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Kevin V. Morris *Editor*

Long Non-coding RNAs in Human Disease

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Preface

In the first post-genomic decade, the ENCODE and FANTOM Consortia have revolutionized our understanding of the human non-protein-coding transcriptome, setting the stage for the next step: harnessing the power and promise of long non-coding RNA to improve the health of humanity through novel therapeutics. This volume attempts to capture a summary of the conceptual revolution that is taking long non-coding RNA from the emerging frontier into a diagnostic and therapeutic territory. The implications of this emerging paradigm portend an entirely new era of therapeutic potential whereby long non-coding RNAs can be harnessed or repressed to modulate gene expression, cellular states and disease.

Sydney, Australia — Kevin V. Morris

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Understanding the Complex Circuitry of lncRNAs at the X-inactivation Center and Its Implications in Disease Conditions

John Lalith Charles Richard and Yuya Ogawa

Abstract Balanced gene expression is a high priority in order to maintain optimal functioning since alterations and variations could result in acute consequences. X chromosome inactivation (X-inactivation) is one such strategy utilized by mammalian species to silence the extra X chromosome in females to uphold a similar level of expression between the two sexes. A functionally versatile class of molecules called long noncoding RNA (lncRNA) has emerged as key regulators of gene expression and plays important roles during development. An lncRNA that is indispensable for X-inactivation is *X-inactive specific transcript* (*Xist*), which induces a repressive epigenetic landscape and creates the inactive X chromosome (Xi). With recent advents in the field of X-inactivation, novel positive and negative lncRNA regulators of *Xist* such as *Jpx* and *Tsix*, respectively, have broadened the regulatory network of X-inactivation. *Xist* expression failure or dysregulation has been implicated in producing developmental anomalies and disease states. Subsequently, reactivation of the Xi at a later stage of development has also been associated with certain tumors. With the recent influx of information about lncRNA biology and advancements in methods to probe lncRNA, we can now attempt to understand this complex network of *Xist* regulation in development and disease. It has become clear that the presence of an extra set of genes could be fatal for the organism. Only by understanding the precise ways in which lncRNAs function can treatments be developed to bring aberrations under control. This chapter summarizes our current understanding and knowledge with regard to how lncRNAs are orchestrated at the *X-inactivation center* (*Xic*), with a special focus on how genetic diseases come about as a consequence of lncRNA dysregulation.

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Abbreviations

lncRNA	Long noncoding RNA
Xist	X-inactive specific transcript
Xic	X-inactivation center
ES cells	Embryonic stem cells
H3K27me3	Histone H3 tri-methylated lysine 27
LINE	Long interspersed nuclear element
YY1	Yin Yang 1
hnRNP U	Heterogenous nuclear ribonucleoprotein U
Xi	Inactive X chromosome
Xa	Active X chromosome
MECP2	Methyl-CpG binding protein 2
MPN/MDS	Myeloproliferative neoplasm and myelodysplastic syndrome
CTCF	11 Zinc finger protein/CCCTF binding factor
hiPSCs	Human induced pluripotent stem cells

Contents

1 Introduction

Our understanding of the human genome keeps increasing as new technological advances allow us to unravel the genome's functional elements. Although mapping of the human genome has been completed, it still remains unclear how certain regions are demarcated as coding regions or regulatory elements and what the vast regions without coding account for. The number of genes annotated dwindled from the previously estimated 35,000–100,000 to 25,000 protein-coding regions, which only occupies 1–2 % of the overall genome (Liang et al. 2000). This finding has

prompted searches elsewhere for unidentified functional elements. Recently, it has become evident that new classes of molecules, noncoding RNAs, are emerging as crucial functional molecules in development, disease, and physiology. Surprisingly, noncoding transcripts were available in abundance while surveying the human genome but had been conveniently overlooked (Carninci et al. 2005). These noncoding RNA transcripts are classified primarily based on the length of the RNA transcripts. Noncoding RNAs generally fall under two main categories with any length below 200 nucleotide-long classified as small noncoding RNA and anything larger than 200 nucleotide-long classified as long noncoding RNA (lncRNA). Furthermore, evidence supporting the involvement of noncoding RNA in various biological processes, for example, gene expression regulation, is rapidly increasing (Wapinski and Chang 2011; Wang and Chang 2011). Indeed, both small and lncRNAs are involved in bringing about epigenetic changes at a particular locus (Peschansky and Wahlestedt 2014). The significance of noncoding RNAs is increasing with regard to their physiological function as well as their association with diseases (Wapinski and Chang 2011; Maass et al. 2014).

One such widely studied lncRNA is *X-inactive specific transcript* (*Xist*), which is indispensable for X chromosome inactivation (X-inactivation). In this chapter, we describe a wide variety of lncRNAs in the *X-inactivation center* (*Xic*), which reside on the X chromosome and are required for the induction of X-inactivation, and discuss how these lncRNAs cooperate in inducing monoallelic *Xist* expression to establish only one inactive X chromosome (Xi) in mammalian females. Finally, the various diseases that arise due to a dysfunctional or skewed X-inactivation, and possible future studies in humans, are discussed. An elucidation of these novel regulators and their interaction networks would provide important insight with respect to the molecular mechanism of diseases such as cancer and help toward designing better therapeutics.

2 X Chromosome Inactivation (X-inactivation)

X-inactivation is a dosage compensation mechanism in female mammals which maintains the balance of X-linked gene expression and is achieved when one of the two available X chromosomes is inactivated at an early stage of embryonic development. Failure of X-inactivation would result in an increased dosage of genes which can alter various pathways pertaining to different vital processes (Lyon 1961; Heard and Disteche 2006; Payer and Lee 2008). In the case of males, only one functional X chromosome exists and is usually referred to as genetic unisomy, whereas in females, two X chromosomes exist and the event of silencing one of them is referred to as functional unisomy. X-inactivation is critical for cellular differentiation, and dysregulation could result in developmental abnormalities (Marahrens et al. 1997). X-inactivation occurs either as imprinted X-inactivation at early embryonic stages and in extraembryonic tissues, wherein the paternal X chromosome is silenced (Takagi and Sasaki 1975; Huynh and Lee 2003; Okamoto

et al. 2004), or as an act of random X-inactivation in which both the paternal and the maternal X chromosome have an equal probability of being inactivated in the epiblast (Monk and Harper 1979; Tan et al. 1993). In imprinted X-inactivation, although the paternal X chromosome undergoes complete inactivation around the blastocyst stage, it is reactivated during the peri-implantation stage in the epiblast lineage which then gives rise to a broad range of tissue types in the fetus (Mak et al. 2004). Subsequently, these cells are prone to another wave of random X-inactivation. Random X-inactivation is thought to occur close to the time of implantation in cells of the epiblast at around embryonic days 4.5–5.5 in mice. Once the Xi is established, it is inherited through all subsequent cell divisions.

X-inactivation is characterized by Xist lncRNA coating the Xi (Clemson et al. 1996). As the coating masks the chromosome completely, this observable phenomenon is referred to as the "Xist cloud." Gene silencing is triggered once *Xist* is upregulated on the future Xi. This designated future Xi goes through the stages of initiation, progression, and maintenance of repressive chromatin states with the aided participation of multiple proteins and machineries (Table 1) (Wutz 2011). Numerous studies have been carried out with respect to dynamic changes occurring on the Xi during X-inactivation. These studies have shown the remarkable changes happening in the epigenetic landscape as well as the chromatin structure during X-inactivation. The epigenetic histone marks accompanying the chromatin state (euchromatin/heterochromatin) are characteristic of its transcriptional activity (active/silent) and influence strongly the chromatin structure. At the onset of X-inactivation, euchromatin markers such as H3K9Ac, H3K4me2, and H3K4me3 are lost, when Xist RNA starts coating the X chromosome, subsequently leading to global H4 hypoacetylation (Keohane et al. 1996; Chaumeil et al. 2002). Meanwhile, loss of RNA polymerase II and nascent transcripts also occurs post-Xist RNA

Table 1 Epigenetic modifications associated with the Xi in mice and humans

Epigenetic modifications	Enzymes	References
H3K27me3	PRC2(Ezh2)	Mak et al. (2002), Silva et al. (2003), Plath et al. (2003)
H2AK119ub1	PRC1(Ring1A/B)	de Napoles et al. (2004), Fang et al. (2004), Plath et al. (2004)
H3K9me2/H3K9me3	Unknown	Heard et al. (2001), Chadwick and Willard (2004)
H3K20me1	Unknown	Kohlmaier et al. (2004), Chow et al. (2007)
H3K20me3	Unknown	Chadwick and Willard (2004)
MacroH2A	–	Costanzi and Pehrson (1998), Csankovszki et al. (1999), Chadwick and Willard (2004)
DNA methylation	Dnmt1, Dnmt3b	Norris et al. (1991), Sado et al. (2000), Gendrel et al. (2012)

This table represents a list of epigenetic modifications accumulated on the Xi during X-inactivation attributing to the repressive state of the entire chromosome. While catalytic enzymes responsible for H3K27me3, H2AK119ub1, and DNA methylation are known, enzymes, which induce other epigenetic modification on the Xi, have not yet been identified

coating (Chaumeil et al. 2006). Interestingly, a whole new array of repressive epigenetic modifications such as H3K27me3, H3K9me2, H4K20me1, and H2AK119ub1 get enriched on the Xi (Wutz 2011). Strikingly, the histone trimethylations on H3K27 and H3K9 occur at different regions and are recognized by different cofactors in the Xi indicating two different populations of repressed heterochromatins in humans (Chadwick and Willard 2004; Nozawa et al. 2013). The kinetics of X-inactivation is tightly associated with the dynamics of histone epigenetic marks brought about by protein complexes such as polycomb repressive complex, PRC1 and PRC2, which catalyze the repressive histone modifications, H2A119ub and H3K27me3, respectively, in an Xist RNA-dependent manner (Mak et al. 2002; Silva et al. 2003; Plath et al. 2003; de Napoles et al. 2004; Fang et al. 2004).

Additionally, the Xi is marked by a series of epigenetic changes such as histone variant macroH2A along with DNA methylation of the CpG islands and promoters (Norris et al. 1991; Costanzi and Pehrson 1998; Sharp et al. 2011). Smchd1 is also involved in delivering the DNA hypermethylation of the CpG islands associated with the Xi, which is required for long-term maintenance of gene silencing (Blewitt et al. 2008; Gendrel et al. 2012). Another group of proteins associated with the Xi is a member of the trithorax group proteins for transcriptional activation, Ash2L, although its functional role on the Xi remains to be elucidated (Pullirsch et al. 2010).

3 X-inactivation Center (Xic)

Early studies of X chromosome truncations and translocations helped identify the X chromosome locus wherein X-inactivation is induced, called an *Xic* (Rastan and Brown 1990; Brown 1991). The initial mapping of the *Xic* was first shown by a series of cytological experiments, which used differential staining of X chromosome material at the metaphase stage of mouse embryos. These showed that chromosomal rearrangements between the X chromosome and autosomes led to random inactivation of the segment hosting the *Xic*. In experiments examining the T16H reciprocal translocation between the X chromosome and chromosome 16, also referred to as the Searle's translocation, only the translocated 16^{X} chromosome was inactivated, predicting the presence of the *Xic* (Takagi 1980; Rastan 1983). Subsequently, truncating one of the X chromosomes at the *Xic* region in embryonic cells resulted in no X-inactivation, suggesting that two *Xic*'s are required for X-inactivation (Rastan and Robertson 1985). Furthermore, when one of the X chromosomes was truncated leaving behind a significant chunk of the *Xic*, termed HD3 translocation, random X-inactivation occurred, indicative of the distal boundary of the *Xic* (Rastan and Robertson 1985). These results thus suggest that the minimum region required for efficiently triggering X-inactivation lies somewhere between the T16H and the HD3 break points, which was followed by further extensive genetic mapping (Keer et al. 1990). While the majority of experiments were established in mice, a similar strategy was used to determine the *XIC* in humans using

mice/human somatic cell hybrids derived from patient samples with human X chromosome translocations and deletions (Brown et al. 1991b). The human equivalent of the mouse *Xic* seems to be highly conserved and spans approximately a 1 Mb region in Xq13. Studies increasingly propose that a minimal region is required for X-inactivation to take place in humans. Any abnormalities arising due to rearrangements in this demarcated region could lead to improper functioning and haywire regulations.

X-inactivation is a phenomenon controlled exclusively by the events occurring at the *Xic*. A series of transgene experiments contributed to delineating the *Xic* region (Heard et al. 1996; Lee et al. 1996; Lee and Jaenisch 1997). In embryonic stem cells, transgenes containing *Xist* can induce silencing in surrounding regions at its insertion site. Experiments showed that a mouse transgene was sufficient to induce Xist RNA expression and coating in *cis* and subsequently silence the LacZ reporter within the transgene (Lee et al. 1996). Copy number and expression levels were also found to play an important role for silencing to take place at the integration site for the genome (Heard et al. 1996). The *Xic* region, including *Xist* and its flanking regions, harbors a number of lncRNAs and consists of a complex interplay between each lncRNA to regulate the monoallelic expression of *Xist* (Fig. 1). Apart from the abundance of both positive and negative lncRNA regulators

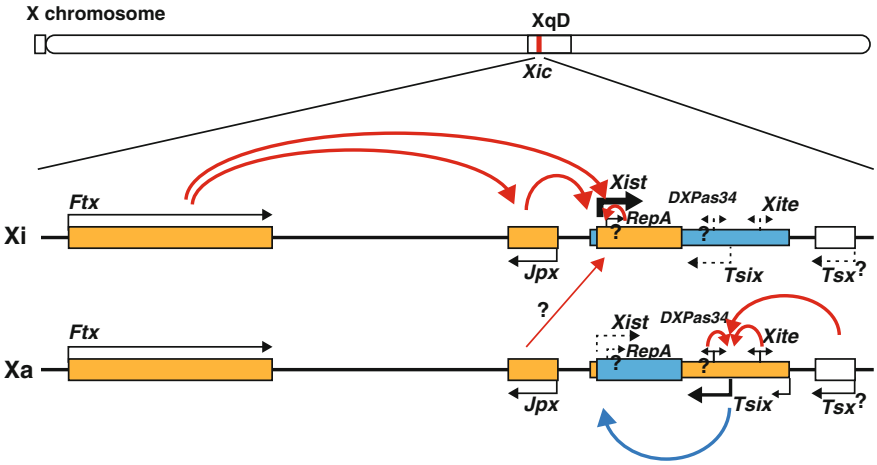

Fig. 1 lncRNAs on the *Xic*. The figure shows the schematic of *Xic* and lncRNAs originating from the locus at the onset of X-inactivation. *Red* and *blue arrows* show the action of lncRNAs as transcriptional activators and repressors, respectively. *Orange box* with *black arrow* indicates the actively transcribed gene and direction of transcription. *Blue box* with *black arrows* by *dashed line* indicates repressed gene. While *Jpx* and *Ftx* are known as escape genes (Tian et al. 2010; Chureau et al. 2011; Kobayashi et al. 2013), expression of *Tsix* and *Xite* persists longer on the Xi at the onset of X-inactivation (Lee et al. 1999a; Ogawa and Lee 2003). Allelic expression of *Tsx, DXPas34,* and RepA has not yet been reported. Although the *Jpx* transgene induces *Xist* expression, it is not clear whether endogenous *Jpx* acts both in *cis* and in *trans* at the initiation of X-inactivation

of *Xist* at the *Xic*, a number of proteins are also involved in this tight regulation (Wutz 2011).

3.1 *Xist*

Xist produces a 17 kb transcript in mice and a 19 kb transcript in humans that is processed by polymerase II, polyadenylated, and retained in the nucleus. The Xist lncRNA is exclusively expressed from the *Xic* on the Xi and a central player of *Xic* function for both imprinted and random X-inactivation (Brown et al. 1991a; Brockdorff et al. 1991; Penny et al. 1996; Marahrens et al. 1997; Wutz and Jaenisch 2000). Xist lncRNA induces chromosome-wide gene silencing through a cascade of epigenetic modifications on the Xi, which is eventually maintained through multiple rounds of cell division. Xist RNA has multiple functional domains spread across its 8 exons including repeat A-F, which are highly conserved in eutherian mammals, enabling its interaction with transcriptional factors, scaffold proteins, and chromatin-modifying proteins (Sado and Brockdorff 2013).

At an initial phase of X-inactivation, it is proposed that transcriptional factors YY1 (Yin Yang 1) act as an anchoring point to bridge between Xist RNA and Xist gene to serve as a nucleation center for Xist RNA spreading across the Xi (Jeon and Lee 2011). Furthermore, since knockdown of hnRNP U (heterogenous nuclear ribonucleoprotein U, also known as SAF-A and SP120), which has a binding affinity to both DNA and RNA, disrupts Xist RNA localization and X-linked gene silencing, hnRNP U acts as a bridge between matrix/scaffold attached region (MAR/SAR) in the Xi and Xist RNA to facilitate spreading of the silencing machinery such as PRC2 across the Xi (Hasegawa et al. 2010). Recent studies to map Xist RNA and its binding partner, PRC2, on the Xi revealed an orderly fashion of Xist RNA and PRC2 spreading, as well as a strong dependency on Xist RNA for the three-dimensional structure of the Xi (Splinter et al. 2011; Engreitz et al. 2013; Simon et al. 2013). Allele-specific ChIP-seq (chromatin immunoprecipitation with deep sequencing) of a catalytic subunit of PRC2, Ezh2, showed $\sim$50 prominent and $\sim$1500 moderate peaks prior to X-inactivation, suggesting that Ezh2 binds at $\sim$50 strong and $\sim$1500 moderate binding sites (Pinter et al. 2012). The Ezh2 binding sites are frequently associated with canonical H3K4me3/H3K27me3 bivalent domain and CpG islands across the X chromosome. Upon differentiation and induction of X-inactivation, Ezh2 binds to an additional >100 strong and $\sim$4000 moderate binding sites which can then induce spreading of H3K27me3 toward neighboring regions. An Xist RNA binding map produced by combining CHART-seq (capture hybridization analysis of RNA targets with deep sequencing) and RAP (RNA antisense purification) data has also revealed that Xist RNA initially binds to gene-rich regions before spreading to distal, gene-poor regions (Engreitz et al. 2013; Simon et al. 2013). The Xist RNA binding profile overlaps heavily with Ezh2 binding and H3K27me3 density, indicating an Xist RNA-dependent deposition of Ezh2 and H3K27me3. To efficiently induce gene silencing

across the entire X chromosome upon differentiation, Xist RNA spreads in a three-dimensional manner away from the *Xic* toward distal binding sites across the Xi (Lieberman-Aiden et al. 2009; Engreitz et al. 2013; Simon et al. 2013). The repeat A region in exon 1 of Xist RNA is presumed to have an important role in Xist RNA spreading across the gene-rich region of the Xi, since deletion of repeat A has resulted in impairment of this process (Engreitz et al. 2013). It was previously suggested that long interspersed nuclear element 1 (LINE1) repeat elements play a role in assisting Xist RNA to spread along the entire X chromosome, as well as supporting the assembly of heterochromatic nuclear structures and propagation of X-inactivation (Lyon 2000; Chow et al. 2010). However, recent reports show that there is less correlation between the Xist RNA binding site and LINE1 repeats that has been previously speculated (Engreitz et al. 2013; Simon et al. 2013).

4 Long Noncoding RNAs and Elements Controlling *Xist* Expression

Xist is the central player of X-inactivation: through neighboring lncRNAs, which are involved in the tight regulation of *Xist* monoallelic expression, *Xist* induces a repressive chromatin state that leads to X-linked gene silencing along the entire X chromosome. Recent advancements in next-generation sequencing and newer techniques have propelled forward the functional analysis of novel lncRNAs in *Xic* and increased our understanding of their function and interactions at the molecular level. Here, we describe several lncRNAs in the *Xic* which regulate *Xist* expression in both positive and negative ways. Tight regulation in the interplay of these lncRNAs is essential for securing the induction of monoallelic *Xist* expression and bringing about X-inactivation in only one of the two X chromosomes in females.

4.1 Negative LncRNA Regulators of Xist

4.1.1 Tsix

The noncoding *Tsix* gene expresses lncRNA antisense to *Xist*; hence, it is named "*Tsix,*" which is *Xist* spelled in reverse order (Lee et al. 1999a). While the Tsix transcript does not coat the X chromosome like its counterpart *Xist* during X-inactivation, it is detected using RNA fluorescence in situ hybridization as a pinpoint signal at both endogenous loci and is expressed in both male and female undifferentiated cells (Lee et al. 1999a). While monoallelic *Tsix* expression coincides with *Xist* silencing on the future active X chromosome (Xa) at the onset of X-inactivation, *Tsix* extinction and *Xist* upregulation also occur on the future Xi, thereby suggesting the antagonistic role of *Tsix* on *Xist* expression. *Tsix* expression finally disappears on both X chromosomes at a later stage of differentiation without

Xist reactivation on the Xa, suggesting that *Tsix* represses *Xist* upregulation at the onset of X-inactivation. *Tsix* has been reported to play a role for *Xist* repression both in imprinted and in random X-inactivation (Lee and Lu 1999; Lee 2000; Sado et al. 2001). In *Tsix* heterozygous mutant female ES cells, a mutation in *Tsix* always leads to the induction of *Xist* expression from the *Tsix*-mutant X chromosome, hence the non-random inactivation of the mutant X chromosome (Lee and Lu 1999). Apart from random X-inactivation, imprinted X-inactivation is controlled by *Tsix* to prevent the maternal X chromosome from undergoing X-inactivation in the extra embryonic tissues. While female embryos carrying a *Tsix* mutation on the maternal X chromosome lead to embryonic lethality due to X-inactivation on both X chromosomes, female embryos carrying a mutated *Tsix* on the paternal X chromosome normally survive to term (Lee 2000; Sado et al. 2001). Since the *Tsix*-mutant male embryo also carries a *Tsix* mutant maternal X, this mutation resulted in embryonic lethality due to X-inactivation. These reports demonstrate that *Tsix* antagonizes *Xist* expression in *cis*. *Tsix* exclusively works to repress *Xist* by modulating the chromatin structure (Navarro 2005; Sado et al. 2005; Sun et al. 2006). Since the insertion of a polyadenylation signal to produce *Tsix* truncation at the site close to the 5′ end of *Xist* effectively abolishes *Tsix* function, *Tsix* transcription across the *Xist* promoter is critical for *Xist* repression (Ohhata et al. 2008).

4.1.2 Xite (X-inactivation Intergenic Transcription) and DXPas34

Xite resides between minor and major *Tsix* promoters and is associated with multiple bidirectional long noncoding transcripts (Ogawa and Lee 2003). Since an *Xite* heterozygous deletion mutation leads to skewed X-inactivation which favors the mutant X chromosome, *Xite* is involved in choosing which X chromosome will be inactivated. Further analysis revealed that *Tsix* is downregulated in the *Xite* deletion mutant in *cis*; thus, *Xite* plays a role in the decision of the Xi by positively promoting *Tsix* expression, which in turn represses *Xist* (Ogawa and Lee 2003). Many models have been proposed with regard to how *Xite* could function in X-inactivation. One model suggests that *Xite* could act as an enhancer for development-specific *Tsix* regulation at the onset of X-inactivation. Frequent association of multiple bidirectional transcripts and DNaseI hypersensitive sites with enhancer elements supports *the Xite* enhancer model (Natoli and Andrau 2012; Lam et al. 2014). Indeed, transient enhancer assays revealed that *Xite* has development-specific enhancer activity in *Tsix* expression (Stavropoulos et al. 2005).

DXPas34, another region associated with bidirectional promoter activity and DNaseI hypersensitive sites, is also a positive regulator of *Tsix* expression (Stavropoulos et al. 2005; Vigneau et al. 2006; Cohen et al. 2007). *DXPas34* is a 1.2 kb CpG-rich region containing a 34-mer tandem repeat residing 750 bp downstream of the major *Tsix* promoter. Transient enhancer assays showed that *DXPas34,* as well as *Xite,* enhanced *Tsix* expression (Stavropoulos et al. 2005). Interestingly, deletion of *DXPas34* leads to repression of the major *Tsix* promoter and activation of *Xist* expression at the onset of X-inactivation, which is followed

by *Tsix* derepression without reversal of X-inactivation. This suggests that *DXPas34* functions as both an enhancer and repressor of *Tsix* in a differentiation-specific manner.

4.1.3 Tsx (Testes-Specific X-Linked Noncoding RNA)

Another noncoding transcript, *Tsx*, located nearly 40 kb from the 3′-end of *Xist*, was found to be specifically expressed in the testes and in a very low concentration in both male and female brains (Simmler et al. 1996; Anguera et al. 2011). Although *Tsx* was initially reported as a potential protein-coding gene, specifically as an encoder of a 144 amino acid protein (Simmler et al. 1996), further investigation has indicated that *Tsx* is likely to be a noncoding gene (Anguera et al. 2011). *Tsx* expresses in ES cells as well as in early embryos and is gradually repressed upon differentiation. The homozygous deletion of *Tsx* in female mice led to a small decrease in fertility, resulting in skewed sex ratios that slightly favored females. Aberrant upregulation of *Xist* along with *Tsix* downregulation was observed in a small population of the *Tsx* mutant cells; thus, *Tsx* might promote *Tsix* expression and indirectly upregulate *Xist* expression (Anguera et al. 2011).

4.2 Positive LncRNA Regulators of Xist

4.2.1 Jpx/Enox

Jpx/Enox is another important lncRNA, which is located 10 kb upstream of *Xist* and is expressed in the antisense direction of *Xist* (Chureau et al. 2002; Johnston et al. 2002; Chow et al. 2003). While transgenes including the X-inactivation hub of lncRNAs such as *Xist*, *Tsix,* and *Xite* could only induce *Xist* activation inefficiently, the additional upstream region of *Xist* restored the induction of *Xist* (Lee et al. 1999b). This suggests that the *Xic* requires an upstream region flanking *Xist*. Subsequently, further analysis showed that *Jpx* is required for the proper expression of *Xist* (Tian et al. 2010; Sun et al. 2013). *Jpx* escapes from X-inactivation and is upregulated during X-inactivation (Tian et al. 2010). When *Jpx* was deleted, no X-inactivation was induced in males; however, *Jpx* heterozygous mutant females exhibited severe phenotypes with massive cell death, significantly impaired *Xist* upregulation, and compromised X-inactivation induction. These defects and Xist expression levels were restored to normal with overexpression of *Jpx* using a transgene. These data thereby suggest that *Jpx* can act in *trans* to activate *Xist*, although the *Jpx* heterozygous mutation shows mildly reduced Xist expression in *cis* (Tian et al. 2010). As *Jpx* overexpression with *Tsix* disruption efficiently induces aberrant X-inactivation even in male differentiating embryonic bodies, both *Jpx* and *Tsix* coregulate *Xist* as an activator and repressor, respectively. That leaves the question of how Jpx RNA promotes *Xist* expression. A recent study has indicated

the unique function of Jpx RNA in *Xist* expression at the initiation of X-inactivation (Sun et al. 2013). In undifferentiated cells, transcription factor CTCF is loaded on the CTCF binding sites within the *Xist* P2 promoter by which *Xist* is repressed. Whereas overexpression of CTCF blocked induction of *Xist* upregulation, this repression was rescued by overexpression of *Jpx*. As CTCF binds to both the *Xist* promoter and Jpx RNA, it is proposed that Jpx RNA replaces CTCF from the *Xist* promoter, which is followed by induction of *Xist* expression on the Xi (Sun et al. 2013). Future work will be needed to elucidate the mechanism by which Jpx RNA selectively replaces CTCF from the *Xist* promoter in the future Xi to induce monoallelic *Xist* expression.

4.2.2 Ftx (Five Prime to Xist)

Ftx is another gene which encodes a long noncoding transcript located in the upstream of *Xist*, a potential activator for *Xist* (Chureau et al. 2002, 2011). *Ftx* escapes X-inactivation in both imprinted and random X-inactivation and, like *Jpx*, is upregulated upon induction of random X-inactivation (Chureau et al. 2011; Kobayashi et al. 2013). Deletion of *Ftx* in male mouse ES cells showed that the expression pattern of X-linked genes in the vicinity of *Ftx* was altered through a significant drop in expression levels. Furthermore, increased methylation at the 5′ CpG island of *Xist* was observed, suggesting a positive role of *Ftx* in *Xist* expression. However, *Ftx* has been reported to be dispensable in imprinted X-inactivation in the mouse embryo (Soma et al. 2014). In spite of targeted deletions of *Ftx*, neither the survival of female embryos nor the expression of *Xist* was affected during the preimplantation period in the *Ftx* mutant mice (Soma et al. 2014). Further investigation would be necessary to conclude whether *Ftx* is essential for random X-inactivation in female mice.

4.2.3 RepA

Apart from the two major isoforms of Xist RNA, known as long and short forms, there is another lncRNA which is derived from *Xist*. The 1.6 kb RepA RNA, which is transcribed from the repeat A region of *Xist*, was identified by a PRC2 immunoprecipitation (Zhao et al. 2008). Multiple roles for RepA RNA have been shown. For instance, RepA embedded within *Xist* is involved in both the recruitment of the PRC2 complex, Ezh2, and the activation of *Xist* (Zhao et al. 2008). Furthermore, the RepA region in Xist RNA has been shown to be a binding region for the Ezh2 protein, while other works have suggested that RepA plays a role in PRC2 complex spreading and H3K27me3 modification across the Xi (Plath et al. 2003; Kohlmaier et al. 2004; Zhao et al. 2008; Engreitz et al. 2013).

The *Xic* harbors a variety of lncRNAs such as *Tsix* and *Jpx* that are directly or indirectly involved in the regulation of *Xist* expression. Since lncRNAs are central players for the proper regulation of X-inactivation, further studies need to be

extensively performed in order to fully explore the roles played by each lncRNA as well as the cooperative molecular mechanism involved in their interaction. With advancing technologies and novel approaches, it is likely that more and more novel noncoding RNAs will emerge, allowing for a deeper understanding of *Xist's* regulation during X-inactivation and its associated epigenetic modifications.

5 X-inactivation and Disease

Maintenance of proper gene dosage in autosome and sex chromosomes is important for ideal development and survival of organisms (Torres et al. 2008). Aneuploidy is referred to as the condition where cells possess an atypical number of chromosomes and is usually detrimental to the organism (Fig. 2a). Although some patients with autosomal aneuploidies can survive, they are at a high risk of congenital

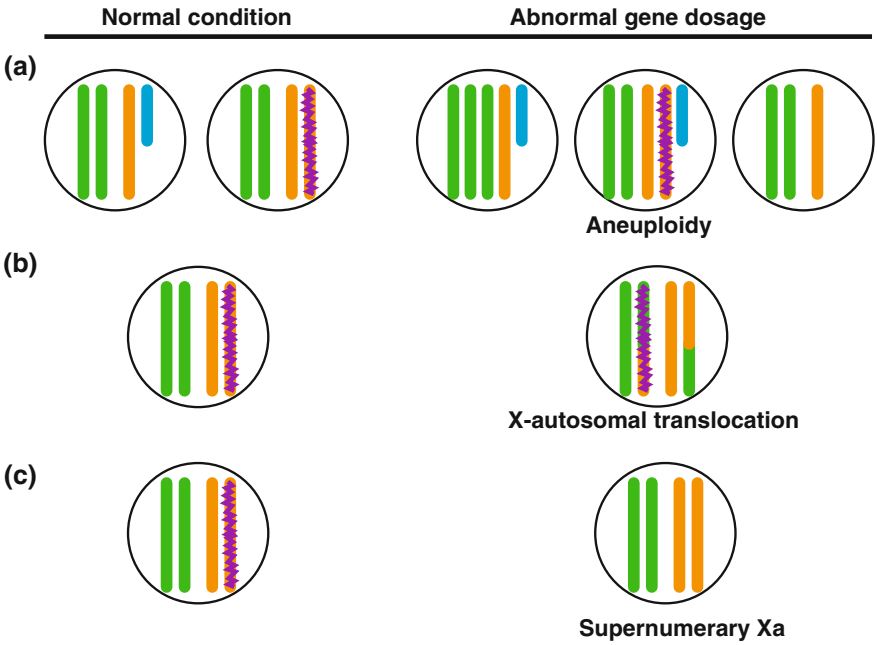

Fig. 2 Genetic and epigenetic failures induce abnormal gene dosage. Deviation of gene dosage leads to detrimental effects on cell survival and development. **a** Aneuploidy with excess or loss of certain chromosome. **b** X-autosomal translocation could induce inactivation of the autosome fused to the X chromosome segment containing the *Xic*. The segment of X chromosome which lacks *Xic* in turn fails to be inactivated. **c** Cells with more than two active X chromosome arise by loss of the Xi followed by duplication of the Xa. Reactivation of the Xi could be a potential cause of a supernumerary Xa. *Green*, *orange,* and *blue bold lines* indicate the autosome, X, and Y chromosomes, respectively. *Purple zigzag line* indicates Xist RNA-induced gene silencing

malformations. Some examples are the trisomies in chromosomes 13, 18, and 21, usually referred to as Patau, Edwards, and Down syndromes, respectively. Turner syndrome and Klinefelter syndrome are diseases that arise due to an absence of one of the two X chromosomes (XO) in females and an extra X chromosome (XXY) in males, respectively (Sybert and McCauley 2004; Groth et al. 2013). While only one X chromosome remains active per each cell as a result of X-inactivation, 15 % of X-linked genes on the Xi in humans are known to be actively transcribed even in this chromosome-wide silenced environment; these are called escape genes (Carrel and Willard 2005). Absence and excess of escape gene expression on the Xi is proposed to be a cause of Turner and Klinefelter syndromes, respectively.

X-inactivation is a complex enforcement system, which tightly controls the X-linked gene balance between the sexes, as well as secures and maintains a perfect ambience for proper cell differentiation and development. When certain segments from the X chromosome corresponding to the *Xic* are translocated to an autosomal region, the autosomal genes around the translocation site might be silenced through XIST RNA spreading coupled with accumulation of repressive epigenetic modifications (Fig. 2b) (Brown et al. 1991b), which potentially leads to haploinsufficiency of autosomal genes (White et al. 1998; Giorda et al. 2008; Van Echten-Arends et al. 2013).

While X-linked genes and autosomal genes are present in two copies in females, subsets of genes are expressed only from a single allele in individual cells. X-inactivation and genomic imprinting have allowed us to better understand the mechanism involved in determining whether a particular gene on a single allele will be expressed or repressed, as recently shown by the attribution of lncRNAs as master regulators of monoallelic expression of *Xist* and imprinted genes (Lee and Bartolomei 2013). The monoallelic expression of female X-linked genes in mammals is crucial for cellular survival and development. In addition, misregulations in proper monoallelic expression of imprinted genes are known to lead to a wide range of diseases such as Beckwith–Weidemann syndrome and Angelman syndrome. Thus, even deviations from normal X-linked gene dosage conditions could potentially give rise to developmental anomalies and disease states (Fig. 2c). In the context of disease conditions induced by dysfunctional X-inactivation, our knowledge remains poor even though the phenomenon is scientifically fascinating.

5.1 X-inactivation and Cancer

While each somatic cell contains a pair of active autosomal chromosomes in mammals, this is not the case in sex chromosomes. When an unfavorable recessive change occurs in an autosomal allele, the secondary chromosome pair can act as a backup to replace the damaged gene; thus, the heterozygous condition might delay or prevent a catastrophic situation. As mentioned previously, mammalian sex chromosomes are characterized as genetic unisomy and functional unisomy in males and females, respectively. Furthermore, the X chromosome is laden with

important genes for cellular differentiation and proliferation, as well as those related to cancer. Thus, genetic changes taking place on the delicate sex chromosomes could be immediately detrimental due to the lack of a backup copy and the higher likelihood that the mutations, when carried forward, may be prone to cancer (Spatz et al. 2004).

Aberrant X-inactivation can bring about local as well as chromosome-wide disturbances of X-linked gene silencing to alter the expression of cancer-related and other genes across the Xi which may lead to tumors (Chaligné and Heard 2014). A loss of *Xist* expression has been reported in many cancer cell lines derived from female breast, cervix, and ovary tumors (Kawakami et al. 2004b; Sirchia et al. 2005; Richardson et al. 2006). In female cancer cells, the frequent disappearance of *Xist*-expressing cells happens by loss of the Xi followed by a duplication of the Xa instead of reactivation of the Xi. In non-cancerous tissue, reactivation of the Xi by loss of *XIST* expression could potentially cause a wide range of derepression of X-linked genes, including cancer-related genes, resulting in abnormalities and diseases. X chromosome reactivation in mice is tightly restricted to happen within the inner cell mass at the blastocyst stage followed by random X-inactivation in the epiblast lineage and during the development of the primordial germ cells (Ohhata and Wutz 2013). Thus, reactivation outside of these periods could lead to detrimental effects. Since *Xist* is constitutively expressed from the Xi in differentiated somatic cells, it is suggestive of *Xist's role* in the maintenance of X-inactivation. However, RT-PCR analysis of the mouse/human somatic hybrid cell lines containing human Xi revealed that gene silencing of *XIC*-lacking human Xi is highly stable, suggesting that no X reactivation takes place on the human Xi without *XIC* once X-inactivation is established (Brown and Willard 1994) Additionally, when *Xist* was conditionally deleted in primary mouse embryonic fibroblast cells, the Xi exhibited maintenance of its unique heterochromatic features such as late DNA replication and hypoacetylation on histone H4 even though histone variant macroH2A disappeared due to its Xist RNA-dependent localization (Csankovszki et al. 1999). Based on these observations, it had long been believed that *Xist* is essential for the initiation of random X-inactivation but dispensable for the maintenance of X-inactivation once the Xi is established. With the recent advantage of high-throughput sequencing and technical improvements, accumulating evidence has indicated otherwise, specifically that depletion of *Xist* RNA from the Xi can induce partial reactivation of a subset of X-linked genes on the Xi (Csankovszki et al. 2001; Zhang et al. 2007). In conditional *Xist*-deleted mouse fibroblast, an assessment of individual gene activity revealed that conditional *Xist* deletion on the Xi led to a slightly increased frequency of reactivation in X-linked GFP and Hprt genes (Csankovszki et al. 2001). This reactivation frequency was largely enhanced by treatment with 5-azadC and trichostatin A, inhibitors for DNA methylation and histone hypoacetylation, respectively, indicating that multiple layers of epigenetic regulation prevent improper reactivation of the Xi. Detailed analysis of conditional *Xist* knockout in dermal and embryonic fibroblast cells also showed that reactivation of the silenced X-linked genes on the Xi happened in a subset of genes (Zhang et al. 2007). Whereas the repressive histone mark H3K27me3 disappeared on the Xi

by conditional *Xist* knockout, a dearth of active histone marker H3K4me2 remained on the Xi as well, suggesting overall chromosome-wide silencing is somewhat maintained without Xist RNA.

A recent study using human induced pluripotent stem cells (hiPSCs) also supports the role of XIST RNA in the stable repression of X-linked genes. Loss of *XIST* expression in hiPSCs is significantly correlated with the upregulation of X-linked oncogenes associated with higher growth rate in vitro and poor differentiation in vivo (Anguera et al. 2012). hiPSCs derived from differentiated cells using the Yamanaka factors (OCT-4, SOX2, KLF4, and c-MYC) or its derivatives hold great potential in regenerative medicine (Takahashi and Yamanaka 2006; Takahashi et al. 2007). However, hiPSCs and human embryonic stem cells (hESCs) are known to be genetically and epigenetically unstable (Kim et al. 2010; Bock et al. 2011; Gore et al. 2011); thus, strict validation of hiPSCs would be necessary for therapeutic purposes. Atypical features of female hiPSCs and hESCs are evident in X-inactivation; it is not currently known why these features are not observed in mouse ES cells (Dvash and Fan 2009; Lessing et al. 2013). They are generally classified into three distinct classes based on the status of X-inactivation and *XIST* expression (Silva et al. 2008; Anguera et al. 2012): Class I lines, which have two Xa's and can undergo X-inactivation very similarly to mouse ES cells; Class II lines, wherein one X is already inactivated by the XIST cloud and cells may be partially differentiated; and finally Class III lines, wherein X-inactivation is already complete, and however, the expression of *XIST* is lost. Analysis across different hiPSC cell lines, especially when comparing Class II with Class III lines, showed loss of *XIST* expression is associated with an upregulation of X-linked genes in the Class III lines. Intriguingly, the upregulated X-linked genes include cancer-related genes such as MAGEA2 and MAGEA6, which are highly expressed in cancers (Rogner et al. 1995). These observations might be a hamper to using hiPSCs as a therapeutic tool. Despite no strong evidence thus far, the changes could be attributed to the loss of *XIST* expression. Conversely, *XIST* expression and presence of the Xi could be used as benchmarks to assess hiPSC quality. Furthermore, it should be noted that culture condition to establish and maintain hiPSCs has been improved to create naive Class I hiPSCs with high efficiency (Tomoda et al. 2012; Gafni et al. 2013).

Despite close association between the overexpression of X-linked genes and supernumerary Xa with many human cancers (Liao et al. 2003; Kawakami et al. 2004b; Pageau et al. 2007), it is not clear whether aberrant X-inactivation and reactivation of X-linked genes are a primary cause or just a consequence of cancer transformation and progression. More recently, our understanding of X-inactivation has advanced from its role in dosage compensation to include higher order functions such as tumor suppression. When a conditional knockout of *Xist* was achieved in the hematopoietic stem cells of mice, highly aggressive forms of hematologic cancer were manifested in a female-specific manner (Yildirim et al. 2013). Histopathological analysis of female mutant mice revealed that *Xist* deletion in the hematopoietic compartment induced myelodysplastic/myeloproliferative neoplasm (also known as mixed myeloproliferative/myelodysplastic syndrome [MPN/MDS])

(Orazi and Germing 2008). A detailed analysis of *Xist* deletion mutant mice showed that *Xist* deletion induced a significant genome-wide gene upregulation, especially in X-linked genes including cancer-related genes, in comparison with autosomal genes. This anomalous gene expression in the *Xist* mutant mice would potentially promote cancerous effects, suggesting a crucial role of *Xist* in not only maintenance of X-inactivation but also suppression of cancer. This is the first report that *Xist* disruption leads to a causal effect on cancer in vivo.

5.2 Skewed X-inactivation and X-linked Diseases

As a consequence of random X-inactivation with an equal probability of either the paternal or maternal originating X chromosome being inactivated, every female's expression profile is a mosaic with cells having either the paternal or maternal Xi. Such mosaicism is usually advantageous for females since it contributes to physiological diversity and eases the deleterious effects of X-linked mutation. For females who have inherited unfavorable mutations on their X chromosome, there is a chance that approximately half of their somatic cells will express genes from the wild-type allele. This orderly process deviates in a subset of individuals, although the skewing varies by age and cell types (Sharp et al. 2000; Hatakeyama et al. 2004; Minks et al. 2008). It is also known that the clonality of cells varies in different female organs, which could also affect the X-linked phenotype in females (Thomas et al. 1988; Bittel et al. 2008). Since X chromosomes in mammalian female somatic cells are functionally unisomy by random X-inactivation, deviation from random X-inactivation ultimately results in a predominance of either the maternal or paternal X chromosome. This predominance could occur either by chance, by selection after primary choice, or in a predetermined fashion due to the presence of genetic elements or mutations that propel selection bias (Belmont 1996; Minks et al. 2008). The X-linked disorder in female carriers, coupled with skewed X-inactivation in favor of the wild-type X, give rise to greater populations of cells in which the mutated X-linked gene is expressed, therefore manifesting as a severe disease condition (Fig. 3) (Migeon 2006). Less severe variations are more likely to occur in females with smaller populations of mutated X-linked genes.

5.2.1 Rett Syndrome

Rett syndrome, an X-linked neurologic disorder which results in severe intellectual disability, primarily affects females and manifests during early childhood, typically occurring between 6 and 18 months of age; prior to onset, development appears to be normal, but is then followed by developmental regression, reduced brain growth, and severe mental retardation (Weaving et al. 2005). In newborn males, the disease is fatal. Rett syndrome symptoms vary from child to child with severity ranging from subtle abnormalities such as loss in muscle tone, difficulty in feeding, and

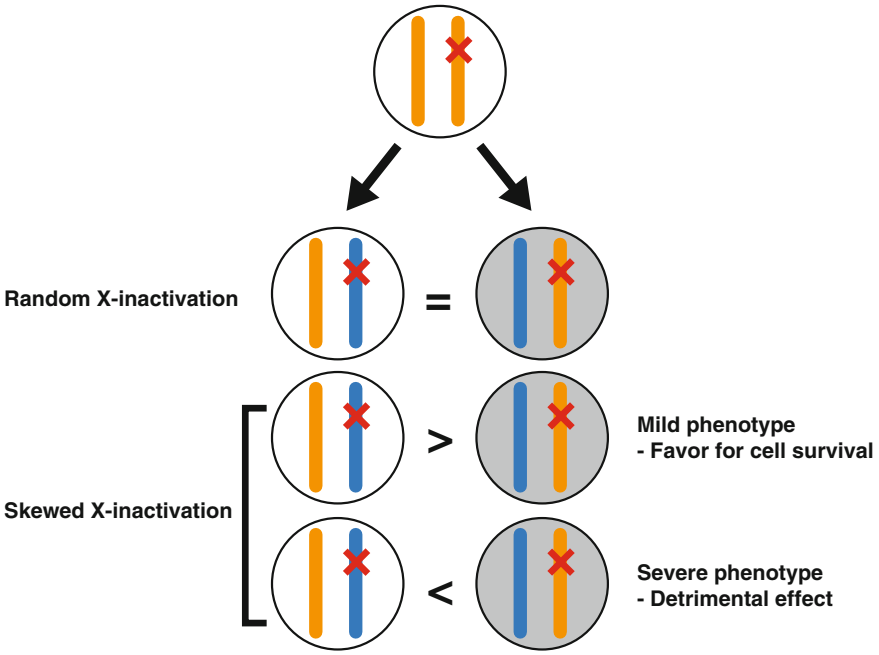

Fig. 3 Skewed X-inactivation and disease phenotype. Female patients carrying a harmful mutation in a single allele show a variety of severity in disease phenotype due to skewed X-inactivation. The skewing of X-inactivation in favor of the wild-type X chromosome specifically leads to a severe phenotype. *Orange* and *blue bold lines* indicate the Xa and Xi, respectively. *Red X* indicates harmful mutation. *White* and *gray circles* show healthy and damaged cells, respectively

jerkiness of limbs to more complex mental and physical abnormalities. Rett syndrome arises as a result of a heterozygous mutation in the X-linked gene encoding ubiquitous methyl-CpG binding protein 2 (MECP2) (Amir et al. 1999). MECP2 functions as a transcriptional regulator by binding to the genome in a DNA methylation-dependent manner. Female Rett syndrome patients possess mosaic wild-type/mutant MECP2 expression as a result of the random nature of X-inactivation in somatic cells. Random X-inactivation could be affected by chance, selection, or other genetic factors during early embryonic development. Skewed X-inactivation in patients with a MECP2 mutation progresses into a wide range of clinical presentations and manifests as Rett syndrome (Camus et al. 1996; Krepischi et al. 1998; Weaving et al. 2003). Interestingly, since Rett syndrome is not accompanied by neurodegeneration, attempts to express a Mecp2 transgene in postmitotic neurons have partially rescued the neurologic symptoms in both immature and mature Mecp2 mutant mice (Giacometti et al. 2007; Guy et al. 2007).

5.2.2 X-inactivation in Other Diseases

Similarly, the relation between skewed X-inactivation and phenotype severity in X-linked disease has been shown in a number of X-linked cutaneous genetic diseases such as incontinentia pigmenti, which is associated with characteristic patterns of lines and swirls appearing on the patient's body called Blaschko's lines (Happle 2006; Sun and Tsao 2008). Blaschko's lines are believed to indicate the migration path of ectodermal skin cells during development illuminated by female cells containing a mix of wild-type and mutant Xi (Happle and Frosch 1985). For example, incontinentia pigmenti caused by heterozygous mutation in X-linked NFkB essential modulator (NEMO) (Smahi et al. 2000) commonly develops vesicles that later progress to verrucous and finally to hyperpigmentation in Blaschko's lines across the trunk. While phenotypic outcome varies in incontinentia pigmenti patients, skewed X-inactivation has been reported in females with heterozygous NEMO mutation (Martinez-Pomar et al. 2005), implicating the correlation between skewed X-inactivation and phenotypic severity of X-linked disease. Skewed X-inactivation is also implicated in X-linked disease conditions such as autoimmune deficiency (Puck et al. 1987), Duchenne muscular dystrophy (Yoshioka et al. 1998; Viggiano et al. 2013), and cancers (Kristiansen et al. 2002, 2005). Clarification of the molecular mechanism that affects probability of skewing toward either X chromosome could help us to develop therapeutic approaches and potential drug targets to improve the condition of these patients.

6 Therapeutic and Diagnostic Applications Using X-inactivation

Characteristic features of Xist RNA-mediated X-inactivation could potentially be harnessed for clinical purposes. Interestingly, the long-range inactivation potential of Xist RNA has already been employed to suppress the extra chromosome 21 in iPS cells established from cells of Down syndrome patients (Jiang et al. 2013). The detrimental condition in Down syndrome arises as a result of three copies of chromosome 21. The manifestations of such an aberration lead to numerous birth defects, stunted growth, reduced intellectual abilities, mental retardation, congenital heart defects, and many physical abnormalities (Mégarbané et al. 2009; Gardiner 2010). To correct gene dosage of chromosome 21, zinc finger nucleases (ZFN) was used to insert an inducible *XIST* into the *DYRK1A* locus on one of the three chromosome 21s of iPS cells derived from a Down syndrome patient. Subsequently, the *XIST* transgene initiated accumulation of the repressive histone markers H3K27me3, H4K20me1, and H2AK119ub1 onto the modified chromosome. This action was associated with hypermethylation of the CpG islands at promoter, and gene repression across the modified chromosome. These results suggest that genes across chromosome 21 undergo chromosome-wide gene silencing by induction of transgenic *XIST* similar to X-linked gene silencing on the

Xi. Most importantly, *XIST* induction from the transgene on the extra copy of chromosome 21 rescued the defects in proliferation and neural development. A similar application could be used to develop potential therapeutics for other diseases with an extra number of chromosomes such as Edwards syndrome (Trisomy 18) and Patau syndrome (Trisomy 13).

Potential application of *XIST* as a biomarker has been proposed in testicular germ cell tumors (TGCTs) (Kawakami et al. 2004a). Plasma samples obtained from patients with TGCTs showed hypomethylation of the 5′-end of CpG sites in *XIST,* while somatic cells showed complete methylation through the CpG sites. The *XIST* gene is silenced on the Xa in males and usually methylated at its 5′-end. Detection of unmethylated *XIST* would be due to the extranumerical X with partial inactivation in TGCT patients (Kawakami et al. 2003). Therefore, the methylation profile of *XIST* might also be used as a potential biomarker for diagnosis of male patients suffering from TGCTs. More recently, advances in the use of *Xist* expression have led to its potential as a biomarker attributing to its high levels in the urine of patients with membranous nephropathy (MN). MN is an autoimmune-induced glomerular nephritis and the most common cause of nephrotic syndrome in humans (Huang et al. 2014). The primary experiments carried out in MN model mice showed significant upregulation of *Xist* and long noncoding gene *Neat1* in tubular epithelial and glomerular cells. Interestingly, *Xist* expression levels detected in urine but not serum of MN mice are strongly correlated with the severity of MN. Significantly, this finding could be applied to identify human patients developing different types of glomerular nephritis, particularly as upregulation levels of *XIST* were detected in the urine, but not in serum samples, of human patients confirmed to have glomerular nephritis. Thus, *XIST* is a potential noninvasive biomarker to detect this disease.

There is support for examining the validity of underutilized molecular markers for diagnostic purposes. In the most recently released data for the GENCODE project (version October 22, 2014, http://www.gencodegenes.org), the number of long noncoding genes in humans is nearly comparable with the number of conventional protein-coding genes and outnumbers miRNA (Henry and Hayes 2012; Hayes et al. 2014). This offers a new variety of possible diagnostic biomarkers to investigate. Thus, combining the expression profiles of several lncRNAs, including *XIST*, might allow us to develop better and more reliable diagnostic tools in the future.

7 Conclusions and Future Perspectives

The discovery of novel lncRNAs and their varied functions is emerging at a high rate owing to the advancement of novel techniques used to detect and investigate them. Thus, our understanding of abundant lncRNAs has increased over the decades. In this chapter, we describe lncRNAs residing within the *Xic* in mice and implicated the role of X-inactivation in the initiation and progression of diverse

disease conditions. To further understand the functional mechanism of lncRNAs in a variety of physiological processes, identification of the protein partners in the ribonucleoprotein complex would be inevitable. Recent introduction of techniques such as CHART, RAP, ChIRP (chromatin isolation of RNA purification), RIP-seq (RNA immunoprecipitation-sequencing), and CLIP (crosslinking and immunoprecipitation) help our understanding of the molecular interaction and function between lncRNA and protein partners (Hafner et al. 2010; König et al. 2010; Chu et al. 2011; Simon et al. 2011). Growing evidence linking pathological conditions and developmental anomalies to lncRNAs is emerging, especially in cancer development and progression. Thus, lncRNAs could become valuable therapeutic targets and promote the development of rapid diagnostic tools. Future studies into X-inactivation, a paradigm of lncRNA-mediated gene regulation, will provide additional insight to the molecular mechanisms behind lncRNA function, which will in turn further contribute to lncRNA research and its clinical and diagnostic applications.

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Long Non-coding RNA *ANRIL* and *Polycomb* in Human Cancers and Cardiovascular Disease

Francesca Aguilo, Serena Di Cecilia and Martin J. Walsh

Abstract The long non-coding RNA *CDKN2B-AS1*, commonly referred to as the *Antisense Non-coding RNA in the INK4 Locus* (*ANRIL*), is a 3.8-kb-long RNA transcribed from the short arm of human chromosome 9 on p21.3 that overlaps a critical region encompassing three major tumor suppressor loci juxtaposed to the *INK4b-ARF-INK4a* gene cluster and the methyl-thioadenosine phosphorylase (*MTAP*) gene. Genome-wide association studies have identified this region with a remarkable and growing number of disease-associated DNA alterations and single nucleotide polymorphisms, which corresponds to increased susceptibility to human disease. Recent attention has been devoted on whether these alterations in the *ANRIL* sequence affect its expression levels and/or its splicing transcript variation, and in consequence, global cellular homeostasis. Moreover, recent evidence postulates that *ANRIL* not only can regulate their immediate genomic neighbors in *cis,* but also has the capacity to regulate additional loci in *trans*. This action would further increase the complexity for mechanisms imposed through *ANRIL* and furthering the scope of this lncRNA in disease pathogenesis. In this chapter, we summarize the most recent findings on the investigation of *ANRIL* and provide a perspective on the biological and clinical significance of *ANRIL* as a putative biomarker, specifically, its potential role in directing cellular fates leading to cancer and cardiovascular disease.

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1 The DNA and RNA Landscape Overlapping Chr9p21 Loci

A growing number of genome-wide association studies (GWASs) have identified specific regions of the human genome with a strong non-random correlation to complex human traits with predisposition to disease (de los Campos et al. 2010). Indeed, several single nucleotide polymorphisms (SNPs) have been identified on the *INK4b-ARF-INK4a* locus located on the human chromosome 9p21 that are tightly related with the increase of cardiovascular disease (CVD) (de los Campos et al. 2010; Gschwendtner et al. 2009) ischemic stroke (Gschwendtner et al. 2009; Matarin et al. 2008), aortic aneurysm (Helgadottir et al. 2008), type II diabetes (Zeggini et al. 2007; Scott et al. 2007), glioma (Shete et al. 2009; Wrensch et al. 2009), and cancer predisposition (Shete et al. 2009; Wrensch et al. 2009; Cunnington et al. 2010; Bishop et al. 2009), among other conditions.

The *INK4b-ARF-INK4a* locus encodes three critical tumor suppressor genes, $p14^{ARF}$ ($p19^{ARF}$ in mice), $p15^{INK4b}$, and $p16^{INK4a}$, all of which play a central role in cell-cycle arrest, thus affecting key cellular processes such as senescence, apoptosis, and stem cells self-renewal by triggering the activities of both retinoblastoma (Rb) and p53 pathways (Gil and Peters 2006; Popov and Gil 2010). Specifically, $p15^{INK4b}$ and $p16^{INK4a}$ target cyclin-dependent kinases CDK4 and CDK6, preventing the binding of these proteins to D-type cyclins and, as a consequence, inhibiting CDK4/6-mediated phosphorylation (inactivation) of retinoblastoma (RB1) family members. In contrast, the unrelated $p14^{ARF}$ protein acts primarily by binding to the E3 ubiquitin-protein ligase MDM2, promoting its degradation, and therefore abrogating MDM2 inhibition of the TRP53 activity (Popov and Gil 2010). The locus contains a fourth gene, methylthioadenosine phosphorylase (MTAP), which has annotated exons overlapping the INK4b-ARF-INK4a locus (Nobori et al. 1996). MTAP catalyzes the phosphorylation of 5′methyladenosine (MTA) in the polyamine pathway, and it has also been associated with cancerogenesis (Behrmann et al. 2003; Schmid et al. 1998).

The long non-coding RNA *ANRIL* (*Antisense Non-coding RNA in the INK4 Locus*) was first identified within the 403-kb germ-line deletion of a French family with a history of melanoma and neural system tumors (Pasmant et al. 2007). *ANRIL* is transcribed as a 3,834-bp lncRNA in the opposite direction from the *INK4b-ARF-INK4a* cluster (Yu et al. 2008), and it shares a bidirectional promoter with $p14^{ARF}$, as the 5′ end of the first exon of *ANRIL* is located 300 bp upstream of the transcription

start site (TSS) of the $p14^{ARF}$ gene. Hence, the expression of both genes is coordinated, and reporter assays have shown a transcriptional activation of this divergent promoter by E2F1 and the insulator CTCF (Sato et al. 2010; Rodriguez et al. 2010). Specifically, CTCF binding is required to maintain the INK/ARF locus in an inducible conformation, which is abrogated upon DNA methylation, having consequences in cancer progression (Rodriguez et al. 2010).

ANRIL transcript contains 20 exons, many of them consisting of LINE, SINE, and *Alu* repetitive elements (Jarinova et al. 2009), that can be alternatively spliced. *ANRIL* transcripts are expressed at very low levels, and the two short forms, both terminating with polyadenylated exon 13, EU741058 (exons 1, 5, 6, 7, 13) and DQ485454 (exons 1–13), and the long form NR_003529 that lacks the exon 13 and terminates with polyadenylated exon 20 (exons 1–20), are the most abundant transcripts. Circular *ANRIL* (c*ANRIL*) isoforms have also been described (Burd et al. 2010), which result from exon skipping events occurring during RNA splicing. Thus, c*ANRIL* show non-sequential linkages between various *ANRIL* exons, appearing species like exons 4–6 and 14–5, to name some examples. A fusion transcript between the MTAP gene and the 3′ end of *ANRIL* has also been identified in cell lines with 9p21 deletion but not in normal cell lines (Burd et al. 2010; Schmid et al. 2000). Many of the *ANRIL* isoforms can coexist in the same cell type although others are tissue-specific (Burd et al. 2010; Folkersen et al. 2009), increasing the complexity of its regulatory mechanism. These alternative splicing events might modify *ANRIL* structure leading to changes not just in *Polycomb* group (PcG) proteins-mediated *INK4b-ARF-INK4a* locus regulation. In fact, the overexpression of exons 13–19 in HeLa cells resulted in the repression of a wide set of genes involved in chromatin architecture remodeling, being Centrosomal protein 290 kDa (CEP290), E1 A binding protein p300 (EP300), and transcription factor 7-like 1 (TCF7L1) the most repressed proteins (Sato et al. 2010). Interestingly, Ras responsive element binding protein 1 (RREB1) and Zinc finger and BTB domain containing 32 (ZBTB32) were upregulated upon *ANRIL* 13–19 overexpression (Sato et al. 2010).

2 ANRIL and Polycomb Group Proteins

The PcG proteins were originally identified in *Drosophila melanogaster*, as transcriptional repressors of homeotic (Hox) genes, required for the correct spatio-temporal expression of developmental regulators along the body axis (Lewis 1978). In most metazoan species, the PcG proteins form two macromolecular repressive complexes named polycomb repressive complex-1 (PRC1) and polycomb repressive complex-2 (PRC2) (Levine et al. 2002). The PRC2 complex consists of three subunits: embryonic ectoderm development (EED), suppressor of zeste 12 (SUZ12), and enhancer of zeste 2 or 1 (EZH2/1), which catalyze the mono-, di-, and trimethylation of lysine 27 of histone H3 (H3K27me1, H3K27me2, and H3K27me3) (Margueron et al. 2008; Shen et al. 2008). H3K27me3 is a signature for chromobox-domain (CBX) protein recognition and PRC1 recruitment. The

PRC1 composition is heterogeneous, depending on the cellular context, and contains several PcG proteins, including one member of the PCGF family (PCGF1-PCGF6) and of the HPH family (HPH1-HPH3), together with chromobox-domain (CBX) protein and RING1a/1b, which catalyze the mono-ubiquitination of H2a on K119 (H2AK119ub1) for the maintenance of silent chromatin (Cao et al. 2005; Wang et al. 2004).

Several long non-coding RNAs have a direct role in recruiting PcG proteins to specific loci to modify the epigenetic chromatin state and thereby to repress gene expression. Some documented examples include *XIST* RNA (Mak et al. 2002; Zhao et al. 2008), *KCNQTLOT1* (Fitzpatrick et al. 2002; Pandey et al. 2008), *HOTAIR* (Rinn et al. 2007) and *ANRIL* (Kotake et al. 2011; Yap et al. 2010). Indeed, *ANRIL* specifically associates with the chromodomain of chromobox homolog 7 (CBX7), a subunit of the PRC1 complex, and participates in CBX7 recognition of H3K27me3 to silence the *INK4b-ARF-INK4a* cluster (Yap et al. 2010). This interaction is abolished after treatment of cell nuclei with the transcriptional inhibitor α amanitin, indicating that *ANRIL* is stably associated with CBX7 as a nascent transcript generated by the RNA polymerase II. Moreover, knockdown of *ANRIL* decreases H3K27me3 levels and it is associated with increased $p16^{INK4a}$ expression, which coincides with a reduction in CBX7 and EZH2 binding at the $p16^{INK4a}$ TSS (Yap et al. 2010). Overall, this mechanism is important for the *INK4b-ARF-INK4a* locus repression in order to control senescence [reviewed by (Aguilo et al. 2011)] (Fig. 1).

On the other hand, *ANRIL* can also interact with the PRC2 component SUZ12 and influence SUZ12 binding to the $p15^{INK4b}$ locus. Thus, depletion of *ANRIL* increases the expression of $p15^{INK4b}$, but not $p16^{INK4a}$ or $p14^{ARF}$, and inhibits cellular proliferation, thereby influencing human disease progression (Aguilo et al. 2011). Recently, RIP sequence (RIP-seq) experiments performed in MonoMac cells in which two specific exon-combinations of *ANRIL* were overexpressed, showed a binding of *ANRIL* with CBX7 and RING1B from PRC1, a binding with the PRC2 subunits EED, JARID2, RBAP46, and SUZ12, and PRC-associated proteins RYBP and YY1 (Holdt et al. 2013).

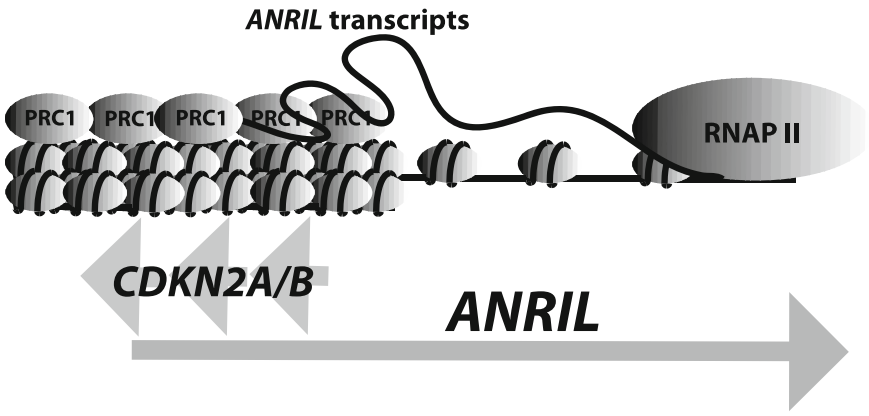

Fig. 1 Illustration of how the *ANRIL* transcript may facilitate polycomb repressive complex 1 to compact chromatin structure of the *INK4b-ARF-INK4a* locus

3 ANRIL and Cardiovascular Disease

CVD covers a wide array of disorders, including diseases of the cardiac muscle and of the vascular system supplying the heart, brain, and other vital organs.

ANRIL locus has been highlighted as the strongest genetic susceptibility locus for CVD, being numerous polymorphisms located in this locus directly associated with increased risk of developing CVD (Cunnington et al. 2010; Folkersen et al. 2009; Holdt et al. 2010; Holdt and Teupser 2012; Liu et al. 2009). In particular, the coronary artery disease (CAD)-associated SNPs are located on chromosome 9p21.3, specifically in a linkage disequilibrium block that does not contain known protein-coding genes, spanning a region of 58-kb named the CAD interval (Guttman et al. 2009). For example, the SNP rs496892-G is linked to atherosclerotic stroke, whereas the rs10757276-G is the lead SNP for CVD risk. These polymorphisms affect the expression of *ANRIL* (Holdt et al. 2010; Congrains et al. 2012), which in turn regulates the expression of downstream genes involved in several atherogenic pathways and/or inflammation response. For example, decreased expression of *ANRIL* transcripts containing exon 13 correlates with decreased expression of adiponectin receptor 1 (ADIPOR1), vesicle-associated membrane protein 3 (VAMP3), and transmembrane protein 258 (C11ORF10) (Bochenek et al. 2013).

Another possibility is that the polymorphism in the CAD interval may affect *ANRIL* splicing and, as a consequence, *ANRIL* structure. Specifically, two SNPs (rs.7341786 and rs7341791) identified in the exon 15, from where most of the c*ANRIL* transcripts arise, were shown to be in linkage disequilibrium with the ASVD-associated SNP rs1075728 and were predicted to increase the ability of exon 15 of acting as splice acceptor. Furthermore, individuals harboring the casual variants mentioned above exhibit a derepressed INK4b-ARF-INK4a expression (Burd et al. 2010), indicating that the alteration of *ANRIL* structure may affect the efficiency of *ANRIL* at repressing the INK4b-ARF-INK4a locus.

Additionally, many of the polymorphisms in the 9p21 locus can also disrupt predicted transcription factor binding sites (Harismendy et al. 2011). For instance, rs564398, one of the SNPs most strongly correlated with *ANRIL* expression, disrupts 'Ras Responsive Element Binding Protein 1' (RREB1) binding site, and the SNP (rs10757278) disrupts the binding of the STAT1 (Signal-transducer and activator of transcription) transcription factor, increasing CVD susceptibility (Harismendy et al. 2011).

The presence of multiple enhancers in this region suggests that the expression of the *INK4b-ARF-INK4a* locus is regulated in a temporal and tissue-specific manner. Thus, some enhancers in the CAD interval appear functional in certain cell types and have cell-type-specific effects. An example is given from the transcription factor STAT1. In physiological conditions, activation of the JAK-STAT pathway is triggered when type II interferons (IFN) bind to their receptor, inducing Janus kinase (JAK) phosphorylation, which in turn phosphorylates STAT family of transcription factors. Upon tyrosine phosphorylation, STAT dimerizes and translocates to the nucleus where it modulates a number of target genes. Although the STATs are

generally associated with transcriptional activation, examples of STAT-dependent transcriptional repression have also been reported (Aaronson and Horvath 2002). Thus, studies in limphoblastoid cell lines (LCL) show that there is a correlation between the CAD risk variants and CDKN2A/B and *ANRIL* expression in lymphocytes (Helgadottir et al. 2008), and that the CAD risk alleles preclude binding of STAT1 at the enhancer ECAD9. STAT1 occupancy on this enhancer correlates with the repression of *ANRIL* expression. On the other hand, IFNγ treatment of endothelial cells (HUVEC) induces STAT1 binding to the same enhancer, which in turn results in increased *ANRIL* expression (Helgadottir et al. 2007).

The Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) cascade is another essential signaling transduction pathway involved in INK4b-ARF-INK4a locus regulation (Malumbres et al. 2000). In fact, it has been shown that the pro-oncogenic Ras protein inhibits *ANRIL* expression and activates $p15^{INK4b}$, suggesting a potential negative regulation of $p15^{INK4b}$ by *ANRIL* (Kotake et al. 2011). Ras has an important role in atherosclerosis progression, promoting vascular senescence and inducing the expression of pro-inflammatory cytokines. In particular, a constitutive activation of Ras is involved in atherogenesis by inducing vascular smooth muscle cell (VSMC) senescence and expression of proinflammatory cytokines (Minamino et al. 2003). Furthermore, activation of ERK and vascular inflammation is associated with VSMC senescence in human atherosclerosis, which suggests that the Ras/Raf/MEK/ERK signaling cascade plays an important role in regulating VSMC lifespan and function in vivo (Minamino et al. 2003).

Previous mechanistic studies postulated that *ANRIL* serve as a scaffold for the chromatin modifying complexes PRC1 and PRC2, mediating the repression in *cis* of the *INK4b-ARF-INK4a* locus (Kotake et al. 2011; Yap et al. 2010). Nonetheless, a recent study revealed that *ANRIL* association with CVD susceptibility can be related to its capability of regulating gene expression in *trans* (Holdt et al. 2013), leading to decreased apoptosis and increased cell proliferation and cell adhesion, characteristic and essential alterations of atherogenesis (Lusis 2000). In particular, *ANRIL*-regulated genes contain an *Alu* repeat motif in their promoters, and the occupancy of CBX7 and SUZ12 is highly enriched ∼150 bp downstream of this Alu motif. Alu repeats are a family of primate-specific short interspersed repeat elements (SINEs) with more than one million copies in the human genome (Lander et al. 2001; Dewannieux et al. 2003) and have been linked with genetic disease (Burns and Boeke 2012). Interestingly, the Alu motif is also present in the *ANRIL* transcript and it is predicted to locate in a central stem-loop-like structure (Holdt et al. 2013), pointing to RNA-chromatin interactions as an effector mechanism (Mercer et al. 2009).

4 ANRIL and Cancer Predisposition

Cancer is a group of more than 100 diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body.

The *INK4b-ARF-INK4a* gene cluster is homozygously deleted or silenced in approximately 40% of human cancers (Iacobucci et al. 2011), being *ANRIL* one of the most frequently altered lncRNAs in cancer development and progression, including ovarian cancers, breast cancer, lymphoblastic leukemia, nasopharyngeal carcinoma, basal cell carcinoma, and gliomas (Shete et al. 2009; Stacey et al. 2009; Turnbull et al. 2010; Pasmant et al. 2011). Moreover, several polymorphisms identified in the *ANRIL* locus show a significant correlation with tumor development (Shete et al. 2009; Wrensch et al. 2009; Bishop et al. 2009). To name a few examples, the SNP rs1063192-C is highly correlated with glioma, the SNP rs1011970-T with melanoma susceptibility (Cunnington et al. 2010), and the SNP Rs564398 increases the risk of lymphoblastic leukemia development (Iacobucci et al. 2011). These polymorphisms alter the expression pattern of *ANRIL* splice variants (Fig. 2), and in consequence, dysregulate the *INK4b-ARF-INK4a* locus expression. A hypothesis for *ANRIL* function is that this ncRNA is composed of several RNA transcript variants such that the accumulation of transcript variants may focalize PRC1 via CBX7 in proximity to the $p16^{INK4a}$ gene promoter to selectively silence $p16^{INK4a}$ (Aguilo et al. 2011). As sequence technology evolves to incorporate higher resolution, we predict that novel isoforms will emerge and specific diseased states will be represented by the presence (or absence) of the transcript variants for *ANRIL* (Fig. 2).

Although the underlying molecular mechanism by which *ANRIL* increases the risk of cancer progression remains ambiguous, it is believed that high expression levels may lead to cancer predisposition. Indeed, it has been reported that *ANRIL* is overexpressed in preneoplastic and neoplastic epithelial tissues (Yap et al. 2010), gastric cancer tissues (Zhang et al. 2014), esophageal squamous cell carcinoma (ESCC) (Chen et al. 2014), and leukemia leukocytes (Cunnington et al. 2010; Yu et al. 2008) compared with the non-tumor tissues. In normal cells, induction of *ANRIL* transcript levels by E2F1 is required for the suppression of $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$ expression at the late stage of DNA damage response, in order to return to physiological cellular levels after the completion of the DNA repair. However, in cancerous cells, aberrant expression of *ANRIL* would cause a blockage of the control of the DNA damage response mechanism, leading to genomic instability, and therefore, tumor progression (Wan et al. 2013).

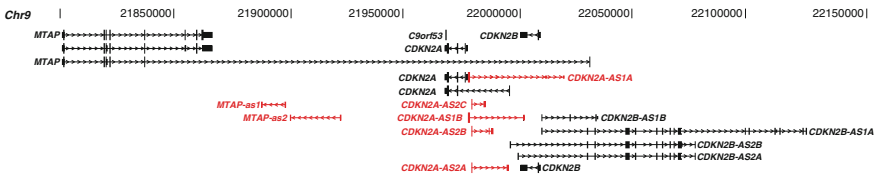

Fig. 2 Comprehensive transcript map overlapping the human *INK/ARF* locus determines the assembly of transcripts by long read- and strand-specific RNA sequencing by ISO-Seq. Samples taken for ISO-Seq analysis are from a single prostate invasive carcinoma specimen and compared with the paired normal prostate duct epithelium. Highlighted in red are novel transcript isoforms identified in the tumor specimen when compared to the normal duct epithelium of the prostate

ANRIL also influences cell proliferation by regulating target genes in *trans*. Hence, in gastric cancer tissues, *ANRIL* cooperates with microRNAs in the epigenetic level by binding to EZH2. Specifically, *ANRIL* silences miR-99a/miR-449a, therefore up-regulating the miR-99a/miR-449a target genes mTOR and CDK6, and as a consequence, up-regulating the CDK6 target gene E2F1 (Zhang et al. 2014). This positive feedback loop could in part account for *ANRIL*-mediated cell growth regulation. On the other hand, in esophageal squamous cell carcinoma tissues, *ANRIL* influences cell growth by repression of the TGFβ/Smad signaling pathway (Chen et al. 2014), although the exact molecular mechanisms of interaction between *ANRIL* and TGFβ1 remain elusive.

Collectively, *ANRIL* could serve as a candidate biomarker for cancer detection, and novel cancer therapies should consider *ANRIL* depletion to specifically target highly proliferative cells. However, despite growing knowledge about *ANRIL* function in cancer and other disease models, a broader understanding of the molecular mechanism of action, and the regulatory pathways, hierarchies and networks in which *ANRIL* and other lncRNA operate, is the essential first step for its therapeutic manipulation.

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Form and Function of Exosome-Associated Long Non-coding RNAs in Cancer

Chris Hewson and Kevin V. Morris

Abstract The recent discovery that long non-coding RNAs (lncRNAs) are functional and are not merely "transcriptional noise" has spawned an entirely new arena of investigation. LncRNAs have been found to be functional in the regulation of a wide variety of genes, including those involved in cancer. Studies have identified that lncRNAs play a role in the development and regulation of cancer and can also act as prognostic markers. Meanwhile, exosomes, which are extracellular particles generated endogenously by cells, have been observed to act as transport vesicles for a variety of biological components, particularly proteins and RNAs. This transportation of biological components has been shown to impact a variety of biological processes including the development of cancer. Collectively, these observations, along with those of several recent studies, suggest that lncRNAs and exosomes may function together to disseminate cell signals that alter and/or control local cellular microenvironments. This review will identify the various roles that lncRNAs and exosomes play in cancer development, as well as the possibility that exosomes may transfer functional lncRNAs between cells as a means of cell-to-cell communication.

Contents

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1 Long Non-coding RNAs

Through the human genome project, it was discovered that only 2 % of the human genome encodes for proteins; however, up to 90 % of the human genome is actively transcribed (Knowling and Morris 2011). These actively transcribed RNAs can be broken down into several different classifications including mRNA, tRNA, miRNA, siRNA and lncRNAs. Long non-coding RNAs are defined (somewhat arbitrarily) as transcripts greater than 200 nucleotides that do not code for proteins. They are generally not as highly conserved when compared to other types of RNA such as mRNA or miRNA (Struhl 2007). When lncRNAs were initially discovered, they were largely dismissed as "transcriptional noise" and thought to serve no particular function; however, further investigation has identified that lncRNAs are able to regulate gene expression using a variety of different mechanisms such as epigenetic regulation or transcriptional regulation (reviewed in Morris and Mattick 2014). LncRNAs have also been associated with various cancers as seen in the lncRNAs HOTAIR (Gupta et al. 2010) and MALAT1 (Gutschner et al. 2013), which opens up the possibility of targeting lncRNA-targeted cancer treatments or screening for them for use as biomarkers.

1.1 LncRNAs and X Chromosome Inactivation

It was originally thought that lncRNAs served no particular function and that they were merely excess transcripts produced during transcription (referred to as "transcriptional noise") (Struhl 2007). However, further investigation has disproved this notion and has shown that lncRNAs exhibit a variety of functions and have been shown to play a role in the epigenetic regulation of several genes (Kung et al. 2013). One example of this is during X chromosome inactivation. In females, one X chromosome is inactivated to ensure only one chromosome is expressed in each cell. In mammals, this is controlled by a cluster of lncRNA loci known as the X-inactivation centre (Brown et al. 1991). From this cluster of loci, a transcript known as the X-specific transcript is produced which is highly expressed during X

chromosome inactivation. This transcript coats the X chromosome in a "cloud" which acts as a scaffold to recruit silencing factors such as Polycomb repressive complex 2 (PRC2). This protein has the ability to methylate histones, primarily at histone 3 lysine 27 (H3K27). This results in the chromosome being remodelled into heterochromatin, thus preventing any transcription from that particular chromosome and ensuring that only one chromosome is actively transcribed. The X-specific transcript itself is also regulated by lncRNAs (Lee 2011).

1.2 LncRNAs Are Regulators of Tumour Suppressor Genes

Phosphatase and tensin homolog (PTEN) is a tumour suppressor protein, which is encoded by the PTEN gene, and it is frequently mutated in a large number of various cancers. For example, it has been demonstrated that in prostate cancer, up to 70 % of cancers have lost a copy of the PTEN gene (Chen et al. 2005). The PTEN gene was recently observed to be regulated by a PTEN pseudogene (PTENpg1), which has two isoforms, α and β. The α isoform of this pseudogene functions by recruiting DNA methyltransferase 3a (DNMT3a) as well as Enhancer of zeste homolog 2 (EZH2) which leads to the chromatin being remodelled resulting in lower expression of the PTEN protein (Johnsson et al. 2013), whereas the β isoform functions to increase PTEN expression by acting along with the PTENpg1 sense pseudogene as a miRNA sponge. This miRNA sponge binds to those miRNAs that are complementary to and/or targeted to the PTEN mRNA. Ultimately, the sponging of PTEN-targeted miRNAs by the PTENpg1 sense/antisense β isoform prevents the miRNAs from binding to the PTEN mRNA, which would normally lead to lowered expression of the PTEN protein (Johnsson et al. 2013).

Other lncRNAs, such as growth arrest-specific 5 (GAS5), have been shown to actively compete against other transcription factors. GAS5 functions by binding to the DNA-binding domain of the glucocorticoid receptor (GR), which prevents glucocorticoid response elements (GRE) from binding to the GR. This affects the transcription of target genes which includes a variety of apoptosis inhibitors such as the cellular inhibition of apoptosis protein 2 gene (cIAP2) (Kino et al. 2010). By preventing the transcription of these apoptosis-inhibiting genes, GAS5 has the capability to make cancer cells susceptible to apoptosis. It has been demonstrated that in prostate cancer cell lines, high levels of GAS5 caused the cells to be far more susceptible to chemotherapeutic agents and radiation (Pickard et al. 2013). This observation suggests that GAS5 could possibly be explored as a possible avenue of cancer treatment, particularly in cases where the cancer is resistant to chemotherapy and radiation. Furthermore, GAS5 expression is very low in several cancer cell lines (such as breast and leukaemia cancer cells), while in normal cells, the expression is much higher. These observations suggest that GAS5 may play a functional role as a tumour suppressor (Mourtada-Maarabouni et al. 2009).

The lncRNA maternally expressed gene 3 (MEG3) is an example of another lncRNA that exhibits tumour-suppressing capabilities. MEG3 functions by

stimulating P53 expression and can also inhibit cell proliferation independent of the p53 protein (Zhou et al. 2007; Zhang et al. 2010). Normally, p53 protein levels are kept low due to its constant degradation via the ubiquitin–proteasome pathway, which is regulated by the mouse double minute 2 homolog (MDM2) gene. MEG3 functions by inhibiting MDM2 expression, thus preventing p53 from being ubiquinated and resulting in higher levels of the p53 protein (Benetatos et al. 2011). The knockout of MEG3 results in the increased expression of vascular endothelial growth factor signalling genes (Gordon et al. 2010). These observations imply that MEG3 inhibits angiogenesis and may have multiple methods of acting as a tumour suppressor. MEG3 is also capable of binding to the PRC2 suggesting that MEG3 may also be able to regulate gene expression via the structural modification of chromatin (Zhao et al. 2010).

1.3 LncRNAs Have the Ability to Promote Cancer

LncRNAs have also been observed to affect the gene expression of tumour-related genes by binding to proteins such as transcription factors. This mechanism is demonstrated by the lncRNA P21-associated ncRNA DNA damage activated (PANDA) which is found slightly upstream of the CDKN1A/p21 locus. PANDA has been observed to exhibit changes in expression in response to DNA damage and activation of the p53 gene (Morachis et al. 2010). PANDA interacts with the transcription factor NF-YA which prevents it from binding to pro-apoptotic genes such as FAS or BIK (Hung et al. 2011). Without the binding of the transcription factor, the pro-apoptotic genes are prevented from being expressed and can cause an increased survival rate within cancer cells.

Studies have observed that lncRNAs can play a variety of different rolls within cancers and can influence tumour metastasis as well as tumour suppression. This is seen in the HOX transcript antisense RNA (HOTAIR). HOTAIR was observed to impact on tumorigenesis and exhibited up to a 2000-fold increase in expression in breast cancer cells relative to normal human breast epithelia (Gupta et al. 2010). This high expression was discovered to be a significant indicator of tumour metastasis and poor patient prognosis. It was also shown that silencing the HOTAIR lncRNA using RNA interference leads to both decreased cell viability and lower cell metastasis, suggesting that particular lncRNAs can have a dramatic impact on the characteristics of cancer cells (Gupta et al. 2010). HOTAIR also functions by epigenetically regulating gene expression via the recruitment of chromatin-modifying complexes such as PRC2 and LSD1. HOTAIR acts as a scaffold for these chromatin-modifying complexes as the 5' end binds to PRC2, while the 3' end binds to LSD1 which leads to chromatin modification and ultimately a change in gene expression via H3K27 methylation and lysine 4 demethylation (Tsai et al. 2010).

Another cancer-related lncRNA is metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) which is expressed endogenously in most human tissue but has been found to be up-regulated in several types of human cancers such as breast

(Guffanti et al. 2009), prostate (Lin et al. 2006) and liver cancer (Lin et al. 2006). MALAT1 was identified as an oncogene that promotes tumorigenesis and therefore is also associated with a high chance of metastasis and a poor patient prognosis (Guffanti et al. 2009). Knockdown of MALAT1 leads to a wide array of phenotypes including the inhibition of angiogenesis, cell cycle progression (Michalik et al. 2014), cell mobility (Tano et al. 2010) and a higher incidence of cell death (Tripathi et al. 2010). MALAT1 functions by regulating the alternative splicing of endogenous target genes (Tripathi et al. 2010). It has also been suggested that MALAT1 may interact with PRC2 to regulate genes epigenetically (Guil et al. 2012). These observations further highlight the importance of the role that lncRNAs play in the regulation of cancer cells.

1.4 Telomere Length Is also Regulated by LncRNAs

Telomere length is another cellular function that has been demonstrated to be regulated by the action of a lncRNA. Telomeres are regions of repeated nucleotide sequences found at the ends of chromosomes, and they act as a barrier to prevent chromosomes from degrading or fusing with each other. However, due to the mechanisms of DNA replication, each time the cell divides and the DNA is replicated, a small fraction of the telomere is lost and the telomere region ultimately becomes shortened. Eventually, after numerous DNA replications, fragments of essential genes are lost which may result in the eventual death of the cell. Cell immortality and limitless replicative potential is one of the hallmarks of cancer, and for this to be achieved, cancer cells must solve the problem of shortening telomeres. This is remedied by the enzyme telomerase, which has the ability to lengthen telomeres. Recent observations have suggested that the lncRNA, TERRA, plays a role in regulating telomerase function. Studies have demonstrated that TERRA inhibits telomerase activity suggesting that it may regulate telomere length negatively. Knockdown of the lncRNA, TERRA, results in shorter telomere length (Redon et al. 2010), and TERRA expression has also been found to be down-regulated in many cancer cells (Schoeftner and Blasco 2008) which further supports this hypothesis. Although the exact functional mechanism behind TERRA is still not well understood, it has been postulated that TERRA may bind to telomere regions to prevent the binding of telomerase. Another hypothesis is that TERRA binds to telomerase itself which causes a conformational change and prevents telomerase from functioning normally (Redon et al. 2010).

2 Exosomes

Exosomes are small membrane vesicles (40–100 nm) with a saucer-like morphology resembling flattened spheres that are endogenously released from cells into the intracellular environment. Exosomes were first observed in mammalian

reticulocytes (immature red blood cells) and found to be released during reticulocyte maturation to erythrocytes. Similar to lncRNAs, exosomes at the time of their discovery were also thought to have no biological significance (Johnstone et al. 1987; Johnstone 2006). The term "exosomes" has previously been used loosely and interchangeably to describe microvesicles. Microvesicles are much larger (up to 1 μm) and are generated via a completely different pathway relative to exosomes. Microvesicles are released when the plasma membrane is shed directly into the extracellular space. Meanwhile, exosomes are secreted when specific endosomal compartments known as multivesicular bodies (MVBs) fuse with the plasma membrane. It is also important to note that the cell is capable of secreting both exosomes and microvesicles simultaneously which can often make the isolation of pure exosomes difficult to impossible (Lee et al. 2011). Exosomes contain a large variety of biological components such as proteins, mRNAs, miRNAs (Rani et al. 2011) and lncRNAs (Spizzo et al. 2012). Since their discovery, exosomes have been demonstrated to impact various biological functions such as cell communication, the immune system and tumour metastasis (Li et al. 2006). Exosomes also have the ability to cross the blood–brain barrier without eliciting an immune response suggesting that they could prove exceedingly useful in developing brain-targeted drug delivery systems (Alvarez-Erviti et al. 2011).

2.1 *Exosome Biogenesis*

Exosomes originate as MVBs that are found within the endocytic cycle of a cell. MVBs are a type of endosome, which are characterized by containing several membrane-bound intraluminal vesicles (ILVs). These vesicles are formed via a direct budding into the lumen of a MVB (Klumperman and Raposo 2014). The formation of these MVBs is achieved by the endosomal sorting complexes required for transport (ESCRT). The ESCRT complex consists of ESCRT-0, I, II and III. ESCRT-0, I and II aid the generation of MVBs by clustering ubiquitinated proteins and then binding them to the membrane of an endosome. The proteins are then absorbed into the endosome via a direct budding into the lumen to form an ILV. ESCRT-III forms a ring-like structure around the vesicles where the proteins are absorbed into the endosome. This prevents any proteins from leaking out into the cytoplasm during the formation of the ILV. Once the proteins have been transferred to the MVB via the ILV, the Vps4-Vta1 proteins remove the ESCRT components from the endosomal membrane, thus completing generation of the MVB (Raiborg and Stenmark 2009; Henne et al. 2011). Silencing of the ESCRT complex has been shown to result in a decrease in the number of exosomes which are secreted by the cell highlighting their importance in exosomes formation (Colombo et al. 2013).

After the formation of the MVB, it needs to be transported to the cellular membrane before it can be secreted as an exosome. While the exact mechanism behind this process is still not well understood, it has been postulated that in order to transport the MVBs to the cell membrane, a combination of cellular framework

such as actin and microtubules along with molecular motors including myosins and kinesins may be utilized (Cai et al. 2007). Once the MVB has reached the cell membrane, it must fuse with the membrane before it is released as an exosome. A family of membrane proteins known as SNAREs mediates this fusion event. A SNARE protein binds to the MVB (v-SNARE), while another one binds to the cell membrane (t-SNARE). The two proteins then bind to each other to fuse the MVB to the cell membrane (Cai et al. 2007). Notably, the Golgi apparatus creates endosomes which can be altered into MVBs. These MVBs can be degraded by the lysosomes or be secreted by the cell as exosomes. Exosomes taken up by recipient cells can also be fused into MVEs and recycled out of the cell, or they can be degraded by the lysosome.

After the MVB is bound to the cell membrane, it can then be ejected from the cell as an exosome. The release of exosomes involves the recruitment of several Rab proteins (family of proteins which belong to the Ras superfamily). Rab proteins bind with the cellular membrane to regulate vesicle budding, vesicle transport and membrane fusion. The proteins Rab27a and Rab27b seem to play pivotal roles in exosome secretion, as the suppression of these proteins results in a reduced number of exosomes released from the cell (Ostrowski et al. 2010). Rab27 has also been found to be associated with the secretion of other organelles from the endocytic pathway, further solidifying the hypothesis that they play a crucial role in the secretion of exosomes (Raposo et al. 2007).

How exosomes are targeted to be taken up by recipient cells remains unknown. It is understood that the binding of exosomes to cell membranes is controlled by cell adhesion molecules used in cell-to-cell interactions such as integrins and intercellular adhesion molecules (ICAM). In order for the exosome to deliver its contents, they are absorbed by the cell and undergo endocytosis. Once the exosome is broken down by the lysosome, its contents can be released into the cell where they may have functional relevance (Record et al. 2014). In this manner, it may be that exosomes are generalized in their targeting of recipient cells, though this notion has not been thoroughly vetted experimentally.

2.2 Exosome Function

It has been hypothesized that exosomes may function as a form of cell-to-cell communication. Due to the fact that proteins and RNA are prone to degradation in the extracellular space, one postulated function for exosomes is that they protect biological compounds from degradation during travel between cells in the extracellular space. Notably, many of the documented exosomal proteins and RNAs have been observed to be functional once absorbed by recipient cells (Valadi et al. 2007). This further solidifies the notion that exosomes are a method of communication utilized by cells to communicate with each other in between the extracellular space (depicted in Fig. 1).

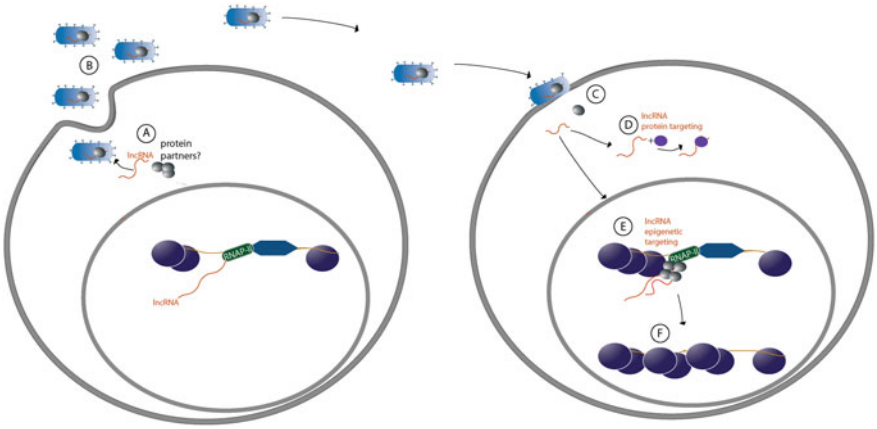

Fig. 1 Exosome-mediated delivery of lncRNAs to target cells. A schematic is shown depicting a generalized model for the spread of lncRNAs from one cell to another via the action of exosomes. *A* The lncRNA may interact with exosome packaging proteins resulting in *B* the release of the exosomes containing candidate lncRNAs. *C* The lncRNA-containing exosomes can then bind and internalize into recipient cells. The lncRNA may then *D* target cellular proteins to affect function or *E* target homology-containing genes and modulate transcription which could lead to *F* stable epigenetic silencing of the lncRNA-targeted gene

2.3 Exosomes Transferring Chemoresistance Between Cells

Resistance to traditional methods of cancer treatments such as chemotherapy or radiation remains to be one of the major hurdles when treating cancer. In order to overcome these hurdles, a more complete understanding of the mechanisms, which allow for this resistance, is required. Many studies have demonstrated that exosomes are capable of playing a key role in regard to cancerous cells obtaining this trait. An example of this is seen through the secretion of Survivin via exosomes. It has been demonstrated that uptake of Survivin via exosomes protects the cell from radiation damage by promoting cell proliferation and improving metastatic potential (Khan et al. 2009). Another study observed that prostate cancer cells which were previously susceptible to docetaxel could obtain resistance to this drug via the exosomal transfer of multidrug resistance protein 1 (MDR-1), a drug transporting glycoprotein which has the ability to pump docetaxel out of the cell. A similar mechanism was observed in the breast cancer cell line MCF-7. MCF-7 cell lines which were previously sensitive to chemotherapeutic agents could inherit chemoresistance via exosomes which originated from drug-resistant variants of the MCF-7 cancer cell line. This occurred due to the miRNAs within the exosomes which were found to knock down various genes such as the important tumour suppressor gene PTEN (Chen et al. 2014).

2.4 Exosomes Impacting Tumorigenesis

Exosomes derived from cancer cells have also been shown to promote tumour invasion and metastasis. Exosomes isolated from highly metastatic variants of melanoma possess the ability to increase the metastatic behaviour of primary tumours via the receptor tyrosine kinase MET (Peinado et al. 2012). Further observations have shown that breast cancer exosomes contain miRNAs as well as the machinery to process precursor miRNAs into mature, functioning miRNAs (such as Dicer, TRBP and Ago2). This leads to a reprogrammed transcriptome, which can induce tumour formation within non-tumourigenic epithelial cells. The inhibition of dicer function within exosomes also demonstrated impaired tumour growth within recipient cells, thus identifying the importance of miRNA processing in regard to exosomes promoting tumorigenesis (Melo et al. 2014).

Previous studies have observed that exosomes can facilitate the transfer of oncoproteins, such as mutant KRAS, between cells to induce tumourigenesis. KRAS is a signalling protein that is essential in many cell-signalling pathways. A mutation in the KRAS gene is a common step in the development of several cancers (Kranenburg 2005). Exosomes originating from colon cancer cells possess the ability to transfer mutant KRAS between cells, resulting in stimulated cell growth, thus increasing the recipient cells chances of becoming tumorigenic (Demory Beckler et al. 2013). Exosomes are also capable of promoting angiogenesis within recipient cells. Glioblastoma cells release exosomes, which contain mRNA, miRNA and proteins that promote angiogenesis. Observations have demonstrated that when these exosomes were taken up by recipient endothelial cells, angiogenesis was stimulated (Skog et al. 2008). Collectively, these observations demonstrate how cancerous cells can utilize exosomes as a method of cell communication in order to induce cancer-like characteristics in healthy cells.

2.5 Exosomes Influencing the Immune System

Exosomes released from cancer cells possess the ability to impact the immune system in order to aid tumour proliferation. Exosomes isolated from lung cancer cells contain miRNAs which prevent toll-like receptors (TLRs) from being expressed in macrophages. The miRNAs bind to the TLR mRNA, which leads to their respective degradation. This results in an increased secretion of pro-inflammatory cytokines from the macrophage and causes tumour cells to spread throughout the body (Fabbri et al. 2012). Another study demonstrated that colorectal cancer exosomes contained Fas ligand, tumour necrosis factor and various other proteins involved in the induction of apoptosis. These exosomes were taken up by recipient T cell initiating apoptosis and death of the recipient cell. This prevents T cells from destroying cancerous cells and ultimately permits tumour proliferation (Huber et al. 2005).

2.6 Exosomes as a Possible Method of Cancer Treatment

Exosomes are currently being explored as a possible tool to treat cancers and a variety of other diseases. Current research is attempting to utilize exosomes as a method of drug delivery as they do not elicit an immune response and are capable of crossing the blood–brain barrier. This was recognized when exosomes derived from dendritic cells within mice were engineered to express Lamp2b, a membrane protein which fuses to neurons. These exosomes were then loaded with a siRNA to knock down GAPDH via electroporation before being injected into the mice. A brain-specific knockdown of GAPDH was observed within the mice as well as a lack of an immune response (Alvarez-Erviti et al. 2011).

The realization that exosomes may have therapeutic use first arose when it was observed that dendritic cells were capable of secreting antigen-presenting exosomes which possessed functional MHC class I and II molecules. These exosomes were able to initiate cytotoxic T cells which resulted in the suppression of tumour growth in vivo within mouse models (Zitvogel et al. 1998). Further investigation demonstrated that these exosomes released from dendritic cells contained heat-shock cognate protein hsc73. This protein is considered to be a key factor in inducing immune responses against cancer cells (Thery et al. 1999). A similar anti-cancer effect was observed from exosomes secreted by endothelial cells. The endothelial cells released exosomes which contained the miRNA miR-503 which when taken up inhibited the proliferation and invasiveness of the breast cancer cell line.

The impact of heat-shock proteins (hsps) in cancer and exosomes has recently been identified in several studies. Hsps are a family of proteins which are activated when the cell is exposed to stressful conditions such as high temperatures (Åkerfelt et al. 2010). Hsps play an important role in the immune system as they bind to antigens and are involved in antigen presentation T cells. Due to this characteristic, hsps are being tested for use as possible immunological adjuvants within vaccines, including cancer vaccines (Bolhassani and Rafati 2008). Previous studies have identified that when B lymphoblastoid cells were exposed to a 42 °C heat shock, the exosomes released by the cells contained higher levels hsps relative to the control (Clayton et al. 2005). A similar effect was observed when a hepatocellular carcinoma cell line was treated with various anti-cancer drugs. It was identified that the drug treatment resulted in an increased level of hsps within the exosomes which were secreted from the treated cancer cells (Lv et al. 2012). Exosomes enriched with hsps have initiated anti-tumour immune responses leading to tumour regression within murine models. These examples identify the importance of heat-shock proteins and exosomes as a possible method of cancer treatment (Cho et al. 2009).

A previous study observed that the tumour suppressor protein PTEN is secreted by cells via exosomes. PTEN functions by regulating the PI3K–AKT pathway which is an essential pathway in cell cycle regulation and if mismanaged can result in high levels of cellular proliferation which can often result in cancer (Vanhaesebroeck et al. 2012). The PTEN protein within the exosomes has been identified as functional when taken up by neighbouring cells, thus ensuring that the

cell proliferation is adequately regulated and preventing any tumours from developing (Putz et al. 2012).

Exosomes are currently undergoing clinical trials within humans for a variety of different cancers. Exosomes containing MAGE 3 peptides were introduced to stage III/IV melanoma patients in an attempt to immunize them. Patients displayed minimal side effects after treatment, and it was demonstrated that exosomes had minimal toxic effects. This study has also served as a proof of concept that exosomes can be produced at a large scale for human therapeutics. This body of work has spawned multiple exosome-based cancer therapies that are currently undergoing clinical evaluation. For example, NCT01159288 is currently undergoing a phase II clinical trial and utilizes exosomes derived from dendritic cells which are loaded with tumour antigens as a vaccine against advanced non-small cell lung cancer. Meanwhile, NCT01344109 is undergoing a pilot study, which involves using exosomes secreted from tumours as diagnostic and prognostic marker for patients undergoing chemotherapy. NCT01779583 is a similar clinical trial where exosomes are being used as a prognostic marker for gastric cancer. While these clinical trials are indicative of the potential use of exosomes as cancer therapeutics, they are merely scratching the surface of their potential. Eventually, human engineered exosomes containing drugs or therapeutic biologicals may enter clinical trials and their full potential may be realized.

2.7 Exosomes and LncRNAs

Recent observations suggest that exosomes may act as transport vesicles for functional lncRNAs which may result in a phenotypic effect within the recipient cell (Kogure et al. 2013) (Fig. 1). The lncRNA TUC339 was identified in exosomes derived from hepatocellular carcinoma (HCC) and shown to be highly expressed in exosomes. Suppression of this lncRNA in cells using RNA interference leads to reduced cell proliferation, clonogenic growth and cellular adhesion (Kogure et al. 2013). This observation suggested that cells utilize exosomes and TUC339 in an attempt to increase cell proliferation of nearby cells. ROR is another lncRNA that was found to be highly overexpressed in exosomes derived from HCC cells treated with doxorubicin. HCC cells were treated with exosomes containing high levels of ROR, and an increased level of chemoresistance was observed. Similarly, knockdown ROR within HCC cells using RNA interference leads to increased sensitivity to chemotherapeutic agents (Takahashi et al. 2014). This implies that cancerous cells may be utilizing lncRNAs and exosomes to improve chemoresistance within neighbouring cells.

Several previously described lncRNAs such as MALAT1, HOTAIR and GAS5 have also been found to be highly expressed within exosomes from HeLa and MCF-7 cells. These previously described lncRNAs play important roles within a variety of cancers suggesting that the cancerous cells are releasing these exosomes to try and induce cancer-like phenotypes within the recipient cells (Gezer et al. 2014).

This also suggests that these lncRNAs are being selectively packaged into exosomes; however, the mechanism behind packaging specific biological contents into exosomes is not well understood at this time. Another possible yet to be explored function of exosome-associated lncRNAs may be to deliver lncRNAs that are capable of directing epigenetic silencing (Fig. 1). While not yet reported for exosome-associated lncRNAs, there are several known lncRNAs capable of controlling transcriptional and epigenetic states (Morris and Mattick 2014). Collectively, the observations presented to date support the notion that exosomes may function as transport elements for lncRNAs and possibly function in cell-to-cell communication.

3 Conclusion

Although once considered transcriptional noise, recent research has identified that lncRNAs play a functional role in gene expression, regulation and cancer (Morris and Mattick 2014). Furthermore, unlike other RNAs such as miRNAs, lncRNAs seem to have several mechanisms or modes of action by which they function. This includes binding to chromatin to promote epigenetic regulation, acting as scaffolds for proteins and acting as a miRNA sponge just to name a few reported functions. Their functional relevance in cancer also presents the possibility of using lncRNAs as diagnostic or prognostic markers as well as cancer therapeutic targets. Exosomes have also been demonstrated to be impactful on cancer and cellular function via the transfer of biological components. The previous examples indicate that they promote tumour-like characteristics, impact the immune system and induce chemoresistance within cells. Indeed, exosomes are currently being assessed as a possible method of targeted drug delivery and are currently undergoing multiple clinical trials. Studies have also provided examples of lncRNAs and exosomes functioning together to control gene expression and cell phenotypes within nearby cells. Considering that most exosome research has focussed on miRNA and proteins, this opens up an interesting new area of research in investigating the functions of lncRNAs within exosomes and those cells targeted by the lncRNA-containing exosomes.

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Long Noncoding RNAs in Lung Cancer

Anna Roth and Sven Diederichs

Abstract Despite great progress in research and treatment options, lung cancer remains the leading cause of cancer-related deaths worldwide. Oncogenic driver mutations in protein-encoding genes were defined and allow for personalized therapies based on genetic diagnoses. Nonetheless, diagnosis of lung cancer mostly occurs at late stages, and chronic treatment is followed by a fast onset of chemoresistance. Hence, there is an urgent need for reliable biomarkers and alternative treatment options. With the era of whole genome and transcriptome sequencing technologies, long noncoding RNAs emerged as a novel class of versatile, functional RNA molecules. Although for most of them the mechanism of action remains to be defined, accumulating evidence confirms their involvement in various aspects of lung tumorigenesis. They are functional on the epigenetic, transcriptional, and posttranscriptional level and are regulators of pathophysiological key pathways including cell growth, apoptosis, and metastasis. Long noncoding RNAs are gaining increasing attention as potential biomarkers and a novel class of druggable molecules. It has become clear that we are only beginning to understand the complexity of tumorigenic processes. The clinical integration of long noncoding RNAs in terms of prognostic and predictive biomarker signatures and additional cancer targets could provide a chance to increase the therapeutic benefit. Here, we review the current knowledge about the expression, regulation, biological function, and clinical relevance of long noncoding RNAs in lung cancer.

Contents

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1 Introduction

1.1 Lung Cancer

Lung cancer is the leading cause of cancer-related deaths worldwide and accounted for 18 % of deaths in 2008 (Jemal et al. 2011; Siegel et al. 2013). Despite great progress in understanding the genomic landscape of lung cancer and the molecular mechanisms involved in lung tumorigenesis, the 5-year overall survival rate remains as low as 16 % (for the USA) and has barely improved in the past 30 years (reviewed in Spiro and Silvestri 2005). A total of 80–90 % of lung cancer deaths can be attributed to long-term exposure to tobacco smoke (U.S. Department of Health and Human Services 2010). A major drawback in lung cancer treatment is the predominantly late diagnosis of the disease, which allows only for restricted therapeutic options with low success rates.

Lung cancer is divided into two major histologically distinct classes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). More than 80 % of lung cancer cases are diagnosed with NSCLC. NSCLC is further histologically classified into the major subtypes of adenocarcinoma (ADC; accounts for $\sim$50 % of cases), squamous cell carcinoma (SCC; accounts for $\sim$40 % of cases), and large cell carcinoma (Langer et al. 2010; Davidson et al. 2013). In addition, biomarker signatures can help distinguishing ADC from SCC. ADC biopsies show characteristic immunostaining for TTF1 (thyroid transcription factor 1) and cytokeratin 7, whereas SCC biopsies are positive for cytokeratin 5, cytokeratin 6, and/or SOX2 (SRY-box 2) and TP63 (tumor protein p63) (reviewed in Chen et al. 2014c). For prognostic and therapeutic reasons, lung cancer staging is applied to monitor the degree of cancer spread from the region of the primary tumor. NSCLC is staged according to the TNM (tumor–node–metastasis) classification which takes into account the size of the primary tumor, involvement of the nearby lymph nodes, and distant metastasis spread (Chheang and Brown 2013).

Comprehensive analyses to unravel the mutational landscape of NSCLC genomes led to the identification of driver mutations in ADC and SCC genomes and helped in the assignment of patients to targeted therapies. Driver mutations occur in

62 % of ADCs (Johnson et al. 2013b), are in general mutually exclusive, and impair physiological function and activity of *i.a.* following genes[1]: *MYC* (via gene amplification), *KRAS*, *EGFR*, *KEAP1*, *ALK* (via fusion with mainly *EML4*), *HER2*, *BRAF*, *MET* (via gene amplification), *MEK1*, *AKT1*, *PIK3CA*, whereas major driver mutations in SCC introduce changes in gene function of *TP53*, *CDKN2A*, *PIK3CA*, *MLL2*, *NFE2L2*, *KEAP1*, *PTEN*, *NOTCH1*, *RB1*, *SOX2* (via amplification) (Cancer Genome Atlas Research 2012; Pillai and Ramalingam 2014; Chen et al. 2014c). Knowledge about the driver mutations enables either direct treatment of target genes or indirect approaches if the target is not druggable in the first place (Chen et al. 2014c). A significant problem of current targeted therapy approaches is the development of treatment resistance in a short time frame, mostly within one year after chronic application of kinase inhibitors (Nguyen et al. 2009; Pallis et al. 2011; Suda et al. 2012; Becker and Xu 2014).

A sought-after alternative for new treatment approaches came from the field of RNA research. MicroRNAs (miRNA) are established small noncoding RNA molecules of 18–25 nucleotides that are estimated to posttranscriptionally regulate the expression of about 60 % of protein-coding genes (reviewed in Winter et al. 2009; Sayed and Abdellatif 2011). Various miRNAs are deregulated in lung cancer. Screening studies uncovered characteristic prognostic and diagnostic lung cancer-associated miRNA signatures from patient tissue and blood (reviewed in Boeri et al. 2012; Liloglou et al. 2014). Additional advantages of miRNAs as biomarkers are their high stability and easy detection. The possibility of target gene regulation via manipulation of miRNAs and the application in personalized medicine is being explored at the moment (reviewed in Zhang et al. 2010a; Esteller 2011; Monroig et al. 2014). Recently, a preclinical study revealed that the combinatorial administration of let-7 and miR-34 tumor-suppressive miRNA mimics negatively affects tumor growth and improves survival in a NSCLC mouse model (Kasinski et al. 2014). As the search for new druggable targets continues and research is moving on, a novel class of RNA molecules gives hope for a more profound understanding of lung cancer: long noncoding RNAs (lncRNA).

[1]*MYC* (avian myelocytomatosis virus oncogene, cellular homolog), *KRAS* (Kirsten rat sarcoma viral oncogene homolog), *EGFR* (epidermal growth factor receptor), *KEAP1* (Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1), *ALK* (anaplastic lymphoma kinase), *EML4* (echinoderm microtubule-associated protein-like 4), *HER2* (human epidermal growth factor receptor 2), *BRAF* (v-raf murine sarcoma viral oncogene homolog B1), *MET* (met proto-oncogene (hepatocyte growth factor receptor)), *MEK1* (MAPK/ERK kinase 1), *AKT1* (v-akt murine thymoma viral oncogene homolog 1), *PIK3CA* (phosphoinositide-3-kinase catalytic subunit alpha), *TP53* (tumor protein p53), *CDKN2A* (cyclin-dependent kinase inhibitor 2A), *MLL2* (myeloid/lymphoid or mixed-lineage leukemia 2), *NFE2L2* (nuclear factor (erythroid-derived 2)-like 2), *PTEN* (phosphatase and tensin homolog), *NOTCH1* (Notch homolog 1, translocation-associated (Drosophila)), *RB1* (retinoblastoma 1).

1.2 Long Noncoding RNAs

The advent of genome tiling arrays and high-throughput sequencing technologies introduced significant changes in the RNA field and put functional RNA molecules back into the spotlight. In-depth analyses of the human transcriptome revealed that >80 % of the genome is transcribed (Djebali et al. 2012; Hangauer et al. 2013) and provide a growing number of novel noncoding transcripts. In the course of the last decade, the class of lncRNAs has gained increasing attention.

LncRNAs comprise a very heterogeneous group of transcripts. In general, lncRNAs are longer than 200 nucleotides and harbor little or no protein-coding potential. More precisely, they can be loosely defined as transcripts that carry out their functions as RNA molecules and do not belong to any other class of small (e.g., miRNA) or structural (e.g., tRNA) RNA molecules (Mercer et al. 2009). Depending on the genomic localization of the nearest protein-coding gene, lncRNAs can be further clustered into transcripts that are expressed in sense, antisense, or bidirectional manner, from intronic or intergenic regions of the genome (Ponting et al. 2009; Mercer et al. 2009; Djebali et al. 2012; Zhang et al. 2014e). Similar to mRNAs, lncRNA expression is subject to transcriptional and epigenetic regulation (Mikkelsen et al. 2007; Guttman et al. 2009; Khalil et al. 2009; Sati et al. 2012). Active (H3K4me3 and H3K36me3) and repressive (H3K27me3) histone marks around the transcription start site of lncRNAs provide information on their transcription status (Sati et al. 2012). However, a clear difference between lncRNAs and protein-coding genes was observed on the level of DNA methylation. In contrast to protein-coding genes, lncRNAs show a higher density of DNA methylation around their transcription start sites independent of their expression status (Sati et al. 2012). LncRNAs are frequently transcribed by RNA polymerase II. Subsequent posttranscriptional processing occurs on many lncRNAs similar to protein-coding genes, including alternative splicing, 5′-capping, and polyadenylation (ENCODE Project Consortium 2012; Derrien et al. 2012). For the most part, lncRNAs are expressed at rather low levels; however, they show high tissue specificity (Mercer et al. 2008; Cabili et al. 2011; Djebali et al. 2012). Notwithstanding that lncRNAs are less well conserved between organisms, their number and diversity help to explain the developmental complexity of higher evolved organisms and suggest significant functional roles in biological processes (Pang et al. 2006; Taft et al. 2007; Mercer et al. 2009).

Indeed, a growing number of publications emphasize the involvement of lncRNAs in a variety of physiological and pathological processes (reviewed in Wang and Chang 2011; Batista and Chang 2013; Lee and Bartolomei 2013). LncRNAs are versatile regulators of genes acting on the epigenetic, transcriptional, and posttranscriptional level. To carry out their functions, lncRNAs can interact with DNA, RNA, and proteins. Because of their frequent deregulation in various types of cancer (Tsai et al. 2011; Shi et al. 2013b; Cheetham et al. 2013; Yang et al. 2014a), lncRNAs have gained special attention in the field of cancer research. There is increasing evidence that lncRNAs play crucial roles in tumor initiation,

progression, and metastasis (reviewed in Gutschner and Diederichs 2012). Moreover, recent observations suggest a crucial role for the well-known cancer driver c-MYC in the regulation of lncRNA expression (Hart et al. 2014), which in turn could drive tumorigenesis. Taking this into account, the emerging field of lncRNAs provides a reservoir of new biomarkers for various diseases, including cancer, and opens up new possibilities for therapeutic approaches.

2 Functional Long Noncoding RNAs in Lung Cancer

Current screening approaches focus on the identification of lung cancer-associated lncRNAs under various conditions. Although many novel lncRNAs have been discovered, their functional characterization and importance awaits further experimental proof. Only few validated and well-characterized lncRNAs with reported deregulation and functions in lung cancer exist today. Beyond that, this chapter comprises lung cancer-associated candidate lncRNAs. This group contains less well-characterized or putative lncRNAs that have been implicated in lung carcinogenesis. In addition, lncRNAs that were well characterized in other cancer entities, but only incidentally linked to lung cancer, could be candidate lncRNAs for further lung cancer-directed research. Since the status of a specific (putative) lncRNA might change quickly and novel lncRNAs are characterized at a fast pace, the presented classification between well-characterized and candidate lncRNAs is only preliminary and subject to changes.

Overall, a manageable number of lncRNAs have been implicated in different aspects of lung tumorigenesis. However, data on the mechanism of action is scarce and in most cases remains elusive. An overview of reported lung cancer-associated pathophysiological functions of lncRNAs is provided in Table 1.

2.1 High-Throughput Expression Screens

Recent screening efforts in lung cancer patient samples and cell lines contributed to the discovery of a plethora of deregulated lncRNAs. NSCLC-associated lncRNAs might be useful to distinguish between ADC and SCC subtypes in patients (Yang et al. 2014b; Zhao et al. 2014; White et al. 2014). Although various novel lncRNAs are deregulated in lung cancer, a more profound analysis revealed that the differential expression of lung cancer-associated lncRNAs (*LCAL*) often extends to other cancer entities (White et al. 2014). In another study, next-generation sequencing of archived FFPE (formalin-fixed paraffin-embedded) tissue revealed that lncRNAs correlate with lung cancer progression and invasiveness (Morton et al. 2014). Long stress-induced noncoding transcripts, termed *LSINCT*, were identified in normal human bronchial epithelial cells exposed to the tobacco carcinogen NNK (nicotine-derived nitrosamine ketone) (Silva et al. 2010). Even lncRNAs that might play a

Table 1 Overview of lncRNA deregulation in NSCLC (or a specific subtype) and biological functions in the context of lung cancer

	Expression	Cell growth				Cell death		Cell metastasis						
	NSCLC	Proliferation[a], cell cycle progression[b]		Colony formation		Apoptosis		Invasion		Migration		EMT gene signatures		References
lncRNA		KD	OE	KD	OE	KD	OE	KD	OE	KD	OE	KD	OE	
MALAT1	↑	–[a,b]		↓		↑[1]		↓		↓		↓		Gutschner et al. (2013a), Eissmann et al. (2013), Shen et al. (2014)
HOTAIR	↑[2]	↑[a]/–[a]	↓	–	↑[3]	↑		↓	↑	↓	↑	–		Nakagawa et al. (2013), Liu et al. 2013b), Ono et al. (2014)
H19	↑			↓, ↓[3]										Kondo et al. (1995), Barsyte-Lovejoy et al. (2006), Chen et al. (2013)
MEG3	↓	↑[a]	↓[a,b]		↓		↑	–[4]						Lu et al. (2013)
GAS5	↓	↑[a]	↓[a,b]	↑	↓		↑		–		–			Shi et al. (2013a)
ANRIL	↑	↓[a,b]	–[a]	↓		↑				↓				Nie et al. (2014a)
SOX2OT	↑ (SCC)	↓[a,b]		↓										Hou et al. (2014)
SPRY4-IT1	↓	–[a]	↓[a]		↓		↑	↑	↓	↑	↓	↑	↓	Sun et al. (2014b)
TUG1	↓	↑[a,b]		↑										Zhang et al. (2014b)
CARLO-5	↑	↓[a,b]						↓		↓		↓		Luo et al. (2014)
CCAT2	↑ (ADC)	↓[a]						↓		↓				Qiu et al. (2014b)
PVT1	↑	↓[a]						↓		↓				(Yang et al. 2014c)
BANCR	↓	–[a]	↓[a]		↓		↑	↑	↓	↑	↓		↓	Sun et al. (2014c)
MVIH	↑	↓[a]	↑[a]					↓	↑	↓	↑			Nie et al. (2014b)
GHSROS	↑		–[a]								↑↓[5]			Whiteside et al. (2013)
GAS6-AS1	↓													Han et al. (2013a)
SCAL1	↑[6]	↓[1]												Thai et al. (2013)

(continued)

Table 1 (continued)

	Expression	Cell growth				Cell death		Cell metastasis						
	NSCLC	Proliferation[a], cell cycle progression[b]		Colony formation		Apoptosis		Invasion		Migration		EMT gene signatures		References
lncRNA		KD	OE	KD	OE	KD	OE	KD	OE	KD	OE	KD	OE	
ZXF1	↑ (ADC)	–[a]						↓		↓				Zhang et al. (2014f)
LncRNA-LET	↓ (SCC)													Yang et al. (2013a)
BC200	↑ (SCC)													Chen et al. (1997)
CUDR	↑													Tsang et al. (2007)

Experimental lncRNA knockout or knockdown (KD) and overexpression (OE) either promoted (↑), inhibited (↓), or did not alter (–) key pathophysiological processes including cell growth, cell death, and metastasis. With respect to the latter, lncRNAs promoted (↑) or reversed (↓) EMT by favoring mesenchymal or epithelial gene signatures, respectively, or did not alter (–) EMT-related gene expression. a—proliferation, b—cell cycle progression. 1—drug induced, 2—also in SCLC, 3—colony formation in soft agar, 4—not specified whether KD, OE, or both performed, 5—ambiguous, cell line-dependent effect, 6—in airway epithelium of cigarette smokers and cancer cell lines

role in the *EGFR* exon 19 deletion in lung ADC were described (Wang et al. 2014c). Furthermore, lncRNAs might be involved in establishing drug resistance to EGFR tyrosine kinase inhibitors in NSCLC (Cheng et al. 2014b). More data on the importance of lncRNAs in lung tumorigenesis is accumulating (Wang et al. 2014e), showing the need for a comprehensive, functional characterization of these transcripts. For instance, initial characterization of *LCALs* implied a regulatory function of *LCAL1* in cell proliferation (White et al. 2014). Depending on the type of screening technology and way of analysis, obtained results on novel transcripts may differ. Therefore, an important task is assigned to the full and reliable annotation of the human genome and the resulting transcriptome. This will help researchers to obtain more consistent results and provide a common basis for further investigations. Currently, the sequence identity of novel transcripts needs to be validated by RACE (rapid amplification of cDNA ends) to ensure that the functional transcript matches the sequences deposited in various databases. It should also be emphasized that many lncRNAs occur in numerous splice variants further requiring the exact definition of the molecules under investigation.

2.2 Well-Characterized Long Noncoding RNAs

2.2.1 MALAT1

MALAT1 (metastasis-associated lung adenocarcinoma transcript 1), also referred to as *NEAT2* (nuclear-enriched abundant transcript 2), is a highly abundant and conserved nuclear lncRNA that is expressed from chromosome 11q13 (Ji et al. 2003). The *MALAT1* nascent transcript is about 8 kb long and was reported to be processed by RNase P resulting in a long, nuclear-retained *MALAT1* transcript and the 61 nucleotides short, cytoplasmic tRNA-like mascRNA (*MALAT1*-associated small cytoplasmic RNA). Although this mascRNA was expressed in many normal human tissues, its function remains unclear (Wilusz et al. 2008).

As one of the first cancer-associated lncRNAs, *MALAT1* quickly gained attention and was assigned a variety of different functions in different cell and cancer models (Gutschner et al. 2013a). *MALAT1* was originally identified as a predictive marker for metastasis and patient survival in early-phase NSCLC (Ji et al. 2003). Furthermore, *MALAT1* expression was a negative prognostic factor in lung cancer of the SCC subtype (Schmidt et al. 2011), clear cell renal cell carcinoma (Zhang et al. 2014c), and pancreatic cancer (Pang et al. 2014) and revealed a predictive potential for hepatocellular carcinoma (HCC) recurrence after liver transplantation (Lai et al. 2012).

Due to the exceptional abundance of *MALAT1* in various tissues and cell lines and the lack of an efficient knockdown system, observations from RNAi (RNA interference)-based cellular assays were of limited reliability. In the lung ADC cell line A549, the most efficient siRNA (small interfering RNA) reduced *MALAT1* expression by 87 % (Gutschner et al. 2011). However, the remaining *MALAT1*

transcript level was still high, namely in the range of the endogenous *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels. Hence, it was unclear whether the remaining *MALAT1* transcripts could compensate for the siRNA-mediated reduction and therefore impair the onset of a phenotype. A zinc finger nuclease-based technique allowed for the first highly efficient knockdown of *MALAT1* in a lung cancer cell line (Gutschner et al. 2011) and confirmed *MALAT1* positive influence on metastasis in vitro and in vivo without affecting cell proliferation (Tano et al. 2010; Gutschner et al. 2013b). *MALAT1* is conserved in mice, and the injection of the *MALAT1*-expressing human EBC-1 lung cancer cells induced tumor formation in mice (Gutschner et al. 2013b). *MALAT1* might be a valuable therapeutic target since administered antisense oligonucleotides (ASO) were able to efficiently reduce *MALAT1* expression in the used mouse model both in human EBC-1 cell-derived tumors and in the surrounding stromal cells and thereby reduced lung cancer metastasis formation (Gutschner et al. 2013b). Recently, elevated levels of *MALAT1* were reported in lung cancer brain metastases (Shen et al. 2014). In this study, *MALAT1* silencing in a highly invasive subline of a brain metastasis lung cancer cell line reduced its migratory and metastatic potential by modulating epithelial-to-mesenchymal transition (EMT) processes.

An impressive number of studies focused on the elucidation of *MALAT1* functions in a variety of cellular and mouse models. Consequently, proposed modes of action for *MALAT1* are manifold. *MALAT1* was reported to interact with and to modulate the activity of serine/arginine (SR) splicing factors, hence influencing gene expression by means of alternative splicing (Tripathi et al. 2010). Supporting these findings, *MALAT1* depletion in normal human diploid fibroblast cell lines resulted in proliferative defects that were partially attributed to changes in alternative splicing of a few key mitotic regulators, among them the transcription factor *B-MYB* (Tripathi et al. 2013). Another study suggested a *MALAT1*-dependent mechanism for proper cell cycle regulation and G2/M phase progression involving hnRNP C (heterogeneous nuclear ribonucleoprotein C). In the presented model, *MALAT1* interactions with nuclear hnRNP C were crucial to facilitate the translocation of hnRNP C to the cytoplasm in the G2/M phase, where it would elicit its regulatory function on the translational level (Yang et al. 2013c). Furthermore, *MALAT1* was also implied in the epigenetic control of gene expression by serving as a subnuclear molecular scaffold and specifically localizing unmethylated PC2 (polycomb 2) proteins into gene activation-promoting interchromatin bodies (Yang et al. 2011b). Epigenetic regulation of a gene signature of genes involved in migration and invasion, but no differences in alternative splicing were found in a genetic loss-of-function model in lung cancer cells (Gutschner et al. 2013b).

Despite the high evolutionary conservation of *MALAT1*, neither in vivo *Malat1* knockout (KO) mouse models nor MEFs (mouse embryonic fibroblast) derived from KO mice reflected the striking effects that were previously described in cell-based and xenograft mouse models (Zhang et al. 2012; Nakagawa et al. 2012; Eissmann et al. 2012). All generated KO mice were viable and fertile and did not present any defects on the level of global gene expression, SR splicing factor activity, or alternative splicing. Therefore, *MALAT1* is not essential for mouse

pre- and postnatal development. In light of these findings, depletion of *MALAT1* might be tolerated by normal cells, making *MALAT1* an interesting cancer drug target—potentially for metastasis prevention therapy in lung cancer. Further investigations of *MALAT1* in cancer are needed to evaluate its therapeutic benefit as biomarker and drug target for patients. In addition, challenging the *MALAT1* KO mouse models with different stressors could provide further insights into the functions of *MALAT1*.

2.2.2 HOTAIR

The lncRNA *HOTAIR* (HOX transcript antisense RNA) is transcribed in antisense direction from the human *HOXC* locus on chromosome 12q13 (Rinn et al. 2007). Because of its 5′- and 3′-domain-dependent interactions with PRC2 (polycomb repressive complex 2) and LSD1 (lysine-specific demethylase 1), respectively, *HOTAIR* was attributed a modular scaffold function (Rinn et al. 2007; Tsai et al. 2010). *HOTAIR* triggered H3K27 (histone 3 lysine 27) trimethylation of the *HOXD* locus on human chromosome 2 by recruiting PRC2 and inducing transcriptional silencing in trans. In addition, *HOTAIR* interaction with LSD1, which is part of the CoREST-REST (RE1-silencing transcription factor) repressor complex, promoted demethylation of H3K4me2 histone marks in the vicinity of the transcription start sites of *HOXD* genes (Tsai et al. 2010).

HOTAIR crucial functions in epigenetic gene regulation have pushed for examining its role in the context of several cancers. Increased *HOTAIR* expression was reported in a plethora of cancerous tissues, including primary breast tumors and metastases (Gupta et al. 2010), HCC (Yang et al. 2011c; Ishibashi et al. 2013), colorectal cancer (Kogo et al. 2011), high-risk gastrointestinal stromal tumors (Niinuma et al. 2012), pancreatic cancer (Kim et al. 2013), nasopharyngeal carcinoma (Nie et al. 2013), esophageal SCC (ESCC) (Lv et al. 2013), NSCLC (Nakagawa et al. 2013; Liu et al. 2013b), glioma (Zhang et al. 2013a), endometrial carcinoma (He et al. 2014a), SCLC (Ono et al. 2014), and ovarian (Qiu et al. 2014a) and cervical cancer (Huang et al. 2014). In most cancer entities, including NSCLC, high *HOTAIR* levels were accompanied by an advanced stage of disease, associated with metastases, and were prognostic for poor patient survival. In NSCLC, *HOTAIR* expression also correlated with a shorter disease-free interval after surgery (Nakagawa et al. 2013).

Supporting a role for *HOTAIR* in lung cancer metastasis, siRNA-mediated downregulation of *HOTAIR* decreased migration and invasion of NSCLC cells in vitro and their metastatic potential in vivo (Nakagawa et al. 2013; Liu et al. 2013b). Furthermore, *HOTAIR* knockdown-induced apoptosis, however, did not alter cell vitality (Liu et al. 2013b). What is more, *HOTAIR* was implicated in invasive and metastatic functions via regulation of MMP2 (matrix metalloproteinase 2), MMP9, and HOXA5 (homeobox protein A5) protein levels, while leaving EMT-promoting protein expression unchanged. In lung ADC cells, high *HOTAIR* expression promoted resistance to cisplatin by reducing P21 protein levels

(Liu et al. 2013c). Further insights into the mechanisms of *HOTAIR* regulation were provided from a COL-1 (type I collagen)-supplemented 3D cell culture system mimicking the tumor microenvironment (Zhuang et al. 2013). In this system, *HOTAIR* expression was induced by COL-1 in lung ADC cells, and this effect was reversed by the application of a neutralizing antibody against the COL-1 receptor α2β1 integrin. Moreover, both the expressions of *HOTAIR* and *COL-1* were elevated in NSCLC patient samples and suggested a contribution of tumor-promoting COL-1 to *HOTAIR* upregulation in vivo. Recently, a role for *HOTAIR* was proposed in SCLC, where high *HOTAIR* levels correlated with increased lymphatic invasion and shorter relapse-free patient survival (Ono et al. 2014).

Altogether, various studies focused on illuminating the mechanism of *HOTAIR* deregulation and function in different cancer entities and cell lines. A functional risk allele in the *HOTAIR* genomic locus was associated with increased *HOTAIR* levels in ESCC, a MYC-dependent upregulation of *HOTAIR* was reported in gallbladder cancer cells, and miRNA- and HuR-dependent *HOTAIR* regulatory processes were described (reviewed in Loewen et al. 2014). Of importance, *HOTAIR* was also involved in the maintenance of stemness and EMT processes in cancer cell lines (Padua Alves et al. 2013). From the clinical point of view, *HOTAIR* was significantly associated with poor prognosis in several cancers and therefore holds high biomarker potential (Deng et al. 2014; Yao et al. 2014). Another study presented promising results on *HOTAIR* availability and prognostic potential from colorectal carcinoma patient blood samples (Svoboda et al. 2014).

In summary, *HOTAIR* is involved in gene regulatory and metastatic processes. However, conditional KO mouse models of *HOTAIR* exhibited only mild skeletal and gene regulatory phenotypes, supporting different roles for *HOTAIR* in vitro, in vivo, and in the context of cancer (Suemori and Noguchi 2000; Schorderet and Duboule 2011; Li et al. 2013). Hence, more detailed analyses on *HOTAIR* regulation and modes of action in the appropriate context will be crucial to resolve discrepancies and allow conclusions about the suitability of *HOTAIR* as a therapeutic target.

2.2.3 H19

H19 is a paternally imprinted gene localized on human chromosome 11p15 and is maternally expressed during embryonic development, but postnatally inactivated in most tissues (Pachnis et al. 1984; Bartolomei et al. 1991; Rachmilewitz et al. 1992; Zhang and Tycko 1992; Gabory et al. 2010). It is located adjacent to the paternally expressed growth factor *IGF2* (insulin-like growth factor 2) (DeChiara et al. 1991; Zemel et al. 1992). In addition to the *H19* lncRNA, the *H19* locus harbors the conserved miR-675 (Cai and Cullen 2007), a long antisense transcript (Berteaux et al. 2008), as well as an antisense protein-encoding transcript (Onyango and Feinberg 2011). Although results from mouse studies attributed tumor-suppressive properties to the *H19* lncRNA (Hao et al. 1993; Isfort et al. 1997; Yoshimizu et al. 2008), an accumulating number of studies point toward an oncogenic potential of

H19. Deregulation of the *IGF2*/*H19* locus was not only observed in pediatric tumors (Reeve 1996; Feinberg 1996; Moulton et al. 1996), but also observed in a variety of adult cancers. Loss of imprinting (LOI) and reactivation of *H19* expression was associated with various cancers including lung (Kondo et al. 1995), cervix (Douc-Rasy et al. 1996), esophageal (Hibi et al. 1996), ovarian (Kim et al. 1998; Chen et al. 2000), head and neck SCC (El-Naggar et al. 1999), osteosarcoma (Ulaner et al. 2003) and bladder cancer (Byun et al. 2007).

Furthermore, *H19* was upregulated by carcinogens in mouse models of bladder and liver cancers (Elkin et al. 1998; Graveel et al. 2001). A comparative study between smokers and non-smokers revealed an LOI-independent upregulation of *H19* in the airway epithelia of smokers (Kaplan et al. 2003). Moreover, treatment of cultured human respiratory epithelial cells with cigarette smoke condensate induced epigenetic changes and increased expression of oncofetal *H19* (Liu et al. 2010). Supporting an oncogenic role, the overexpression of *H19* and *MDIG* (mineral dust-induced gene) was associated with poor survival of lung cancer patients and smokers with lung cancer, respectively (Chen et al. 2013). Elevated *MDIG* levels were observed as a result of exposure to mineral dusts and associated with lung cancer (Zhang et al. 2005; Lu et al. 2009). MDIG was implicated in the demethylation of H3K9me3 in the promoter region of *H19* and thereby promoted its expression (Chen et al. 2013).

Another report provided evidence for the c-MYC-dependent upregulation of *H19* and simultaneous downregulation of *IGF2* in various cell lines. C-MYC selectively increased *H19* transcription from the maternally derived allele (Barsyte-Lovejoy et al. 2006). Of note, neither the imprinting status of *H19* nor *IGF2* genomic locus was affected. On the functional level, RNAi-mediated knockdown of *H19* in lung and breast cancer cell lines decreased their colony formation ability and anchorage-independent growth. What is more, elevated *H19* levels correlated with *c-MYC* levels in node-negative breast cancer and NSCLC patient samples and supported cell-based results (Barsyte-Lovejoy et al. 2006). Overall, the presented experimental evidence supports an oncogenic role of *H19* in lung cancer development.

Further insights into the mechanisms underlying *H19* regulation and function were provided from additional cellular and mouse models. In breast cancer cells, E2F1-induced expression of *H19* was linked to cell cycle progression (Berteaux et al. 2005). Additionally, several studies implied *H19* involvement in metastasis and EMT processes of cancer cells (Zhang et al. 2013b; Luo et al. 2013; Matouk et al. 2014). In an HCC cell line and in human bladder carcinoma cells, *H19* expression was induced under hypoxic stress conditions (Matouk et al. 2007). SiRNA-mediated *H19* knockdown in an HCC cell line caused deregulation of several genes involved in angiogenesis, survival, and tumorigenesis under hypoxic conditions. Although *H19* knockdown in HCC cells did not influence cell proliferation in vitro, mouse xenograft models presented significantly impaired tumor growth in vivo (Matouk et al. 2007). In addition, *H19* knockdown reduced anchorage-independent colony formation after recovery from hypoxia, reduced

viability of hypoxic cells, and modulated expression of genes that are involved in survival and tumorigenesis under hypoxic stress.

A follow-up study provided more details on the relationship between TP53, HIF-1α (hypoxia-inducible factor 1 alpha) and *H19* regulation in various cells, including lung cancer cell lines (Matouk et al. 2010). Under hypoxic conditions, HIF-1α promoted *H19* upregulation. However, in a *TP53* wild-type genetic background, TP53 was able to counteract *H19* expression, possibly by triggering degradation of HIF-1α. In summary, only the combination of non-functional or absent TP53 and elevated HIF-1α levels significantly increased *H19* expression in vitro and in vivo pointing toward an oncogenic role for *H19*, particularly in the context of TP53 aberrant cancers.

Another functional analysis in gastric cancer cells revealed that ectopic overexpression of *H19* enhanced cell proliferation (Yang et al. 2012). Conversely, RNAi-directed knockdown of *H19* decreased cell proliferation and increased apoptosis. *H19* was proposed to interact with TP53 and was able to impair TP53-driven luciferase expression in a cell-based assay. Hence, *H19* might contribute to proliferative and apoptotic phenotypes by modulating TP53 activity in gastric cancer cells.

Summed up, deregulation of *H19* expression might occur by genomic, epigenetic, transcriptional, or posttranscriptional mechanisms. Early mouse models revealed that ectopic *H19* overexpression was prenatally lethal (Brunkow and Tilghman 1991), while homozygous knockout mice were viable and heterozygous knockout mice with a maternally inherited *H19* deletion only presented increased birthweight due to LOI of *Igf2* locus (Leighton et al. 1995). Hence, future studies should be aware of *H19* different functions in physiological and pathological processes and carry out investigations in the right context.

2.2.4 MEG3

MEG3 (maternally expressed gene 3), also named *GTL2* (gene trap locus 2), was originally discovered in mice and resides on the human chromosome 14q32 (Schuster-Gossler et al. 1998; Miyoshi et al. 2000). In the course of a study that focused on the identification of imprinted genes from mouse distal chromosome 12, *Meg3* was isolated as a paternally imprinted, maternally expressed gene (Miyoshi et al. 2000). Noteworthy, uniparental disomies of human chromosome 14, and the syntenic mouse chromosome 12, were linked to disease phenotypes and abnormalities in imprinting (Miyoshi et al. 2000). Further studies uncovered that the *Meg3* and *Dlk1* (delta-like 1) genes are part of an imprinted gene cluster that is also conserved in humans (Schmidt et al. 2000). Similar to *H19* and *Igf2*, imprinting occurs reciprocally on the maternally expressed *Meg3* and the paternally expressed growth-promoting gene *Dlk1* and is crucial for proper gene expression (Schmidt et al. 2000; Lin et al. 2003; Kagami et al. 2010).

Similar to its mouse homolog, the human *MEG3* locus gives rise to various alternatively spliced, non-protein-encoding transcripts (Miyoshi et al. 2000; Zhang

et al. 2010b). *MEG3* isoforms are expressed during embryonic development and exhibit tissue and cell type specificity (Schuster-Gossler et al. 1998; Zhang et al. 2003, 2010b). Noteworthy, predicted *MEG3* secondary structures revealed the existence of three conserved motifs in all isoforms and supported a structure-dependent functional role in TP53 activation (Zhou et al. 2007; Zhang et al. 2010b).

In the context of tumor development and progression, *MEG3* was attributed tumor-suppressive functions. Various cancers, including lung cancer, were associated with a decrease or loss of *MEG3* expression (reviewed in Benetatos et al. 2011). In most cases, *MEG3* deregulation was either attributed to a copy number loss at 14q32 or occurred as a consequence of CpG hypermethylation in functionally crucial genomic regions upstream of the *MEG3* gene, in particular concerning the intergenic differentially methylated region (IG-DMR) of the *DLK1/MEG3* locus (Zhao et al. 2005; Gejman et al. 2008; Benetatos et al. 2011; Zhou et al. 2012).

Loss of *MEG3* expression was first described in pituitary tumors, and its overexpression in human cancer cell lines impaired cell proliferation and growth (Zhang et al. 2003). Furthermore, *MEG3* was downregulated in a microarray-based screen for cigarette smoke condensate-induced transcriptome changes in human bronchial epithelial cells (Hu et al. 2009). Treatment of cells with the demethylating compound azacytidine reactivated *MEG3* expression and indirectly pointed toward the epigenetic nature of cigarette smoke condensate-mediated *MEG3* silencing. As a conclusion, loss of *MEG3* expression could be an early step in cell transformation leading to lung cancer. Indeed, *MEG3* was downregulated in NSCLC tissue (Lu et al. 2013). Low *MEG3* levels correlated with an advanced pathological stage and increased tumor size and were prognostic for poor patient survival. Concerning its biological functions, *MEG3* overexpression in lung cancer cell lines enhanced apoptosis and impaired proliferation and colony formation ability. However, *MEG3* did not influence the invasion behavior of cells. Additionally, *MEG3* reduced tumor growth in a mouse model. In more detail, a *MEG3*-dependent decrease in MDM2 (mouse double minute 2 homolog) and increase in TP53 protein levels suggested that *MEG3* acts in a TP53-dependent manner. These findings are in accordance with previous studies that demonstrated both TP53-dependent and TP53-independent *MEG3* functions (Zhou et al. 2007; Zhang et al. 2010b, c; Wang et al. 2012a).

Additional valuable insights into *MEG3* regulation and function were provided from KO mice (Lin et al. 2003; Takahashi et al. 2009; Zhou et al. 2010). Although the observed phenotype characteristics differ between multiple studies, they all agree on an unequal contribution of maternally versus paternally inherited *MEG3* loci to embryonic development. A microarray-based analysis of gene expression in *Meg3*-null mouse brains revealed alterations in genes related to angiogenesis, brain development, and postnatal brain functions (Gordon et al. 2010). Subsequent validation confirmed increased activation of genes associated with the VEGF (vascular endothelial growth factor) pathway and enhanced microvessel formation in *Meg3*-null mouse brains. These observations support a tumor-suppressive function of *Meg3* by regulating angiogenesis, a hallmark of cancer (Hanahan and Weinberg 2011). Further research is necessary to determine the exact phenotypes of *Meg3*

knockout and to clarify the influence of *Meg3* transcription on *Dlk1-Meg3* imprinting control and on the regulation of downstream maternally expressed genes.

Because of its prognostic properties and tumor-suppressive functions, research on *MEG3* could significantly deepen our understanding of tumorigenesis. Since *MEG3* transcripts could be functional in a TP53-dependent and TP53-independent context, follow-up studies need to illuminate associated pathways and corresponding mechanisms of action. In addition, functionally relevant *MEG3* isoforms need to be identified in each tumor entity. Future therapeutic approaches could consider the restoration of endogenous *MEG3* expression by DNA demethylating agents or the application of functional *MEG3* mimics to counteract tumor growth and trigger apoptotic processes.

2.2.5 GAS5

GAS5 (growth arrest-specific transcript 5) was originally identified from a cDNA library of enriched RNAs in growth-arrested mouse fibroblasts and is expressed from human chromosome 1q25 (Schneider et al. 1988; Coccia et al. 1992). Further characterization of the *GAS5* transcripts and locus identified the expression of 10 C/D box snoRNAs (small nucleolar RNA) from its intronic regions (Raho et al. 2000; Smith and Steitz 1998; Hirose and Steitz 2001). Only, the snoRNAs and the *GAS5* 5′-end sequence, which is a characteristic of the 5′-terminal oligopyrimidine (5′TOP) class of genes, are conserved between human and mouse (Smith and Steitz 1998). Hence, the biological function of the *GAS5* host gene was initially tightly linked to its snoRNAs. Nonetheless, mature *GAS5* itself was upregulated during growth arrest, and its overexpression sensitized mammalian cells to apoptosis inducers and independently induced apoptosis in some cell lines (Mourtada-Maarabouni et al. 2009). In line with these findings, a mechanism by which *GAS5* functions in growth control was proposed. *GAS5* acted as a decoy for the glucocorticoid receptor (GR) by directly interacting with its DNA-binding domain and thus competing with the binding to glucocorticoid response elements (GRE) on the DNA (Kino et al. 2010). As a consequence, *GAS5* prevented the activation of glucocorticoid-responsive genes causing reduced cell metabolism, growth arrest, and sensitizing cells to apoptosis. In addition, *GAS5* was able to suppress the transcriptional activation of target genes through several other steroid receptors (Kino et al. 2010). Of importance, the *GAS5* riborepressor function was independent of the *GAS5* locus-encoded snoRNAs.

Due to its physiological role in growth arrest and apoptosis, it seemed likely that *GAS5* expression played a role in cancer as well. *GAS5* was associated with tumor-suppressive functions and was significantly downregulated in breast cancer (Mourtada-Maarabouni et al. 2009), renal cell carcinoma (Qiao et al. 2013), and NSCLC tissue (Shi et al. 2013a). Low *GAS5* levels were prognostic for poor patient survival in gastric cancer (Sun et al. 2014a), HCC (Tu et al. 2014), colorectal cancer (Yin et al. 2014), and cervical cancer (Cao et al. 2014). Most of these studies in

addition to various cell-based reports agreed on *GAS5* antiproliferative and pro-apoptotic functions. Of importance for lung cancer research, *GAS5* downregulation in NSCLC patient samples also correlated with advanced TNM stage and increased tumor size (Shi et al. 2013a). Furthermore, *GAS5* ectopic overexpression in lung cancer cell lines resulted in reduced E2F1, but increased P21 and TP53 protein levels. The influence of *GAS5* on E2F1 and P21 protein levels was confirmed in gastric cancer cell lines (Sun et al. 2014a) and indicated a mechanism for a regulatory effect of *GAS5* on cell proliferation.

Concerning the regulation of cellular *GAS5* levels, starvation of cells, drug-induced cell growth arrest, the inhibition of nonsense-mediated decay pathway (Mourtada-Maarabouni and Williams 2013), and the inhibition of PI3K (phosphatidylinositol 3-kinase) in combination with mTOR (mammalian target of rapamycin) (Pickard and Williams 2014) were able to contribute to *GAS5* upregulation. Moreover, miR-21 was able to posttranscriptionally downregulate *GAS5* levels and vice versa, and *GAS5* impaired miR-21 expression (Zhang et al. 2013d). A negative correlation between *GAS5* and miR-21 expression was further confirmed in breast cancer tissue and cell lines (Zhang et al. 2013d). Furthermore, it was speculated that epigenetic alterations might play a role in *GAS5* deregulation in lung cancer cells (Shi et al. 2013a).

From the clinical point of view, the mechanism of action of *GAS5* in the regulation of proliferation and sensitization of cells to apoptosis provides interesting therapeutic possibilities. Since *GAS5* expression is mostly lost in cancer cells, either a drug-induced reconstitution of endogenous *GAS5* expression or the application of *GAS5*-mimic RNAs that contain *GAS5* functional domain(s) could be envisioned to counteract tumor growth. Furthermore, *GAS5* was identified as a prognostic marker for overall patient survival in tumors of different origins. Nonetheless, it remains to be elucidated whether *GAS5* would also be a promising independent prognostic marker in lung cancer.

2.2.6 ANRIL

ANRIL [antisense noncoding RNA in the *INK4* (inhibitor of cyclin-dependent kinase 4) locus] was originally discovered by analysis of a 403-kb large germline deletion on chromosome arm 9p21 encompassing the *p15*/*CDKN2B* (cyclin-dependent kinase inhibitor 2B)-*p16*/*CDKN2A*-*p14*/*ARF* (alternative reading frame of *INK4A* gene) gene cluster in a family with predisposition to cutaneous malignant melanoma (CMM) and neural system tumors (NST) (Pasmant et al. 2007). *ANRIL* consists of 19 exons and gives rise to multiple isoforms that are transcribed in a tissue-specific manner (Pasmant et al. 2007; Folkersen et al. 2009). Owing to its genomic location in a cluster of three tumor suppressor genes, research on the *ANRIL* locus and transcripts was mainly carried out in the context of various diseases and cancers. Genome-wide association studies (GWAS) uncovered various disease-associated SNPs (single nucleotide polymorphisms) within chromosome 9p21 locus (Cunnington et al. 2010; Uno et al. 2010; Pasmant et al. 2011; Wiggs

et al. 2012; Chen et al. 2014b). A significant correlation of allelic *ANRIL* expression with SNPs conveying susceptibility to coronary artery disease, type 2 diabetes, and cancer, including melanoma and glioma, was noted (Cunnington et al. 2010; Johnson et al. 2013a). In contrast, fewer risk SNPs are associated with *p15/CDKN2B* and *p16/CDKN2A* expressions (Cunnington et al. 2010). This observation implied an independent role for *ANRIL* transcripts in diseases. Furthermore, SNPs correlated inversely with the expression of *ANRIL* and *p15/CDKN2B* (Cunnington et al. 2010). Further studies shed light on the mechanisms by which *ANRIL* controls epigenetic silencing of *p15/CDKN2B* and *p16/CDKN2A in cis*. Direct interactions between *ANRIL* and SUZ12 (suppressor of zeste 12 homolog), a member of the PRC2, promoted H3K27 trimethylation and silencing of the *p15/CDKN2B* gene (Kotake et al. 2011). In addition, silencing of *p16/CDKN2A* was mediated by direct interactions between *ANRIL* and CBX7 (chromobox 7), which is part of the PRC1 (polycomb repressive complex 1) (Yap et al. 2010).

Recently, elevated *ANRIL* levels were reported in ESCC (Chen et al. 2014a), gastric cancer (Zhang et al. 2014a), and NSCLC patient samples (Nie et al. 2014a). *ANRIL* upregulation in gastric cancer and NSCLC correlated with advanced TNM stage and increased tumor size. Furthermore, increased *ANRIL* levels were prognostic for poor patient survival in both cancer entities. Consistently, siRNA-mediated knockdown of *ANRIL* reduced cell proliferation in ESCC, gastric cancer, and lung cancer cell lines. However, the underlying mechanisms varied. In ESCC patient samples, an inverse correlation between *ANRIL* and *TGFβ1* (transforming growth factor beta 1) mRNA levels was observed (Chen et al. 2014a). TGFβ1 was implicated in the upregulation of *p15/CDKN2B* and induction of subsequent cell cycle arrest (Hannon and Beach 1994; Robson et al. 1999). Hence, the *ANRIL*-mediated downregulation of *TGFβ1* mRNA and protein levels was proposed to contribute to *p15/CDKN2B* reduction and enhanced cell proliferation (Chen et al. 2014a).

In gastric cancer, *ANRIL* recruited the PRC2 complex to repress transcription of miR-99a and miR-449a in trans (Zhang et al. 2014a). These miRNAs hold crucial roles in the regulation of the mTOR and CDK6 (cyclin-dependent kinase 6) pathways. Consequently, the application of miRNA mimics induced a cell cycle arrest at the G0/G1 phase and induced apoptosis in gastric cancer cell lines. The *ANRIL*-mediated reduction of miR-449a levels was followed by an accumulation of CDK6, which in turn phosphorylated and inactivated the Rb protein. Subsequent release of E2F1 transcription factor enabled cell cycle progression. In a positive feedback loop, E2F1 directly interacted with the *ANRIL* promoter to increase its expression (Wan et al. 2013; Zhang et al. 2014a). The relevance of this mechanism was underscored by an inverse correlation between *ANRIL* and miR-99a and miR-449a expressions in gastric cancer patient samples (Zhang et al. 2014a). Moreover, reduced miR-449a expression in lung cancer was associated with a shorter disease-free survival of patients, and increased miR-449a expression impaired proliferation of lung cancer cells in vitro and in vivo (Ren et al. 2014). Nonetheless, it remains to be elucidated whether *ANRIL*-promoted mechanisms of action in the context of gastric cancer and lung cancer are similar.

Knockdown of *ANRIL* in lung cancer cell lines not only decreased proliferation in vitro and in vivo, but also reduced colony formation and migration, while increasing apoptosis (Nie et al. 2014a). Noteworthy, overexpression of *ANRIL* did not influence proliferation in a human bronchial epithelial cell line. In accordance with previous studies, *ANRIL* knockdown was accompanied by increased *p15*/*CDKN2B* and *p16*/*CDKN2A* levels. However, because this effect was not observed in all evaluated lung cancer cell lines, a more complex mechanism of action was hypothesized. Indeed, a direct interaction between *ANRIL* and the PRC2 promoted H3K27me3-suppressive marks in the promoter region of *P21* and *KLF2* (krüppel-like factor 2) in trans (Nie et al. 2014a). In support of this data, an inverse correlation between *ANRIL* and *KLF2* transcription factor expression was noted in NSCLC patient tissue. Furthermore, KLF2 overexpression promoted antiproliferative and pro-apoptotic cellular responses (Nie et al. 2014a). Hence, *ANRIL* might in part exert its effects by epigenetic silencing of the tumor suppressor proteins P21 and KLF2 in lung cancer.

In summary, the *ANRIL* locus is associated with a plethora of disease-associated risk SNPs, including lung cancer (Timofeeva et al. 2012). *ANRIL* functions as an epigenetic regulator of various genes *in cis* and in trans. A recent study revealed that Alu elements within *ANRIL* transcripts convey specificity in the genome-wide trans-regulation of genes (Holdt et al. 2013). *ANRIL*-mediated recruitment of PRC1 and PRC2 occurred via complementary binding to Alu motifs in the promoters of target genes. Furthermore, *ANRIL* holds a crucial role in silencing tumor suppressor genes and therefore represents an interesting therapeutic target. Considering the complexity of the *ANRIL* genomic locus and the complex network of targeted genes, the benefit of future approaches about targeting *ANRIL* for cancer therapy must be carefully evaluated.

2.2.7 SOX2OT

The lncRNA *SOX2OT* (SRY-box-containing gene 2 overlapping transcript) was discovered in a study focusing on the involvement of mutations in the *SOX2* gene in anophthalmia (Fantes et al. 2003). The *SOX2* monoexonic gene resides within an intron of the multiexonic, conserved *SOX2OT* genomic locus in the chromosome region 3q26, and is transcribed in the same orientation. The highly conserved *SOX2* gene encodes a high-mobility-group transcription factor that is spatiotemporally controlled and plays a crucial role in the establishment and maintenance of pluripotency in vertebrate stem cells as well as neural, retina, and lens development in mice (Amaral et al. 2009). In humans, *SOX2* loss of heterozygosity is linked to ocular and neural abnormalities (Fantes et al. 2003; Kelberman et al. 2006; Sisodiya et al. 2006). Moreover, amplification of the chromosomal region 3q26 was reported in SCC of the lung, esophagus, head and neck, and cervix (Balsara and Testa 2002; Bass et al. 2009; Hussenet et al. 2010; Schrock et al. 2014) and revealed *SOX2* as a novel oncogene in SCC tumorigenesis (Hussenet and du Manoir 2010).

The similar expression patterns of *SOX2* and *SOX2OT* might be explained by their shared genomic locus. Significant upregulation of *SOX2OT* and coexpression with *SOX2* were reported in esophageal SCC, lung SCC, and lung ADC tissues from patients (Shahryari et al. 2014; Hou et al. 2014). Noteworthy, *SOX2OT* levels were significantly higher in lung SCC compared to lung ADC samples (Hou et al. 2014). Furthermore, *SOX2OT* overexpression was a negative prognostic marker for NSCLC patient survival (Hou et al. 2014). Insights into the mechanism of *SOX2OT* function were obtained from studies in lung cancer cell lines. Here, siRNA-mediated knockdown of *SOX2OT* induced a G2/M cell cycle phase arrest via reduction of EZH2 (enhancer of zeste 2) mRNA and protein levels and modulation of transition-regulating genes. In addition, cell proliferation and colony formation ability of lung cancer cell lines were reduced (Hou et al. 2014). A recent report demonstrated the enhanced coexpression of *SOX2* and *SOX2OT* in estrogen receptor-positive invasive breast cancer using data from the TCGA database (Askarian-Amiri et al. 2014). Ectopic overexpression of *SOX2OT* in MDA-MB-231 cells increased *SOX2* mRNA and protein expressions (Askarian-Amiri et al. 2014).

In contrast to observations from human cancers, *Sox2ot* was found to be expressed in some mouse tissues where *Sox2* was absent, indicating an independent role for *Sox2ot* in vertebrate development (Amaral et al. 2009). This hints at a more complex relationship between *SOX2* and *SOX2OT* depending on the developmental and cellular context and awaits further investigation. Its clinical importance, genomic location, and first mechanistic insights into *SOX2OT* function make it an interesting molecule for further research.

2.2.8 SPRY4-IT1

SPRY4-IT1 (sprouty 4 intronic transcript 1) resides within the second intron of the *SPRY4* gene on chromosome 5q31 and is an unspliced, mainly cytoplasmic lncRNA that was initially identified in adipose tissue and is not conserved beyond primate genomes (Khaitan et al. 2011). SPRY4 is an inhibitor of the MAPK (mitogen-activated protein kinase) signaling pathway acting upstream of RAS (rat sarcoma) activation and inhibiting active GTP-RAS formation (Leeksma et al. 2002). Tumor-suppressive functions of SPRY4 were described in NSCLC cell lines (Tennis et al. 2010). A common reduction of *SPRY4* mRNA and protein levels in NSCLC cell lines was observed. General cell growth and anchorage-independent growth, migration, and invasion were reduced in stably *SPRY4*-expressing NSCLC cell lines. In addition, *SPRY4* expression was linked to the reversal of EMT (Tennis et al. 2010).

A recent study claimed equally important functions for the lncRNA *SPRY4-IT1* in NSCLC tumorigenesis (Sun et al. 2014b). *SPRY4-IT1* RNA levels were reduced in 94 % of paired NSCLC patient samples compared to adjacent non-cancerous tissue. Of clinical importance, low *SPRY4-IT1* expression levels held independent prognostic value for poor patient survival. The reduction in *SPRY4-IT1* transcript levels was at least partially caused by EZH2-mediated epigenetic gene silencing.

High expression of EZH2 in NSCLC further supported these results (Behrens et al. 2013). Further characterization of *SPRY4-IT1* lncRNA by means of transient overexpression in NSCLC cell lines revealed its inhibitory effect on cell proliferation, migration, and invasion while increasing apoptosis (Sun et al. 2014b). In analogy with the functions of the SPRY4 protein, *SPRY4-IT1* was also implicated in the modulation of EMT. It is worth noting that neither *SPRY4-IT1* overexpression nor knockdown introduced significant changes in *SPRY4* expression. This implies that *SPRY4-IT1* lncRNA exerts its functions independent of the SPRY4 protein.

In contrast to NSCLC, *SPRY4-IT1* lncRNA was upregulated and associated with pro-tumorigenic properties in melanoma, ESCC, and clear cell renal cell carcinoma (Khaitan et al. 2011; Xie et al. 2014; Zhang et al. 2014d). RNAi-based knockdown of *SPRY4-IT1* in melanoma cells induced cell growth arrest, inhibited invasion, and increased apoptosis (Khaitan et al. 2011). In ESCC and clear cell renal cell carcinoma, high *SPRY4-IT1* levels were an independent prognostic factor of poor patient survival (Xie et al. 2014; Zhang et al. 2014d). In vitro assays indicated reduced cell proliferation, migration, and invasion upon *SPRY4-IT1* knockdown in both cancer types. In another study, *SPRY4-IT1* upregulation was described in severe preeclamptic placenta and functionally implicated in reduced cell migration and proliferation, but enhanced apoptosis of a trophoblast cell line (Zou et al. 2013).

Altogether, *SPRY4-IT1* might function as an independent prognostic marker in different cancers and represents a putative therapeutic target as EMT regulator in NSCLC.

2.2.9 TUG1

TUG1 (taurine upregulated gene 1) was initially identified in a genomic screen for upregulated genes in response to taurine treatment in developing mouse retinal cells (Young et al. 2005). Interestingly, *Tug1* existence was not limited to the mouse genome, but a highly conserved homolog was also present in human (on chromosome 22q12), rat, dog, and cow genomes. RNAi-mediated knockdown of *Tug1* in the developing mouse eye implied a role in normal photoreceptor development in mice (Young et al. 2005). *Tug1* knockdown was accompanied by altered gene expression of different photoreceptor genes. Since *Tug1* expression was not only observed in mouse developing retina, but also observed in brain and other tissues of the adult organism, a putative function in diseases of other tissues stands to reason. Indeed, *TUG1* lncRNA was deregulated in a variety of human cancers. In contrast to a general upregulation of *TUG1* in bladder urothelial carcinomas, osteosarcoma, and ESCC (Han et al. 2013b; Zhang et al. 2013c; Xu et al. 2014), a general downregulation in NSCLC patient samples (Zhang et al. 2014b) was observed.

With regard to lung cancer research, *TUG1* downregulation in NSCLC patient tissue was associated with advanced TNM stage and tumor size and was an independent prognostic factor of poor patient survival (Zhang et al. 2014b). *TUG1* was reported to be a TP53-inducible lncRNA, and its knockdown in lung cancer cell lines increased proliferation in vitro and tumor growth in vivo, possibly by

accelerating cell cycle progression. Furthermore, *TUG1* interaction with PRC2 was involved in epigenetic silencing of the *HOXB7* gene (Zhang et al. 2014b). This conclusion was supported by the inverse correlation between *TUG1* and HOXB7 protein levels in NSCLC patient samples. Since HOXB7 is known to promote cell proliferation through AKT and MAPK pathway activation, knockdown of *HOXB7* consequently reduced activating phosphorylation of key genes. In summary, *TUG1* was proposed to modulate NSCLC cell growth indirectly through AKT and MAPK signaling pathways via epigenetic regulation of the *HOXB7* locus (Zhang et al. 2014b).

An earlier contribution to clarify *TUG1* mechanism of action described *TUG1* function as molecular scaffold in polycomb bodies (Yang et al. 2011b). In response to mitogenic signals, *TUG1* was implied in the negative regulation of cell growth by interacting with methylated PC2 protein and relocating growth control transcription units to the gene-repressive environment of the polycomb bodies. In contrast, the *MALAT1* lncRNA interacted with unmethylated PC2 protein and was proposed to relocate growth control transcription units to the activating gene environment of the interchromatin granules, thus promoting cell growth.

Since *TUG1* was upregulated in all so far reported cancer entities except NSCLC, the mechanisms underlying *TUG1* function are expected to differ from those in the context of lung cancer. In bladder urothelial carcinoma and osteosarcoma cells, *TUG1* knockdown inhibited cell proliferation and promoted apoptosis (Han et al. 2013b; Zhang et al. 2013c). Similar observations were made in ESCC, where *TUG1* knockdown also reduced proliferation, possibly by inducing a cell cycle arrest, and additionally reduced migration (Xu et al. 2014).

As a conclusion, the elucidation of *TUG1* function and molecular mechanisms in lung cancer revealed a complex pathway of *TUG1* action. Although *TUG1* seems to act like a tumor suppressor in NSCLC, the opposite was observed in other cancer entities. This discrepancy can only be resolved by a more profound investigation of *TUG1* mechanism of action in due consideration of the cancer tissue of origin.

2.2.10 CCAT1, CCAT1-L, and CARLO-5

The biexonic lncRNAs *CCAT1* (colon cancer-associated transcript 1), *CCAT1-L* (*CCAT1* long isoform), and *CARLO-5* (cancer-associated region long noncoding RNA 5) are transcribed from the (-) strand of the 8q24.21 genomic region and show significant overlap in their sequences (Nissan et al. 2012; Xiang et al. 2014; Kim et al. 2014b) (Table 2).

CCAT1, *CCAT1-L*, and *CARLO-5* were significantly upregulated in colon cancer patient tissue (Nissan et al. 2012; Xiang et al. 2014; Kim et al. 2014b). Additionally, *CCAT1* was upregulated in ovarian cells (Liu et al. 2013a), and *CARLO-5* was increased in prostate cancer patient tissue and in NSCLC patient tissue (Kim et al. 2014b; Luo et al. 2014). *CCAT1* is highly upregulated in premalignant conditions as well as in the complete adenoma–carcinoma sequence of

Table 2 Relationship between CCAT1, CCAT1-L, and CARLO-5 according to GRCh37/hg19 assembly

LncRNA	Genomic position	Transcript length (nt)	Original references
CCAT1	chr8:128,219,629-128,231,333	2613	Nissan et al. (2012)
CCAT1-L	chr8:128,217,038-128,231,333*	5204[a]	Xiang et al. (2014)
CARLO-5	chr8:128,220,431-128,231,175	1652	Kim et al. (2014b)

The lncRNAs are transcribed from the (–) strand of chromosome 8

[a]*CCAT1-L* sequence is extended at the 3'end as compared to *CCAT1*. The presented genomic position of *CCAT1-L* was determined according to the provided 3'RACE result (Xiang et al. 2014). The resulting transcript length is as stated

colon cancer tumorigenesis and cancer-associated lymph node tissues and therefore contains high biomarker potential (Nissan et al. 2012; Alaiyan et al. 2013).

Elevated *CARLO-5* levels in NSCLC patient samples were associated with advanced pathological stage and lymph node metastasis and were an independent prognostic marker for poor patient survival (Luo et al. 2014). The cellular function of *CARLO-5* in NSCLC was linked to enhanced proliferation, metastasis, and invasion, possibly by modulating the EMT process. Results on the functional level significantly overlap with observations in gastric cancer (Yang et al. 2013b) and colon carcinoma (Kim et al. 2014b; He et al. 2014b).

Interesting mechanistic insights into lncRNA function were mainly provided from colorectal cancer, where transcription takes place from a super-enhancer region upstream of the MYC gene (Hnisz et al. 2013). The level of *CARLO-5* expression in normal colon tissue correlated with the cancer-associated SNP variant rs6983267 in the *MYC* enhancer region (Kim et al. 2014b). Chromosome conformation capture (3C) method revealed long-range interactions between the *CARLO-5* promoter and the 8q24 enhancer region encompassing rs6983267. These interactions might hint at a regulatory role of the rs6983267-containing enhancer region for *CARLO-5* expression. Concerning the *CARLO-5* function in colon cancer, RNAi-mediated knockdown reduced cell proliferation by modulating the cell cycle (Kim et al. 2014b). In addition, reduced *CARLO-5* levels impaired colony formation of colon cancer cell lines on soft agar and diminished tumor incidence in xenograft mouse models. In another report, the *CCAT1-L* isoform was reported to regulate *MYC* transcription *in cis* (Xiang et al. 2014). Application of the 3C method implied long-range DNA interactions via CTCF-mediated chromosome looping between *CCAT1-L* and *MYC* genomic loci. Furthermore, *CCAT1-L* interacted with CTCF and this interaction depended on the 3' additional *CCAT1-L* sequence (including nucleotides 2655–3959) that is not present in *CCAT1*. In terms of function, *CCAT1-L* was able to modulate CTCF binding to chromatin and thus exerted its regulatory properties on *MYC* transcription through regulation of chromosomal organization (Xiang et al. 2014). Overexpression of *CCAT1-L* in mouse xenograft studies increased tumor size and supported a tumorigenic effect in vivo. This was most likely linked to the upregulation of c-MYC expression *in cis* (Xiang et al. 2014). Another relationship between *CCAT1* and c-MYC was reported earlier

in gastric cancer, where c-MYC transcription factor upregulated *CCAT1* expression by direct interaction with an E-box element in the *CCAT1* promoter region (Yang et al. 2013b).

In the future, it will be crucial to further investigate the function and relationship between mentioned overlapping transcripts. For *CCAT1* and *CCAT1-L*, even a different way of biogenesis was hypothesized (Xiang et al. 2014). Also, further studies need to consider that super-enhancers are tumor type specific and absent in normal cells (Hnisz et al. 2013). Therefore, the reason for differential regulation and mechanism of action for described lncRNAs might not only depend on their biogenesis and sequence, but also depend on the cell-, tissue-, and tumor-specific genomic features. In summary, initial observations on the prognostic potential and function of *CARLO-5* in NSCLC provide an interesting basis for future research.

2.2.11 CCAT2

CCAT2 (colon cancer-associated transcript 2) is expressed from a highly conserved genomic region within 8q24.21 and was first described to be highly transcribed in microsatellite-stable colorectal cancer (Ling et al. 2013b). This lncRNA encompasses the rs6983267 SNP, which was previously associated with enhanced risk for to colon, ovary, prostate, and inflammatory breast cancer (Haiman et al. 2007; Tomlinson et al. 2007; Ghoussaini et al. 2008; Bertucci et al. 2012). In NSCLC patients, an elevated *CCAT2* level was associated with the ADC subtype and was able to predict lymph node metastasis only in combination with the serum biomarker CEA (carcinoembryonic antigen) (Qiu et al. 2014b). Initial approaches to elucidate the function of *CCAT2* in NSCLC imply a role in cell proliferation, migration, and invasion.

Another report focused on the significance and predictive potential of *CCAT2* overexpression in breast cancer (Redis et al. 2013). In breast cancer, where *CCAT2* functioned in an rs6983267-independent manner, *CCAT2* expression predicted shorter metastasis-free survival and overall survival, but only for a particular subgroup of patients with lymph node-positive disease that have received adjuvant CMF (cyclophosphamide/methotrexate/fluorouracil) chemotherapy (Redis et al. 2013). The original report in colon cancer provided most mechanistic insights into *CCAT2* function so far (Ling et al. 2013b). Here, the characterization of *CCAT2* revealed that the rs6983267 risk allele "G" produced higher levels of RNA compared to the "T" allele in vitro. Moreover, *CCAT2* lncRNA regulated the transcription of *MYC* and promoted cancer growth, metastasis, and chromosomal instability in microsatellite-stable colon cancer. In addition, a relation between *CCAT2* and WNT signaling was proposed, supporting and complementing previous data on a WNT transcription factor binding site including rs6983267 (Tuupanen et al. 2009; Pomerantz et al. 2009). Whether the rs6983267 SNP plays any role or influences *CCAT2* expression in lung cancer has not been addressed yet.

In summary, a more profound analysis of *CCAT2* function and potential contribution of rs6983267 SNP to lung cancer pathogenesis will shed light on the prognostic and therapeutic value of *CCAT2* for future clinical applications.

2.2.12 PVT1

The human *PVT1* (plasmacytoma variant translocation 1) locus resides telomeric to the *MYC* gene on chromosome 8q24.21 and was originally discovered as a site of chromosomal translocations and frequent viral integrations in B- or T-cell lymphomas (Erikson et al. 1983; Tsichlis et al. 1983; Webb et al. 1984; Villeneuve et al. 1986). In human Burkitt's lymphoma, the most common translocations involve the *MYC* gene and the immunoglobulin heavy-chain locus [t(8;14)] and account for ~85 % of cases. In contrast, the variant translocations involve *PVT1* and either the immunoglobulin kappa [(t(2;8)] or lambda light-chain locus [t(8;22)] and account for ~15 % of cases (Shtivelman et al. 1989; Huppi et al. 2012). Moreover, novel *PVT1* fusion genes were identified in SCLC (Pleasance et al. 2010; Iwakawa et al. 2013), medulloblastoma (Northcott et al. 2012), multiple melanoma (Nagoshi et al. 2012), gastric cancer (Kim et al. 2014a), and acute myelogenous leukemia (Chinen et al. 2014). Although the *PVT1* locus harbors conserved transcriptional activity in human, mouse, and rat, an expression of various alternatively spliced, non-protein-encoding transcripts along with a lack of sequence conservation between species was noted (Shtivelman et al. 1989; Tsichlis et al. 1989; Shtivelman and Bishop 1990; Huppi et al. 1990). Furthermore, the miR-1204–miR-1208 cluster (Huppi et al. 2008) and various disease-associated SNPs reside within the *PVT1* locus (reviewed in Huppi et al. 2012). Despite frequent coexpression of *PVT1* and *MYC* in various cancers, *PVT1* transcripts and -derived miRNAs were also implicated in MYC-independent functions, supporting an additional function of *PVT1* as lncRNA and reservoir for short regulatory RNAs.

In colorectal cancer, HCC, and NSCLC, *PVT1* overexpression was a negative prognostic marker for patient survival (Takahashi et al. 2014; Wang et al. 2014a; Yang et al. 2014c). In the context of NSCLC, *PVT1* upregulation was associated with advanced histological grade and lymph node metastases (Yang et al. 2014c). RNAi-mediated knockdown of *PVT1* in one lung cancer cell line reduced cell proliferation, migration, and invasion (Yang et al. 2014c). To further investigate the role of *PVT1* transcripts in lung cancer, a profound analysis of transcript variants and experimental support of the described *PVT1* biological functions in additional lung cancer cell lines would be required. In addition, PVT1's mechanism of action needs to be defined to fathom the use of *PVT1* as a therapeutic target.

Additional insights into *PVT1* regulation and function were provided from studies in other diseases and cancer entities. Although *PVT1* and *MYC* expression levels differed in normal human tissues, MYC overexpression in transformed cell lines correlated with high *PVT1* expression (Carramusa et al. 2007). By experimental evidence, a c-MYC transcription factor binding site was defined in the promoter of *PVT1* (Carramusa et al. 2007). In another report, a TP53 transcription

factor binding site was characterized in the *PVT1* promoter region (Barsotti et al. 2012). Moreover, a TP53-dependent increase of *PVT1* and miR-1204 expressions was observed upon exposure of various cell lines to the DNA-damaging agent, daunorubicin. Overexpression of miR-1204 subsequently induced cell death and opposed so far described *PVT1* pro-tumorigenic functions (Barsotti et al. 2012). In ovarian and breast cancer cell lines with *PVT1* amplification and overexpression, RNAi-based *PVT1* knockdown inhibited proliferation and induced apoptosis (Guan et al. 2007). Importantly, this effect was not observed in cell lines without amplification or overexpression. In contrast, *MYC* knockdown had no effect on apoptosis, emphasizing a different role for *PVT1* and *MYC* in ovarian and breast cancers (Guan et al. 2007). In pancreatic cancer cells, *PVT1* upregulation was linked to a decrease in cellular sensitivity to the cytidine analog, gemcitabine (You et al. 2011). In colorectal cancer cell lines, RNAi-based *PVT1* knockdown reduced proliferation and invasion and increased apoptosis (Takahashi et al. 2014). High *PVT1* expression was also detected in fetal mouse liver and HCC tumors (Wang et al. 2014a). Here, *PVT1* positively influenced cell proliferation in vitro and in vivo and enhanced cell cycle progression. *PVT1* promoted stem cell-like properties in hepatoma cells. Furthermore, *PVT1* was implicated in the regulation of NOP2 nucleolar protein stability by a mechanism that remains elusive (Wang et al. 2014a). In summary, proliferative and stem cell-like properties were proposed to be controlled by *PVT1*- and NOP2-dependent processes in HCC. Furthermore, *PVT1* was a candidate gene promoting susceptibility to end-stage renal disease (ESRD) attributed to type 1 diabetes and type 2 diabetes (Hanson et al. 2007; Millis et al. 2007). A follow-up study demonstrated *PVT1* upregulation in normal human mesangial cells under hyperglycemic conditions and suggested a role in partially TGFB1-independent extracellular matrix protein regulation in the context of diabetic nephropathy (Alvarez and DiStefano 2011).

Despite its many MYC-independent functions in vitro, *Pvt1* locus gain in concert with *Myc* gain was necessary to promote cancer in MMTVneu [gain (Myc, Pvt1, Ccdc26, Gsdmc)] mouse models in vivo (Tseng et al. 2014). *PVT1* inhibited MYC phosphorylation and degradation and thus enhanced its oncogenic function by increasing MYC protein stability in cell lines. In fact, more than 97 % of tumors with 8q24 copy number gain concertedly displayed amplification of both, the *MYC* locus and the *PVT1* locus (Tseng et al. 2014). This study raised hope for putative clinical applications where targeting of *PVT1* could regulate MYC protein abundance.

Altogether, various transcript variants, a miRNA cluster, and disease-associated SNPs originate from the *PVT1* locus. In addition, the *PVT1* locus harbors translocation break sites generating different fusion transcripts. Hence, an important task will be to determine the functional *PVT1* transcripts and respective sequences or domains conveying pathogenic effects. Despite its genomic location telomeric to the *MYC* gene, *PVT1* transcripts are functional in a MYC-dependent and MYC-independent manner. Further studies need to confirm whether described

PVT1 functions and mechanisms of action might be general contributors to human tumorigenesis or additional, tissue-specific functions need to be defined. Research on the *PVT1* locus is challenging and however might be rewarded by revealing further prognostic and therapeutic options, also with regard to the *MYC* oncogene.

2.2.13 BANCR

BANCR (BRAF-activated noncoding RNA) is transcribed from the (-) strand of chromosome 9q21 and was discovered by a high-throughput RNA sequencing screen aiming at the identification of changes in primary patient melanocyte transcriptome profiles linked to oncogenic $BRAF^{V600E}$ expression (Flockhart et al. 2012). A recent study in NSCLC reported a significant downregulation of *BANCR* in patient tissue (Sun et al. 2014c). Low *BANCR* levels correlated with increased TNM stage and increased tumor size and lymph node metastasis. In addition, low *BANCR* expression was an independent prognostic marker for poor patient survival. Cell-based experiments implied an HDAC3 (histone deacetylase 3)-dependent epigenetic silencing of the *BANCR* gene in cancer cells. Transient overexpression of *BANCR* in cell lines reduced cell viability, migration, and invasion and promoted apoptosis, whereas RNAi-mediated *BANCR* knockdown increased cell migration and invasion (Sun et al. 2014c). Supporting its role in cell migration, mouse xenograft studies revealed that *BANCR* overexpression reduced the number of metastatic lung tumor nodules in vivo. First insights into a more precise mechanism highlighted the regulation of EMT-associated genes by *BANCR* and provided a basis for further research (Sun et al. 2014c).

In contrast to NSCLC, *BANCR* was significantly upregulated in malignant melanoma, colorectal carcinoma, and papillary thyroid carcinoma tissue (Wang et al. 2014d; Guo et al. 2014; Flockhart et al. 2012). In the original study on *BANCR*, an influence of the lncRNA on melanoma cell migration via regulation of CXCL11 chemokine expression levels was observed (Flockhart et al. 2012). Another report in malignant melanoma pointed out *BANCR* function in the regulation of cell proliferation in vitro and in vivo, possibly by dephosphorylating and inactivating Raf-1, JNK, and ERK1/2 (Li et al. 2014)—mediators of the cellular response to growth signals. In colorectal carcinoma, *BANCR* expression levels correlated with TNM stage and lymph node metastasis (Guo et al. 2014). Analysis of the prognostic potential was not provided yet. Similar to previous studies, *BANCR* was also suggested to influence cell migration in colorectal cancer, probably via modulation of EMT (Guo et al. 2014). In papillary thyroid carcinoma, *BANCR* knockdown reduced cell proliferation and moderately induced apoptosis in a cell line (Wang et al. 2014d). No effect on cellular metastatic potential was observed. Furthermore, initial attempts to concretize *BANCR* function in papillary thyroid carcinoma hint at the regulation of autophagy-related processes (Wang et al. 2014d).

In summary, *BANCR* deregulation in cancer seems to depend on the tissue of origin. However, most studies agree on a major role for *BANCR* in modulating EMT. Importantly, somatic *BRAF* mutations occur in a wide range of human cancers (Davies et al. 2002). $BRAF^{V600E}$ mutations occur particularly in melanoma and colorectal carcinoma, where *BANCR* deregulation was already described. Additional *BANCR* functions might be revealed from future investigations of other, not yet appreciated, cancer entities harboring $BRAF^{V600E}$ mutations. Notwithstanding that there has been some progress in describing the functional properties of *BANCR*, more effort is necessary to unravel the mechanisms underlying *BANCR* function in the process of tumorigenesis.

2.2.14 MVIH

The lncRNA *MVIH* (lncRNA associated with microvascular invasion in hepatocellular carcinoma) is located on human chromosome 10q22 in an intron of the *RPS24* (ribosomal protein S24) gene and partially overlaps with *RPS24* exonic region. It was initially discovered to be upregulated in microarray data aiming at a comparison of lncRNA expression in hepatitis B virus (HBV)-related HCC and paired non-tumor samples (Yang et al. 2011a). In a follow-up study, the *MVIH* lncRNA was characterized as an independent transcript, and its overexpression was confirmed in HCC patient tissue (Yuan et al. 2012). Of clinical significance, *MVIH* upregulation was linked to microvascular invasion, advanced TNM stage, and decreased recurrence-free and overall survival of patients. Furthermore, *MVIH* was an independent predictive marker for poor recurrence-free survival of patients after hepatectomy. On the functional level, *MVIH* overexpression increased tumor growth and intrahepatic metastases in a mouse model (Yuan et al. 2012). In vitro assays supported a pro-angiogenic function of *MVIH* that could partially be explained by reduced secretion of the antiangiogenic factor PGK1 (phosphoglycerate kinase 1).

In agreement with the study in HCC, *MVIH* was upregulated in NSCLC patient samples and correlated with advanced TNM stage, tumor size, and lymph node metastasis (Nie et al. 2014b). Additionally, high *MVIH* levels were prognostic for poor overall survival of patients. RNAi-mediated knockdown of *MVIH* in lung cancer cell lines reduced proliferation, migration, and invasion. These effects were reversed upon ectopic *MVIH* overexpression and partly explained by *MVIH* regulatory influence on MMP2 and MMP9 protein levels.

In summary, first promising insights into *MVIH* prognostic potential in NSCLC and biological function were provided. Further research is necessary to uncover the mechanism of action and provide supportive evidence for lncRNA *MVIH* biomarker properties.

2.3 Candidate Functional Long Noncoding RNAs

2.3.1 GHSROS

The *GHSROS* (growth hormone secretagogue receptor opposite strand) monoexonic lncRNA is transcribed from the first intron of the *GHSR* gene on chromosome 3q26 and was first characterized in the context of lung cancer (Whiteside et al. 2013). In contrast to NSCLC tumor patient samples, most normal human tissues exhibited a lower expression of *GHSROS*. In lung cancer cell lines, *GHSROS* overexpression enhanced migration, while it decreased cell migration in a normal bronchoepithelial cell line. Obtained results might indicate context- and cell type-specific functions of *GHSROS*. This study provided a basis for further investigations that need to confirm *GHSROS* upregulation in a bigger set of NSCLC patient tissues and also investigate GHSROS' potential as biomarker and therapeutic target. Furthermore, the relationship between lncRNA and host gene awaits clarification and might provide valuable insights into lncRNA function.

2.3.2 GAS6-AS1

GAS6-AS1 [growth arrest-specific 6 (GAS6) antisense RNA 1] is a putative lncRNA localized on chromosome 13q34 and transcribed antisense to the *GAS6* gene (Han et al. 2013a). The *GAS6-AS1* putative lncRNA was downregulated in NSCLC patient tissue and its expression negatively correlated with lymph node metastasis and advanced TNM stage (Han et al. 2013a). In addition, low *GAS6-AS1* expression was an independent prognostic marker of poor overall survival in patients. Moreover, an inverse correlation between *GAS6-AS1* and *GAS6* expressions was reported in NSCLC tissue.

Since no basic characterization of *GAS6-AS1* was provided, its assignment to the class of lncRNAs is preliminary. Furthermore, additional studies need to illuminate *GAS6-AS1* relationship with the *GAS6* gene and provide insights into *GAS6-AS1* function and mode of action.

2.3.3 SCAL1

SCAL1 (smoke and cancer-associated lncRNA 1) is transcribed from chromosome 5q14 and was first characterized as XLOC_004924 in a human long intergenic noncoding RNA (lincRNA) catalog based on RNA-seq data from 24 human tissues and cell lines (Cabili et al. 2011). Here, *SCAL1* expression was highest in thyroid tissue and fifth highest in lung tissue. Aiming at the identification of differentially regulated lncRNAs that convey aggressive malignant characteristics in lung cancer, an RNA-seq screening approach identified *SCAL1* to be the most strongly upregulated lncRNA in the metastatic lung cancer cell line CL1-5 compared to the

noninvasive cell line CL1-0 (Thai et al. 2013). This data was consistent with two publically available datasets from the NCBI Sequence Read Archive (SRA) with a significant upregulation of *SCAL1* in smokers. Furthermore, *SCAL1* was upregulated in airway epithelium cell cultures after exposure to cigarette smoke extract and in many lung cancer cell lines (Thai et al. 2013). The NRF2 [nuclear factor erythroid 2 (NF-E2)-related factor 2]-KEAP1 pathway, which is one of the protective pathways of lung epithelial cells against smoke and oxidative stress, was implied in *SCAL1* regulation. More specifically, ChIP data supported transcriptional regulation of *SCAL1* by NRF2. In conclusion, *SCAL1* could be a novel NRF2 downstream regulator of genes for oxidative stress protection (Thai et al. 2013). Noteworthy, a previous GWAS identified the SNP rs933688, which resides within *SCAL1* genomic locus, to be significantly associated with ever versus never smoking status of subjects (Caporaso et al. 2009).

Accumulating evidence supports a role for the *SCAL1* locus in cigarette smoke-associated tissue changes. Nonetheless, it remains the subject of future studies to provide a profound experimental analysis of *SCAL1* locus and putative transcript variants, more detailed functional analyses, and insights into its potential as biomarker and therapeutic target. Also, at the moment the relationship between the discovered SNP and the lncRNA *SCAL1* is unclear and awaits further elucidation.

2.3.4 ZXF1

The *ZXF1* (named after its discoverer Zhou Xue-Feng) putative lncRNA resides in chromosome 10q23 and was recently discovered by microarray screening in the ADC subtype of NSCLC (Zhang et al. 2014f). High *ZXF1* expression status correlated with advanced pathological stage and lymph node metastasis. *ZXF1* expression could be a potential negative prognostic marker for patient survival. Initial RNAi-based experiments implied a role for *ZXF1* in migration and invasion, but not in proliferation (Zhang et al. 2014f). Although *ZXF1* might hold interesting properties, no basic characterization of this putative lncRNA was undertaken, that is, no data on sequence analysis, coding potential, and genomic features was provided. However, this would be a crucial step toward a comprehensive and reliable analysis in the future.

2.3.5 LncRNA-LET

The *LncRNA-LET* (lncRNA low expression in tumor) is a monoexonic lncRNA that is expressed from an intron of the *NPTN* (neuroplastin) gene on chromosome 15q24 and was discovered in a microarray screen comprising HBV-related HCC tissues and corresponding non-tumor control tissues (Yang et al. 2011a). In the follow-up study, *LncRNA-LET* was characterized and confirmed to be underexpressed in the majority of the HCC, colorectal cancer, and lung SCC patient tissues (Yang et al. 2013a). The mechanism of *lncRNA-LET* downregulation was of epigenetic nature.

Specifically, hypoxic conditions induced HDAC3 to repress *lncRNA-LET* expression by deacetylation of the *lncRNA-LET* promoter region in HCC cell lines. Furthermore, *lncRNA-LET* is associated with NF90 (nuclear factor 90) and was implied in increased NF90 protein ubiquitination and subsequent degradation. Given that the NF90 protein favors hypoxia-induced cancer cell invasion, the process of HCC cell invasion might partially be regulated by the *lncRNA-LET*/NF90/HIF-1α axis (Yang et al. 2013a). Indirect evidence for the interplay between hypoxia, *lncRNA-LET*, NF90, and metastatic HCC was further provided from HCC patient tissue samples, where the hypoxic marker carbonic anhydrase 9 was inversely correlated with *lncRNA-LET* levels, *lncRNA-LET* was downregulated in metastatic HCCs, and NF90 protein levels were higher in metastatic HCCs (Yang et al. 2013a).

Low *lncRNA-LET* levels in gallbladder cancer even held a prognostic potential for poor patient survival (Ma et al. 2014). *LncRNA-LET* downregulation by hypoxia and *lncRNA-LET* inhibitory effect on cell invasion under hypoxic and normoxic conditions were confirmed in gallbladder cells (Ma et al. 2014). In addition, *lncRNA-LET* tumor-suppressive functions were linked to the induction of a G0/G1 cell cycle phase arrest and apoptosis under hypoxic conditions.

So far, interesting insights into *lncRNA-LET* function were provided in HCC and gallbladder cancer cells. With respect to lung cancer research, it would be interesting to illuminate the prognostic and therapeutic potential in lung SCC, where *lncRNA-LET* was already described to be deregulated. In addition, it would be worth to investigate *lncRNA-LET* function in lung cancer on the basis of the presented studies.

2.3.6 BC200

BC200 (brain cytoplasmic 200 nt) is a 200-nt brain-specific, cytoplasmic lncRNA with sequence homology to the left monomer of Alu repeats and a special tripartite domain structure, similar to rodent *BC1* RNA (Sutcliffe et al. 1984). *BC200* is transcribed by polymerase III and was identified to exhibit a strong and selective expression pattern in primate neural tissue, but not in other normal tissues (Watson and Sutcliffe 1987; Tiedge et al. 1993; Martignetti and Brosius 1993). The investigation of *BC200* expression in different human tumor tissues by Northern Blot revealed, among others, a detectable signal in five out of eleven lung SCC tissues and no signal in both examined lung ADC tissue samples (Chen et al. 1997). Until now, no further studies followed up on *BC200* deregulation in lung cancer. The first step here would be to estimate the extent of BC200 deregulation in lung cancer by a large-scale analysis in patient samples compared to non-tumor samples.

In fact, research on *BC200* in human cancers is scarce. Nonetheless, a study in invasive breast carcinoma revealed the prognostic potential of high *BC200* expression (Iacoangeli et al. 2004) and highlighted the importance of *BC200* outside of neural tissue. Most reports, however, focused on elucidating *BC200* function and *BC200-RNP* composition in primate brain. Here, *BC200* (and rodent *BC1*

RNA) was implied in local control of translation (Kondrashov et al. 2005; Lin et al. 2008) and hence might play a role in synaptic plasticity (Smalheiser 2014).

2.3.7 UCA1, CUDR/UCA1a

UCA1 (urothelial carcinoma associated 1) and *CUDR* (cancer upregulated drug resistant), also named *UCA1a,* are variant transcripts derived from chromosome 19p13 and show a significant overlap in their sequences (Wang et al. 2012b). *UCA1* (1.4 kb) was first discovered as a highly specific and sensitive biomarker for the diagnosis of bladder transitional cell carcinoma (TCC) from urine sediment (Wang et al. 2006). First functional characterizations of *CUDR* (2.2 kb) implied a role in increased resistance of cancer cells to drug-induced apoptosis (Tsang et al. 2007; Wang et al. 2012b).

Both *UCA1* and *CUDR* exhibit tissue specificity and were upregulated in human embryonic tissue and a limited set of normal tissues (Wang et al. 2008; Tsang et al. 2007), but also in cancer cell lines from different origin and cancerous patient tissue (Wang et al. 2006, 2012b; Kaneko et al. 2011; Fang et al. 2014; Tian et al. 2014; Han et al. 2014; Tsang et al. 2007).

Of importance for lung cancer research, *CUDR* was reported to be upregulated in four out of five paired lung cancer patient tissues (Tsang et al. 2007). Results from other cancer models implicated *CUDR* and *UCA1* in enhanced proliferation and metastatic potential of cancer cells. Transcriptional regulation of *UCA1* in bladder cancer cells might occur via ETS-2, C/EBPα, or HIF-1α (Wu et al. 2013a; Xue et al. 2014a, b). More detailed analysis of *UCA1* function suggested that *UCA1* might regulate bladder cancer cell proliferation by inhibiting BRG1 (brahma-related gene 1) functions (Wang et al. 2014b).

In summary, *UCA1* and *CUDR* seem to modulate crucial pathways, mainly in bladder cancer. No studies on *UCA1* or *CUDR* were conducted in lung cancer cells, and the only patient sample set that was used for analysis of *CUDR* deregulation in lung cancer tissue was very limited in size. Hence, research concerning these lncRNAs in lung cancer is in very early stages, and it remains to be elucidated what role they play in lung tumorigenesis.

3 Long Noncoding RNAs—Prospects for Novel Cancer Diagnostics and Therapeutics

Lung cancer is a heterogeneous disease with a low 5-year overall survival rate, mainly owing to the late stage of diagnosis and fast onset of chemoresistance. There is an urgent need for new prognostic and predictive biomarkers, as well as alternative therapeutic options. New biomarkers should ideally be of high sensitivity and specificity, stable, easy, and fast to detect, and easily available with noninvasive

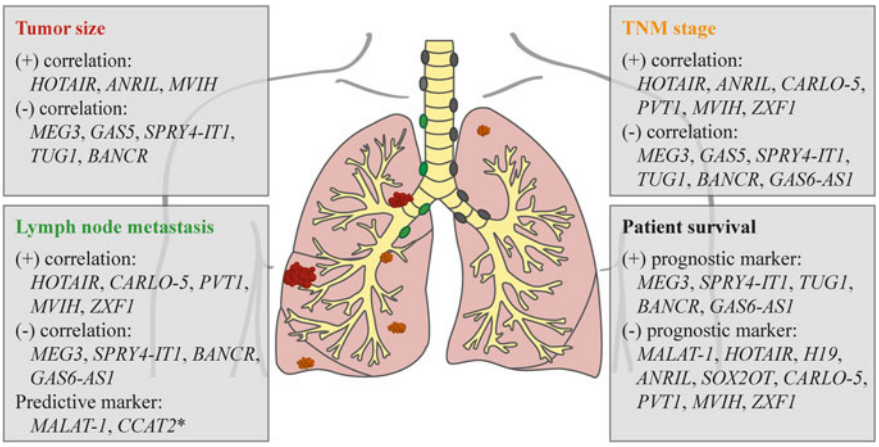

Fig. 1 Correlation between lncRNA expression and clinicopathological parameters in NSCLC. LncRNA expression was associated positively (+) or negatively (−) with tumor size, lymph node metastases, and staging and was either a positive (+) or negative (−) prognostic marker for patient survival. LncRNAs for which no information was available or that did not correlate with presented parameters are not included. * only in combination with CEA levels

methods from blood, plasma, urine, or sputum. Most of the lung cancer cases are associated with long-term exposure to tobacco smoke. Hence, there is a special interest in the early detection of tumorigenic processes, especially in high-risk groups.

LncRNAs are a new class of functional RNA molecules with many advantageous features with respect to cancer detection and therapy. Their expression can be very tissue specific and is deregulated in a variety of cancers of different origin. In the previous chapter, lncRNA candidates implicated in lung tumorigenesis were reviewed. Many of these lncRNAs hold biomarker potential (Fig. 1). Furthermore, lncRNAs could be used in a combinatorial approach to establish reliable prognostic gene signatures and more accurately predict the patients' benefit from the available therapeutic options.

Up to now, most studies examined lncRNAs from patient tissues that were removed by invasive methods. However, new hope was raised by the identification of circulating lncRNAs that are easily available from body fluids (Tinzl et al. 2004; Wang et al. 2006; Panzitt et al. 2007; Reis and Verjovski-Almeida 2012). Circulating RNAs are thought to be released as a result of tumor cell secretion processes, also including apoptosis and necrosis, and are protected from degradation due to packaging into small, membrane-covered vesicles such as exosomes, microparticles, and apoptotic bodies. Good examples for noninvasive diagnostic markers of prostate cancer and bladder TCC are the lncRNAs *PCA3* (*DD3*) and *UCA1*, respectively. Both allow a highly sensitive and specific diagnosis of respective cancer types from urine samples (Tinzl et al. 2004; Wang et al. 2006).

However, research on circulating lncRNAs is scarce, and initial studies have just started to describe their occurrence and features. Therefore, comparative screening studies are necessary to explore the availability of circulating lncRNAs from body fluids and might open up new possibilities for early detection of cancer. An important future task is attributed to the standardization of lncRNA detection techniques. In combination with standardized sample taking and processing, a direct comparison of results between different studies would be possible.

The clinical value of lncRNAs is not only limited to their biomarker potential, but reaches beyond into the field of therapeutic targets. LncRNAs are key regulators of many physiological and pathological processes; they are functional as scaffolds, decoys, guides, and enhancers and are versatile gene regulators on epigenetic, transcriptional, and posttranscriptional levels. Development of novel lncRNA-targeting therapies is currently hampered by a general lack or insufficient mechanistic insights into lncRNA function. Elucidating the mechanism of action, however, is a crucial step toward the design of anticancer therapeutics. High-throughput loss-of-function screens will be essential to establish the functional significance of the numerous lncRNAs that are deregulated in lung cancer.

Putative lncRNA-targeting approaches will highly depend on lncRNA function and might be based on (i) siRNAs, shRNAs, or siPOOLs, (ii) antisense oligonucleotides (ASO) or GapmeRs, (iii) ribozymes or deoxyribozymes, (iv) aptamers, (v) lncRNA mimics, or (vi) small molecules (Li and Chen 2013; Ling et al. 2013a; Wahlestedt 2013; Hannus et al. 2014). As a first example of therapeutic targeting of a lncRNA in lung cancer, the reduction of *MALAT1* levels by ASOs efficiently reduced lung cancer metastasis in a mouse model (Gutschner et al. 2013b). Consequences of *GAS5* glucocorticoid receptor decoy function could be reversed by overexpression of *GAS5* GR-binding mutants in a cell line model (Kino et al. 2010).

Another challenge is presented by the delivery of lncRNA-targeting therapeutics to respective tumor sites. Current approaches to deliver miRNA and siRNA therapeutics are based on nanoparticles or lipids and were suitable to counteract tumor growth in lung cancer mouse models (Chen et al. 2010; Wu et al. 2011, 2013b; Trang et al. 2011). Recently, a delivery system for antisense oligomers was presented that specifically targets the acidic tumor microenvironment (Cheng et al. 2014a). Despite initial success, solving the challenges of stability, immunogenicity, bioavailability, and delivery of lncRNA therapeutics to their target tissue will be another tremendous task for the future.

4 Concluding Remarks

A worldwide effort on genome-wide sequencing and reliable gene annotation in publicly available databases has profoundly changed our view on the complexity of the human genome and its derived transcripts. Fundamental cellular processes can be regulated by lncRNAs, a novel and highly diverse class of functional RNA

molecules. LncRNAs might act simultaneously as biomarkers and therapeutic targets, opening up a new world of opportunities for cancer diagnosis and treatment. In lung cancer, there is a compelling need for early diagnosis and alternative treatment options to improve patient survival. There is increasing evidence for lncRNA deregulation, involvement in pro- and antitumorigenic processes, and biomarker function. Only few lncRNAs have been thoroughly characterized; for most presented lncRNAs, the mechanism of action remains elusive. However, this would be a key point toward the design of novel targeted therapies. As a conclusion, additional in-depth functional studies on lncRNAs are urgently required to allow their placement into the context of pathologic processes and to assess their suitability for lung cancer treatment. Despite some discrepancies and open questions, lncRNA research is progressing fast and offers encouraging future perspectives for diagnosis and therapeutic intervention against cancerous diseases.

Disclosure

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Pseudogene-Expressed RNAs: Emerging Roles in Gene Regulation and Disease

Dan Grandér and Per Johnsson

Abstract Pseudogenes have for long been considered as non-functional relics littering the human genome. Only now, it is becoming apparent that many pseudogenes are transcribed into long noncoding RNAs, some with proven biological functions. Here, we review the current knowledge of pseudogenes and their widespread functional properties with an emphasis on pseudogenes that have been functionally investigated in greater detail. Pseudogenes are emerging as a novel class of long noncoding RNAs functioning, for example, through microRNA sponging and chromatin remodeling. The examples discussed herein underline that pseudogene-encoded RNAs are important regulatory molecules involved in diseases such as cancer.

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1 Introduction

Tens of thousands of long noncoding RNAs (lncRNA) have been identified, and some are now emerging as important regulatory molecules (Carninci et al. 2005; Djebali et al. 2012; Morris and Mattick 2014). Although the vast majority of these transcripts still remain to be functionally investigated, it now stands clear that lncRNAs are involved in a large variety of processes including, but not limited to, epigenetic remodeling (Gupta et al. 2010), imprinting (Latos et al. 2012), chromatin structure (Hacisuleyman et al. 2014), RNA stability (Gong and Maquat 2011), and microRNA sponging (Poliseno et al. 2010). Based on genomic organization and relation to coding genes, lncRNAs are divided into several subclasses such as long intergenic noncoding RNAs (lincRNAs), antisense RNAs (asRNA), sense overlapping RNAs, and sense intronic RNAs. In addition to these main categories, numerous other subclasses, such as transcribed ultra-conserved regions (Mestdagh et al. 2010), enhancer RNAs (Kim et al. 2010), and transcribed pseudogenes (Pei et al. 2012), have been proposed. However, as far as we know today, there is no evidence of difference in function among various classes of lncRNAs.

Repetitive elements comprise large parts of mammalian genomes (Levy et al. 2007; Margulies et al. 2005). Already in 1950, pioneering work by Barbara McClintock suggested the presence of mobile genetic elements when her studies on maize revealed the existence of transposable elements (McClintock 1950). A few decades later, a similar moving element was discovered when a truncated version of the 5S DNA, that still contained homology with the native gene, was reported and named a pseudogene (Jacq et al. 1977). Pseudogenes are the result of duplicated genes, which have lost their protein-coding capacity through molecular events such as point or frameshift mutations (Fig. 1). Pseudogenes were originally considered mainly as 'junk DNA,' and their function has until recently been largely unknown. Since its original discovery in 1977, approximately 14,000 pseudogenes have been reported in the human genome arising from several regulatory protein-encoding genes well known to be involved in diseases, such as p53 (Zakut-Houri et al. 1983), OCT4 (Takeda et al. 1992), PTEN (Dahia et al. 1998), and BRCA1 (Puget et al. 2002). However, functional investigations of most pseudogenes have remained limited, but the recent development of genomewide platforms has led to the discovery that many pseudogenes are transcriptionally active (Pei et al. 2012; Kalyana-Sundaram et al. 2012). Previously underappreciated functions for this group of genes are now emerging, and a few pioneering studies suggest pseudogenes to maintain critical functions in gene regulation.

Here, we review the current work on pseudogene-expressed lncRNAs and their involvement in gene regulation and human disease. We also highlight areas that have not yet been well investigated and hope this will motivate additional research and increase our understanding of the regulatory functions of pseudogenes.

2 Pseudogenes

Pseudogenes are abundant in the human genome (Pei et al. 2012), but only a subset of these has been reported to be transcriptionally active (Pei et al. 2012; Kalyana-Sundaram et al. 2012; Frith et al. 2006). Moreover, only a handful of those transcripts have been assigned any function. While some protein-coding genes such as *PTEN* have a single pseudogene (Dahia et al. 1998), other highly expressed genes are remarkably rich in pseudogenes and may have over 100 distinct pseudogenes (Pei et al. 2012). This is exemplified by the ribosomal protein L121 which is reported to have 143 pseudogenes (Balasubramanian et al. 2009) and GAPDH that has 68 pseudogenes (Liu et al. 2009).

Based on how the pseudogene was generated from its ancestral gene, pseudogenes are divided into three main categories: (1) processed pseudogenes, (2) unprocessed pseudogenes, and (3) unitary pseudogenes.

1. Processed pseudogenes reflect messenger RNAs (mRNAs) that have been spliced, reverse transcribed, and integrated into a new genomic location. As a consequence of this process, introns as well as other regulatory elements such as enhancers and promoter elements have normally been lost during the process of pseudogenization (Fig. 1a).
2. Unprocessed pseudogenes are the result of duplicated genes. These pseudogenes maintain intronic as well promoter elements (Fig. 1b). Unprocessed pseudogenes may also be the result of partial duplications, meaning that some genomic regions of the parental gene may be missing.

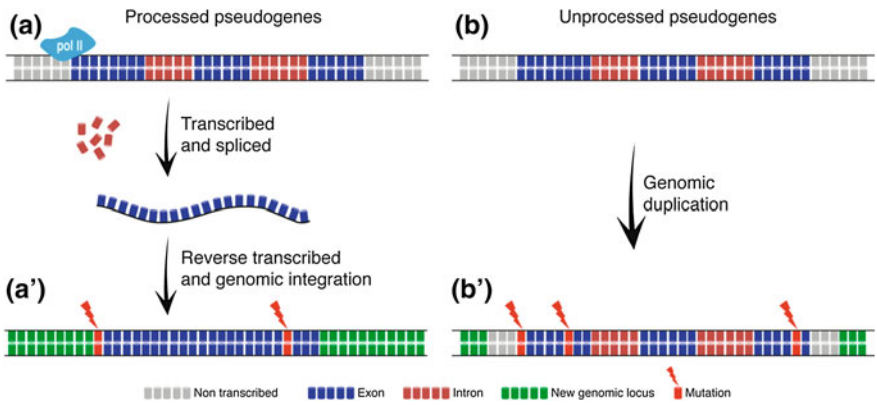

Fig. 1 The process of pseudogenization. **a** A protein-coding gene is transcribed and spliced into mRNA. The mature mRNA is reverse transcribed and **a'** integrated into the genome at a new genomic locus. The protein-coding capacity is lost through various mutations. **b** A protein-coding gene is duplicated into a new genomic locus together with its introns and promoter elements. **b'** The protein-coding capacity is lost through various mutations

3. Unitary pseudogenes refer to previously active genes, which have become inactive through various mutations. Thus, the process of inactivation is similar as for processed and unprocessed pseudogenes, but there is no prerequisite for the duplication process to take place.

The large majority of the ~14,000 human pseudogenes (10,532) represent processed pseudogenes, while 2945 unprocessed and 163 unitary pseudogenes have been identified (Pei et al. 2012). Although the processed pseudogenes are clearly more numerous than the unprocessed pseudogenes, this is not reflected with regard to the number of transcriptionally active pseudogenes. The ENCODE consortium has reported 876 processed pseudogenes and 531 unprocessed pseudogenes to be transcriptionally active (Pei et al. 2012). This is likely due to the fact that unprocessed pseudogenes are normally duplicated together with the ancestral promoter region, while the expression of processed pseudogenes is highly dependent on whether it is integrated into a transcriptionally active or inactive genomic locus. The unitary pseudogenes only constitute a small fraction of all pseudogenes, but one of them, the *XIST/Xist* gene, has been widely investigated for its involvement in dosage compensation and X-chromosome inactivation (Brown et al. 1992; Brockdorff et al. 1992; Duret et al. 2006; Penny et al. 1996).

The excess of processed pseudogenes in the human lineage is considered to be the result of a burst in retrotranspositional activity in ancestral primates taking place about 40 million years ago (Ohshima et al. 2003; Zhang et al. 2004). Interestingly, a similar event took place in the mouse lineage about 36 million years ago (Liu et al. 2009). Since the common ancestor of human and mouse diverged about 70 million years ago, most processed pseudogenes are distinct between the two species and largely lineage specific. Although the group of pseudogenes is overall divergent and poorly conserved among species, some show traces of evolutionary constraints, possibly suggesting functional importance (Pei et al. 2012).

2.1 Expression of Pseudogenes in Cancer Tissues

Characterization and functional investigation of pseudogenes is often challenging due to close sequence similarities with their ancestral coding genes. In addition, the presence of numerous pseudogenes for a single protein-coding parental gene may complicate such investigations even further. Kalyana-Sundaram and coworkers (2012) undertook this challenge and developed a pipeline for the identification of transcribed pseudogenes in cancer samples. Using a total of 293 samples from 13 different tissue types including 248 cancer and 45 samples from normal tissues, 2082 pseudogenes were found transcribed from a total of 1437 different parental genes, implying that some protein-coding genes have multiple, actively transcribed, pseudogenes. While some pseudogenes were ubiquitously expressed in most

samples investigated, others were highly tissue specific. Noteworthy, an interesting subset of 248 pseudogenes showed specific expression in cancer cells. In particular, the unprocessed pseudogene ATP8A2-ψ was studied in more detail in breast cancer. The *ATP8A2* gene has been related to stress response and proliferation (Khoo et al. 1997), and its pseudogene was found highly expressed in breast cancer tumors with luminal histology, while tumors with a basal histology presented very low expression (Kalyana-Sundaram et al. 2012). Interestingly, siRNA-induced knockdown of ATP8A2-ψ inhibited proliferation and reduced cell migration as well as invasion by a yet-uncharacterized molecular mechanism.

2.2 The *OCT4* Pseudogenes

OCT4 (POU5F1) is a transcription factor with a critical role in maintaining pluripotency and self-renewal (Niwa et al. 2000; Pesce and Scholer 2001). The presence of the first *OCT4*-related pseudogene was reported already in 1992 (Takeda et al. 1992) and has since then been expanded to a total number of six OCT4 pseudogenes (Pain et al. 2005). Transcriptional investigations have been undertaken, and several of them are reported to be transcribed (Suo et al. 2005; Zhao et al. 2011).

Initially, Kastler et al. (2010) reported high expression of OCT4pg1 in prostatic carcinoma. Similarly, OCT4pg1 was also found amplified and overexpressed in gastric cancer, an event which was correlated with an aggressive phenotype and poor survival (Hayashi et al. 2013). Importantly, knockdown of OCT4pg1 confirmed a role of this pseudogene in promoting tumor growth, while its overexpression had anti-apoptotic effects and triggered the expression of several growth factors (Hayashi et al. 2013).

In addition to OCT4pg1, OCT4pg4 has also been related to carcinogenesis (Wang et al. 2013). Survival analysis showed that high expression of OCT4pg4 correlated with poor prognosis in patients with hepatocellular carcinoma (Wang et al. 2013). Molecular characterization suggested that *OCT4* and OCT4pg4 share a miRNA-binding site for miRNA-145 (Xu et al. 2009). Increased expression of OCT4pg4 consequently released *OCT4* from miRNA-145-mediated suppression, thereby increasing the expression of *OCT4* and promoting growth and tumorigenicity (Fig. 2a) (Wang et al. 2013).

In contrast to OCT4pg1 and OCT4pg4, OCT4pg5 was found to encode an asRNA that acts as a negative regulator of *OCT4* expression (Hawkins and Morris 2010). The OCT4pg5 asRNA recruits the histone methyltransferase EZH2 to the *OCT4* promoter. EZH2 catalyzes trimethylation of histone 3 Lys27 (H3K27me3), and transcription of *OCT4* is consequently suppressed by the action of OCT4pg5 asRNA (Fig. 2b). Intriguingly, OCT4pg5 asRNA was also found to epigenetically modify the expression of OCT4pg1 and a complex interplay between the numerous pseudogenes and the *OCT4* gene was therefore suggested.

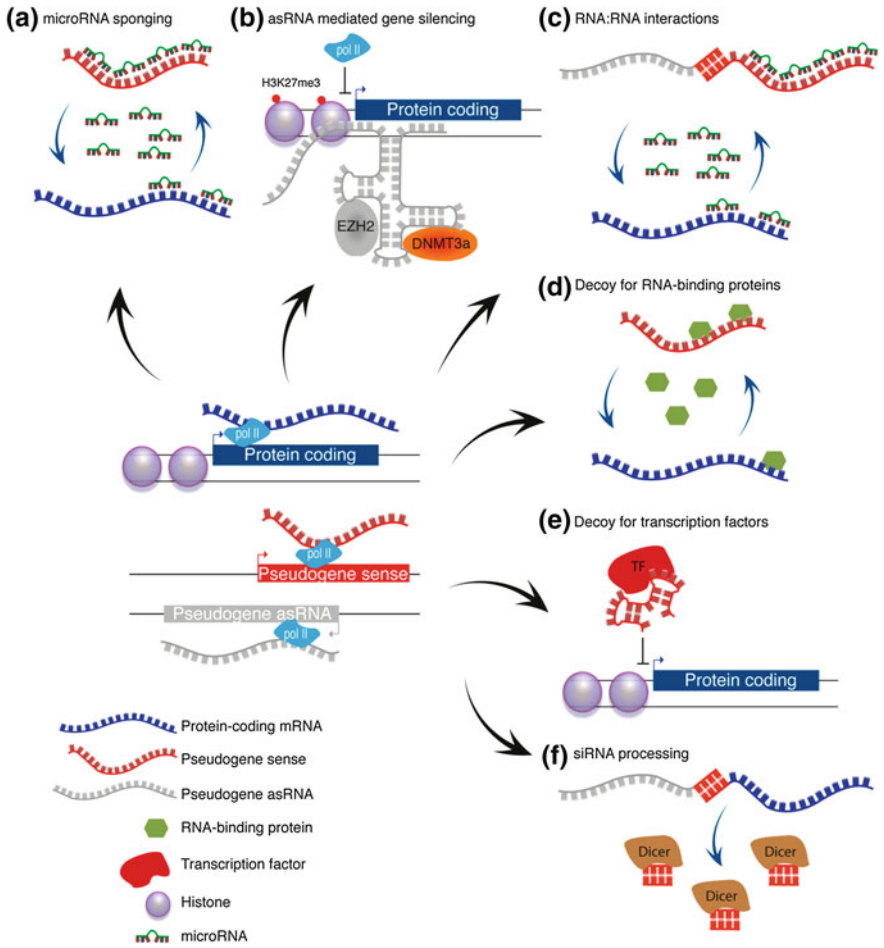

Fig. 2 Various mechanisms of pseudogene-mediated regulation. **a** The pseudogene sense acts as a microRNA sponge (Balasubramanian et al. 2009; Liu et al. 2009; Pain et al. 2005). **b** The pseudogene asRNA recruits chromatin-remodeling factors to its parental gene (Hawkins and Morris 2010; Johnsson et al. 2013). **c** The pseudogene sense transcript is stabilized through RNA–RNA interactions with its corresponding asRNA transcript, consequently affecting miRNA sponging (Johnsson et al. 2013). **d** The pseudogene sense acts as a decoy for RNA-binding proteins (Chiefari et al. 2010). **e** The pseudogene transcript associates with DNA-binding transcription factors, thereby interrupting the DNA binding capacity (Rapicavoli et al. 2013). **f** RNA–RNA interactions are formed between the protein-coding mRNA and the pseudogene asRNA. Dicer processes these dsRNA structures into siRNAs (Watanabe et al. 2008; Tam et al. 2008)

OCT4 is normally predominantly expressed in embryonic stem cells, but its expression has also been reported in differentiated cells such as peripheral blood mononuclear cells (Zangrossi et al. 2007; Tai et al. 2005). Concerns that pseudogene-expressed transcripts could interfere with the interpretation of *OCT4* expression have

been raised, and as a result, primer sets which exclusively amplify the protein-coding transcripts have been designed and evaluated (Liedtke et al. 2007). This highlights that protein-coding genes and their corresponding pseudogene(s) should preferentially be investigated in parallel in order to avoid unspecific artifacts from the corresponding pseudogene and vice versa.

2.3 The PTEN Pseudogene

The PTEN pseudogene, PTENpg1 (also called PTENp1, PTENΨ), is a processed pseudogene highly homologous to the tumor suppressor gene *PTEN*. In contrast to the numerous *OCT4* pseudogenes, only one pseudogene has been reported for *PTEN* (Dahia et al. 1998). The protein-coding *PTEN* is regulated by numerous miRNAs, and in a study by Poliseno et al. (2010), it was found that PTENpg1 retained the same miRNA biding sites as *PTEN*. PTENpg1 was found to possess a regulatory function through its ability to compete for these miRNAs, thus acting as a decoy for *PTEN*-related miRNAs (Fig. 2a). Reduced expression of PTENpg1 released these miRNAs, which instead targeted *PTEN*, thus causing reduced PTEN protein expression (Poliseno et al. 2010). In a subsequent study, partial deletion of the PTENpg1 locus was reported in human melanoma (Poliseno et al. 2011). Unexpectedly, *PTEN* was found codeleted (partial as well as complete) in the majority of melanoma samples, possibly suggesting a role for PTENpg1 beyond acting as decoy for *PTEN*-related miRNAs.

The regulatory function of PTENpg1-expressed transcripts was found to be more complex than originally anticipated when a study by Johnsson et al. (2013) reported that PTENpg1 also encodes two asRNA isoforms: PTENpg1 asRNA alpha and beta. The alpha isoform shares the greatest sequence overlap with *PTEN* and acts as a negative regulator for transcription of *PTEN*. Through sequence homology, PTENpg1 asRNA alpha recruits the DNA methyl transferase DNMT3a and EZH2 to the *PTEN* promoter, whereby transcription is suppressed through the formation of H3K27me3 (Fig. 2b). In contrast, the beta isoform lacks the capacity to act on the *PTEN* promoter. Instead, the PTENpg1 asRNA beta forms RNA–RNA interactions with the PTENpg1 sense transcript. This RNA–RNA interaction stabilizes PTENpg1 sense, consequently affecting its ability to sponge *PTEN*-related miRNAs (Fig. 2c). Taken together, the PTEN–PTENpg1 pathway acts as a network where transcription as well as translation is controlled by the action of several long and short ncRNAs (Johnsson et al. 2013). Although the involvement of PTENpg1 asRNA(s) has not yet been investigated in human disease, subtle variations of *PTEN* are known to associate with cancer (Alimonti et al. 2010) and it is reasonable to envision that both transcriptional and translational regulation are required for maintaining proper expression of *PTEN*.

2.4 The HMGA1 Pseudogenes

The HMGA1 protein belongs to the high-mobility group of proteins, which can act as both negative and positive regulators of transcription [reviewed in (Fusco and Fedele 2007)]. Although HMGA1 does not directly have transcriptional activity, the protein binds to the minor groove of AT-rich DNA sequences where it interacts with the transcriptional machinery, thereby altering the chromatin structure and transcriptional activity (Thanos and Maniatis 1992; Grosschedl et al. 1994; Reeves and Nissen 1990). The protein is expressed at low levels in normal cells, but overexpression has been reported in transformed cells (Pierantoni et al. 2003; Chieffi et al. 2002; Chiappetta et al. 1996; Giancotti et al. 1985) and correlates with a malignant phenotype and poor prognosis (Fusco and Fedele 2007; Pegoraro et al. 2013; Chiappetta et al. 1995). The oncogenic properties of the high-mobility group of proteins include several different mechanisms, for example, induction of cyclin A (Tessari et al. 2003) and repression of p53-induced apoptosis (Pierantoni et al. 2001, 2007; Frasca et al. 2006).

Esposito and coworkers (2014) identified seven pseudogenes for the *HMGA1* gene. Two of these, HMGA1p6 and HMGA1p7, presented high sequence overlap and conserved miRNA-binding sites with the parental gene. Induced expression of HMGA1p6 and HMGA1p7 increased proliferation and cell migration, while knockdown induced apoptosis. Mechanistically, it was found that HMGA1p6 and HMGA1p7 function as miRNA sponges, thus regulating the expression of *HMGA1* (Fig. 2a). Transgenic mice were engineered to overexpress HMGA1p7, and mouse embryonic fibroblasts (MEFs) from these transgenic mice presented an increased growth rate when contrasted to wild-type MEFs (Esposito et al. 2014).

In addition to carcinogenesis, HMGA1 has also been associated with insulin resistance and type 2 diabetes. The insulin receptor (IR) is important for a proper response to insulin, and lack of expression impairs the cellular response to insulin (Taylor et al. 1992, 1994). HMGA1 promotes expression of the IR (Brunetti et al. 2001), and reduced expression of HMGA1 is a risk factor for type 2 diabetes (Foti et al. 2005). The underlying mechanisms disrupting the expression of HMGA1 were until recently largely unknown, but involvement of HMGA1p7 has been suggested (Chiefari et al. 2010). In contrast to acting as a miRNA sponge, HMGA1p7 may also act as a decoy for the protein αCP1. Under normal conditions, αCP1 stabilizes the expression of *HMGA1* mRNA by interacting with a C-rich element in its 3'UTR. Upon increased expression of HMGA1p7, αCP1 translocates to the pseudogene whereby the stability of *HMGA1* mRNA decreases (Fig. 2d). The findings presented by Chiefari et al. (2010) suggest a mechanism where pseudogenes may act as decoys for proteins in addition to microRNAs.

2.5 Lethe Regulates Pro-inflammatory Signaling

Pseudogene-encoded lncRNAs have also been reported to be involved in inflammatory signaling. Pro-inflammatory signaling by stimulation with TNFα was found to regulate the expression of hundreds of lncRNAs, including 54 pseudogenes (Rapicavoli et al. 2013). The ribosomal protein Rps15a has several annotated pseudogenes, and one of them, Rps15a-ps4 (also named Lethe), was found induced upon inflammatory signaling. Lethe is a nuclear localized lncRNA, which interacts with the RelA subunit of NF-KB. This RNA–protein interaction impairs the DNA binding capacity of RelA, consequently interfering with gene activation and acting as a negative regulator of NF-KB signaling (Fig. 2e). While these studies were primarily carried out in MEFs, exogenous expression of mouse Lethe in the human cell line HEK293T was interestingly found to interact with RELA (Rapicavoli et al. 2013).

2.6 Acquired Pseudogenes During Cancer Development

Repetitive DNA such as long interspersed elements (LINE) and Alu repeats is among the most common sequences in the human genome (Batzer and Deininger 2002). Approximately 20 % of the human DNA is derived from LINE1 elements, the vast majority (>99 %) considered 'dead' and transcriptionally inactive due to mutations and epigenetic inactivation (Lander et al. 2001; Hata and Sakaki 1997; Phokaew et al. 2008). A functional and mobile LINE1 element contains two open reading frames encoding a reverse transcriptase (Mathias et al. 1991) and an endonuclease (Feng et al. 1996), both needed for retrotransposition (Moran et al. 1996). Interestingly, processed pseudogenes are thought to be by-products of such activity (Esnault et al. 2000). Retrotransposition has recently been reported to occur in somatic cells (Baillie et al. 2011; Coufal et al. 2009; Muotri et al. 2010) and intriguingly also in cancer cells (Cooke et al. 2014).

In a study covering 660 cancer samples, 2.6 % were found to have acquired somatic pseudogenes during carcinogenesis (Cooke et al. 2014). This notion was most prevalent in lung (19 %) and colorectal cancer (18 %) and is remarkable consistent with reports suggesting increased retrotransposition of LINE1 elements in these types of tumors (Solyom et al. 2012; Lee et al. 2012; Iskow et al. 2010). It is therefore possible that the acquired pseudogenes are a consequence of the increased activity of retrotransposition. While many of the somatically acquired pseudogenes are expected to be passenger events during cancer progression, some might indeed prove functionally important, for example, in driving genetic instability of coding genes. Although many pseudogenes were found integrated within introns, others were integrated within 3'UTRs or the first exon of protein-coding genes, therefore potentially influencing expression of the targeted gene. In support of this notion, the PTPN12 pseudogene was found integrated within the promoter and first exon of the tumor suppressor gene *MGA* in the adenocarcinoma cell line NCI-H2009. Noteworthy, this integration disrupted expression of *MGA* (Cooke et al. 2014).

Importantly, in some tumors, it was also found that both the primary and the metastatic sample contained the pseudogene, thus suggesting that the pseudogenization had been taking place already during early cancer development.

2.7 Pseudogene-Encoded asRNAs Are Substrates for siRNAs

The work presented on the PTENpg1 and OCT4pg5 pseudogenes suggests that one function of pseudogene-expressed asRNAs is to act as scaffolds for chromatin-remodeling factors (Hawkins and Morris 2010; Johnsson et al. 2013). In addition, pseudogene asRNAs have also been linked to posttranscriptional regulation through RNA interference (RNAi) (Watanabe et al. 2008; Tam et al. 2008). Two studies focusing on small RNAs in mouse oocytes suggest pseudogene asRNAs to form double-stranded RNA (dsRNA) sense–asRNA structures that serve as Dicer substrates (Fig. 2f). Surprisingly, these dsRNA structures were not formed between the pseudogene sense and asRNA transcripts, but instead between the pseudogene asRNA and the mRNA of its protein-coding parental gene. Although the authors present Dicer-dependent processing of the pseudogene-asRNA-generated siRNAs, the biogenesis and function of such endogenous siRNAs still remain largely unknown (Watanabe et al. 2008; Tam et al. 2008).

Endogenous pseudogene-associated siRNAs have also been proposed in human cells. Mapping of small RNAs from deep sequencing data identified a subset of small RNAs that aligned to pseudogenes (Chan et al. 2013). Although these are candidates for endogenous siRNAs, further validation is needed in order to establish their function.

2.8 Pseudogenes; a Novel Source of Trans-Acting asRNAs?

Thousands of genes have been associated with asRNAs (Chen et al. 2004; Engstrom et al. 2006; Katayama et al. 2005), and important regulatory proteins such as $p15^{INK4B}$ (Yu et al. 2008), p 53 (Mahmoudi et al. 2009) and Zeb2 (Beltran et al. 2008) have been reported to be regulated by such transcripts. Well-studied examples such as the interplay between *Xist* and *Tsix* during X-chromosome inactivation (Lee et al. 1999; Sado et al. 2001), the insulin-like growth factor 2 receptor (*Igf2r*) (Latos et al. 2012; Wutz et al. 1997; Sleutels et al. 2002; Stoger et al. 1993), and HOTAIR (HOX antisense intergenic RNA) (Gupta et al. 2010; Rinn et al. 2007) have served as groundbreaking examples of lncRNA- and asRNA-mediated gene regulation.

The genomic arrangement of sense–asRNA pairs gives the impression that they act primarily on each other, and asRNA-mediated gene regulation has mainly been considered as *cis*-acting mechanisms. However, this may have been an oversimplified model, and a number of recent reports, including pseudogene-expressed

asRNAs, challenge the *cis*-acting mechanism of asRNAs (Johnsson et al. 2014). Although several reports suggest thousands of pseudogenes to be transcriptionally active (Frith et al. 2006; Pei et al. 2012; Kalyana-Sundaram et al. 2012), no such comprehensive data are available for pseudogenes transcribed in the asRNA direction. To date, investigation of such transcripts mainly relies on case-by-case studies.

The first evidence of pseudogene asRNAs was presented in 1992 when Zhou et al. (1992) found the human DNA topoisomerase I pseudogene to be associated with asRNA transcription. A few years later, in 1997, a similar discovery was reported also in mouse for the Fgfr-3 pseudogene (Weil et al. 1997). However, no function was assigned for either of these transcripts, and it was not until investigations of the neuronal nitric oxide synthase gene (nNOS) pseudogene in the mollusk *Lymnaea stagnalis* that it became evident that such transcripts might maintain function (Korneev et al. 1999). Pseudo-nNOS contains a 150 bp long inverted repeat, which was found to form a duplex with nNOS and prevent its translation in a mechanism still not well investigated.

In an initial attempt to identify pseudogene asRNAs, expressed sequence tags (ESTs) were used and indeed, 87 pseudogenes were found to have asRNAs using this approach (Muro and Andrade-Navarro 2010). Similar as for sense transcribed pseudogenes, different regions of the pseudo-asRNAs were noted to harbor various degrees of sequence conservation. The region sharing homology with the parental gene was found to contain the lowest degree of mutations, thus suggesting there is selection for maintaining complementarity between pseudogene asRNAs and their parental genes (Muro and Andrade-Navarro 2010).

3 Conclusions and Future Perspectives

Pseudogenes have for long been considered as non-functional relics littering the genome. Only now, it is becoming clear that many pseudogenes are transcribed (Pei et al. 2012; Kalyana-Sundaram et al. 2012; Frith et al. 2006) and that at least some maintain regulatory functions (Poliseno et al. 2010; Hawkins and Morris 2010; Johnsson et al. 2013). Although more functional investigations are needed in order to better understand their exact role in cellular physiology, a handful of investigations suggest their involvement during pathogenesis of diseases such as cancer (Johnsson et al. 2013; Poliseno et al. 2011). In addition, characterization of pseudogenes is also of great importance due to the high sequence overlap with their parental genes (Liedtke et al. 2007). This overlap may potentially impede functional studies of the parental genes where expression, mutational as well as methylation analysis could be affected by the presence of pseudogenes.

A growing body of evidence suggests thousands of pseudogenes to be transcribed as sense transcripts. Several studies present such transcripts to act as sponges/decoys for miRNAs and proteins, thus suggesting multifaceted networks between miRNAs, lncRNAs, mRNAs, and proteins (Fig. 2). However, similar genomewide studies are currently lacking for the presence of pseudogene asRNAs.

Strand-specific RNA sequencing should be a good starting point and could bring much information regarding the extent of pseudogene asRNAs and asRNA-mediated regulation. To date, only a few intriguing reports reveal the involvement of pseudogene asRNAs in gene regulation and the extent of such regulation remains to be investigated. While asRNA-mediated regulation has mainly been considered to function by *cis*-acting mechanisms, pseudogene asRNAs may expand this model and increase the complexity of sense–asRNA regulation into *trans*-acting mechanisms. Pseudogene asRNAs may, for example, allow for independent transcriptional regulation and may also show greater flexibility to evolve since there are no constraints from the protein-coding sense counterpart.

In general, functional investigations of the great majority of the tens of thousands of annotated lncRNAs have proved challenging and the function of most lncRNAs currently remains unknown. Low expression, tissue specificity, and often poor conservation between species have hampered the interpretation of function. Similar difficulties will be faced also with regard to pseudogene-encoded lncRNAs. The starting point for functional investigations of pseudogenes has often been the parental gene. While this is a valid approach, there are indeed examples illustrating that pseudogenes may have other functions that are not directly associated with their ancestral gene (Rapicavoli et al. 2013). In addition, one of the major challenges within this field will be to face the high sequence overlap with the parental gene and for some, the presence of numerous closely related pseudogenes (Balasubramanian et al. 2009; Liu et al. 2009; Pain et al. 2005).

The work presented and discussed within this review supports pseudogenes to maintain important regulatory functions. Many subgroups of lncRNAs have been proposed over the last $\sim$10 years, and based on the growing evidence presented in this review, pseudogene-expressed RNAs should be considered to be added to the growing list of lncRNAs.

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Functional Long Non-coding RNAs in Vascular Smooth Muscle Cells

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Abstract Increasing evidence shows that long non-coding RNAs (lncRNAs) are not "transcriptional noise" but function in a myriad of biological processes. As such, this rapidly growing class of RNAs is important in both development and disease. Vascular smooth muscle cells are integral cells of the blood vessel wall. They are responsible for relaxation and contraction of the blood vessel and respond to hemodynamic as well as environmental signals to regulate blood pressure. Pathophysiological changes to these cells such as hyperproliferation, hypertrophy, migration, and inflammation contribute to cardiovascular diseases (CVDs) such as restenosis, hypertension, and atherosclerosis. Understanding the molecular mechanisms involved in these pathophysiological changes to VSMCs is paramount to developing therapeutic treatments for various cardiovascular disorders. Recent studies have shown that lncRNAs are key players in the regulation of VSMC functions and phenotype and, perhaps also, in the development of VSMC-related diseases. This chapter describes our current understanding of the functions of lncRNAs in VSMCs. It highlights the emerging role of lncRNAs in VSMC proliferation and apoptosis, their role in contractile and migratory phenotype of VSMCs, and their potential role in VSMC disease states.

Abbreviations

VSMCs	Vascular smooth muscle cells
Ang II	Angiotensin II
lncRNA	Long non-coding RNA
miRNA	microRNA
CVDs	Cardiovascular diseases
HCASMCs	Human coronary artery smooth muscle cells.

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1 Introduction

Vascular smooth muscle cells (VSMCs) are integral components of the blood vessel wall. These highly specialized cells are responsible for contraction and relaxation of the vasculature in response to many signals and cues, including hemodynamic alterations, mechanical injury, growth factors, and ligand–receptor signaling (Owens 1995; Mack 2011; Lacolley et al. 2012). VSMCs are important for maintaining normal blood pressure, vessel integrity and function, and perturbations of their fully differentiated contractile states can contribute to the development and onset of vascular diseases. Specifically, inappropriate VSMC proliferation, cell growth, migration, and inflammatory signaling contribute to cardiovascular diseases (CVDs). For example, hyperproliferation and migration of VSMCs have been shown to lead to lesion formation in restenosis, atherosclerosis, and hypertension (Brasier et al. 2002).

Early work on VSMCs uncovered many protein signaling pathways, including classical G protein-mediated pathways and receptor tyrosine kinases, which regulate the response of VSMCs to environmental cues and growth factors. These include angiotensin II (Ang II) and platelet-derived growth factor (PDGF) (Mehta and Griendling 2007; Berk and Corson 1997). In recent years, it has become clear that non-protein mechanisms and post-transcriptional mechanisms, such as those mediated by small non-coding RNAs called microRNAs (miRNAs) (Maegdefessel et al. 2015) and long non-coding RNAs (lncRNAs), also function in normal and diseased VSMCs (Leung et al. 2013; Bell et al. 2014; Leung and Natarajan 2014). Non-coding RNAs have been at the forefront of research due to increasing evidence of their involvement in several cellular processes, their dysregulation in diseased states, and their potential to be novel therapeutic targets in the treatment of various diseases. In this chapter, we will briefly discuss the known roles of lncRNAs and then cover the recent literature that uncovers functions of lncRNA in VSMCs.

2 Non-coding RNAs

Since the discovery of miRNAs, it has become clear that non-coding RNAs can function in post-transcriptional regulation of gene expression (Arasu et al. 1991; Wightman et al. 1991; Lee et al. 1993; Bartel 2009). miRNAs are 20–25 nucleotide non-coding RNAs which regulate the stability and/or translation of specific target mRNAs based upon sequence specificity with the target 3′ UTR. These small RNAs have been shown to be important for normal development and act as fine-tuners of gene expression. Abnormal levels of miRNAs have been associated with numerous diseases including CVDs (Small and Olson 2011; Kataoka and Wang 2014). In VSMCs, miRNAs are important for many processes and phenotypes. For example, miR-143 and miR-145 have been shown to regulate normal VSMC differentiation and contractility (Cordes et al. 2009). The upregulation of miR-221 and miR-222, which target mRNAs of two key cyclin-dependent kinase inhibitors, p27Kip1 and p57Kip2, results in the migration and proliferation of VSMCs and reduces the expression of contractile genes (Liu et al. 2009).

miRNAs can also function as part of the response to VSMC growth and inflammatory cues such as Ang II signaling. Ang II is a small polypeptide hormone that regulates many processes in the vessel wall including vasoconstriction, inflammation, fibrosis, and cellular states (Mehta and Griendling 2007). Ang II signaling mediated by its type 1 and type 2 receptors results in the activation of signaling cascades which rapidly result in gene expression changes and ultimately physiological, as well as pathophysiological responses in VSMCs, including proliferation, fibrosis, and inflammation (Berk and Corson 1997). Recently, non-coding RNAs have been shown to mediate this Ang II response in VSMCs. Specifically, Ang II signaling induces upregulation of miR-132 and miR-212, which target PTEN (phosphatase and tensin homolog) mRNA (Jin et al. 2012). This repression of PTEN in VSMCs furthermore causes induction of pro-inflammatory monocyte chemoattractant protein-1 (MCP-1). In addition, increase in miR-132 enhances activation of CREB (cyclic AMP-responsive element binding protein) through increased phosphorylation (Jin et al. 2012).

Dysregulation of certain miRNAs in VSMCs can also contribute to increased inflammation related to the development and progression of diabetic vascular complications. Under diabetic conditions, miR-125b upregulates the pro-inflammatory response of VSMCs by targeting and downregulating key repressive histone methyltransferases (Villeneuve et al. 2010). These data also highlight crosstalk between non-coding RNAs and epigenetic mechanisms in chromatin. Similarly, miR-200 family members upregulated in VSMCs of diabetic mice also enhance the expression of inflammatory genes by targeting the E-box repressor Zeb1 to relieve repression (Reddy et al. 2012). Clearly, non-protein-coding RNAs are emerging as important regulators in VSMC functions, and the dysregulation of miRNAs can contribute to VSMC dysfunction leading to development of disease. Since miRNAs are usually highly conserved, are well preserved in biological fluids and formalin-fixed sections, and can be targeted by various antisense strategies, they are

increasingly exploited as novel biomarkers or therapeutic targets for various diseases, including CVDs and diabetic vascular complications (Kato and Natarajan 2014; Kataoka and Wang 2014).

2.1 Long Non-coding RNAs

After the discovery of miRNAs, additional high-throughput sequencing efforts characterized thousands of more non-protein-coding RNAs that are longer than 200 nucleotides and are subsequently classified as lncRNAs. Some members of this class of RNAs are similar to protein-coding RNAs as they are processed by RNA polymerase II, 5′ capped, and can be also 3′ polyadenylated, but lack distinct open reading frames (Cabili et al. 2011; Guttman et al. 2010; Khalil et al. 2009). LncRNAs are generally expressed at much lower levels than protein-coding RNAs (Khalil et al. 2009). In contrast to miRNAs that have a distinct role in targeting mRNAs, members of this class of RNA have many diverse molecular and biological functions (Fig. 1) (Wapinski and Chang 2011; Moran et al. 2012) and are

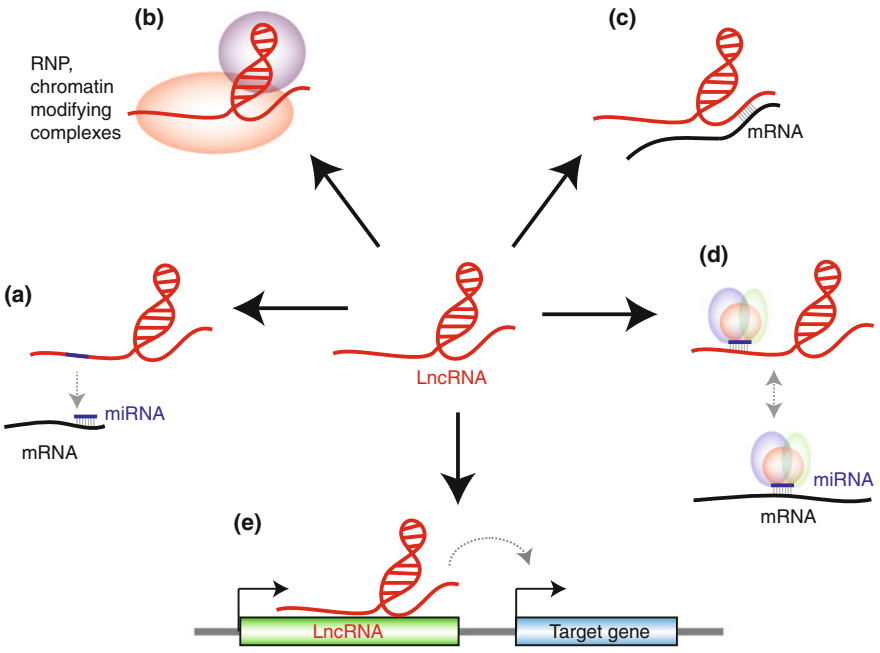

Fig. 1 Molecular roles of lncRNAs. LncRNAs have myriad of molecular functions. Known roles include the following: **a** host transcripts for miRNAs, **b** molecular scaffolds for protein complexes known as ribonucleoproteins and chromatin remodeling complexes, **c** regulators of mRNA stability, **d** competitors of mRNAs targeted by miRNAs, and **e** *cis* regulators of gene expression

important for many if not all aspects of cell biology. The earliest known function of this class of RNAs is in the regulation of transcription of local genes. *Xist*, transcribed on the X chromosome in mouse and humans, functions to regulate the expression of genes on the X chromosomes in the process of X inactivation (Brown et al 1991; Penny et al. 1996). *Xist* RNAs, which are highly expressed from one of the two X chromosome copies, coat the local inactivated X and interact with Polycomb Repressive Complex 2 (PRC2) silencing complex to silence local gene transcription (Froberg et al. 2013; Zhao et al. 2008). Since the characterization of *Xist*, additional lncRNAs have been identified using high-throughput sequencing technologies and were found to be important for the regulation of gene transcription.

The first two lncRNAs found to regulate gene transcription include *p15AS* and *p21 antisense* (Morris et al. 2008; Yu et al. 2008). The former was shown to regulate the transcription of the overlapping *p15* and the latter to regulate the transcription of *p21*, both by suppressing promoter activity. Other lncRNAs have also been found to interact with chromatin modifying complexes. One of these is *HOTAIR* transcript which interacts with both the PRC2 complex and the lysine-specific 1A/REST corepressor/RE1-silencing transcription factor (LSD1/REST/CoREST) (Tsai et al. 2010). Additional lncRNAs, such as *linc-p21*, have also been found to act as scaffolds for other types of proteins including hnRNPs (Huarte et al. 2010). Some lncRNAs were found to regulate local gene regulation through *cis*-acting function. In particular, enhancer-like RNAs were classified as lncRNAs which affect local transcription of nearby genes (Orom et al. 2010). One such RNA, *ncRNA-a7*, regulates a neighboring gene, *Snai1,* which is an important gene in cellular migration and the development of cancer (Orom et al. 2010). Further investigations have also described a set of lncRNAs called enhancer RNAs (eRNAs) which affect many biological processes including macrophage biology, p53-targeted gene expression, and estrogen receptor alpha-targeted gene expression (Melo et al. 2013; Li et al. 2013; Lam et al. 2013). These lncRNAs can interact with a variety of regulators involved in the control of local transcription including chromatin-modifying complexes and transcriptional activators (Fig. 1). One key molecular function of these lncRNAs is interacting and recruiting key protein complexes to local DNA.

It has also been demonstrated that lncRNAs affect gene expression via several post-transcriptional regulatory mechanisms (Fig. 1). They can function as competing RNAs which can deplete miRNAs from their target RNAs. For example, in muscle differentiation *linc-MD1,* RNA competes with two miRNAs, miR-135 and miR-133, which target *MEF2C* and *MAML1* mRNAs, respectively. With the expression of *linc-MD1*, miR-135 and miR-133 are titrated from *MEF2C* and *MAML1* mRNAs and prevented from inducing mRNA degradation (Cesana et al. 2011). Aberrant expression of *linc-MD1* has been found in patients with Duchenne muscular dystrophy, highlighting the importance of lncRNAs in muscular disorders. In addition to modulating levels of miRNAs, lncRNAs can also serve as host genes of miRNAs (Fig. 1). It is estimated that 10 % of lncRNAs host miRNAs (Consortium et al. 2007; Kapranov et al. 2007). Transcription of lncRNAs can thus

directly alter the level of miRNAs. There is also evidence that lncRNAs can directly interact with mRNAs to affect their stabilization. For example, *TINCR* (Terminal differentiation-induced ncRNA) binds to target RNAs through a 25-nucleotide motif sequence and regulates the stability of its targets. Lack of this interaction results in abnormal epidermal differentiation (Kretz et al. 2013). In recent years, lncRNAs have been increasingly implicated in various disease states and hence evaluated as potential therapeutic targets (Kataoka and Wang 2014; Kato and Natarajan 2014).

3 LncRNAs in Vascular Smooth Muscle Cells

The study of lncRNAs in VSMCs has been relatively underexplored compared to other tissues types. Since VSMC growth and differentiation is critical for normal and pathophysiological states of the vessel wall, a study of lncRNAs could shed new insights into their roles in VSMC biology and functions. Here, we describe recent studies which have just begun to uncover the role of lncRNAs in these very important cell types and their potential role in human disease (Fig. 2).

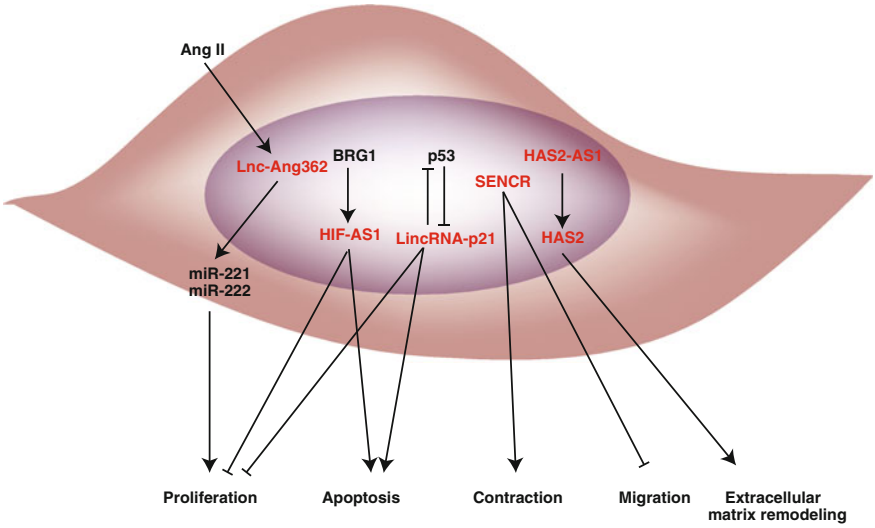

Fig. 2 VSMC processes regulated by LncRNAs. LncRNAs reported to date (in *red*) that function in VSMC proliferation, apoptosis, contraction, and migration. Lnc-Ang362, regulated by Ang II, is the host gene for miR-221 and miR-222, which regulate VSMC proliferation. BRG1 regulates HIF-AS1 inducing apoptosis and reducing cell proliferation. p53 and lincRNA-p21 regulate each other to promote or reduce cell proliferation and apoptosis. SENCR transcripts promote VSMC contraction and reduce VSMC migration. HAS2-AS1 regulates HAS2 transcription to promote extracellular matrix remodeling in VSMCs

3.1 Rats

One of the first studies to investigate lncRNAs in VSMCs described lncRNA mediation of Ang II signaling in rat VSMCs (Leung et al. 2013). In this study, we performed genome-wide chromatin profiling of two post-translational histone modifications associated with active transcription, histone H3 lysine 4 trimethylation (H3K4me3), and histone H3 lysine 36 trimethylation (H3K4me36), along with transcriptome profiling. These parallel experiments allowed us to comprehensively characterize lncRNAs that are expressed in rat VSMCs as well as those that are differentially expressed under Ang II treatment. In total, 466 lncRNAs were found to be expressed in control and/or Ang II-treated VSMCs, and of those, 29 lncRNAs were significantly regulated by Ang II. We further investigated the role of one novel lncRNA, *Lnc-Ang362*, which is located in proximity to miR-221 and miR-222. These two proximal miRNAs are co-expressed and have been shown to be involved in the response of VSMC to Ang II. Two key features led us to hypothesize that this lncRNA and two miRNAs are co-regulated as follows: (1) *Lnc-Ang362* is upregulated in response to Ang II which is concordant with the expression of the two miRNAs and (2) the chromatin profile for this locus indicated one RNA polymerase II initiation site for the lncRNA and the miRNAs (i.e., one H3K4me3-enriched locus at the 5′ end of *Lnc-Ang362* locus and continuous H3K36me3 enrichment across the locus including the *miR-221* and *miR-222* loci). To investigate the potential of these RNAs to be co-regulated, short interfering RNAs (siRNAs) were employed to reduce the levels of *Lnc-Ang362*. In response to the siRNA-mediated reduction of *Lnc-Ang362*, the two miRNAs were downregulated. *Lnc-Ang362* was therefore classified as the host transcript for the two miRNAs, and these investigations uncovered a novel mechanism by which Ang II regulates the expression of these two miRNA. The siRNA-mediated reduction of the *Lnc-Ang362* was also able to reduce VSMC proliferation which is likely to occur through the downstream action of the two miRNAs. As noted earlier, these miRNAs have been shown to be involved in VSMC proliferation and the development of neointimal lesions (Liu et al. 2009).

These data highlight the importance of key lncRNAs in VSMC biology and the influence of lncRNAs in the response of VSMCs to environmental cues. Furthermore, *Lnc-Ang362* is just one of the many lncRNAs that are regulated by Ang II in rat VSMCs (Leung et al. 2013) which indicates that several other unidentified lncRNAs may also be important for Ang II biology, other related growth factor actions, and ultimately the regulation of VSMC functions pertinent to CVD. Unlike miRNAs, lncRNAs display lesser conservation across species. Hence, the lncRNAs expressed in rat VSMCs must be further examined for similar expression profiles and actions in human VSMCs to determine relevance to human CVD.

3.2 Humans

Since the first study with rat VSMCs, a few studies have been performed to investigate the role of lncRNAs in human VSMCs and their potential influence on VSMC differentiation as well as the development of human vascular disease. Initial studies focused on roles of previously identified lncRNAs, including H19 (Han et al. 1996) and ANRIL (Congrains et al. 2012), on human VSMC function and atherosclerosis development. Recently, it was discovered that expression of the non-coding natural antisense transcript for hyaluronan (HA) synthase 2 (HAS2-AS1) in human atherectomy specimens correlates directly with lesion severity (Vigetti et al. 2014). HAS2-AS1 partially overlaps with HAS2 exon 1 and promoter regions, was initially identified in several tumor cell lines (Chao and Spicer 2005), and has previously been shown to stabilize HAS2 mRNA in renal proximal tubular epithelial cells (Michael et al. 2011). HAS2 is responsible for HA synthesis, and previous studies have implicated HA vascular deposition with extracellular matrix remodeling, vessel wall thickening, and neointima formation (Riessen et al. 1996; Chai et al. 2005). Because HAS2 is upregulated by *O*-GlcNAcylation (Vigetti et al. 2012), Vigetti et al. sought to identify a possible role for HAS2-AS1 in CVD. Interestingly, they found that HAS1-AS1 enrichment was required for HAS2 upregulation in human aortic smooth muscle cells upon *O*-GlcNAcylation, but not through mRNA stability as previously identified in other cell types. Rather, induction of *O*-GlcNAcylation caused NFκB-dependent accumulation of HAS-AS1 transcripts, which induced chromatin opening at the promoter of HAS2 allowing increased HAS2 transcription. This novel mechanism of HAS2-AS1 function in VSMCs highlights the varying physiological roles lncRNAs can have are dependent on tissues in which they are expressed.

Only within the past year have researchers forayed into the subject of human VSMC-selective lncRNAs. Bell and colleagues investigated novel lncRNAs with potential functions in human VSMCs (Bell et al. 2014). Using RNA sequencing, they first identified lncRNAs that were enriched in human coronary artery smooth muscle cells (HCASMCs). One of these lncRNAs, a multi-exonic lncRNA named *SENCR*, resides within the first intron of *FL1* in an antisense orientation. There are two distinct isoforms of *SENCR,* with *SENCR_V1* exhibiting much broader expression than *SENCR_V2*. Further analyses using high-resolution RNA FISH revealed that the transcript is cytoplasmic and depicts low levels of expression in human umbilical vein endothelial cells (HUVECs). To investigate the potential function of *SENCR*, Bell and colleagues knocked down the transcript using siRNAs. This knockdown of *SENCR* did not influence the expression of *FL1,* indicating that the lncRNA, unlike *Lnc-Ang362,* does not act in *cis*. To identify the function of *SENCR* in an unbiased manner, the investigators performed RNA sequencing of HCASMCs after *SENCR* knockdown. They discovered that with the reduction in *SENCR* transcripts, many contractile genes, including those associated with regulation of *MYOCD*, an important transcriptional regulator of VSMC contractile gene expression, were downregulated and cell migration genes were

upregulated. Phenotypically, HCASMCs with reduced expression of *SENCR* displayed increase in cell migration in scratch wound and Boyden chamber assays. Therefore, these studies demonstrated that *SENCR* is likely to be involved in maintaining a normal, non-motile contractile phenotype in SMCs, thereby uncovering a novel lncRNA-mediated mechanism in regulating VSMC contractility.

Whereas lncRNAs can mediate normal functions including contractile gene expression in VSMCs, it is also becoming clear that aberrant levels of lncRNAs are associated with aberrant cell growth and disease progression in human cells. Below are recent examples of VSMC-expressed lncRNAs related to human vascular disease.

Apoptosis of VSMCs in the aortic media can lead to thoracic aortic aneurysms (TAA). Wang and colleagues therefore examined the expression of Brahma-related gene 1 (BRG1), a component of the SWI/SNF chromatin remodeling complex and a mediator of apoptosis in VSMC, in aortic specimens from TAA patients and found that BRG1 was expressed at significantly higher levels in TAA specimens compared to control (Wang et al. 2014). Further, overexpression of BRG1 in cultured VSMCs caused an increased rate of apoptosis, higher levels of apoptosis-promoting gene caspase 3, downregulation of anti-apoptotic gene Bcl2, and concomitant decrease in VSMC proliferation. Since BRG1 controls gene expression by altering chromatin remodeling and structure, the researchers investigated the potential that lncRNAs were serving as regulators of chromatin remodeling through BRG1. 95 apoptosis-related lncRNAs were screened for expression changes upon modulation of BRG1 levels. LncRNA *HIF1-AS1* expression was modulated by changes in BRG1 levels. Knockdown of *HIF1A-AS1* in VSMCs caused lower caspase 3 levels and increased Bcl2 expression, as well as increased cell proliferation rate. These data suggest that *HIF1-AS1* may play a role in the pathology of TAA and VSMC dysfunction.

In addition to aneurysms, lncRNAs expressed in VSMCs can function in the development of atherosclerosis. In investigating the role of p53, Wu and colleagues characterized the potential role of a lncRNA named *lincRNA-p21*, a member of the p53 pathway which is known to interact with p53 repressive complex hnRNP-K to cause reduction of many p53 targets (Huarte et al. 2010; Wu et al. 2014). The authors initially examined atherosclerotic plaques from ApoE-/- mice fed a high fat diet and found reduced levels of *lincRNA-p21* transcripts when compared to wild-type mice. Further, inhibition of *lincRNA-p21* transcript expression increased cell proliferation, improved viability, and decreased apoptosis in both RAW264.7 mouse macrophage cell line and human VSMCs (HVSMC). Global gene expression analysis after *lincRNA-p21* transcript knockdown revealed downregulation of many p53 targets, indicating a role for this lncRNA in regulating p53 activity. Indeed, RNA immunopreciptation experiments show a direct interaction between lincRNA-p21 and the p53 antagonist MDM2. Furthermore, p53-specific ChIP-seq revealed lincRNA-p21 negatively affects the recruitment of p53 to its target promoters and enhancers. Interestingly, given that *lincRNA-p21* is a transcriptional target of p53 itself, these data suggest a negative feedback loop in the *lincRNA-p21*/p53 axis. In vivo experiments using the murine carotid artery injury model showed

that injection of *lincRNA-p21* siRNA-expressing vector can cause a dramatic increase in the severity of neointima formation, including intima-media thickness, increased Ki67+ prevalence, and decreased apoptosis. Consistent with in vitro studies, p300/p53 interaction was reduced, while MDM2/p53 interaction increased, causing repression of p53 target genes. The expression of *lincRNA-p21* was found to be reduced by 50 % in human coronary artery tissues collected from patients suffering from coronary artery disease and atherosclerosis demonstrating relevance to human disease (Wu et al. 2014).

4 Conclusions

LncRNAs have been in the forefront of molecular biology in recent years due to their numerous biological functions and potential as novel therapeutic targets. As such, we have begun to understand their molecular functions and how these processes affect the development of disease. VSMCs are important cells that mediate normal vascular processes as well as the development of vascular diseases such as restenosis, hypertension, and atherosclerosis. As highlighted in this review, lncRNAs are novel mediators of normal VSMC processes such as contraction and migration, as well as in VSMC dysfunction in response to pathophysiological stimuli such as Ang II. We are also beginning to learn that abnormal lncRNA expression can be associated with human vascular disease, implying that lncRNAs are perhaps mediating the development and onset of CVDs. There is a flurry of recent reports demonstrating the involvement of several lncRNAs in cardiac hypertrophy, heart failure, and heart functions (Klattenhoff et al. 2013; Grote et al. 2013; Ishii et al. 2006; Han et al. 2014) which are not discussed in this review.

Future investigations into the role of lncRNAs expressed in human diseased vascular tissue biopsies have the potential to illuminate new VSMC-specific, lncRNA-based biomarkers and mechanisms that could someday be translated into new treatment options for CVD. Furthermore, examination of single nucleotide polymorphisms (SNPs) within or near lncRNA genomic sites can potentially provide important genetic information to complement data emanating from genome-wide association studies of human CVDs. This is because such SNPs can alter the biological and epigenetic mechanisms of actions of these non-coding RNAs to influence the expression of disease-related genes.

The study of lncRNAs in VSMCs is still in its infancy, and many questions remain as to the degree by which these transcripts affect VSMC function and whether they can be effective therapeutic targets in the treatment of diseases associated with Ang II or growth factors, VSMC dysfunction, and CVDs. However, with the characterization of additional lncRNAs in VSMCs and those related to CVDs, at the very least, they may be used as biomarkers for clinical diagnosis and prognosis. Recently, there have been approaches to effectively modulate lncRNA levels by various chemical approaches in vitro that can be extrapolated to in vivo models (Kato and Natarajan 2014; Kataoka and Wang 2014). Some antisense

oligonucleotide-based techniques have been able to modulate lncRNA levels in mouse models (Wheeler et al. 2012), pointing toward a potential therapeutic strategy or experimental technique for studying lncRNAs in vivo. Overall, lncRNA research, while still in the early stages, represents a fast moving and novel area of investigation, and future studies will further illuminate our understanding of VSMC biology that could in turn help exploit these intriguing molecules for therapeutic purposes.

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Long Noncoding RNAs as Targets and Regulators of Nuclear Receptors

Charles E. Foulds, Anil K. Panigrahi, Cristian Coarfa, Rainer B. Lanz and Bert W. O'Malley

Abstract Intensive research has been directed at the discovery, biogenesis, and expression patterns of long noncoding RNAs, yet their biochemical functions have remained elusive for the most part. Nuclear receptors that interpret signaling mediated by small molecule hormones play a role in regulating the expression of some long noncoding RNAs. More importantly, these RNAs have also been shown to effect hormone-affected gene transcription regulated by the nuclear receptors. In this chapter, we summarize the current knowledge that has been acquired on hormonal signaling inducing expression of long noncoding RNAs and how they then may act in *trans* or in *cis* to modulate gene transcription. We highlight a few of these noncoding RNA molecules in terms of how they may impact hormone-driven cancers. Future directions critical for moving this field forward are presented, with a clear emphasis on the need for better biochemical approaches to address the mechanism of action of these exciting RNAs.

Contents

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1 Introduction

Approximately, only 1.2 % of the human genome encodes protein (Bejerano et al. 2004), and ~90 % of the mammalian genome may be transcribed (Willingham and Gingeras 2006; Carninci et al. 2005; Kapranov et al. 2007; Amaral et al. 2008). Besides messenger RNAs (mRNAs) encoding proteins that have been the most widely studied, different classes of noncoding RNAs (ncRNAs) are also expressed in mammalian cells (Cech and Steitz 2014; Sharp 2009; Prensner and Chinnaiyan 2011). The most abundant of these are the "housekeeping" ncRNAs (e.g., rRNAs and tRNAs, small nuclear and nucleolar RNAs, and telomerase RNA) that play critical structural/enzymatic roles in translation, splicing, and chromosome end maintenance, respectively. MicroRNAs (miRNAs) and related small RNAs that are roughly 22 nucleotides in length represent a class of ncRNA regulators that generally play a repressive role on targeted mRNA substrates (Bartel 2009). While not as well characterized, it is clear that ncRNAs greater than 200 nucleotides are expressed in mammalian cells and some of these "long noncoding RNAs" or lncRNAs have been shown to have regulatory functions in modifying gene transcription (reviewed in Wilusz et al. 2009; Goodrich and Kugel 2009; Bonasio and Shiekhattar 2014; Cech and Steitz 2014; Yang et al. 2014; Ulitsky and Bartel 2013; Sun and Kraus 2014; Ottaviani et al. 2014; Quinodoz and Guttman 2014; Vance and Ponting 2014). In general, these lncRNAs often resemble mRNAs, except for protein-coding capacity, as they are transcribed by RNA polymerase II (Pol II), spliced, and polyadenylated. The biogenesis of lncRNAs has been extensively reviewed elsewhere (Sun and Kraus 2013, 2014). It should be noted that lncRNAs can be transcribed intergenically [these are called large intergenic ncRNAs or lincRNAs (Ulitsky and Bartel 2013)], within introns of coding genes, antisense to a given coding gene, and also from enhancers [these are termed eRNAs (Lam et al. 2014)]. As cited in the above reviews, lncRNAs are known to play regulatory roles in biological process ranging from imprinting (e.g., H19) and X chromosome

inactivation (e.g., XIST) to regulation of steroid hormone-mediated transcription (e.g., SRA and GAS5)—a topic covered later in this chapter. Importantly, lncRNAs can act either in *cis* to affect neighboring coding genes on the same chromosome (e.g., XIST) or in *trans* to affect coding genes on different chromosomes (e.g., SRA). lncRNAs can exert either coactivator or corepressor functions on gene transcription, as will be shown using SRA RNA as a paradigm.

Nuclear receptors (NRs) constitute a family of transcription factors (TFs) and are grouped together based on their zinc (Zn) finger DNA-binding domains (DBDs). Humans express 48 such NRs, ranging from steroid hormone binders (estrogen receptors (ERs), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR)) to ones binding other ligands such as thyroid hormone (TRs), vitamin D (VDR), retinoic acid (RARs), bile acids (FXRs), oxysterols (LXRs), and fatty acids (PPARs) to ones considered "orphans" as their ligands have yet to be discovered. Steroid hormone receptors homodimerize, whereas the latter class forms heterodimers with retinoid X receptor (RXR) (reviewed extensively in Tsai and O'Malley 1994; Mangelsdorf et al. 1995; Evans and Mangelsdorf 2014). After many years of work from multiple laboratories, a general model for hormone action for gene transcription has emerged (reviewed in McKenna and O'Malley 2002; see Fig. 1). Although largely extranuclear, a small population of the unliganded NR can exist in a complex with corepressors bound to specific DNA sequences called hormone response elements (HREs) in the chromatin (Fig. 1a). Within the steroid receptors, GR, AR, PR, and MR recognize a similar palindromic HRE-containing AGAACA, whereas ER binds a different palindromic AGGTCA sequence as its HRE. After a hormone enters a cell, it binds its respective NR (e.g., estradiol (E2) for ERs), translocates to the nucleus, and exchanges corepressor complexes for coactivator multi-protein complexes that bring enzymatic activity to the gene to allow histone modifications (such as acetylation and methylation), activating phosphorylation events, and to help facilitate Pol II recruitment (reviewed in Lonard and O'Malley 2005, 2006, 2007; Fig. 1b). Some of the ligand-recruited coregulators are actually repressive, such as RIP140 with ERα in breast cancer cells (Foulds et al. 2013). NRs recruit coregulators via two distinct domains called activation functions (AFs) in their *N*- and *C*-termini (Fig. 1c). The *C*-terminal AF2 domain contains the ligand-binding domain (LBD) and recruits many coregulators via hormone binding. The *N*-terminal AF1, on the other hand, provides transcriptional activation that is ligand-independent possibly, by being a target of phosphorylation mediated by kinase signaling (reviewed in Trevino and Weigel 2013).

The focus of this chapter is to highlight published data on a class of lncRNAs whose expression itself is regulated by hormone signaling and those lncRNAs that appear to modulate gene transcription mediated by NRs. We will conclude the chapter by highlighting lncRNAs that may play a role in hormone-associated cancers and with future directions for studying lncRNA/NR cross talk.

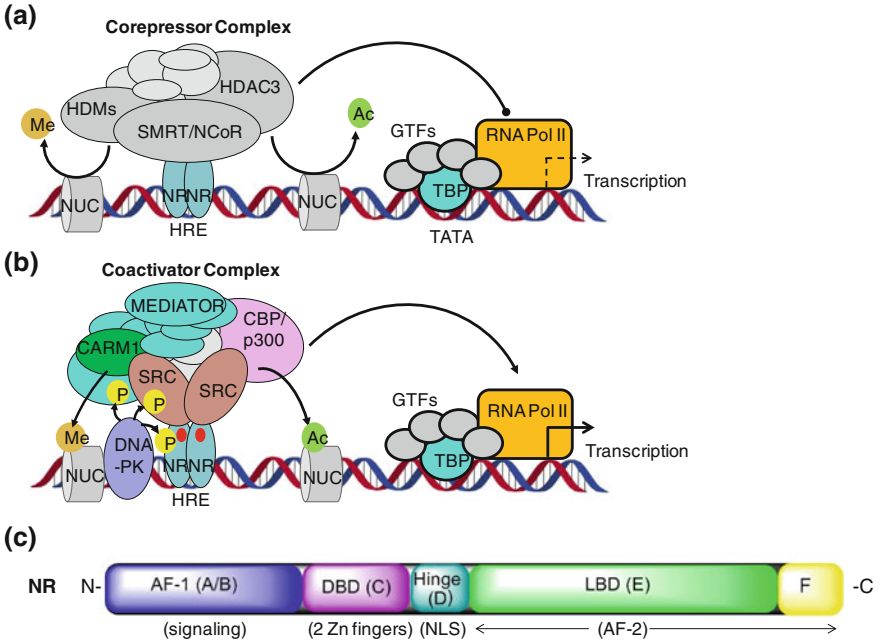

Fig. 1 Nuclear receptors (*NRs*) modulate gene transcription in response to hormonal signaling. **a** In the absence of a hormonal signal entering a cell, a NR dimer binds its specific DNA sequence [called generically a hormone response element (*HRE*)]. The dimer can be a homodimer (e.g., ERα) or a heterodimer with a RXR (e.g., PPAR–RXR). Unliganded NRs recruit multi-protein corepressor complexes containing NCoR/SMRT, HDAC3, and histone demethylases (*HDMs*). HDAC3 removes transcriptionally activating acetylation (*Ac*) from histone tails present in a nucleosome (*NUC*), while HDMs remove methyl groups (*Me*). Collectively, these actions repress RNA pol II-mediated gene transcription from promoter elements such as the TATA box. Note that general TFs are denoted as GTFs, TATA-binding protein is TBP, and the dotted arrow indicates the transcription start site. **b** Once a cell encounters a hormone, it passes through the cell membrane and then is bound by its cognate NR (e.g., ERα binds estradiol) that dimerizes and binds its cognate HRE in the nucleus. Liganded NRs (illustrated with a *red dot*) exchange corepressor complexes for multi-protein coactivator complexes. These complexes contain steroid receptor coactivators (*SRCs*) as the platforms for recruiting other coactivators such as histone acetyltransferases (e.g., CBP/p300), histone methyltransferases (e.g., CARM1), and the Mediator complex. Importantly, CBP/p300 and CARM1 "write" transcriptionally activating "marks" on histone tails (such as H3K27Ac and H3R17Me2a, respectively). Mediator is thought to serve as a bridging factor between the coactivator complex and the RNA pol II transcriptional machinery. Recently, DNA-dependent protein kinase (*DNA-PK*) has been suggested to activate MED1 in the Mediator complex, SRCs, and ERα by phosphorylating particular serine/threonine residues (*P*) in each protein (Foulds et al. 2013). Collectively, these actions facilitate RNA pol II-mediated gene transcription. **c** A generalized schematic of the protein domains present in a NR—A/B, C, D, E, and F. The protein binds specific HRE-containing DNA via its zinc finger-containing DNA-binding domain (DBD, C domain). NRs recruit coactivator proteins via two activation function (*AF*) domains: the ligand-dependent AF-2 containing both the ligand-binding domain (*LBD*, E domain) and another domain F at the extreme *C*-terminus; and the ligand-independent AF-1 that is responsive to kinase signaling pathways. A "Hinge" region (D domain) is located *C*-terminal to the DBD and contains the nuclear localization signal (*NLS*)

2 Long Noncoding RNA (lncRNA) Expression Regulated by Nuclear Receptor Signaling

Since it has been suggested in the prior literature that many lncRNAs may be transcribed by Pol II based on Pol II occupancy at their promoter regions and that the lncRNAs bear 5′- 7-methylguanosine caps and 3′-poly A tails (e.g., Guttman et al. 2009), different laboratories have begun to investigate if any of these RNAs are regulated by NR signaling. Two general approaches have been taken: (1) targeted analysis of a particular lncRNA by reverse transcription qPCR (RT-qPCR) and (2) high-throughput methods, such as microarray profiling and global nuclear run-on sequencing (GRO-seq) developed by Core and colleagues (Core et al. 2008). We compiled existing data in the literature for lncRNAs that were induced or repressed as a function of hormone (estrogen, androgen, progesterone, and corticosteroid) or loss of NR (AR, VDR, or Rev-Erb proteins) (Table 1). As the majority of data exist for lncRNA expression as a function of the steroid hormones, estrogen or androgen (also reviewed in Ottaviani et al. 2014; Sun and Kraus 2013, 2014), we will focus on these data below.

Bona fide lncRNAs such as HOX transcript antisense RNA (HOTAIR), metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), nuclear-enriched abundant transcript 1 (NEAT1), and cDNA clone number 19 isolated from a fetal hepatic library (H19) had their expression affected by E2 administration to ERα-expressing MCF-7 human breast cancer cells and VCaP or LNCaP human prostate cancer cells. In many cases, but not all, the effect of E2 was shown to be dependent on ERs either by performing RNA interference-mediated knockdown of the ER or by adding fulvestrant to cells to enhance ER turnover. Two new lncRNAs, TC0101441 and TC0101686, whose expressions were affected by E2 and ERα in human ovarian cancer cell lines, have been reported (Qiu et al. 2014). After we independently analyzed the GRO-seq data reported by (Hah et al. 2011) in which they treated MCF-7 cells without or with E2 for 40 min, we discovered 965 lincRNAs up-regulated and 743 down-regulated by E2 (p-value < 0.05, fold change exceeding 1.5). We found induction of 11 and repression of five antisense lncRNAs by E2. We also found 12 lncRNAs, which are also present in two human lncRNA databases, LNCipedia (Volders et al. 2013) and lncRNome (Bhartiya et al. 2013), to be affected by E2. The functional significance of these lincRNAs, antisense lncRNAs, and 12 lncRNAs is still uncertain as to whether any of them may affect ERα-mediated transcription. More recently, Sun and colleagues reported that knockdown of two newly identified lncRNAs (lncRNA152 and lncRNA67) reduced expression of a subset of E2-induced genes and proliferation of MCF-7 cells (Sun et al. 2015). It is also clear that E2 treatment of MCF-7 cells induces the expression of many enhancer RNAs (eRNAs) (Hah et al. 2011, 2013; Li et al. 2013). The eRNAs will be further discussed in Sect. 4 of this chapter.

We also report lncRNAs affected by androgen [either the natural hormone dihydrotestosterone (DHT) or synthetic R1881] or loss of AR in Table 1, including *bona fide* lncRNAs such as H19, PCAT18, CTBP1-AS, prostate cancer antigen 3

Table 1 Long noncoding RNAs whose expression is hormonally regulated

lncRNA	Cell Line/organ	Hormone	NR	Effect	Reference
HOTAIR	MCF-7; rat MG	E2, DES	ERα, ERβ	Induction	Bhan et al. (2014)
	MCF-7	E2	ERα, ERβ	Induction	Bhan et al. (2013)
MALAT-1	MCF10a; MCF-7; MDA-MB-231	E2	Not ERα	Repression	Zhao et al. (2014b)
NEAT1	VCaP	E2	ERα	Induction	Chakravarty et al. (2014)
TC0101441; TC0101686	SKOV3; PEO1	E2	ERα	Induction; Repression	Qiu et al. (2014)
lincRNAs	MCF-7	E2	n.d.	Induction (965); Repression (743)	Our analysis of 40 min E2 data published by (Hah et al. 2011)[a]
eRNAs	MCF-7	E2	ERα	Induction	Li et al. (2013) and Hah et al. (2011, 2013)
	LNCaP	DHT	AR	Induction	Wang et al. (2011) and Hsieh et al. (2014)
	Mouse macrophages		Rev-Erbα, Rev-Erbβ	Repression	Lam et al. (2013)
Antisense ncRNAs	MCF-7	E2	n.d.	Induction (11); Repression (5)	Our analysis of 40 min E2 data published by Hah et al. (2011)[a]
12 lncRNAs:	MCF-7	E2	n.d.		Our analysis of 40 min E2 data published by Hah et al. (2011)[a]
BCYRN1				Repression	
DIO3OS				Induction	
DLEU2				Repression	
FKBP1A-SDCBP2				Induction	
FTX				Repression	
HAR1B				Induction	
LOC147976				Repression	
MIR205HG				Repression	
MIR7-3HG				Induction	

(continued)

Table 1 (continued)

lncRNA	Cell Line/organ	Hormone	NR	Effect	Reference
MIRLET7DHG				Repression	
NKAPP1				Repression	
PVT1				Induction	
H19	OVX mice; MCF-7	E2, Corticosteroid, P4	ERα, PR-A	Induction (E2; Cort.) Repression (P4)	Adriaenssens et al. (1999); our analysis of 40 min E2 data published by Hah et al. (2011)[a]
	rat PRO; LNCaP	DHT	n.d.	Repression	Berteaux et al. (2004)
Intronic (39)	LNCaP	R1881	AR demonstrated for asMYO5A	Induction (29); Repression (10)	Louro et al. (2007)
PCA3	LNCaP	DHT	AR	Induction	Ferreira et al. (2012)
PCAT18	LNCaP	DHT	AR indirectly	Induction	Crea et al. (2014)
CTBP1-AS	LNCaP; VCaP	DHT, R1881	AR	Induction	Takayama et al. (2013)
PCGEM1	human PRO; LNCaP	R1881	n.d.	Induction	Srikantan et al. (2000)
PlncRNA-1	Knockdown of AR in LNCaP or LNCaP-AI	None	AR	Repression	Cui et al. (2013)
23 lncRNAs	$VDR^{-/-}$ mouse keratinocytes	None	VDR	Induction (16); Repression (7)	Jiang and Bikle (2014)

Note *MG* mammary gland, *PRO* prostate; *OVX* ovariectomized, *E2* 17β-estradiol; *DES* diethylstilbestrol, *GEN* genistein, *DHT* dihydrotestosterone, *R1881* methyltrienolone, and *P4* progesterone

[a]Our statistical cutoff was p-value < 0.05, fold change > 1.5; *n.d.* not directly determined

(PCA3), prostate cancer gene expression marker 1 (PCGEM1), and PlncRNA-1 (also called CBR3-AS1). PCA3 and PCGEM1 will be further discussed in Sects. 3.3 and 5.2 of this chapter. Androgen induces an antisense transcript of the gene encoding *C*-terminal binding protein 1 (termed CTBP1-AS) (Takayama et al. 2013). CTBP1-AS was shown to repress the level of the CTBP1 transcription by recruitment of the PSF repressor and histone deacetylases. This action, in turn, led to increased transcription of AR target genes, as the CTBP1 protein is a corepressor of AR. Besides AR-activating expression of PlncRNA-1, knockdown of this lncRNA resulted in decreased AR mRNA, suggesting a feedback regulatory loop (Cui et al. 2013). In addition, it has been reported that the expression of 39 intronic lncRNAs was affected by androgen (Louro et al. 2007). It is also clear that DHT treatment of LNCaP cells also induces the expression of many eRNAs (Wang et al. 2011; Hsieh et al. 2014), which we will further discuss in Sect. 4 of this chapter.

3 lncRNAs as Effectors of Nuclear Receptor (NR) Function in *Trans*

3.1 SRA Modulation of NR Transcriptional Activity

The steroid receptor RNA activator (SRA) was cloned in 1999 from a yeast two-hybrid assay initially searching for proteins that bound the AF-1 of human PR (Lanz et al. 1999). It was one of the first regulatory lncRNAs to be described (e.g., only H19 Brannan et al. 1990 and XIST Brown et al. 1991 were reported earlier). However, unlike H19 and XIST that act in *cis* to inactivate neighboring genes at the *Igf2* imprinted locus and on the X chromosome, respectively, SRA acts in *trans* to modulate NR transcriptional activity as described below.

Three different SRA cDNAs were reported by Lanz et al. (1999); these contained stop codons and thus would not be predicted to make any protein. One of them, SRA isoform I, encoded an 875 nucleotide RNA (AF092038 in Genbank). SRA was demonstrated to be a spliced, polyadenylated transcript. Experiments with the protein translation inhibitor cycloheximide confirmed SRA coactivates the synthetic glucocorticoid dexamethasone-bound GR on a HRE reporter gene in transfected HeLa cells. Besides coactivating liganded GR, other steroid receptors displayed SRA coactivation in the presence of their cognate ligand on their respective HRE-containing reporter gene (Fig. 3a). It was additionally revealed that the AF1 function of steroid receptors was coactivated by SRA lncRNA. Biochemical gel filtration experiments showed that SRA RNA existed in distinct ribonucleoprotein complexes and that it coimmunoprecipitated with the NR coactivator, steroid receptor coactivator 1 (SRC-1), in select fractions.

Subsequently, structure–function analyses on the full-length SRA ensued. Figure 2a displays the predicted RNA folding using mfold (Zuker 2003). Using a combination of chemical and RNase digestion probing, the actual secondary structure of the SRA RNA molecule has been solved. SRA has 25 distinct helices

comprising four discrete domains (Novikova et al. 2012). Importantly, stem-loop structures from the mfold predictions were mutated to disrupt the hybrids from forming and then tested for coactivation of PR. It was shown that five distinct stem-loop structures in SRA (termed STR 1, 7, 9, 10, 11) were identified as being important for coactivation (Lanz et al. 2002; see Fig. 2a, b). Subsequently, Zhao and colleagues discovered that STR5 was important for SRA's ability to coactivate mouse RARγ on a reporter and endogenous target gene (Zhao et al. 2004). Importantly, a pseudouridine synthase (Pus 1), an enzyme known to isomerize

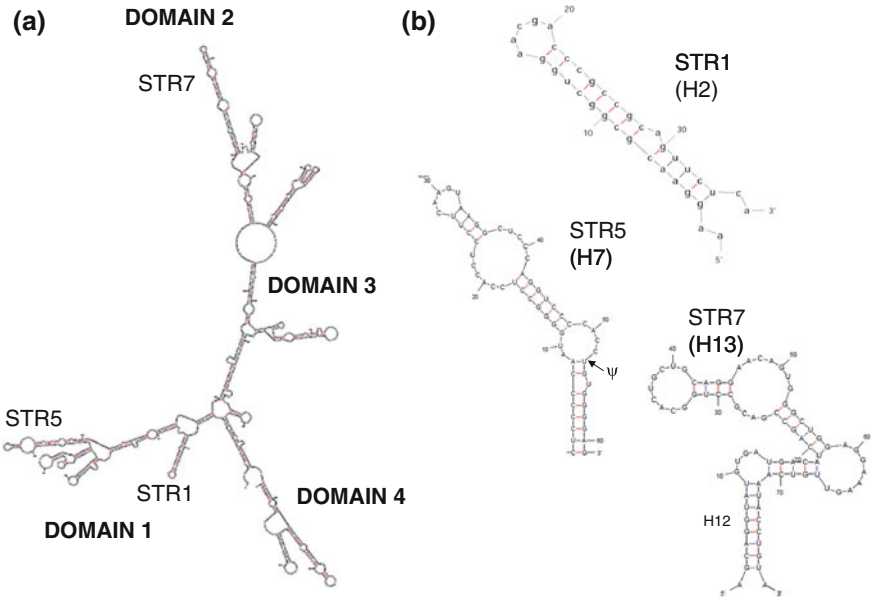

Fig. 2 SRA RNA as a modular functional lncRNA. **a** Predicted RNA fold of full-length 875 nucleotide SRA (AF092038 in Genbank, Lanz et al. 1999) with numbering of predicted stem-loops (STRs) as per (Lanz et al. 2002). STR1 and 7 were shown to be functional by mutagenesis using progesterone receptor (PR)-dependent luciferase reporter assays (Lanz et al. 2002), while STR5 was shown to be functional in reporter assays in another study (Zhao et al. 2007). Predicted secondary structures were performed with the mfold Web server (Zuker 2003). Chemical/enzymatic probing (Novikova et al. 2012) determined the actual four domain SRA RNA structure. STR1 and 5 are within Domain 1, while STR7 residues in Domain 2. **b** Close-up views of three different functional SRA secondary structures. These substructures were recently defined by chemical/enzymatic probing (Novikova et al. 2012), revealing their different stem-loops, and bulges. STR1, 5, and 7 were defined as helices H2, H7, and H13, respectively, according to Novikova et al. (2012). The uridine in STR5 that is modified to pseudouridine in MCF-7 cells (Zhao et al. 2007) is indicated by ψ. These stem-loop RNA structures were drawn using mfold based on the structural data from (Novikova et al. 2012). As SHARP binds to H12-H13 of SRA (Arieti et al. 2014), this helix is also presented

uridine to pseudouridine in tRNAs, was shown to bind the first zinc finger in the DBD of RARγ and posttranscriptionally modify SRA. Pus1, RARγ, and SRA RNA were shown by immunoprecipitation–RT-PCR experiments to form a complex, which is modeled in Fig. 3b. It was later shown that the related Pus enzyme, Pus3, can also pseudouridylate SRA RNA and that at least 7 sites of modification of SRA in MCF-7 cells were detected (Zhao et al. 2007). Importantly, this study also showed that mutation of one of the modification sites (U206; see STR5 in Fig. 2b) to an adenine resulted in a hyper-pseudouridylated and dominant-negative SRA RNA. Zhao et al. (2007) also published that SRA RNA appeared both nuclear and cytoplasmic in MCF-7 cells. Recently, STR1 of SRA has been reported to bind components of the RNA-induced silencing complex (RISC), including Dicer, TRBP, and PACT to promote coactivation of steroid receptors on HRE-containing reporter and endogenous target genes (Redfern et al. 2013; Fig. 3c). This study also raises the intriguing question of whether any interplay between NRs, SRA, and miRNAs may exist.

Originally thought of as only a coactivator, SRA RNA has since been shown to play repressive roles as well. For example, the corepressors SHARP (Shi et al. 2001) and SLIRP (Hatchell et al. 2006) have been shown to use their RNA recognition motifs (RRMs) to directly bind SRA helices H12-H13 (Arieti et al. 2014) and H13 (STR7) (Hatchell et al. 2006), respectively, recruiting HDAC-containing NCoR/SMRT and NuRD corepressor complexes to promoters and also likely sequestering SRA away from SRC-1-containing coactivator complexes (Fig. 3d). These actions lead to repression of HRE-containing genes as listed in the figure. Based on ChIP assays, *pS2* and *MTIIa* promoters are two likely targets for SLIRP repression of SRA coactivation of liganded NRs (Hatchell et al. 2006). Recently, data from Vicent and colleagues suggested that SRA RNA may play a repressive role with unliganded PR by being part of a HP1γ/LSD1 corepressor complex that additionally contains COREST/KDM5B/HDAC1/2 (Vicent et al. 2013; Fig. 3e). This corepressor complex is suggested to be anchored to target genes via HP1γ bound to histone H3 lysine 9 trimethylated (H3 K9me3) "marked" nucleosomes. SRA was shown to directly interact with PR, HP1γ, and LSD1 in this complex, but it is not known which SRA sub-structures are actually important for these binding events. In the presence of progesterone, MSK1 kinase becomes activated and phosphorylates histone H3 serine 10, thereby promoting dismissal of the repressive HP1γ–LSD1 complex (Vicent et al. 2013).

SRA has been shown to coactivate other NRs in *trans*, including TR (Xu and Koenig 2004), VDR (Hatchell et al. 2006), PPAR (Xu et al. 2010; Hatchell et al. 2006), and SF-1/Dax-1 (Kelly et al. 2010; Xu et al. 2009) on reporter genes bearing their respective HREs. SRA also has been reported to coactivate non-NR TFs, including MyoD (Caretti et al. 2006) and Notch (Jung et al. 2013) as part of a complex with the p68 RNA helicase. Additionally, SRA RNA was shown to interact with p68 RNA helicase and CTCF for insulator function at the *Igf2/H19* imprinted region (Yao et al. 2010). Consistent with these observations that SRA RNA may play a more global role, SRA RNA knockdown in HeLa and MCF-7 cells lead to both up-regulated and down-regulated genes, including some,

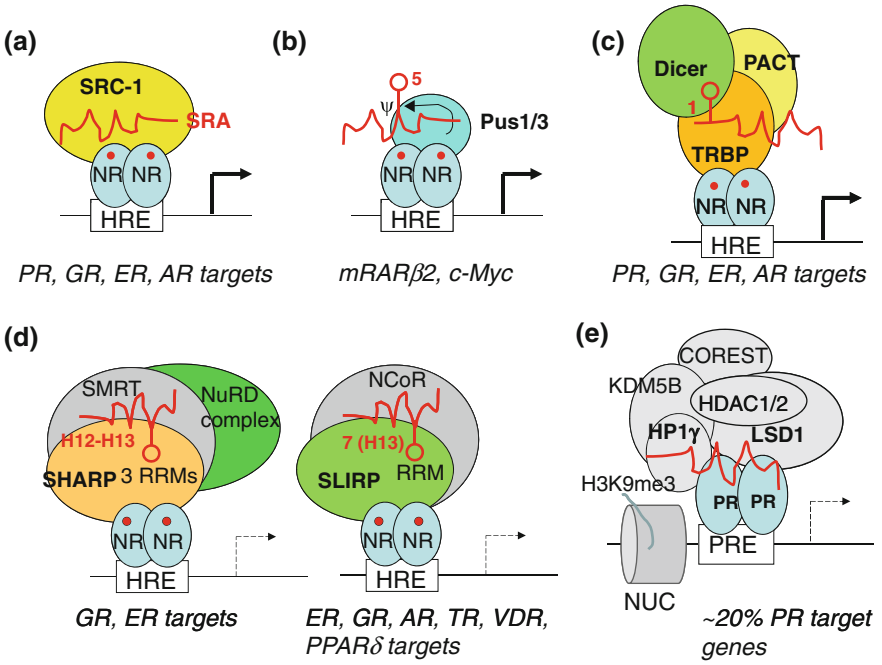

Fig. 3 SRA structures STR1, 5, and 7 act as scaffolds for assembly of distinct coregulatory complexes that modulate gene transcription in response to hormonal signaling. **a** SRA (depicted as *red wavy line*) as an RNA coactivates steroid hormone receptors bound to HREs as part of an SRC-1 complex (Lanz et al. 1999). Five different predicted structures, including STR1 and 7, were shown to be critical for PR-mediated luciferase reporter assays (Lanz et al. 2002), but which one(s) is critical for SRC-1 interaction is unknown. Later it was shown that SRA additionally coactivates other NRs, such as TR (Xu and Koenig 2004), VDR (Hatchell et al. 2006), PPARs (Hatchell et al. 2006; Xu et al. 2010), and orphan NRs, such as SF-1 and Dax1 (Xu et al. 2009). Ligand bound to nuclear receptor (NR) is illustrated with a *red dot*, and transcription is indicated with an arrow. **b** Pseudouridylation of SRA STR5 at a particular uridine U206 (indicated by ψ; see Fig. 2b; Zhao et al. 2007; Huet et al. 2014) and six other uridines by pseudouridine synthase Pus1 and/or Pus3 enzymes results in an ribonucleoprotein complex promoting transcription from genes with HREs. Pus1 directly binds the DNA-binding domain of RARγ (Zhao et al. 2004). This complex may exist on the *mRARβ2* and *c-Myc* promoters (Zhao et al. 2004, 2007). **c** TRBP, PACT, and Dicer, components of the RNA-induced silencing complex, form a hormone-dependent complex with SRA STR1. These three proteins augment SRA-mediated coactivation of steroid hormone receptors. This complex may exist on HRE-containing genes, such as *PSA,* leading to transcriptional activation (Redfern et al. 2013). **d** RRM domains of SHARP (Shi et al. 2001) and SLIRP (Hatchell et al. 2006) proteins directly bind SRA H12-H13 (Arieti et al. 2014) and H13 (STR7) (Hatchell et al. 2006), respectively, recruiting HDAC-containing NCoR/SMRT and NuRD corepressor complexes to promoters and also likely sequestering SRA away from SRC-1-containing coactivator complexes. These actions lead to repression of HRE-containing genes as listed in the figure. **e** SRA plays a repressive role with unliganded PR by being part of a HP1γ/LSD1 corepressor complex that additional contains COREST/KDM5B/HDAC1/2 (Vicent et al. 2013). This corepressor complex is anchored to target genes via HP1γ binding histone H3 K9me3-"marked" nucleosomes (NUC). SRA directly interacts with PR, HP1γ, and LSD1 in this complex, but it is not known which SRA sub-structures are important for these binding events. In the presence of progesterone, MSK1 kinase becomes activated and phosphorylates H3S10, thereby promoting dismissal of the repressive HP1γ–LSD1 complex (Vicent et al. 2013)

but not all, GR and ERα targets (Foulds et al. 2010). In two different studies, SRA knockdown in HeLa, MCF-7, or T47D cells did not affect neighboring genes on chromosome 5, arguing against SRA action in *cis* (Foulds et al. 2010; Vicent et al. 2013).

Thus, SRA RNA is thought of as an lncRNA that plays a "scaffolding" role with a variety of TFs, analogous to what Tsai and colleagues have proposed for HOTAIR binding PRC2 and LSD1 corepressors via different domains (Tsai et al. 2010). SRA RNA may adopt a distinct conformation to promote assembly or stability of each respective complex highlighted in Fig. 3. Only one study has presented direct evidence for SRA RNA being bound at any of its endogenous target genes, namely the PR target gene *STAT5A* in T47D cells using ChOP (chromatin oligoaffinity precipitation) assays (Vicent et al. 2013). Although with additional methodologies discussed in Sect. 6.2 of this chapter, it should be feasible to define more direct SRA RNA target genes in the future.

It has been reported that the human SRA genomic locus on chromosome 5 can also make a protein product as a result of alternative mRNA splicing retaining the first exon (Emberley et al. 2003; Kawashima et al. 2003). Exactly what the protein termed "SRAP" may do in cells is unclear, although it has been proposed to bind SRA RNA and inhibit its coactivation of MyoD (Hube et al. 2011). However, these data have not been reproducible in another laboratory (McKay et al. 2014), thereby leaving the function of SRAP unclear. Nevertheless, SRA RNA represents an interesting example of a "bifunctional" RNA (Dinger et al. 2011) that acts as a NR coregulator as an lncRNA and also be used as a template for protein synthesis.

3.2 GAS5 Repression of Select Steroid Receptor Transcriptional Activity

Growth-arrest specific 5 (GAS5) is another well-characterized lncRNA of 598 nucleotides that acts in *trans* to repress some, but not all, steroid receptors (Kino et al. 2010). GAS5 RNA appeared both nuclear and cytoplasmic in HeLa cells (Kino et al. 2010). However, its mechanism of action is quite different than SRA as will be detailed below. GAS5 was shown to bind GR, AR, PR, and MR in a hormone-dependent manner, but not ERα or PPARδ, and to repress transcription of reporter genes containing the GRE/ARE/PRE elements. As mentioned in the **Introduction**, these three steroid receptors bind with high affinity a similar palindromic HRE-containing AGAACA that ERα and PPARδ do not. Furthermore, from mapping experiments, the DNA-binding domain, but not other domains of GR, was responsible for interaction with the 3′ end of GAS5 (nucleotides 400–598). This region of GAS5 was predicted to form six stem-loop structures and hairpin #5 (nucleotides 546–566) formed a double-stranded RNA that resembled a GR DNA

binding site (GRE). Importantly, mutation of the hairpin disrupting the predicted "GRE mimic" antagonized GAS5 repression of GR activity and also had no effect on GR occupancy of the endogenous GR target gene *cIAP2*. This model was further supported by showing that increasing GAS5 expression repressed transcription of *cIAP2* (and other GR targets) and decreased GR occupancy at the GREs of that target gene in transfected HeLa cells. Also, in vitro binding assays with GR revealed alteration in the dissociation constant (K_d) when hairpin #5 of GAS5 was mutated. Thus, GAS5 forms a discrete stem-loop structure that mimics the double-stranded GRE, so that GAS5 may act as a "GRE decoy" to compete for select steroid receptors binding to DNA (Kino et al. 2010).

Biologically, it was shown that GAS5 accumulates in cells starved of growth factors and represses GR activation of target genes such as *cIAP2* (encoding an inhibitor of apoptosis), thereby promoting apoptosis (Kino et al. 2010). GAS5 overexpression also reportedly causes apoptosis in many adherent cell lines (Mourtada-Maarabouni et al. 2009).

3.3 Controversial lncRNAs Associated with the Androgen Receptor (AR)

It is known that PCGEM1 lncRNA expression is induced upon androgen treatment of LNCaP cells and that it is overexpressed in human prostate tumors (Srikantan et al. 2000; Table 1). Additionally, prostate cancer-associated noncoding RNA 1 (PRNCR1) was reported to be up-regulated in prostatic neoplasia, and its knockdown in prostate cancer cells reduced viability and expression of an AR-dependent reporter gene (Chung et al. 2011). These findings prompted Yang and colleagues to investigate whether these two lncRNAs may play a role in prostate cancer by affecting AR transcriptional activity (Yang et al. 2013). They reported the following key findings: (1) both lncRNAs are present at higher levels in prostate tumors than in benign samples, (2) both lncRNAs appear to be associated with AR in "RNA immunoprecipitation" (RIP) assays in a DHT-enhanced manner, and (3) knockdown of these lncRNAs using antisense deoxynucleotides reduced the transcription of many AR target genes. They proposed a model in which PRNCR1 binds at the Hinge region of AR initially and recruits the methyltransferase DOT1L for subsequent methylation of the AR AF1 that then recruits PCGEM1 to promote DNA looping for transcription.

While this model is conceptually quite appealing, the failure of another laboratory to reproduce some of the above key findings gives caution in conclusively stating how these two lncRNAs actually function in prostate cancer (Prensner et al. 2014c). Specifically, Prensner et al. did not find an association of PRNCR1 with prostate cancer, and neither of the lncRNAs were associated with AR in RIP assays using the same AR antibody. Future work is needed to resolve what these two lncRNAs may actually be doing.

One recent study suggests a new function for PCGEM1—by serving as a coactivator of c-Myc; this lncRNA up-regulates the expression of key enzymes in several tumor cell metabolic pathways, regardless of hormone or AR status (Hung et al. 2014). Specifically, PCGEM1 was shown to directly bind c-Myc, to localize to c-Myc target gene promoters, and to enhance c-Myc transcriptional activity.

4 Other lncRNAs as Potential Regulators of NR Function

4.1 Progesterone Receptor Gene Promoter Antisense Transcripts

There is another kind of lncRNA that derives not from an intergenic region but instead from the promoter region of a gene, is antisense in its orientation, and can regulate the expression of the gene from which it is derived. Antisense transcripts derived from the promoter of the *PR* gene (official symbol: *PGR*) can span over 70 kilobases (kb) of this genomic region and are spliced (Schwartz et al. 2008). Several sets of synthetic anti-gene RNA (agRNA) duplexes targeting these PR antisense transcripts have been demonstrated to result in either an increased or decreased PR protein level in transfected breast cancer cells. Interestingly, the PR antisense transcripts contribute to differential expression of PR in MCF-7 and T47D cells (Janowski and Corey 2010). However, since no natural short RNAs (e.g., miRNAs) have been identified that target the PR antisense transcripts, the biological relevance of these promoter-derived antisense lncRNAs awaits elucidation. The prevalence of promoter-derived *cis*-based antisense transcription in the human genome and its relevance to NR function also need further exploration.

4.2 Enhancer RNAs (eRNAs) Transcribed from ERα, AR, and Rev-Erb DNA-Binding Sites

Defying the popular belief, The ENCODE consortium has revealed pervasive transcription of the human genome (Birney et al. 2007; Djebali et al. 2012); the vast majority of these transcripts derives from intergenic regions and does not code for proteins. Interestingly, RNA pol II also maps to vast stretches of extragenic regions enriched with the characteristics of active enhancers (see below), revealing vast arrays of enhancer-derived transcripts called enhancer RNAs (eRNAs) (De Santa et al. 2010; Kim et al. 2010). Using the GRO-seq methodology to specifically identify and map nascent transcripts (Core et al. 2008), Hah and colleagues revealed robust, rapid, and transient bursts of eRNA production in MCF-7 cells upon

estradiol (E2) treatment (Hah et al. 2011). The GRO-seq analyses revealed E2-stimulated transcription from nearly a third of the MCF-7 genome; these transcripts comprise not only of protein-coding genes, but also of a variety of ncRNAs synthesized by all the three RNA polymerases (Hah et al. 2011). Notable among these were bidirectional eRNAs synthesized from ERα-bound enhancers.

Subsequently, DHT-stimulated eRNA synthesis at AR-bound enhancers was reported in human LNCaP cells (Wang et al. 2011; Hsieh et al. 2014), while the NRs, Rev-Erbα and Rev-Erbβ, have been shown to repress eRNA synthesis in mouse macrophages (Lam et al. 2013).

Several features of eRNAs set them apart from the more "conventional" lncRNAs. They largely lack splicing, undergo exosome-dependent turnover, and are not polyadenylated (Andersson et al. 2014), although DHT-induced eRNA from the *KLK3* (also called PSA, prostate-specific antigen) regulatory locus was reported to be polyadenylated (Hsieh et al. 2014). Additionally, the majority of eRNAs are much shorter in length, variously reported to be 3–5 kb (Hah and Kraus 2014), <2 kb (Kim et al. 2010), or with a median length of 346 nt (Andersson et al. 2014). The majority of eRNAs are bidirectional in nature; while some represent a set of divergent transcripts with a median gap of ∼180 bp between the transcription start site (TSS) of the plus and minus strands (Andersson et al. 2014), other sets of eRNAs can have sequence overlaps (Hah et al. 2013). The inter-TSS region invariably houses the enhancer sequences. Definitive proteomic and epigenomic signatures correlating eRNA production have been identified (see below), while no such features have been proposed for other lncRNAs in general.

Enhancers in a given cell type can be broadly classified into two groups: transcriptionally "active" (i.e., producing eRNAs and activating the target gene) and inactive (Li et al. 2013). This dichotomy is well exemplified by the population of enhancers in MCF-7 cells that gain ERα occupancy upon E2 treatment. Nearly half of the 3,191 ERα-bound enhancers in MCF-7 cells produce eRNAs detectable by GRO-seq upon brief E2 treatment (Hah et al. 2013). Generally, the active enhancers exhibit binding of the coactivator p300 and RNA pol II (Heintzman et al. 2009; Andersson et al. 2014; De Santa et al. 2010), key pioneering TFs such as FOXA1, and steroid receptor coactivator (SRC) family members (Wang et al. 2011). It has been recently suggested that eRNA production from enhancer regions is dependent on huge complexes containing multiple TFs recruited to the enhancers in *trans* (i.e., independent of their respective DNA-binding domains) (Liu et al. 2014b). The flanking regions on both sides of the active enhancers are relatively nucleosome-deficient and lack CpG islands (Andersson et al. 2014). However, these regions nevertheless are highly enriched with certain histone H3 tail modifications (H3K4me1 and H3K27ac) (Wang et al. 2011; Kim et al. 2010; De Santa et al. 2010; Heintzman et al. 2009). These features are greatly subdued in inactive enhancers (Plank and Dean 2014; Hah et al. 2013).

4.3 eRNAs as Simple "Marks" of Active Enhancers or as Components of Enhancer–Promoter Looping

Post-E2 treatment, ERα occupancy is greater on active enhancers than on the inactive ones (Li et al. 2013). Among all the lncRNAs, the eRNAs are the only class that invariably undergoes up-regulation upon E2 stimulation (Hah et al. 2011). Moreover, in both estrogen and androgen signaling, the level of expression of eRNAs correlates positively with the expression level of neighboring protein-coding genes contained on the same chromosomal region (Hah et al. 2011, 2013; Li et al. 2013; Wang et al. 2011). Therefore, it is safe to assume that eRNAs are linked to AR/ERα-mediated activation only, and not with repression. Consistent with this hypothesis, rhythmic circadian oscillations in several eRNAs have been recently reported that mirror the rhythmicity of expression of the neighboring target genes in mouse liver (Fang et al. 2014).

However, critical questions concerning eRNAs remain, such as what is the role of the eRNAs and how might they mechanistically affect promoter-driven transcription and/or enhancer–promoter communication? Active enhancers, producing eRNAs, are more likely to interact with the corresponding gene promoter via chromatin looping [as measured by chromosome conformation capture (3C) (Hagege et al. 2007) or related assays], as compared to inactive enhancers (Hah et al. 2013). These data suggest that eRNA production may have mechanistic relevance to activation of the neighboring target protein-coding gene. The simplest approach to address these questions would be to knockdown eRNAs and assay target gene expression and chromatin looping. Two recent studies employing MCF-7 or LNCaP cells reported a reduction in ERα or AR target gene expression and enhancer-promoter looping when the cells were transfected with eRNA-targeting small-interfering RNAs (siRNAs) (Li et al. 2013; Hsieh et al. 2014). Using an artificial tethering model, Li and colleagues further proposed that E2-induced eRNAs stimulate promoter-driven transcription in *cis*, while Hsieh and colleagues argue that DHT-induced eRNAs can activate select genes residing on different chromosomes, suggesting some *trans* effects. While Li and colleagues present evidence supporting a physical interaction between E2-induced eRNAs and the cohesin complex (Li et al. 2013), Hsieh and colleagues suggest that the DHT-induced eRNAs bind AR and the MED1 subunit of the Mediator complex and recruit them to the target promoter (Hsieh et al. 2014). Thus, these two studies suggest that eRNA–protein complexes mediate activation of NR target genes.

However, the experimental strategy adopted by these two studies raises questions. E2 elicits rapid and robust but transient expression of eRNAs in MCF-7 cells, which peak at 40 min post-treatment and start diminishing thereafter (Hah et al. 2011, 2013). Li and colleagues (Li et al. 2013) transfected MCF-7 cells with siRNAs or locked nucleic acid (LNA) oligonucleotides targeting specific eRNAs for two days and then stimulated the cells with E2 for 1 h prior to harvesting the

cells. Clearly, the siRNAs and the LNAs were introduced into cells to degrade a target that may already be reduced in its level. It is doubtful that the siRNAs and LNAs, which were already present in the cell at the time of E2 induction, would have caused destruction of the nascent eRNAs as they were being synthesized. The same problem also clouds the report by Hsieh et al. (2014) detailing DHT-induced eRNA function in LNCaP and VCaP cells. Development of in vitro transcription assays supplemented with/without defined eRNAs may be the better approach in the future to address eRNA function.

It is interesting that the features of active enhancers as discussed above remain mostly unaltered upon inhibition of global Pol II transcription by flavopiridol and that the enhancer–promoter looping also remains unaffected (Hah et al. 2013). These data suggest that the epigenomic and proteomic features of active enhancers, as well as enhancer–promoter communication, are established prior to transcription initiation and that progressive RNA synthesis is not required for maintenance of these features. These results have led Hah and colleagues (Hah et al. 2013) to consider eRNAs simply as "marks" for enhancer activity (Fig. 4a).

However, it is difficult to visualize that the massive and pervasive transcription from the enhancers would merely be a "mark" for enhancer activity and that it would have no functional role in gene regulation. Thus, an alternative hypothesis for eRNAs is that they have an active role in transcription regulation, by forming competent ribonucleoprotein complexes either in *cis* (Fig. 4b) or in *trans* (Fig. 4c) that ultimately links the enhancer with the promoter. While enhancer–promoter interaction correlates with promoter-driven transcription, a definitive causal relationship has not yet been demonstrated. In this context, it is worth noting that the target genes of the inactive enhancers, which neither produce eRNAs nor loop to the promoter, remain dormant (Fig. 4d).

Among the steroid hormones, only E2 and DHT have been reported to induce eRNA synthesis in human cells. In light of the fact that other non-hormonal signaling events have been shown to induce eRNA expression in various mammalian cell types and tissues, we expect that other hormones such as glucocorticoid and progesterone and their respective NRs will also induce specific sets of eRNAs.

5 lncRNAs as Suggested Players and/or Biomarkers in Hormone-Associated Cancer

Perhaps the best studied human pathologies associated with hormone-regulated lncRNAs or those that functionally interact with NRs are hormone-associated cancers. We will highlight below a few of the best examples of lncRNAs that may be involved in breast and prostate cancer progression/metastases.

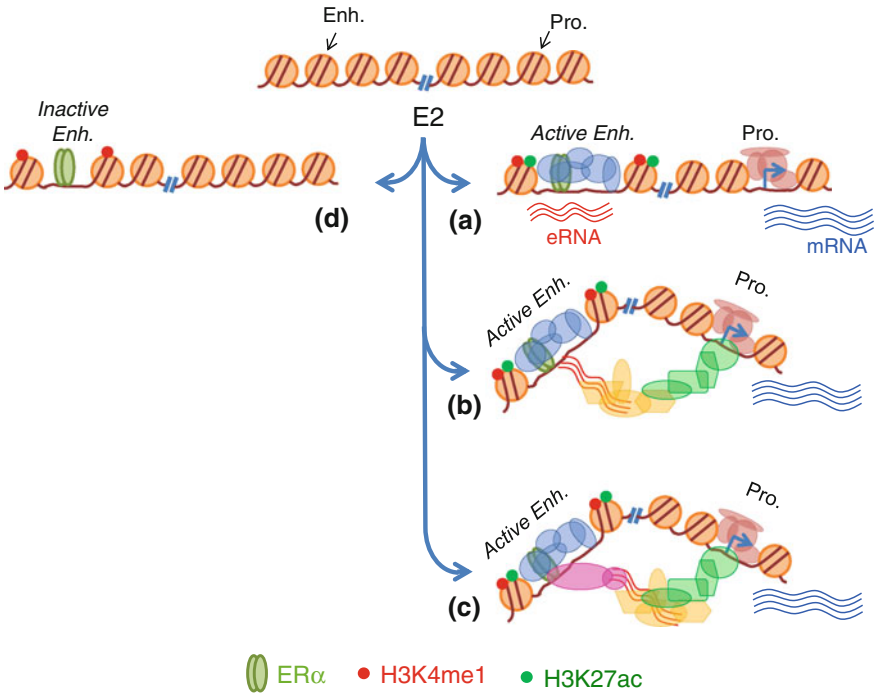

Fig. 4 Possible role of enhancer RNAs (eRNAs) in chromatin looping for activation of promoter-driven transcription. **a** E2 stimulation leads to ERα binding to the enhancer (Enh.), which promotes transcription from the promoter (Pro.). eRNAs (*red curved lines*) produced at the enhancer may be released from the template and take no part in chromatin looping and/or production of promoter-driven transcripts (mRNA; *blue curved lines*). These may simply serve as a "mark" of an "active" enhancer. **b** eRNAs may remain tethered to the template and form ribonucleoprotein complexes to nucleate chromatin looping in *cis*. **c** Released eRNAs may form ribonucleoprotein complexes to nucleate chromatin looping in *trans*, such complexes may "sea-fare" through the nucleoplasm to contact far away chromatin sites on other chromosomes. Chromatin looping—formed either in *cis* or *trans*—may or may not be mechanistically linked to mRNA production. **d** Inactive enhancer. Despite ERα binding here upon E2 stimulation, no eRNA is made, and the target promoter is not activated

5.1 lncRNAs in Breast Cancer

There have been several recent reviews on lncRNAs that may play a role in breast cancer, and as this topic could be a chapter itself, we suggest the following reviews for additional information: (Shore and Rosen 2014; Hansji et al. 2014; Ye et al. 2014). Recently, lncRNA profiling efforts have been used to define lncRNAs enriched with a particular breast cancer subtype (e.g., ERα-positive luminal A and B, ERα-negative basal, and HER2-enriched) (Su et al. 2014; Zhao et al. 2014a; Ding et al. 2014). It remains to be seen how much these high-throughput catalogs will be able to inform the clinic, but these data are a first step in that process. In

addition to these profiling experiments, individual lncRNAs have been studied for their possible involvement in breast cancer, and below, we highlight four of the better characterized examples.

SRA lncRNA that coactivates ERα and PR transcriptional activities has been suggested to be a player in breast cancer (as well as uterine and ovarian cancers), as it is overexpressed in tumors compared to normal tissues (Leygue et al. 1999; Murphy et al. 2000; Lanz et al. 2003) and affects the growth of certain hormone-sensitive breast cancer cell lines (Cooper et al. 2009). Interestingly, mice overexpressing human SRA lncRNA in their mammary glands display increased epithelial hyperplasia, but did not develop tumors, due to compensating enhanced apoptosis (Lanz et al. 2003). SRA knockout mice have been established (Liu et al. 2014a), but they have not yet been crossed with mice representing a breast cancer model to ascertain whether the loss of SRA may affect tumorigenesis. Finally, knockdown of SRA in ER-negative MDA-MB-231 cells reduced cell migration and invasion (Foulds et al. 2010), so it is formally possible that SRA may promote metastasis in ER-negative breast cancers.

While HOTAIR and H19 lncRNAs have not been reported to functionally interact with NRs, their expression is increased with E2 treatment (Table 1). These data suggest they might play a role in hormone-dependent breast cancer. It has been reported that HOTAIR is overexpressed and promotes cell migration and invasion in metastatic ERα-positive breast cancer cells (Gupta et al. 2010). Furthermore, HOTAIR has been suggested to be an independent prognostic marker of metastasis in ERα-positive breast cancer (Sorensen et al. 2013). Unlike HOTAIR, H19's role in breast cancer is controversial—while it is overexpressed in breast cancer (Adriaenssens et al. 1999) and its forced overexpression in MCF-7 cells stimulated proliferation (Sun et al. 2015) suggestive of an oncogenic function, it may also act as a tumor suppressor in other cellular settings (Gabory et al. 2010).

Unlike SRA and HOTAIR that appear oncogenic, GAS5 appears to be a tumor suppressor in breast cancer. GAS5 overexpression leads to apoptosis in breast cancer cell lines, and compared to matched normal controls, GAS5 expression is down-regulated in breast tumors (Mourtada-Maarabouni et al. 2009).

5.2 *lncRNAs in Prostate Cancer*

There have been several recent reviews on lncRNAs that may play a role in prostate cancer, and a reader wanting more information should review the following: (Ronnau et al. 2014; Bolton et al. 2014; Cheng et al. 2013; Walsh et al. 2014). Below, we will highlight some of the best studied lncRNAs in prostate cancer.

Similar to the findings in breast cancer cells, SRA and GAS5 lncRNAs, which are known modulators of AR function (see above), appear to be oncogenic and tumor-suppressive, respectively, in prostate cancer. SRA promotes the growth of LNCaP and DU145 prostate cancer cell lines (Agoulnik and Weigel 2009). GAS5 is down-regulated in prostate cancers (Romanuik et al. 2010), and its overexpression stimulates apoptosis in prostate cell lines (Pickard et al. 2013).

PCGEM1, as mentioned above in Sect. 3.3, may or may not functionally interact with AR. However, its expression is induced with androgen (Table 1). It also has been shown to promote prostate cancer cell proliferation (Petrovics et al. 2004) and to inhibit apoptosis (Fu et al. 2006). PCGEM1 is overexpressed in 41 % of Caucasian-American and 68 % of African-American patients, respectively (Petrovics et al. 2004).

Finally, there are three lncRNAs highly expressed in prostate cancers that we wish to highlight—PCA3, SChLAP1, PCAT-1, although none of them interact with AR in RIP assays (Prensner et al. 2013). PCA3 is well known to be overexpressed in prostate cancer versus normal tissues (de Kok et al. 2002). PCA3 knockdown in LNCaP cells inhibits cell growth and viability and expression of some AR target genes, although the mechanism for the latter is unclear (Ferreira et al. 2012). PCA3 can be detected in urine samples, and the Progensa PCA3 test is FDA-approved for men presenting with elevated serum PSA and a negative biopsy (reviewed in Hessels and Schalken 2009). Thus, an lncRNA can be a prognostic biomarker, and time will tell whether others mentioned above will also be similarly utilized in the clinic [e.g., MALAT1 may be another urinary biomarker for prostate cancer (Wang et al. 2014)]. SChLAP1 (second chromosome locus associated with prostate-1) was reported to play critical roles in prostate cancer cell invasiveness and metastasis in vivo (Prensner et al. 2013). This lncRNA has elevated expression in prostate cancer cases with poor prognosis. Mechanistically, SchLAP1 interacts with the SNF5 subunit of the SWI/SNF chromatin remodeling complex and inhibits its genomic localization to target genes associated with tumor suppression. PCAT-1 (prostate cancer-associated transcript (1)) was discovered from a RNA-Seq experiment of 102 prostate tumors/cell lines (Prensner et al. 2011). It is up-regulated only in prostate cancer and can promote prostate cancer cell proliferation. PCAT-1's effect on proliferation may be due to repression of the tumor suppressor gene *BRCA2* (Prensner et al. 2014b) and stabilization of c-Myc protein (Prensner et al. 2014a) via posttranscriptional mechanisms. Clearly, more studies are needed to elucidate how cancer-associated lncRNAs exert their biological properties.

6 Future Directions

6.1 Knockout Studies

One way to understand the biological functions of lncRNAs is to create knockouts for conducting loss-of-function experiments. There have been several instances where lncRNA genes have been manipulated; these studies have provided invaluable insights into the functions of 13 different lncRNAs (reviewed in Li and Chang 2014). It is a challenge to effectively delete such long stretches of the genome as the lncRNA genes. Recently, the efficacy of the CRISPR/Cas9 system in creating knockouts for an lncRNA up to 12 kb (AK023948) has been demonstrated

(Ho et al. 2014). Such technology can be potentially employed, and improvised, to carry out knockout screens for lncRNAs in large scale. However, it is possible that producing an lncRNA may not be the only cellular function of the concerned genomic region, and consequences of the deletion of the entire lncRNA gene may become difficult to interpret. Therefore, we would instead suggest transcript knockdown strategies in which genomic deletions are informed by some structure–function data. For example, if bases within a particular stem-loop are critical for binding an effector protein, then these sequences should be deleted, not the entire locus.

6.2 Genome-Wide Localization and Proteomics

To impart their cellular function, lncRNAs could potentially form ribonucleoprotein complexes and communicate with other proteins and/or chromatin. Also, various lncRNAs could potentially bind select chromatin regions and regulate the local chromatin structure and function. Therefore, one way to understand cellular roles of lncRNAs is to elucidate their chromatin-wide occupancy. Several recent studies have developed four techniques to identify chromatin binding sites for specific lncRNAs (reviewed in Vance and Ponting 2014). These methods are called ChOP (defined above), chromatin isolation by RNA purification (ChIRP) (Chu et al. 2011), CHART (capture hybridization analysis of RNA targets), and RAP (RNA antisense purification). All of these protocols rely on capture of the lncRNA from cross-linked chromatin extracts by hybridization to biotinylated antisense oligonucleotides. Bound genomic DNA regions are then identified by either qPCR or high-throughput sequencing. Using the CHART technique, West and colleagues mapped the binding sites of the ~8 kb MALAT1 and ~3.7 kb NEAT1 lncRNAs in MCF-7 cells (West et al. 2014). While both MALAT1 and NEAT1 mapped to actively transcribed chromatin regions enriched with pro-transcriptional histone modifications, NEAT1 is specifically localized to E2-inducible genes, and its occupancy is correlated positively with E2 induction of genes such as *GREB1*.

As an extension of CHART, West and colleagues used mass spectrometry to discover proteins bound to MALAT1 and NEAT1 lncRNAs after reversal of the cross-links (West et al. 2014). Additional protocols for discovery of proteins associated with lncRNAs involve either cell-free methods, such as biotinylated RNA pulldown of proteins from nuclear extract (Marin-Bejar and Huarte 2015), or cotransfection of mammalian cells with a vector expressing a particular lncRNA fused to multiple MS2-phage coat protein-binding sites and a vector expressing a FLAG-tagged MS2-phage coat protein. After formaldehyde cross-linking, cells are lysed and lncRNA–protein complexes can be isolated by anti-FLAG antibody beads (Gong and Maquat 2015). Using a variation of this latter approach without cross-linking, we isolated DDX5 (p68 RNA helicase) and YBX1 (YB-1) from HeLa cells as preferential binders to a 6x MS2-tagged SRA ncRNA as compared to the 6x MS2 RNA tag alone (C.E. Foulds and B.W. O'Malley, unpublished

observation). Both of these proteins have been reported in other cell systems to bind SRA RNA (Caretti et al. 2006; Honig et al. 2002). Elucidation of chromatin-wide occupancy and binding proteins of more lncRNAs—especially those relevant to hormone signaling—will uncover invaluable information about their function.

6.3 Structure–Function Studies

Unlike DNA, lncRNA molecules assume distinct three-dimensional structures formed by a series of stem-loop structures (e.g., SRA RNA in Fig. 2). These structures may be pivotal for the RNA's enzymatic activity as is the case with ribozymes (Cech 2002) or may be critical in establishing unique ribonucleoprotein complexes. As lncRNAs presumably will have distinct lowest energy structures, elucidation of their structures will be important for understanding their functions. This assumption has recently been vindicated in three independent studies. Recent data from Hudson and colleagues have provided more biochemical and structural support further extending the GAS5-GR interaction model highlighted above in Sect. 3.2 (Hudson et al. 2014). Namely, dissociation constants for the purified DBDs of AR, PR, GR, and MR binding Gas5 were determined, and the authors found that mutation of a single glycine (G439E) located in the first zinc finger of GR abolished Gas5 binding. X-ray crystallography of hairpin #5 confirmed its helical nature and that it could act as a "GRE decoy." GR-GAS5 amino acid–base contacts were mapped by performing hydroxyl radical "footprinting" and by analyzing nuclear magnetic resonance (NMR) "chemical shifts"—much like what has been done for TF: DNA interactions in the past. Finally, a single nucleotide mutation in GAS5 (G549A) was shown to abolish steroid receptor–GAS5 interaction and prevented GAS5-induced apoptosis. In the second study, Arieti and colleagues (Arieti et al. 2014) have elucidated the three-dimensional structure of the RRMs of SHARP (see Sect. 3.1), and by RNA binding assays, they defined two helices of SRA (H12-H13) that mediated the interaction with the RRMs. Finally, Huet and colleagues (Huet et al. 2014) further characterized how human Pus1 protein binds SRA RNA and modifies it. Specifically, they validated that the STR5 (also called H7; Fig. 2) is the minimal region of SRA for Pus1 binding and modification and determined the three-dimensional structure of the catalytic domain of Pus1 and modeled how STR5 of SRA binds it. Additional structure–function studies of lncRNAs involved in NR signaling are needed to better understand the "rules of engagement."

6.4 Posttranscriptional Modifications

Hundreds of posttranscriptional modifications in RNAs have been characterized that regulate downstream cellular roles of the respective RNA molecules in many different ways. These include regulation of transcript stability, protein–RNA

interaction, nuclear export–import control, splicing, and transcript editing (Li and Mason 2014). However, only a single RNA modification is currently known to affect NR function—pseudouridylation of SRA RNA by Pus enzymes (Zhao et al. 2004, 2007) as described in Sects. 3.1 and 6.3. However, pseudouridylation profiling of HeLa cells has recently revealed two pseudouridines in MALAT1 and one in the 7SK lncRNA (Carlile et al. 2014). Data from additional profiling experiments in breast/prostate cells with/without hormone treatment might suggest new lncRNA modifications made during NR signaling.

Notable among other modifications of RNA is N6-methyladenosine (m6A). Recent studies have mapped m6A levels transcriptome-wide using a technique called m6A-seq in human HepG2 cells, and 250 lncRNAs were found to be m6A-methylated, including lincRNAs and antisense lncRNAs (Dominissini et al. 2012). Furthermore, m6A-seq of mouse embryonic stem cells revealed 117 lncRNAs as being m6A-methylated, including NEAT1 and MALAT1 (Batista et al. 2014). How m6A modification may affect the activities of these lncRNAs is not presently clear. Interestingly, a writer (METTL3), an eraser (FTO), and readers (YTHDF2, ELAV1) of the m6A RNA modification are involved in diseases such as prostatitis, polycystic ovarian syndrome, and breast cancer (Liu and Pan 2015), where NR signaling plays critical roles. In our view, it is highly likely that dysregulation of the lncRNA m6A methylome may significantly affect NR signaling, and this possibility needs to be explored in the future.

7 Conclusions

Profiling lncRNA expression changes as function of hormone and tumor type may allow the identification of "biomarkers" for certain human diseases, such as hormone-driven cancers. The next challenge will be to move beyond "prognostication" and define which of the identified lncRNAs might really be "drivers" of disease and whether pharmacologically realistic approaches to inhibit the expression and/or activity of these lncRNAs can be achieved. For this to be a future possibility, we propose that more studies are needed to better define how the expression of lncRNAs is modulated. Perhaps more importantly, additional biochemical/structural studies may elucidate how their activities could effectively be targeted (as RNA interference and antisense deoxynucleotide approaches involving hybridization to the lncRNAs may not be clinically effective) and the generation of small molecules capable of disrupting lncRNA: protein interactions should not be discounted.

It would serve the field working on regulatory lncRNAs that may modulate NR transcriptional activity to think in terms of what the transcription factor (TF) field did years ago. Namely, we propose that that many lncRNAs, like TFs, are "modular" in nature. In other words, lncRNAs are made up of different structural domains that work together by nucleating different protein–RNA interactions. Additionally, lncRNAs are likely targeted to discrete genomic regions (although

most likely through protein tethering). After finding their genomic sites of action, TFs recruit coregulatory complexes for modulating gene transcription. Likewise, current data suggest that some lncRNAs may function as "scaffolds" for nucleation and/or stabilization of distinct coregulatory complexes bound with NRs to affect transcription in a hormone-dependent manner. Clearly, more structural details of how a particular stem-loop in an lncRNA binds a coregulator are desperately needed. Finally, TFs often undergo posttranslational modifications, such as phosphorylation and ubiquitination, for modulation of their activity. The finding that SRA RNA is posttranscriptionally modified by pseudouridylation suggests that additional lncRNAs may have this modification and/or additional RNA base modifications, such as m6A, to create an "active" regulatory RNA. As suggested above, understanding these modifications may be critical for pharmacological targeting of lncRNA activity in the future.

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Noncoding Transcriptional Landscape in Human Aging

Marina C. Costa, Ana Lúcia Leitão and Francisco J. Enguita

Abstract Aging is a universal phenomenon in metazoans, characterized by a general decline of the organism physiology associated with an increased risk of mortality and morbidity. Aging of an organism correlates with a decline in function of its cells, as shown for muscle, immune, and neuronal cells. As the DNA content of most cells within an organism remains largely identical throughout the life span, age-associated transcriptional changes must be achieved by epigenetic mechanisms. However, how aging may impact on the epigenetic state of cells is only beginning to be understood. In light of a growing number of studies demonstrating that noncoding RNAs can provide molecular signals that regulate expression of protein-coding genes and define epigenetic states of cells, we hypothesize that noncoding RNAs could play a direct role in inducing age-associated profiles of gene expression. In this context, the role of long noncoding RNAs (lncRNAs) as regulators of gene expression might be important for the overall transcriptional landscape observed in aged human cells. The possible functions of lncRNAs and other noncoding RNAs, and their roles in the regulation of aging-related cellular pathways will be analyzed.

Contents

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1 Introduction

Aging is a universal and multifactorial process in complex living systems, characterized by a general decline of the organism physiology associated with an increased risk of mortality and morbidity. Due to its intrinsic complexity, models for studying organism aging are often inadequate and partial, being difficult to distinguish between causes and consequences of the aging phenomenon. At the phenotype level, aged organisms show a characteristic panoply of features always related to their physiological deterioration (Madrigano et al. 2012). However, the molecular mechanisms underlying this phenotype are far to be globally understood.

In complex organisms, aging appeared to be caused by the individual cell aging. In several types of tissues, the function of somatic cells declines with age. The term senescence was applied to these cells that ceased to divide in culture, based on the speculation that their behavior recapitulated organism aging. Consequently, cellular senescence is sometimes termed cellular aging or replicative senescence. Global aging of an organism is directly related to the individual cell aging. Increasing data suggest that cell aging is not merely an accumulation of damage, but an accumulation of damage associated with an altered transcriptional profile (Kato et al. 2011). There is not likely to be a single gene responsible for aging. Rather, a complex network of genomic interactions probably exists, which currently remains unknown. In order to support this idea, a coherent and integrative view has recently emerged with the major goal of studying the genetic mechanism subjacent to cell aging by combining systems biology with Genomics and Proteomics (Madrigano et al. 2012).

Spatial genome organization can critically affect gene expression in aging. While it is well known that chromatin composition can directly shape gene activity, three-dimensional chromatin organization is also emerging as an important gene regulation mechanism in aging (Collado et al. 2007). There are many ways by which chromatin interactions could be regulated: first, by modifying the DNA itself with cytosine methylation and consequently altering protein association (Fraga et al. 2007). Chromatin contacts could also be regulated by controlling access to

DNA sequences with post-translational histone modifications (PTMs), the use of histone variants or by altering nucleosome positioning. Similarly, post-translational modification or changes in expression level of non-histone chromatin-binding proteins could represent important mechanisms to regulate chromatin contacts. Additionally, noncoding RNAs (ncRNAs) and their protein complexes could regulate the three-dimensional architecture of our genome. ncRNAs are a broad class of RNAs consisting of structural (rRNAs, tRNAs, snRNAs, snoRNAs, etc.), regulatory (miRNAs, piRNAs, etc.), and of sense/antisense transcripts, whose functions remain mostly uncharacterized (Mattick 2009). RNA is an ideal molecule to regulate biological networks, since it encodes sequence information and possess a great structural plasticity. The intrinsic relevance of ncRNAs in the regulation of genomic output has been rapidly unveiled during the last decade (Kato et al. 2011; Liao et al. 2011). However, the functional elements in the primary sequence of the majority of ncRNAs that determine their regulatory role remain unknown.

This chapter will analyze the molecular aging events and discuss the possible role of small and long ncRNAs in the regulation of pathways and processes related to aging at the cellular level, emphasizing their importance as modulators of the aging-mediated deterioration of cell physiology.

2 Pathways and Key Topics of Human Cellular Aging

2.1 Molecular Damage as a Driving Factor for Cell Aging

In humans, aging is thought to correlate with a recession in function of its cells and tissues, namely immune and neuronal cells (Grolleau-Julius et al. 2010; Lu et al. 2004). At the tissue and organ level, aging can be also characterized by the accumulation of senescent cells. Senescence is a physiological process in which normal cells cease to divide and can be induced by nutrient starvation (replicative senescence), DNA damage, telomere shortening, or by the expression of some genes (oncogene-induced senescence) (Lopez-Otin et al. 2013). In normal tissues, senescent cells are part of a mechanism devoted to the tissue regeneration which selectively eliminates damaged and dysfunctional cells. Recently, Muñoz-Espín and Serrano (2014) proposed that the accumulation of senescent cells in aged tissues could be the result of the lack of proper clearance of damaged cells by the immune system.

At the cellular level, aging is characterized by the presence of increasing amounts of molecular damage, which leads to a physiological imbalance and decline of cell metabolic functions. How and when cellular functions begin to decline due to aging is unknown; however, this decline is founded within a molecular basis. It is difficult to determine whether this molecular damage is the main cause of aging, but its presence can be related to the impairment of the control mechanisms that happens during organism aging (Rattan 2008). Seminal research

by Wulf and coworkers showed early in the 1960s that the aging tissues and cells are unbalanced for the production of RNA molecules (Wulff et al. 1962). The authors postulated that the accumulation of mutations at the DNA level during aging would lead to the production of faulty RNAs, responsible at least in part for the aged phenotype. DNA damage leads to a misreading of the genetic information, and consequently to the possibility of faulty transmission of its message (Fukada et al. 2014). The correct functionality of the repair systems that correct DNA lesions has been related to an increased life span in mouse models (Brenerman et al. 2014).

The main sources of molecular damage during aging in DNA, RNA, proteins, and other biomolecules such as lipids come from free radicals or oxidative chemicals that can be originated either internally or externally to the cell (Fig. 1). In this context, the mitochondrial respiratory chain is responsible for the generation of reactive oxygen species (ROS), which include some free radicals, hydrogen peroxide, and the very reactive superoxide anion (Poyton et al. 2009). Extrinsic factors such as radiation and UV light can also trigger the production of ROS and free radicals. ROS are extremely reactive species, able to covalently modify many macromolecules, altering their functional and structural properties, and being responsible by the so-called oxidative stress (Nunomura et al. 2012; Di Domenico et al. 2010). Healthy cells harbor different mechanisms to destroy the free radicals and ROS in order to avoid their oxidative action over biomolecules, mainly based on the antioxidant molecules.

Enzymes such as superoxide-dismutase, catalase and thioredoxin and small organic molecules such as glutathione, are defense systems against the oxidative action of reactive chemical species (Mari and Cederbaum 2001; Sims-Robinson et al. 2013; Fukui and Zhu 2010). When the molecular damage is already caused, the disturbed biomolecule must be either repaired or destroyed. DNA molecules are typically repaired by several complex mechanisms involving macromolecular complexes assembled at the damaged *loci*, which are globally triggered in the presence of specific DNA lesions (Huen and Chen 2010; Lord and Ashworth 2012). In the unlikely event of an unrepaired DNA lesion, a global DNA damage response is activated and the cell will enter a cell-cycle arrest phase or become senescent in order to ensure genome maintenance and stability (Tian et al. 2014). DNA damage-induced senescence is also a natural mechanism to protect cells against cancer, but its relationship with the aging process is still not clear (Lieberman 2008; Tian et al. 2014). In mouse models during aging, some tissues appeared to be more prone to be enriched in senescent cells induced by DNA damage or telomere shortening (Wang et al. 2009). The same phenomenon is observed in human progeroid syndromes of accelerated aging like Werner's syndrome, where genetic mutations disrupt totally or partially the molecular machinery responsible for the genomic integrity (Pichierri et al. 2001). RNA molecules can also be targets of oxidative damage during aging as described previously in neural cells (Nunomura et al. 2012). These alterations would lead to faulty transcription and an imbalance in the cellular RNA content (Fig. 1). When the affected molecules are ncRNA transcripts, defects in their regulatory activities are also expected.

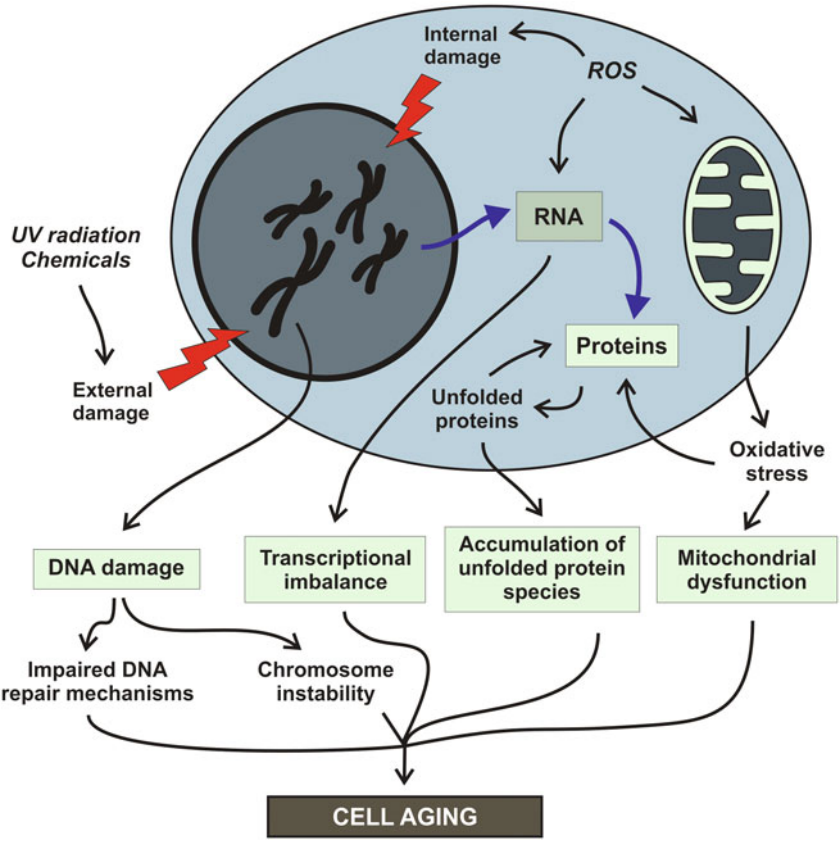

Fig. 1 Molecular damage induced by internal or external factors contribute to the cellular aging process, founded in four pillars: DNA damage, transcriptional imbalance, accumulation of unfolded proteins and mitochondrial dysfunction. The molecular imbalance observed in aged cells is mainly triggered by chemical or physical stress, produced from either internal or external sources. Chemical stress can be originated by external chemicals or by the reactive oxygen species (*ROS*) formed as a consequence of the cellular oxidative metabolism

Proteostasis, understood as the maintenance of a functional proteome, also declines with aging (Perez et al. 2009). A functional and healthy proteome is related to the chemical integrity of its components and their proper folding into a 3D space. Many cellular and external factors can challenge the proteome to cause protein instability or misfolding. Among them, the stress that lead to covalent modifications such as oxidation, the translational errors, and the presence of genome mutations are the most frequent. Misfolding can affect globular proteins or their domains when those have a consistent three-dimensional structure. In consequence, proteins lacking stable structure often denominated as intrinsically disordered proteins or IDPs are less sensitive to cellular stress and mutations (Light et al. 2013). Accumulation of misfolded proteins have negative consequences to the cell, since

mutated and destabilized proteins often expose hydrophobic regions that tend to aggregate or to interact with cellular structures (Chiti et al. 2003; Stefani and Dobson 2003).

Time-dependent decline in protein functions during aging induces a stress over the physiological mechanisms devoted to the clearance of faulty protein molecules, mainly the proteasome and the lysosomes (Miller et al. 2014; Taylor et al. 2011). Intermediate quality control sensors and effectors, also known as protein chaperones, are also submitted to pressure during aging due to the accumulation of unfolded proteins (Brehme et al. 2014; Taylor et al. 2011). In model systems such as *Caenorhabditis elegans,* recent work demonstrated that the levels of ribosomal and mitochondrial proteins were decreased in aged worms, supporting the notion that proteostasis is altered during organism aging (Liang et al. 2014). Moreover, mitochondrial enzymes of the Kreb's cycle and electron transport chain were diminished in aged animals, being consistent with the observed age-associated energy impairment (Ben-Zvi et al. 2009). Also in *Drosophila*, impaired proteasome function promoted aging phenotypes and reduced life span among individuals (Tsakiri et al. 2013). In humans, proteostasis networks centered in the protein chaperones have been characterized in relationship with neurodegenerative and aging-related diseases (Brehme et al. 2014). Interestingly, the mass spectrometry characterization of the proteome of human cells during aging also showed a consistent picture of decreased levels of proteins involved in cell death, cell differentiation and organization, response to stress, translation, RNA metabolism, and proteostasis control during aging (Waldera-Lupa et al. 2014).

2.2 Aging-Related Metabolic Pathways

Despite its multifactorial nature, aging is regulated by specific metabolic pathways including hormone-regulated signaling cascades and environmental nutrient sensing systems (Barzilai et al. 2012). The main cellular pathways involved in the control of life span in complex organisms are summarized in Fig. 2. All these pathways together form an entangled and interconnected regulatory framework which is part of the aging hallmarks (Lopez-Otin et al. 2013).

Insulin, insulin-like growth factor, and mTOR pathways showed crucial roles over organism life span, being highly conserved among species (Greer and Brunet 2008). IGF and insulin pathways are activated via their cognate membrane receptors inducing a signaling cascade centered in the AKT family of protein kinases that is related to a reduction in life span in model organisms (Miyauchi et al. 2004). On the other hand, the nutrient-dependent activation of mTOR pathway induces a metabolic alteration toward cell growth upon regulation of catabolism mediated by autophagy (Kapahi et al. 2010). Inhibition of this pathway extends life span in model organisms and confers protection against a wide range of age-related pathologies (Johnson et al. 2013). Autophagy, a well-characterized process that protects cell integrity by removing the damaged cell components is impaired during

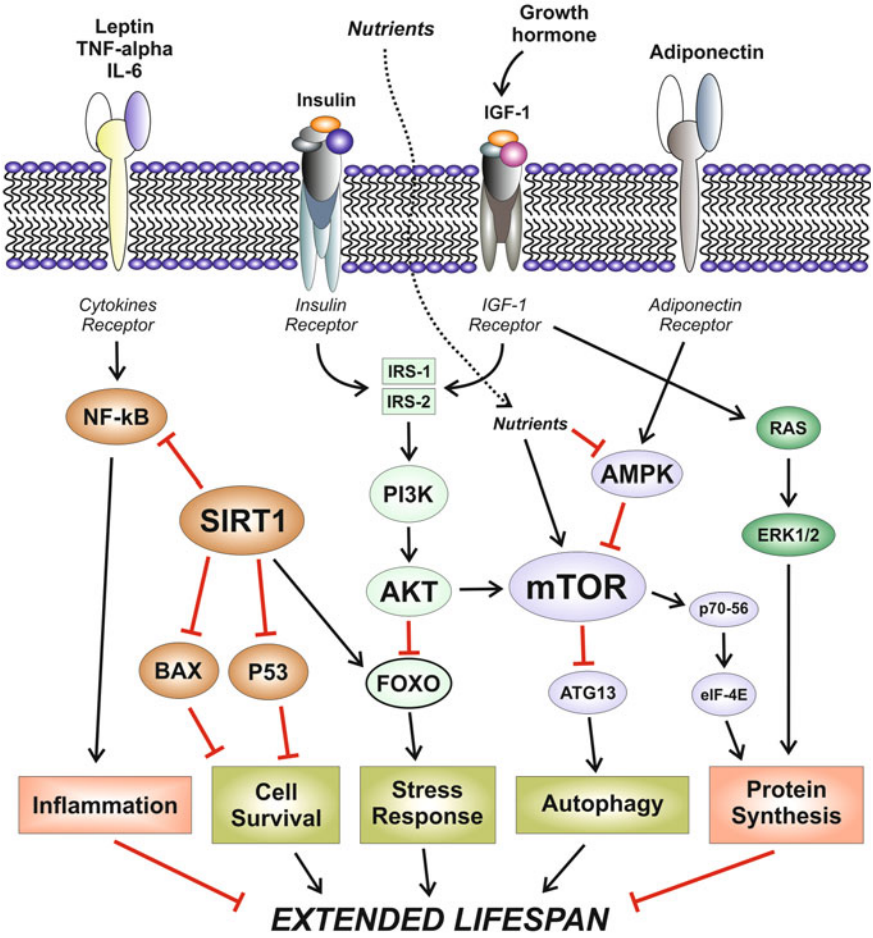

Fig. 2 Metabolic pathways involved in the control of life span in humans. Nutrients and different external signals can act as triggers for pathways that ultimately control the cell fate under diverse biological circumstances

aging leading to the accumulation of molecular damage. This phenomenon has been observed in model organisms and in some human tissues (Carnio et al. 2014; Zou et al. 2014). Moreover, aging can be also considered as a chronic low-intensity inflammation state, where cytokine activation of the NF-kB pathway plays an important role. This cytokine-mediated activation is extremely relevant in the global aging process of a particular organism since it can be related to the accumulation of senescent cells and their secretory phenotype which can collaborate to the tissue function impairment (Coleman et al. 2013). Additional modulators of cell survival like sirtuins which are responsible for an extended life span in complex organisms, as well as for the introduction of more complexity into the aging-related pathways (Michan 2014).

Within this context, the changes in the expression of genes encoding proteins involved in aging-related metabolic pathways have been used as quantifiable biomarkers and possible causes of aging. Next-generation sequencing technologies have improved the resolution and information obtained from transcriptional data related to aging. To date, multiple studies profiled age-related transcriptional changes in mouse and human cells revealed important insight into the molecular mechanisms of aging. Namely, a set of age-regulated genes were identified, including genes associated with immunity and the inflammatory response, metabolic energy and degradation pathways, and extracellular matrix components (de Magalhaes et al. 2009). A parallel approach relied on genetics to search for single-gene mutations that extend life span in model organisms. These studies found that mutations affecting genes of the insulin signaling pathway increase the life span of *C. elegans* (Kenyon et al. 1993), *Drosophila* (Satomura et al. 2001; Clancy et al. 2001), and mice (Bluher et al. 2003; Holzenberger et al. 2003). Despite such striking evolutionary conservation, the genes that appear differentially expressed in mutant nematodes, flies, and mice tend to be species-specific (McElwee et al. 2007), highlighting the importance of investigating biological processes rather than individual genes to understand the molecular mechanisms underlying aging. More recent work has further contributed to pinpoint an intimate interplay between age-related transcriptional changes including those observed in the noncoding genome, alterations in chromatin structure and epigenetic modifications, and persistence of irreparable DNA lesions in chromosomal and mitochondrial DNA (Burgess et al. 2012).

3 Small Noncoding RNAs in the Aging Context

Noncoding (nc) RNAs represent an additional layer of gene regulation implicated in aging (Jung and Suh 2012). The ncRNAs are a remarkably diverse universe of RNAs that are not templates for protein synthesis but can regulate their expression in the context of human physiology and pathology. Several classes of small (typically 20–30 nucleotides) and long (>200 nucleotides) ncRNAs have been identified and shown to act as key regulators of protein gene expression in several biological processes (Grammatikakis et al. 2014; Di Leva and Croce 2013; Jung and Suh 2012). The medical relevance of ncRNAs is well established, particularly for some of the small members of the group such as the miRNAs (Esteller 2011). The vast majority of miRNAs act as posttranscriptional repressors of protein gene expression by binding the untranslated regions (UTRs) of target mRNAs. The miRNA regulatory effect over a selected transcript is relatively mild and could be described as a "fine-tuning" mechanism of post-transcriptional regulation (Grosshans and Filipowicz 2008). In contrast, a single miRNA could act over hundreds of different mRNAs, constituting an overall control layer that modulated the products of gene expression. Taking into account this fact, it is very tempting to relate miRNAs and their mechanism of action with global cell phenomena such as differentiation,

senescence, cancer, or aging (Lafferty-Whyte et al. 2009; Bates et al. 2009b). MiRNAs have been implicated in many biological and pathological processes, ranging from development to cancer and life span (Jung and Suh 2012). Among them, the miRNA Lin-4 was first shown to regulate life span in *C. elegans* (Boehm and Slack 2005); lin-4 was subsequently found to be part of a group of miRNAs that change in expression as animals grow older (Ibanez-Ventoso et al. 2006). More recently, additional miRNAs were identified that influence life span in *C. elegans* both positively and negatively (de Lencastre et al. 2010). Age-related changes in miRNA expression were also reported in mouse brain (Inukai et al. 2012) and in human peripheral blood mononuclear cells (Noren Hooten et al. 2010). Also in model systems, a particularly interesting case is the Ames dwarf mouse, a mouse that shows increased delay in the onset of aging: miR-27a has been described as a main regulator of some intermediate metabolic enzymes that are related to the delayed aging of these animals (Bates et al. 2009a).

Moreover, the identification of mRNAs regulated by these miRNAs is further providing clues to understand how alterations in miRNA expression can contribute to the age-associated physiological decline. For instance, miR-146a, which is highly expressed in aged mice, down-regulates the expression of IL-1β and IL-6 leading to a lack of response of macrophages to proinflammatory stimuli (Jiang et al. 2012). Other aging-related pathways such as Wnt-mediated signaling (Vinas et al. 2013) and insuling/IGF-1 regulatory axis (Jordan et al. 2011) are also under the control of miRNAs. Interestingly, miRNAs are also regulatory players that can respond to hormonal stimuli, constituting feedback regulatory loops that ensure the tight control of metabolic signals (Martin et al. 2012).

Recent studies suggested that miRNAs and their biogenesis could control specifically the aging process by targeting several apparently unrelated genes. The nuclear work supporting this evidence has been performed using specific tissue or organs from aging mice. For instance, some murine miRNAs such as miR-93 and miR-214 have been found to be up-regulated in extremely old liver tissues (Li et al. 2009). Defects in the biogenesis of miRNAs have been also related to the induction of a senescence phenotype (Mudhasani et al. 2008), and the regulatory activities of miRNAs over cell aging and senescence-related pathways were proposed to act as pro- and antilongevity factors (Murphy 2010; de Lencastre et al. 2010). The term senescence was coined to describe cells that cease to divide in culture, assuming that this behavior recapitulates organism aging. However, several lines of evidence argue that replicative senescence and cell aging are not overlapping processes (Wennmalm et al. 2005; Bai et al. 2011). MiRNAs are also able to revert some senescence phenotypes induced by oncogenic factors such RAS (Borgdorff et al. 2010) and also to actively induce senescence and aging phenotypes in a variety of cell and organism models (Tazawa et al. 2007; Li et al. 2011; Liu et al. 2012).

It is well known that miRNAs can be actively secreted by cells, being detectable in all biological fluids. The mechanistic reasons for this phenomenon are far to be completely understood, but probably the secreted miRNAs could function as slow-action hormones able to regulate gene expression within cells located in organs or tissues far from they were synthesized (Creemers et al. 2012). Circulating

miRNAs have been also considered as powerful biomarkers for the diagnosis and prognosis of several human conditions including aging (Weilner et al. 2013). Several authors proposed an active role for circulating miRNAs during aging, for instance acting as modulators of the chronic inflammatory phenotype observed in aged individuals (Olivieri et al. 2013b). Recently, a group of up-regulated age-related circulating miRNAs has been identified in mouse models. This group of circulating miRNA appeared to be up-regulated in aged animals and this effect can be reverted by caloric restriction. The genes targeted by this cohort of age-modulated circulating miRNAs are predicted to regulate biological processes linked to the phenotypic manifestations of aging, including metabolic changes, demonstrating the growing importance of this circulating regulators and their roles in the global context of organisms aging (Dhahbi et al. 2013).

4 Regulatory Long Noncoding RNAs as Modulators of Aging Metabolic Pathways

4.1 Common Functional Features of lncRNAs Within the Aging Transcriptional Landscape

In addition to miRNA genes, the human genome contains over 15,000 long noncoding RNA genes (lncRNAs) (Volders et al. 2013; Bu et al. 2012). This class of RNAs are by definition >200 bp in length, lacking significant protein-coding capacity. Their synthesis and structure are similar to protein-coding mRNAs, as they contain introns, have their 5′ and 3′ ends capped, and are frequently poly-adenylated. These transcripts have initially been suggested to represent only the bystander's transcription within protein-coding regions. However, histone markers of active transcription have been identified them outside protein-coding regions (Guttman et al. 2010). A subset of lncRNAs can be highly cell- and tissue-specific (Guttman et al. 2011) and show precise temporal specific patterns of expression as well as a certain degree of evolutionary conservation (Cabili et al. 2011). Meanwhile, only a small number of thousands of known noncoding RNAs have been implicated in a specific biological function.

Loss-of-function experiments have provided further evidence of lncRNAs functional importance on the regulation of gene expression patterns that control cell pluripotency, differentiation and survival, as well as epithelial-to-mesenchymal transition (Beltran et al. 2008). They also act as regulators of development and morphogenesis (Ulitsky et al. 2011), chromosomal dosage compensation (Tian et al. 2010), control of imprinting (Sleutels et al. 2002), cell-cycle regulation, and alternative splicing (Tripathi et al. 2010). LncRNAs exert the regulatory function in *cis*, modulating nearby genes on the same allele, or in *trans* by affecting genes at long genomic distances (Court et al. 2011). LncRNAs also interact with genomic DNA as well as RNA, and they function as flexible molecular scaffolds for the

recruitment of chromatin modifying enzymes and transcription factors (Saxena and Carninci 2011; Gupta et al. 2010), driving their correct localization to genomic DNA targets. LncRNAs have also been shown to regulate the activity of other ncRNAs, specifically miRNAs, by acting as "sponges" that titrate miRNAs away from natural mRNA targets (thereby acting as competing endogenous RNAs; ceRNAs) (Cesana et al. 2011). However, the role of lincRNAs in cell aging needs to be further investigated.

Despite the lack of experimental data, an altered expression pattern of the noncoding transcriptome is also expected in aging. In fact, lncRNAs are known to be involved in the control and regulation of cell fate decisions, including cell lineage commitment (Lin et al. 2014) and stemness (Guttman et al. 2011). Similar regulatory circuits based on the ncRNAs have been proposed to be on the basis of the age-dependent evolution of some human diseases such as cognitive disorders (Qureshi and Mehler 2011). In a small number of cases, the noncoding transcriptome was used to characterize the aging process and their phenotypic consequences (Chang et al. 2013). Also very recently, a specific cohort of lncRNAs has been characterized as implicated in replicative cell senescence (Abdelmohsen et al. 2013). However, in the majority of the studied cases, the relationships between lncRNAs and aging can only be depicted by the particular regulatory action exerted over a gene or an aging-related pathway, and not over the global aging process (Fig. 3). Moreover, these regulatory mechanisms have been frequently characterized outside the aging phenomenon itself and related to other biological problems such as cell differentiation, lineage commitment, or cancer (Table 1).

4.2 lncRNAs and DNA Damage

As already discussed, the cell capacity to respond to DNA damage is essential to avoid the deleterious accumulation of functional mutations during aging (Jackson 2009). Several lncRNAs have been recently characterized as regulator of the cellular DNA damage response. One of the initial evidences of the regulatory role of a lncRNA on the DNA damage response was observed for RoR, a strong negative regulator of P53. Interestingly, and unlike other P53 regulators such as MDM2 which causes an ubiquitin-mediated P53 degradation, the lncRNA RoR suppresses the translation of P53 protein by direct interaction with hnRNPI (Zhang et al. 2013).

Other lncRNAs acting as modulators of the DNA damage response (DDR) include the CDKN2B antisense transcript also known as ANRIL (Wan et al. 2013b). Globally, ANRIL contributes to the maintenance of cellular responses triggered by DNA damage, via its regulation of cell-cycle checkpoints, apoptosis, and DNA repair (Wan et al. 2013b). ANRIL is transcriptionally activated by E2F1, and functions as homeostatic regulator by inhibiting P53 protein and thus bringing down the DNA damage response. In the particular case of cancer cells, the aberrant expression of this lncRNA would imbalance the DDR and eventually cause the blockage of this defense mechanism (Wan et al. 2013b).

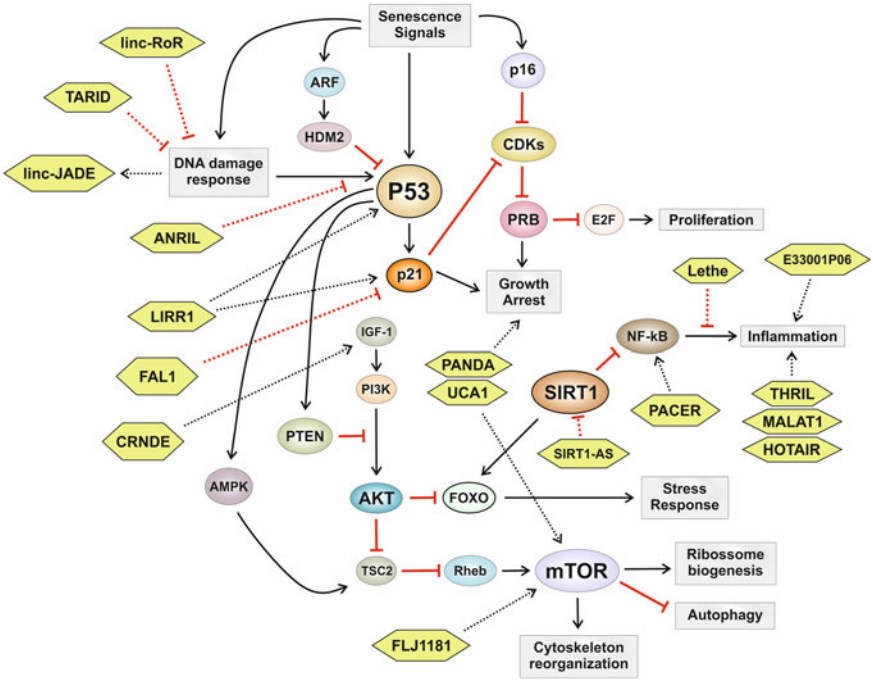

Fig. 3 Potential regulatory role of selected lncRNAs within the aging pathways. Aging-related pathways are schematically represented using only the main key players, connected with lines to pinpoint their regulatory relationships. The lncRNAs are depicted in *solid hexagons*, connected with their regulated targets by *dotted lines*

The role of chromatin structure in DDR has been extensively studied, including chromatin modifications. Recently, the lncRNA-JADE which is induced after DDR has been characterized as an inducer of histone H4 acetylation. The histone acetylation is ensured via activation of the closing coding gene JADE1, a component of the HBO1 histone acetylation complex (Wan et al. 2013a).

More recently, a group of long intergenic radiation-responsive ncRNAs (LIRRs) have been shown to have an important role in the p53-mediated DDR. The expression of these lncRNAs is induced after a radiation-induced cell injury. A member of this family, LIRR1, has been characterized as an important regulator of the DDR. Its overexpression in human cells led to a decreased expression of several DNA repair proteins, an activation of p53, induction of p21 expression, and a cell-cycle G1 phase arrest (Jiao et al. 2015).

Other lncRNAs potentially involved in the mechanisms of DNA damage repair are TARID, which has been characterized as a regulator of DNA demethylation involved in base excision repair (Arab et al. 2014) and PCAT-1, a lncRNA identified in prostate cancers which negatively regulates the homologous recombination mechanism via repression of the tumor suppressor BRCA2 (Prensner et al. 2014).

Table 1 Long noncoding RNAs with demonstrated regulatory activity over processes and pathways involved in cellular and organism aging

lncRNA	Process	Comments	Reference
ANRIL	DNA damage	ANRIL is transcriptionally up-regulated by the transcription factor E2F1 in an ATM-dependent manner following DNA damage	Wan et al. (2013b)
CCAT2	Wnt signaling	CCAT2 interacts with TCF7L2 resulting in an enhancement of WNT signaling activity	Ling et al. (2013)
CRNDE	Insulin pathway	lncRNA regulated by insulin/IGFs and related to nuclear transcripts involved in the modulation of cellular metabolism	Ellis et al. (2014)
E330013P06	Insulin pathway	Its overexpression in macrophages from type 2 diabetic mice induced inflammatory genes	Reddy et al. (2014)
ERIC	DNA damage	Inhibition of ERIC expression increased E2F1-mediated apoptosis in a negative feedback loop that modulates E2F1 activity	Feldstein et al. (2013)
FAL1	Senescence	Association by the epigenetic repressor BMI1 and modulation of expression of CDKN1A	Hu et al. (2014)
FLJ11812	mTOR pathway	Derived from the 3′ untranslated region (3′UTR) of TGFB2, it could bind with miR-4459 targeting ATG13 (autophagy-related 13)	Ge et al. (2014)
GAS5	Apoptosis	Promoted apoptosis by PI3 K/mTOR inhibition	Pickard and Williams (2014)
HOTAIR	Inflammation	IL-6 up-regulates HOTAIR in an autocrine manner, contributing to the EMT and defining a link between inflammation and EMT in malignant cell transformation	Liu et al. (2015)
	Senescence	Up-regulated in senescent cells as a mechanism to prevent premature senescent	Yoon et al. (2013)
	Wnt signaling	Repressed by Wnt/β-catenin signaling	Carrion et al. (2014)
JADE	DNA damage	Transcriptionally activates Jade1, a key component in the HBO1 histone acetylation complex	Wan et al. (2013a)
Lethe	Inflammation	Selectively induced by proinflammatory cytokines via NF-κB or glucocorticoid receptor agonist, and functions in negative feedback signaling to NF-κB	Rapicavoli et al. (2013)
LIRR1	DNA damage	Regulation of DNA damage response in a p53-dependent manner	Jiao et al. (2015)
IL7R	Inflammation	Regulation of inflammatory mediators by epigenetic control of promoters	Cui et al. (2014)
MALAT1	Inflammation	The cross talk between MALAT1 and p38 MAPK signaling pathways is involved in the regulation of endothelial cell function and inflammation	Liu et al. (2014)

(continued)

Table 1 (continued)

lncRNA	Process	Comments	Reference
NEAT1	Senescence	Interaction with AUF1 to preserve genomic integrity and prevent premature senescence	Yoon et al. (2014)
lncRNA-p21	Wnt signaling	Promotes cell apoptosis by suppression of the β-catenin signaling pathway and elevation of the pro-apoptosis gene Noxa expression	Wang et al. (2014a)
PACER	Inflammation	It associates with p50, a repressive subunit of NF-kappaB and occludes it from the COX-2 promoter, potentially facilitating interaction with activation-competent NF-kappaB p65/p50 dimers	Krawczyk and Emerson (2014)
PANDA	Senescence	It interacts with senescence-promoting genes via PCR1 and PRC2	Kumar et al. (2014)
PCAT-1	DNA damage	Expression of PCAT-1 produces a functional deficiency in homologous recombination through repression of BRCA2 gene	Prensner et al. (2014)
RoR	DNA damage	RoR inhibits p53-mediated cell-cycle arrest and apoptosis in response to DNA damage	Zhang et al. (2013)
Sirt1-AS	Sirtuin pathways	Overexpression of Sirt1 AS lncRNA increased the levels of Sirt1 protein	Wang et al. (2014b)
TARID	DNA damage	TARID interacts with both the TCF21 promoter and GADD45A (growth arrest and DNA-damage-inducible alpha), a regulator of DNA demethylation	Arab et al. (2014)
THRIL	Inflammation	THRIL expression was correlated with the severity of symptoms in patients with Kawasaki disease, an acute inflammatory disease of childhood	Li et al. (2014a)
UCA1	mTOR pathway	UCA1 activates mTOR to regulate HK2 through both activation of STAT3 and repression of microRNA143	Li et al. (2014b)
	Senescence	Oncogenic-induced senescence by interaction by sequestering of hnRNPA1 and stabilization of CDKN2A-p16INK complex	Kumar et al. (2014)
	Wnt signaling	UCA1 positively regulates expression of wingless-type MMTV integration site family member 6 (Wnt6) in human bladder cancer cell lines	Fan et al. (2014)

4.3 lncRNAs and Inflammation

Human aging is characterized by a low-degree chronic inflammatory state, being a significant risk factor for morbidity and mortality in elderly individuals. The etiology of human chronic inflammation during aging remains unknown; however, the identification of pathways and modulators that control this phenotype is important in order to understand whether specific treatments that control inflammation can be beneficial to elderly people (Franceschi and Campisi 2014). In this context, the role of noncoding RNAs, inflammation, and aging has been extensively explored for the case of the miRNAs and reviewed elsewhere (Olivieri et al. 2013a). On the other hand, the evidences of the regulatory role of lncRNAs on the inflammation process are relatively more recent and derived from isolated observations.

Probably, one of the first lncRNAs characterized as a modulator of the inflammatory signals is Lethe. This mouse lncRNA is selectively induced by proinflammatory cytokines via NF-kappaB or glucocorticoid receptor agonists, and functions as a negative regulator in a feedback signaling to NF-kappaB. Lethe is able to interact with the RelA subunit of the NF-kappaB, inhibiting the RelA binding to the DNA targets and their activation (Rapicavoli et al. 2013). Interestingly, Lethe decreases with the organism aging, which is associated with an increase in the proinflammatory signals mediated by NF-kappaB pathway in several human tissues (Maqbool et al. 2013; Sriram et al. 2011).

Another lncRNA, PACER (p50-associated COX-2 extragenic RNA), has been recently characterized as a modulator of the inflammation also within the cancer context; however, its regulatory functions could be extended far from this disease to the overall inflammatory phenotype observed in aging (Krawczyk and Emerson 2014). PACER lncRNA is able to interact with p50, a repressive subunit of the NF-kappaB leading to an activation of competent NF-kappaB p65/p50 dimers. This mechanism will further enable the recruitment of histone acetyltransferases, a genome-wide histone acetylation, and RNApol II initiation complex assembly, constituting a global modulator of the inflammatory process (Krawczyk and Emerson 2014). In the same context, a lncRNA transcript which partially overlaps the gene encoding the interleukin-7 receptor alpha-subunit (IL7R) designated as lnc-IL7R has been characterized as a modulator of the inflammatory response via epigenetic regulation of the promoters of several inflammatory mediators (Cui et al. 2014). Indirect evidences have also linked the role of MALAT1 (Liu et al. 2014) and HOTAIR (Liu et al. 2015) lncRNAs to the regulation of the inflammatory response.

Also in the case of acute inflammatory events, the role of lncRNAs is becoming to be relevant. Recently, Li and coworkers have characterized a group of around 160 lncRNA founded to be differentially expressed upon innate activation of THP1 macrophages (Li et al. 2014a). Among them, a lincRNA called THRIL (TNFalpha

and hnRNPL related immunoregulatory lincRNA) was required for expression of many immune response genes including cytokines and transcriptional and post-transcriptional regulators of TNFalpha expression (Li et al. 2014a). The authors were also able to correlate the levels of THRIL lncRNA with the severity of the symptoms of acute inflammatory diseases as Kawasaki syndrome.

4.4 Regulation of Senescence by lncRNAs

Senescence is an essential process to understand organism aging, since aged tissues have the tendency to accumulate senescent cells. The senescent phenotype can be reached by several biological routes involving different external stimuli and signaling cascades (Munoz-Espin and Serrano 2014). Early work by Gorospe's laboratory showed that human senescent cells are characterized by a specific pattern of differentially expressed lncRNAs when compared to replicative cells. (Abdelmohsen et al. 2013). Further work has described different lncRNAs involved in the modulation of the senescence process.

HOTAIR lncRNA is clearly up-regulated in senescent cells, being associated with ubiquitin ligases to constitute a platform for protein ubiquitination. In senescent cells, HOTAIR helps to ubiquitinate Ataxin-1 and Snurportin-1, accelerating their degradation and preventing premature senescence (Yoon et al. 2013). Another lncRNAs such as UCA1 is involved in a more directed control of the senescence process. In fact, the lncRNA UCA1 is able to bind and sequester hnRNPA1, stabilizing the CDKN2A-p16INK complex and inducing senescence (Kumar et al. 2014). Interestingly, down-regulation of NEAT1, a lncRNA located in nuclear paraspeckles, has been also related to the induction of replicative senescence since it controls the overall nuclear organization (Yoon et al. 2014).

Also recently, Kumar and coworkers characterized a lncRNA called PANDA that is able to differentially interact with polycomb repressive complexes (PRC1 and PRC2) and the transcription factor NF-YA to promote or suppress senescence. In proliferation cells, the scaffold-attachment protein factor SAFA and the PANDA lncRNA recruit polycomb complexes to repress senescence-promoting genes (Puvvula et al. 2014).

In this context, study of several types of tumors and their development allowed the identification of an additional lncRNA denominated as FAL1 (Focally amplified lncRNA on chromosome 1), which was overexpressed in cancers with poor outcome. Molecular characterization of FAL1 transcript determined its ability to interact with the epigenetic repressor BMI1 to modulate the transcription of some genes including CDKN1A. FAL1 overexpression in tumors maintains the cells in the proliferative state. In consequence, FAL1 can be considered as a classical oncogene, mainly because of its ability to repress p21, a CDK inhibitor which is an inductor of senescence (Hu et al. 2014).

4.5 Regulatory LncRNAs and the Insulin Pathway

The insulin/IGF-1 metabolic axis is an essential regulatory pathway that is involved in organism development and aging. In fact, the levels of growing hormone and IGF-1 declined with aging. Low peripheral levels of IGF-1 are associated with increased aging-dependent risk of several conditions such as sarcopenia and osteoporosis (Barzilai et al. 2012). Moreover, in humans the aging process is accompanied by a phenomenon known as "insulin resistance" (IR), characterized by a lack of response of insulin receptors across the body. The IR syndrome is compensated by a hyperinsulinemia which can be considered as a risk factor for age-related diseases (Erol 2007). Epigenetic factors, including the regulatory effects of the noncoding transcriptome, could be potential modulators of this age-dependent decline of the insulin signaling pathway (Koerner et al. 2012).

One of the first lncRNAs characterized as a direct global regulator of the insulin signaling pathway is CRNDE. This lncRNAs has been firstly characterized as an overexpressed noncoding transcript in human colorectal cancer, being able to promote metabolic changes to support the aerobic glycolytic metabolism in cancer cells. Selective knockdown of CRNDE lncRNA by RNAi experiments affected the expression of many genes, which showed correlation with insulin/IGF-1 signaling pathway components and responses, including lipid and sugar metabolism (Ellis et al. 2014).

Other recently characterized lncRNAs included E330013P06, a mouse lncRNA up-regulated in macrophages obtained from diet-induced insulin-resistant type 2 diabetic mice, but not in type 1 diabetic mice. Reddy and coworkers determined that this lncRNA must constitute a link between insulin and inflammation pathways, since its knockdown inhibited the expression of inflammatory genes induced by diabetic stimuli (Reddy et al. 2014).

4.6 Regulatory lncRNAs in the WNT, mTOR, and Sirtuin Pathways

Aging-related metabolic pathways are closely related to those observed as dis-regulated in tumors, empowering the propensity of elderly people to suffer cancer. For instance, WNT and downstream effectors regulate processes that are relevant for cancer progression such as cell senescence and death which are also significant for complex organism aging (Anastas and Moon 2013). Some lncRNAs have been characterized recently as possibly involved in the regulation of WNT signaling pathway. The most relevant is probably lncRNA-p21, a long noncoding RNA which represses the WNT/β-catenin signaling axis (Wang et al. 2014a). Inversely, CCAT2 a lncRNA related to metastases in colon cancer has shown to be an enhancer of WNT signaling activity. Its mechanism of action involves a direct

interaction with the TCF7L2 transcription factor, being itself also a downstream target of WNT (Ling et al. 2013).

Additional aging-related pathways such as mTOR signaling are also susceptible to the modulation exerted by lncRNA. In this context, the work by Li and coworkers proposed a new role for the UCA1 lncRNA (Li et al. 2014b). Experimental evidences linked the molecular regulatory mechanism of UCA1 lncRNA to the glucose and energy metabolism. This lncRNA is able to induce the expression of hexokinase 2 (HK2) in tumor cells by a mechanism that involves the activation of mTOR pathway (Li et al. 2014b). Regulation of mTOR signaling in the context of aging is related in part with autophagy. Also very recently, a lncRNA designated as FLJ1181 and derived from the 3′-UTR of the TGFB2 gene was characterized as a complementary endogenous ncRNA (ceRNA) involved in the regulation of autophagy via mTOR (Ge et al. 2014). Complementary endogenous RNAs or ceRNAs are lncRNAs which sense and capture miRNAs, acting as sponges that remove miRNAs from their action places. FLJ1181 binds miR-4459 which is a regulator of the autophagy-related 13 protein (ATG13). In consequence, FLJ1181 is a mTOR activator which acts as a link with the autophagy process (Ge et al. 2014).

Sirtuins are a wide group of enzymes with deacylase or mono-ADP ribosyl-transferase activity, classically related to cell differentiation processes and also with aging and extended life span in complex organisms (Liu and Sun 2011; Mantel and Broxmeyer 2008). Some recent evidences have pointed out the possible role of ncRNAs in the regulation of sirtuin activity. Wang and coworkers identified a natural antisense transcript (NAT) derived from divergent antisense transcription of Sirt1 gene (Wang et al. 2014b). This NAT has been characterized in myogenic differentiation of mouse model cells and showed regulatory activity of the Sirt1 gene. Due to their ubiquity, diversity of functions, and inter-species conservation, NATs are good functional candidates to be studied in within the context of the aging process (Werner 2013).

5 Conclusions and Further Perspectives

Eukaryotic genomes are pervasively transcribed into hundreds of RNA transcripts, many of them without evident capacity for coding proteins. The degree of organism complexity strongly correlates with the relative proportion of noncoding DNA in their genomes. Noncoding RNA transcripts have been pointed out as essential modulators of many biologically relevant processes. The ability of noncoding RNAs to regulate biological processes is mainly related to the intrinsic nature of the RNA molecules, able to carry sequence information as DNA but also to fold into complex structures and to have catalytic activity as proteins.

The specific role of some families of ncRNAs such as miRNAs and lcnRNAs is starting to be unveiled. As described along this review, some of the evidences pointing specific ncRNAs to their regulatory functions within the aging context are

still circumstantial and in the majority of the cases extracted in an indirect fashion using aging-related diseases or cancer. Taking into account the complexity of the pathways and regulatory events involved in human aging, a single "master regulator" is not expected. Moreover, the accumulation of data obtained with the study of specific metabolic pathways involved in aging clearly suggests the presence of an important regulatory layer modulating aging-related processes which is ensured by the action of specific ncRNAs. Indeed, the aberrant ncRNA expression could be a new factor contributing to aging and aging-associated conditions in humans. The presence of aberrantly expressed ncRNAs in aging-related diseases opens room for RNA-based therapeutics using oligonucleotide-based drugs.

Our knowledge of the roles and rules of the noncoding transcriptome within the human aging context is still in its infancy, with only a few examples of miRNAs and lncRNAs characterized as regulators of aging-related pathways. One of the main weaknesses to develop functional aging studies is the lack of strong models of the process, which is more relevant in the case of the ncRNAs since they are not conserved across species. Future trends need to be focused in the development of new aging models, but also on the dissection of the molecular mechanism underlying the action of the already characterized ncRNAs and in the discovery of new relevant ones. The use of new techniques to characterize the function and structure of the genome at its output will be essential to understand the particular role of each ncRNA in the complex aging landscape. In this context, the combination of chromosome conformation capture techniques with the determination of structural features of the transcribed RNAs will open a new field of research to understand the wide range of functional genomic changes associated with the aging process and the role of ncRNAs in the regulation of these events.

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lncRNAs in Stress Response

Saba Valadkhan and Alberto Valencia-Hipólito

Abstract All living organisms sense and respond to harmful changes in their intracellular and extracellular environment through complex signaling pathways that lead to changes in gene expression and cellular function in order to maintain homeostasis. Long non-coding RNAs (lncRNAs), a large and heterogeneous group of functional RNAs, play important roles in cellular response to stressful conditions. lncRNAs constitute a significant fraction of the genes differentially expressed in response to diverse stressful stimuli and, once induced, contribute to the regulation of downstream cellular processes, including feedback regulation of key stress response proteins. While many lncRNAs seem to be induced in response to a specific stress, there is significant overlap between lncRNAs induced in response to different stressful stimuli. In addition to stress-induced RNAs, several constitutively expressed lncRNAs also exert a strong regulatory impact on the stress response. Although our understanding of the contribution of lncRNAs to the cellular stress response is still highly rudimentary, the existing data point to the presence of a complex network of lncRNAs, miRNAs, and proteins in regulation of the cellular response to stress.

Contents

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1 Introduction

Living systems have evolved highly sophisticated molecular networks to monitor changes in intracellular and extracellular environment and launch complex responses against harmful disturbances to maintain cellular homeostasis and viability. These cellular responses, collectively termed the cellular stress response, are induced upon exposure to a wide variety of stimuli including changes in temperature, altered oxidative state, ischemia, harmful radiations, and changes in availability of nutrients. The stressful stimuli can originate from the environment, or result from organismal responses to diseases such as cancer or infection, which result in stressful conditions including hypoxia, acidosis, and oxidative stress among others (Dandekar et al. 2015). Further, some stressful conditions such as hypoxia occur during normal development (Saunders 1966; Caniggia et al. 2000). The cellular response to each stressful condition is tailored to mitigate the damage induced by the specific stressor and reprioritize appropriate aspects of cellular function in order to maintain homeostasis under suboptimal conditions. As a final safeguard, failure to curtail the stress-induced damage will activate programmed cell death pathways to eliminate the irreversibly impaired cells in order to ensure organismal viability.

As mentioned above, the cellular response to stress involves the activation of complex signaling pathways, many of which lead to transcriptional cascades aiming to alter the cellular transcriptome and proteome to optimally combat the damage induced by the stressor. This response also frequently includes reprioritization of cellular gene expression away from normal housekeeping expression pattern toward optimal expression of stress response factors (Johnson and Barton 2007; Velichko et al. 2013; Liu and Qian 2014; Espinosa-Diez et al. 2015). The result is profound changes in the composition of cellular RNAs and proteins, many aspects of which have been studied and shown to play critical roles in alleviating the impact of stress. While the study of the function of many proteins and miRNAs in stress response has provided us with a basic understanding of the regulation of this critical aspect of cellular homeostasis (Sunkar 2010; Leung and Sharp 2010; Majmundar et al. 2010; Richter et al. 2010; Cannizzo et al. 2011; Arrigo and Pulliero 2015), the role of a large class of functional cellular transcripts, the long non-coding RNAs (lncRNAs), has remained largely unstudied (Amaral et al. 2013).

1.1 lncRNAs Are an Abundant and Functionally Diverse Class of Cellular Transcripts

The discovery of the extent of non-coding transcription from higher eukaryotic genomes is perhaps the most significant outcome of the high-throughput transcriptome analysis efforts (Rinn and Chang 2012; Morris and Mattick 2014). Indeed, while less than 2 % of the genome in mammalians codes for protein-coding transcripts, a much larger fraction is transcribed into tens of thousands of long RNAs that do not seem to have protein-coding capacity (ENCODE Project Consortium et al. 2012; Clark et al. 2013). lncRNAs are a highly heterogeneous class of transcripts, and some can be tens of thousands of nucleotides long and include a large number of unspliced and non-polyadenylated transcripts (Djebali et al. 2012; ENCODE Project Consortium et al. 2012; Engelhardt and Stadler 2015). Although originally an arbitrary lower length limit of 200 nucleotides was proposed, it should not be applied too strictly as it mainly serves to distinguish lncRNAs from the small non-protein-coding cellular RNAs such as snRNAs, snoRNAs, and miRNAs which have distinct functions and modes of action (Clark and Mattick 2011; Rinn and Chang 2012; Mattick and Rinn 2015). While the recent availability of high-throughput transcriptome analysis technologies has revealed the existence of tens of thousands of lncRNAs, it is likely that many more such transcripts remain to be discovered. This is partly due to the fact that a large fraction of lncRNAs, unlike their protein-coding counterparts, are expressed in a strongly cell type- and state-specific manner (Rinn and Chang 2012; Amaral et al. 2013; Gloss and Dinger 2015). In fact, almost every RNA-seq experiment of sufficient depth can potentially yield novel lncRNAs that are highly specific to the cell type and experimental conditions of the study. Further, a significant fraction of protein-coding loci yield alternatively transcribed or processed RNAs that lack protein-coding capacity and thus are categorized as lncRNAs (Carninci et al. 2005; Djebali et al. 2012; ENCODE Project Consortium et al. 2012).

Due to the large number and relatively recent discovery of the vast majority of lncRNAs, most of them remain unstudied. However, emerging evidence from functional analysis of a small number of lncRNAs indicates the involvement of this class of transcripts in virtually every aspect of cellular function (Wapinski and Chang 2011; Rinn and Chang 2012; Moran et al. 2012; Clark et al. 2013; Young and Ponting 2013; Amaral et al. 2013; Ulitsky and Bartel 2013; Dey et al. 2014; Yang et al. 2014b; Kapusta and Feschotte 2014). Many lncRNAs seem to be predominantly or even exclusively nuclear-localized, and consistently, their expression mostly impacts various aspects of nuclear events including regulation of the epigenetic state of chromatin and transcription (Rinn and Chang 2012; Amaral et al. 2013; Rinn 2014; Quinodoz and Guttman 2014). Indeed, many lncRNAs associate with chromatin modifying complexes and likely affect the genomic localization or other aspects of function of these complexes (Khalil et al. 2009; Hung and Chang 2010; Rinn and Chang 2012). In addition to the identity of the proteins with which a lncRNA interacts, the genomic locus of transcription of

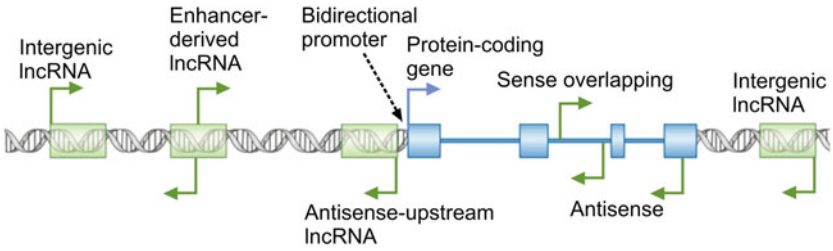

Fig. 1 Diverse genomic origins of lncRNAs. The genomic position of lncRNAs relative to other genes is shown. The broken arrows mark the location of transcription start sites and direction of transcription

lncRNAs can yield crucial mechanistic insights into their potential function (Fig. 1). Many lncRNAs fall into the "intergenic" category of lncRNAs, as they arise from genomic loci that are located far from other annotated genes. In contrast, other lncRNAs either overlap with other genes, or are located in the vicinity of other genes without overlapping with them or with their promoter or 3′ processing sequences. In many studied cases, the expression of such "vicinal" intergenic lncRNAs affects the expression of their neighboring genes through transcriptional interference or epigenetic regulation (Valadkhan and Nilsen 2010; Rinn and Chang 2012; Mattick and Rinn 2015). Other lncRNA genes can overlap the genic region or promoter/3′ processing sequences of protein-coding genes or other non-coding RNA genes in the sense or antisense orientation (Fig. 1). Such RNAs can originate from a promoter within an exon or intron of the overlapped gene, or from promoters located within the 3′ processing sequences of the overlapped gene or even further downstream. The expression of studied overlapping genes frequently affects the biogenesis or function of the other genes in the locus through several mechanisms, including epigenetic regulation of the activity of the entire locus, transcriptional interference, or masking of functionally critical elements through basepairing to the other transcripts originating from the overlapped locus (Valadkhan and Nilsen 2010). Transcription from the so-called bidirectional promoters is another frequently observed conformation of lncRNA loci (Fig. 1) (Adachi and Lieber 2002; Uesaka et al. 2014; Wakano et al. 2012), and in a number of studied examples, one member of the promoter-sharing pair regulates the expression of the other RNA (Wei et al. 2011; Uesaka et al. 2014). Finally, many lncRNAs are transcribed from promoters in enhancer loci, and emerging evidence suggests that these transcripts play important roles in the function of the enhancers from which they originate (Fig. 1) (Lam et al. 2014; Gardini and Shiekhattar 2015). Thus, analysis of the locus of a lncRNA may help guide the study of its potential cellular function. As will be discussed in the following sections, a number of studied lncRNAs are involved in regulation of diverse aspects of stress response in all kingdoms of life, including in bacteria, unicellular eukaryotes, plants, and animals (Amaral et al. 2013). In many

of the examples discussed below, the genomic location of the lncRNA shows a clear correlation with its function, indicating the importance of the analysis of lncRNA loci in the study of their function.

1.2 The Importance of Being RNA

Expansion of the non-coding transcriptome in higher eukaryotes has led to the evolution of RNA-mediated regulatory networks in nearly every aspect of cellular function. Indeed, it has been noted that the rise in RNA-mediated regulation is concomitant with the evolution of complexity (Taft et al. 2007). As a biological macromolecule, RNA has many unique properties which complement those of proteins and makes it particularly suitable for many cellular functions. For example, RNAs can easily recognize and bind to another nucleic acid molecule. Through the formation of RNA–RNA basepairing interactions, a regulatory RNA can sequester its target RNA, or stabilize it, or mask its cognate sequence from other regulatory elements such as proteins or other RNAs (e.g., miRNAs). Through base triplex formation with DNA and simultaneous interaction with proteins, RNAs can help increase the local concentration of proteins in a certain genomic locus (Rinn and Chang 2012; Rinn 2014).

These RNA-mediated regulatory and targeting strategies are particularly beneficial considering the much lower energetic requirement for RNA synthesis, compared to the synthesis of a protein which also involves the energetic cost of translation and folding. This can be especially helpful in cases of starvation stress or other metabolic stresses, when lowering the energetic cost of induction of stress response is highly desirable. Further, some regulatory RNAs, such as intronic lncRNAs, originate from otherwise degraded products of gene expression (Ayupe et al. 2015). Further, it has been shown that mammalian promoters are inherently bidirectional; however, the RNAs transcribed in one of the two possible directions are frequently degraded immediately after being made (Almada et al. 2013; Ntini et al. 2013; Marquardt et al. 2014; Grzechnik et al. 2014). Interestingly, some cellular lncRNAs are the result of stabilization of such "waste" RNAs generated in bidirectional promoters (Adachi and Lieber 2002; Uesaka et al. 2014; Wakano et al. 2012), further reducing the energetic cost of lncRNA-mediated regulation. Such RNAs also constitute an ideal means for the regulation of their promoter sharing transcript, since they can easily function as a homing scaffold for the recruitment of regulatory protein factors, eliminating the need for protein-mediated recognition of genomic loci (Rinn and Chang 2012; Rinn 2014). In addition to the energetic cost of the extra steps involved in protein synthesis, generating an RNA regulator is much less time-consuming than making a protein. Thus, in cases such as stress response when launching a rapid cellular protective response is of paramount importance, use of RNA confers a significant advantage.

Further, RNAs can evolve much faster than proteins, as point mutations or small deletions/insertions in the sequence of a functional RNA will much less frequently lead to a catastrophic negative impact compared to a protein. Further, the modular nature of RNAs results in very different evolutionary constraints on functional RNA genes, such that despite lack of sequence conservation, the function of the RNA may remain conserved (Pang et al. 2006; Ulitsky et al. 2011). Finally, the majority of cellular proteins are over 100 amino acids long, requiring an open reading frame of 300 nucleotides or more, plus additional regulatory sequences at their 3′ and 5′ UTRs. Functional RNAs can be much smaller than this size; thus, in organisms with compact genomes such as bacteria, or wherever parsimony of size is important, RNAs can be very attractive regulatory options. In the sections that follow, description of the lncRNAs involved in response to various stresses provides examples in which evolution has taken advantage of the unique properties of RNAs for generating highly efficient and specific stress response networks.

2 Role of Non-coding RNAs in Bacterial Stress Response

The impact of non-coding RNAs on stress in bacteria has been known for over four decades with a wealth of studies uncovering the mechanism of action of many bacterial regulatory RNAs (Ikemura and Dahlberg 1973; Storz et al. 2011). The non-coding bacterial RNAs, referred to as small RNAs (sRNAs), are often conserved, range between 50 and 300 nucleotides in length and are ubiquitous, even to the point of outnumbering protein regulators (Storz et al. 2011). Many of these RNAs play important roles in bacterial stress response. The majority of studied sRNAs act in trans via forming short, imperfect basepairing interactions to their target RNAs and often mask ribosome binding sites to block translation (Waters and Storz 2009; Storz et al. 2011). In Gram-negative bacteria, this class of regulatory RNAs often associates with the Hfq RNA-binding protein (Storz et al. 2011).

Trans-acting sRNAs play important roles in bacterial response to a diverse variety of stresses, for example, two abundant non-coding RNAs in *E. coli*, MicA and RybB, are critical factors in the σE stress response which monitors and repairs the outer membrane of the bacteria. When envelope homeostasis is perturbed, MicA and RybB act as post-transcriptional repressors with both distinct and shared targets including several abundant porins, thus complementing the transcriptional activation function of the σ^E protein (Gogol et al. 2011). Another RNA, OxyS RNA, is a stable abundant transcript induced in *E. coli* in response to oxidative stress. It regulates the expression of several genes, including transcriptional regulators, and helps protect the cells against oxidative damage (Altuvia et al. 1997). In Staphylococcus aureus, another sRNA named RsaE accumulates in late exponential growth and interacts with the 5′ region of opp3A mRNA, which encodes an ABC transporter component. RsaE prevents the formation of the ribosomal initiation complex on opp3A and helps in downregulation of metabolism when carbon sources become scarce (Bohn et al. 2010).

Translation of two key bacterial stress response proteins, the stress response sigma factor σ^S and H–NS, a histone-like nucleoid protein, are also regulated by a sRNA named DsrA. This RNA is induced at low temperatures in *E. coli* and forms a complex with Hfq. DsrA forms basepairing interactions with its target genes which in the case of the mRNA coding for σ^S relieves an intramolecular secondary structure that blocks ribosome access to the ribosome binding site of the mRNA, thus allowing translation (Sledjeski et al. 1996; Lease et al. 2004; Večerek et al. 2010). Additional examples of the function of trans-acting sRNAs in stress response include roles in iron deficiency stress (RyhB) (Massé et al. 2005), elevated glycine (GcvB), glucose level changes (Spot42 and CyaR), and high glucose phosphate levels (SgrS) (reviewed in Waters and Storz 2009). Last but not least, trans-acting bacterial RNAs also play a pivotal role in response to viral infections and the presence of foreign DNA. A well-studied class of such RNAs are the CRISPR RNAs, which target bacteriophages and plasmids and likely also play a role in silencing genes from other mobile elements (Barrangou et al. 2007; Marraffini and Sontheimer 2008).

Another class of sRNAs originate from the opposite strand of their target genes, and unlike the trans-acting RNAs, which are often only induced under certain conditions, they are constitutively expressed. Antisense RNAs are transcribed from the loci of a significant number of bacterial genes and regulate their overlapping RNAs through diverse mechanisms including changing the translation efficiency or stability of their target gene, or by interfering with its transcription (Thomason and Storz 2010; Georg and Hess 2011). A number of studied antisense sRNAs regulate target mRNAs that code for proteins that are toxic in higher levels. It has been proposed that such RNAs, by regulating the level of the toxic protein, help adjust the rate of growth of bacteria under stress conditions to allow the cells to repair or otherwise adapt to their new environment (Kawano et al. 2007; Unoson and Wagner 2008). In addition to the regulation of the rate of bacterial growth, antisense RNAs can play direct roles in cellular response to diverse stressful stimuli. For example, an antisense sRNA, IsrR (iron stress-repressed RNA), is an antisense RNA transcribed from the opposite strand of the IsiA (iron stress-induced protein A) locus in Cyanobacteria and negatively regulates this gene. During iron deficiency, IsrR is repressed, allowing for induction of IsiA, which forms a giant ring structure around photosystem I, thus regulating photosynthesis (Dühring et al. 2006).

A third group of sRNAs, including the CsrB and 6S RNAs of *E. coli*, act via binding to protein targets and modifying their RNA-binding or enzymatic activity (Babitzke and Romeo 2007; Wassarman 2007). In addition to the regulatory RNAs discussed above, bacteria also take advantage of RNA motifs often located at the 5′ end of their mRNAs to regulate the expression of the gene containing the motif. Such motifs, named riboswitches, have been extensively reviewed elsewhere (Breaker 2011).

3 lncRNAs in Eukaryotic Stress Response

3.1 *lncRNAs in Hypoxic Stress: lnc-ing Stress to Cancer*

In addition to physiological hypoxia occurring during embryonic development (Saunders 1966; Caniggia et al. 2000) and at high altitudes, hypoxia plays an important role in a number of human pathologies, including ischemic stroke and many solid tumors (Beasley et al. 2002; Kaidi et al. 2006; Semenza 2012a; Oh et al. 2012; Luo et al. 2014; Erickson et al. 2015; Chang et al. 2015). The hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2, Fig. 2) are transcription factors which act as key mediators of the hypoxic response. HIF-1 and HIF-2 differ in one of their subunits, HIF-1α and HIF-2α, which have similar DNA-binding and dimerization domains but distinct transactivation domains. Consequently, while they share many of their target genes, each of them also induces several unique

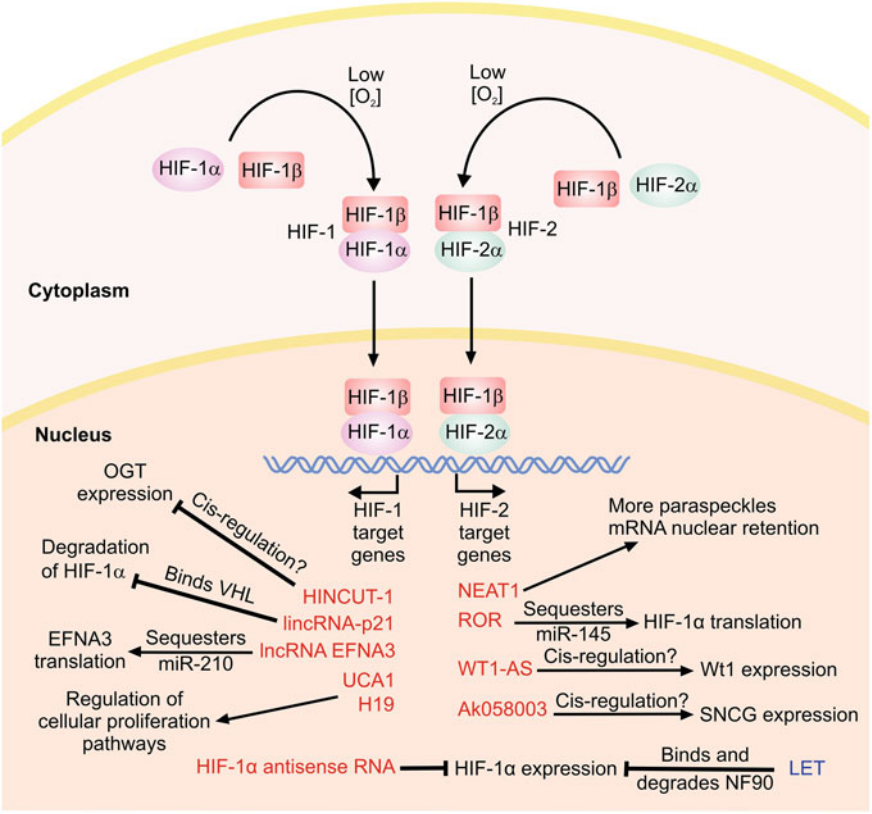

Fig. 2 Simplified schematic summary of the role of lncRNAs in the hypoxia response pathway. The hypoxia-induced and repressed lncRNAs are shown in *red* and *blue* font, respectively

targets. During hypoxia, HIF-1α and HIF-2α form dimers with HIF-1β to form the active HIF-1 and HIF-2 complexes, which will translocate to the nucleus and bind to hypoxia response elements (HREs,5′-RCGTG-3′) in the promoters or enhancers of hypoxia-responsive genes (Fig. 2) (Kaelin and Ratcliffe 2008; Majmundar et al. 2010). Hypoxia-induced genes, in turn, play important roles in the regulation of cellular metabolism, survival/apoptosis pathways, proliferation, angiogenesis, and several aspects of tumorigenesis including migration, invasion, and metastasis (Majmundar et al. 2010; Semenza 2012b). Indeed, activation of HIF1 and HIF2 is associated with a more aggressive tumor phenotype and poor prognosis in many cancers (Yang et al. 2011, 2013b).

3.1.1 HIF-Induced lncRNAs and Their Role in Hypoxic Response

The transcriptional cascade induced by HIF proteins involves the induction of a large number of both protein-coding RNAs and lncRNAs (Choudhry et al. 2014), including a number of studied RNAs such as NEAT1, UCA1, linc-ROR, and H19 among others (Choudhry et al. 2014; Yu et al. 2015; Yang et al. 2015; Chang et al. 2015). In a high-throughput study of the transcriptome of hypoxic and normoxic MCF-7 human cells, Choudhry et al. (2014) identified a large number of lncRNAs, including many previously unannotated ones, that were induced in response to hypoxia. These RNAs included many antisense lncRNAs, which showed coordinated regulation with the genes they overlapped. Many hypoxia-induced lncRNAs showed proximity to previously identified HIF-1 and HIF-2 binding sites (Choudhry et al. 2014), suggesting that they were directly regulated by these transcription factors. Another high-throughput study using microarrays targeting the transcribed ultraconserved regions of the human genome identified ∼60 putative lncRNAs that were differentially expressed between hypoxic and normoxic cells in a HIF-dependent manner (Ferdin et al. 2013). One of the identified RNAs, which originated from an intron of the *O*-linked *N*-acetylglucosamine (GlcNAc) transferase (OGT) gene, seems to correspond to a hypoxia-stabilized intronic RNA of ∼400 nucleotide length. This RNA, named HINCUT-1/uc.475, may play a role in regulation of the function of its host gene, which is also overexpressed in response to hypoxia and in epithelial cancers, and plays an important role in tumor formation (Ferdin et al. 2013). A third high-throughput study used lncRNA microarrays to analyze changes in the pattern of gene expression in cortex of rats subjected to an hour of cerebral ischemia. Similar to the two previous studies, the results indicated significant changes in the non-coding transcriptome, with over three hundred and fifty RNAs upregulated and over eighty showing significantly reduced expression (Dharap et al. 2012). It will be interesting to compare the three high-throughput datasets obtained by the above studies to determine the degree of overlap of the hypoxic response in different tissues/cell lines and under slightly different conditions.

In addition to the global studies of the lncRNA expression patterns, several functional studies have analyzed the role of individual lncRNAs in the hypoxic

response. Study of the function of NEAT1, a nuclear-localized lncRNA which participates in the formation of paraspeckles, has shown that it is directly induced by HIF-2 (Choudhry et al. 2015). In a number of breast cancer cell lines and solid tumors, hypoxia-mediated induction of NEAT1 led to an increase in the number of paraspeckles. This, in turn, led to changes in subcellular localization of some RNAs, increased cellular proliferation and survival, and a decrease in apoptosis, thus providing a mechanistic explanation for the observed correlation between the expression level of NEAT1 in breast cancer and poor survival (Choudhry et al. 2015) (Fig. 2).

Another hypoxia-induced lncRNA, lincRNA-p21, is induced by HIF-1 and interacts with both HIF-1α subunit of HIF-1 and the von Hippel–Lindau (VHL) protein, which acts as a ubiquitin E3 ligase (Yang et al. 2014a). In the absence of hypoxia, HIF-1α is hydroxylated followed by ubiquitination by VHL, leading to its rapid degradation. On the other hand, during hypoxia, hydroxylation of HIF-1α is inhibited, leading to its accumulation and formation of the active HIF-1 complex. Through interaction with both HIF-1α and VHL, lincRNA-p21 disrupts the interaction of the two proteins, leading to the accumulation of HIF-1α even in the presence of oxygen (Yang et al. 2014a). Through induction of expression of glucose transporters, glycolytic enzymes and additional metabolic enzymes, activation of HIF-1 leads to upregulation of glycolysis and downregulation of oxidative phosphorylation during normoxia, which is observed in many tumor cells (Bartrons and Caro 2007). This metabolic adaptation results in easy availability of precursors for de novo nucleotide and lipid synthesis and minimizes the reactive oxygen species (ROS) generated in the mitochondria, leading to a growth advantage for cancer cells. As expected, the level of lincRNA-p21 was found to be proportional to tumor growth level in mouse xenograft models (Yang et al. 2014a). In addition to its HIF-1-mediated effect on cancer cells, lincRNA-p21 has been shown to be induced by p53 and plays an important role in repression of p53-dependent transcriptional response in human cells (Huarte et al. 2010; Zhang et al. 2014a), thus acting as a bifunctional pro-survival factor in tumor cells. Interestingly, another non-coding RNA, miR-210, is also involved in repression of mitochondrial respiration during hypoxia (Chan et al. 2009), pointing to the presence of an RNA regulatory network in metabolic changes occurring during the hypoxic response (also see below). As will be discussed below, lincRNA-p21 is also induced in response to genotoxic stress in human cancer cells (Özgür et al. 2013). Although the mechanism of this induction has not been studied, it is likely resulting from the activation of p53 transcriptional response, indicating a significant degree of lncRNA-mediated cross talk between different stress response pathways.

Studies on a number of other hypoxia-induced lncRNAs similarly indicate the exploitation of lncRNA-mediated aspects of the hypoxic response by tumor cells. Urothelial cancer-associated 1 (UCA1), also named cancer upregulated drug resistant (CUDR), which was discovered as a marker for bladder cancer (Wang et al. 2006), is another HIF-1-induced lncRNA (Xue et al. 2014). Studies in human cancer cell lines have indicated the contribution of UCA1 to proliferation, migration, invasiveness, and enhanced survival of bladder cancer cells under hypoxia

(Wang et al. 2006, 2008a; Yang et al. 2012a; Xue et al. 2014). Similarly, H19 lncRNA, an imprinted lncRNA and miRNA precursor which is known to play an important role in regulation of cancer-related pathways, is induced during hypoxic stress (Hao et al. 1993; Matouk et al. 2007; Yoshimizu et al. 2008; Keniry et al. 2012; Yang et al. 2012b; Guo et al. 2014; Matouk et al. 2014). Interestingly, induction of H19 by HIF-1 during hypoxia is strongly inhibited by p53 (Matouk et al. 2010).

As mentioned above, many protein-coding RNAs have non-coding isoforms that originate through the use of alternative promoters, alternative 3′ end processing signals, or alternative splicing events (Djebali et al. 2012; ENCODE Project Consortium 2012). An example of such lncRNAs in hypoxia has been recently reported in the Ephrin-A3 (EFNA3) locus, which encodes for a cell surface protein involved in modulating cellular adhesion and repulsion and cancer metastasis. Several non-coding isoforms of EFNA3 arise from alternative, HIF-regulated promoters and alternative 3′ processing sites during hypoxia, leading to the accumulation of the Ephrin-A3 protein (Gómez-Maldonado et al. 2015). These lncRNAs act through sequestration of miR-210, which is induced by hypoxia and negatively regulates the translation of Ephrin-A3 mRNA (Kulshreshtha et al. 2007; Fasanaro et al. 2008; Huang et al. 2009) (also see above), thus relieving the post-transcriptional inhibition and resulting in an increase in the level of Ephrin-A3 protein. As expected, overexpression of the EFNA3 lncRNAs led to enhanced metastatic ability in breast cancer cells (Gómez-Maldonado et al. 2015).

3.1.2 Hypoxia-Induced Epigenetic Changes Regulate the Expression of Functional lncRNAs

In addition to direct induction by HIF-1 or HIF-2 transcription factors, many lncRNAs are expressed during hypoxia as a result of hypoxia-mediated epigenetic changes or secondary to activation of other pathways and play critical roles in the regulation of the hypoxic response. An interesting example of such RNAs is linc-RoR (regulator of reprogramming), which is upregulated in response to hypoxia and shows increased expression in hypoxic regions in tumors (Takahashi et al. 2014). Linc-RoR functions at least partially through sequestering miR-145, which negatively regulates the translation of HIF-1α by targeting p70S6K1 (RPS6KB1) (Xu et al. 2012; Wang et al. 2013). Thus, expression of linc-RoR leads to an increase in HIF-1α protein level (Takahashi et al. 2014), leading to potentiation of the hypoxic response. In another study, lincRNA-ROR was found to exert a strong negative regulation on p53 translation in a manner that requires hnRNP I, thus enhancing cellular survival (Zhang et al. 2013a). Interestingly, linc-RoR was abundantly found in extracellular vesicles released by hepatocellular cancer cells during hypoxia, likely acting as an intercellular signal to promote cellular survival during hypoxia (Takahashi et al. 2014).

In addition to linc-RoR, lncRNA-LET (lncRNA low expression in tumor) (Yang et al. 2011) also plays an important role in regulation of HIF-1α. Unlike the

previously discussed examples of hypoxia-regulated lncRNAs, lncRNA-LET is downregulated during hypoxia via repressive epigenetic changes at its promoter through the action of hypoxia-induced histone deacetylase 3 (HDAC3), which itself is a HIF-1α-regulated gene (Yang et al. 2013a). When expressed, lncRNA-LET associates with nuclear factor 90 (NF90), enhancing its degradation by inducing its ubiquitination through an unknown mechanism. As NF90, a double-stranded RNA-binding protein, is involved in biogenesis of many mRNAs including HIF-1α (Kuwano et al. 2010), an increase in NF90 degradation leads to post-transcriptional downregulation of HIF-1α protein level. Thus, lncRNA-LET, together with NF90, HIF-1α, and HDAC3 constitute a positive feedback loop that likely plays an important role in initiation and resolution of the hypoxic response. As expected, reducing the level of lncRNA-LET led to increased metastatic activity in human tumor cells (Yang et al. 2013a). In addition to the regulation of expression of HIF-1α by trans-acting lncRNAs, two antisense lncRNAs, one overlapping the first (5′aHIF-1α) and the other overlapping the last exon of HIF-1α (3′aHIF-1α), are expressed from the locus of HIF-1α gene (Bertozzi et al. 2011). 5′aHIF-1α was induced in response to camptothecin, and its upregulation was associated with a decrease in the level of HIF-1α, suggesting the possibility of a regulatory function for the antisense RNA (Bertozzi et al. 2011). The expression of 3′aHIF-1α is induced in response to hypoxia, likely mediated by binding sites for HIF-1/HIF-2 complexes in its promoter (Thrash-Bingham and Tartof 1999; Uchida et al. 2004). Knockdown of 3′aHIF-1α prevented the hypoxia-induced decrease in HIF-1α mRNA, likely through the loss of a destabilizing effect exerted by this antisense RNA (Uchida et al. 2004).

In addition to global regulation of the hypoxic stress response, two examples of local, *cis*-regulation by hypoxia-induced lncRNAs have been reported. One study focused on WT1-AS lncRNAs, which originate from the first intron or the promoter region of the WT1 protein-coding gene in an antisense orientation and are alternatively spliced, generating a number of distinct transcripts. At least some of these transcripts are exported to the cytoplasm, where they form basepairing interactions with their complementary region on the first exon of WT1 protein-coding mRNA (Dallosso et al. 2007). The expression of WT1-AS lncRNAs is partially regulated by changes in methylation of a CpG island in intron 1 of WT1 gene, which occurs during hypoxia (Malik et al. 2000; Dallosso et al. 2007; McCarty and Loeb 2015). This leads to an increase in the expression of both WT1 and WT1-AS transcripts. Interestingly, shRNA-mediated downregulation of WT1-AS leads to reduced WT1 level, suggesting *cis*-regulation of the expression of the protein-coding gene by its non-coding antisense overlapping transcripts, although shRNA-mediated silencing of the locus must be ruled out. Since WT1 is overexpressed in a number of cancers including leukemias, this lncRNA-mediated regulation may contribute to the development or progression of these malignancies (McCarty and Loeb 2015). Another report pointed to the potential *cis*-regulation of the metastasis-related γ-synuclein (SNCG) gene by the hypoxia-induced lncRNA AK058003 originating about 10 Kb away in an antisense divergent conformation (Wang et al. 2014b). The expression of the two genes showed a strong correlation in gastric cancer clinical

samples and cell lines, and knockdown of the lncRNA led to reduced SNCG expression and increased methylation of the CpG island near its promoter (Wang et al. 2014b) (Fig. 2).

3.2 *lncRNAs in Oxidative Stress*

In mammalian cells, oxidative stress leads to a transcriptional cascade targeting many protein-coding genes involved in regulation of the cellular redox state (Ma 2010). In addition, expression of a large number of genes is altered in response to oxidative stress via post-transcriptional mechanisms including regulation of RNA stability and translation (Abdelmohsen et al. 2008). Until recently, contribution of the non-coding transcriptome to the oxidative stress response had remained largely unexplored.

The first study of the impact of oxidative stress on global gene expression pattern in mammalians was recently reported by Giannakakis et al. (2015). High-throughput RNA-seq analysis of human fibroblasts treated with hydrogen peroxide indicated that except the known oxidative stress response genes that were upregulated, the vast majority of the protein-coding genes had reduced cellular levels (Giannakakis et al. 2015). In contrast, the non-coding transcriptome was strongly upregulated, together with a large number of novel transcripts. Over a thousand intergenic and antisense RNAs and RNAs arising from bidirectional promoters were detected, with the majority of stress-induced RNAs belonging to the latter group (Giannakakis et al. 2015). Many of the induced novel RNAs were predominantly nuclear and did not show significant protein-coding capacity; however, among those with higher predicted protein-coding capacity, many associated with polysomes to a significant extent (Giannakakis et al. 2015). The predominance of transcripts originating from bidirectional promoters suggests a large degree of gene-specific fine-tuning during the response to oxidative stress, as many such transcripts affect the expression of their promoter-sharing neighboring transcript.

In addition to the above study, the upregulation of a number of novel transcripts that are likely to be lncRNAs in response to the induction of oxidative stress by hydrogen peroxide treatment has been reported (Tani and Torimura 2013; Tani et al. 2014). However, very few oxidative stress-induced lncRNAs have been subjected to functional analysis. One studied RNA is lncRNA gadd7 (growth arrested DNA-damage inducible gene 7), which was originally discovered as a non-coding DNA damage response gene with a central role in the regulation of the G1/S checkpoint following DNA damage (Hollander et al. 1996; Liu et al. 2012). Liu et al. (2012) could show that after UV irradiation, gadd7 binds to TAR DNA-binding protein (TDP-43) and induces its dissociation from cyclin-dependent kinase 6 (Cdk6) mRNA, leading to degradation of Cdk6 mRNA. As Cdk6 is a key factor in the regulation of G1/S transition of the cell cycle, gadd7-mediated regulation of this key cell cycle step after genotoxic stress contributes to the

maintenance of genomic fidelity. Interestingly, in addition to genotoxic stress, gadd7 is induced by lipotoxicity in a manner that depends on the ROS and is required for lipotoxicity-mediated and oxidative stress-mediated cell death (Brookheart et al. 2009). Indeed, depletion of gadd7 resulted in reduction in lipid-induced ROS and ROS-induced endoplasmic reticulum stress (Brookheart et al. 2009). While the mechanism of action of gadd7 in the context of lipid-mediated oxidative stress is not known, it is possible that by interacting with TDP-43, which regulates the biogenesis of a large subset of cellular mRNAs, gadd7 controls the stability of key proteins in multiple stress response pathways.

Many human diseases are associated with oxidative stress, and a causal relationship is proven or strongly suspected in a large subset of human diseases (Galea et al. 2012; Dandekar et al. 2015). A study of the exfoliation syndrome (XFS), a systemic fibrillinopathy, found a lncRNA transcribed antisense to the XFS-associated LOXL1 gene (Hauser et al. 2015). Interestingly, the genomic region with strongest disease association lies upstream of this antisense lncRNA, LOXL1-AS1, and mutations in this region affect its expression. LOXL1-AS1 is strongly induced in response to oxidative stress, and although the impact of its expression on cellular homeostasis after stress is not known, it is possible that dysregulation of LOXL1-AS1 expression by disease-causing mutations plays an important role in XFS pathogenesis (Hauser et al. 2015).

3.3 The Role of lncRNAs in Genotoxic Stress and the DNA Damage Response

To identify the lncRNAs that are differentially expressed in response to DNA damage, Mizutani and colleagues computationally screened a library of human cDNAs for novel potentially non-coding transcripts (Mizutani et al. 2012). Among the identified putative lncRNAs, twenty-five were nuclear-localized, and several showed expression in multiple human tissues. After the treatment of HeLa cells with mitomycin C or doxorubicin, two completely distinct subsets of the putative lncRNAs showed differential expression in response to the two genotoxic agents (Mizutani et al. 2012). While the function of these RNAs has not yet been studied, the lack of overlap between lncRNAs induced in response to the two genotoxic agents is intriguing and points to the specificity of the response of the non-coding transcriptome to each DNA damage mechanism. Further evidence for this high level of specificity was provided by another report, in which the expression of a number of well-studied lncRNAs was analyzed in two human cell lines after the induction of DNA damage using bleomycin and γ-radiation (Özgür et al. 2013). Interestingly, similar to the results of Mizutani et al. (2012), differentially expressed lncRNAs showed a high level of cell type and genotoxic agent specificity (Özgür et al. 2013). For example, ANRIL and GAS5 were mainly induced in irradiated cells, while HOTAIR, MALAT1, lincRNA-p21, ncRNA-CCND1, and MEG3

seemed to mostly respond to bleomycin treatment (Özgür et al. 2013, see also Chaudhry 2013). A third study, similarly, showed a high level of specificity in the pattern of induction of lncRNAs in response to genotoxic agents. Analysis of the expression of a set of candidate lncRNAs in two human glioma cell lines after treatment with resveratrol or two concentrations of doxorubicin indicated that not only the pattern of induction of lncRNAs was genotoxic agent specific, but it was also dose-dependent (Liu et al. 2015b). While the results of the above studies are certainly thought-provoking, neither study involved an unbiased, comprehensive study of the changes in global gene expression pattern in response to the DNA damage inducing agents. Comparison of the results of high-throughput studies using unbiased techniques such as RNA-seq or whole-genome tiling arrays (e.g., Silva et al. 2010) can shed light on the extent and physiological significance of specificity and overlap between the transcriptomic changes induced in response to the different genotoxic agents.

While based on the above data, there seems to be a significant degree of specificity in the response of the non-coding transcriptome to each genotoxic agent, there is nevertheless some degree of overlap. For example, one of the differentially expressed RNAs described in the above studies, ncRNA-CCND1, was originally identified as a heterogeneous group of single-stranded, low copy number RNAs that were expressed in response to ionizing irradiation from the regulatory regions upstream of the promoter of the CCND1 locus (Wang et al. 2008b). These RNAs seemed to recruit the RNA-binding protein FUS/TLS to the CCND1 promoter, resulting in transcriptional repression through inhibition of the CREB-binding protein (CBP) and p300 histone acetyltransferase activities by FUS/TLS (Wang et al. 2008b). Another radiation-induced lncRNA, PARTICLE (promoter of MAT2A-antisense radiation-induced circulating lncRNA), is transcribed from a bidirectional promoter that also gives rise to the MAT2A gene, which encodes the catalytic subunit of methionine adenosyltransferase (O'Leary et al. 2015). PARTICLE forms a triple helix with the CpG island at the promoter of MAT2A and binds and recruits the repressive chromatin modifying complexes G9a and PRC2, resulting in downregulation of MAT2A expression (O'Leary et al. 2015). Such *cis*-acting mechanisms observed at the CCND1 and MAT2A loci provide an elegant and efficient means for fine-tuning of expression of individual genes in response to stressful stimuli. Similar mechanisms are likely to be involved in the regulation of many other critical genes during the cellular response to genotoxic agents and other stressful stimuli.

A third example of regulation by neighboring RNAs is provided by lncRNA-JADE, which was shown to be induced in an ATM (ataxia telangiectasia mutated)-dependent manner in response to a radiomimetic drug, neocarzinostatin, that generates double-stranded breaks in DNA (Wan et al. 2013a). The induction of expression of this lncRNA leads to transcriptional induction of its neighboring gene, Jade1, which is a component of the HBO1 (human acetylase binding to ORC1) histone acetylation complex (Wan et al. 2013a). Mechanistic analyses have indicated that lncRNA-JADE physically binds Brca1, which in turn stimulates the interaction of Brca1 with the p300/CBP complex, leading to the induction of

expression of Jade1 gene (Wan et al. 2013a). Another lncRNA, ANRIL/CDKN2B-AS1, is also induced after DNA damage in an ATM/E2F1-dependent manner from a locus in chromosome 9p21 which overlaps the CDKN2B/p15/INK4b gene in an antisense orientation (Wan et al. 2013b). Further, the transcription start site of ANRIL is less than 500 nucleotides away from that of the CDKN2A/p16/INK4a/ARF, and some isoforms of CDKN2A may even overlap the first exon of ANRIL in antisense orientation. Transcriptional induction of ANRIL results in suppression of expression of CDKN2A and CDKN2B at the later stages of DNA damage response, contributing to the termination of the DNA damage response (Wan et al. 2013b).

As mentioned above, lincRNA-p21 plays an important role in hypoxia (Yang et al. 2014a). However, in an interesting example of cross talk between different stress response pathways, this lncRNA is also induced in response to the DNA damaging agent doxorubicin and through its inhibitory effect on p53 function blocks doxorubicin-induced apoptosis (Huarte et al. 2010). A more recent study has also indicated a role for lincRNA-p21 in the induction of ER stress (Ning et al. 2015). Another lncRNA, which similar to lincRNA-p21 originates from the vicinity of p21/CDKN1A locus, is also induced after doxorubicin treatment in a p53-dependent manner (Hung et al. 2011). Functional analysis of this lncRNA, named PANDA, indicated that it interacts with the alpha subunit of nuclear transcription factor Y (NF-YA), which is involved in inducing the expression of apoptotic genes. Association with PANDA prevents NF-YA from binding its target genes, leading to impaired apoptosis (Hung et al. 2011). Thus, both lincRNA-p21 and PANDA act in RNA-mediated negative feedback loops to regulate p53 activity.

3.4 lncRNA in Heat Stress

The heat shock response involves global adjustments to diverse cellular processes in order to improve survival under hyperthermia, including general repression of transcription, RNA processing and translation, and the selective expression of heat shock proteins (HSP) and other chaperones (Yost and Lindquist 1986; Calderwood 2005; Lakhotia 2012; Audas and Lee 2015). In addition, it is also a part of cellular defense mechanisms against other stresses, including ischemia (Arya et al. 2007; Brown 2007; Richter et al. 2010; Vabulas et al. 2010; Lakhotia 2012). In eukaryotes, the transcription factor HSF1 (heat shock factor 1) plays a key role in the activation of the heat shock response. During hyperthermia, monomeric HSF1 is released from its interaction with HSPs and other chaperone proteins and forms a homotrimer which translocates to the nucleus (Fig. 3). Once in the nucleus, the trimeric HSF1 binds specific sequence motifs, named the heat shock elements (HSEs) in the promoters of its targets genes including HSPs. The induced HSP proteins, in turn, resequester HSF1 in a negative regulatory feedback loop. In addition to transcriptional induction of the HSP genes, the stability of these transcripts is also improved through decreased deadenylation via interactions involving

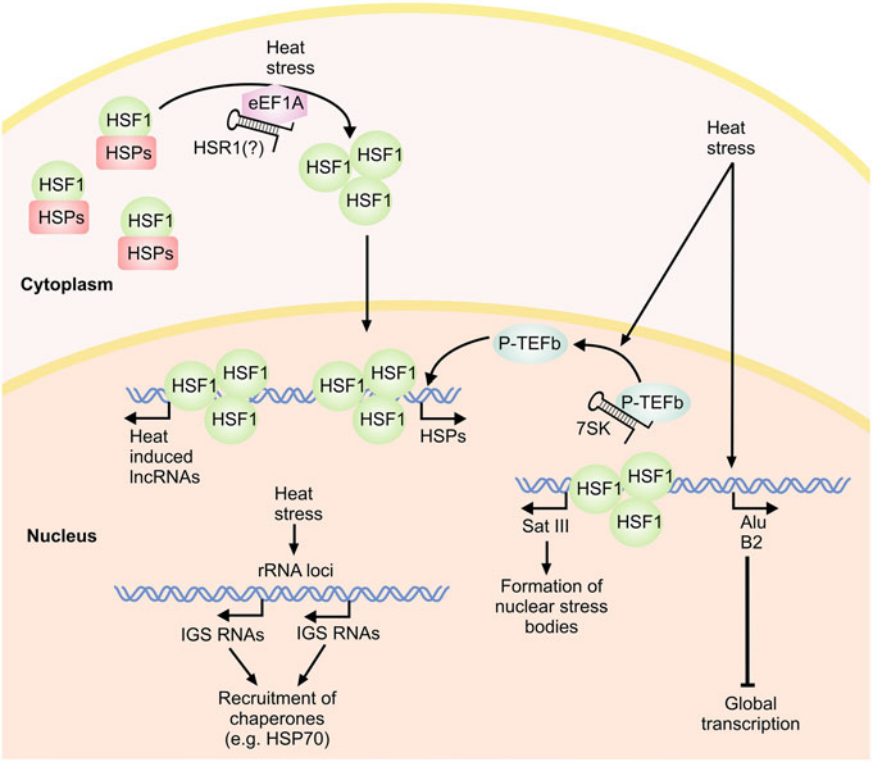

Fig. 3 lncRNAs in the heat shock pathways

AU-rich domains (AREs) in their 3′ UTRs (Moseley et al. 1993; Dellavalle et al. 1994). In contrast, the cellular level of most non-HSP transcripts is reduced during heat stress through destabilization or sequestration in stress granules or *p*-bodies (Buchan and Parker 2009). Although our understanding of the role of lncRNAs in the heat shock response remains highly rudimentary, several studies have highlighted the importance of this class of RNAs in regulation of the heat shock response (Place and Noonan 2014; Audas and Lee 2015).

One of the earliest reports on the involvement of lncRNAs in the heat shock response centered on a RNA named heat shock RNA-1 (HSR1) which interacted with the translation elongation factor eEF1A and regulated the activation of HSF1 by heat shock (Shamovsky et al. 2006). HSR1 was found to be required for HSF1 activation in vitro and in cultured cells after heat stress. Although Shamovsky et al. (2006) reported that HSR1 is expressed in both human and hamster cells (Shamovsky et al. 2006; Shamovsky and Nudler 2009), efforts at defining the locus of the RNA on the reference human and rodent genomes were unsuccessful (Kim et al. 2010). Analysis of the sequence of HSR1 indicated strong similarities to the

bacterial genomes (Kim et al. 2010; Choi et al. 2015), indicating its bacterial origin. Despite the current confusion about the origin of HSR1, the fact that bacterial sequences can strongly affect the regulation of the eukaryotic heat shock response can point to a hitherto unknown layer of lncRNA-mediated host–pathogen interactions during bacterial infections.

As mentioned above, an important aspect of heat shock response is a general transcriptional repression affecting nearly all cellular transcripts except the heat shock response-regulated genes. Several studies suggest that this repression is at least partially lncRNA mediated. It has been known that the expression of RNA polymerase III transcripts Alu RNA (in humans) and B2 RNA (in mice), which are derived from short interspersed elements (SINEs), is strongly upregulated following certain cellular stresses, including heat shock (Liu et al. 1995; Li et al. 1999). Both B2 and Alu RNAs directly associate with RNA polymerase II (Pol II) and transcriptional complexes formed at promoters both in cell-free systems and in cultured cells. This association leads to a transcriptional block by preventing Pol II from contacting the promoter during closed complex formation (Allen et al. 2004; Mariner et al. 2008; Yakovchuk et al. 2009). In these structurally altered transcriptional complexes, Pol II is held on DNA through contacts with DNA-binding transcriptional proteins in an inactive conformation (Mariner et al. 2008; Yakovchuk et al. 2009). More recent studies indicate that B2 RNA can also specifically block the phosphorylation of the Pol II *C*-terminal domain (CTD) by TFIIH, providing an additional mechanism by which B2 RNA regulates transcription in response to heat shock (Yakovchuk et al. 2011). Interestingly, B2 and Alu RNAs do not show any sequence homology, but their induction and function in response to heat shock are highly similar. This transcriptional repression function seems to be specific to only a subset of SINE-derived RNAs, as human scAlu RNA and mouse B1 RNA also associate with Pol II but do not repress its activity in vitro (Mariner et al. 2008).

An additional aspect of the involvement of Alu elements in heat shock response stems from the presence of HSF-binding sequences within Alu elements. Indeed, a significant fraction of the transcripts that show differential expression in response to heat shock contain Alu elements that harbor HSF-binding sites (Pandey et al. 2011). The Alu elements integrated within the host transcript can be in the sense or antisense orientation relative to the direction of transcription of the host gene. The presence of sense Alu elements harboring HSF-binding sites was associated with upregulation of the host gene in response to heat shock (Pandey et al. 2011), likely through acting at the DNA level as a landing pad for the recruitment of HSF1. A subset of the antisense Alu elements that contained HSF-binding sites, similarly, resulted in heat shock-induced transcription of RNAs; however, the transcriptional activity triggered by these elements occurred in the antisense orientation relative to the direction of transcription of the host gene. These antisense transcripts, in turn, resulted in reduced cellular level of the sense host gene (Pandey et al. 2011), most likely via direct basepairing to the sense RNA or induction of epigenetic changes. The above results provide an elegant example of mass regulation of gene expression through the use of repeat elements.

While B2 and Alu RNAs mediate transcriptional repression by blocking the initiation step, another Pol III-derived non-coding RNA, 7SK RNA, seems to participate in the induction of stress response genes under heat shock and other stressful conditions including genotoxic stress (Chen et al. 2008). In the absence of heat shock, the elongation phase of transcription is stimulated by the kinase activity of P-TEFb (positive transcription elongation factor b) leading to hyperphosphorylation of Pol II CTD (Lis et al. 2000). 7SK RNA and its associated proteins act as a repressor of the P-TEFb complex by binding and sequestering over half of all cellular P-TEFb complexes under normal conditions (Nguyen et al. 2001; Peterlin et al. 2012). During hyperthermia, almost all cellular P-TEFb complexes are released from 7SK due to a conformational change in the RNA (Chen et al. 2008), making them available to the active transcriptional complexes forming on the heat shock response genes, thus promoting highly efficient transcriptional activation of these critical loci (Lis et al. 2000; Peterlin et al. 2012) (Fig. 3).

3.4.1 Functional Role of Heat Shock-Induced lncRNAs

The drosophila hsr-omega (hsrω) non-coding RNA is perhaps the most extensively studied heat shock-induced non-coding transcript (Prasanth et al. 2000; Jolly and Lakhotia 2006; Lakhotia et al. 2012; Lakhotia 2012; Audas and Lee 2015). The hsrω nuclear transcripts and several cellular proteins including the heterogeneous nuclear RNA-binding proteins (hnRNPs) colocalize in nuclear structures named the omega speckles, which coalesce during heat shock into speckles overlapping the genomic locus of hsr-omega (Prasanth et al. 2000; Jolly and Lakhotia 2006). The hsrω nuclear RNAs regulate the localization, trafficking, and availability of hnRNPs and other proteins found in the omega speckles and play a critical role in thermo-tolerance and recovery from heat shock in drosophila (Prasanth et al. 2000; Jolly and Lakhotia 2006; Lakhotia et al. 2012).

In mammals, the satellite III repeat sequences found in the pericentromeric region of a number of chromosomes are transcribed into non-coding RNAs which accumulate in response to several stressful stimuli including heat stress (Jolly et al. 2004; Rizzi et al. 2004; Valgardsdottir et al. 2005; Jolly and Lakhotia 2006; Valgardsdottir et al. 2008; Eymery et al. 2010). It thought that in the absence of heat stress, the Pol II-transcribed satellite III RNAs are rapidly degraded by cellular RNA interference mechanisms in a dicer-dependent fashion (Jolly and Lakhotia 2006). After induction of their transcription by HSF1 during heat shock, the sat III transcripts remain associated with their loci and form the nuclear stress bodies, which recruit HSF1 and several hnRNPs and splicing factors during heat exposure (Denegri et al. 2001, 2002; Metz et al. 2004; Jolly and Lakhotia 2006). The sat III RNAs may play a role in the maintenance of the chromatin structure of the repeat-rich satellite III loci or, similar to hsrω RNAs, may nucleate the formation of a regulatory protein repository during stress (Jolly and Lakhotia 2006; Lakhotia 2012). Transcription of non-coding RNAs from another repeat-rich locus, the pericentromeric satellite 2 loci, is also strongly induced during heat shock response

in an HSF1-dependent manner (Tilman et al. 2012). While the impact of this transcriptional activity on cellular homeostasis during heat shock is not known, it may play a role in tumor progression in cancers. The heat shock response is upregulated in many tumors, and the HSF1-mediated activation of transcription from satellite 2 loci results in demethylation of these loci (Tilman et al. 2012). Demethylation of the satellite 2 regions, in turn, has been shown to favor chromosomal rearrangements and progression of the cancerous phenotype (Tilman et al. 2012). Interestingly, heat shock regulatory elements have been found in the telomeric repeats of fly species leading to telomeric puffing and transcription under heat shock conditions (Martinez et al. 2001); however, the physiological significance of this transcriptional activity is currently unknown. In addition to inducing changes in methylation marks on repeat-containing genomic regions, heat stress also results in changes in epigenetic marks elsewhere in the genome, including methylation marks associated with imprinting. It has been shown that the methylation marks in a subset of imprinted lncRNAs such as the paternally imprinted H19 and Igf-2r are altered in heat-stressed blastocyst stage mouse embryos compared to controls (Zhu et al. 2008).

Another lncRNA-mediated aspect of the heat shock response is nucleolar remodeling, which involves the recruitment of several regulatory and chaperone proteins including HSP70 to the nucleoli (Kotoglou et al. 2009; Boulon et al. 2010; Bański et al. 2010a, b). It is thought that the recruitment of chaperones, co-chaperones, and other regulatory proteins to the nucleoli provides a protective mechanism for the nucleoli during stress (Kotoglou et al. 2009; Boulon et al. 2010). Interestingly, heat stress results in the induction of expression of a number of lncRNAs from specific intergenic spacer (IGS) loci positioned between ribosomal genes (Audas et al. 2012; Jacob et al. 2013). These lncRNAs, which are transcribed by Pol I from the same strand as the rRNA at IGS 22 and 16 loci, mediate the recruitment of HSP70 to the nucleoli during stress. Surprisingly, other types of stress, such as acidosis, also lead to the expression of similar lncRNAs from other IGS loci and recruitment of HSP70 and several additional proteins including VHL (Audas et al. 2012; Jacob et al. 2012; Jacob et al. 2013), suggesting the extensive use of lncRNAs as protein recruitment scaffolds in the nucleoli during the stress response. As mentioned above, VHL plays an important role in restriction of HIF-1 activity, and thus, its sequestration by IGS RNA loci points to a physiological cross talk between acidosis and hypoxic responses. In addition to the ISG RNAs, a number of lncRNAs antisense to the ribosomal RNA promoter or pre-rRNA itself are transcribed from the ribosomal RNA loci in response to serum starvation and growth arrest (Bierhoff et al. 2010, 2014). These RNAs induce chromatin compaction via trimethylation of histone H4 lysine 20 at these loci in a manner that seems to involve the formation of a DNA:RNA triplex in order to downregulate the synthesis of ribosomal RNAs during starvation and quiescence (Bierhoff et al. 2010, 2014).

3.5 Role of lncRNAs in Other Stress Response Pathways

The role of lncRNAs in a number of important cellular stress response pathways including osmotic stress, ER stress, and starvation has remained almost entirely unknown. To our knowledge, no high-throughput analysis of the global changes in the non-coding transcriptome in response to these three stressful stimuli has been reported in mammalians, despite their critical physiological and clinical importance. However, a number of reports have described the outcome of functional study of individual lncRNAs involved in these three stress pathways. A study in yeast has revealed a role for antisense non-coding transcription during osmotic stress (Nadal-Ribelles et al. 2014; Solé et al. 2015). The stress-activated protein kinase (SAPK) p38/Hog1 induces the expression of tens of stress response genes after osmotic stress, including a number of lncRNAs (Nadal-Ribelles et al. 2014). One of the Hog1-induced lncRNAs is transcribed from the antisense strand of CDC28, which has an important role in regulation of the cell cycle in yeast. This antisense RNA, named Cdc28 lncRNA, originates from the 3′ UTR of CDC28 gene near a Hog1 binding site in this region. Interestingly, induction of transcription of the Cdc28 lncRNA promoted the formation of a DNA loop between the transcriptional start site of CDC28 and its 3′ UTR, allowing the 3′ UTR-bound Hog1 to induce the expression of CDC28 gene. This action of Cdc28 lncRNA was mediated in *cis*, likely through nascent or tethered Cdc28 lncRNA transcripts, and resulted in accumulation of CDC28, which in turn primes the cells to reenter the cell cycle after resolution of the stress (Nadal-Ribelles et al. 2014).

BACE1 (β-site amyloid-β precursor protein cleaving enzyme 1) is a transmembrane enzyme that participates in amyloid-β generation. BACE1 mRNA and protein expression are regulated by an antisense lncRNA, BACE1-AS, which overlaps the sixth exon of BACE1 mRNA in human (Faghihi et al. 2008). BACE1-AS is upregulated in response to a variety of cellular stressors, including ER stress, heat shock, and oxidative stress, resulting in increased BACE1 mRNA stability through the formation of an RNA duplex (Faghihi et al. 2008; Nogalska et al. 2010). In addition, BACE1-AS masks the binding site of miR-485-5p on BACE1 mRNA, further increasing the cellular level of BACE1 protein (Faghihi et al. 2010). Thus, BACE1-AS acts as an RNA-mediated link through which cellular stresses can affect the formation of amyloid-β and ultimately the progression of neurodegenerative disorders.

Finally, the expression of the growth arrest-specific 5 (Gas5) non-coding RNA is induced in response to starvation and has been shown to suppress the glucocorticoid-mediated induction of a number of key target genes, including those inhibiting apoptosis. Mechanistic studies have suggested that Gas5 RNA forms a structure that mimics that of the glucocorticoid response elements in the genome. Through this molecular mimicry, Gas5 binds to the DNA-binding domain of the glucocorticoid receptor as a competitive inhibitor, thus blocking the transcriptional activity of the glucocorticoid receptor (Kino et al. 2010).

3.6 lncRNAs and Stress in Plants

The plant genomes, similar to that of mammalians, harbor a large number of lncRNAs that perform a wide range of functions in response to different stressful stimuli (Boerner and McGinnis 2012; Li et al. 2014; Wang et al. 2014a; Bai et al. 2015; Liu et al. 2015a; Ariel et al. 2015; Xuan et al. 2015). Several studies have defined the extent of lncRNA transcriptional response following stressful stimuli in plants and have studied the role of individual lncRNAs in the context of stress (Amor et al. 2009; Xin et al. 2011; Wu et al. 2012; Lembke et al. 2012; Qi et al. 2013; Zhang et al. 2013b, 2014b; Zhu et al. 2014; Wunderlich et al. 2014; Csorba et al. 2014; Di et al. 2014; Bazin and Bailey-Serres 2015; Wang et al. 2015; Aversano et al. 2015). Since a number of recent reviews have provided excellent summaries and discussion of our current state of knowledge of the role of plant lncRNAs in stress (Liu et al. 2015c; Shafiq et al. 2015; Chekanova 2015), in the interest of space, a similar discussion is not included in this review.

4 Concluding Remarks

Although our knowledge of the role of lncRNAs in stress response is still in its infancy, existing studies have pointed to a critical and ubiquitous role for lncRNAs in stress response in all kingdoms of life. As discussed above, unique properties of RNAs make them highly suitable for function during the stress response. While the vast majority of currently identified stress-responsive lncRNAs have not been functionally studied, it is very likely that the list of stress-responsive lncRNAs and the processes which they regulate will significantly grow in near future. Due to technical shortcomings, many bona-fide lncRNAs, which originate in introns, or are short-lived and expressed at very low copy numbers, and unspliced RNAs are frequently filtered during high-throughput studies as noise. However, recent improvements in sequencing depth are likely to at least partially address this issue (Mercer et al. 2014). Further, transcribed pseudogenes, which fall under the category of lncRNAs, can play critical roles in cellular function as exemplified by a number of reports (Poliseno et al. 2010; Johnsson et al. 2013); however, as a group, they remain highly understudied. Improving our understanding of the role of all classes of lncRNAs in stress response will be highly fruitful, as the stress response pathways are known to be involved in pathogenesis of a wide range of human diseases from cancer to neurodegeneration (Romano et al. 2010; Facecchia et al. 2011; Ramalingam and Kim 2012; Gabr and Al-Ghadir 2012; Luca et al. 2015; Saito et al. 2015; Ng et al. 2015). Further, considering the cell type- and state-specific nature of lncRNA expression, it is likely that many members of this class of RNAs can be used as diagnostic or prognostic markers in human diseases (Di Gesualdo et al. 2014).

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Expression Specificity of Disease-Associated lncRNAs: Toward Personalized Medicine

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Abstract Long noncoding RNAs (lncRNAs) perform diverse regulatory functions in transcription, translation, chromatin modification, and cellular organization. Misregulation of lncRNAs is found linked to various human diseases. Compared to protein-coding RNAs, lncRNAs are more specific to organs, tissues, cell types, developmental stages, and disease conditions, making them promising candidates as diagnostic and prognostic biomarkers and as gene therapy targets. The functional annotation of mammalian genome (FANTOM) consortium utilizes cap analysis of gene expression (CAGE) method to quantify genome-wide activities of promoters and enhancers of coding and noncoding RNAs across a large collection of human and mouse tissues, cell types, diseases, and time-courses. The project discovered widespread transcription of major lncRNA classes, including lncRNAs derived from enhancers, bidirectional promoters, antisense lncRNAs, and repetitive elements. Results from FANTOM project enable assessment of lncRNA expression specificity across tissue and disease conditions, based on differential promoter and enhancer usage. More than 85 % of disease-related SNPs are within noncoding regions and are strikingly overrepresented in enhancer and promoter regions, suggestive of the importance of lncRNA loci at these SNP harboring regions to human diseases. In this chapter, we discuss lncRNA expression specificity, review diverse functions of disease-associated lncRNAs, and present perspectives on their potential therapeutic applications for personalized medicine. The future development of lncRNA applications relies on technologies to identify and validate their functions, structures, and mechanisms. Comprehensive understanding of genome-wide interaction networks of lncRNAs with proteins, chromatins, and other RNAs in regulating cellular processes will allow personalized medicine to use lncRNAs as highly specific biomarkers in diagnosis, prognosis, and therapeutic targets.

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Abbreviations

CAGE	Cap analysis of gene expression
ChIP	Chromatin immunoprecipitation
ENCODE	Encyclopedia of DNA elements
eRNAs	Enhancer RNAs
FANTOM	Functional annotation of mammalian genome
GWAS	Genome-wide association study
lncRNAs	Long noncoding RNAs
miRNAs	MicroRNAs
TSS	Transcription start sites
RNA-seq	RNA sequencing

Contents

1 Introduction

1.1 Widespread Transcription of LncRNA and LncRNA Annotation

A decade ago, genome-wide expression studies in mammals, using high-density tiling array or massive sequencing of full-length complementary DNAs, identified widespread transcription of tens of thousands of long non-protein-coding RNAs (lncRNAs), which are commonly defined as RNAs longer than 200 nucleotides, and have no coding potential for long peptides (Carninci et al. 2005; Bertone et al. 2004). With a rapid increase in sequencing depth and in the number of tissues, cell types, and organisms being sequenced, the list of lncRNAs is steadily growing. The ENCODE project (ENCyclopedia Of DNA Elements) estimated that 80.4 % of the human genome participates in at least one biochemical event in at least one cell type (Bernstein et al. 2012) and at least 74.7 % is transcribed (Djebali et al. 2012). Assembled transcripts from over 4.5 billion uniquely mapped reads in an RNA-seq data set of 23 human tissues under multiple conditions, with additional annotation information of known spliced expressed sequenced tags (ESTs), cDNAs and genes, could be mapped to 85.2 % of the genome (Hangauer et al. 2013). A recent ab initio assembly of over 43 Tb of sequence from 7256 RNA-seq libraries shows that there are at least two times more lncRNAs (68 %) than coding RNAs and the majority of these lncRNAs (79 %) were so far unannotated (Iyer et al. 2015).

The annotation of reproducible lncRNA loci (also called lncRNA genes in GENCODE) is rapidly growing to a comparable number to that of coding genes. In GENCODE annotation, the number of lncRNA loci increased from 9277 in 2012 (Derrien et al. 2012) to 15,900 lncRNA loci (version 22, October 2014). Current continuous attempts to annotate lncRNAs have produced several large systemic databases of lncRNAs such as lncRNAtor (Park et al. 2014), LNCipedia (Volders et al. 2015), and NONCODE (Xie et al. 2013). The numbers of lncRNA transcripts from these databases differ, largely subjective to criteria used for defining lncRNAs, sequencing depth, and diversity of sequenced samples. For example, while there are 56,018 human lncRNA loci in NONCODE version 4.0, the lncRNAtor version 1.0 contains 14,051 lncRNA gene units. Current unpublished analysis of RNA-seq and CAGE data in FANTOM5 suggests transcription of 45–50,000 lncRNA loci in the human genome (Unpublished results).

The debate on whether or not lncRNAs are the products of transcription noise is moving toward how lncRNAs produce functions (Pennisi 2014). Many lncRNAs possess key features distinct from transcription noise, including active transcription regulation as indicated in high frequency and conservation of transcription factor binding sites in lncRNA promoters, high precision of transcription in splicing processes, a similar range of half-lives, higher sequence conservation than random intergenic regions, lncRNA expression profiles associated with profiles of mRNA, and increasing evidence of specific lncRNA biological functions especially those related to diseases (Derrien et al. 2012; Pennisi 2014; Necsulea et al. 2014; Guttman

et al. 2009; Mercer et al. 2008). However, current knowledge of lncRNA functions remains limited to approximately 287 functionally characterized lncRNA loci, as documented in the lncRNAdb database (Quek et al. 2014).

1.2 Diverse Functions and Mechanisms of Action of LncRNAs

LncRNAs utilize diverse mechanisms of action by interacting with most types of binding partners, including RNA, DNA, and proteins. These interactions may vary according to cell types, tissues, and organs. Comprehensive reviews on lncRNA functions and mechanisms are available elsewhere (Morris and Mattick 2014; Li and Chang 2014). In this section, we exemplify potential links between regulatory functions of lncRNAs in transcription, translation, chromatin modification, and cellular organization to diseases.

For transcriptional regulation, lncRNAs act either on *cis* or *trans* by forming complex with proteins to regulate expression, often by controlling chromatin states (Rinn and Chang 2012). The X-inactive-specific transcript (Xist) recruits Polycomb repressive complex (PRC2) to inactivate X chromosome, and deletion of Xist resulted in X chromosome reactivation, promoting hematologic cancer in mice (Yildirim et al. 2013). Linc1992 THRIL [TNFα and Heterogeneous nuclear ribonucleoprotein L (hnRNPL) Related Immunoregulatory LincRNA] binds to hnRNPL and TNFα promoter to upregulate TNFα transcription (Li et al. 2014). LncRNA CCAT1-L (Colon cancer-associated transcript) is abundant in colorectal cancer cell lines or patients' mucosa samples, whereas it is undetectable or lowly expressed in other cell types or control samples (Xiang et al. 2014). CCAT1-L regulates long-range chromatin looping between MYC (v-myc avian myelocytomatosis viral oncogene homolog) promoter and enhancers to increase MYC expression and stimulates tumorigenesis (Xiang et al. 2014). LncRNA can also regulate expression at translational level. The antisense lncRNA of the ubiquitin carboxyl terminal hydrolase gene (Uchl1) is exported to the cytoplasm and facilitates the binding of the sense Uchl1mRNA to active polysomes for more efficient translation (Carrieri et al. 2012). The Uchl1 protein is a potential therapeutic target for treatment of Parkinson's disease (Liu et al. 2002). Several lncRNAs are known as essential organizing factors of the nucleus, for example, NEAT1 (Nuclear-enriched abundant transcript 1), and Xist and Malat1 (metastasis-associated lung adenocarcinoma transcript 1) (Hirose et al. 2014; Rinn and Guttman 2014; Mao et al. 2011). H19 lncRNA and miR-675 are derived from a same genomic locus and both contribute to gastric cancer, but by two different pathways to suppress two different genes, namely ISM1 (isthmin 1, angiogenesis inhibitor) and CALN1 (calneuron 1) (Li et al. 2014). LncRNAs also act as regulators of signaling pathways. For example, downregulation of the lncRNA low expression in tumor (LET) inversely affects expression and stability of genes in hypoxia signaling network, which may contribute to hepatocellular carcinoma (HCC) metastasis (Yang et al. 2013). Leukemia-induced noncoding activator RNA (LUNAR) enhances

IGF1R (insulin-like growth receptor factor 1) expression, contributing to maintenance of the IGF1 pathway and promoting T cell acute lymphoblastic leukemia (T-ALL) growth (Trimarchi et al. 2014).

1.3 LncRNA Expression Specificity

LncRNAs are highly specific to cell type, organs, and species. An RNA-seq study on 15 cell lines showed that 29 % of all lncRNAs were transcribed in only one cell type, and that only 10 % expressed in all cell lines, whereas 53 % of protein-coding mRNAs were constitutively transcribed in all cell lines (Djebali et al. 2012). The majority of lncRNAs are expressed at low levels spanning five to six orders of magnitude, from 10^{-2} to 10^{3} Reads Per Kilobase per Million mapped reads (rpkm) for non-polyadenylated lncRNAs or 10^{-2} to 10^{4} rpkm for polyadenylated lncRNAs (Djebali et al. 2012). LncRNAs are organ-specific, with the highest number of lncRNAs distributed in testes (55 % for young lncRNAs and 46 % for old lncRNAs), followed by neural and liver tissues (Necsulea et al. 2014). In previous studies, in the mouse, lncRNAs were also abundantly detected during early development and organogenesis (Carninci et al. 2003), which cannot be extensively sampled in human. In the GENCODEv7 database, at least 30 % of lncRNAs are found transcribed only within primate linage (Derrien et al. 2012). A de novo study using RNA-sequencing data of 11 tetrapod species showed that in a combined data set containing 13,533 lncRNAs, commonly detected in at least three out of 11 species, 81 % were primate-specific (Necsulea et al. 2014). It is evident that the level of sequence conservation for lncRNA primary sequences is low (Guttman et al. 2009; Yue et al. 2014; Fort et al. 2014). For example, only approximately 15 % of human lncRNAs have homologs in mouse (Yue et al. 2014). Interestingly, in human, significant evidence of purifying selection for SNPs within lncRNAs was obtained, suggestive of lineage-specific functions of human lncRNAs (Ward and Kellis 2012). Although still in its infancy, lncRNAs structural studies produce emerging evidence for a high level of secondary structural conservation of lncRNA functional domains, such as those in MEG3, SRA1, and HOTAIR (Mercer and Mattick 2013).

2 LncRNAs Derived from Enhancers, Promoters, and Repetitive Elements—Insights from FANTOM Promoter Analysis

2.1 A Promoter-Centric Approach to Identifying and Characterizing LncRNA Transcription

In the FANTOM project, CAGE sequencing of 5′ capped RNAs at transcription start sites (TSS) was used to generate expression data for activity analysis of

enhancers and promoters of both coding and noncoding RNAs (Takahashi et al. 2012; Kanamori-Katayama et al. 2011). CAGE differential expression analysis at single nucleotide level enables discovery of alternative promoter usage in different tissues and developmental stages (Carninci et al. 2006; Haberle et al. 2014). Quantitative expression of promoter regions from CAGE data allows ab initio motif activity response analysis of transcription factors, allowing construction of transcriptional network (Suzuki et al. 2009; Akalin et al. 2009). An example of such study is the comprehensive regulatory circuitry in differentiation and growth arrest of human monocytic cell line using CAGE time-course data (Suzuki et al. 2009).

Promoter-centric analysis of capped RNAs has identified and characterized expression of major classes of lncRNAs (Fig. 1). FANTOM3 discovered

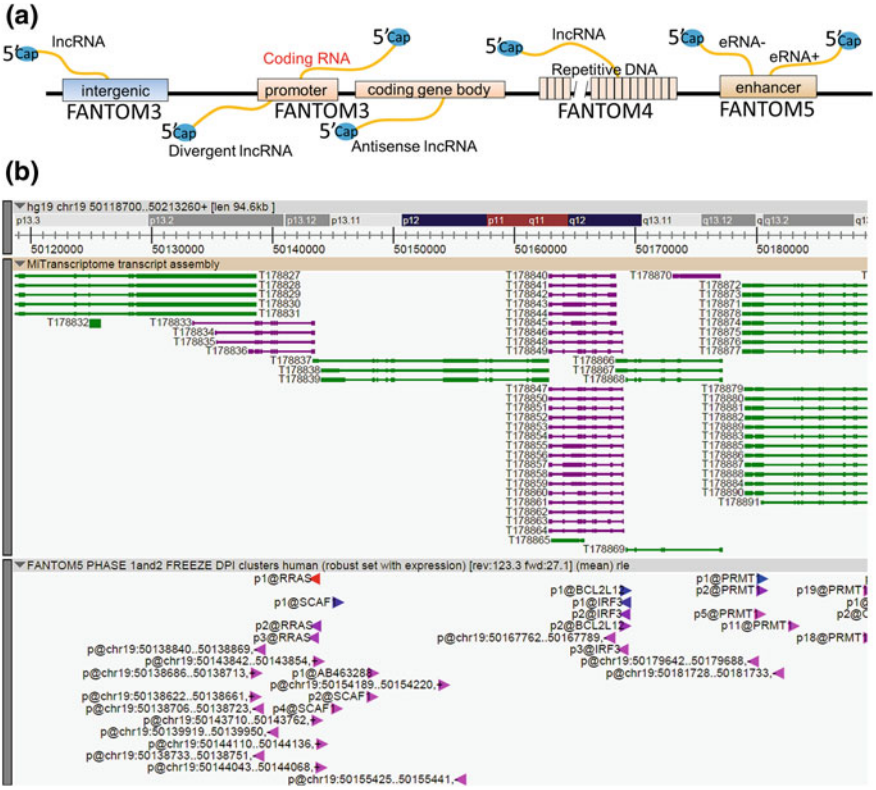

Fig. 1 **a** CAGE technology applied in FANTOM project (http://fantom.gsc.riken.jp/5/) leads to detection of several large classes of lncRNA, including lncRNAs derived from promoters, enhancers, repetitive elements, and antisense RNAs. **b** CAGE sequencing quantifies directional transcription of coding/noncoding genes at different promoters. CAGE data were from FANTOM5 human pooled robust promoter data set (http://fantom.gsc.riken.jp/5/data/). CAGE clusters are shown as *directional arrows*, with *names* and *colors* representing different promoter clustering levels. Reference transcripts (*green bars* for sense and *purple bar* for antisense transcripts) were from MiTranscriptome database (Iyer et al. 2015) using ZENBU for visualization (Severin et al. 2014)

genome-wide transcription events of lncRNAs (Carninci et al. 2005), especially the widespread production of antisense RNAs (Katayama et al. 2005). Following this success, FANTOM4 revealed at least 6–30 % of the capped transcripts detected in a sequencing library were transcribed from repetitive regions in human and mouse genome (Faulkner et al. 2009). Further, FANTOM5 comprehensively studied transcripts derived from regulatory elements, resulting in the most comprehensive promoter atlas and enhancer atlas (Forrest et al. 2014; Andersson et al. 2014). Moreover, from investigating enhancer and promoter activities of 19 human and 14 mouse time-course differentiation samples, FANTOM5 found a temporally coordinated transcription pattern of enhancers, promoters, and transcription factors (Arner et al. 2015). In this cascade of time-course transcription under different stimuli, enhancer RNAs were first transcribed, followed by transcription of regulatory genes, then by other responsive genes.

2.2 *LncRNA Expression Specificity: Insights from Quantified Human Promoterome*

LncRNAs are highly developmental and tissue-specific and are not readily detected in many biological cell lines and tissues. From sequencing of 975 human samples including 573 primary cell samples, 152 postmortem tissues, and 250 cancer cell lines, the FANTOM5 project produced a comprehensive human cell-type-specific promoterome atlas, covering activity of 185,000 promoters, which represent promoters of 91–94 % coding and noncoding known genes (Forrest et al. 2014). Across this large collection of tissues and cell types, 20 % of promoters were found expressed in more than 50 % of all samples (ubiquitously expressed), while 80 % were considered cell-type-specific (expressed in fewer than 50 % of all sequenced samples). The large FANTOM5 promoter expression data set with a diverse collection of samples from various diseases enables sample ontology enrichment analysis (SOEA), which associates promoters to disease ontology terms (Forrest et al. 2014). For each CAGE promoter, SOEA tests the overrepresentation of disease ontology terms in a ranked list of samples based on expression of the promoter. Applying this approach for all 127,645 human CAGE peaks revealed that a large proportion of transcribed RNAs in human are enriched in immune system, especially in monocytes and bone marrows (Forrest et al. 2014). A similar sample set enrichment analysis (SSEA) approach was performed at transcript level using data from 7256 RNA-seq libraries identified 7942 lncRNAs stringently associated with cancer or cell linage specificity or both (Iyer et al. 2015).

Through quantitative promoter analysis of different tissues in mouse and human, Carninci et al. (2006) first revealed that alternative promoter usage is common and that differential promoter usage is tissue-specific. More and more studies have shown relevance of this phenomenon to diseases and development. Alternative transcription start sites were found associated with colorectal tumors (Thorsen et al. 2011),

or in macrophage responses to activating reagents (Carninci et al. 2006), or in early zebra fish embryonic development (Haberle et al. 2014). At genome-wide level, the promoterome atlas shows that approximately 80 % of human promoters are cell-type-specific, while only 6 % can be considered as housekeeping promoters (Forrest et al. 2014). Moreover, 51 % of human promoters showed changing activities over time during time-course differentiation, including stem cells, progenitors, differentiated cells, and cells under different stimulating conditions (Arner et al. 2015). Similarly, 13 % of expressed enhancers displayed changing activities during the time-course.

2.3 Cell-Specific Enhancer Usage

Defining active enhancers by a double-CAGE-peak pattern appears to be more accurate than traditional enhancer-detection approaches by ChIP sequencing (Chromatin immunoprecipitation) and by sequencing of DNase I hypersensitive sites (DHSs) (Andersson et al. 2014). This approach quantifies enhancer transcription level, and thereby enabling assessment of cell-specific enhancer usage and their activity. In vitro enhancer validation assay showed that to identify cell-specific enhancers in monocytes, B cells, and T cells, the method based on enhancer CAGE transcription activity was more consistent than using chromatin accessibility information obtained from sequencing DHSs or ChIP sequencing (Andersson et al. 2014). CAGE data also allow correlation of enhancer activities to promoter activities for target genes of enhancers. Applying this method, FANTOM5 produced a comprehensive collection of ~44,000 active enhancers, which displayed bidirectional transcription across a diversity of human tissues and cell states (Anderson et al. 2014). In general, lncRNAs derived from active enhancers are non-polyadenylated (90 %), not spliced (95 %), short, not overlapping downstream mRNAs or lncRNAs, and unstable (Andersson et al. 2014; Core et al. 2014). Further, binding activity of putative transcription factors to domains within enhancers and promoters can be assessed by motif response activities analysis (MARA) (Suzuki et al. 2009).

A genome-wide binding profiling showed that enhancer RNAs act to increase chromatin accessibility by transcriptional regulatory complexes to defined genomic regulatory regions, contributing to cell-type-specific transcriptional regulation (Mousavi et al. 2013). The lncRNA CCAT1-L, which is transcribed from a super-enhancer cluster located at 515 kb upstream of the MYC gene, regulates long-range binding of enhancers to MYC promoters, thereby upregulating MYC transcription in colorectal cancer (Xiang et al. 2014). The transcription repressors, Rev-Erb nuclear receptors, suppress macrophage gene expression by downregulating lncRNA expression of distal enhancers, which are macrophage lineage determining factors (Lam et al. 2013).

2.4 LncRNAs Derived from Repetitive Elements

FANTOM4 project identified 250,000 retrotransposon-derived transcription start sites, accounting for 6–30 % of capped transcripts in a cell (Faulkner et al. 2009). Depending on estimation approaches, approximately 30–50 % of human DNA is constituted by repetitive elements, which consist of short interspersed noncoding element (SINEs), long interspersed noncoding element (LINEs), long terminal repeat elements (LTRs), and other less common types of repetitive elements. The ENCODE project found 18 % of CAGE-defined TSS overlapping repetitive regions. Shannon entropy analysis of expression uniformity, a measure of tissue specificity (Schug et al. 2005), showed that transcripts from repetitive regions were more narrowly expressed than those from genic regions, suggestive of higher cell-line specificity for repetitive RNA (Djebali et al. 2012). The higher cell specificity was also found in transposable element (TE) containing lncRNAs (Kelley and Rinn 2012). Transcription from LTR has recently been reported to produce functional lncRNAs. Knockdown of a subset of these lncRNAs reduced expression of multiple gene markers of pluripotency (Fort et al. 2014). TE composition analysis of 9241 lncRNA found that 83 % of these lncRNAs contain transposable elements, occupying 41.9 % total length of lncRNA regions (Kelley and Rinn 2012). Retrotransposon TSS within protein-coding genes can drive alternative transcription initiation of these genes (Kelley and Rinn 2012). In addition, 35 % of retrotransposon-associated TSS are tissue-specific, two times higher than that for other TSS types (17 %) (Faulkner et al. 2009).

Transposable elements (TEs) commonly found in lncRNAs may act as binding domains of lncRNAs, a theory called repeat insertion domain of lncRNA (RIDL) hypothesis (Johnson and Guigo 2014). For example, a nuclear-enriched lncRNA (antisense Uchl1) containing an embedded inverted SINEB2 repeat accelerates protein translation of the sense protein-coding gene Uchl1, which is associated with neurodegenerative diseases (Carrieri et al. 2012). The SINE B2 domain was shown to be an essential functional domain of the AS-Uchl1 (Carrieri et al. 2012). LncRNAs containing TEs are involved in a wide range of cellular functions. A hybrid lncRNA derived from integration of LINE1 (Long interspersed element 1) and the X gene of an integrated Hepatitis B virus (HBx) was detected in 23.3 % of HBV-associated hepatocellular carcinoma tumors, and correlated with poorer survival, possibly acting via Wnt/β-catenin signaling pathway (Shukla et al. 2013; Lau et al. 2014). Another example of a functional repetitive RNA is the telomeric RNA, known as TERRA (Telomeric Repeat containing RNAs). Transcription of repetitive regions at telomere ends produces lncRNA TERRA, which is essential in telomere length regulation, telomere recombination, and telomere end damage repair (Azzalin and Lingner 2014; Yu et al. 2014). An example on interaction of repeat containing lncRNAs with DNA for transcription regulation is the C_0T-1 RNA. LINE1 DNA, which comprises approximately 17 % of human genome, is widely transcribed to generate C_0T-1 stable lncRNA (Hall et al. 2014). C_0T-1 RNAs is transcribed from euchromatin regions tightly associate with chromatins in *cis*,

preventing chromosome condensation, a function similar to the Xist lncRNA (Hall et al. 2014). Many more potential functions of different types of TE containing RNAs remain to be explored.

3 LncRNAs and Human Diseases

3.1 LncRNAs with Strong Links to Human Diseases

By curating data from above 500 publications, a database of experimentally verified lncRNA-related diseases shortlisted 321 lncRNAs, which are associated with 221 diseases (http://cmbi.bjmu.edu.cn/lncrnadisease) (Chen et al. 2013). Among various types of diseases, most commonly, lncRNAs are found related to cancer. At least six lncRNAs have been shown to be involved in prostate carcinogenesis, three of which are highly prostate-specific, including prostate cancer antigen 3 (PCA3), prostate cancer gene expression marker 1 (PCGEM1), and prostate cancer-associated lncRNA transcript 1 (PCAT1) (Walsh et al. 2014). The prostate cancer-associated lncRNA transcript-1 (PCAT-1) represses BRCA2 tumor suppressor by post-transcriptional repression of its 3' UTR in a similar way to microRNA-like or competitive-endogenous RNAs, but not by epigenetic (Prensner et al. 2014). HOX antisense intergenic RNA (HOTAIR) lncRNA is involved in several types of cancers including gastric adenocarcinoma, colorectal cancer, and breast cancer. HOTAIR recruits PRC2 to specific loci for trimethylation of Histone 3 Lysine 27 (H3K27me3) and represses a series of genes (Gupta et al. 2010). Human colorectal cancer-specific CCAT1-L lncRNA promotes long-range chromatin looping between MYC promoter and its enhancers (Xiang et al. 2014). More cancer-associated lncRNAs are being discovered. A recent large-scale ab initio transcriptome analysis of 27 cancer types in different tissues and organs found 7942 lncRNAs statistically associated with cancer and/or linage in human (Iyer et al. 2015).

Besides cancer, lncRNAs are found associated with a range of other disease types, in which two top categories are cardiovascular diseases and neurodegenerative diseases (Chen et al. 2013) (Fig. 2). For example, the antisense APOA1-AS represses the sense APOA1 mRNA, resulting in reduction of the Apolipoprotein A-1 protein, a main component of high-density lipoprotein (HDL) in plasma (Halley et al. 2014). In addition, lncRNAs appear to be important for cognitive functions as brain is the second most common organ expressing the highest number of lncRNAs and many lncRNAs are specific for mammals and primates (Necsulea et al. 2014; Morris and Mattick 2014). The upregulation of the natural antisense lncRNA BACE1-AS (β-secretase-1) increases BACE1 stability and thus maintaining high level of BASE1 enzyme, which may lead to pathophysiology in

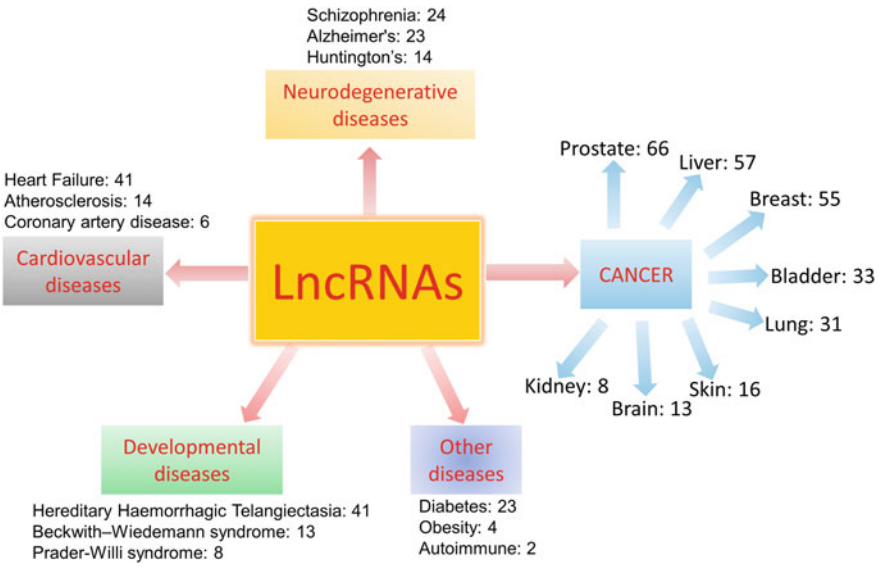

Fig. 2 Various types of diseases found associated with lncRNAs. The number shown for each type of disease is the number of lncRNAs found associated with the disease by experimental evidence on interactions, epigenetics, mutation, expression, and genomic location. The figure was produced by the authors using statistics from the lncRNA Disease database (updated 2014 June 14th) (Chen et al. 2013)

Alzeimer's disease (Faghihi et al. 2008). ANRIL is involved in type-2 diabetes and coronary artery diseases. The lncRNA ANRIL can cross talk with microRNAs at epigenetic levels. ANRIL binds to PRC2 and epigenetically represses miR-99a/miR-449a, thereby controlling mTOR and CDK6/E2F1 pathways (Zhang et al. 2014). LncRNAs are also involved in immune response processes, as in the case of a lncRNA overlapping 3'UTR region of the interleukin-7 receptor α-subunit gene (lnc-IL7R), which when being repressed reduced trimethylation of H3K27 at proximal promoter regions of inflammatory mediators, diminishing LPS-induced inflammatory responses (Cui et al. 2014). LncRNAs bind to STAT3 in the cytoplasm and promote STAT3 phosphorylation, which is essential for dendritic cell differentiation and T cell activation (Wang et al. 2014). For development and growth diseases, the lncRNA IPW (imprinted gene in the Prader-Willi syndrome region), which is normally transcribed from a paternal allele on chromosome 15, interacts with G9A methyltransferase to maintain H3K9me3 state at the DLK1-DIO3 region on chromosome 14 to repress maternally expressed genes (MEGs) (Stelzer et al. 2014). The aberrant upregulation of MEGs may contribute to Prader-Willi phenotypes.

3.2 *Known LncRNAs from Enhancers and Repetitive Elements with Significant Association to Human Diseases*

Exons of lncRNAs contain two times higher the number of disease-associated SNPs than those in exons of coding RNAs (Iyer et al. 2015). Notably, lncRNAs are on average longer than coding RNAs, so the higher number of SNPs in lncRNAs may be partly attributed to the larger sizes. Among 301 known cancer-linked SNPs, 88 % are at introns or intergenic regions (Cheetham et al. 2013). The rs944289 SNP is in noncoding region and is linked to the downregulation of a 3.2 kb downstream thyroid-specific lncRNA PCTSC3 (papillary thyroid carcinoma susceptibility candidate 3) which is a possible tumor suppressor (Jendrzejewski et al. 2012). Andersson et al. (2014) found significantly more disease-related SNPs in promoters and enhancers than in exon regions or in random sequence of a wide range of cell types and diseases. As a proof of concept, the authors used in vitro luciferase assay to show that reduced enhancer activities due to two SNPs within enhancers are associated with diabetes and Crohn's diseases (Andersson et al. 2014). The cancer-associated variant, rs6983267, regulates expression of an adjacent lncRNA CARLo-5 (cancer-associated region long noncoding RNAs) via long-range interaction between MYC enhancer and CARLo-5 promoter, which correlates with increased cancer susceptibility (Kim et al. 2014).

A noticeable number of lncRNAs derived from or containing repetitive elements are found involved in pluripotency and immune responses. A lncRNA chimera human-viral transcript derived from an integrated genomic region of Hepatitis B virus gene X to a LINE1 site enhances Hepatocellular carcinoma tumor proliferation via Wnt/β-catein signaling pathway and promotes metastasis via epithelial to mesenchymal transition (Lau et al. 2014). Transcription of transposable elements, especially those originated from endogenous retroviruses (ERV), is a part of pluripotency regulation network (Kunarso et al. 2010). Long terminal repeat derived transcripts, particularly those belong to endogenous retrovirus families, are found enriched in human, mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) compared to mouse epithelial fibroblasts (MEFs), human fetal dermal fibroblast (HDF-f), B lymphocytes, and T lymphocytes (Fort et al. 2014). Interestingly, a primate-specific endogenous retrovirus (HERVH) binds naive-pluripotency transcription factor LBP9 to drive transcription of hESC-specific alternative and chimeric transcripts, with over 10 % being lncRNAs, to regulate pluripotency (Wang et al. 2014).

Repetitive lncRNA such as TERRA RNA at telomere ends is required for recruiting telomerase complex and is needed for telomerase protection (de Silanes et al. 2014; Porro et al. 2014). A novel response class, consisting of lncRNAs and small ncRNAs, named as DDRNAs, is needed for site-specific DNA repair and may act in recruitment of DNA damage repair complexes (Francia et al. 2012). Overexpression of PCAT1 lncRNA impairs DNA damage repairs (Prensner et al.

2014). RNA can form hybrids with complementary DNA, which then act as template for homologous recombination and DNA damage repair (Keskin et al. 2014).

3.3 *LncRNAs as Biomarkers for Diagnosis, Prognosis and as Targets for Gene Therapy*

Screening of lncRNAs for potential therapeutic targets is being developed. Most lncRNA loci have been identified and genome-wide lncRNA differential expression analysis starts to reveal hundreds of potential candidates. For example, an RNA-sequencing expression analysis of a noninvasive lung cancer cell line (CL1-0) and a more metastatic prone sub-clone (CL1-5) identified 111 lung cancer-associated lncRNAs, which include candidates with experimental evidence support such as the lung cancer metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and the lncRNA *SCAL1* (*smoke and cancer-associated lncRNA-1*) (Thai et al. 2013).

Several lncRNAs have been shown as promising biomarkers in diagnosis and prognosis. Overexpression of lncRNA PCAT-1 by 50 % or more may be a prognostic indicator for colorectal cancer progression (Ge et al. 2013). HOTAIR is a predictor of tumor metastasis and survival in breast cancer progression. Approximately 125-fold overexpression of HOTAIR was found in more than one-third of all primary tumors studied (Gupta et al. 2010). An analysis from public database for 2255 patients suggests that HOTAIR expression level is strongly correlated with hazard ratio for esophageal squamous cell carcinomas and colorectal cancer (Deng et al. 2014). Applying CAGE for 50 matched human hepatocarcinoma liver samples, Hashimoto et al. (manuscript submitted) found 43 LTR-derived lncRNAs strongly upregulated in more than 50 % of cancer tissues. Kaczkowski et al. (manuscript submitted) compared FANTOM CAGE expression in 216 different cancer cell lines with corresponding primary cell lines and identified a core set of pan-cancer biomarkers, including enhancer RNAs and RNAs from repetitive elements.

LncRNAs have been shown as promising therapeutic targets. Knockdown of the lncRNA-JADE represses histone H4 acetylation in DNA damage response pathway and reduces breast tumor growth in vivo in mice (Wan et al. 2013). In a test for a potential therapeutic intervention to Angelman syndrome, antisense oligonucleotides were successfully applied to knockdown UBE3A-ATS transcripts, allowing the expression of paternal Ube3a in neuron both in vitro and in vivo, which in turn recovers UBE3A ligase protein expression and mediates some cognitive deficits in Angelman mouse model (Meng et al. 2014). Intratumoral injection of a plasmid carrying a toxin produced under the control of the lncRNA H19 promoter was applied to reduce tumor size in bladder, ovarian, and pancreatic cancer. In a clinical trial phase 2 for diphtheria toxin-ABC-819, 33 % complete ablation of bladder cancer tumor and 66 % prevention of new tumors in the first 3 months were reported (Gofrit et al. 2014).

3.4 *Strategies for Perturbation of Disease-Associated LncRNAs*

For selection of lncRNA perturbation technologies, a collection of lncRNA knockdown options are available. These include both traditional reagents such as antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), new classes of inhibitory molecules such as AntagoNAT oligonucleotides (single-stranded gapmer LNAs-modified with phosphorothioate backbone) to knockdown natural antisense transcripts (NATs), and precise genome-editing nuclease technologies, most commonly including the use of chimeric nucleases Transcription Activator Like Effector Nucleases (TALENs), Zinc Finger Nucleases (ZFNs) and Clustered Regulatory Interspaced Short Palindromic Repeat (CRISPR)/Cas-based RNA guided DNA nucleases (Gaj et al. 2013; Takahashi and Carninci 2014; Kim and Kim 2014). Use of ASOs was reported to produce effective knockdown of lncRNA in vivo by 50–80 % for MALAT1 in human and mouse (Gutschner et al. 2013). In human and monkey liver cell lines and in intravenous injection experiments on African green monkeys, downregulation of APOA1-AS by ASOs enhanced expression in APO (Apolipoprotein) gene cluster, including APOA1, APOC3, and APOA4 (Halley et al. 2014). ANRIL (antisense noncoding RNA in the INK4 locus) recruited PCR2 complex to specifically repress mir99a and mir449a in gastric cancer, while siRNA knockdown of ANRIL decreased expression of mRNA targets of these two miRNAs (Zhang et al. 2014). Knockdown of the low abundance antisense brain-derived neurotrophic factor (BDNF-AS) by SiRNA increased BDNF mRNA and BDNF protein levels in hippocampal neurospheres (Modarresi et al. 2012). Retroviral transduction could stably overexpress HOTAIR to several 100-fold in human breast cancer cell lines (Gupta et al. 2010). Application of AntagoNATs for transiently upregulating expression of sense protein-coding genes in a locus-specific manner opens a new pharmacological strategy to expression perturbation (Modarresi et al. 2012). Knockout of MALAT1 by 1000-fold in human lung tumor cells was achieved using zinc finger nucleases, creating an efficient loss-of-function model (Gutschner et al. 2013). Overexpression of lncRNA CCAT1-L by 15- to 30-fold using TALENs showed that upregulation of CCAT1-L lncRNA enhanced MYC expression (Xiang et al. 2014). Efficient deletion of a large 23-kb fragment within the lncRNA Rian was achieved by CRISPR/Cas9 system in mice (Han et al. 2014).

Selection of lncRNA knockdown targets needs to consider molecular mechanism of actions of the targets. For example, the targets can be different if the lncRNA directly participates in regulation, or if their transcription process is needed to generate chromatin context for regulating transcription of other genes (Bassett et al. 2014; Latos et al. 2012). A number of lncRNAs form complexes with epigenetic factors, which repress cancer suppressor genes by modifying chromatin state. For these targets, repressing RNA–protein complexes such as HOTAIR-PRC2, ANRIL-CBX7 (chromobox homolog 7), PCAT-1-PRC2, and H19-EZH2 (enhancer of zeste homolog 2) may specifically reactivate cancer suppressor genes (Fatemi et al. 2014). The interdependence of the lncRNA and protein in these complexes suggests

that the endogenous levels of proteins and lncRNAs may decide target selection, i.e., it may be more effective to use small molecule inhibitors to inactivate the protein in the case that the lncRNA is abundant and vice versa (Gupta et al. 2010). On the other direction, for more direct tumor suppressing lncRNAs such as TUG1 (taurine upregulated gene 1) (Zhang et al. 2014) and PINT (p53 induced transcript) (Marin-Bejar et al. 2013), direct upregulation of those lncRNAs may reduce tumor growth. A database for putative coding genes affected by lncRNA knockdown or overexpression, for instance the lncRNA2Target database, is useful for lncRNA target selection (Jiang et al. 2014). In addition, selection of lncRNAs should take into account half-lives of targeted lncRNAs, for which the current understanding is still limited. Most lncRNAs produced from bidirectionally balanced transcription are suppressed post-transcriptionally by ribo-nucleolytic RNA exosomes complex (Andersson et al. 2014). In a microarray stability assay for 823 lncRNAs in mouse Neuro-2a cells, Clark et al. (2012) showed that lncRNA half-lives are similar in range of coding RNA, and over 6 % of lncRNAs are highly stable (>12 h).

Furthermore, selecting knockdown regions within lncRNA targets should consider structure and functional domains. LncRNAs commonly comprise two exons, fewer compared to mRNAs (Derrien et al. 2012), but exons of lncRNAs can be large in size, requiring careful selection and combination of targeted knockdown regions. However, structure of lncRNAs is still poorly understood, due to lack of high-throughput biophysical and biochemical tools for RNA structure analysis (Mortimer et al. 2014). Similarly, functional domains of lncRNAs are still not well studied due to technological constraints. Although repeat insertion domains present in most lncRNAs may act as binding domains of many lncRNAs to proteins and DNA, these repetitive domains may be more challenging to be specifically and effectively knockdowned (Kelley and Rinn 2012; Johnson and Guigo 2014). Recent development of domain-specific chromatin isolation by RNA purification (dChIRP) technology enables investigation of binding sites of single RNA domain in RNA–RNA, RNA–DNA, and RNA–protein interactions (Quinn et al. 2014). Such technology will provide useful parameters for selecting knockdown regions within lncRNAs. Another important challenge for developing lncRNA generic therapy is about specificity, efficiency, and immunogenicity of gene-delivery strategies. In-depth discussion about this challenge can be found elsewhere (Takahashi and Carninci 2014).

4 Perspectives

4.1 Studies of LncRNA Structure and Their Interactions with RNA, DNA, and Proteins

Recent advances in high-throughput experimental structure sequencing methods, e.g., structure-seq, in combination with computational modeling starts to produce rich information of secondary in vivo structure of tens of thousands transcripts

(Wan et al. 2014; Ding et al. 2014). The information may aid the design of knock-down targets to avoid stably folded RNA regions. However, these techniques are constrained by low resolution, which is usually not sufficient to predict functional domains of lncRNAs. In contrast, recent development of domain-specific chromatin isolation by RNA purification (dChIRP) enables thorough study of a functional domain of lncRNAs in pair-wise interactions of RNA–RNA, RNA–DNA, and RNA–protein, yet the throughput is low (Quinn et al. 2014). More advanced combination of computational biology and experimental approaches will increase resolution and throughput of RNA structure, which will advance lncRNA functional studies. More specialized tools are being rapidly developed for (1) RNA–protein interactions such as cross-linking immunoprecipitation CLIP-seq, with various protocols such as PAR-CLIP, HITS-CLIP, and iCLIP (Sugimoto et al. 2012; Hafner et al. 2010), for (2) chromosome organization by chromosome conformation captures (3C, 4C, 5C, Hi-C, Chia-PET, and 6C) (de Wit and de Laat 2012), for (3) RNA–DNA interaction by capture hybridization analysis of targets (CHART) (Simon et al. 2011) or chromatin isolation by RNA purification (ChIRP) (Chu et al. 2011) and a modified version of ChIRP to study domain-specific RNA–DNA interaction (dChIRP) (Quinn et al. 2014), for (4) RNA–RNA interaction by cross-linking ligation and sequencing of hybrids (CLASH) (Helwak and Tollervey 2014) or by RNA antisense purification (RAP-RNA) (Engreitz et al. 2014), and in situ labeling technologies and imaging (Chakraborty et al. 2012). Remarkably, application of dChIRP can decipher the lncRNA architecture and functions at domain-specific level and can detect pair-wise RNA–RNA, RNA–protein, and RNA–DNA interactions (Quinn et al. 2014). Combination of high resolution mapping of RNA–chromatin interaction sites using RNA antisense purification (RAP) with chromosome conformation capture (Hi-C) and computational modeling could revealed the mechanism of the lncRNA Xist to spread along the X chromosome by utilizing three-dimensional conformation of the genome (Engreitz et al. 2013).

4.2 Personalized Medicine

With the great magnitude of expression specificity, lncRNAs may be molecules of choice for future personalized medicine. Large-scale studies in FANTOM projects establish that tissue and disease specificity are important characters of lncRNAs. More insights on potential RNA therapies are discussed elsewhere (Takahashi and Carninci 2014). Promoter design can play a vital role in optimization of non-viral gene expression therapy to reduce inflammatory responses, to increase tissue specificity, and to increase expression levels (Pringle et al. 2012; Hyde et al. 2008). For example, complete removal of CpG dinucleotides in enhancer/promoter regions of non-viral expression vector administered in cystic fibrosis treatment resulted in stronger and longer expression of transgenes with undetectable inflammatory responses (Hyde et al. 2008). For this type of application, the use of FANTOM5-rich database of promoter usage with tissue specificity, promoter

structure, and promoter activities can help increase efficacy and specificity of therapeutic vectors (Forrest et al. 2014).

LncRNAs will provide additional options for gene therapies. The consensus number of distinct molecular targets of FDA-approved drugs (Food and Drug Administration) until 2006 was as low as 324, in which 266 (or 82.1 %) targets are human-genome-derived proteins (Overington et al. 2006). The total estimated number of druggable coding genes in a human genome is limited to approximately 2000–3000 genes (Russ and Lampel 2005). Expansion of the potentially druggable targets may need to include lncRNAs. Importantly, since disease-associated SNPs present more frequently in transcribed regions encompassing enhancers, promoters, and lncRNAs, the interpretation of genome-wide association studies (GWAS) should take into account these regulatory elements (Gong et al. 2014). Primate-specific SNPs found in lncRNA exons carry significantly higher selective constraint than those in intergenic regions (Necsulea et al. 2014). For therapeutic application of lncRNAs to be approved in clinical settings, it is likely that the effects of lncRNA perturbation should be characterized at regulatory network level. While functions and mechanisms of lncRNA are still poorly characterized, caution has to be taken on their application. The Progensa™ PCA3 urine test (Gen-Probe Inc., San Diego, CA, USA) using lncRNA prostate cancer antigen 3 (PCA3) as a marker for prostate cancer was approved by FDA. However, a recent assessment by the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group found that the current data are insufficient to support the clinical validity of PCA3 test for diagnosis and management of prostate cancer, unless further supporting evidence is available (Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group 2014). Better understanding of possible effects of lncRNA perturbation on cellular processes and availability of more clinical data will foster lncRNA therapeutic applications.

5 Conclusion

LncRNAs play important regulatory roles, and misregulation of lncRNAs is found associated with various diseases. Genome-wide sequencing of capped RNAs in FANTOM project enables promoter-centric analysis of transcription contributed to discovery of major lncRNA classes such as those derived from bidirectional promoters, enhancers, repetitive regions, and antisense RNAs. Moreover, by quantifying activities of regulatory sequences for lncRNAs, including promoters and enhancers, high level of lncRNA expression specificity can be found between individuals, organs, tissues, and cell types. From genome-wide CAGE sequencing of multitude of systematically classified primary cell samples, tissues and cell lines within FANTOM project, differential promoter and enhancer usage of lncRNAs can be linked to disease ontology terms (Forrest et al. 2014; Andersson et al. 2014). The lncRNA expression specificity in relation to human diseases makes lncRNAs promising candidates for biomarkers in diagnosis and prognosis, and for targets in

therapeutic treatments. This is important because lncRNA candidates add more options to the current limited number of druggable genes as well as limited use of disease-related SNPs from GWAS studies. Interestingly, a majority of SNPs lie within lncRNA, promoter, and enhancer regions, which open possibility to make use of information from adjacent lncRNAs and regulatory genomic regions such as promoters and enhancers to better link SNPs to diseases. Combined use of lncRNAs, coding genes, and SNPs may bring personalized medicine closer to clinical applications in the near future. Some challenges to be solved require high-throughput technologies for studying structure and interaction network, and technologies for effective perturbation of lncRNA expression.

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