Vascular Pharmacology 114 (2019) 23–30

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Review

The function of long non-coding RNAs in vascular biology and disease

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ARTICLE INFO

Keywords:
lncRNA
Angiogenesis
Vascular injury and remodeling
Endothelial cells
Vascular smooth muscle cells

ABSTRACT

In recent years, it has been revealed that majority of the genome is transcribed in a cell- and context-specific manner into a vast array of RNA transcripts that do not encode proteins. Increasing evidence suggests that non-coding RNAs, especially long non-coding RNAs (lncRNAs) are essential regulators of gene expression and other cellular processes, including in the cardiovascular context. In this review, we discuss lncRNAs and their function during endothelial and vascular smooth cell differentiation, function and homeostasis as well as their role in vessel wall injury response and vascular disease pathophysiology. Although our understanding of lncRNAs is still emerging, these examples reveal important insights on how lncRNAs may ultimately be used in clinic as therapeutic targets for cardiovascular disease.

1. Introduction

Until recently, efforts to understand cellular differentiation and function have mostly relied on interrogation of the protein coding genome. However, over recent years, with the help of large-scale RNA-sequencing projects and technological advances, thousands of short and long non-coding RNAs have been identified revealing that most of the genome is in fact transcribed but only 1–2% of those transcripts encode proteins [1, 2]. Thus, our understanding of the non-coding genome and its impact to regulate cellular identity and function has dramatically magnified.

Long non-coding RNAs (lncRNAs) are defined as transcripts longer than 200 nucleotides and like messenger RNAs (mRNAs), they are typically transcribed by RNA polymerase II and generally spliced and polyadenylated but they have no or low coding potential. LncRNAs mostly exhibit cell type and developmental stage specific expression pattern and they are shown to play key roles in regulating diverse and large class of cellular functions. Based on genomic location with respect to nearby protein-coding genes, lncRNAs can be classified into sense, antisense, intronic, intergenic (lincRNA) and enhancer (eRNA) lncRNAs. On the other hand, functional annotation of lncRNAs is dramatically more complex. LncRNAs manifest specific and diverse subcellular localizations in different cell types depending on their molecular function. Unlike microRNAs, lncRNA function is not limited to other RNA interactions. Rather, lncRNAs have been shown to also interact with protein and DNA, exhibiting very diverse molecular functions in different cellular compartments [3]. Firstly, by interacting with proteins, lncRNAs can act as scaffolds for protein complexes [4], guides for targeting protein complexes such as chromatin modifiers to specific sites [5, 6], signaling molecules to spatiotemporally regulate cellular processes [7], and molecular decoys to sequester away proteins from chromatin [8, 9] depending on their subcellular localization. Secondly, nuclear lncRNAs are also implicated in regulating chromatin states by directly interacting with genomic DNA to recruit epigenetic modifiers to a specific locus [10, 11]. Several other lncRNAs such as *XIST*, *AIRN* and *H19* bind neighboring genomic loci to initiate genomic imprinting [12–14]. Finally, via direct RNA interaction, lncRNAs regulate protein coding transcripts by controlling their splicing [15], stability [16, 17], and translation [18, 19]. In an indirect way to regulate transcript levels, lncRNAs have also been shown to sequester miRNAs from their endogenous targets and act as molecular sponges [20, 21]. Similarly, a subset of lncRNAs post-transcriptionally regulate its target genes

Abbreviations: lncRNAs, Long non-coding RNAs; MLN, Myoregulin; SENCR, Smooth muscle and endothelial cell-enriched migration/differentiation associated lncRNA; MYOSLID, MYOcardin-induced smooth muscle long non-coding RNA inducer or differentiation; MLK1, MYOCD related transcription factor A; MALAT1, Metastasis associated lung adenocarcinoma transcript 1; HLI, Hind limb ischemia; PRC, Polycomb repressive complex; MEG3, Maternally expressed 3; GAS5, Growth arrest-specific 5; TUG1, Taurine upregulated 1; MIAT, MI-associated transcript; TIE1, Tyrosine kinase containing immunoglobin and epidermal growth factor homology domain-1; PAH, Pulmonary arterial hypertension; CAD, Coronary artery disease; SMILR, Smooth muscle-induced lncRNA enhances replication; HAS2, Hyaluronan synthase 2; RNCR3, Retinal non-coding RNA3; ANRIL, Antisense noncoding RNA in the INK4 locus; TGFβ2-OT1, Transforming growth factor beta 2 overlapping transcript 1; SHR, Spontaneously hypertensive rats; Ang II, Angiotensin II; TAA, Thoracoabdominal aortic aneurysm; HUVECs, Human umbilical vein endothelial cells; HCASMCs, Human coronary artery smooth muscle cells; PASMCs, Pulmonary artery smooth muscle cells; HAOSMCs, Human aortic smooth muscle cells; HSVSMCs, Human saphenous vein smooth muscle cells; HAOECs, Human aortic endothelial cells

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https://doi.org/10.1016/j.vph.2018.06.004
Received 26 February 2018; Received in revised form 4 June 2018; Accepted 5 June 2018

indirectly by acting as precursors of miRNAs [22, 23]. As well as miRNA precursors, lncRNAs can also code for short peptides. For example, toddler/ELABELA is a short-secreted peptide, regulating APJ/Apelin signaling [24], while in two independent studies, micropeptides DWORF and myoregulin (MLN) were shown to mediate muscle contractility by controlling Ca^{2+} levels [25, 26]. Although some lncRNAs are shown to be evolutionarily conserved [27], large number of lncRNAs do not exhibit the same level of conservation constraint compared to protein-coding transcripts [28]. On the other hand, many lncRNAs are located at syntenic genomic regions and display conserved expression patterns between species in a cell-type specific fashion due to similarities in their regulatory and promoter elements [29].

A growing number of lncRNAs are now also implicated in endothelial and vascular smooth cell differentiation and function, however, our knowledge in understanding how they regulate cellular processes to maintain homeostasis and response to vascular injury is very limited at this juncture. We provide an overview of lncRNAs that are involved in vessel biology, including in the disease context to elucidate the potential of these regulatory molecules as therapeutic targets.

2. LncRNAs in vessel differentiation and function

LncRNAs have been shown to play an important role during cell fate specification by tightly regulating cell type specific gene expression [5, 10, 30]. Surveying how global gene expression patterns change during endothelial cell (EC) commitment and differentiation, Kurian et al. identified 1924 dynamically regulated lncRNAs [31]. The study initially relied on an efficient differentiation methodology that converted human pluripotent stem cells into mesoderm-derived vascular progenitors and terminally differentiated ECs, providing a "lncRNA catalogue" that provided candidate RNA species that may be important for EC differentiation and cell identity. Using very strict sets of criteria, they then narrowed the list into three lncRNAs that exhibited cell/differentiation stage specific expression patterns, have significant sequence conservation between vertebrates and reside in genomic loci that are near known cardiovascular regulatory elements. The lncRNA *AC104461 (TERMINATOR)* was expressed during very early development and morpholino (MO) mediated loss-of-function led to massive cell death, developmental delays and severe cardiovascular defects [31]. On the other hand, *LINC00261 (ALIEN)* was shown to be expressed only in cardiovascular progenitors and to regulate genes that are associated with angiogenesis and blood vessel development. Abrogation of *LINC00261* function caused defective vascular and cardiac patterning in zebrafish embryos [31]. *AGAP2-AS1 (PUNISHER)* was shown to be expressed only in terminally differentiated ECs and was required for effective cardiovascular patterning during zebrafish development. Loss of *AGAP2-AS1*.

activity in human umbilical vein endothelial cells (HUVECs) caused defective EC function, including abnormal tube formation/branching and impaired low density lipoprotein uptake [31]. However, further investigation is required to determine the precise molecular function of those lncRNAs during EC differentiation and how they lead to such profound effect on cell fate and function in the cardiovascular system.

Another lncRNA that has a function during early vessel development is termed smooth muscle and endothelial cell-enriched migration/differentiation associated lncRNA (*SENCR*). Although it was initially characterized as a lncRNA enriched in human coronary artery smooth muscle cells (HCASMCs) and HUVECs [32], its expression was also regulated during human pluripotent stem cell derived EC differentiation. Overexpression of *SENCR* during EC differentiation potentiated mesodermal and endothelial cell commitment [33]. Likewise, *SENCR* was required for maintenance of endothelial [33] and smooth muscle cell [32] characteristics such as tube formation ability and contractility, respectively. Repression of *SENCR* expression was associated with coronary artery disease and critical limb ischemia [33]. Besides *SENCR*, only several other lncRNAs are implicated in vSMC differentiation and function. One of the recently characterized vSMC specific lncRNA is *MYOSLID* (MYOcardin-induced smooth muscle long non-coding RNA, inducer or differentiation) [34]. This lncRNA was identified in a study performed to discover novel transcripts that are regulated by MYOCD, the master regulator of vSMC differentiation program [35, 36]. In addition to MYOCD, *MYOSLID* is direct transcriptional target of TGFβ1, another potent activator of human vSMC differentiation [37]. Both knockdown and overexpression studies revealed that *MYOSLID* stimulated vSMC differentiation and attenuated cell proliferation and migration. The molecular function of *MYOSLID* in vSMCs is twofold: First, *MYOSLID* regulates MLK1 (MYOCD related transcription factor A) nuclear translocation by modulating stress fiber assembly and F-actin formation. Subsequently, nuclear MLK1 transcriptionally induces genes associated with vSMC identity and contractility. Secondly, *MYOSLID* regulates TGFβ1-induced SMAD2 phosphorylation although this functional connection seems to be indirect and needs to be further investigated.

3. LncRNAs in vessel function and angiogenesis

Dysregulation blood vessel function and complex biological processes such as angiogenesis will lead to serious consequences in the cardiovascular diseases (CVD) setting, in tumors, and in non-healing wounds. Stimulation of angiogenesis is essential in wound repair, treatment of regenerative diseases and tissue engineering. Therefore, a detailed understanding of regulatory pathways in vessel biology and during angiogenesis is vital for potential therapeutic approaches.

Recent studies highlight the potential roles of lncRNAs to regulate EC & vSMC specific function and homeostasis (Fig. 1). Transcriptional profiling of HUVECs performed by Michalik et al. identified several lncRNAs that exhibit EC specific expression pattern [38]. One of the well-studied lncRNAs in cancer context, *MALAT1* (metastasis associated lung

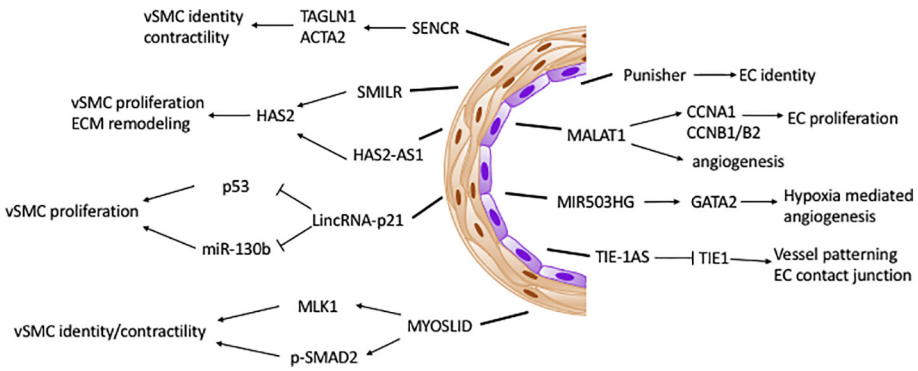

Fig. 1. Examples of lncRNA expression and function in endothelial and vascular smooth muscle cells. Purple area denotes endothelial cells, orange area represents vSMCs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adenocarcinoma transcript 1) was identified in this screen and has shown to have an essential role in angiogenesis. Both siRNA and GapmeR mediated knockdown of *MALAT1* caused significant reduction of cell cycle progression and increased angiogenic sprouting in-vitro. Similarly, in-vivo, *MALAT1*$^{-/-}$ knock-out mice exhibited impaired vascularization of the neonatal retina during development, such as reduced vessel density and delayed vessel extension. Furthermore, loss of *MALAT1* in hind limb ischemia (HLI) model caused declined blood flow recovery [38]. Interestingly, however, *MALAT1* is dispensable for normal development of mice [39, 40], suggesting that *MALAT1*, which is highly responsive to hypoxia in HUVECs [38], may have a more fundamental role in regulation of endothelial function under stress and in diseased conditions. *MALAT1* regulates mRNA splicing by controlling splicing factor phosphorylation [8], yet, no significant alternative splicing was observed in HUVECs lacking *MALAT1* [38]. On the other hand, *MALAT1* was also shown interact with PRC2 (polycomb repressive complex 2) components to regulate cell cycle progression [41, 42]. Thus, it was proposed that *MALAT1* regulates endothelial function by controlling expression of cell cycle regulators [38] however the exact molecular function of *MALAT1* during angiogenesis remains to be determined.

Maternally expressed 3 (*MEG3*) is expressed wide variety of cells including endothelial and vascular smooth muscle cells as wells as tumor tissues [38, 43–45]. In *MEG3* knockout mice brains, angiogenic genes were upregulated causing increased microvessel density [46]. *MEG3* regulates EC function and vessel growth in-vitro, as shown in numerous studies [47–50] however, each study proposed different molecular mechanism to explain the angiogenic function of *MEG3*. For example, it was suggested that, even though *MEG3* is a nuclear enriched RNA [28, 51], it may act as a molecular decoy against miR-9, miR21 and miR140-5p [47, 48, 50]. Furthermore, several studies suggested *MEG3's* function in regulating AKT or Notch signaling [47, 49]. Also, in other cellular contexts, *MEG3* regulated TGF-beta pathway by interacting PRC2 complex [51]. Together, it is still not clear the molecular mechanisms of *MEG3* regulation on EC function and angiogenesis. Interestingly, out of several highly EC enriched lncRNAs, *MEG3* is the only lncRNA that was upregulated during cardiovascular aging and its expression was strongly correlated with age in cardiac atria samples [52]. In addition, *MEG3* controlled proliferation by regulating cell cycle associated proteins in pulmonary artery SMCs (PASMCs). This was achieved by activation of p53 signaling pathway by inducing p53 nuclear translocation [43].

GAS5 (Growth arrest-specific 5), a widely-studied tumor suppressor lncRNA, has important functions for vessel function, remodeling, VSMC differentiation and exosome mediated EC-VSMC crosstalk [53, 54]. In HUVECs and human aortic smooth muscle cells (HAOSMC), loss of *GAS5* affected their proliferation, migration and improved cell viability [53]. Similarly, *GAS5* regulated proliferation, migration, cell cycle and apoptosis of human saphenous vein smooth muscle cells (HSVSMCs) by interacting with Ca^{2+}-dependent phospholipid binding protein Annexin A2 [55]. However, the binding site and the molecular mechanisms triggered with this interaction remain unknown. Although *GAS5* is a well-known molecular decoy for glucorticoid receptor in cancer [56], it was alternatively shown to control β-catenin signaling activity by affecting β-catenin nuclear translocation to regulate EC and VSMC function [53]. In a different study, *GAS5* regulated TGF-β signaling by competitively interacting with SMAD3, preventing binding to its target gene promoters, including vSMC markers [54].

3.1. LncRNAs in transcriptional regulation of EC identity

Endothelial cell identity is maintained by tightly regulated gene expression networks. Recent studies revealed that dynamic but strict transcriptional and epigenetic changes modulate vascular cell lineages during development and regeneration [57–59]. To identify epigenetically controlled lncRNAs in ECs, Leisegang et al. investigated lncRNAs that are dysregulated in HUVECs lacking histone methylase JARID1B [60]. *MANTIS* was the most robustly induced lncRNA in this screen. In HUVECs and pulmonary artery endothelial cells (PAECs), knockdown of *MANTIS* caused attenuated tube formation, sprouting and defects in shear stress response. *MANTIS* regulated endothelial cell function and expression of angiogenesis related genes such as NR2F2, SOX18 and SMAD6 by acting as a scaffold in SWI/SNF complex and modulated its nucleosome remodeling activity by binding to BRG1 [60]. In disease context, *MANTIS* expression was also inversely correlated with JARID1B expression levels. For example, in lungs of pulmonary arterial hypertension patients, *MANTIS* was repressed while JARID1B was induced. On the other hand, during atherosclerosis regression and vascular regeneration, *MANTIS* was induced whereas JARID1B was repressed [60].

There are several lncRNAs reported to regulate angiogenesis by regulating vascular endothelial growth factor, VEGF. Taurine Upregulated 1, *TUG1*, is highly expressed in HUVECs and other ECs from different vascular beds [38], as well as human tumors [61–63] and it is highly responsive to hypoxia [38]. *TUG1* regulated (tumor-induced) angiogenesis, proliferation, migration, tube formation both in-vitro and in-vivo by acting as molecular sponge for miR-299 and miR-34a-5p so that expression of VEGFA, direct target of these miRs, was fine-tuned during angiogenesis [64, 65]. Similarly, *HOTAIR* promoted angiogenesis by regulating VEGFA expression both directly as transcriptional level and indirectly by upregulating GRP78-Ang2-VEGFA axis [66]. Finally, in another study, *MIAT* (MI-associated transcript) was shown to modulate endothelial cell expression, including VEGF by acting as a competing endogenous RNA against miR-150-5p [67].

Endothelial nitric oxide synthase (eNOS) has key role in regulating EC hemostasis and function [68]. *LEENE* (lncRNA that enhances eNOS expression) is one of the lncRNAs that are reported to regulate eNOS expression in ECs both in-vivo and in-vitro [69]. LEENE is expressed at an enhancer region that is proximally associated with eNOS promoter. Through this direct chromatin interaction, LEENE acts as a guide to recruit RNA polymerase II to eNOS locus to enhance nascent eNOS transcript production, thereby regulating eNOS regulated NO bioavailability and EC function [69]. Similarly, *STEEL* (spliced-*t*ranscript *e*ndothelial-*e*nriched lncRNA) was recently shown to regulate eNOS and KLF2, another key transcription factor regulating EC identity and homeostasis [70], expression via poly-ADP ribosylase (PARP1) interaction [71]. Moreover, *STEEL* promotes the number of microvessels in-vivo, induce angiogenesis and shear stress responsiveness in-vitro [71].

Hypoxia is one of the principal factors that induce angiogenesis in ECs through VEGF signaling [72]. To determine lncRNAs that may be responsible for this process, Fiedler et al. globally studied transcriptomes of HUVECs cultured under normoxic and hypoxic conditions. Two of the selected lncRNAs from this study, *MIR503HG* and *LINC00323*, were shown to regulate hypoxia-mediated-angiogenesis by controlling levels of key angiogenic transcription factor GATA2 [73]. In another study, *MEG3* and *LINC00657* were also significantly upregulated upon hypoxia [38]. However, the molecular mechanism behind this regulation is still unknown.

LncRNAs are often expressed in a species-specific manner and lack sequence conservation [74], however, numerous lncRNAs are located at syntenic genomic regions and display similar promoter properties [29, 75] to exhibit conserved cell-type specific expression and potentially have conserved function between species. One such EC-specific lncRNA was characterized in a study performed by Keguo et al. Although *tie-1AS* was initially identified in zebrafish, it is located antisense to *tie1* (tyrosine kinase containing immunoglobin and epidermal growth factor homology domain-1) in zebrafish, mouse and human [76]. Sequence conservation between 3 species is only 28.5% but its expression is highly conserved in endothelial cells across species. In zebrafish, *tie-1AS* fine-tuned *tie1* transcript levels by direct interaction for proper vessel patterning during development. In human, similarly, TIE1 was regulated by *TIE-1AS* and TIE1-*TIE-1AS* imbalance in placentas was implied to cause vascular malformations [76].

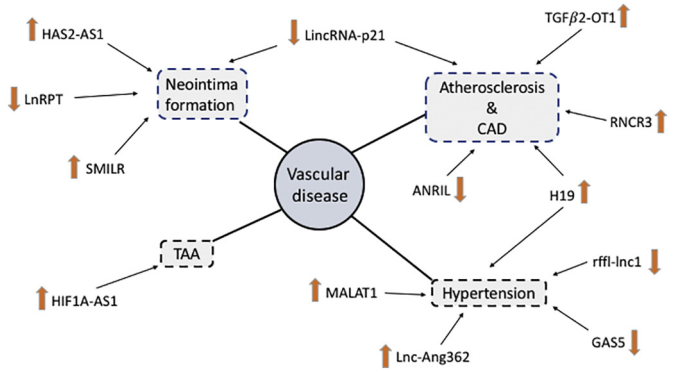

Fig. 2. Examples of lncRNAs that are associated with vascular injury response and disease. Arrows indicate the direction of differential expression (up- or down-regulation) in the disease state.

4. LncRNAs in vascular injury and disease

The integrity of blood vessels is critical for vascular homeostasis. Injury to blood vessel will lead to vessel function imbalance and vascular remodeling. Vascular remodeling is a multistep process that causes structural and functional changes to both endothelial and smooth muscle cells and drive neointima formation by altering cell proliferation, migration and apoptosis, and modulating the overall extracellular matrix composition in the vessel [77–79]. The abnormalities in vessel structure and function can then lead to initiation and progression of various vascular pathologies such as atherosclerosis, hypertension, pulmonary arterial hypertension (PAH), coronary artery disease (CAD) and diabetic retinopathies. Molecular mechanisms that regulate vascular injury induced vascular remodeling and pathological responses are extensively studied [80–82]. Yet, the association between vascular diseases and lncRNAs is only recently appreciated. Here, we provide an overview of lncRNA functions that are correlated to pathological vascular diseases (Fig. 2).

4.1. LncRNAs in neointima formation after vessel injury

Excessive proliferation and migration due to inflammation and vSMC phenotypic switching is the hallmark of neointima formation in many vascular diseases, including pulmonary arterial hypertension (PAH). To study the lncRNAs that are regulated in inflammatory and proliferative pathways in vSMC, Ballantyne et al. performed RNA-seq analysis of HSVSMCs treated with IL1α and PDGF, pathological mediators of vascular injury [83]. *SMILR* (Smooth muscle-induced lncRNA enhances replication) was one of the most differentially expressed lncRNAs compared to unstimulated cells. Knockdown of *SMILR* significantly reduced vSMC proliferation while overexpression of *SMILR* caused an increase [83]. Circulating *SMILR* level was correlated with inflammatory C-reactive protein thus it may have a prognostic value. Also, its expression was increased in unstable atherosclerotic plaques compared to relatively healthy adjacent sections [83]. Similarly, *HAS2-AS1*, a lncRNA neighboring *SMILR*, was also induced in human atherectomy samples derived from severe lesions and aortas from atherosclerotic mice [84]. Interestingly, both lncRNAs were shown to regulate HAS2 (Hyaluronan Synthase 2) levels. HAS2 is responsible for hyaluronan synthesis and shown to contribute to extracellular remodeling, vessel wall thickening and neointima formation during progression of vascular disease [85, 86]. BRAF-activated noncoding RNA (BANCR) is another recently characterized lncRNA that is also upregulated in atherosclerotic plaques however its molecular pro-proliferative function was attributed to JNK mediated signaling pathways in vSMCs [87].

Chen et al. identified 95 lncRNAs that were differentially regulated in PDGF stimulated hyperproliferative rat PASMCs compared to unstimulated cells [88]. *LnRPT* (lncRNA regulated by PDGF and transforming growth factor β) was one of the significantly repressed upon PDGF treatment in both time and dose dependent manner. Knockdown of *LnRPT* caused augmented vSMC proliferation while overexpression blocked it [88]. In rats with monocrotaline induced PAH, *LnRPT* was also downregulated [88]. RNA-seq analysis in *LnRPT* knockdown cells revealed that Notch signaling pathway components *notch3* and *jag1* were highly upregulated. Supporting this observation, suppression of Notch signaling rescued the pro-proliferative phenotype of *LnRPT* knockdown [88]. In a more directed study correlating to PAH disease model, *LncRNA-TCONS_00034812* was found to be significantly repressed in pulmonary arteries of rats with hypoxia induced PAH [89]. Similar to *LnRPT*, *TCONS_00034812* knockdown promotes the vSMC proliferation in PASMCs [89] however additional work is required for both studies to clarify the functional relevance in human PAH pathophysiology.

LincRNA-p21 is another lncRNA that regulates neointima formation after vascular injury by repressing cell proliferation and stimulating apoptosis in vSMCs both in-vitro and in-vivo [90]. Its expression was dramatically repressed in both human and mice atherosclerotic plaques and loss of *LincRNA-p21* resulted in neointima hyperplasia in carotid artery injury model [90]. There are several mechanisms proposed to explain molecular function of *lincRNA-p21*. *LincRNA-p21* is direct transcriptional target of p53 [91] and in a feedback loop, lincRNA-p21 modulated transcriptional activity of p53 in vSMCs and regulate proliferation and apoptosis associated genes by interacting MDM2, a component of p53 associated regulatory complex [90]. In a different study, *lincRNA-p21* was shown to directly bind miR-130b and act as endogenous sponge in vSMCs [21]. miR-130b function is involved in several vascular cell phenotypes associated with PAH manifestation such as excessive cell proliferation and robust extracellular matrix (ECM) stiffening by regulating STAT3/miR-204 axis and YAP/TAZ signaling respectively [92, 93].

4.2. LncRNAs in atherosclerosis and coronary artery disease

Atherosclerosis is a multistep process where dysregulated interaction between ECs, vSMCs and macrophages leads to inflammation, lipid deposition, vSMC proliferation, EC dysfunction and ECM remodeling in artery wall. This eventually causes formation of plaques made up of fat, cholesterol and calcium, and therefore narrowing arteries and restricting blood flow. CAD is the result of plaque formation in coronary arteries. Several lncRNAs are reported to be involved in this process, including plaque initiation and disease progression.

Retinal non-coding RNA3 (*RNCR3*, *LINC00599*) is a lncRNA that is associated with atherosclerosis related vascular dysfunction [94]. *RNCR3* was expressed in both HUVECs and vSMCs at basal levels while ox-LDL stress induces *RNCR3* expression further. Both in in-vitro and in-vivo, loss of *RNCR3* led to reduced cell viability, and cell proliferation. In mice and human atherosclerotic plaques, *RNCR3* expression was induced dramatically. Moreover, loss of *RNCR3* in *Apoe*$^{-/-}$ mice caused elevated total cholesterol and triglyceride and inflammatory factor release in the plasma, thereby augmenting the atherosclerosis progression. Mechanistically, *RNCR3* acts as a signaling mediator, carried from ECs to vSMCs via extracellular vesicles. Competing endogenous RNAs (ceRNAs) are natural decoys with shared miRNA binding sites thereby de-repressing the targeting transcripts by competing for common miRNA pools [95, 96]. At molecular level, *RNCR3* acts as a ceRNA against miR-185-5p, forming a feedback loop with KLF2 [94], a flow responsive transcription factor that controls endothelial identity and vascular identity [70]. Overall, *RNCR3's* atheroprotective function suggest potential therapeutic potential.

There is increasing evidence from GWAS studies suggesting links between lncRNA single nucleotide polymorphisms (SNPs) and genetic risk factors for vascular pathologies [97–99]. One of the most robust CVD susceptibility locus was found at chromosome 9p21. Homozygotes for the associated risk allele have increased risk of coronary heart

disease by 30–40% [99]. Similarly, the estimated risk of MI of the carriers of the 9p21 risk haplotype is much greater than non-carriers [98]. Interestingly, no protein coding genes are located in this locus, however the core haplotype pattern is detected flanking last exon of *ANRIL* (antisense noncoding RNA in the INK4 locus). *ANRIL* is highly expressed in ECs and vSMCs [97] and exhibit tissue-specific splice variants [100]. Remarkably, the effects of *ANRIL* on gene expression is splice variant specific, suggesting different splice variants regulate diverse cellular processes [101]. Additionally, expression levels of *ANRIL* was directly correlated in atherosclerotic plaques with severity of the pathology [102]. Initially, *ANRIL* was shown to regulate the neighboring gene expression in cis by interacting and modulating PRC1& PRC2 repressive complex [6, 103] however, *ANRIL* also regulates gene networks that are important in atherogenesis such as proliferation, cell adhesion and apoptosis, in trans, by acting a scaffold for PRC complex thru its Alu elements [104]. In a different study, *ANRIL* was shown to regulate miR-44a/miR-449a-mTOR-CDK6 axis, thereby modulating cell cycle associated transcription factor E2F1 [105]. In addition to linear transcripts, *ANRIL* has circular splice variants that are associated with atherosclerosis risk [106]. circANRIL functions as a atheroprotective molecule by acting as a scaffold in pre-ribosomal complex and regulates pre-rRNA maturation by interacting PES1 [107]. Similarly, initial findings on Chinese Han cohorts suggested that *lincRNA-p21* polymorphisms may influence on individual susceptibility to CAD [108]. In a parallel study, *lincRNA-p21* was decreased in patients with CAD [90], supporting this observation although more detailed and broader genome-wide association studies (GWAS) studies need to be performed to further validate the genetic component of *lincRNA-21*-CAD association.

H19 is another lncRNA that is strongly associated with CVD risk factors, such as high blood pressure and CAD through GWAS [109, 110]. *H19* is a well-studied imprinted lncRNA that is important in cell differentiation and growth. Although *H19* expression is generally limited to early development, it was highly expressed in neointima of rat carotid artery after vascular injury [111], and rabbit atherosclerotic plaques [112] suggesting that *H19* may have a role in vSMC phenotypic changes induced by neointima and plaque formation. Moreover, *H19* was induced in hypoxic conditions and mouse hindlimb ischemia model. Knockdown of *H19* in human aortic ECs (HAOECs) caused impaired tube formation, increased apoptosis and G1/S cell cycle arrest due to induced p21/CDKN1A [113]. *H19* has several proposed molecular mechanisms regulating cell proliferation in cancer cells however its vSMC relevant function is still ambiguous. For example, nuclear *H19* mediated proliferation by interacting with EZH2/PRC2, and repressing E-Cad expression [114]. On the other hand, miR-675, which is encoded in *H19* gene, repressed expression CDK6, a key regulator of cell cycle [22]. In a more relevant cardiovascular context, *H19* is responsible for controlling cardiac fibrosis by regulating DUSP5/ERK1/2 axis [115]. Let-7 family miRs have important atheroprotective role in vascular disease progression [116]. *H19* acts as a ceRNA against let-7, affecting transcript levels of CVD associated let-7 target genes [117, 118].

A similar ceRNA mechanism has also been proposed for lncRNA *TGFβ2-OT1* (Transforming growth factor beta 2 overlapping transcript 1) regulating atherosclerosis pathophysiology such as autophagy and inflammation. *TGFβ2-OT1* was initially discovered in 3BDO-induced autophagy screen in HUVECs [119]. In a subsequent study, *TGFβ2-OT1* expression levels was shown to be regulated by LPS and oxLDL-induced inflammation through NUPR1 and TIA1 mediated RNA processing [120]. In turn, *TGFβ2-OT1* acted as a ceRNA by directly interacting with miR-3960, miR-4488 and miR-4459 and thereby indirectly regulating the transcript levels of miRNA target genes such as CERS1, NAT8L, and LARP1, respectively. *TGFβ2-OT1* then promoted autophagy and inflammation related protein synthesis such as SQSTM1 through LARP1 activity and activated RELA & CASP1 [120].

4.3. LncRNAs in hypertension

Hypertension is associated with structural changes of the arterial wall, effecting ECs, vSMCs and ECM of the blood vessel. Although many protein coding genes and miRNAs are linked to hypertension pathophysiology [121, 122], not many lncRNAs were reported regulating the progression of the disease, at least not to this juncture. Conversely, *GAS5's* involvement in hypertension, as well as vascular remodeling, atherosclerosis and other vascular pathologies is well established. Wang et al., showed that *GAS5* expression was repressed in spontaneously hypertensive rats (SHR), a genetic hypertension model, and in plasma of human hypertension patients. In the same study, it has been shown that *GAS5* is involved in hypertension mediated microvascular dysfunction including capillary degeneration and leakage [53]. Moreover, in *GAS5* knockdown SHRs, higher systolic, diastolic and mean arterial blood pressure was observed. In same animals, medial thickness and luminal diameter was affected in various vessels, suggesting that *GAS5* knockdown affected hypertension induced arterial remodeling [53] and worsened the overall hypertensive phenotype [53]. In a different vascular disease context, *GAS5* may have a role in progression of atherosclerosis, indicated by increased *GAS5* levels in atherosclerotic plaques [123]. In a very recent study, it was suggested that *GAS5* is carried as a cargo during macrophage-EC crosstalk via exosomes upon atherosclerotic stimuli and mediates apoptosis of both cell types [124]. In an unrelated study, *GAS5* was induced in varicose great saphenous veins (GSV) compared to control veins [55] revealing multifaceted role of *GAS5* in vessel function and homeostasis.

In a more directed approach, Leung et al. studied gene networks that are regulated by Angiotensin II (Ang II), mediator of hypertension, in rat vSMCs [125]. Out of 24 novel lncRNAs that are differentially regulated by Ang II stimulation, *Lnc-Ang362* (*MIR222HG*) was further investigated. *Lnc-Ang362* is host gene for miR-221 and miR-222, that are associated with vSMC proliferation, neointimal hyperplasia, Ang II induced-EC migration, and inflammation [23, 126]. *Lnc-Ang362* controlled cell proliferation in vSMCs by regulating expression of Mcm7 [125], a key component of DNA replication complex during cell cycle [127], likely through miR-221 and miR-222 function. On the other hand, whether *Lnc-Ang362* has a standalone function during disease progression is still a mystery.

Multiple GWAS studies and quantitative trait locus (QTL) analysis performed in inbred Dahl salt-sensitive (S) and *S. Lewis* (LEW) congenic rats revealed an association of hypertension and short QT intervals to ~42.5 kb highly conserved homologous locus found at human chromosome 17 and rat chromosome 10 [128–130]. In rats, the susceptibility locus contains a non-variant protein coding gene rififylin (*rffl*) and a lncRNA, *rffl-lnc1,* exhibiting a 19 bp polymorphism in Dahl S and S.LEW congenic rats. Targeted CRISPR/Cas9 approaches revealed that loss of *rffl-lnc1* caused short QT-intervals and elevated blood pressure [131]. Furthermore, a 19 bp knock-in S.LEW congenic rats with restored deletion polymorphism rescued the aberrant QT-interval and blood pressure phenotype [131]. However, the molecular mechanism of *rffl-lnc1* and the effects of reported polymorphism on disease pathology remains to be determined.

In a recently study, Lin et al. compared circulating lncRNA profiles between hypertensive patients and healthy controls to study hypertension-associated lncRNAs [132]. *LncRNA-AK098656* was one of the most significantly upregulated lncRNAs in plasma of hypertensive patients. Similarly, ectopic overexpression of human *AK098656* in rats caused narrowed resistance artery and development of spontaneous hypertension. Gain- and loss-of-function studies in HASMCs revealed that *AK098656* promotes vSMC synthetic phenotype by inducing cell proliferation and migration [132]. At molecular level, *AK098656* binds to MYH11 (myosin heavy chain-11) and FN1 (fibronectin-1) proteins to promote their degradation partly in lysosome mediated degradation pathway [132] however direct correlation of this function to hypertensive pathophysiology still needs to be studied.

Other vessel diseases are also associated with lncRNA function. For example, in a microarray screen performed on serum of thoracoabdominal aortic aneurysm patients (TAA), *HIF1A-AS1* was identified as the most differentially expressed lncRNA compared to serum of healthy individuals [133]. Increased apoptosis of vSMCs in aortic wall is major etiology of TAA progression [134]. Consistent with this, in-vitro knockdown of *HIF1A-AS1* promoted cell proliferation and inhibited apoptosis in vSMCs [133, 135] however, the molecular mechanism behind *HIF1A-AS1* regulating apoptosis and mediating TAA pathophysiology remain to be studied.

5. Therapeutic applications of lncRNAs

Given the important role of lncRNAs in vascular injury and vessel regeneration, lncRNA therapeutics can provide novel interventions in vascular disease context. There are several potential advantages and disadvantages to use lncRNAs in therapy. On the positive side, first, since lncRNA itself is the functional molecule, targeting lncRNAs as an intervention is likely more straightforward than intervening with a protein to affect its function as that protein may elicit additional other functions. Secondly, as explained earlier, many lncRNAs exhibit tissue or disease specific expression pattern associated with different pathologies. Due to this specificity, lncRNAs have great potential by reducing possible side effects of systemic interventions; however, therapy still relies on the ability to efficiently target the lncRNA in a define tissue efficiently, a problem associated with much of the vascular system. Many lncRNAs are also functional in tissues and cellular processes in addition to vascular setting, thus compromising selectivity of a given therapeutic. For example, although *H19* function is associated with CVD, it also has fundamental function to regulate cancer initiation, progression and metastasis [136]. Therefore, before using lncRNAs in therapy, their expression and function should ideally be fully and carefully dissected to prevent undesired side-effects.

LncRNAs offer potential to diagnose and treat vascular diseases, however there are still many issues that restrict use of lncRNAs in clinical setting. First and foremost, most lncRNAs are not evolutionarily conserved between human and existing animal models, making identification and testing human lncRNAs in clinical setting is challenging in many cases – a clear engagement with regulators will be important for preclinical pathways to trial to have clarity. Second, delivery of lncRNA therapeutics are far from accurate. Optimal delivery route, tissue specific and subcellular compartment directed delivery are still needs to be optimized. Next, little is yet known about molecular kinetics of any lncRNA function. For efficient targeting, duration of treatment and dosage adjustment, toxicity and pharmacokinetic analysis are needed for each lncRNA.

There are several different lncRNA targeting approaches that can potentially be used in therapy. Most methods are based on therapeutic silencing of lncRNAs, using RNAi, antisense oligo based aptamers (ASOs) and GapmeRs. While RNAis are widely used to knock-down cytoplasmic RNAs, GapmeRs are more suitable for nuclear lncRNAs. Although there are several human and non-human primate clinical trials utilizing RNAis [137, 138], their efficacy and safety are still being tested. Future loss-of-function approaches may also include employing small molecules that would interfere possible RNA-protein interaction or conformation, interfering with the lncRNA function [139]. Overexpression of lncRNAs that have cardio- or athero-protective roles as therapeutic intervention is much more complicated. Because of their length, tissue specific delivery through cell membrane and potential toxicity is the limiting step. Although adeno associated virus (AAV) delivery approaches are very promising with miRNAs and protein coding transcripts [140–143], efficacy of lncRNA delivery is still needs to be tested. However, recently, first RNA drugs gain FDA approval (Spinraza for spinal muscular atrophy treatment [144] and Mipomersen for treatment of hypercholesterolemia [145]) and many more progress to phase 3 trials. Undeniably, with advances in RNA therapeutics, the potential to use RNA drugs as novel medicines for cardiovascular diseases is not too far-fetched.

Acknowledgements

A.H·B is funded by the British Heart Foundation BHF one CH/11/2/28733Chair of Translational Cardiovascular Sciences and the European Research Council (VASCMIR)338991.

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