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SPOTLIGHT REVIEW

Non-coding RNAs as regulators of gene expression and epigenetics

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Abstract Genome-wide studies have revealed that mammalian genomes are pervasively transcribed. This has led to the identification and isolation of novel classes of non-coding RNAs (ncRNAs) that influence gene expression by a variety of mechanisms. Here we review the characteristics and functions of regulatory ncRNAs in chromatin remodelling and at multiple levels of transcriptional and post-transcriptional regulation. We also describe the potential roles of ncRNAs in vascular biology and in mediating epigenetic modifications that might play roles in cardiovascular disease susceptibility. The emerging recognition of the diverse functions of ncRNAs in regulation of gene expression suggests that they may represent new targets for therapeutic intervention.

Keywords Non-coding RNA • ncRNA • Vascular biology • Epigenetics • Gene regulation

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1. Introduction

Recent high-throughput transcriptomic analyses have revealed that eukaryotic genomes transcribe up to 90% of the genomic DNA.[1] Only 1–2% of these transcripts encode for proteins, whereas the vast majority are transcribed as non-coding RNAs (ncRNAs). Evolutionarily, the repertoire of protein-coding genes has remained relatively static, whereas the amount of non-coding sequences has markedly increased along with the complexity of the organism.[2] A biological basis for this observation is supported by the growing evidence of the functionality of these transcripts. The fact that most putative ncRNAs are expressed at substantially lower levels than mRNAs further suggests that these RNAs mainly fulfil regulatory functions. Indeed, there is increasing evidence for regulatory roles of ncRNAs during development[3–5] and in response to stress and environmental stimuli.[6–9] A major goal of contemporary molecular biology is to identify and functionally characterize the full spectrum of ncRNAs with respect to normal physiological functions and roles in human diseases.

ncRNAs can be divided into infrastructural ncRNAs and regulatory ncRNAs. Constitutively expressed infrastructural ncRNAs include ribosomal, transfer, small nuclear, and small nucleolar RNAs. Regulatory ncRNAs can be classified into microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and long non-coding RNAs (lncRNAs).[10] In addition, a novel class of promoter-associated RNAs (PARs) and enhancer RNAs (eRNAs) has been recently described.[8,9,11] In this review, we will focus on highlighting the characteristics and biological roles of the regulatory RNAs, with particular emphasis on their roles in vascular biology. The potential therapeutic applications of regulating ncRNA expression will also be discussed.

2. Types of ncRNAs and their biological roles

2.1 MicroRNAs

MiRNAs are evolutionarily conserved, small single-stranded molecules (20–24 nucleotides), that have been postulated to regulate the expression of ~50% of the genes in a cell at the post-transcriptional level.[12] In contrast to other endogenous small RNAs, miRNAs derive from transcripts forming distinctive hairpin structures (*Table 1*). Processing of the hairpin into the mature miRNA by Drosha and Dicer[13,14] allows interaction with Argonaute (Ago) proteins to form RNA-induced silencing complex (RISC).[15,16] Strand selection for RISC is dictated by the thermodynamic stabilities of the two duplex ends: the strand having its 5′ terminus at the less stably base-paired end of the duplex is favoured.[12] The miRNAs then pair with mRNAs, most favourably to the 3′ untranslated region (UTR), to guide their translational repression or deadenylation and degradation (*Table 1*).[13,15,17,18] Challenging this view, a recent report by Guo *et al.*[19] suggested that destabilization of the target mRNA is the

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Table 1 Regulatory ncRNAs produced from eukaryotic genomes and their characteristics and functions

Type	Long name	Length (nt)	Characteristics	Function
miRNA	Micro RNA	20–24	Pri-miRNA produced in the nucleus as capped and polyadenylated ssRNA with a imperfectly paired stem-loop structure	Perfect complementarity: Ago2-mediated cleavage of mRNA
			Processing by Drosha and Dicer lead to a production of mature dsRNA with exact ends	Non-perfect complementarity: Suppression of translation or mRNA degradation (deadenylation, decapping, and exonucleocytic degradation)
			Effector phase occurs primarily in the cytoplasm mediated by Ago proteins	Minor functions in transcriptional silencing and translational activation
piRNA	PIWI-interacting RNA	24–31	Precursor ssRNA, which is modified to contain 3′-terminal 2′-*O*-methyl	Silencing of transposable elements in the germline
			Strong preference for uridine at the 5′ end	
siRNA	Small interfering RNA	20–24	Canonical form long, linear, perfectly base-paired dsRNA	Perfect match: endonucleocytic cleavage
			Processed by Dicer into mature siRNA with heterogenous end composition	Non-perfect match or endonuclease-inactive RISC: translational repression or exonucleocytic degradation
			Effector functions occur primarily in the cytoplasm supported by Ago proteins	Induction of heterochromatin formation
				Silencing of the same locus from which they are derived
PAR ([a]PASR, TSSa-RNA, tiRNA, PROMPT)	Promoter-associated RNA	16–200	Weakly expressed ssRNAs	Partly unknown but indications of transcriptional regulation (example interaction with Polycomb group of proteins)
			Short half-life	
			Bidirectional expression reflecting PolII distribution	
eRNA	Enhancer RNA	100–9000	ssRNA produced bidirectionally from enhancer regions enriched for H3K4me1, PolII and coactivators such as p300	Mostly unknown but plays a role in transcriptional gene activation
			Short half-life	
			Evolutionarily conserved sequences	
			Dynamically regulated upon signalling	
			Expression correlates positively with nearby mRNA expression	
lncRNA	Long non-coding RNA	>200	Precursor ssRNA	Chromatin remodelling
			Many lncRNAs are subject to splicing, polyadenylation, and other post-transcriptional modifications	Transcriptional regulation
			Mostly nuclear RNAs but a subset also located in the cytoplasm	Post-transcriptional regulation (splicing, TF localization)
			Not evolutionary conserved with the exception of large intergenic ncRNAs, lincRNAs (H3K4me3-H3K36me3 signature)	Precursors for siRNAs
				Component of nuclear organelles (paraspeckles, nuclear speckles)

[a]PASR, promoter-associated small RNA; TSSa-RNA, transcription start site-associated RNA; tiRNA, transcription initiation RNA; PROMTs, promoter upstream transcript.

predominant reason (≥84 %) for reduced protein levels by endogenous miRNAs. In addition to its classical roles, miRNAs have also been shown to regulate gene expression through promoter targeting and translational activation.[12,20,21] Interestingly, the former is likely to involve epigenetic mechanisms.[21]

The first observations establishing the significance of miRNAs in the regulation of vascular biology came from experimental studies disrupting the function of Dicer and Drosha in miRNA biogenesis.[22–24] Dicer-deficient mice died early during development due to defects in blood vessel formation.[22] Similarly, knockdown of Dicer and Drosha *in vitro* results in reduction in endothelial cell migration, capillary sprouting, and tube formation.[23,24] These studies paved the way for an explosion of new studies exploring the roles of individual miRNAs in angiogenesis and in the pathogenesis of vascular diseases. Indeed, numerous miRNAs have been shown to participate in angiogenic processes, making them interesting therapeutic tools. In this respect, miRNAs can be divided into two groups: pro-angiogenic miRNAs or anti-angiogenic miRNAs. Pro-angiogenic miRNAs include miR-17–92,[25] miR-27b, Let-7,[23] miR-126,[26,27] miR-130a,[28] miR-210,[29] miR-378,[30] and miR-296,[31] whereas anti-angiogenic miRNAs include miR-15b, miR-16,[32] miR-221/222,[33] miR-328,[34] miR-92a,[35] and miR-214.[36] Deregulation of miRNA expression is also implicated in many vascular diseases. For example, the roles of miRNA-1,[37,38] -23,[39] -133,[40] and -208[41] in cardiac hypertrophy, miR-1[42] and -328[43] in arrhythmia, miRNA-29[44] and -133[45] in cardiac fibrosis, and miR-1, -21,[46] and miR-15 family[47] in cardiac ischaemia have been established.

2.2 Piwi-interacting RNAs

PiRNAs are small ncRNAs of 24–31 nt in size named for their ability to form complexes with Piwi proteins of the Argonaute family.[48] PiRNAs have a 2′-O-methyl modification on the nucleotide at the 3′ end and usually a uridine at the 5′ end (*Table 1*).[48] PiRNAs were first discovered in *Drosophila* as repeat-associated siRNAs (rasiRNA), which show complementarity to a variety of transposable and repetitive elements.[49] The primary role of these small RNAs has been shown to be suppression of transposon activity during germ line development.[50,51] Single-stranded precursors give rise to antisense (AS) piRNAs, which then recognize and target the cleavage of transposons by associated PIWI-proteins. This generates additional sense piRNAs arising from the target transposon sequence. This 'ping-pong' cycle goes on to increase the abundance of piRNAs and transposon silencing.[50,51]

Unlike *Drosophila* piRNAs, more that 90% of mammalian piRNAs map uniquely in the genome and cluster to a small number of loci.[52–54] However, transposon control also occurs in mammals during spermatogenesis through *de novo* DNA methylation.[55] PiRNAs have been mostly uncovered in the germline but growing evidence suggests that their defensive function extends into somatic cells.[56,57] Supporting this, a recent study proposed a role for piRNAs in the regulation of the cell cycle of mesenchymal stem cells.[58]

2.3 Small interfering RNAs

The canonical siRNA is a linear, perfectly base-paired dsRNA, which is processed by Dicer into 20–24 nt siRNAs that direct silencing when loaded onto RISC. They mediate post-transcriptional silencing similar to miRNA silencing. Compared with miRNAs, guide strand recognition is indistinguishable, but it is still unclear if all siRNA sequences are capable of effectively guiding all RNA silencing functions.[59] In addition to post-transcriptional gene silencing (PTGS), siRNAs have also been found to direct sequence-specific transcriptional gene silencing by increasing epigenetic marks characteristic of heterochromatin (*Table 1*).[59,60]

SiRNAs were first observed during transgene-induced silencing in petunia[61] followed by studies in *Caenorhabditis elegans*.[62,63] Initially, RNA interference (RNAi) was considered to be a natural defence mechanism that used exogenous siRNAs to protect organisms from viruses, but it soon became evident that endogenous siRNAs (endo-siRNAs) also play a role in regulating genome functions. In this respect, transposons and repetitive elements were first discovered as the source of endo-siRNAs, suggesting that they may play a similar role as piRNAs in suppressing transposon activity.[64,65]

Another group of endo-siRNAs consists of natural AS transcripts (NATs). NATs can be divided into different categories based on their orientation to the protein-coding gene: head-to-head (overlapping 5′ ends), tail-to-tail (overlapping 3′ ends) or fully overlapping.[66] An interesting subgroup of NATs is composed of AS-transcribed pseudogenes.[67,68] Approximately half of all mammalian protein families include pseudogenes, with greatest enrichment found in ribosomal and housekeeping families of genes.[69] A high degree of pseudogenization is also exhibited by genes of the SH3_1 (Src homology 3), homeobox, Gp_dh_N/C (glyceraldehyde 3-phosphate dehydrogenase, NAD-binding domain/C-terminal domain), and collagen families implicated in vascular development, homeostasis, and disease.[69]

2.4 Long ncRNAs

The majority of the non-protein-coding transcripts belong to the group of lncRNAs, which are arbitrarily considered as >200 nt in length (*Table 1*).[10] However, many of these lncRNAs can also act as primary transcripts for the production of short RNAs, making the categorization of this group of ncRNAs ambiguous. Most lncRNAs are characterized by nuclear localization, low expression, low level of sequence conservation and are composed of both poly A+ and poly A− transcripts.[70,71] LncRNAs can be classified according to their proximity to protein coding genes placing them into five categories: sense, AS, bidirectional, intronic, and intergenic.[10]

Recently, a subgroup of lncRNAs, named large intergenic non-coding RNAs (lincRNAs), was described based on distinctive chromatin signature that marks actively transcribed genes.[72,73] LincRNAs are marked by trimethylation of lysine 4 of histone H3 (H3K4me3) at their promoter and trimethylation of lysine 36 of histone H3 (H3K36me3) along the transcribed region. In contrast to most lncRNAs, lincRNAs exhibit a high conservation between different species. LincRNAs have been suggested to guide chromatin-modifying complexes to specific genomic loci and this way participate in the establishment of cell type-specific epigenetic states.[72,73] The most well-described examples are involved in epigenetic gene silencing, exemplified by the role of X-inactive specific transcript *Xist* in X-chromosome inactivation and *H19* or *Air* in genomic imprinting.[10] The *H19* gene encodes a 2.3-kb ncRNA, which is highly expressed during embryogenesis but shut off in most tissues after birth.[74] Environmental factor, such as maternal undernutrition, has been shown to regulate the expression of *H19* in a sex-specific manner; maternal low-protein diet was shown to cause abnormalities in male but not female mice blastocysts.[75] Interestingly, this mechanism could potentially contribute to the different susceptibility of cardiovascular diseases between male and female.[76] Apart from embryogenesis, it has been shown to play roles in tumour development by promoting the expression of genes involved in metastasis and angiogenesis.[74]

The majority of lncRNAs are transcribed as complex networks of overlapping sense and AS transcripts with respect to protein-coding loci.[77] In humans, 61% of transcribed regions show evidence of AS transcription suggesting a role for AS ncRNAs in transcriptional regulation of the overlapping mRNA.[78] Already a decade ago, it was shown that mRNA stability of hypoxia inducible factor alpha (*HIF-1α*), a physiological regulator of angiogenesis, is modulated by an AS HIF (*aHIF*) transcript complementary to the *HIF-1α* 3′UTR.[79,80] Prolonged hypoxia or *aHIF* overexpression was found to trigger the decay of the *HIF-1α* mRNA.[81] Interestingly, HIF also upregulates *aHIF* expression through a hypoxia response element present in the promoter region of *aHIF* thus generating a negative feedback loop.[81]

Subsequent studies have also discovered an AS mRNA to endothelial nitric-oxide synthase (*eNOS*), termed *sONE*, which participates in the regulation of endothelial cell-specific gene expression.[82] Moreover, *sONE* mediates the post-transcriptional down-regulation of *eNOS* during hypoxia.[83] As the down-regulation of *eNOS* may play a role in the aetiology of vascular diseases such as pulmonary arterial hypertension, ways to interfere with this interaction could bear potential for therapeutic purposes.

Recently, an AS RNA produced from the tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (*tie-1*) locus was also identified as a transcriptional repressor with potential implications

in the control of vascular development.[84] The *tie-1 AS* was shown to form a duplex with *tie-1* mRNA leading to down-regulation of gene expression. Overexpression of *tie-1 AS* lncRNAs resulted in defects in endothelial cell junctions and tube formation. Moreover, the levels of *tie-1 AS* were found to be 5–10-fold higher in human vascular anomaly samples compared with normal tissue suggesting a role in the aetiology of vascular disease. Whether AS mechanisms regulate a broader class of genes with endothelial-restricted pattern of expression forms an interesting area of future research.

2.5 Enhancer RNAs

Another class of ncRNA that has received much recent attention is found expressed at enhancer regions. The size of eRNAs has been shown to range from 0.1 to 9 kB, with an average size of 800 nt.[9,11] This situates most of the eRNAs to the category of lncRNAs but owing to their specific histone methylation signature typical of enhancers, they are discussed separately (*Table 1*).

High-throughput sequencing studies of RNA and immune-precipitated chromatin (ChIP) have defined the following characteristics of eRNA transcripts (*Table 1*). (i) eRNAs are produced from regions defined by high enrichment of monomethylation on lysine 4 of histone 3 (H3K4me1) and low enrichment of H3K4 trimethylation (H3K4me3).[8,9] (ii) These regions are enriched for RNA polymerase II (PolII) and transcriptional co-regulators, such as the p300 co-activator. (iii) Transcription of eRNAs initiates from PolII-binding sites and elongates bidirectionally. (iv) DNA sequences encoding eRNAs are evolutionarily conserved. (v) Enhancer-associated RNA transcripts have a short half-life. (vi) RNA transcripts are dynamically regulated upon signalling, and (vii) are positively correlated to levels of nearby mRNA expression.[8,9] These latter characteristics have been described in the neuronal cell response to membrane depolarization and in macrophage response to lipopolysaccharide and γ-interferon.[8,9] A recent global run-on sequencing (GRO-Seq)[85] in human fibroblasts data reveals that eRNAs are also a prominent feature of vascular genes exemplified by fibroblast growth factor 2 (*FGF-2*) and vascular endothelial growth factor C (*VEGF-C*) (*Figure 1*).

Although still speculative, several lines of evidence suggest a functional role for eRNAs as transcriptional activators. Reports of enhancer-transcribed RNAs date back to as early as 1992, when Tuan *et al.*[86,87] discovered RNA transcripts at the locus control region (LCR) of the *beta-globin* locus. The LCR is defined by four erythroid-specific DNAseI hypersensitive sites (HS1–4) and temporally regulates globin genes within the cluster throughout development. But how does the LCR, transcribed 10–50 kb upstream of its target genes, control transcription of the globin gene cluster? In a transient reporter assay, levels of eRNA were decreased by insertion of a transcriptional terminator downstream of the HS2 enhancer. This lead to reduced reporter gene activity driven by the *epsilon-globin* promoter.[88] In another line of experiments, ChIP studies demonstrated PolII recruitment to both HS2 enhancer and adult *beta-globin* promoter.[89] When elongation of PolII was inhibited pharmacologically, PolII enrichment decreased at the *beta-globin* promoter, but not at the HS2 enhancer.[89] This suggests that the recruitment of PolII to promoter, but not to the enhancer, is dependent on RNA synthesis. One may speculate that RNA synthesis from the enhancer is required for promoter PolII recruitment. A recent report further provided evidence of eRNA's functionality by targeting non-coding transcripts using RNAi.[11] Depleting eRNA led to a gene-specific decrease in mRNA expression and DNA segments encoding eRNA were sufficient to induce transient reporter activity. Moreover, RNA-mediated enhancer activity appears to be sequence-specific; while keeping the transcription start site (TSS) intact, substituting eRNA with other open reading frames led to decreased enhancer activity.[11] This indicated that transcription at the enhancer alone is insufficient for enhancer activity. Collectively, these studies propose a possible transcriptional activation role for eRNA.

Many questions arise as more functional evidence for eRNAs emerge. Kim *et al.*[8] demonstrated that the absence of an intact promoter abolishes eRNA transcription. What is the relationship between promoter and enhancers? Are the low-abundance eRNAs transcriptional noise or are they byproducts of the moving PolII along intervening DNA from enhancers to promoter?[90] Do eRNAs maintain an open chromatin state and modulate promoter and enhancer interactions? Do eRNAs serve as platforms for RNA-binding transcription factors and participate in the establishment of cell-type-specific enhancer signature?[91,92] Addressing these functional and mechanistic questions should lead to an improved understanding of the role of enhancers in the control of gene expression in different cell systems.

2.6 Promoter-associated RNAs

Similar to the discovery of eRNAs, various genome tiling and high-throughput sequencing methods have unveiled the diverse class of ncRNA linked at promoters. These RNAs can be classified based on their size—ranging from small RNA species of 16–36 to 200 nt (*Table 1*).[70,93–95] Longer >200 nt RNAs have also been described, but it is unclear whether these are precursors of shorter ncRNAs.[70,93] They can also be characterized by their location; some are expressed near TSSs, whereas others are expressed from upstream elements of the promoter.[96] Furthermore, these RNAs are found expressed in sense and divergent orientation with respect to the TSS. Most of these RNAs are associated with highly expressed genes, while themselves being weakly expressed and exhibiting short half-lives. In fact some of these transcripts were discovered when the RNA degradation machinery was either depleted or functionally deficient.[96,97]

Increasing number of studies are beginning to connect PARs with transcriptional activation and repression.[93,98–100] Studying this group of ncRNAs presents a big challenge since conventional tools like DNA deletion or mutation may interrupt regulatory elements or alternative TSS. Attempts have been made to understand functions of PARs by modulating their levels in cells. Transfection of synthetic RNAs designed to target promoter regions of *E-cadherin*, vascular endothelial growth factor A (*VEGF-A*) and *p21* increased expression of these genes in human, non-human primates, and rodent cells.[101–103] More commonly, however, promoter-targeted siRNAs lead to the repression of the downstream genes, empasizing emerging roles of PARs in transcription.[98,103] PARs are also a general feature of vascular genes as significant colocalization of GRO-Seq tags and the hallmark of active promoters H3K4me3 is found for these genes as illustrated for *FGF-2* and *VEGF-C* (*Figure 1*).

There is a growing body of work unravelling the mechanisms by which PARs participate in the transcriptional regulation. For example, most target genes of the repressive Polycomb group (PcG) protein complex exhibit low levels of the repressive histone mark histone 3 trimethyl lysine 27 (H3K27me3), while being associated with histone marks for transcriptional initiation such as RNA Pol II and H3K4me3. These characteristics suggest that the PcG

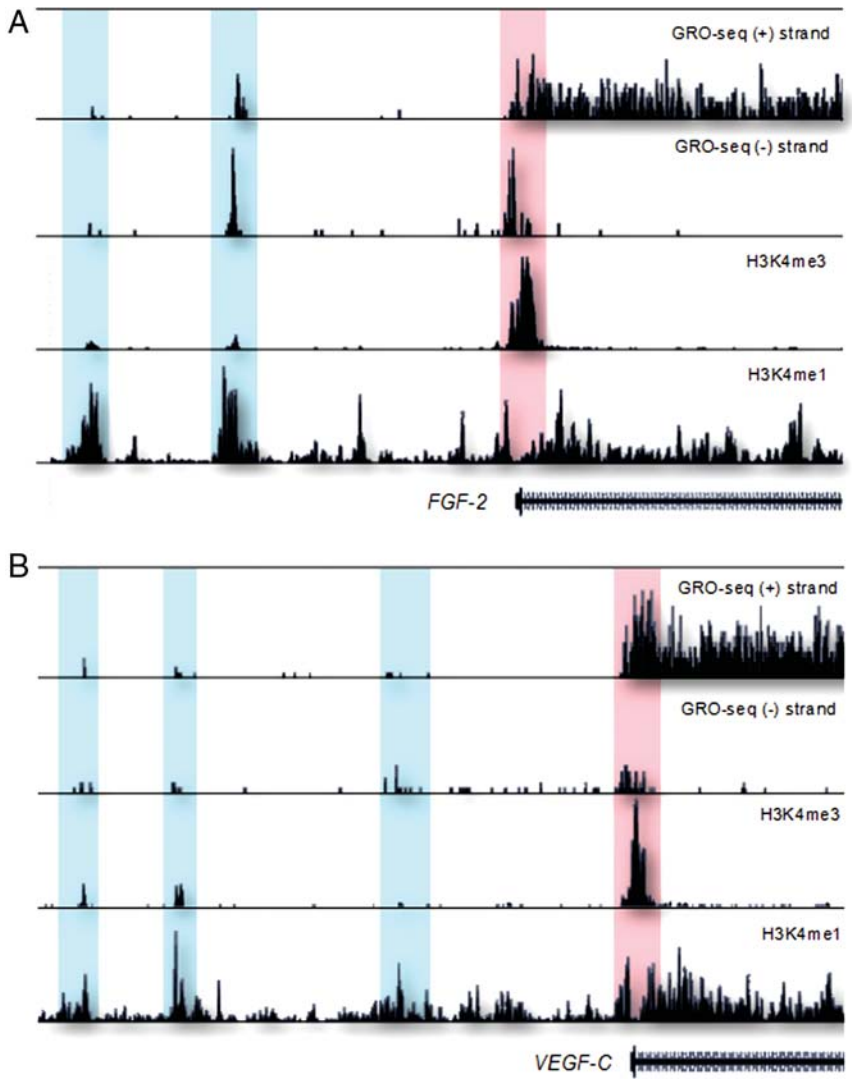

Figure 1 Potential promoter-associated RNAs and enhancer RNAs produced upstream of (*A*) fibroblast growth factor 2 (*FGF-2*) and (*B*) vascular endothelial growth factor C (*VEGF-C*) genes in human IMR90 cells.[85] The promoter-associated RNAs (highlighted in red) colocalize with H3K4me3 histone mark, whereas the eRNAs (highlighted in blue) are revealed by their overlap with H3K4me1 mark.[145] Regions upstream of transcription start site (TSS) for *FGF-2* and *VEGF-C* are 50 and 100 kb, respectively. The *y*-axis indicates the number of sequencing tags.

target gene promoters have adopted a poised state that allows their rapid induction upon cellular responses. Interestingly, short RNAs of 50–200 nt in length originate from the promoters of PcG target genes in primary T cells and embryonic stem cells. Components of the PcG complex bind to stem loop structures of these RNAs and mediate transcriptional repression in *cis*. These short RNAs are lost upon activation, offering a model in which dissociation of PcG is in the sequence of rapid induction of poised genes.[104]

3. Mechanisms mediating transcriptional regulation and epigenetics

3.1 Chromatin remodelling

Understanding how ncRNAs regulate transcription has become an area of intense research. LncRNAs have been proposed to regulate transcription by recruiting chromatin-remodelling complexes, which in turn mediate epigenetic changes.[10] Epigenetics refers to heritable changes in phenotype and gene expression caused by mechanisms other than the changes in DNA sequences. The repressive PcG is one of the most well-described transcriptional complexes that initiate and maintain epigenetic changes. PcG is characterized as two multiprotein complexes—polycomb repressive complex 1 (PRC1) and 2 (PRC2).[105] Components of PRC2 trimethylate H3K27, establishing the silent chromatin state. Components of PRC1 bind H3K27me3 and ubiquitinate lysine 119 on histone 2A. Interestingly, components of PRC1 and PRC2 are also RNA-binding proteins.[4,106–108] Locus-specific silencing mediated by PcG might thus be guided by bound lincRNAs. A classic example for this model is X-chromosome inactivation—PcG binds to ncRNA *XIST* expressed on the targeted X-chromosome and initiates epigenetic silencing by trimethylation of H3K27 in *cis* (*Figure 2B*). PcG also mediates transcriptional repression through interaction with

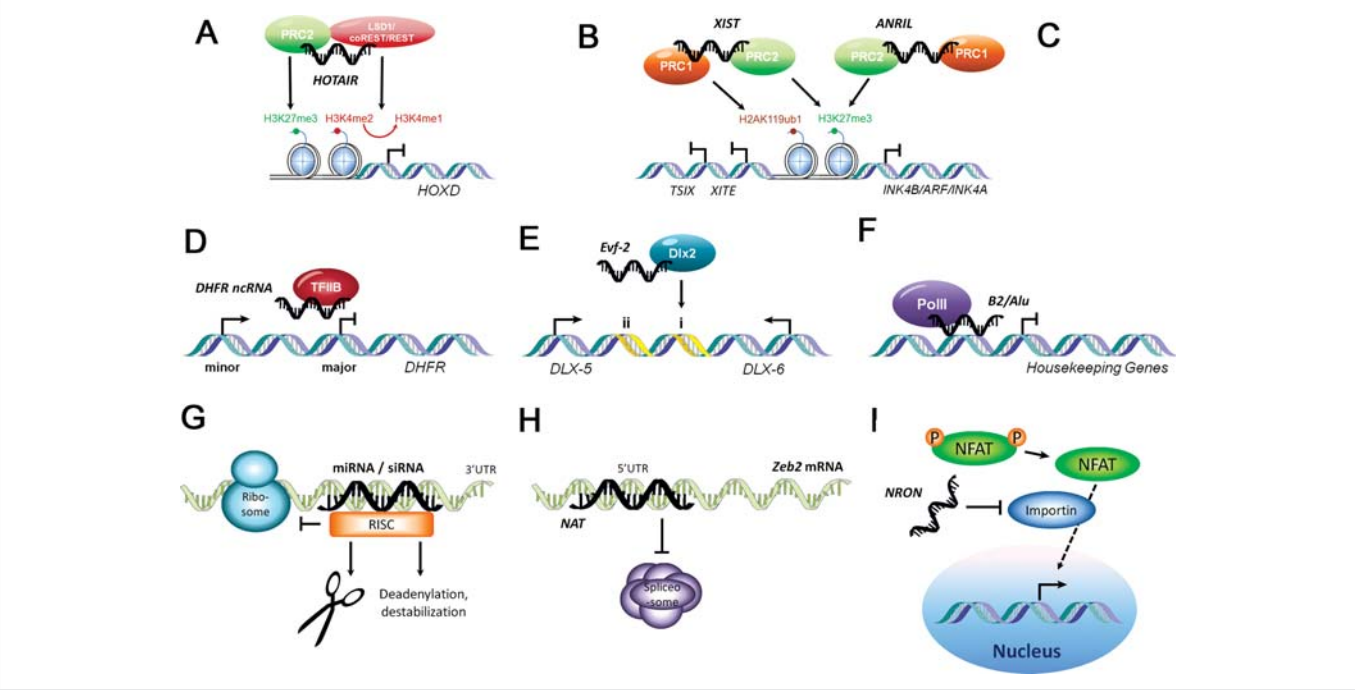

Figure 2 Mechanisms for regulation of epigenetics and gene expression by non-coding RNAs. NcRNAs can function as modulators of epigenetics through (*A* through *C*) chromatin remodelling or regulate gene expression at (*D* through *F*) transcriptional or (*G* through *I*) post-transcriptional level. (*A*) A 5′ domain of *HOTAIR* binds polycomb repressive complex 2 (PRC2), whereas a 3′ domain of *HOTAIR* binds the LSD1/CoREST/REST complex. This allows *HOTAIR* to coordinate histone H3 lysine 27 methylation and lysine 4 demethylation at the *HOXD* locus in *trans*. (*B*) In *cis* recruitment of PRC2 by *Xist* antisense RNA and appearance of H3K27me3 along the inactive X chromosome are among the earliest events in X inactivation. Recruitment of PRC1-mediated H2AK119ub1 parallels the recruitment of PRC2. (*C*) Similarly, antisense non-coding RNA *ANRIL* represses the expression from *INK4b/ARF/INK4a* locus by recruiting and retaining PRC1 and PRC2 complexes in *cis*. (*D*) LncRNA transcribed from the minor promoter of dihydrofolate reductase (*DHFR*) froms a triplex together with the transcription factor TFIIB and the major promoter leading to the dissociation of the preinitiation complex. (*E*) Enhancer region (i and ii) of *Dlx5/6* generates an lncRNA *Evf-2* which forms a complex with homeodomain protein Dlx-2 to activate transcription. (*F*) Transcription of *B2* and *Alu* RNAs is induced upon heat-shock. They inhibit mRNA synthesis by disrupting contacts between RNA polymerase II and promoter DNA. (*G*) Small interfering RNAs (siRNAs) and microRNAs (miRNAs) are incorporated into RNA-induced silencing complexes (RISCs) that target specific mRNAs for cleavage, translational repression or destabilization depending on the extent of sequence complementarity. (*H*) Natural antisense transcript (NAT) prevents the binding of the spliceosome to the 5′UTR of the *Zeb2* mRNA. This leads to retention in an intron containing internal ribosomal entry site (IRES), which is dispensable for the translation of Zeb2 protein. (*I*) The nuclear trafficking of nuclear factor of activated T cells (NFAT) is inhibited by the interaction of non-coding repressor of NFAT (*NRON*) with proteins of the importin-beta superfamily.

histone deacetylases and exerts long-lasting silencing by CpG methylation through interaction with DNA methyltransferase 3 alpha.[109,110]

Recent studies have further highlighted the mechanistic roles of ncRNAs in PcG-mediated transcriptional regulation. PcG was first described to silence the homeotic (*Hox*) genes in *Drosophila melanogaster* during development. *HOX* antisense intergenic RNA (*HOTAIR*), a 2.2 kb lincRNA expressed in *HOXC* cluster, is required for PcG-mediated silencing of the *HOXD* cluster in *trans* (*Figure 2A*).[4,111] A recent study demonstrated that the 5′ end of *HOTAIR* is bound by PRC2, whereas the 3′ end is bound by the LSD1/REST/CoREST complex (Lysine-specific demethylase 1; RE-1 silencing transcription factor; corepressor or REST). LSD1 represses gene expression by removing dimethyl mark from H3K4. In essence, *HOTAIR* enables tethering of two distinct repressive complexes to chromatin for coupled H3K27 methylation and H3K4 demethylation.[112]

While *HOTAIR* represents a model for ncRNA-dependent PcG function, an estimated 20% of lincRNA expressed in human cells are bound by PcG, suggesting that a similar strategy may be more widely applicable.[72] Indeed, PcG has been implicated in tumourigenesis by repression of *INK4B/ARF/INK4A* tumour suppressor genes (inhibitors of CDK4 and CDK6; alternative reading frame of INK4A).[113–116] An Antisense Non-coding RNA in the INK4 Locus, or *ANRIL*, is expressed in this locus and recruits PcG repressive complexes to repress these tumor suppressor genes in *cis* (*Figure 2C*).[117–119] Polycomb repressive complex 1 component chromobox 7 (CBX7) can bind *ANRIL* and H3K27me3. Mutation in either binding domains for RNA or H3K27me3 impairs repression of *INK4A/ARF/INK4B*. Cells harbouring these mutations have reduced proliferative capacity in colony forming assays.[119] Intriguingly, *ANRIL* is the only transcript found to localize at the chromosome region 9q21 associated with cardiovascular disease susceptibility.[120] Indeed, a recent study demonstrated that disease-associated genetic variants decrease the efficiency of *ANRIL* splicing and subsequent production of circular RNA species, thereby influencing PcG-mediated *INK4/ARF* repression and atherosclerosis susceptibility.[121]

3.2 Transcriptional regulation

Many lncRNAs have also been demonstrated as negative regulators of transcription. An illustrative example of this is the regulation of dihydrofolate reductase (*DHFR*).[122] The gene encoding *DHFR* contains a major and a minor promoter, the latter being silenced in quiescent cells. The lncRNAs generated from the minor promoter bind both the major promoter (triplex formation) and the general transcription factor IIB leading to the dissociation of preinitiation complex (*Figure 2D*).[122]

Recently, p53 was shown to activate the expression of numerous lincRNAs.[123] One such RNA, called lincRNAs-p21 was shown to be essential for the guidance of heterologous nuclear ribonucleoprotein K to the promoters of genes repressed by p53 thus playing an important role in cellular response to apoptotic signals. The exact mechanism by which *lincRNA-21* contributes to repression is still unknown but it might act by a similar mechanism as *DHFR*.[123] Interestingly, the specificity of p53-mediated activation of lincRNAs can be further regulated by *MEG3* ncRNA, which might modulate the binding of p53 on the promoter of its target genes.[124] Knock-out studies in mice have highlighted the important role of *MEG3* in the control of vascularization in the brain.[125]

NcRNAs can also serve as transcriptional coactivators as illustrated by the 3.8 kb polyadenylated *Evf2* ncRNA (*Figure 2E*). *Evf2* is transcribed from an ultraconserved region in *Dlx5/6* locus, and it forms a complex with the homeodomain-containing protein Dlx2.[126] This Dlx2–*Evf2* complex functions cooperatively as transcriptional activator of *Dlx5/6* expression in an enhancer-specific manner.[126] The same group followed up with an *in vivo* study by generating a mouse model where *Evf2* expression is interrupted by insertion of polyadenylation sequences. Interestingly, disruption of *Evf2* ncRNA increased *Dlx5/6* expression. Reintroduction of *Evf2* partially rescued the phenotype; but when a higher amount of *Evf2* was reintroduced, expression of *Dlx5/6* was further increased as seen in the reporter assay. This result suggests a complex scenario where dosage of *Evf2* ncRNA may have different effect on target genes.[127]

By regulating transcription, ncRNAs can be viewed as sensors of environmental signals, exemplified by two ncRNAs, mouse *B2* RNA and human *Alu* RNA transcribed from short interspersed sequence elements. These ncRNAs have been found to repress mRNA transcription in response to heat shock. They do so by preventing PolII from establishing contacts with the promoter around TATA box during the first step of transcription initiation called closed complex formation (*Figure 2F*).[128] These studies together with other examples of ncRNA transcription factor complexes—like heat-shock RNA-1/heat-shock transcription factor 1,[129] ncRNA steroid receptor RNA activator (SRA)/nuclear receptors,[130] SRA/master regulator of muscle differentiation MyoD[131]—illustrate the emerging role of ncRNA in regulating transcriptional responses to external and developmental stimuli through interaction with transcription factors.

3.3 Post-transcriptional regulation

ncRNAs are also implicated in the regulation of post-transcriptional processing, such as splicing, transport, translation, and degradation. The best characterized mechanism is no doubt the PTGS mediated by siRNAs and miRNAs through RNAi pathway (*Figure 2G*).[48] As discussed earlier, both types of RNAs influence the expression of genes by regulating the stability or translation of mRNAs. What separates these two is the fact that siRNAs silence the locus from which they are derived, whereas miRNAs regulate different genes.[48]

An increasing number of metazoan genes are being found to have naturally occurring AS transcripts.[66] AS transcripts overlapping exon–intron boundaries can mask the splicing sites thus enabling alternative splicing. For example, the expression of *Zeb2* relies on the splicing of the internal ribosomal entry site (IRES)-containing intron, which is dependent upon the expression of AS transcript (*Figure 2H*).[132] Similar mechanisms have also been described for *c-erb2*[133] and more recently natriuretic peptide precursor.[134]

Other ncRNAs regulate the transcription by controlling the subcellular localization of transcription factors. One such lncRNA is called non-coding repressor of nuclear factor of activated T cells (NFAT) (*NRON*), which regulates the nuclear trafficking of NFAT (*Figure 2I*).[135] Upon stimulation, NFAT is dephosphorylated by the calcium-regulated phosphatase calcineurin and localized to the nucleus, where it becomes transcriptionally active. The role of *NRON* seems to be to prevent the translocation of dephosphorylated NFAT thus modulating its activity. As calcineurin signalling and NFAT activation play a critical role in the development of cardiovascular and skeletal muscle[136] and coronary angiogenesis,[137] it remains to be seen if this system plays a role in cardiovascular disease processes.

3.4 Other potential mechanisms and perspectives

No study yet delivers a definitive explanation of the role of PARs or the functions of eRNAs.[8,9,11,50,64–66,85,94] Are they just a result of spurious transcriptional noise or a result from RNA Pol II molecules failing to elongate? The prevailing view at present suggests a role for these ncRNAs in maintaining the chromatin landscape poised for regulation. Recently, targeting of eRNAs by synthetic siRNAs was shown to decrease the expression of neighbouring protein-coding genes, supporting their functional roles.[11] Also, as discussed earlier, transfection of synthetic promoter-associated small RNAs most often leads to a reduction in the expression of the overlapping mRNA promoter.[138] Han *et al.*[99] showed that low-copy PARs (extended 5′ UTR) are required for RNA-directed epigenetic gene silencing in human cells. Divergent transcription spanning the promoter could thus be involved in the regulation of mRNA expression. This is supported by another piece of evidence by Morris *et al.*[98] who showed that *p21* AS RNA maintains a low level of epigenetic silencing by recruitment of Ago1 and H3K27me3 to the promoter. Subsequently, suppression of AS RNA transcription allows enhanced transcription of the sense/mRNA. According to this model, dysregulation of gene expression in disease conditions could well be due to imbalance in bidirectional transcription. Controlling transcription of the PARs and eRNAs could allow cells to fine tune gene expression, processes which can be foreseen to be harnessed for therapeutic purposes.

In addition, eRNAs could play a structural role in bringing the enhancer areas together with the promoter region by chromatin looping.[139] On a genome-wide level, this would suggest a function for eRNAs in maintenance of the three-dimensional conformation of chromosomes by bringing widely separated functional elements into close spatial proximity. Indeed, deep sequencing of chromatin-associated RNAs in human fibroblast cells provided first evidence of the role of ncRNAs in fine-tuning of the chromatin architecture.[140] Moreover, recent studies exploiting the latest genomics approach of chromosome conformation capture (3C) demonstrated that sections containing genes co-regulated during the cell cycle and genes containing the same DNA motifs at their promoter regions

tend to associate in a statistically significant manner.[141] It is tempting to speculate that genes induced by environmental stimuli, also displaying induction of eRNAs[9] could associate together through the interplay of ncRNAs and transcriptional co-regulators.

Similarly, we could ask do lncRNAs have master regulatory functions or could their role also be to provide fine-tuning? How do proteins interact with lncRNAs and how does this interaction specify the functional outcome? The non-conserved sequence of lncRNAs could suggest that conserved secondary structure is the key to its functions.[10] This is further supported by the fact that many lncRNAs with low sequence similarity are associated with Polycomb proteins.[4,118] One can envisage the secondary structure being responsible for interaction with the protein partner but also in the recognition of DNA elements or histone marks. The consequences of lncRNAs binding to a protein partner could then be to modulate its activity, ability to bind other co-regulators, or recognize binding motifs. Illustrative example of such mechanism is the regulation of cyclin D1 (*CCND1*) by a ncRNA generated from the 5′ regulatory regions of the gene.[100] The *CCND1* ncRNA is upregulated in response to genotoxic stress, which in turn enables allosteric modulation of the activity of an RNA-binding protein, translocated in liposarcoma (TLS). The modified TLS inhibits the enzymatic activities of CBP/p300 which subsequently represses the *CCND1* mRNA expression.

Many questions still remain unanswered but the ever growing evidence strongly points to a central role of ncRNAs in gene regulatory programmes. Shedding light to these intricate and complex roles of ncRNAs will no doubt be a major objective for future investigations.

4. Conclusions

The continual discovery of new regulatory ncRNA species suggests that we are only just beginning to understand their complexity and functions. Nevertheless, it has already become evident that much of their biological and molecular functions are associated with the control of epigenetic pathways, transcription, translation, and turnover.

There is increasing evidence that epigenetic pathways may control vascular endothelial gene expression and modulate cardiovascular disease susceptibility.[76] Two recent publications have already described a causative role for epigenetic alterations in the progression of heart failure.[142,143] Owing to the extensive roles of ncRNAs in the regulation of gene expression they may well serve as novel diagnostic markers for vascular diseases. For example, the use of miRNAs as biomarkers for cardiovascular disease diagnosis has already been proposed.[144] We can expect this to be expanded to the other regulatory ncRNAs when their role in vascular disorders becomes fully established by genome-wide association studies. Furthermore, the potential for therapeutic applications can be imagined. Therapy using small RNAs that target ncRNA transcripts, such as eRNAs or PARs, may represent a new way to treat disease conditions caused by epigenetic changes. The emphasis to fully characterize the mechanism of ncRNA-based gene regulation will no doubt lead into the development of novel therapies for cardiovascular diseases.

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