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The emerging role of pseudogene expressed non-coding RNAs in cellular functions

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Abstract

A paradigm shift is sweeping modern day molecular biology following the realisation that large amounts of “junk” DNA”, thought initially to be evolutionary remnants, may actually be functional. Several recent studies support a functional role for pseudogene-expressed non-coding RNAs in regulating their protein-coding counterparts. Several hundreds of pseudogenes have been reported as transcribed into RNA in a large variety of tissues and tumours. Most studies have focused on pseudogenes expressed in the sense direction, but some reports suggest that pseudogenes can also be transcribed as antisense RNAs (asRNAs). A few examples of key regulatory genes, such as *PTEN* and *OCT4*, have in fact been reported to be under the regulation of pseudogene-expressed asRNAs. Here, we review what are known about pseudogene expressed non-coding RNA mediated gene regulation and their roles in the control of epigenetic states.

Keywords

pseudogenes; lncRNAs; epigenetic control; transcription

Small RNA modulate of transcription

Studies over the last decade suggest that similar to plants and yeast, RNA can play a role in controlling gene transcription by the targeted recruitment of epigenetic silencing complexes to homology-containing loci in the human genome (reviewed in (Hawkins and Morris 2008)). Many of the early observations involving RNA directed transcriptional gene silencing (TGS) suggested that antisense non-coding RNAs can function in controlling the recruitment of silent-state epigenetic complexes to the genome (Weinberg, Villeneuve et al. 2006). While these initial observations were carried out with small antisense non-coding RNAs, particular key cellular protein components were found to be involved in the process,

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including DNA methyltransferase 3A (DNMT3a), Histone Deacetylase 1 (HDAC-1), and Enhancer of Zeste (EZH2) (Jeffery and Nakielnny 2004; Weinberg, Villeneuve et al. 2006; Suzuki, Juelich et al. 2008; Ross, Suetake et al. 2010; Holz-Schietinger and Reich 2012). Interestingly, these protein complexes, HDAC-1, DNMT3a and EZH2 have been found to be integral in epigenetic silencing (Fuks, Burgers et al. 2001; Vire, Brenner et al. 2005), but how these protein complexes localize to targeted loci in the cell had, until recently, been largely unknown.

While several studies of exogenously introduced small antisense non-coding RNA directed control of gene transcription have been carried out (reviewed in (Morris 2009) it has, until recently, remained unknown as to what extent endogenous RNAs were active in human cells in controlling gene transcription and epigenetic states.

Endogenous long antisense RNAs are involved in transcription and epigenetic states

Studies carried out in the early 1990's suggested that antisense transcripts might be functional in regulating gene expression in cells (Spicer and Sonenshein 1992). In early 2003 evidence began to emerge suggesting that endogenous antisense transcripts might be functional in human cells and disease (Tufarelli, Stanley et al. 2003). While these early studies defined the presence of antisense non-coding RNAs with their protein-coding counterpart it was later studies that began to disentangle the mechanistic insights into how antisense long non-coding RNAs (lncRNAs) function. In these studies it was determined that several protein-coding tumor suppressor genes exhibit bidirectional transcription, with both sense and antisense transcripts being found spanning the respective gene (Morris, Santoso et al. 2008; Yu, Gius et al. 2008). Interestingly, these antisense transcripts appeared to be involved in controlling the promoter and ultimately transcription of their sense counterparts (Morris, Santoso et al. 2008; Yu, Gius et al. 2008). The suppression of these long antisense RNAs (asRNAs) resulted in the de-repression and transcriptional activation of their protein-coding counterpart (reviewed in (Weinberg and Morris 2013)). These observations suggested that one possible role for asRNAs in human cells might be in the control and modulation of gene transcription and epigenetic states.

More recent studies have found that pseudogenes also express asRNAs and that some of these asRNAs are active transcriptional and epigenetic modulators of their protein-coding counterparts. First observed with the Oct4 pseudogene 5 (Oct4-pg5), long asRNAs emanating from the pseudogene appeared to be controlling the transcriptional expression of the Oct4 protein-coding gene (Hawkins and Morris 2010). This antisense transcript was found to associate with Nucleolin and PURA (Hawkins and Morris 2010). Nucleolin has already been implicated in trafficking of proteins and possibly ncRNAs around the cell (Ginisty, Sicard et al. 1999) and PURA is a DNA-binding protein, capable of acting as both a repressor and activator of gene expression (Mittler, Butter et al. 2009). While these data implicated pseudogene asRNAs in transcriptional and epigenetic control of gene expression, connections between those proteins involved in small RNA directed TGS and longer forms of endogenous non-coding RNAs remained elusive.

Direct evidence of a role for long asRNAs in controlling gene transcription has emerged from observations with the PTEN pseudogene. The PTEN pseudogene, (PTENpg1), is a pseudogene expressed from chromosome 9, while its protein-coding counterpart, PTEN, is expressed on chromosome 10 (Steck, Pershouse et al. 1997; Teng, Hu et al. 1997). The PTEN tumour suppressor gene is a negative regulator of the Akt/PKB signalling pathway, which is epigenetically altered in many cancers (Xing 2010). PTEN functions predominantly as a tumor suppressor gene whose expression is key to genomic stability (Yin and Shen 2008). Recent observations by Johnsson et al. found that PTENpg1 expresses a long asRNA and that this asRNA is an active transcriptional modulator of PTEN (Johnsson, Ackley et al. 2013). While this study was similar to previous studies with p15 (Yu, Gius et al. 2008), p21 (Morris, Santoso et al. 2008), Oct4 (Hawkins and Morris 2010) and BDNF (Modarresi, Faghihi et al. 2012), it shed light on the mechanism of action and demonstrated that the PTENpg1 asRNA directly interacts with DNMT3a. It was also observed that EZH2 was required for PTENpg1 asRNA to epigenetically modulate PTEN (Johnsson, Ackley et al. 2013). These observations suggest that long asRNAs arising from pseudogene loci may be guiding epigenetic remodelling complexes around the genome to impart transcriptional control of gene expression (Figure 1A). Indeed, the notion of long non-coding RNAs acting as scaffolds is quickly emerging.

Non-coding RNAs as guide and scaffold complexes

Some lncRNAs have been found to act as adaptors on which molecular machinery can be assembled. Initially, these lncRNA scaffolds were thought to be linker molecules involved in facilitating protein complex assembly, however more recently the term has been used to describe all higher-order RNA organisational structures. In addition to their functions as part of ribonucleoprotein (RNP) complexes, scaffolds can also act as tethering molecules between two distal regions of DNA. RNA molecules may be termed scaffolds whether they interact with either a single type of molecule such as TERC in the RNP telomerase, or form a complex web between multiple molecules, such as the lncRNA HOTAIR (Hox antisense intergenic RNA). HOTAIR and its associated complex appears to be a bimodal complex consisting of a lncRNA and several proteins exhibiting opposing functions such as the repressive PRC2 and the activating LSD1, all tethered in one complex (Khalil, Guttman et al. 2009; Gupta, Shah et al. 2010; Tsai, Manor et al. 2010; Chu, Qu et al. 2011)(Figure 1B). Many other lncRNAs are known to also function in *trans* such as Tsix, an antisense transcript that evolved from a pseudogenization of the XIST locus (Duret, Chureau et al. 2006; Do, Han et al. 2008), and ANRIL of the INK4b/ARF/INK4a locus (Yap, Li et al. 2010).

Once assembled, these scaffolded complexes can be guided to target sites as a result of either the lncRNA target homology (as is the case with PTENpg1) or via protein specificity. Though it should be made clear that it remains unknown as to whether the lncRNA first binds proteins and recruits them to target loci or if the lncRNA associates first with homology containing target loci and leads to the subsequent recruitment of the particular protein complexes.

Another example of a scaffolding function for lncRNAs can be found in Telomerase. Telomerase is a RNP complex consisting of a specialised Telomerase reverse transcriptase (TERT) paired with a lncRNA termed Telomerase RNA (TERC).

Telomerase is found in almost all eukaryotes and functions to preserve genome stability by adding DNA repeat sequences to chromosome ends. TERC interacts with TERT in a specific manner to provide a template for telomerase repeat sequences (Lustig 2004). The association between TERC and TERT forms secondary structures essential to the fidelity of telomere synthesis as well as telomerase enzyme stability (Lustig 2004; Collins 2008). The manner in which TERC and TERT associate is highly specific, as mutations in this scaffolding complex have been shown to contribute to diseases including cancer and aplastic anaemia (Yamaguchi, Baerlocher et al. 2003; Artandi and DePinho 2010).

Another scaffolding lncRNA is HOTAIR. HOTAIR is a lncRNA encoded in the HOXC gene cluster that is involved in the epigenetic regulation of HOXD and many other genes through chromatin remodelling (Rinn, Kertesz et al. 2007; Tay, Blythe et al. 2009; Tsai, Manor et al. 2010). HOTAIR is a RNA scaffold for the chromatin remodelling complexes PRC2 (polycomb repressive complex 2) and LSD1/CoREST/REST (Tsai, Manor et al. 2010; Qi, Xu et al. 2013). PRC2 binds to the 5' domain of HOTAIR and contains the H3K27 methylase EZH2, SUZ12 and EED (Rinn, Kertesz et al. 2007; Tay, Blythe et al. 2009; Gupta, Shah et al. 2010; Qi, Xu et al. 2013) and act to repress gene expression. Whereas LSD1/CoREST/REST, a complex also found associated with HOTAIR, binds to the 3' domain and is involved in the demethylation of H3K4me2 (Tsai, Manor et al. 2010; Qi, Xu et al. 2013; Shiau, Trnka et al. 2013) and functions as an activator. Following the RNA-mediated assembly of these chromatin-remodelling complexes, HOTAIR acts as a guide to direct them to their target loci (Figure 1B). The genes at the targeted loci are transcriptionally silenced through enzymatic H3K27 methylation and presumably activated by H3K4me2 demethylation (Qi, Xu et al. 2013). HOTAIR overexpression has been observed in primary and metastatic breast tumours resulting in altered gene expression and increased tumour invasiveness and metastasis (Gupta, Shah et al. 2010). HOTAIR is a classic example of a lncRNA, which acts both as a scaffold and a guide (Figure 1B).

Long non-coding RNAs as Decoys

Long non-coding RNAs are thought to also be capable of acting as decoys to DNA-binding proteins such as transcription factors, chromatin modifying proteins or enhancers. Through sequence homology to the target gene, these RNAs act as bait to their specific effector proteins, binding them and preventing their interaction with a target gene (Hung and Chang 2010). This interaction results in repression of the original gene target by lncRNA occupation of positive transcriptional signal. A lncRNA behaving in this manner can be defined into the functional class of a decoy RNA. Decoys such as the lncRNAs Gas5 and PANDA appear to play a central role in transcriptional regulation of many genes (Kino, Hurt et al. 2010; Hung, Wang et al. 2011). More recently such a decoy effect has been observed with DNA methyltransferase 1 (DNMT1), whereby the lncRNA bound DNMT1 and repressed the ability of DNMT1 to maintain CpG methylation, ultimately resulting in

activation of the previously CpG methylated targeted genes expression (Annalisa Di Ruscio, Maria Eugenia Figueroa et al. 2013)(Figure 2A).

Another example of a lncRNA acting as a decoy can be found with the glucocorticoid receptor (GR). Glucocorticoid receptors are transcription factors, almost ubiquitous in mammalian cells, which are activated by the binding of a glucocorticoid ligand. They play a vital role in regulating genes involved in cell growth, metabolism, and survival (Schneider, King et al. 1988; Kino, Hurt et al. 2010). Glucocorticoid response elements (GREs) are transcriptional activation sites located in the promoters of target genes that are bound by GR to promote gene expression (Chrousos and Kino 2005; Kino, Hurt et al. 2010). A recently described ~600 nucleotide lncRNA named growth arrest-specific 5 (Gas5) exhibits a secondary hairpin structure which appears to have the ability to bind the DNA-binding domain of GR (Kino, Hurt et al. 2010). This binding sequesters GRs, obviating their access to GREs, and causing repression of GR-mediated gene expression (Kino, Hurt et al. 2010). Gas5 is a typical example of an RNA decoy as it titrates the GR from target DNA, which is the GRE, and causes down regulation of transcriptional signals that would otherwise arise from these GR-regulated genes.

PANDA (p21 associated ncRNA DNA damaged activated) is another example of a decoy lncRNA. PANDA is a ~1.5 kilobase lncRNA that is polyadenylated and 5'-capped. It is transcribed antisense to the CDKN1A promoter in the form of a single exon. The expression of CDKN1A and PANDA are induced by p53 following DNA damage. CDKN1A mRNA codes for the protein p21, which is responsible for cell cycle arrest, while PANDA is involved in cell survival by repressing the genes responsible for apoptosis. Apoptotic genes associated with p53, such as FAS and PUMA, are expressed following binding of the transcription factor NF-YA to their promoters. PANDA function as an RNA decoy by binding to the NF-YA transcription factor. This binding ultimately inhibits NF-YA from binding to transcriptional promoters leading to the suppression of genes involved in apoptosis (Hung, Wang et al. 2011).

Long non-coding RNAs can also act as decoys and bind microRNAs. MicroRNAs (miRNAs) have been observed to play instrumental roles in the complex choreography of genetic expression patterns. They exhibit functions in critical cellular processes, including cell differentiation, apoptosis and cycling events and function by binding targeted messenger RNAs and recruiting RNA induced silencing complexes (RISC) to protein-coding messenger RNAs, resulting ultimately in either degradation of the messenger RNA or suppression of protein translation from the messenger RNA template (Agrawal, Dasaradhi et al. 2003; Meister, Landthaler et al. 2004). The fine balance by which these RNAs regulate expression is paramount to normal cellular growth and function, as disruptions to miRNA expression patterns have been linked to disease, such as cancer (Lu, Qian et al. 2005; Care, Catalucci et al. 2007; Chang, Wentzel et al. 2007). To elucidate functions of specific miRNAs, antisense oligonucleotides to miRNAs have been used (Stenvang, Petri et al. 2012); however more recently, miRNA inhibitors have been developed that can be expressed from vectors within cells. These vectors produce products that are designed to out-compete endogenous mRNAs for their miRNA targets and are known as sponge RNAs

(Ebert, Neilson et al. 2007). Essentially the sponge RNAs are acting as decoy's pulling miRNAs away from their intended mRNA target.

Decoy functional RNAs were first observed with the lncRNA highly up-regulated in liver cancer (HULC) (Wang, Liu et al. 2010) and later with the PTEN pseudogene sense transcript (PTENpg1) (Poliseno, Salmena et al. 2010). These non-coding RNAs have been termed competing endogenous RNAs (ceRNAs) (Salmena, Poliseno et al. 2011), and appear to function by sequestering miRNA/RISC complexes away from protein-coding messenger RNAs (Figure 2B). Other examples of endogenously expressed lncRNAs acting as ceRNAs or sponges are loc285194 and linc-RoR (Johnsson 2013; Liu, Huang et al. 2013; Wang, Xu et al. 2013), which like HULC and PTENpg1 have been shown to display sponge activity. Similar to PTEN and PTENpg1 (Poliseno, Salmena et al. 2010), the oncogene KRAS also possesses a pseudogene (KRASP1) that is able to act as a miRNA sponge. The 3' UTR of KRASP1 contains binding sites for mir-143 and let-7 family members. When the 3' UTR of KRASP1 is overexpressed, KRAS mRNA is more highly expressed as is presumably the KRAS protein. This indicates that KRASP1 is able to prevent KRAS repression by binding to KRAS targeting miRNAs (Poliseno, Salmena et al. 2010), subsequently bolstering KRAS protein expression in the cell. The Oct4 pseudogene 5 sense transcript might also function as a sponge to affect Oct4 expression (Hawkins and Morris 2010), in a manner similar to Oct4-pg4 sponging miR-145 (Wang, Guo et al. 2013), though to date no studies have definitively determined this eventuality for OCT4-pg5. Interestingly, these pseudogene expressed ceRNAs appear strikingly similar in their function to circular RNAs. First described in the testis in the early 1990's (Capel, Swain et al. 1993), circular RNAs now appear to also be involved in sequestering protein complexes, including miRNAs, away from target messenger RNAs (Hansen, Wiklund et al. 2011; Hansen, Jensen et al. 2013) to affect gene expression.

Collectively, these examples of lncRNAs suggest that they can function as competitive inhibitors of miRNA binding, making them decoys by their own right (Figure 2B). Sponge RNAs contain sequence complementarity to their miRNA targets and sequester them, preventing their association with messenger RNAs and therefore resulting in translational activation of gene expression.

Technical challenges of studying lncRNAs

To date the great majority of work carried out on lncRNAs has been defining that they exist (Bernstein, Birney et al. 2012; Djebali, Davis et al. 2012) and that they are generally non-coding (Banfai, Jia et al. 2012). Most of these studies have been based on deep sequencing studies and bioinformatic analysis, including differential expression analysis (Mazar, Sinha et al. 2010). While these analyses have been telling with regards to possible functions; e.g. lncRNAs are present and differentially expressed, they do not prove function. Studies to delineate the various functions of lncRNAs have relied on either suppression (Carninci, Kasukawa et al. 2005; Morris, Santoso et al. 2008; Hawkins and Morris 2010; Johnsson, Ackley et al. 2013; Saayman 2014) or over-expression (Yu, Gius et al. 2008; Khalil, Guttman et al. 2009; Johnsson, Ackley et al. 2013; Saayman 2014) of the candidate lncRNAs. While these types of analysis can be informative with regards to those genes

affected by the particular lncRNAs, deep analysis such as chromatin immunoprecipitation analysis (ChIP) of lncRNAs containing biotin dUTPs (Johnsson, Ackley et al. 2013; Saayman 2014) or small antisense oligonucleotides containing biotin linked moieties and homology to particular lncRNAs, to determine lncRNA target binding sites is needed (Hawkins and Morris 2010; Chu, Quinn et al. 2012). Moreover, combining lncRNA CHIPs in conjunction with mass spectrometry analysis to determine lncRNA protein binding partners (Hawkins and Morris 2010; Chu, Quinn et al. 2012), is appearing to be one of the more powerful methods for discerning new and underappreciated functions of lncRNAs. Indeed, recent studies using biotin labelled antisense lncRNA from HIV determined that HIV functions in a manner similar to the PTENpg1 antisense lncRNA, interacting with DNMT3a to direct epigenetic silencing and possible viral latency (Saayman 2014). Knockout studies of lncRNAs may also prove useful in discerning phenotypes (Sauvageau, Goff et al. 2013), but such approaches may prove problematic for in vivo studies when either the transcript of interest is human specific (Johnsson, Lipovich et al. 2013) or when assessing lncRNAs that are functional in *cis*, e.g. knockout of the lncRNA may affect the protein-coding gene or enhancer like elements associated with the lncRNA and the *cis* locus. While only a handful of lncRNA have been interrogated in such a detailed manner, it is clear that what has been revealed to date is only the proverbial tip of the iceberg and that many more strange and under-appreciated functions abound for lncRNAs.

Conclusion and final remarks

RNA is an essential molecule of life; not only for its role in providing a message through which proteins can be formed but it is also an integral part of the cells regulatory and signalling mechanisms. Traditional studies have focused on proteins as the main effector molecules in transcriptional control of genes however it is now becoming evident that this view needs to be altered to accommodate the burgeoning role that non-coding RNAs may play in the life of the cell. While there are many examples of protein effectors such as transcription factors, enhancers and silencers, lncRNAs appear to be playing a subtler and possibly specific role in the fine-tuned regulation of genes involved in essential cellular processes. Of note only a few of the documented functions involving gene expression were discussed here. Indeed, lncRNAs can also affect protein translation, transcript splicing and RNA degradation to name just a few other roles whereby lncRNAs appear functional (Reviewed in (Ponting, Oliver et al. 2009; Johnsson, Lipovich et al. 2013)).

While much remains to be determined with regards to lncRNA form and function, it is becoming clear that interruptions to some lncRNA-mediated regulatory pathways appear to be involved in cellular deregulation and the progression to cancer. While protein-directed transcription is still vital, lncRNAs may have been introduced through evolution as secondary regulators involved in gene expression. At their simplest, lncRNAs can be signals or guides, interacting with a single protein or molecule to direct cellular changes. However, more intricate epigenetic regulation can be achieved through assembly of multi-component complexes consisting of RNA, proteins and DNA. The sophisticated interplay between these molecules lncRNAs, proteins, and chromatin, provides a level of regulation that to date is only becoming appreciated and suggests that complex interactions are operative in regulating gene expression.

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Figure 1. Non-coding RNAs acting as guides and scaffolds

(A) Some lncRNAs can bind chromatin remodelling complex proteins such as DNMT3a and possibly also EZH2 and recruit the particular complexes to homology containing target loci whereby stable epigenetic gene silencing can be initiated (Johnsson, Ackley et al. 2013; Saayman 2014). (B) Some lncRNAs, such as HOTAIR can function in a bimodal fashion recruiting disparate protein complexes to target loci, ultimately functioning as both a guide and scaffolding (Gupta, Shah et al. 2010; Tsai, Manor et al. 2010).

Figure 2. Non-coding RNAs acting as decoys

(A) Long non-coding RNAs can function in the nucleus and bind transcription factors or DNA methyltransferase 1 (DNMT1, shown) and affect the ability of CpG methylation to persist (Annalisa Di Ruscio, Maria Eugenia Figueroa et al. 2013). (B) Long non-coding RNAs can function cytoplasmically as sponges to soak up proteins and miRNA/RISC complexes away from their intended mRNA targets. The result of this sponging can be enhanced stability and translation of the miRNA targeted mRNA.