1* in *cis* by forming a ribonucleoprotein repressor complex [91]. This lncRNA, tethered to the 5' regulatory region of *CCND1*, acts as a ligand and recruits the RNA-binding protein TLS (translated in liposarcoma). TLS, allosterically modulated by *ncRNACCND1*, inhibits histone acetyltransferases resulting in inhibition of *CCND1* transcription.

There is extensive evidence of inactivation of the cyclinD/p16/Rb tumor suppressor pathway in breast cancer, which is often characterized by cyclin D1 overexpression and amplification, *p16<sup>INK4a</sup>* silencing, and/or Rb loss. It is not yet known whether *ANRIL* or *ncRNACCND1* play a role in inactivating this pathway in breast cancer.

**LSINCT5**—*LSINCT5* (long stress-induced non-coding transcript 5) is a 2.6 Kb polyadenylated lncRNA expressed from the negative strand, that is localized in the nucleus, and overexpressed in breast and ovarian cancer cell lines and tumors [92]. Knockdown experiments revealed that *LSINCT5* enhances cellular proliferation. Interestingly, following knockdown, another nuclear lncRNA, *NEAT-1* as well as PSPC1 (paraspeckle component 1) are downregulated. These results are suggestive of a potential role of *LSINCT5* in paraspeckle formation, but this needs to be explored further.

**BC200**—*BC200*, also known as *BCYRN1* (brain cytoplasmic RNA 1), is a 200 nt long ncRNA selectively expressed in the nervous system and usually not detected in somatic cells other than neurons. It is, however, overexpressed in several solid cancers including breast cancer [93]. *BC200* RNA is expressed at high levels in invasive breast carcinomas, but is barely detectable in normal tissue or in benign tumors [94]. Interestingly, in ductal carcinomas in situ (DCIS), significant *BC200* expression is associated with high nuclear grade. This suggests that *BC200* may be a useful marker for early detection of breast cancer and that the presence of *BC200* RNA in early lesions might have utility as a prognostic indicator of tumor progression.

### LncRNAs and p53

The *p53* tumor suppressor is the most commonly altered gene in human breast cancer. It is mutated in about 30–40% of all human breast tumors with a much higher frequency associated with poor clinical outcome and basal-like breast cancers. *P53* is an example of an increasing number of genes that encode both RNAs and proteins with distinct functions. Thus, while *p53* is best known for its function as a transcription factor, *p53* mRNA also has evolved the additional function of regulating the MDM2 protein [95].



As a transcription factor, p53 also regulates a number of ncRNAs, including both miRNAs and lncRNAs. For example, p53 regulates both miRNA processing [96] as well as the transcription of miRNAs, such as miR-34 family members [97] and miR-200c [98, 99]. Furthermore, p53 regulates the expression of specific lncRNAs (discussed in detail below), such as those transcribed from the regulatory sequence upstream of *CDKN1A/p21*, a well-known p53 transcriptional target.

**LincRNA-p21**—A lincRNA located 15 kb upstream of *CDKN1A*, named *lincRNA-p21*, is induced following the activation of p53 in murine tumors [100]. *LincRNA-p21* represses p53 target genes by binding to hnRNP-K, a repressor protein that binds the promoters of genes involved in p53 signaling. *LincRNA-p21* is further required for the induction of apoptosis. It has not been reported whether there is a conserved and functional human ortholog corresponding to lincRNA-21.

**PANDA**—DNA damage induces five lncRNAs from the *CDKN1A* locus [13]. One of these, located approximately 5 kb upstream of the *CDKN1A* transcriptional start site, is *PANDA* (P21 associated ncRNA DNA damage activated). *PANDA* is a 1.5 kb 5'-capped and polyadenylated non-spliced lncRNA that is transcribed antisense to *CDKN1A* following p53-dependent induction. Functionally, *PANDA* interacts with the NF-YA transcription factor, preventing it from occupying target gene promoters, and thus limiting the expression of pro-apoptotic genes including *FAS*, *PUMA*, and *NOXA*. *PANDA* knockdown significantly sensitized human fibroblasts to apoptosis following treatment with doxorubicin. This suggests that in parallel to p53-mediated induction of *CDKN1A* to regulate cell cycle arrest, *PANDA* is a p53 effector that acts independently of p21 to promote cell survival by impeding apoptosis. Interestingly, the gain-of-function p53 (p.Arg273His) mutant, observed in Li-Fraumeni syndrome lacks the ability to induce *CDKN1A*, while maintaining the ability to induce *PANDA*. Similar observations were reported in metastatic ductal carcinomas. This may be important because in many cancers including breast cancer, gain-of-function p53 missense mutations rather than just inactivating mutations are frequently observed. This has led to the speculation that these mutations may have been selected for at least in part as a function of increased cell survival due to their ability to induce *PANDA*.

**MEG3**—Maternally expressed gene 3 (*MEG3*) is an imprinted lncRNA located on chromosome 14q32 and, as its name implies, is expressed exclusively from the maternal allele. *MEG3* has been shown to activate p53 and facilitate p53 signaling, including the enhancement of p53 binding to target genes [101]. *MEG3* overexpression in meningioma, hepatocellular carcinoma, and breast cancer cell lines leads to suppression of cell proliferation [102–104]. *MEG3* is downregulated in human tumors and hypermethylated in leukemias and pituitary tumors [105, 106], but its role in breast cancer has not been established.

### Steroid Receptors and Co-activators

Steroid hormones and their receptor play major roles in the etiology, progression, and treatment of breast cancer. Greater than 70% of breast cancers are estrogen receptor (ER) positive and/or progesterone receptor (PR) positive. LncRNAs have been recently shown to be important as regulators, targets, and co-activators of steroid receptor signaling.

**Estrogen Receptor**—Global nuclear run-on and sequencing (GRO-seq) has been used to explore the rapid effects of estrogen treatment on the entire transcriptome of MCF-7 human breast cancer cells [107]. Estrogen signaling directly regulates a large fraction of the transcriptome including not only protein-encoding genes, but also noncoding transcripts.

These transcripts include divergent (transcribed in the opposite direction from transcriptional start sites, also produced at enhancers), antisense, and intergenic transcripts. A large number of previously undetected estrogen-regulated intergenic transcripts were identified, many of which are located proximal to ER binding sites.

**Progesterone Receptor**—Antisense RNA transcripts have also been identified overlapping the PR promoter [108]. Synthetic antigene RNAs (agRNAs) complementary to the PR promoter, rather than binding DNA, form a complex with and recruit AGO2 to the antisense transcript that overlaps the promoter. These agRNAs can modulate the expression of PR. miRNA mimics predicted to target the PR promoter and inhibit PR expression have also been identified [109]. Interestingly, the antisense RNA transcript serves as the target for these mimics in inhibiting PR transcription.

These findings clearly have a much broader significance than just the regulation of PR expression, which is important in mammary gland development and breast cancer. Non-coding transcripts may overlap with many other gene promoters. Thus, the recognition and interplay of miRNAs and lncRNAs at promoters may provide a general mechanism for regulating gene transcription.

**SRA**—The *SRA* gene encodes both noncoding as well as protein-generating RNA isoforms. *SRA* RNA is highly expressed in a number of solid tumor types including breast, ovarian, and uterine tumors [61, 110]. *SRA* is a spliced, polyadenylated transcript that has been shown to enhance ligand-activated steroid receptor transcription on luciferase reporters and is found in SRC-1/SRC-2 coactivator complexes [20, 111]. *SRA* RNA is capable of interacting with and coactivating a large number of nuclear receptors (steroid and non-steroid) and other transcription factors. Thus, it may act as a scaffold in assembling coregulator complexes. Interestingly, *SRA* RNAs that retain intron-1 are noncoding, and are expressed at higher levels in invasive breast cancer cell lines (MDA-MB-231 and MDA-MB-468) as compared to non-invasive cell lines (MCF-7, T47D, and BT20). Non-transformed MCF10A cells express the lowest levels [112]. Higher expression of *SRA* RNAs that retain intron-1 is observed in clinical breast tumor samples with higher PR levels [113]. siRNA knockdown has been used to deplete *SRA* RNA in two human cancer cell lines (HeLa and MCF7 cells) followed by genome-wide expression analysis to determine target genes [114]. The majority of significantly changed genes following knockdown were downregulated, suggesting that *SRA* RNAs play a larger role in co-activation than co-repression. Similar knockdown experiments were performed in E2-treated and -untreated MCF7 cells. Surprisingly, despite *SRA*'s putative role as an ER coactivator, only a small number of direct ER target genes were affected by *SRA* depletion in estradiol-treated MCF-7 cells indicating that *SRA* serves as a coactivator for only a minor subset of ER targets. Overall, these target gene expression data is suggestive of roles for *SRA* in glucose uptake, cellular signaling, T<sub>3</sub> hormone generation, and invasion/metastasis. *SRA* depletion in MDA-MB-231 cells reduced invasiveness and expression of genes consistent with the invasive phenotype. In agreement with the knockdown results and consistent with its role as a coactivator, overexpression of *SRA* was shown to co-activate certain target promoters and enhance the activity of some co-regulatory proteins. As mentioned previously, transgenic overexpression of human *SRA* in the murine mammary gland results in increased epithelial hyperplasia [61]. These mice fail to develop palpable tumors, however, likely due to enhanced apoptosis in the preneoplastic lesions. Together, while these data suggest that *SRA* RNA is playing an oncogenic role in mammary tumorigenesis, the precise mechanisms involved remain to be elucidated.

**GAS5**—*GAS5* is an lncRNA existing as multiple splice isoforms containing up to 12 exons. Following overexpression in HeLa cells *GAS5* modulates cell survival and metabolism [16].

It functions by serving as a RNA transcript decoy that prevents the glucocorticoid receptor (GR) from binding to target genes. Mechanistically, the 3' end of *GAS5* contains a stem-loop structure resembling the GR DNA-binding element and this interacts with the GR DNA-binding domain. Upon stimulation with dexamethasone, a GR agonist, *GAS5* is sufficient to repress GR-induced genes, such as *cIAP2*. By the same means, *GAS5* also seems to regulate other receptors including the androgen, mineralcorticoid, and progesterone receptors.

In breast tumors, *GAS5* transcript levels were significantly reduced relative to adjacent unaffected normal breast epithelial tissues [115]. Decreased expression of *GAS5* and *RNU44*, an intronic snoRNA contained within the *GAS5* transcript, were associated with a poor prognosis in breast cancers [116]. When overexpressed in breast cancer cell lines *GAS5* induced apoptosis and suppressed proliferation [115]. These data are suggestive of a tumor suppressor role for *GAS5* in breast cancer.

### Breast Cancer Metastasis

Metastatic disease is the major cause of mortality from breast cancer. Metastasis is an inefficient multi-step process which involves local invasion of the primary tumor cells and entry into the vasculature, survival in the circulation, invasion into other tissues, establishment of micrometastases, and finally the growth of secondary tumors. While miRNAs have been shown to play major roles in both the promotion and suppression of metastasis (reviewed elsewhere in this issue), the role of lncRNAs is only just starting to be explored. However early indications suggest that lncRNAs will also be of major importance.

**HOTAIR**—One lncRNA that recently has been shown to play a key role in breast cancer metastasis is *HOTAIR* (Hox transcript antisense intergenic RNA). *HOTAIR* is expressed from the *HOXC* locus and was the first lncRNA shown to be acting in *trans*. *HOTAIR* binds to and targets the PRC2 complex to the *HOXD* locus [29]. Furthermore, *HOTAIR* functions as a RNA scaffold containing two main functional domains. The 5' domain of *HOTAIR* binds PRC2, whereas a 3' domain binds the LSD1/CoREST/REST H3K4 demethylase complex [117], thus bridging two repressive complexes in order to coordinate their functions in gene silencing. Enforced *HOTAIR* expression in epithelial cancer cells induces genome-wide retargeting of PRC2 leading to widespread changes in repressive (H3K27me3) and active (H3K4me3) chromatin marks resembling those found in embryonic fibroblasts. This results in more invasive and metastatic cells. Thus, it is not surprising that *HOTAIR* expression is predictive of breast cancer survival [15]. Finally, Chromatin Isolation by RNA Purification (ChIRP) followed by DNA sequencing has been employed recently to map the genome-wide occupancy of three lncRNAs including *HOTAIR* [118]. These results suggest that *HOTAIR* binds to focal target sites in the genome, and is responsible for recruiting PRC2 to these sites. PRC2 then spreads from these focal target sites along the chromosome in a *HOTAIR*-independent manner. The mechanisms, however, by which *HOTAIR* recognizes these sites remain to be established.

### Long Meets Short

As mentioned above with reference to the role of *linc-MD1* in muscle differentiation [19], lncRNAs can regulate gene expression through their interactions with miRNAs by acting as decoys for target mRNAs. Another example of this mechanism is the lncRNA *HULC* (highly upregulated liver cancer transcript), which binds to and inhibits miR-372 in liver cancer [119].

**PTENP1**—miRNAs have been shown to be major regulators of the *PTEN* tumor suppressor and thus contribute to cell transformation through aberrant PI3K/AKT pathway activation.

*PTENP1*, a pseudogene of *PTEN* is able to compete for miRNA binding sites with *PTEN* [120]. Accordingly, *PTENP1* displays a tumor suppressive role in in vitro assays and is genomically lost in cancer. A similar relationship is also found between *KRAS* and its pseudogene, *KRASIP*. Overexpression of the *KRASIP* 3' UTR results in increased *KRAS* mRNA abundance and an increase in cellular proliferation. These findings show that transcribed pseudogenes can serve as decoys for miRNAs that target protein-encoding mRNA transcripts of their cognate genes. Not only pseudogenes and long non coding transcripts can play this role, but protein-encoding RNA transcripts also can act as ceRNAs (competing endogenous RNAs). These can communicate and co-regulate each other by competing for miRNAs that target both transcripts [121, 122]. Several putative *PTEN* ceRNAs have been identified and validated. This includes the *ZEB2* transcript, which can modulate *PTEN* protein levels and thus PI3K/AKT signaling in a miRNA-dependent, protein-coding-independent manner [121].

Not surprisingly, miRNAs can also target lncRNAs just as they do mRNAs [123]. miR-155 has been shown to target transcribed ultraconserved regions (UCRs), regions of the genome that are completely conserved between orthologous regions in the human, rat, and mouse genomes [124]. We are just beginning to understand these complex networks containing all combinations of RNA:RNA interactions and their ultimate impact on gene expression. Thus, it is likely that similar mechanisms may be operable and play an important role in breast cancer.

## Genetic Association

Genetic association studies have recently uncovered a number of single nucleotide polymorphisms (SNPs) associated with susceptibility to common diseases including breast cancer. While most of these studies have initially focused on coding genes many of these polymorphisms may be found in regulatory regions and regions containing non-coding RNAs. With this in mind recently discovered SNPs in lncRNAs are associated with prostate cancer risk [125]. In addition, among six SNPs that are located within UCRs, two were significantly associated with familial breast cancer risk [126]. At present the significance of variations in the sequence of lncRNAs as compared with protein-coding genes will be difficult to verify experimentally.

## Clinical Significance, Prognostic Implications, and Therapeutics

lncRNAs that show dramatic differences in expression between tumors and normal tissues may also be detected in body fluids. Thus, they potentially represent promising prognostic biomarkers for the diagnosis and treatment of breast cancer. As an example, the lncRNA *PCA3/DD3* is being tested as a potential biomarker for early detection in prostate cancer [127, 128]. *PCA3* originally was discovered in a differential display analysis comparing normal prostate and tumor samples [129]. As mentioned previously, lncRNAs like *HOTAIR* and *BC200* may represent promising prognostic and/or predictive biomarkers for breast cancer.

lncRNAs also have the potential to be useful as therapeutic agents or targets. Currently epigenetic cancer therapy uses HDAC and DNMT inhibitors to induce genome-wide modifications of histone acetylation and DNA methylation, respectively, but these are relatively non-specific approaches. An alternative approach of targeting RNA-protein interfaces with a focus on cancer specific lncRNAs may allow for site-specific epigenetic modulation and the development of cancer-specific therapeutics [130].

lncRNAs can be targeted with synthetic siRNAs or miRNAs. However, because of their more extensive secondary structures as compared to mRNAs, it is likely that many siRNAs

will need to be screened in order to achieve successful knockdown [131]. Another way to target lncRNAs is to utilize oligonucleotides that bind and disrupt RNA-protein interactions. These “antagolincns” potentially can be selected for using a method such as systematic evolution of ligands by exponential enrichment (SELEX) [131]. Finally small molecules can be screened for their interaction with lncRNAs. For successful application of these approaches, more insight into lncRNA structure and function will be required.

Substantial technical hurdles, including the development of specific delivery systems, must be overcome before it will be feasible to rationally design and utilize synthetic RNA molecules as novel strategies to target tumor cells. However, one might envision that the same way that *GASS* serves as a decoy for the GR, the activity of transcription factors might be regulated using RNA molecules designed to form hairpin structures mimicking DNA transcription factor-binding sites. Likewise, synthetic RNA sponges can be designed that contain miRNA-binding sites to sequester and reduce the expression level of oncogenic miRNAs. With analogy to lncRNAs like *HOTAIR* it should also be possible to rationally design chimeric RNA molecules that possess the RNA structural motifs to recruit chromatin modifying complexes in order to regulate specific gene expression. At present all of these approaches represent wishful thinking, but they provide a future direction for lncRNA research.

## Perspectives on LncRNAs and Breast Cancer

In summary, lncRNAs function in many important oncogenic and tumor suppressor pathways. This includes playing important roles in the regulation of two important tumor suppressor pathways often perturbed in cancers, the RB pathway and the p53 pathway. The extensive association of lncRNAs with chromatin modifying complexes is of particular interest with regards to cancer. Mutations in proteins involved in chromatin modification have been a recurrent theme in recent cancer sequencing studies, e.g. mutations have been identified in components of chromatin modifying complexes including Ezh2 in B-cell lymphoma [132], MLL2 and MLL3 in medulloblastomas [133], and MEN1 in pancreatic neuroendocrine tumors [134]. When cancer sequencing studies turn their attention to noncoding portions of the genome, it is highly likely that mutations will be found in lncRNAs that interact with these protein complexes. Targeting the interaction between lncRNAs and chromatin modifying complexes, rather than these ubiquitous proteins themselves, therefore, may represent a promising new approach for cancer specific therapeutics.

LncRNAs have been shown to extensively bind and regulate protein complexes. Their interactions with chromatin modifying complexes and transcription factors are at present best understood. This has led some to hypothesize that lncRNA functions may extend well beyond their roles in the nucleus associated with gene regulation. They may also modulate signal transduction pathways by linking signaling proteins to specific downstream target effectors thus regulating how the signals are propagated in a context specific manner [135]. If true, this could open up a new paradigm for looking at how signal transduction pathways are wired in cancer and also provide novel therapeutic approaches. Finally, genes that encode bi-functional RNAs like *SRA* and *TP53*, as well as mRNAs like *ZEB2* that can act as ceRNAs, challenge the existing paradigm classifying RNAs as either coding or noncoding. It is becoming increasingly clear that coding-independent functions possibly can be bestowed on any RNA.

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## Abbreviations

<b>miRNA</b>	microRNA
<b>lncRNA</b>	long/large noncoding RNA
<b>lincRNA</b>	long/large intergenic noncoding RNA
<b>PRC2</b>	polycomb repressive complex-2
<b>CoREST</b>	co-repressor for REST
<b>REST</b>	repressor element-1 (RE1) silencing transcription factor
<b>MLL1</b>	mixed lineage leukemia 1
<i>Xist</i>	X-inactive specific transcript
<i>RepA</i>	Repeat A
<i>Kcnq1ot1</i>	Kcnq1 overlapping transcript 1
<i>Airn</i>	antisense Igf2r RNA noncoding
<b>mESC</b>	mouse embryonic stem cell
<b>Oct4</b>	octamer-binding transcription factor 4
<b>Sox2</b>	SRY (sex determining region Y)-box 2
<b>EGO</b>	eosinophil granule ontogeny
<b>HOTAIRM1</b>	HOX antisense intergenic RNA myeloid 1
<i>LincRNA-EPS</i>	lincRNA erythroid prosurvival 1
<i>Evf2</i>	embryonic ventral forebrain 1
<b>GABA</b>	gamma-aminobutyric acid
<i>Tug1</i>	taurine-upregulated gene 1
<b>RNCR</b>	retinal noncoding RNA
<b>MIAT</b>	myocardial infarction associated transcript
<b>ceRNA</b>	competing endogenous RNA
<b>ANCR</b>	anti-differentiation noncoding RNA
<b>SRA</b>	steroid receptor RNA activator
<b>MMTV</b>	mouse mammary tumor virus
<b>PINC</b>	pregnancy induced noncoding RNA
<i>Zfas1</i>	Znfx1 antisense RNA 1
<i>mPINC1.0</i>	mouse pregnancy induced noncoding RNA 1.0 Kb
<i>mPINC1.6</i>	mouse pregnancy induced noncoding RNA 1.6 Kb
<b>RbAp46</b>	retinoblastoma associated protein 46
<b>Znfx1</b>	zinc finger, NFX1-type containing 1
<b>snoRNA</b>	small nucleolar RNA
<b>TCGA</b>	the cancer genome atlas
<b>Shh</b>	sonic hedgehog

<b>BMP</b>	bone morphogenetic protein
<b>MALAT1</b>	metastasis associated lung adenocarcinoma transcript 1
<b>ER</b>	estrogen receptor
<b>PR</b>	progesterone receptor
<b>AGO2</b>	argonaute 2
<b>UCR</b>	ultraconserved region
<b>GWAS</b>	genome-wide association study

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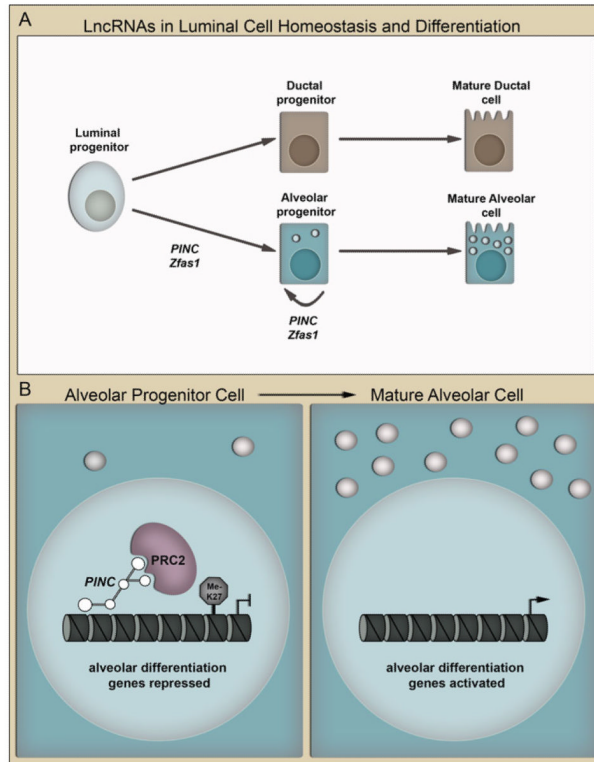
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**Figure 1.** LncRNAs regulate mammary epithelial differentiation. **a** A schematic diagram illustrates a mammary epithelial luminal progenitor and its progeny. *PINC* and *Zfas1* may contribute to alveolar cell fate commitment. *PINC* and *Zfas1* inhibit terminal alveolar differentiation thereby maintaining alveolar progenitor cells. **b** A hypothetical model shows the role of *PINC* in alveolar progenitor cells. A *PINC*-PRC2 complex targets and represses alveolar differentiation genes by methylating histone H3 at lysine 27 (me-K27). In cells lacking *PINC*, the repressive marks are removed and alveolar differentiation genes are transcribed to induce terminal differentiation

**Table 1****LncRNAs involved in mammary gland development and tumorigenesis**

Mammary Gland Development		
LncRNA	Developmental Expression/Role	Mechanism
<i>PINC</i>	Downregulated during lactation and by lactogenic differentiation, knockdown and overexpression studies show that <i>PINC</i> inhibits lactogenic differentiation (unpublished data). Also regulates cell cycle of mammary epithelial cells [63].	Interacts with PRC2, unknown targets (unpublished data)
<i>Zfas1</i>	Downregulated during lactation and by lactogenic differentiation, negatively regulates proliferation and lactogenic differentiation as shown by knockdown of <i>Zfas1</i> [62].	Unknown
Breast Cancer		
LncRNA	Clinical Expression/Role	Mechanism
<i>HOTAIR</i>	Independent predictor of breast cancer survival. Promotes invasion and metastasis [15].	Interacts with PRC2 and LSD1/CoREST/REST complexes in gene silencing [29, 117].
<i>H19</i>	Highly expressed in stromal cells of breast tumors and associated with the presence of ER and PR [85]. Upregulated by E2F1 and promotes cell cycle progression [86].	Unknown
<i>LSINCT5</i>	Overexpressed in breast and ovarian cancer cell lines and tumors. Enhances cellular proliferation [92].	Unknown
<i>BC200</i>	Overexpressed in several solid tumors including breast. In DCIS lesions, <i>BC200</i> expression is associated with high nuclear grade [93, 94].	Unknown
<i>SRA</i>	High expression of noncoding <i>SRA</i> RNA in invasive breast cancer cell lines and PR+ clinical breast cancer samples [112, 113].	Interacts with and coactivates a large number of nuclear receptors and transcription factors [20, 21, 111].
<i>GAS5</i>	Low expression in breast cancers associated with poor clinical outcome [116].	Acts as a decoy that prevents GR from binding to its targets [16].

This table contains a summary of lncRNAs that are expressed in the mammary gland and have been shown to regulate mammary development as well as lncRNAs that are misregulated in breast cancer and are thought to contribute to tumor initiation and/or progression