2017

Activating the Chromatin by Noncoding RNAs

Laham-Karam Nihay

Mary Ann Liebert Inc

info:eu-repo/semantics/article
info:eu-repo/semantics/acceptedVersion
© Mary Ann Liebert, Inc.
All rights reserved
http://dx.doi.org/10.1089/ars.2017.7248

https://erepo.uef.fi/handle/123456789/4341

Downloaded from University of Eastern Finland's eRepository
Activating the chromatin by non-coding RNAs

Nihay Laham-Karam1, Pia Laitinen1*, Tiia A. Turunen1*, Seppo Ylä-Herttuala1,2,3

1 A.I. Virtanen Institute, University of Eastern Finland, Kuopio, Finland
2 Heart Center, Kuopio University Hospital, Kuopio, Finland
3 Gene Therapy Unit, Kuopio University Hospital, Kuopio, Finland
* These authors contributed equally to the work.

Running title: ncRNA activating the chromatin

Correspondence should be addressed to N.L-K. / S.Y-H.
Address: Yliopistonranta 1E, 70210 Kuopio, Finland
Phone: +358 40 355 3292
Email: nihay.laham-karam@uef.fi / seppo.ylaherttuala@uef.fi

Key words: non-coding RNA, chromatin, transcriptional activation, siRNA, miRNA, IncRNA, piRNA

Word count (excluding references and figure legends)- 8,095 (abstract 250)
Reference numbers- 168
Number of greyscale illustrations- 3
Number of colour illustrations- 3 (online 1 and hardcopy 2)
Abstract

**Significance:** The extent and breadth of transcription has recently been uncovered and this has revealed an extensive array of non-coding RNAs (ncRNAs). The biological role and significance of these ncRNAs have been realised and to date it appears that ncRNA may have many important regulatory functions. ncRNAs are multifaceted and they induce a complexity of different types of transcriptional and post-transcriptional regulation including gene activation.

**Recent Advances:** Association of ncRNAs with gene activation is an important finding. Not only enhancer RNA (eRNA) but other types of ncRNAs, including small RNA (sRNA), long non-coding RNA (lncRNA), micro RNA (miRNA) and PIWI-associated RNA (piRNA) have also been implicated in gene activation. Interestingly, they often coincide with histone modifications that favour an open chromatin. In addition, these ncRNAs can recruit key factors important for transcription including RNA polymerase II. They may directly bind the genomic DNA or act as scaffolds, alternatively they may loop the chromatin to enhance transcription.

**Critical Issues:** Although the role of small activating (sa)RNAs has been considerably studied, the roles of miRNAs and piRNAs in gene activation still need to be substantiated and issues of specificity require further studies.

**Future Directions:** The ncRNA field is coming out of its infancy and we are gaining a global picture of the importance of ncRNAs. However, detailed mechanisms of action of the different ncRNAs are still to be determined. This may reveal novel ways of transcriptional regulation which will facilitate our ability to utilise these regulatory pathways for research and therapeutic purposes.
Laham-Karam N.

**Introduction**

With the advent of high throughput genomic technologies, our knowledge of the transcriptional landscape has changed, such that we now recognise that the majority of the genome is transcribed (28,29). This has also altered our view of the RNA world and non-coding RNAs (ncRNAs). Different types of ncRNAs have been defined, mainly based on their size and some according to their function (see table 1). These include micro RNA (miRNA), enhancer RNA (eRNA), PIWI-interacting RNA (piRNA), small RNA (sRNA), long non-coding RNA (lncRNA) and promoter-associated RNA (paRNA). These ncRNAs (Figure 1) can arise from different parts of the genome, either from unidirectional or bidirectional transcription, as is the case for eRNA and paRNAs, and have variable stability and longevity [reviewed in (48,111,118,120)]. Interest in the ncRNAs has grown with increasing evidence of functionality and biological relevance. The ncRNAs are all found in the nucleus but not exclusively, for example miRNA and siRNA also have their effects in the cytoplasm and some lncRNA can also be found there. The nuclear localisation and level of expression of ncRNAs has supported a regulatory role for these RNAs. The type and means of ncRNA regulation are still being defined and although ncRNAs can often impact negatively on gene expression, as is often the case with miRNA, supporting evidence has also been accumulated that suggests a role for ncRNA in activation of gene expression. This has mainly been due to induced changes at the chromatin that allow for increased transcription. In this review, we will concentrate on ncRNAs that activate gene expression, including sRNA, lncRNA, eRNA, piRNA and miRNA. We will present evidence of their involvement in gene activation and discuss what is known to date about the mechanisms of their activity.

**Small RNA**

sRNAs are short RNA sequences of 19-27 nucleotides (nt). The most well-known of these are the small interfering RNA (siRNA), which are processed from perfectly based double-stranded RNA (dsRNA) by DICER ribonuclease III (DICER) within the RNA interference (RNAi) system (Figure 2; (30)). In the cytoplasm, the siRNA associate with Argonautes (AGOs) and they silence gene expression by degrading perfectly complimentary target messenger RNAs (mRNA). The siRNAs can also be delivered from exogenous sources and this has been widely used to regulate gene expression. Although the siRNAs mainly function in the cytoplasm, sRNAs can also have nuclear targets which are often associated with gene.

**Activity of saRNA in vitro and in vivo**

sRNAs targeting promoters were first observed in plants (84) and subsequently in *Drosophila* (98) and mammalian cells (58). The first reports demonstrated that when expressed (84,98) or introduced into the cell (87), these promoter-targeted sRNA inhibited gene expression and this was termed transcriptional gene silencing (TGS) and distinguished it from the post-transcriptional gene silencing (PTGS) mediated by siRNA targeting the mRNA. However, already in 2004 Kuwabara et al (58) identified a small non-coding dsRNA that could activate neuron-specific genes at the transcriptional level and this was followed by Li et al (69) demonstrating activation of gene expression by delivery of sRNA targeting different gene promoters. These sRNA are now often referred to as small activating RNA (saRNA). This ability of saRNA to increase gene expression has now been reported for many different genes including those of the vascular endothelial growth factor (VEGF) family (59,69,131), receptors (39,44,79,122), transcription factors (39,109,137,144,145), cancer related genes (16,39,69,70,99,104,108,161) and other biologically important genes (148,157,158; summarised in Figure 3). Likewise, saRNA have been tested in cell lines from different
species and found to activate gene expression (39), suggesting conservation of this system in mammalian cells. Although, some saRNA can be active in various cell types, others demonstrate differential activity (69,80,131). The differential activity of these saRNA is likely to be a result of distinct pre-established methylation states of the gene in question, since the responsiveness of a cell line to a saRNA can be altered with chemically-induced demethylation of the cell (69,130).

The observed range of activation by saRNAs has on average varied between 2-15 fold depending on the gene promoter targeted. Recently even higher activation has been demonstrated for a type I promoter, indicating that there might be differences in the level of activity depending on the type of promoter that is targeted (141). Often the activation has been observed on both the mRNA and protein level but not always (59). This observation is in accordance with recent estimates that only approximately 40% of the protein variance is explained by mRNA expression and that the protein translation rate is the dominant factor in the protein variance (121), and is likely to be due to the interplay of other post-transcriptional and translational regulation. An important feature of promoter targeted saRNAs is that they are able to increase the expression of multiple isoforms of a gene as has been demonstrated for VEGF-A (69,130), thereby maintaining the physiological equilibrium of perhaps functionally distinct isoforms.

Functional output of saRNA

Importantly, saRNA-mediated increased gene expression has been correlated with functional outputs. Specifically, saRNA regulating cancer-related genes have reported decreased viability (55,69,105,145), inhibition of proliferation (16,156,158), invasion and/or migration of cancer cells (145,158). Alternatively, saRNA have been used to decrease resistance of cancer cells to treatment (70,157). In addition, following saRNA-mediated increase of Progesterone receptor (PR) (44), VEGF-A (34) or transcription factors (eg Kruppel-like factor [KLF4] or Myc) (137,149), activation of downstream genes of the targeted gene product were also observed.

In vivo studies of saRNA have also been encouraging in the context of functional activation. Turunen et al (131) first demonstrated that activating small hairpin (sh)RNA targeting the mouse VEGF-A promoter were able to induce increased vascularity in a hindlimb ischemia mouse model. Likewise, the same saRNA was also effective in reducing the infarct size in a mouse heart infarction model (130). In vivo, functional activity has also been observed in rodent cancer models where saRNA were associated with either smaller tumour size and/or extended animal survival (50,110,165) in addition to decreased metastasis (66). Furthermore, saRNA-enhanced expression of inducible nitric oxide (iNOS) in the cavernous tissue improved intracavernous pressure in streptozotocin induced diabetic rats (148).

Targeted promoter sites and saRNA design

Some mystery still exists regarding the promoter areas that can be targeted by saRNA. Notably, not all saRNAs that have been designed have demonstrated activity in regulating gene promoters (39,59,69,161). The most successful targeting has occurred in the promoter area -1000bp to -100bp from the transcription start site (TSS), however more distal hotspots have also been reported (82). Often, known transcription factor binding sites, GC rich areas (CpG) and repeat elements are avoided in the design of the exogenous saRNA. These are intended to limit interference with known transcription mechanisms or to avoid off-
target effects that may result from targeting repeat elements. Algorithms to predict active sites have at best reported a 50% success (2). This is not surprising since shifting the saRNA sequence by as little as one base pair on the target site is enough to abolish its activity (44). A better promoter targeting efficiency will come as we gain better understandings of the mechanisms of saRNA activation.

In addition to the place of targeting, the actual sequence and chemical properties of the saRNA are also an important consideration. Some effort has gone into characterisation of the saRNA sequence requirements and kinetics (44,104,152). Although there are some similarities to siRNA design, this is by far not absolute. We and others (146), recommend the following features for saRNA design, a duplex of 19nt that has a GC content of 40 to 60% (too high GC content may hinder AGO processing of the RNA) and 5’ base-pairing biased towards asymmetric thermodynamic stability. Sequence mismatches are often not tolerated in saRNA (44,149,161), particularly in the 5’ end of the guide RNA where there is an absolute requirement for sequence complementarity to the targeted area (82,149). Furthermore, modifications of RNA duplexes to improve stability or resistance to endonucleases, have not been tolerated on the 5’ guide strand of saRNA, however modifications including Locked nucleic acid (LNA) and 3’ biotin as well as 2’F and 2’-O-methyl of the saRNA backbone were tolerated to varying degrees (79,82,104,149,152).

Since saRNAs can be generated as duplexes then either the sense or the antisense can be chosen as the guide strand to mediate activity, whereas the passenger strand is targeted for degradation. Examples of both usage of sense and antisense strands have been shown mainly through mutation and loss of function assays (149). The choice of the guide strand is thought to depend on its thermodynamic nature, favouring an asymmetric stability of the RNA (8). In particular, AGO2 loading is dependent on the comparative thermodynamic nature of the RNA 5’ and 3’ ends, such that saRNA with lower stability at the 5’ end relative to the 3’ are preferentially loaded. Recently, Portnoy et al (106) correlated the loading of either strand to their 5’ thermodynamic stabilities. However, these same authors demonstrated that loading into AGO2 did not guarantee activity, indicating that other factors influence the choice of the active strand as well.

**Mechanisms of saRNA activation**

More information about the mechanisms of saRNA activation have appeared in the literature in recent years (summarised in Figure 4). saRNAs have demonstrated some dependence on RNAi factors, those required for RNA processing and loading. In particular, they have been associated with a requirement for AGO, especially AGO2 (18,69,104,122,131). AGO2 has not only been localised to the nucleus (3,18,31) but importantly AGO2 enrichment at saRNA target site has been demonstrated, indicating the possibility that saRNA guides the AGO2 to the promoter region (37,82,106). AGO2 has also been shown to associate with either the promoter DNA or the corresponding antisense transcripts (18,106,122). Interestingly however, AGO2 cleavage activity, that is its main function in RNAi, does not appear to be necessary for saRNA activation (18,78,79,122). AGO2 activity at the chromatin level is still unclear. Is ncRNA loaded in AGO2 recruiting histone modifiers or is it impacting on transcriptional units? In Droshophila, it has been shown that AGO2 associates with chromatin preferentially at transcriptionally active sites and that it interacts with core transcription machinery; in particular, in conjunction with DICR 2 it can impact on RNA polymerase II (RNA polII) positioning on promoters (13). Perhaps saRNA-guided localisation of AGO2 at the promoter can affect RNA polII dynamics, and thereby saRNA alters the transcriptome.
The evidence to date supports transcriptional activation by saRNA, and as such the chromatin needs to be in a state amenable for transcription, that is in an ‘open’ state. Chromosomal DNA is normally wound on histone comprising nucleosomes, different modifications of the histones determine the level of interaction between the DNA and the nucleosome and subsequently the level of openness of the chromatin (reviewed in (95,102)). For transcription to occur unwinding of the chromosomal DNA must take place and as such this is associated with specific histone modifications. These types of modifications of epigenetic marks at the targeted promoter site have been associated with saRNA activation. In particular, enrichment of histone modifications characteristic of active marks such as, H3K4me2/3 and H3K27ac or loss of repressive marks, such as H3K9me3 and H3K27me3 were correlated with saRNA activation (44,59,69,78,131,161). In addition, inhibition of histone methylation and deacetylation by specific inhibitors reduced the activation achieved by saRNA (44,58). However, this correlation of histone modifications with saRNA activation can be the consequence rather than the mechanistic cause. One putative mechanism that was quickly dismissed for saRNA is alterations in DNA methylation at the targeted promoter. These changes were not observed for activating sRNA (69), although changes in DNA methylation had been observed with silencing sRNA (43,83). To date, what has been lacking in the chain of evidence for saRNA mediating histone modifications is sufficient experimental demonstration of saRNA-mediated recruitment of histone modifying enzymes. Only recently, Matsui et al (78) demonstrated that saRNA activation of Cyclooxygenase-2 (Cox-2) promoter required expression of WD40 repeat-containing protein (WDR5), which is a protein scaffold to stimulate histone methyltransferase. Furthermore, Portnoy et al (106) demonstrated co-precipitation of CTR9 and polymerase associated factor (PAF)1 following saRNA activation, both of which participate in a larger complex that interacts with histone modification enzymes (168).

In addition to modifying the chromatin to allow for transcription to take place, saRNAs may also activate gene expression by recruiting transcriptional units to the promoter sites. On the whole, active saRNAs have induced enrichment of RNAPII at the targeted promoters (18,37,79,103). It was also demonstrated that this RNA polII enrichment occurs in areas at or flanking the saRNA target site (37,82,106) as well as at the proximal promoter (106) including TSS (37). Furthermore changes in the different phosphorylated forms of RNA polII have been demonstrated with saRNA activation of a targeted promoter. Specifically, RNA polII with a Ser2 phosphorylation at the C-terminal domain (CTD) residues, which is indicative of an elongating RNA polII, was found enriched at the 5’-end and throughout the gene body of a saRNA-targeted promoter. Furthermore, this corresponded to a decline in the ‘paused’ form of RNA polII that is RNA polII Ser5 phosphorylation (106). It was also demonstrated that the sites of RNA polII recruitment, mediated by saRNA activation, were co-localised with nucleosome depletion near the TSS or at the proximal promoter and enhancer (141). This observation led the authors (141) to suggest that AGO2-loaded saRNA induces nucleosome depletion which would then allow for RNA polII binding in those regions and subsequently increase transcription.

In addition to RNA polII recruitment to saRNA-targeted promoters, recruitment of transcription factors have also been reported, in particular NFKappaB and CAMP responsive element binding protein (CREB)1 enrichment at the COX2 promoter following activation by saRNA(78). Likewise, activation by saRNA was also associated with specific heterogeneous nuclear ribonucleoproteins (hnRNPs) either by co-precipitation studies (122) or as identified by saRNA pull-down and identification of associated proteins with mass spectrometry (37). In the later study, it was observed that the hnRNP bound to the saRNA thereby localising to the target site. Loss of function analysis demonstrated significant albeit partial dependence of saRNA activity on the presence of hnRNP (37). In similar pull-down experiments, additional factors associated with a saRNA were identified, in particular saRNA activation of the p21 promoter was also
associated with transcriptional activators CTR9 and RNA helicase A (RHA) (106). Interestingly, these factors were enriched at the saRNA promoter target site and the corresponding TSS, furthermore knockdown of CTR9 and RHA significantly affected the saRNA activity. Since RHA is a nuclear RNA/DNA helicase II, which can directly bind to DNA, recruit RNA polII and bridge to transcription factors, and RNA polymerase-associated protein CTR9 homolog (CTR9) is a component of the PAF1 complex (PAF1C) which is involved in transcription initiation and elongation, then it was proposed that saRNA facilitates the assembly of RNA-induced transcriptional activation complex (106).

Clues to the mechanism of saRNA activation might come from better knowledge of the saRNA-target molecule. A number of studies have addressed the question as to whether the saRNA bind their target site on the genomic (g)DNA or activate by binding a corresponding promoter RNA (sense or antisense; Figure 5). saRNA mutation studies (see above) have demonstrated the requirement for target-specific sequences but these of course cannot delineate if sequence recognition is occurring via gDNA or RNA binding. Likewise, induction of mutations in the gDNA of targeted sites also reduced the activity of a saRNA (82). However, this too could not differentiate the saRNA binding since gDNA mutations would consequently affect antisense RNA sequences. Nevertheless, in several studies binding of saRNA to their gDNA target sites (p21, E-cadherin and PR) was exhibited by co-precipitation of targeted-gDNA with biotinylated-saRNA (37,82,106). Furthermore, the requirement of this binding for saRNA activation of the PR promoter was verified in a cell clone that carried a mutation in the targeted gDNA binding site that resulted in both reduced saRNA activity and reduced gDNA co-precipitation with the saRNA (82). These are in contrast to previous observations of saRNA association with the PR antisense promoter transcript and not the PR promoter DNA (122), albeit in different cell lines. Likewise, promoter sense RNA have been detected as saRNA targets (78).

Debate has been ongoing as to the possible role of the promoter RNA transcripts in particular the antisense in the activation mechanisms of saRNA. Although the initial studies proposed an involvement of promoter antisense RNA in the activation by saRNA (18,79,122) more recent studies have negated a role for the promoter antisense (82). Initial proposals for a role for the promoter antisense were either based on association of the promoter antisense with the saRNA (122) or; association of AGO with the antisense transcript following saRNA treatment (18,79,122). Furthermore, a knockdown study of the PR promoter antisense RNA resulted in reversal of saRNA-activation of the PR promoter (122). In these studies no reduction in antisense RNA following saRNA treatment or degradation of the antisense RNA by AGO was detected (18,79,122), therefore putative mechanisms did not rely on changes in antisense RNA as had been suggested (86). The role of the antisense overlapping the promoter was subsequently proposed as a shuttle, specifically it was proposed that saRNA shifted the interacting proteins from the promoter to the antisense thereby remodelling the protein interactions at the promoter. This too was suggested for saRNA activation mediated via the promoter sense RNA (78). However, not all promoters have a corresponding antisense transcript, for example Hu et al (37) were unable to detect non-coding transcripts for the p21 promoter and yet they observed saRNA activation of that promoter, likewise Voutila et al (137) were unable to detect antisense transcripts for the KLF promoter. Consistently, lack of changes in the antisense have been noted with saRNA activation for different gene promoters (18,79,122,152,161). More recent studies interrogating the role of the antisense in saRNA activation, demonstrated that siRNA knockdown of the overlapping antisense did not attenuate the saRNA activity on the PR promoter (82). It is possible that multiple mechanisms can be activated by the saRNA depending on the molecular targets that are available, as such the debate on the role of the promoter non-coding transcripts on saRNA activation will only be resolved once the entire mechanisms are elucidated (164).
Specificity

One of the issues of saRNA activity is the specificity of its action. There are two aspects to the non-specific effects that have likewise been a concern in the siRNA field [reviewed in (42)]: 1) Activation of the innate immunity by dsRNA and; 2) Activation of miRNA-like mechanisms that mediate post-transcriptional silencing due to binding of sRNA to sequences of partial homology. Activation of either of these can result in off-target effects (88,154) and potentially complicate interpretation of saRNA activation (105).

In the siRNA field, it was known early on that double-stranded RNA molecules can induce type I-Interferons (IFNs) and thereby induce the expression of IFN-responsive genes [reviewed in (113)]. Subsequently, this IFN induction may then be responsible for the observations of gene activation by saRNA, and not specific effects of the saRNA on the gene promoter target site. To address this issue, assessment of the IFN response activation has been carried out and to date no significant changes in expression of IFN-regulated genes have been reported with saRNA treatment (69,79,137). In addition, type I-IFN-treatment did not affect E-cadherin or p21 expression (69) nor did induction of IFN by poly-inosinic-polycytidylic acid (poly I:C) affect Low density lipoprotein receptor (LDLR) expression (79), although these same genes were induced by a corresponding saRNA. Chemical modifications of the saRNA also can improve their specificity and reduce their IFN activation (46).

Since the saRNAs are only 19-24 nt then there is a potential for partial homology binding to sequences other than the targeted site. In doing so saRNA may activate miRNA-like mechanisms targeting other RNA transcripts. This is more difficult to track and requires more global assessment of gene expression such as micro-array analysis, as has been performed for shRNA regulating VEGF-A (40), or deep sequencing to determine altered genes following saRNA treatment. Of course, saRNA activation of any particular gene promoter may not be influenced by off-target effects occurring in the cell (105), likewise not all promoter-targeted sRNA induce off-target effects (127), however this is an important check in saRNA applications.

Long non-coding RNAs in chromatin regulation

To date, knowledge of the specific functional roles of lncRNA is still lacking. This is partly due to the breadth of this class of IncRNAs. IncRNAs have been defined based mainly on their size and non-protein coding function, and it is not likely that all of these transcript share similar functions or mechanisms, and some of them may be only by-products of transcription. Some attempts have been made to re-categorize these transcripts; Mukherjee et al. for example defined seven different RNA classes based on the metabolism (e.g. synthesis, processing and degradation) of the transcripts (90). As expected, in these RNA classes mRNAs and IncRNAs differed in some properties, such as the rate of transcription and degradation. However, two of the new classes contained both mRNAs and IncRNAs, suggesting maybe similar processing pathways. Some studies have shown that IncRNAs can also code for peptides with biological function, such as the 46 amino acids peptide that is structurally similar to sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) inhibitors phospholamban and sarcopilin (4). This peptide was shown to directly interact with SERCA, decreasing Ca2+ reuptake (4). The same research group also found another muscle-specific micrpeptide that is 34 amino acids in size which enhanced SERCA activity (91). Many IncRNAs have computationally annotated open reading frames (ORF) but not so much translational activity as mRNAs (12). Computational prediction
identified IncRNA ORFs as less functional with poorer start codon and short ORF, thus resulting in less activation and small peptides (92). The studies so far that have described individual peptides encoded by IncRNAs, have described only short micropeptide formation. It also seems that IncRNA-ORFs are relatively rare considering how much of the genome is transcribed into ncRNAs. New technologies will provide platforms to generate more information about IncRNAs and their function. For example screening using Clustered regularly interspaced short palindromic repeats (CRISPR) was recently used to identify the IncRNAs that have specific function in cell growth in human cells (75). This study, like many before, showed also that the IncRNA functions are highly cell-type specific.

Transcriptional gene regulation on the chromatin level has been indicated for some IncRNAs. Many IncRNAs have been shown to induce gene silencing, often by recruiting the polycomb repressor complex (PRC2) to their loci (51). A very well-known example is the X-inactive specific transcript (XIST) that in female cells participates in the inactivation of the X chromosome gene expression (10,24). However, on the other X chromosome the XIST expression needs to be restricted in order to maintain active gene expression on one of the chromosomes. IncRNA TSIX was identified to be expressed antisense to XIST and therefore repressing it on the active chromosome (64). Recently the mechanisms have been characterized in more detail and it has been shown that TSIX recruits chromatin-modifying enzymes that induce H3K36 trimethylation on the XIST promoter (93). The process of X chromosome inactivation seems to be very complex and the mechanism is not yet resolved. Another IncRNA, X active coating transcript (XCAT) was found expressed specifically in humans from the active X chromosome and later studies have shown that it antagonizes XIST accumulation in early stage of X chromosome inactivation (134,135). Likewise regulation of Homeobox A cluster (HOXA) genes have been linked to histone modifications, in particular IncRNA Gm15055 in mouse embryonic stem cells was shown to recruit PRC2 to HOXA gene cluster and thus maintain stable, repressive H3K27me3 levels on their promoter (74). This study interestingly showed by 3C method that the Gm15055 directly interacts with multiple sites on HOXA gene cluster. A very well-known example of IncRNAs in chromatin regulation is the HOTAIR RNA transcript, which was first characterized in 2007 (112). HOTAIR is expressed from the HOXC locus and mediates the repression of HOXD locus by interacting with PRC2, thus mediating the methylation of H3K27 at the locus. However, a recent study showed that the gene silencing induced by HOTAIR can be independent of PRC2 binding, where only tethering of HOTAIR to chromatin was able to lead to gene silencing events, after which PRC2 can be recruited to the loci (107).

Even though the role of IncRNAs in transcriptional gene silencing has been more widely studied, an increasing amount of evidence also supports a functional role for IncRNA in gene activation. There are many mechanisms by which this transcriptional activation may occur (summarized in Figure 6). The molecular mechanisms have not yet been characterized in detail but an increasing number of publications on the topic suggests a high correlation between specific IncRNA expression levels and gene regulatory events (54,56,132). Many studies have been based on global characterization of expression in response to different cellular status, associating specific IncRNA expression changes with certain disease (such as cancer or, cardiovascular disease; 125,129,133). These studies offer insights into the possible use of IncRNA expression as a biomarker for such disease states. However, they often tell little about the actual function of such RNA transcripts. Further characterization has been done in some cases by overexpression or knockdown experiments to provide more details into the mechanism of action, but deeper understanding of the action of different IncRNAs has not yet been reached.

A study by Ørom et al identified already in 2010 that IncRNAs played a role in regulating the neighboring genes (96). This study found that knocking-down many of these IncRNAs resulted in decreased expression of
the neighboring protein-coding gene. These lncRNAs were serving as “enhancers” for the protein-coding genes, even though the chromatin signature was different than with typical enhancer. These lncRNAs were also not transcribed bidirectionally as eRNAs, and they were also more often polyadenylated. A later study by the group showed that these activating enhancer-like ncRNAs act by bringing the Mediator complex to the vicinity of their neighboring genes via chromatin looping (60).

lncRNAs have been shown to transcriptionally silence genes by recruiting repressive enzymes and factors to their loci. Likewise, recruitment of histone modifiers has been observed with lncRNAs in transcriptional gene activation. Hoxb genes for example have been shown to be activated by HoxB1nc lncRNA transcript that binds to Hoxb genes, this followed by recruitment of the enzyme complex Setd1a/MLL1 which maintains H3K4me3 histone modification levels (26). Moreover, deletion of HoxB1nc by CRISPR genome editing impaired Hoxb gene expression, but also chromatin looping. lncRNA transcripts may therefore serve an important role in the three dimensional organization of chromatin. An interesting finding in HOXA gene regulation was lncRNA HOTAIRM1, which has been identified as a biomarker in many cancer types [e.g. (27,140)]. This lncRNA was found to have different variants that can either repress or activate HOXA gene expression in different cell types (150). These transcripts all originate from the same HOTAIRM1 gene and influence H3K4me3 and H3K27me3 histone marks. The basal level of HOTAIRM1 expression and histone methylation marks determined the effects that the lncRNA had on the activation: in cells with higher expression of HOTAIRM1, the H3K27me3 levels were lower and the HOTAIRM1 function was mediated by only increased H3K4me2 levels. In another cell type, where the basal level of HOTAIRM1 was lower, the promoters were marked with high levels of both H3K4me3 and H3K27me3 and the induction of HOTAIRM1 expression led to significantly lower levels of H3K27me3 on HOXA genes, as well as to increased H3K4me3 methylation at HOXA1 and HOXA2 gene promoters. This study also showed direct chromatin interaction of HOTAIRM1 with the HOXA genes. The lncRNA variants recruited UTX/MLL or PRC2 complexes to activate or silence, respectively, HOXA gene expression. Another example is the HOTTIP transcript that is expressed from the HOXA locus. HOTTIP activates HOXA genes by binding directly to WDR5 and through chromosomal looping, can bring the WDR5/MLL complex to target gene loci (147). This leads to increased levels of H3K4me3 and activates the transcription of these HOXA genes.

Interesting feature of lncRNA action has been their ability to guide genes to active compartment of the nucleus. lncRNAs were demonstrated to participate in the nuclear organization, as was seen for example with lncRNA Nuclear Paraspeckle assembly transcript 1 (NEAT1) that structurally enables paraspeckle formation (20). A study by Yang et al. showed that specific lncRNAs contribute to the translocation of E2F1-regulated growth control genes to active nuclear compartment upon serum stimulation (162). This was dependent on histone demethylase KDM4C. Two different lncRNAs were important for this regulation, taurine up-regulated 1 (TUG1) and NEAT2. The promoters that were found to interact with TUG1 were associated with a corepressor complex and histone modifications that are considered marks of repressed gene expression. On the other hand, NEAT2 was found to interact with promoters that had histone modifications that imply active gene transcription and was shown to bring these genes to transcriptionally active nuclear compartment. These studies imply that lncRNAs play an important role in forming transcriptionally repressive or active nuclear chromatin compartments. Acting as scaffolds, lncRNAs recruit many different types of chromatin modifying enzymes to their associated genes thereby directly affecting gene regulation.

In addition to their role as scaffolds, lncRNAs can also activate genes by sequestering away repressive proteins and miRNAs. In T-cells derived from immune thrombocytopenic purpura patients, lncRNA Maternally expressed 3 (MEG3) expression was increased and was shown to bind miR-125a-3p (67). This
Laham-Karam N.

group had previously shown that miR-125a-3p was associated with pathogenesis of the disease by regulating C-X-C motif chemokine ligand 13 (CXCL13), and so now suggested that MEG3 is regulating the levels of this miRNA. Interestingly, DNA hypomethylation of MEG3 promoter and a corresponding increase in expression of MEG3 and another miRNA cluster have also been detected in atherosclerotic lesions (1), suggesting that MEG3 may play a role in different diseases. Another example of IncRNA activation potential is that of muscle-specific IncRNA, Inc-MD1, which has been shown to directly interact with miRNAs miR-133 and miR-135. This ‘sponging’ interaction acts to sequester the miRNAs and thereby regulate muscle-specific gene expression of transcription factors Mastermind-like protein (MAML)1 and Myocyte enhancer factor 2C (MEF2C) (14). In Duchenne muscular dystrophy cells the level of Inc-MD1 was decreased and the expression levels of MAML1 and MEF2C were altered. The studies of these competing endogenous RNAs imply that IncRNAs participate in a complex network of regulatory events to achieve necessary gene expression for the cells, and can direct tissue specific gene expression. Many of these functions in sponging other factors have been so far shown in the cytoplasm, but similar ncRNA function may be found within nucleus. IncRNA HOXA11-AS was shown to bind PRC2, Lysine-specific histone demethylase 1 (LSD1) and DNA methyltransferase (DNMT)1 in the nucleus and to regulate protease, serine 8 (PRSS8) and KLF2, while it also functioned as a competing RNA in the cytoplasm by interaction with miR-1297 (126).

IncRNAs have also been shown to induce signaling pathways through direct interaction, whereby they activate not only a few specific targets in the genome but induce more widespread downstream effects. IncRNA NRB2 was shown to activate kinase signaling by direct interaction with AMP-activated protein kinase alpha (AMPKα) subunit following energy stress (76). The system worked in a feed-back loop where AMPK pathway increased NRB2 expression. Both IncRNAs CCHE1 and Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) were shown to participate in the activation of ERK/MAPK kinase pathway in hepatocellular carcinoma (101) and in neuronal cell differentiation (15), respectively. In stroke, neuronal death is increased by activation of p53 that was induced by IncRNA MEG3 that directly binds p53 (160). Together these examples show that IncRNAs can initiate cascades of cellular function by direct interaction and activation of kinases or transcription factors.

IncRNAs act also in derepression of genes, activating poised genes. For example in S. cerevisiae, metabolic GAL genes were activated by GAL IncRNAs from the same cluster (22). The IncRNAs promote faster recruitment of RNA PolII and their expression decreases the binding of co-repressor protein Cyc8, thus inducing the activation of GAL genes when environmental conditions are suitable. Studying the mechanism further, Cloutier et al found that this de-repression occurs through R-loop formation between the IncRNA and GAL gene cluster DNA (21). This mechanism of action of IncRNAs enables fast induction of gene expression for certain critical genes. ncRNAs are likely to play a role in rapid responses to environmental stimuli since RNA transcription may occur more instantly than increasing production of proteins, such as transcription factors that would then further regulate other genes.

The regulation of gene expression is a complex network of multiple factors. The role of IncRNAs in this process is emerging and the functions of IncRNAs seem versatile. The expression of many individual IncRNAs has been associated with different disease states, but also with developmental processes (5,120,139). Fully understanding the action of IncRNAs will be important for comprehending the cellular processes concerning both basal cell functions, as well as therapeutic benefits in modifying the expression of these transcripts.
Enhancer RNA

Although eRNAs may resemble lncRNAs in size, ranging from 0.1-9Kb, they are a distinct class of ncRNAs, distinguished by their associated histone profile (25). The eRNAs are often, but not exclusively, derived from bidirectional transcription following RNA polIII recruitment and binding to enhancer regions within the genome (Figure 1; reviewed in (62)). These enhancer regions are characterised by enrichment of H3K4me1 and low H3K4me3, they are bound by lineage-determining transcription factors, as well as enriched occupancy of RNA polll and other transcriptional co-regulators. Importantly, enhancers regulate transcription at gene promoters. The majority of eRNA transcripts are 5’ capped, unspliced, not polyadenylated, have short half-lives, but are also dynamically regulated following signalling and correlate with increased expression of neighbouring genes (Table 1; (53)). Although there has been some debate as to whether eRNAs have a functional role in regulating transcription or are coincident to enhancer transcription, growing evidence suggests that they do indeed function in transcriptional activation (reviewed in (62,115)). This has included knockdown studies of eRNA, either by siRNA-mediated RNAi (61,81,96), or antisense oligonucleotide-activation of RNase H degradation (71), which led to a specific decrease in the corresponding mRNA expression. Equally, gain-of-function studies of eRNA using assays tethering eRNA to the promoter or enhancer sites, also demonstrated transcriptional activation (71,81).

The mechanisms by which eRNAs mediate their function are still to be defined. Enhancer regions loop to their corresponding promoter, this looping is mediated in part by Mediator and cohesins (47,60,143). Enhancer looping may facilitate eRNA tethering to the promoter. Alternatively eRNA may participate in mediating the DNA looping. It has been shown that eRNAs are enriched preferentially at enhancers that are actively looping to promoters or other enhancers (73,117). In addition, knockdown of eRNA of the estrogen receptor-α enhancer, concomitantly resulted in the reduction of both enhancer-promoter interaction and gene expression (71), suggesting that eRNA may initiate or stabilise enhancer looping likely by interacting with Mediator and the cohesin complex (36,60). Other knockdown studies of eRNAs have also demonstrated that eRNAs may facilitate RNA polIII recruitment specifically to the gene promoter, as has been demonstrated for the MyoD gene (89). The effect of eRNAs on RNA polIII are likely to be multifactorial, since it has also been shown that eRNAs can facilitate the release of RNA polIII at poised genes into productive elongation (119). Most recently, eRNAs were also shown to interact with the transcriptional activators CBP/p300 to stimulate modifications in histone acetylation, that are characteristic of active enhancers (7).

Our knowledge and understanding of eRNA has at large followed advances in high-throughput transcriptomic analysis and it is likely to improve with new tools and methods that detect spatial and temporal changes with greater sensitivity, as has recently been demonstrated (85). These may change our view of eRNA function and strengthen the evidence that supports a functional role for eRNAs in transcriptional activation.

PIWI-associated RNA in chromatin activation

PIWI-associated RNAs (piRNAs) are small ncRNAs that are traditionally considered to act in silencing transposons. Especially during spermatogenesis (100) they bind AGO/Piwi family of proteins and direct the methylation of DNA. In addition, piRNAs have also been shown to regulate Heterochromatin Protein 1 (HP1) activity and H3K9 methylation (151). Besides transcriptional gene silencing, piRNAs have been associated with miRNA-like functions in PTGS, specifically during late spermatogenesis (33).
Already in 2007, piRNAs in association with Piwi-proteins were shown to transcriptionally activate gene expression by inducing epigenetic changes in the chromatin of *D. melanogaster* (163). This study showed that also piRNAs may have a dual role in gene regulation, where they act as a guide to chromatin for either activating or repressing enzyme complexes. piRNAs can be processed from other longer non-coding RNA transcripts and a study by He et al (35) showed that IncRNA and snoRNA –derived piRNAs activated TNF-related apoptosis-inducing ligand (TRAIL) gene expression. In this study, the piRNA pi-sno75 induced H3K4 methylation while decreasing H3K27 methylation at the promoter of the proapoptotic factor TRAIL, in addition recruitment of the histone methyltransferases macromolecular MLL/COMPASS-like complex to the piRNA site on chromatin was observed. Some other studies have also suggested that 22G-RNAs, that are derived from the piRNA genes, activate gene expression by association with AGO protein CSR-1 and bind to nascent RNA transcripts in *C. elegans*, affecting the epigenetic status of the chromatin (19,123). This function is similar to piRNA-mediated silencing of transposons and other elements in the genome and was suggested that the pathway mediates trans-generational ON/OFF-expression memory for germline genes (153).

**MicroRNAs: Biogenesis, function and proposed mechanism for activation**

miRNAs are small (20-24 nt) ncRNAs which in spite of their small size have notable roles in numerous cellular functions (138), such as cell proliferation and cell apoptosis (9,159). After the first miRNA, let-7, was discovered in *C.elegans* in 1993 (65), many different miRNAs have been annotated and some of their regulatory roles have been studied both in plants and animals.

miRNA genes are spread across the genome, they can be found in intronic and exonic sequences or be intergenic. They can be found either in the sense or antisense orientation (Figure 1). Furthermore, miRNAs are often found in clusters and from one RNA transcript, one or more mature miRNAs can be generated (6). miRNA biogenesis (Figure 2) can be divided into two main steps: the formation of the miRNA transcripts in the nucleus and the maturation of miRNAs in the cytoplasm (57). Most often, miRNA biogenesis starts with RNA polIII mediated transcription which leads to the formation of primary miRNA (pri-miRNA). pri-miRNA is then processed into precursor miRNA (pre-miRNA) by Drosha ribonuclease III (Drosha) enzyme. This is not absolutely required since some of the intronic miRNAs are so small that they bypass Drosha-mediated processing (116) and are instead cleaved by spliceosomes into pre-miRNAs (57). The hairpin pre-miRNA is then transported from the nucleus to the cytoplasm by Ran-GTP-dependant exportins (6). In the cytoplasm, DICR enzyme cleaves pre-miRNAs into double-stranded structures which are then further processed into single-stranded mature miRNAs.

To become functional, mature miRNAs must be loaded into the RNA-induced silencing complex (RISC). RISC contains many different proteins including AGOs. The AGOs are vital for miRNA-mediated regulation as they form a suitable environment for miRNA-mRNA binding and therefore support miRNA function. Zamudio et al. (167) showed that the loss of AGOs in mouse embryonic stem cells leads to the destabilization of miRNAs. In the context of RISC, miRNAs act in the cytoplasm to induce PTGS. Generally, this is initiated through binding of miRNAs to the 3′untranslated regions (3′UTRs) of their target mRNAs. miRNAs recognize their target mRNAs based on the seed sequence which is the first 2-8 nt from the 5′end of the mature miRNA. Depending on the extent of the complementary, target mRNAs are either degraded or inhibited (114).

In addition to their role in PTGS, miRNAs were also found to activate gene translation in the cytoplasm. Vasudevan et al. showed in 2007 that miR-369-3 can activate tumor necrosis factor–α (TNFα) expression by
recruiting AGOs and other proteins to 3'UTRs of target mRNAs (136). There is also some evidence that miRNAs can regulate gene expression by binding the 5'UTR of the mRNAs. In mouse ES cells, miR-10a was shown to bind to the 5'UTR of the mRNA encoding ribosomal protein leading to the induction of ribosomal protein expression (97). Similar regulation was found in human liver cells, where miR-122 enhanced viral replication by binding the 5'end of viral genome (45). Although the canonical view of miRNAs is that they function in the cytoplasm, recent research has also suggested a role for miRNAs in the nucleus. This idea has been supported by studies that have found mature miRNAs in the nuclear fractions of the cells. For example, miR-29b was shown to preferentially localize in the nuclear fractions of human Hela cells (41). It has been proposed that nuclear miRNAs are processed in the same way as canonical miRNAs and only after maturation in the cytoplasm, they are transported back to the nucleus by importins (155). In addition to mature miRNAs, AGOs and other RISC components are also found in the nuclear fraction of the cells and are functionally active in the nucleus (31).

As mature miRNAs were found in the nucleus of the cells, it raised the question of the function of the nuclear miRNAs. Small RNAs had been known to repress gene expression at the gene promoter level in *C. elegans* (30). Mette et al. also showed in plants that small dsRNAs can silence their target gene expression by inducing DNA methylation on gene promoters (84). miRNA-mediated silencing at the transcriptional level was shown by Omato and Fujii in 2004, as human immunodeficiency virus (HIV)-1 transcription in human T-cells was reduced by miR-N367 induction (94). In another study, miR-320 was linked with epigenetic modifications of the RNA polIII subunit D (POLR3D) gene promoter leading to the downregulation of that gene (52). However, the newest studies suggest that some miRNAs can actually also activate gene expression in the nucleus. Potential miRNA target sites have also been found in the introns of nascent mRNAs, non-coding RNAs, and coding sequences (17).

Bioinformatic studies showed that there are 800 000 potential miRNA target sites (seed matches) in over 27 000 gene promoters (166). A functional activity of miRNA activating gene expression by targeting gene promoters has been demonstrated for a number of promoters. miR-373 was shown to have target sites on E-cadherin and CSDC2 gene promoters, and over-expression of miR-373 or its precursor form induced the expression of corresponding genes (103). Likewise, Wang et al. also showed that p21 expression can be induced by miR-370-5p which targets its promoter, in human bladder cancer cells and tissues (142). Equally, miR-205 was demonstrated to have target sites on promoters of interleukins-24 and -32 (IL-24 and IL-32) in human prostate cancer cells (77). After transfection of pre-miR-205, IL-24 and IL-32 expression levels multiplied, compared to control transfection levels, both at the mRNA and protein levels. Further evidence that supports a functional role for miRNA targeting gene promoters was provided by Huang et al. who not only demonstrated that miR-744 targets the cyclin B1 (Ccnb1) promoter to activate gene expression, but also demonstrated the decrease in Ccnb1 expression following siRNA knockdown of either enzymes needed for miRNA biogenesis (such as DICER and DROSHA Ribonuclease III) or RISC components (including AGO1 and AGO2) (38).

As miRNA target sites are found also in non-coding RNA transcripts, miRNA-mediated regulation of genes could also happen indirectly via regulation of the gene-associated ncRNAs. Indeed, this was demonstrated in lung cancer cells where two target sites for miR-589 were found on IncRNA that overlap the COX-2 promoter (78). When miR-589 was bound to IncRNA, COX-2 expression was induced. Interestingly, miR-589 binding sites were not perfectly complementary to IncRNA as in mRNA targeting by miRNAs.
Bioinformatic studies have shown a correlation between miRNAs expression and histone modifications characteristic of an open chromatin (128). miRNA may activate gene promoters by altering the promoter histone marks. In support of this, Majid et al. have demonstrated enrichment of open histone marks on IL-24 and IL-32 promoter areas following activation by miRNA-205 (77). Likewise, overexpression of miR-744 that targets the Ccnb1 promoter lead to enrichment of open histone mark H3K4me3 at the TSS of Ccnb1 (38). The evidence for additional functional mechanisms for miRNA activity in the nucleus is still sparse, however this will be fueled by the increasing interest in nuclear miRNAs.

Conclusion

Already various species of ncRNAs have been identified and characterized, and the research is increasingly focused on the mechanisms of action of these new types of gene expression regulators. The biological importance of the ncRNAs is increasingly appreciated, and what is clear is that these RNA transcripts are not just noise, but play an important role in gene regulation. As research of the ncRNAs continues, the classification of ncRNA types may need to be refined. Already we see that classification of ncRNAs by size is overly simplistic as is the case for the IncRNAs that serve multiple and different roles in the cells and act by distinct mechanisms. The function and the mechanisms of ncRNA activity, in particular activation by ncRNA, have not yet been defined for all the ncRNA types. However, despite the paucity in detailed molecular mechanisms, what is clear is that ncRNA can activate the chromatin, either by inducing histone modifications to open up the chromatin, by increasing the recruitment of transcriptional elements, or by looping the chromatin to alter transcription. The net yield of these modifications is increased transcriptional activity. Transcription as an event takes place more rapidly than protein production, offering the cells an option for a more instant response to changes in the environment. When environmental variation induces cellular signaling, transcription is the faster reaction to the stimulus and ncRNAs may be more potent and rapid regulators of genes that are required for environmental responses. From the evolutionary respect, the ncRNAs are more prone to mutations or new variations of the sequence since they are not restricted by codon rules as the protein-coding sequences. This means that the ncRNAs are more poised to sequence diversity and modifications can be achieved quickly for the fine-tuning of gene expression.

For the IncRNAs, the mechanisms of action in gene activation have so far been mainly associated with either guiding proteins and factors to the transcription site (cis-acting) and sequence dependent loci (trans-acting), or binding miRNAs and proteins to preventing them from binding their target sequences. IncRNAs are transcribed and processed in a similar way as mRNAs so it is not surprising that already some IncRNAs have been observed to be translated into peptides, and also some mRNAs have been associated with IncRNA-like functions in the nucleus. Processing of IncRNA, such as splicing, also creates variability in the transcript sequences that may direct different secondary structure formation. This can impact on the IncRNA interactions with proteins, other RNAs or the genome, and can increase the potential roles for any one IncRNA. Transcriptional regulation is not the only function identified for these RNAs, as they seem to have even structural roles for example in the maintenance of the nuclear matrix, cytoskeleton and extracellular matrix structure. In a similar manner, IncRNAs may play a part in coactivator and/or corepressor complexes and act potentially to guide the protein complex to its target site, or purely to maintain the correct structure or to affect the assembly of the transcription factor complex.
The usefulness of saRNA and miRNA that target promoters and thereby activate gene expression is clear both as a research tool and for therapeutic potential. In particular, for saRNA that can modify simultaneously different isoforms of a single gene, the therapeutic potential for potent molecules that would benefit from temperate regulation is evident. Likewise, miRNA that are able to simultaneously target multiple promoters or other ncRNAs that participate in the regulation of gene expression, may be valuable. An additional attractive feature of these ncRNA is not only their sequence specificity but also their differential activity in varying cell types. To date much of the evidence on saRNA has been derived from exogenous saRNA with few examples of endogenous saRNA. However, immunoprecipitation studies of AGO-associated sRNAs have revealed fragments of diverse origin (11) including many promoter-proximal RNA (167), therefore it is likely that we are yet to realise the significance of nuclear saRNAs and miRNAs in activating gene expression. Whether by inducing histone modifications to open the chromatin, or by increased recruitment of transcriptional elements, or by looping of the chromatin to alter transcription, ncRNA play an important role in the regulation and activation of the chromatin.
Laham-Karam N.

Acknowledgements

We would like to sincerely thank Jyrki Äikäs for his kind assistance in the preparation of the figures. This work was supported by the Finnish Academy Centre of Excellence and the ERC Advance grant.

List of Abbreviations

AGO  Argonaute
CRISPR  Clustered regularly interspaced short palindromic repeats
dsRNA  double-stranded RNA
eRNA  enhancer RNA
HIV  human immunodeficiency virus
hnRNPs  heterogeneous nuclear ribonucleoproteins
iNOS  inducible nitric oxide synthase
IncRNA  long non-coding RNA
mRNA  messenger RNA
miRNA  micro RNA
ncRNA  non-coding RNA
nt  nucleotides
paRNA  promoter-associated RNA
piRNA  PIWI-interacting RNA
PRC2  polycomb repressor complex
PTGS  Post-transcriptional gene silencing
RISC  RNA-induced silencing complex
RNAi  RNA interference
RNA pol  RNA polymerase
sRNA  small RNA
saRNA  small activating RNA
siRNA  small interfering RNA
TGS  transcriptional gene silencing
Laham-Karam N.

TSS transcriptional start sites

UTR untranslated region
References


Laham-Karam N.


Laham-Karam N.


Laham-Karam N.


Laham-Karam N.


Laham-Karam N.


Laham-Karam N.


Laham-Karam N.
Transcription factors
- NK3 homeobox1 (NKL3-1; 39)
- Kruppel-like factor (KLF4; 145)
- Myc proto-oncogene (Myc; 137)
- Nanog homeobox (NANOG; 149)
- CCAAT/enhancer binding protein alpha (CEBPA; 109)

Cell cycle and cancer related genes
- P21 (CDKN1A; 69)
- Tumour protein p53 (TP53; 39)
- PRKC apoptosis WT1 regulator (PAWR; 39)
- Wilms tumour 1 (WT; 39)
- Hypermethylated in cancer 1 protein (HI1C; 99)
- Phosphatase and tensin homolog (PTEN; 70)
- Mouse cyclin B1 (CCNB1; 39)

Receptors, transporters and enzymes
- Progesterone receptor (PR; 44)
- Major vault protein (MVP; 44)
- Chemokine C-X-C receptor 4 (rcSCR4; 39)
- Low density lipoprotein receptor (LDLR; 79)
- Vascular endothelial growth factor-R1 (FLT1; 59)
- Na/I symporter (NIS; 257)

Growth factors and cellular adhesion
- Vascular endothelial growth factor-A (VEGFA; 69, 131)
- Vascular endothelial growth factor-C (VEGFC; 59)
- E-cadherin (CDH1; 69)
- Vezatin (VEST; 158)
- Dihydropyrimidinase like 3 (DPYSL3; 66)
A. Promoter DNA

B. Promoter antisense RNA

C. Non-coding RNA