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Non-coding RNA and Antisense RNA. Nature's Trash or Treasure?

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Abstract

Although control of cellular function has classically been considered the responsibility of proteins, research over the last decade has elucidated many roles for RNA in regulation of not only the proteins that control cellular functions but also for the cellular functions themselves. In parallel to this advancement in knowledge about the regulatory roles of RNA there has been an explosion of knowledge about the role that epigenetics plays in controlling not only long-term cellular fate but also the short-term regulatory control of genes. Of particular interest is the crossover between these two worlds, a world where RNA can act out its part and subsequently elicit chromatin modifications that alter cellular function. Two main categories of RNA are examined here, non-coding RNA and antisense RNA both of which perform vital functions in controlling numerous genes, proteins and RNA itself. As the activities of non-coding and antisense RNA in both normal and aberrant cellular function are elucidated, so does the number of possible targets for pharmacopeic intervention.

Keywords

antisense RNA; non-coding RNA; epigenetic; transcription; gene silencing

RNA, Regulatory Nucleic Acid?

Recent research using techniques such as Genomic tiling arrays and cDNA sequencing has allowed transcription in the human genome to be studied with a degree of accuracy and resolution previously unattainable. The ENCODE project identified several functional elements in the Human genome and demonstrated that RNA can be processed to yield both short and long RNAs, which can overlap the 5' and 3' ends of protein coding regions [1]. Although approximately 90% of the Human genome is transcribed [2] the ENCODE project demonstrated that a surprisingly small amount actually encodes protein (~2% of the eukaryotic genome encodes protein coding genes (mRNA)), therefore, a vast number of transcripts appear which are non-protein coding RNAs (ncRNAs).

The identification of these long or short ncRNAs effectively split DNA transcription into two camps, coding and non-coding transcripts [3] where traditional non-coding transcripts are viewed to be ribosomal RNA (rRNA) and transfer RNA (tRNA), both of which play an

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essential function in protein translation. As for the other ncRNAs, which can be either long (>200bp) or short, there is disagreement as to whether these ncRNAs are transcriptional “noise”: nevertheless there is growing evidence that ncRNAs play an important role in cellular function [3] as is the case for the aforementioned tRNA and rRNA.

In general, the more complex an organism, the greater its number of ncRNAs [4]. Supporting this varied complexity is the observation that the processing of longer RNA to shorter RNA to yield unique secondary and tertiary structures that participate in cellular processes has long been known to occur in the presence of [5] and absence of protein [6,7]. The enticing possibility that although the level of protein coding transcripts between organisms is similar, the ultimate control of cellular function may be through interactions between proteins and ncRNA, which is corroborated by the fact that the majority of chromatin modifying complexes do not have DNA binding capacity [8] and therefore, must utilize a third party in binding to DNA. This is in stark contrast to transcription factors, which specifically recognize and bind DNA sequences and do not require extrinsic factors.

Classically it has been thought that control of gene expression is largely due to the action of transcription factors but ncRNAs have also recently been shown to exert their control over gene transcription *via* several different pathways, transcriptional gene silencing (TGS) through the targeted recruitment of epigenetic silencing complexes to particular loci (reviewed in [9,10]), through post-transcriptional gene silencing (PTGS); degradation of transcriptionally active mRNAs as exhibited in RNAi, siRNA and miRNA, and also via STAU-1 mediated RNA decay process [11]. Control of gene expression by ncRNA is evolutionary sensible; the response is rapid due to the speed of ncRNA production in the vicinity of the gene and the energy costs to the cell are much lower due to the lack of protein synthesis.

Epigenetics and ncRNAs

Epigenetics is the study of the underlying changes in phenotype that are caused by alterations to the expression of the genome by chemical modification of the DNA molecule. Notably, these chemical changes to the DNA do not alter the sequence context of the DNA. Although several forms of epigenetic regulation exist, the two main forms of interest for this review are the (1) addition of chemical groups to specific bases, as with DNA methylation and (2) the local alterations of histones, such as targeted methylation at particular lysines that affects the accessibility of the surrounding genomic DNA to the transcriptional machinery. These epigenetic controls have been shown to be capable of being passed onto daughter cells as shown by changes to the *IGF2* gene that are persistent across familial generations six decades later [12]; an effect that has also been noted in mice [13,14]. One obvious question, which arises from such observations, is whether or not there is a link between the large amount of transcribed ncRNA and the regulation of genome modification *via* epigenetics. Given that high-throughput sequencing revealed transcription in ~90% of the genome, including RNAs transcribed at a low-levels and not rapidly degraded [2]; suggests there must be a function for these ncRNAs, *i.e.* this is an energy cost to the cell and these transcripts have been retained over successive generations, so one would expect that they play some, as yet unknown, beneficial role. Although we currently cannot exclude the fact that the mere act of transcription of ncRNA is an archaic remnant of cellular activity and is due to the presence of a promoter region; growing evidence suggests that at least some of these ncRNA molecules play specific roles in eukaryotic cells and gene expression (reviewed in [9]). The comprehensive role that ncRNA plays in this epigenetic control remains to be fully elucidated but if recent observations are any indication, [15,16,17,18,19,20,21,22], ncRNAs in human cells might be active regulators involved in

controlling gene expression *via* the targeted recruitment of epigenetic complexes to various loci in the genome.

Expressed ncRNAs can show clear evolutionary conservation [23] and many emanate from gene promoter regions, which tend to be more conserved than protein-coding genes [16] but also exhibit little conservation of expressed regions between different species [24,25], [16]. These observations suggest a level of retention in the machinery of the cell and a possibly harkening to a role in gene level control. It should be noted that although ncRNAs can be found in polyadenylated, unadenylated or bimorphic forms [26] and do not contain classical ORFs longer than 100 amino acids; some might in fact encode small peptides [27]. Such an eventuality could add even more layers of complexity to the cell than have been previously appreciated.

Antisense RNA and non-coding RNA: The Yin and Yang of gene control

Some of the most studied ncRNA to date have been the long intergenic non-coding RNAs (lincRNAs), which are a heterogeneous group of transcripts involved in epigenetic control of the cell that range in size from ~300 nucleotides to several thousands. Currently the human catalog of lincRNAs is thought to be around 3,300 although the true number may be closer to 4,500 [28]. Often associated with these ncRNA is an antisense RNA (asRNA) that contains a sequence complementary to the ncRNA and thus may afford the cell another layer of genetic regulation.

To date the most studied and well understood lincRNA is the 17,000 nucleotide transcript Xist, which is involved in X chromosome inactivation (for an in-depth review see [29]). Of prime importance in X-inactivation is the X inactivation center (XIC in humans, Xic in mice), which contains at least two ncRNA, the aforementioned XIST (XIST humans and Xist in mice) and its asRNA Tsix. Expressed early on in embryonic development, Xist is weakly expressed by both X chromosomes until cell differentiation when an yet-to-be determined key factor triggers up-regulation of Xist transcription from the future inactive chromosome by progressive coating of the chromosome from the XIC outwards [30]. In humans this randomly coats one of the two X chromosomes in females whereas in mice the Xist locus on the maternal X chromosome is always repressed and thus, the maternal X chromosome is always active giving rise to an X_{active} and X_{inactive} [31]. Upon differentiation the histone modification of the active and inactive become significantly altered with the inactive X chromosome exhibiting more repressive chromatin modification, which is thought to play a role in recruitment of proteins, while the active X chromosome exhibits silencing of the Tsix asRNA promoter due to a lack transcriptional machinery recruitment. The result of these eventualities is the alteration of the expression of Xist and the coating of one of the chromosomes by the ncRNA causing inactivation of those chromosome associated genes due to the loss of histone modification by acetylation and methylation [32]. This coating of the chromosome ensures an equal dosage of gene expression between X-linked genes of males and females. In mice, this inactivation has been shown to require an interaction between the 5' of Xist, named RepA [33] and the Polycomb Repressive Complex 2 (PRC2, a complex containing histone methyltransferases (HMTases), Enhancer of Zeste (EZH2, a H3K27 histone methyltransferase) and SUZ12 or G9A (both of which are H3K9 histone methyltransferases)).

Therefore, in order to maintain an active X chromosome, control of the Xist ncRNA is critical. In mice this is achieved by the action of the antisense RNA Tsix. Notably, in Humans Tsix appears to either truncated or absent [34]. Tsix is a 40Kb antisense transcript (that is further processed to smaller transcripts [35]) and found to be expressed from the X controlling element at the opposite strand of Xist and thus overlaps the 3' end of Xist gene

[36]. Unlike Xist, Tsix shows high levels of transcriptional machinery and epigenetic marks in undifferentiated cells [37]. Tsix acts in *cis* to regulate the role of Xist in X inactivation by repressive marking of the Xist promoter. The repressed state of Xist exhibits low levels of (H3K4) methylation and (H3K9) acetylation whilst also exhibiting higher levels of the repressive H3K9 and CpG DNA methylation [37]. Due to these epigenetic marks the Xist RNA cannot be detected by either ChIP or nuclear run on transcription [38,39] and ensures that the chromosome is not coated in Xist; although doubt still surrounds this hypothesis [40] it appears most likely that regulation of Xist is due to a shift from a heterochromatin state to a euchromatin state. Interestingly, the human TSIX contains only ~50% sequence homology to its murine cousin, but retains many similarities in that it is expressed in embryonic derived cells, produces a ncRNA and is initiated downstream 3' of XIST. As the human TSIX transcript does not appear to overlap the 5' region of XIST, such an eventuality rules out a direct interaction with the XIST promoter. What role human TSIX exactly plays remains to be determined. Some have postulated that the human TSIX is simply an evolutionary carry over [34].

Although not as well studied as X-inactivation, one area of epigenetics that utilizes ncRNA that are antisense to their gene counterpart is gene imprinting, where genes are expressed in a manner that is dependant upon their paternal or maternal origin. Some of the first genes to be identified as imprinted were the insulin-like growth factor type-2 receptor (Igf2r), Igf2 and H19 locus [41,42,43]. H19 is a tumor-suppressor gene and produces a long ncRNA (although considerably shorter than Xist) that is 2.3kb long, capped and polyadenylated [44]. The H19 ncRNA is transcribed exclusively from the maternal allele during development of the embryo but down-regulated after birth, whilst the IGF2 gene is expressed exclusively from the paternal allele [43].

Of major importance to genetic imprinting are imprinting-coding regions (ICR), which are differentially methylated regions in the genome that control the local transcription. The ICR for H19/ IGF2 is located approximately 2kb upstream of the H19 promoter and controls expression of IGF2 interaction with chromatin insulator protein, CTCF. The H19 ncRNA is maintained by post-transcriptional regulation and is mainly localized to the cytoplasm but has also been identified in the nucleus [45,46]. Its primary functions are varied with one function being implicated in RNA regulation [47]. The H19 transcript is associated with polysomes and may regulate IGF2 expression in *trans* [48], tumor suppression [49] and oncogenesis, particularly in breast cancer [50]. Mechanistically, it has been proposed that the H19 ncRNA competes for the binding region in the promoter of overlapping genes and therefore prevents binding of transcription factors [51]. Importantly, unlike Xist, H19 does not silence genes but rather differentially modulates their expression.

Recently, Berteaux and colleagues identified a novel antisense RNA to H19 [52]. The 120Kb antisense transcript called 91H, was found to be stabilized in breast cancer cells, overexpressed in human breast tumors and exhibiting a role in the regulation of IGF2 *in trans*. Expressed on the maternal allele, 91H is found at a higher concentration in the nucleus (>80%) than the cytoplasm unlike the sense RNA (H19), which is equally distributed between the nuclear and cytoplasmic compartments of the cell. It has also been shown that all cancerous cell lines examined for breast cancer exhibited higher levels of 91H than normal breast tissue due to an 8-fold increase in the half-life stability of the transcripts. In order to determine if 91H acts in a contrary manner to H19, as with Xist and Tsix, its transcript was silenced using RNA interference (RNAi). Interestingly, the suppression of 91H by RNAi did not cause any notable change to the imprinting of H19 on the maternal allele but caused a *trans* down-regulation of the IGF2 gene from the paternal allele. This observation suggested a role of 91H in the recruitment of chromatin modifying enzymes to

the maternal allele and thus when 91H is knocked down the IGF2 gene is down-regulated due to epigenetic modifications, in a similar manner to p15 [22].

Imprinted genes are frequently regulated by ncRNAs and tend to be *cis*-regulated with associations to chromatin-modifying complexes. One of the best-known examples of this is the long antisense ncRNA AIR, which acts in *cis* to interact with promoter chromatin and the H3K9 histone methyltransferase G9a to cause gene suppression *via* hypermethylation [53]. There is also the well characterized 2,200 nucleotide lincRNA, HOTAIR, a *trans*-acting long intergenic ncRNA located antisense to the *HOXC* locus (Chromosome 12) that interacts with the Polycomb Repressive Complex 2 (PRC2) and results in H3K27 trimethylation of *HOXD* locus (Chromosome 2)[54] and the subsequent suppression of *HOXD* genes in *trans*; suggesting that HOTAIR associates with and guides chromatin modifying complexes to their target [54].

These examples are the traditional imprinting genes that have been well studied and provided the first concrete evidence that non-coding RNAs can play a role in gene expression (reviewed in [55]). One commonality with these loci is their utilization of the endogenous epigenetic regulatory machinery in the regulation of the intended targeted loci. Unfortunately, these complex imprinted loci and the role ncRNAs as well as asRNAs play in their respective regulation, has been somewhat overlooked. The implications from these works have in essence been relegated to the “peculiar aspects” of cellular differentiation. A recent body of evidence has emerged suggesting that ncRNAs can play a role in the regulation of gene transcription in differentiated cells [20]. These observations, suggesting that at least one endogenous mechanism may be operative and involved in the control of gene transcription in differentiated cells offers significant insights into the possibility of specifically controlling protein-coding genes at the transcriptional level. Such an opportunity may provide the framework for a new class of pharmacopeia, as shown in figure 1. Antisense therapy is undergoing rapid development; the first asRNA drug to be approved by the FDA was Fomivirsen (previously supplied under the brand name Vitravene, Isis Pharmaceuticals). Used in the treatment of viral inflammation of the retina of the eye by cytomegalovirus (CMV retinitis), common amongst patients with compromised immune systems. Fomivirsen is a 21-nucleotide oligonucleotide that is stabilized by phosphorothioate linkages. Administered by intravitreal injection, Vitravene binds to its complimentary sequence in the CMV mRNA and prevents translation. Paramount to the success of future therapies is the stability of the oligonucleotides used for antisense therapy; especially for RNA therapeutics. With this in mind, RNA (and DNA) can be highly modified by inclusion of numerous modifications which include modification of the ribose chemistry (2'-Fluoro [56,57], 2'-Amino, 2'-O-Methyl [58] and locked nucleic acid (LNA)), modification of the phosphate backbone as with phosphorothioate and the inclusion of non-ribose backbones as with peptide nucleic acid (PNA) and phosphorodiamidate morpholino oligo (PMO). Together, these modifications increase the molecules resistance against endo- and exo-nucleases. Recent antisense work has targeted miRNA and their role in disease states [59] although, as shown in figure 1, there are currently a wide variety of asRNA drugs in development for numerous disease states including cancer, cardiovascular disease, neurodegenerative diseases, infectious diseases and inflammatory diseases.

There has been debate as to whether ncRNA cause or repress disease; in the case of HOTAIR, expression levels increase approximately 125-fold during the progression of breast cancer, eventually leading to altered gene expression, specifically epigenetic silencing of tumor suppressor genes [60]. Additionally, the antisense ncRNA ANRIL (antisense non-coding RNA in the INK4 locus) [61] was also recently identified as a risk locus for diseases including gliomas [62] and basal cell carcinomas [63]. Notably, out of all Human tissues the brain and the testes produce the greatest quantity of unique ncRNA and it is in these regions

that there would be predicted to be a number of diseases linked to ncRNA and asRNA in Humans [64]. Currently the human catalog of lincRNAs is thought to be around 3,300 although the true number may be closer to 4,500 [28]. In Homo sapiens there are 108 ncRNA that have experimentally verified and published (lincRNA database [65]). Further streamlining of the data shows that 34 of these related are to disease states and of these 11 are related to asRNA (with 41 asRNA in the database). At present there is no overwhelming bias towards ncRNA and asRNA disease states in either the brain or testes but with approximately 2% of the predicted ncRNA studied it remains plausible that there are many interesting discoveries to be made.

The ncRNA FMR4 (2.4Kb) and its antisense ASFMR1 are both silenced in the genetic disease fragile X syndrome [66] and, of particular interest to an aging population, is the recent discovery that the enzyme β -secretase-1 (BACE1) is regulated *via* mRNA interactions with an antisense transcript (BACE1-AS). BACE1-AS is a 2kb transcript that is produced on the opposite strand of the BACE1 locus and is present in two different forms in humans and mice which, as with other ncRNA, are polyadenylated; suggesting that they are targets for RNA Polymerase II but sequencing indicates that they contain no protein coding ORF. Upon encountering stressors, the cell upregulates the amount of BACE1-AS transcribed and subsequently through interactions with its target mRNA, the amount of amyloid precursor protein being converted to A β 1-42 increases concordantly with the asRNA level [67]. So it would appear that both ncRNA and asRNA may indeed play an important role in disease states.

The role of asRNA in pluripotency

The medical benefits of stem cell research make understanding regulation of pluripotency an area of great interest for modern researchers; for asRNA to be involved in this regulation would be an exciting development. Recent work involving mouse cells has shown that Oct4 and Nanog, two transcription factors critical for self-renewal processes in undifferentiated stem cells, directly target and regulate the transcriptional expression of two lincRNAs [68]. Further study in human cells has show that Oct4 is further regulated by the action of a pseudogene expressed antisense ncRNA that regulates the transcription of Oct4 [18]. As discussed above for various other antisense and ncRNA, the Oct4 asRNA was not only antisense and overlapping to the Oct4 promoter but also found to lack a polyadenylated tail and, most intriguingly, to regulate Oct4 transcription by targeted recruitment of various epigenetic remodeling proteins to the Oct4 promoter [18]. The results for the antisense ncRNA regulation of Oct4 reflect those observed for the respective antisense ncRNAs associated with p15, p21 and HOX loci [20,22,54]. Based on these bodies of work, a model has been developed, whereby the long antisense ncRNAs are expressed either in *cis* or *trans*, and function in *trans* to both target homology containing loci as well as recruit particular proteins to the homology containing targeted loci (Figure 2). Such a model implies one role for long antisense ncRNAs is to both bind epigenetic remodeling proteins as well as to guide them to the appropriate target loci in the genome and instigate epigenetic remodeling (Figure 2). It appears that not all ncRNAs regulate their sense counterpart in a discordant manner; as proposed after analysis of data from the RIKEN consortium analysis of RNA-based regulation in mouse cells (Functional Annotation Of Mammalian Genome, FANTOM) [69].

Concordant regulation [70] was observed when antisense ncRNAs were degraded by RNAi and the net result was the activation of the ncRNA-targeted gene. It has been proposed that concordant regulation is active due to RNA/RNA interactions of defined stoichiometries in the cytoplasm. When these interactions are perturbed by RNAi a resultant increase in sense/mRNA expression and gene activation is observed (personal communication based on [70]). One such example of concordant regulation is the stem cell genesis gene, Nanog. Like Oct4

[18], Nanog has specific pseudogenes that express long antisense ncRNAs that are unadenylated but unlike Oct4, Nanog does not appear to share homology its promoter. When Nanog antisense ncRNAs were suppressed, concordant regulation of Nanog was observed [71] which was surprising as this discordant regulation acted in the inverse manner as known discordantly regulated genes (reviewed in [18] and [72]). Several insights have been gleaned from these works. Taken together the Oct4 discordant and Nanog concordant model systems might be telling us something important about endogenous RNA circuits and gene regulation. No doubt when one considers now the plethora of ongoing RNA interactions in the cell, such as these observations whereby pseudogene expressed antisense ncRNAs are actively regulating their respective mRNA expressing counterpart in either a positive or negative manner (i.e. concordant or discordant manner respectively), indicates a far more complex landscape of RNA based regulation than had been previously appreciated.

It is evident that both small and long antisense ncRNAs are able to direct epigenetic control of gene transcription and recent observations have begun to craft a modified version of the previously held dogma that only transcription factors can control gene expression. The new model intercalates the action of ncRNAs and asRNA in the control of gene expression and indicates there is multiple layers of RNA based regulation, which would include RNA/RNA, RNA/protein, RNA/DNA based interactions. Due to its ability to act as a template for protein synthesis it is most likely that the basal strata of RNA directed regulation of gene expression is likely to function at the level of transcription. This fundamental layer of RNA based regulation may have some striking similarities and functional properties involved in the mechanism that dictates euchromatin *vs.* heterochromatin transitions in the nucleus. Certainly work with imprinted genes and X-inactivation, which involves significant compaction of the ncRNA targeted loci, would suggest many similar components involved in the basal modes of action [55,73].

The regulation of transcription, *via* ncRNA targeted epigenetic controlling mechanisms, may in fact be the essence of endogenous gene regulation and the fundamental core regulator of protein production. One could envision the stoichiometric ratios of particular ncRNAs are active in regulating gene transcription, but that these ncRNAs are themselves regulated indirectly by other ncRNAs and directly through interactions with RNA antisense to its sequence. Such regulation of a cell would add, in theory, an incredibly deep layer of complexity that would be extremely difficult to extrapolate into regulatory pathways. Therefore, it is probably wise to think of these interactions as part of a larger “ecosystem” of interactions.

Although complex, it is worth noting that it has been shown that modulating both ncRNA and asRNA regulatory networks might afford us the opportunity to control both single gene/protein expression and possibly yet-to-be discovered larger complex networks. Theoretically such knowledge could allow for increased gene specific targeting capabilities to be developed as well as studies on larger gene regulatory networks to be carried out.

Overall, it is becoming clear that what was once considered the trash of the cell is becoming treasure. As the body of work behind ncRNA and asRNA continues to grow the possibility increases that new mechanisms of action for classical diseases may be found and that a modern era is dawning for treatment of these diseases by regulating gene expression through manipulation of RNA-interacting pathways. Antisense is starting to make sense after all.

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We highlight and review the regulation of cellular process by ncRNA. How these ncRNA cause epigenetic changes that can be passed onto daughter cells. We then investigate the role that ncRNA plays in gene imprinting and disease states. The review highlights that RNA that is antisense to many of these ncRNA is present, and plays a regulatory role in controlling gene expression.

Figure 1. Current antisense RNA drugs in development
Data taken from Isis Pharmaceuticals, Antisense Pharma and AVI BioPharma.

Figure 2. Endogenous ncRNA directed epigenetic regulation of gene transcription
(A) Long antisense ncRNAs can be expressed in *cis* or **(B)** *trans* where they **(C)** fold into complex structures that can **(D)** contain both target homology to other loci or the bidirectionally transcribed loci in the genome as well as possess an ability to bind epigenetic remodeling proteins. The ncRNA directed epigenetic complexes could then **(E)** target the silencing of the corresponding homology containing loci, which can result in **(F)** heterochromatinization of the targeted site.