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Non-coding RNAs in Vascular Disease

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Abstract

Purpose of review—Non-coding RNAs (ncRNAs) have gained the attention of molecular biologists and clinicians alike due to increasing evidence implicating their role in many biological processes and in the development of diseases. In addition to small microRNAs (miRNAs) that play major roles in post-transcriptional regulation of gene expression, more recently long ncRNAs (lncRNAs, > 200 nucleotides) are recognized as being intimately involved in key cellular processes including transcription and mRNA expression, and having functions in cellular development, differentiation, and development of disease. lncRNAs represent a diverse class of RNAs with many known and likely yet to be discovered functions. This review aims to summarize emerging roles of lncRNAs in vascular development and disease.

Recent Findings—lncRNAs have been recently described to play a role in vascular development, lineage commitment and in mesoderm differentiation into heart. Additionally, lncRNAs have been associated with Angiotensin II actions, and with vascular diseases including coronary heart disease and atherosclerosis. miRNAs, well studied in various vascular diseases, have also been recently shown to be differentially expressed in biofluids of patients with vascular disease and mediate cell-cell communication.

Summary—lncRNAs may mediate many different pathways in growth factor actions, vascular development and disease and are worthy of further investigation because of their potential to serve as novel therapeutic targets.

Keywords

lncRNAs; vascular disease; miRNAs

A. Introduction

The central dogma of molecular biology originally presented the direction of information within a cell to be from DNA to RNA to protein [1]. However, it has become apparent that there are many complex layers of gene regulation that comprise epigenetic mechanisms which include regulatory, non-coding RNAs. These RNAs are emerging as important players in all aspects of cell biology including RNA transcription, stability, and translation. Advances in genomic sequencing technologies and data from several genomics consortia

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have demonstrated that the majority of the genome is transcribed into RNA, much of which is non-coding. Regulatory non-coding RNAs (ncRNAs) include small ncRNAs like microRNAs (miRNAs), as well as long ncRNAs (lncRNAs). Since there have been many reviews covering the role of miRNAs in various vascular diseases, this review will mainly focus on the emerging new roles of lncRNAs.

In 2009, Guttman and colleagues cataloged more than a thousand lncRNAs in both mouse and humans across many tissue types. For this analysis, they characterized all genomic regions that contain histone H3-lysine trimethylation (H3K4me3) and H3K36me3, two chromatin modifications associated with actively transcribed regions of the genome. In parallel, the authors assessed the presence of transcripts originating from these loci and, by assessing their protein-coding potential, they classified many to be transcripts that do not code for proteins. They further found a number of these lncRNAs capable of regulating expression of key genes including those that encode key transcription factors in embryonic stem cells [2]. While this catalog of lncRNAs exists, it is clear that lncRNAs are more complex in that they are highly tissue specific and can be induced by specific conditions. With the advent of next-generation sequencing, many more lncRNAs have been classified and characterized with very diverse functions. It is therefore important to determine their roles in vascular diseases since they could be utilized as novel therapeutic targets.

B. Molecular Functions of long non-coding RNAs

Recently, lncRNAs, which are classified as non-coding RNAs greater than 200 nucleotides long, have gained the attention of the molecular biology field. These RNAs can be processed like protein-coding mRNAs which includes Pol II processing, 5' capping, and 3' polyadenylation [3-5]. In general, lncRNAs are expressed at much lower levels compared to mRNAs. Previously described as “junk” transcripts, more and more evidence shows that lncRNAs function in many aspects of “normal” cell biology including embryonic stem cell development and differentiation. Genome-wide studies in multiple tissues have shown that lncRNAs are very cell-type specific [3] and therefore represent an epigenetic layer. Additionally, recent studies show that lncRNAs can be important mediators in cancers and other diseases including vascular disease. Indeed the human ENCODE project has defined > 9000 lncRNAs in at least 12 tissues and further classification may uncover more lncRNAs [6]. lncRNAs, as a class, have many diverse actions in the nucleus and cytoplasm that can regulate gene expression and cell functions [7,8] (Figure 1).

Cis-acting transcriptional function

One of the first described functions of lncRNAs is in regulating transcription of local genes from the locus in which they are transcribed. One of the first lncRNAs to be discovered, *Xist* transcript, functions in *cis* to establish and maintain X-inactivation [9]. Transcription of *Xist* locus results in the local spreading of the *Xist* RNA across the inactivated X chromosome [10]. *Xist* RNA further recruits components of the PRC2 silencing complex through a specific motif, Repeat A, at its 5' end [11]. Recruitment of the PRC2 complex results in histone H3 lysine 27 trimethylation which cause transcriptional silencing across the inactivated X chromosome. Interestingly, the transcription of *Xist* RNA on the activated X chromosome is repressed by the transcription of an antisense transcript of the *Xist* locus

known as *Tsix* [12]. The transcription of *Tsix* specifically regulates the function of the *Xist* promoter [12]. Interestingly, many more lncRNAs, in addition to *Xist* and *Tsix* RNAs have been found to regulate X inactivation. Together, *Xist* and *Tsix* RNAs are models for two types of *cis* regulation: 1) local transcription of a lncRNA recruits chromatin modifying complexes and regulates gene expression in *cis* and 2) transcription of an antisense lncRNA regulates the transcription of the sense RNA.

With the advent of sequencing technologies many more lncRNAs have been described which function in *cis*. Using genome-wide approaches, Orom and colleagues described enhancer-like RNAs which regulate the transcription of neighboring genes [13]). One in particular, *ncRNA-a7*, regulates the transcription of *Snai1* which is important for the cellular migration. More recently, a class of ncRNAs called enhancer RNAs (eRNAs) has been shown to play a role in various biological processes including p53-targeted gene expression, Estrogen receptor (ER) alpha-targeted gene expression, and macrophage biology [14-16]. eRNAs have been shown to be necessary for transcription of target genes and drive the transcription of neighboring genes. In mouse macrophages, Lam et. al. demonstrated that Rev-Erbalpha and Rev-ErbBeta, two nuclear receptors that function as transcriptional repressors, recruit the NCoR-HDAC3 complex to target genes by inhibiting the transcription of eRNAs at enhancer sites [16]. Downregulation of these eRNAs resulted in the reduction of mRNAs from neighboring loci. These data indicate that eRNAs can mediate specific transcriptional pathways including gene expression in macrophages.

Trans-acting transcriptional function

As noted above with *Xist*, lncRNAs can interact with protein complexes and regulate gene expression. One mode is through interaction with chromatin modifying complexes such as PRC2. LncRNAs can also recruit two different chromatin modifying complexes to the same genomic site. Tsai and colleagues showed that *HOTAIR* RNA specifically interacts with the PRC2 complex and the LSD1/CoREST/REST complex, at its 5' end and 3' end, respectively [17]. This indicates that lncRNAs may act as a scaffold to recruit different protein complexes to the same site in a sequence-specific manner. This observation suggests that the transcripts themselves, and not DNA-binding transcription factors, may direct the function of chromatin modifying proteins which can affect local transcription. In addition to chromatin modifying proteins, lncRNAs can also interact with other proteins to regulate transcription. For example, *linc-p21*, which is a p53 targeted gene upstream from the *CDKN1A* locus, can affect the transcription of other p53 target genes through its interaction with heterogeneous nuclear ribonucleoprotein (hnRNP-K)[18]. Thus, it is clear that lncRNAs can interact with an array of proteins including those that affect transcription.

Competing RNAs

In addition to regulating transcription, lncRNAs have been found to function as endogenous decoys for miRNAs. For example, *linc-MD1* RNA, which is important for muscle differentiation, contains sites that can be bound by two miRNAs, miR-135 and miR-133 miRNAs [19]. The former miRNA targets MEF2C transcripts and the latter targets MAML1 and regulates myoblast differentiation. The levels of *linc-MD1* RNA ultimately determine the effectiveness of the two miRNAs and the levels of MEF2C and MAML1. Reduced

levels of *linc-MDI* RNA are found in patients with Duchenne Muscular Dystrophy. LncRNAs with similar functions as *linc-MDI* RNA have been termed competitive endogenous RNA (ceRNA).

Stabilization of mRNAs

Recently it was shown that lncRNAs can also directly interact with mRNAs to regulate their expression. Terminal differentiation-induced ncRNA (TINCR) regulates stability of target mRNAs by directly binding to mRNAs through a 25 nucleotide motif [20]. The function of *TINCR* RNA, which is involved in epidermal differentiation and expression of target mRNAs, requires stau1 (STAU1) protein, a known RNA-binding protein. This data suggests that lncRNAs can interact with specific proteins to engage mRNAs and ultimately regulate their expression.

Altogether lncRNAs, as a class, have very diverse roles in the cell (Figure 1) regulating many processes in the nucleus and in the cytoplasm.

C. Emerging roles of miRNAs as biomarkers of vascular disease

Since the discovery of miRNAs over 20 years ago, these small non-coding RNAs have been very well-studied and have been found to be involved in both vascular development and disease (for a review, see [21]). One of the recent and emerging areas of interest in regard to miRNA biology has been the role of miRNAs in cell-cell communication locally and at a distance. They are also recently suggested to be transported from donor to acceptor cells and function as ligands of toll-like receptors [22]. miR-143/145 has been shown to be packaged into microvesicles for communication between endothelial cells and smooth muscle cells [23]. This shows that miRNAs may function in a non-cell autonomous manner, be able to avoid degradation, and function at distant sites. Indeed, more recent studies have discovered circulating RNAs in the bloodstream which may function in a “long-distance” manner to regulate gene expression [24]. Many circulating RNAs have been found to be increased or decreased in cardiovascular diseases such as acute myocardial infarction (AMI) and coronary artery disease (CAD) [25-29]. Recently, circulating p53-responsive miRNAs have been identified in the blood stream and are indicators of heart failure subsequent to an AMI [29]. Patients with atherosclerotic CAD display elevated levels of miR-17, miR-126, miR-92a, which are expressed in endothelial cells, and miR-145, which is expressed in vascular smooth muscle cells [28]. Additional investigations into circulating miRNAs that may distinguish heart failure patients from normal individuals led to the identification of four miRNAs that can classify the two groups [30]. Altogether, along with the already existing evidence, it is increasingly clear that miRNAs function by canonical as well as novel mechanisms in cardiac-related diseases and may be useful as biomarkers of cellular and pathophysiological states.

D. Functions of LncRNAs in vascular development and disease

Using RNA-sequencing, Klattenhoff and colleagues were able to define a heart-specific lncRNA in mouse called *Braveheart* (*Bvht*). Specifically, they found that *Bvht* RNA functions in vascular lineage commitment, driving mesoderm to ultimately become

cardiomyocytes. *Bvht* RNA associated with the PRC2 complex, specifically interacting with SUZ12 [31], and was required for the expression of many cardiac lineage commitment genes including *MesP1*. Interestingly, the genomic target of *Bvht*-PRC2 complex was not identified, leading to the possibility that *Bvht* RNA functions to compete with PRC2 binding at target sites critical for differentiation.

Similar to *Bvht* RNA, another lncRNA, *Fendrr* RNA was discovered to be expressed in the lateral mesoderm of mouse embryos [32]. *Fendrr* RNA was shown to interact with the Trithorax group/Mixed lineage leukemia (TrxG/Mll) complex and with the PRC2 complex. Loss of *Fendrr* in mice results in the loss of lateral mesoderm differentiation into heart and body wall, which results in embryonic lethality. Interestingly, *Fendrr* RNA activity decreases H3K27me3 and/or increases H3K4me3 at promoters of target genes by recruitment of either PRC2 or TrxG/Mll complex. As with *HOTAIR* RNA, it seems that lncRNAs may act to coordinate two distinct chromatin modifications which may define gene activity and vascular development.

Amongst the more well-known loci defined by genome-wide analysis studies to be involved in the development of coronary artery disease is the INK4b/ARF/INK4a locus. Specifically, this locus contains a few human variants associated with coronary artery diseases and atherosclerosis [33,34]. This locus has been shown to be critical for cell cycle progression and cell growth, and well-characterized as a locus that plays a role in the development of cancer. Molecular analysis of this locus revealed an antisense lncRNA called *ANRIL*, also known as *CDKN2B-AS*. This particular RNA can interact with the PRC1 and PRC2 components which are required for transcriptional repression of the locus [35,36] (Figure 2A). Interestingly, *ANRIL* splice variants can be in both a linear as well as circular form [37-39]. The expression of the circular isoforms has been associated with distinct cardiovascular disease (CVD) risk-alleles suggesting that the function of these isoforms is associated with the development of CVDs [37]. In vascular smooth muscle cells (VSMC), knockdown of *ANRIL* affects the expression of pathways involved in proliferation [34]. More recently, circular RNAs have been described to function in regulating miRNAs [40,41]. The exact function of circular *ANRIL* in the development of CVDs remains to be determined.

Using RNA-sequencing and ChIP-sequencing analysis of H3K4me3 and H3K36me3, we recently identified for the first time several lncRNAs that are expressed in rat VSMCs and those that are regulated by Angiotensin II, a peptide hormone that plays a crucial role in VSMC functions and in atherosclerosis, hypertension and kidney diseases [42]. We found that one particular lncRNA, *Lnc-Ang362*, functions as a host transcript for miR-221 and miR-222, two miRNAs that function in cell proliferations (Figure 2B). Indeed, loss of *Lnc-Ang362* results in a reduction in the expression of these two miRNAs and a decrease in proliferation of VSMCs, thus demonstrating a functional role for this lncRNAs in VSMC and Angiotensin II actions. Notably, the mouse and human genomes both contain evidence of *Lnc-Ang362* based on transcriptome and chromatin modification data. These data indicate that lncRNAs may be further processed into miRNAs to modulate actions of key vasoactive growth factors and the progression of vascular diseases.

Evidence shows that a long non-coding RNA, *PVT1*, or plasmacytoma variant translocation 1, is expressed in renal mesangial cells under hyperglycemic conditions. Knockdown of *PVT1* in mesangial cells led to decreases in the expression of key genes such as *PAI-1*, collagen and fibronectin which are associated with fibrosis and diabetic nephropathy, a major microvascular complication of diabetes ([43]).

Thus, accumulating evidence suggests that lncRNAs may also play an important role in the development of vascular disorders either directly via as yet unclear mechanisms, or by serving as hosts of functional miRNAs.

E. Future of non-coding RNAs

As we begin to learn more about lncRNAs, it is clear that this particular class of RNAs contain many subclasses[44]. For example, there are those that can interact with one or many different chromatin modifying complexes. Within this group, some lncRNAs can recruit the complexes to specific sites (e.g. *HOTAIR* RNA), whereas others compete for the chromatin complexes (e.g. *Bvht* RNA). In contrast, lncRNAs may be involved in miRNA biogenesis and function, acting as miRNA host RNAs (e.g. *Lnc-Ang362* RNA) or as endogenous decoys (*linc-MD1* RNA). While there may be different function of lncRNAs, it is clear that they are more complex than miRNAs, very diverse, and may function in all aspects of cellular biology within the nucleus and cytoplasm. One of the current challenges in the field of lncRNA is understanding how lncRNA structure is associated with its function [8]. Furthermore, whereas many lncRNAs seem to interact with specific chromatin modifying complexes, and some with the same protein, there has been little evidence of sequence conservation. This may be due to the fact that lncRNAs interact with proteins through similar folded domains that we have yet to determine. The discovery that circular RNAs exist and may be associated with specific disease will also influence the studies of lncRNAs[37,41,45,46].

Another avenue that remains relatively unexplored is whether GWAS loci contain lncRNAs. In recent years there has been many loci described to be associated with diseases including cardiovascular diseases, however, not many have been distinctly characterized. One of the main reasons is the location of the variants, most which are distal from protein-coding genes and non-exonic regions [47]. As we begin to define additional intergenic regions that can yield lncRNAs, it would be useful to examine whether these regions coincide with those associated with specific diseases as with the *ANRIL* locus.

Within the miRNA field, there has been great interest and increasing success in using circulating and urinary excreted miRNAs as biomarkers for specific states [21,48,49]. With vascular diseases, upregulated or downregulated levels of miRNAs in plasma have been discovered and are promising diagnostic markers [25-29]. Indeed minimally invasive diagnostic procedures such as blood draw to assess the level of these miRNAs in the clinic are invaluable in determining a patient's condition. Furthermore, quantifying miRNAs is a relatively quick procedure that would yield almost immediate results. With respect to lncRNAs, their presence in biofluids or value as biomarkers is not yet clear. Furthermore,

targeting and inhibiting lncRNAs *in vivo* is more challenging than with miRNAs, although some data is emerging [50]. Poor sequence conservation poses another challenge.

F. Conclusions

Given the rapidly increasing literature in this field, we anticipate learning more about the chemistry, biology, and cellular functions of lncRNAs as well as their roles in human vascular and other diseases, which in turn can be used to harness their potential as biomarkers or therapeutic targets.

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Key Points

- The class of long non-coding RNAs (lncRNAs) is a functionally diverse set of non-protein coding RNAs that regulate many processes including transcription and RNA expression.
- Emerging evidence suggests that lncRNAs function in vascular biology and may contribute to vascular diseases including atherosclerosis and hypertension.
- Circulating ncRNAs in biofluids may be used as biomarkers of specific states.
- Non-coding RNAs could be valuable novel therapeutic targets for vascular diseases.

Figure 1. Diverse Functions of LncRNAs

LncRNAs can have diverse functions including serving as: A) recruiters of transcription factors such as chromatin modifying complexes (cis or trans function), B) modular recruiters, acting as a scaffold for multiple complexes (cis or trans), C) regulators of sense transcription (cis function), enhancers of local transcription (cis function), E) stabilizers of messenger RNAs, F) competitors of mRNAs targeted by miRNAs, and G) hosts of miRNAs. Light blue lines represent mRNAs, green miRNAs, and black lines represent lncRNAs.

A. *ANRIL* B. *Lnc-Ang362* **Figure 2. LncRNAs in vascular diseases**

A) *ANRIL*, also known as *CDKN2B-AS* (antisense) transcript recruits PRC complex in *cis* and regulates the transcriptional repression of *CDKN2A* and *CDKN2B*. Single nucleotide variants within the *ANRIL* locus are associated with many diseases including coronary artery disease. B) *Lnc-Ang362* locus, marked by H3K4me3 at the transcriptional start site and H3K36me3 through the gene body in vascular smooth muscle cells (nucleosomes highlighted with red and light blue, respectively, encodes an Angiotensin II (Ang II) regulated lncRNA. *Lnc-Ang362* RNA is the host transcript for two miRNAs, miR-221 and miR-222 which regulate VSMC proliferation, and ultimately mediates the response to Ang II.