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LncRNAs in vascular biology and disease

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ABSTRACT

Accumulating studies indicate that long non-coding RNAs (lncRNAs) play important roles in the regulation of diverse biological processes involved in homeostatic control of the vessel wall in health and disease. However, our knowledge of the mechanisms by which lncRNAs control gene expression and cell signaling pathways is still nascent. Furthermore, only a handful of lncRNAs has been functionally evaluated in response to pathophysiological stimuli or in vascular disease states. For example, lncRNAs may regulate endothelial dysfunction by modulating endothelial cell proliferation (e.g. *MALAT1, H19*) or angiogenesis (e.g. *MEG3, MANTIS*). LncRNAs have also been implicated in modulating vascular smooth muscle cell (VSMC) phenotypes or vascular remodeling (e.g. *ANRIL, SMILR, SENCR, MYOSLID*). Finally, emerging studies have implicated lncRNAs in leukocytes activation (e.g. *lincRNA-Cox2, linc00305, THRIL*), macrophage polarization (e.g. *GAS5*), and cholesterol metabolism (e.g. LeXis). This review summarizes recent findings on the expression, mechanism, and function of lncRNAs implicated in a range of vascular disease states from mice to human subjects. An improved understanding of lncRNAs in vascular disease may provide new pathophysiological insights and opportunities for the generation of a new class of RNA-based biomarkers and therapeutic targets.

1. Introduction

Impaired vascular remodeling contributes to a wide variety of cardiovascular disease states including atherosclerosis, percutaneous coronary or peripheral interventions, vein graft disease, organ transplantation, among others. Accumulating studies have identified cell- or stage-specific pathophysiological mechanisms in the macro- and microvasculature that may underlie susceptibility to vascular disease. For example, beside its implication in "classical" systemic chronic inflammatory diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus or gout, vascular inflammation contributes also to progression of more complex diseases such as atherosclerosis [1], diabetes [2] or cancer [3]. Although the role of inflammation in atherosclerosis has been identified over 150 years ago by Virchow [4], only recently has the "inflammation hypothesis" in atherosclerosis been specifically tested using an anti-inflammatory drug targeting IL-1β (canakinumab), whereby recurrent cardiovascular events were reduced in the canakinumab treatment group independent of changes in lipid levels [5]. However, major mechanistic gaps in the understanding of regulatory pathways involved in homeostasis of the vessel wall in response to pathophysiological stimuli remain and contribute to the lack of targeted therapeutics in a range of vascular disease states.

The recent recognition that only about 1.5% of the human genome encodes proteins, has opened new opportunities to better understand regulatory pathways in vascular health and disease [6,7]. Interestingly, a large quantity of the genome is transcribed at some point during development [8]. Postnatally, the majority of biologically active RNAs that cannot be translated into proteins are long non-coding RNAs (lncRNAs) measuring > 200 nucleotides in length and display mRNA-like characteristics such as being 5′-capped, spliced, and polyadenylated. In contrast to microRNAs, which bind to the 3′-UTR of target genes to mediate translational repression thereby altering the biology of diverse disease states [9–12], lncRNAs have emerged as powerful biological regulators by modulating numerous cellular processes, according to their cellular localization, in the nucleus or the cytoplasm (Fig. 1). For example, lncRNAs localized in the nucleus can regulate transcription by guiding or sequestering transcription factors (TF) [13], inducing histone modifications, guiding chromatin remodeling complexes to the correct chromosomal locations [14], or acting as enhancer RNAs [15]. Other studies demonstrated that lncRNAs may regulate nucleocytoplasmic shuttling of TFs such as nuclear factor of activated T cells (NFAT) [16] or alternative splicing of pre-mRNAs [17]. In the cytoplasm, lncRNAs can regulate mRNA stability and control translational events [18], sponge miRNAs [19], and act as a scaffold for proteins complexes [20]. Further regulatory functions may include stabilization of ribonucleoprotein (RNP) complexes

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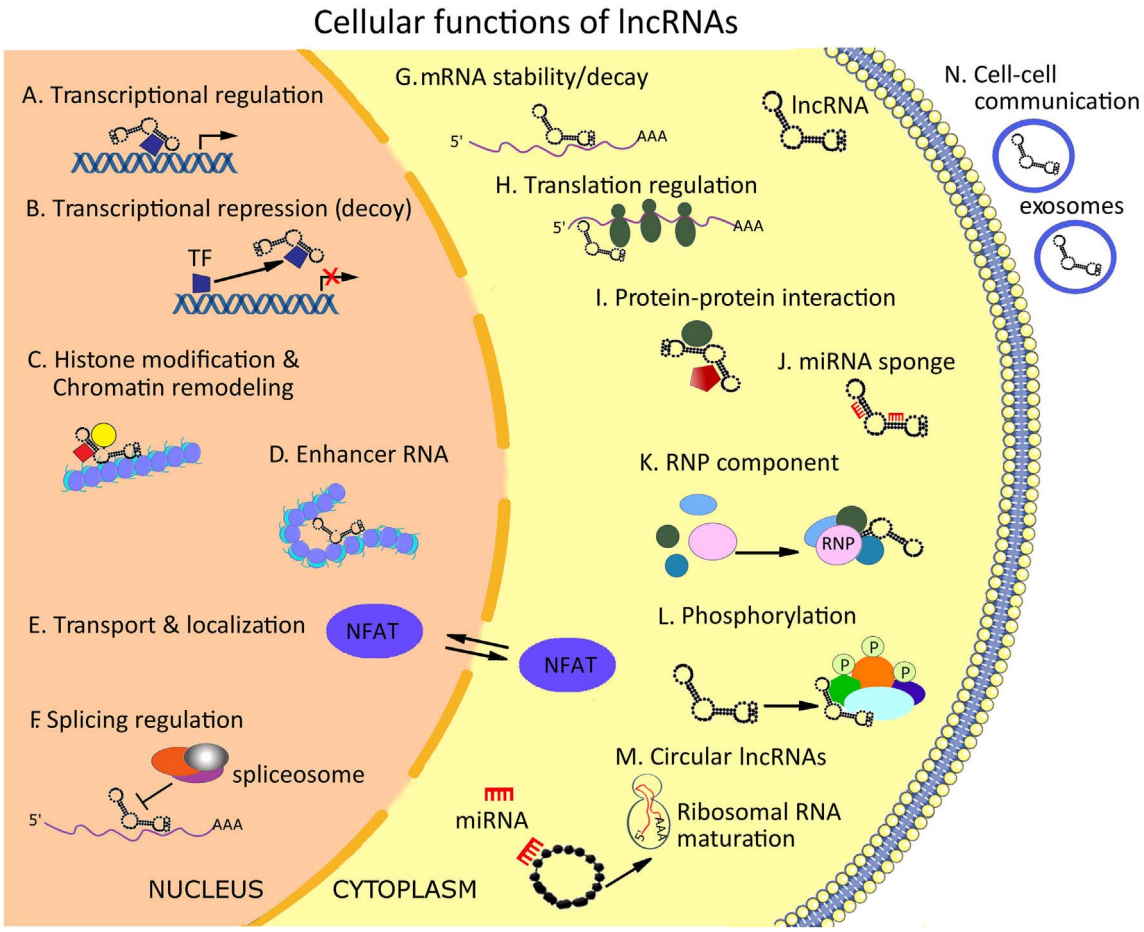

Fig. 1. Cellular functions of long non-coding RNAs (lncRNAs).
LncRNAs regulate gene expression by multiple mechanisms. Nuclear-localized lncRNA can guide transcription factors (TF) or protein complexes to specific sites in the genome (A) or sequester the TF and repress their function (B). They can induce histone modifications and guide chromatin remodeling complexes to the correct chromosomal locations (C) or induce chromosomal looping to increase association between enhancer and promoter regions (D). LncRNAs can regulate nucleocytoplasmic shuttling (E) of nuclear factor of activated T cells (NFAT) or alternative splicing of pre-mRNAs (F). In the cytoplasm, lncRNAs can regulate mRNA stability (G) and control translational events (H), sponge miRNAs (J) and act as a scaffold for proteins complexes (I). Further regulatory functions may include stabilization of ribonucleoprotein (RNP) complexes (K) or protein phosphorylation and activation of signaling pathways (L); Circular lncRNAs are formed by RNA splicing and were observed to act as miRNA sponges, or regulate the maturation of ribosomal RNAs (M). Finally, some lncRNAs are released in exosomes or microvesicles, potentially facilitating cell-to-cell communication (N).

[21] or protein phosphorylation and activation of signaling pathways [22]. In addition, lncRNAs can be circularized by RNA splicing (circular lncRNAs) and act as miRNA sponges [23], or regulate the maturation of ribosomal RNAs [24]. Finally, some lncRNAs are released in exosomes or microvesicles, potentially facilitating cell-to-cell communication [25,26]. However, the role of lncRNAs in vascular biology and disease remains poorly understood [27–29]. This review summarizes examples of lncRNAs and their regulatory effects on diverse biological processes important to the macro- and microvasculature in health and disease (Fig. 2).

2. LncRNAs and endothelial dysfunction

Impaired endothelial function has been linked to a variety of acute and chronic inflammatory disease states. For example, in response to both biochemical (e.g. IL-1β, modified-LDL) and biomechanical (e.g. disturbed blood flow) stimuli, endothelial activation is among the earliest processes involved in atherosclerotic lesion initiation [30]. Consequently, expression of adhesion molecules (e.g. VCAM-1, *E*-Selectin) and secretion of chemokines (e.g. MCP-1, fractalkine) facilitates the recruitment of leukocyte subsets into the vessel wall [31]. Chronic endothelial dysfunction may lead to loss of endothelial integrity predisposing to vascular inflammation and atherosclerosis [32]. Accumulating studies highlight an emerging role for lncRNAs in regulating endothelial dysfunction (Table 1).

Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is a lncRNA highly expressed in endothelial cells in both the macro- and microvasculature. It has been shown to regulate endothelial cells (ECs) inflammation, angiogenesis, and response to oxidative stress [33–35]. *MALAT1* knockdown decreases ECs proliferation by inhibiting cell cycle progression, decreasing the number of cells in S-phase under basal or hypoxic conditions and after vascular endothelial growth factor (VEGF) stimulation. *MALAT1* silencing reduced the S-phase cyclins CCNB1, CCNB2, and CCNA2, while increasing the cell cycle inhibitory genes p21 and p27Kip1 [33]. In contrast, *MALAT1* overexpression increases the retinal EC proliferation rate [34] and inhibits apoptosis induced by oxygen-glucose deprivation and reoxygenation in human brain microvascular endothelial cells [36]. *MALAT1* silencing also decreased phosphorylated p38 levels in retinal ECs and the glucose-induced upregulation of IL-6 and TNFα through activation of SAA3 in ECs [35]. In a different study, following oxygen-glucose deprivation and reoxygenation in brain microvascular ECs, lentiviral knockdown of MALAT1 decreased PI3K activities and the activation of Akt phosphorylation, and increased cell apoptosis and caspase 3 activity, suggesting a potential role of *MALAT1* in cerebral ischemia/reperfusion [36]. Consistent with these in vitro observations, pharmacological inhibition of *MALAT1* in mice undergoing hindlimb ischemia reduced blood flow recovery and capillary density, verifying studies that *MALAT1* controls

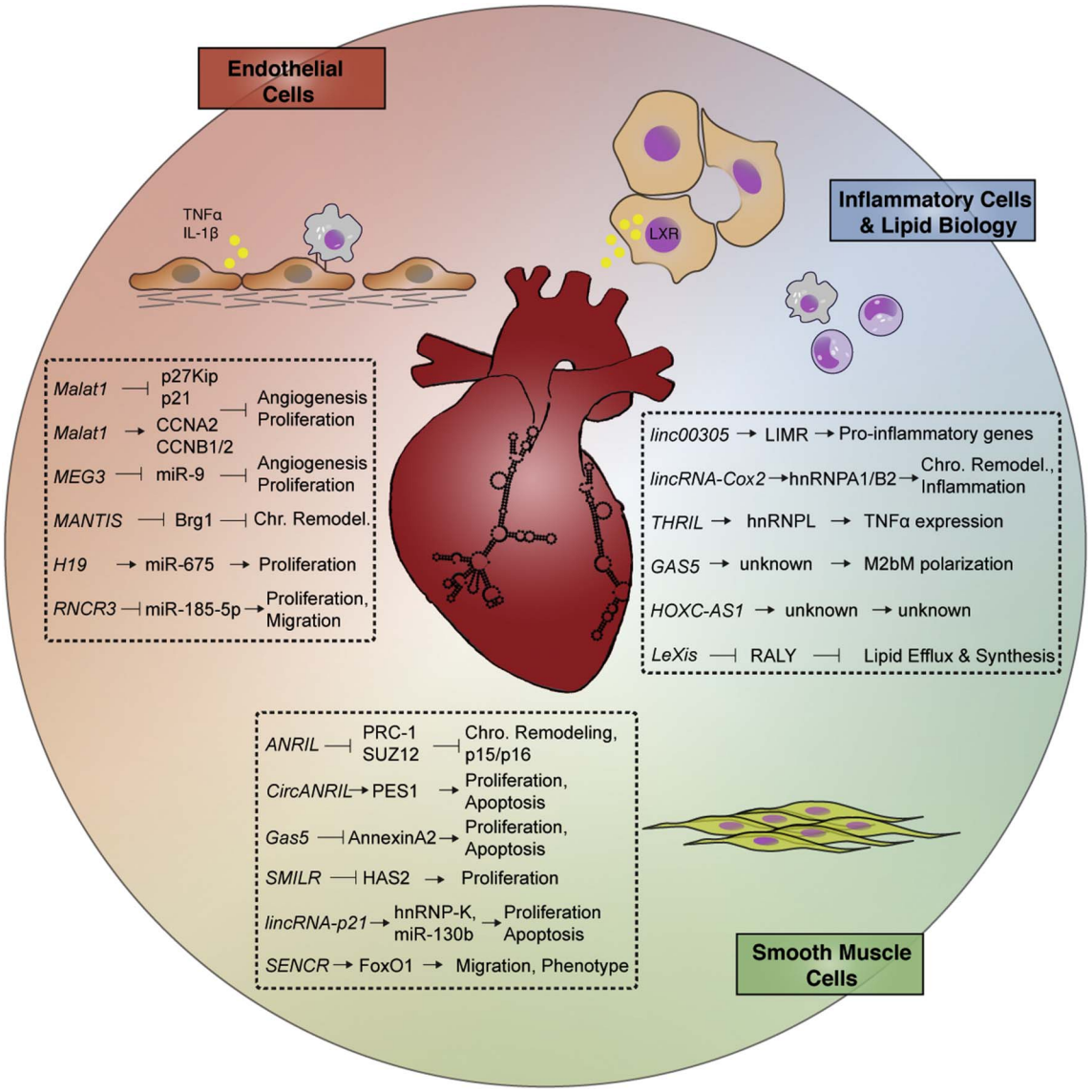

Fig. 2. LncRNAs implicated in vascular disease. Highlighted lncRNAs involved in endothelial cell biology, vascular smooth muscle cell proliferation, leukocyte inflammation, and lipid metabolism.

EC proliferation and vessel outgrowth in vitro and in vivo [33]. MALAT1 also protects the endothelium against ox-LDL-induced dysfunction via upregulating the expression of the miR-22-3p target genes CXCR2 and AKT, hence acting as a miRNA sponge [37]. In streptozotocin-induced diabetic rats, intraocular injection of *MALAT1* shRNA alleviated vascular leakage induced by hyperglycemia, decreased the number of apoptotic retinal cells, and significantly reduced retinal inflammation [34]. Phenotypically, MALAT1-KO mice showed a delayed vessel extension in the retina and a reduction of the vessel density when compared with wild-type littermates while the number of proliferating ECs was significantly reduced [33]. While the molecular mechanisms mediating *MALAT1's* angiogenic effects has not been clarified, a recent study has indicated that MALAT1 may serve as a potential endogenous sponge for miR-26b, regulating EC autophagy and survival [38]. Because the related family member miR-26a harbors a very similar seed sequence to miR-26b, a known anti-angiogenic miRNA in diabetic wound healing and post-MI repair [39,40], future studies will be of interest to assess angiogenic regulation by the *MALAT1-miRNA-26a axis*. Collectively, these studies highlight an important role for MALAT1 in regulating EC homeostasis, angiogenesis, and vascular inflammation.

Maternally expressed gene 3 (*MEG3*) is a lncRNA that regulates angiogenesis and diabetes-related microvascular dysfunction [41]. *MEG3* expression was decreased in retinal ECs upon oxidative stress and high glucose stimulation in vitro and in the retinas of STZ-induced diabetic mice in vivo. *MEG3* silencing in vivo exacerbates retinal vessel dysfunction, as observed by increased microvascular leakage, severe capillary degeneration, and inflammation. In retinal ECs *MEG3* knockdown increased their proliferation, migration, and tube formation capacity by activating the PI3K/Akt signaling pathway. Mechanistically, *MEG3* acts as a miRNA sponge in vascular ECs by negatively regulating miR-9, a key player in angiogenesis and proliferation [42]. Alternatively, *MEG3* expression is induced in senescent human ECs and *MEG3* knockdown rescued the age-induced impairment of angiogenesis. Moreover, in mice undergoing hindlimb ischemia, *MEG3* inhibition augmented blood flow recovery [43]. Finally, Meg3-KO mice showed increased expression of genes regulated by VEGF. Indeed, the Meg3-null embryos showed increased cortical microvessel density, suggesting the important role of *MEG3* in angiogenesis and vascularization [44].

MANTIS (lncRNA n342419) is a lncRNA initially observed at low levels in patients with idiopathic pulmonary arterial hypertension (IPAH) and in a rat PAH disease model. In contrast, it was induced in ECs isolated from human glioblastoma patients as well as in carotid

Table 1
List of lncRNAs potentially implicated in endothelial dysfunction and their regulatory mechanisms. ECs: endothelial cells; ESCs: embryonic stem cells; EPCs: endothelial progenitor cells; PBMCs: peripheral blood mononuclear cells; VSMCs: vascular smooth muscle cells.

lncRNA	Target cell type	Regulatory effect	Mechanism	Reference
ALT1	ECs	Controls ECs cell cycle and proliferation	Targeting ACE2 and Cyclin D1	[54]
ASncmtRNA-2	ECs	Induced in vascular aging and senescence	Potentially non-canonical precursor of hsa-miR-4485 and hsa-miR-1973	[55]
cANRIL	VSMCs and PBMCs	Atheroprotection; induces vascular cell apoptosis	Not investigated	[56]
FLJ11812	ECs	Regulates autophagy	Binding to miR-4459 and targeting ATG13	[57]
H19	ECs; VSMCs	Increases proliferation and decreases apoptosis; regulated in hypoxia	Host gene for miR-675, targeting PTEN; activates p38-MAPK and NF-kB signaling pathways	[46–50]
HIF1A-AS2	ECs	Promotes angiogenesis in hypoxia conditions	Sponging miR-153-3p	[58]
HOTAIR	ECs; PBMCs	Decreased in ECs from athero plaques; regulates ECs proliferation and migration	TSLP activates HOTAIR transcription through PI3K/AKT-IRF1 pathway	[59]
HOTTIP	ECs	Regulates ECs proliferation and migration	Wnt/β-catenin pathway	[60]
IGF2-AS	ECs	Increased in myocardial microvascular endothelial cells of diabetes rat model; controls angiogenesis	Not investigated	[61]
LINC00341	ECs	Anti-inflammatory effects	LINC00341 guides EZH-2 to the promoter region of VCAM1	[15]
LINC00305	ECs	Regulates hypoxia-induced apoptosis	Sponging of miR-136	[19]
LOC100129973	ECs	suppression of apoptosis	sponging 4707-5p and miR-4767	[62]
MALAT1	ECs	Controls ECs proliferation and cell cycle; inhibits apoptosis; protects the endothelium against ox-LDL-induced dysfunction; controls vascular homeostasis in diabetic rats and mice undergoing hindlimb ischemia	Sponge for miR-22-3p; controls p38 and AKT phosphorylation and signaling pathways	[33–40]
MANTIS	ECs	Controls ECs migration, angiogenic sprouting, and tube formation	Interacts with Brg1 and regulates SMAD6, COUP-*TFII*, SOX18	[45]
MEG3	ECs	Controls vascularization and angiogenesis, EC proliferation, and senescence	Sponge for miR-9; activates the PI3K/AKT signaling pathway	[41–44]
MIAT	ECs	Regulates angiogenesis and EC function in diabetes	ceRNA for miR-150-5p	[63]
PINC	ECs	EC apoptosis; Kawasaki disease	Not investigated	[64]
RNCR3	ECs	Atherosclerosis; EC proliferation and migration; due to RNCR3 exonic overlap with miR-3078 and miR-124a, it is unclear if phenotypes are related to RNCR3 or regulation by these microRNAs	ceRNA for miR-185-5p, forming a feedback loop with KLF2;	[51]
SENCR	ESCs	CAD; ECs proliferation, migration and angiogenesis	SENCR regulates ESC differentiation into EC	[65]
SIRT1-AS	EPCs	EPCs senescence, proliferation, and migration	Sponge for mir-22, (relieving miR-22-induced SIRT1 (downregulation)	[66]
TGFB2-OT1	ECs	Regulates autophagy and inflammation	ceRNA for Mir3960, Mir4488 and Mir4459	[67]
TUG1	ECs	ECs apoptosis, atherosclerosis	Potentially a sponge for miR-26a	[68]

arteries of *Macaca fascicularis* subjected to atherosclerosis regression diet [45]. MANTIS is localized to the nucleus and its expression is controlled by the histone demethylase JARID1B, suggesting a chromatin regulatory function. Functional silencing of *MANTIS* by oligonucleotide delivery (siRNAs or GapmeRs) or CRISPR/Cas9-mediated deletion inhibited EC migration, angiogenic sprouting, and tube formation in vitro and in vivo in mice injected with matrigel-embedded human umbilical vein endothelial cells (HUVECs). Mechanistically, *MANTIS* also interacts with Brg1 and regulates SMAD6, COUP-*TFII*, and SOX18, which are all implicated in angiogenesis modulation.

Polymorphisms in the *lncRNA H19* was originally associated with CAD risk in a Chinese population [46]. Recent studies have shown that plasma levels of *H19* can be an independent predictor for CAD [47]. Expression of *H19* was significantly increased in the plasma of patients with atherosclerosis compared to healthy volunteers, and was also expressed higher in atherosclerotic plaques *of ApoE−/−* mice [48]. Overexpression of *H19* in HUVECs increased their proliferation while decreasing apoptosis by regulating p38-MAPK and NF-κB signaling pathways [48]. In contrast, inhibition of *H19,* a hypoxia-regulated lncRNA, decreased HUVEC growth, inducing their accumulation in G1 phase of the cell cycle [49]. Recently, lncRNA *H19* was observed to play a role in arterial restenosis, as it is overexpressed in the neointima of balloon-injured arteries [50]. As *H19* is a host gene for miR-675, gain-of-function studies have revealed that overexpression of H19 increases vascular smooth muscle cell (VSMC) proliferation rate by targeting PTEN in a miR-675-dependent manner [50]. Future knockdown studies in atherosclerotic-prone disease models will be important to verify the therapeutic potential of *lncRNA H19*.

Retinal non-coding RNA3 (*RNCR3*), also known as *LINC00599*, is a lncRNA recently implicated to play a role in both atherosclerosis and diabetes mellitus [51–53]. Increased levels of *RNCR3* were observed in human and mouse aortic atherosclerotic lesions. *RNCR3* knockdown in ApoE−/− mice accelerated the development of atherosclerosis, increased LDL plasma levels, and in turn regulated the inflammatory response. *RNCR3* knockdown also reduced the proliferation and migration and accelerated apoptosis of ECs and VSMCs in vitro, suggesting that *RNCR3* inhibition might impair EC regeneration in injured arteries. Mechanistically, *RNCR3* was suggested to function as a competing endogenous RNA (ceRNA) by decreasing the concentration of miR-185-5p, ultimately resulting in the de-repression of Kruppel-like factor 2 (KLF2), a transcriptional factor conferring an endothelial vasoprotective phenotype. This regulatory mechanism was observed by the same group in two different studies on retinal vascular dysfunction in diabetes mellitus [52,53]. However, in all the above studies, an important interpretation issue will still need to be resolved. Because two of the four isoforms of *RNCR3* have exonic overlap with microRNA-3078 and microRNA-124a-1, it is not clear if the observed phenotypes after *RNCR3* knockdown can be attributed to these miRNAs or to *RNCR3* alone. Future studies will need to definitively establish whether the above described phenotypes are indeed independent of miR-3078 and miR-124.

3. LncRNAs and vascular injury

Maladaptive vascular remodeling contributes to a wide range of cardiovascular procedures including percutaneous coronary or

Table 2
List of lncRNAs potentially implicated in vascular injury and their regulatory mechanisms. VSMCs: vascular smooth muscle cells; HSVSMCs: human saphenous vein smooth muscle cells; PASMC: pulmonary aortic smooth muscle cells; CASMCs: coronary artery smooth muscle cells; HAVSMCs: human aortic vascular smooth muscle cells.

lncRNA	Target cell type	Regulatory effect	Mechanism	Reference
ANRIL	HAVSMCs	Independent risk factor for CAD; controls VSMC proliferation	Binds to CBX7 and SUZ12, components of the PRC-1 and PRC-2 respectively	[20,76,81–84]
cANRIL	VSMCs; macrophages	Atheroprotection; impaires ribosome biogenesis; inhibits proliferation and induces apoptosis	Binds to PES1, a 60S–preribosomal assembly factor and induces p53 activation and nucleolar stress	[24]
Gas5	VSMC	Regulates vascular remodeling in hypertension; accelerates VSMC proliferation and migration	Regulates AnnexinA2; decreases miR-21 and increases one of its targets, PTEN	[92–96]
HAS2-AS1	HASMC	SMC homeostasis	Altering the chromatin structure around the HAS2 proximal promoter via O-GlcNAcylation and acetylation	[99]
HIF1A-AS2	VSMC	Thoracic aortic aneurysms; controls proliferation and apoptosis	Interaction with BRG-1	[100]
HOTAIR	VSMC	Downregulated in STAA (sporadic thoracic aortic aneurysm);	Regulates extracellular matrix remodeling	[101]
HypERLinc	Pericytes	Role in idiopathic pulmonary arterial hypertension; heart failure	ER stress regulator	[102]
Linc-p21	VSMC, macrophages and HUVECs	Promote apoptosis and repress proliferation	Associates with hnRNP-K to repress hundreds of genes in the p53 pathway; feedback mechanism: association with MDM2 to depress p53; endogenous sponge for miR-130b; binds to RelA mRNA regulating NFκB	[85–88]
Lnc-Ang362	VSMCs	VSMC proliferation	Host transcript for miR-221 and miR-222,	[103]
LnRPT	PASMCs	PASMC proliferation	Inhibits the genes Notch3, Jag1, CCNA2	[104]
MEG3	PASMCs	Regulates PASMCs cell cycle, proliferation and migration	Regulates p53 pathway	[105]
MYOSLID	CASMCs	Promotes VSMC differentiation and inhibits proliferation; actin stress fiber formation	Abrogates TGF-β1–induced SMAD2 phosphorylation; modulate nuclear translocation of MKL1	[22]
SENCR	VSMCs	Increases proliferation, inhibits migration	Decreases FoxO1 and its binding to H3 histone; regulates myocardin	[65,89–91]
SMILR	HSVSMCs	Regulates proliferation; decreased in athero plaques	Decreases the expression of proximal gene HAS2	[98],
TCONS_34812	PASMC	Proliferation and apoptosis	Increase the expression of TF Stox1	[106]
TUG1	VSMCs	VSMC homeostasis	TUG1 supports the interaction of EZH2 and α-actin, and their co-localization	[107]
XR007793	VSMCs	Hypertension, VSMC proliferation and migration	STAT2, LMO2, IRF7	[108]

peripheral interventions, vein grafts for coronary artery bypass surgery, fistulas for dialysis access, and organ transplantation. In response to mechanical injury often initiated by endothelial denudation or injury, endothelial cells, platelets, and leukocytes release a variety of growth factors (e.g. PDGF-BB, TGF-β1), cytokines (e.g. IL-1, IL-6, and IL-8), chemokines (e.g. MCP-1), metalloproteinases (e.g. MMP-9), and prothrombotic mediators (e.g. thrombin) that cooperatively induce the proliferation of vascular smooth muscle cells of the medial layer to form a significant component of the neointima of the damaged vessel wall. Negative medial and adventitial remodeling may result in late lumen loss, restenosis, or complete occlusion of the vessel wall [69,70,71]. VSMCs also play a prominent role in chronic inflammatory disease states such as atherosclerosis, hypertension, aneurysm formation, and pulmonary artery hypertension. Genetic lineage tracing studies in atherosclerotic models have implicated that VSMCs undergo phenotypic switching to cells that exhibit macrophage-like features with loss of VSMC marker identity [72]. Accumulating studies have implicated a growing list of lncRNAs in vascular smooth muscle cell biology, providing potential new levels of functional regulation, mechanistic insights, and targets for therapy in a range of conditions (Table 2).

Genome-wide association studies (GWAS) have identified that the INK4 locus on chromosome 9p21.3 harbors multiple single nucleotide polymorphisms (SNPs) linked to coronary artery disease (CAD) susceptibility [73,74,75], atherosclerosis [76], aortic aneurysm [77], ischemic stroke [75], type II diabetes [78], and specific cancer subtypes [79,80]. Antisense to this locus lies the lncRNA *ANRIL* (antisense ncRNA in the INK4 locus). The increased risk of SNPs associated with CAD is independent of all known CAD risk factors, suggesting that *ANRIL* may regulate a different biological pathway relevant for atherosclerosis [76]. Two *ANRIL* transcripts (EU741058 and NR_003529) were found significantly increased in peripheral blood mononuclear cells and human atherosclerotic plaque tissues from CAD patients as compared to healthy subjects, while the most abundant isoform, DQ485454, remained unaffected [81]. Consistent with this, loss-of-function studies with siRNAs targeting different exons (exon 1/19) in human SMCs induced different regulatory effects, suggesting that different splicing variants of *ANRIL* might play distinct roles in cell physiology [82]. Mechanistically, Yap et al. showed in chromatin fractions that *ANRIL* directly binds to CBX7 and SUZ12, components of the polycomb repression complex-1 (PRC-1) and PRC-2, respectively [20,83]. Inhibition of *ANRIL* disrupts the binding of PRC-1 and PRC-2 at the INK4 locus, increasing the mRNA levels of $p15^{INK4b}$ and $p16^{INK4a}$, two of the genes encoded by the INK4 locus and limiting cellular life span [20,83,84]. Adding another layer of complexity, a recent study revealed that ANRIL circularization, resulting from exon skipping events during RNA splicing, activates a different cellular mechanism conferring atheroprotection [24]. CircANRIL binds to PES1, an essential 60S-preribosomal assembly factor, impairing pre-rRNA processing and ribosome biogenesis in macrophages and VSMCs. As a consequence, circANRIL induces p53 activation and nucleolar stress, resulting in the inhibition of proliferation and induction of apoptosis. Although ANRIL is an independent risk factor for CAD, future studies will be informative to assess the functionality of ANRIL in relevant disease models.

Reduced levels of *lincRNA-p21* were observed in PBMCs and artery tissues of patients with CAD and in the aortic plaques of ApoE−/− mice, as compared to artery tissues of control patients and in C57BL/6 control mice, respectively, thus suggesting a potential role of this lncRNA in disease development [85]. Recently, the G-A-A-G haplotype of *lincRNA-p21* was found to be associated with a decreased risk of CAD and MI, particularly among premature CAD/MI in the Chinese Han population [86]. In vitro studies in VSMCs and mouse mononuclear macrophage cells showed that *lincRNA-p21* induces apoptosis and represses cell proliferation [85]. Moreover, in the same study in vivo inhibition of *lincRNA-p21* resulted in enhanced neointimal hyperplasia in response to carotid artery injury in mice. Mechanistically, *lincRNA-p21* expression is regulated by p53, and it physically associates with hnRNP-K to repress hundreds of genes in the p53 pathway [85]. Transcriptomic analysis revealed that *lincRNA-p21* inhibition deregulated many p53 targets. *LincRNA-p21* also binds to mouse double minute 2 (MDM2), an E3 ubiquitin-protein ligase that represses p53 in physiological conditions. The association of *lincRNA-p21* with MDM2 de-represses p53, enabling p53 to interact with p300 and to bind to the promoters/enhancers of its target genes, hence participating in a feedback mechanism [85]. In addition, *lincRNA-p21* promoted cell apoptosis and induced cell cycle progression by acting as an endogenous sponge for miR-130b in vascular endothelial cells [87]. *LincRNA-p21* has also been implicated in chronic vascular inflammation in patients with rheumatoid arthritis (RA) who expressed lower levels of *lincRNA-p21* and increased levels of phosphorylated p65 (RelA), a marker of NF-κB activation. In contrast, patients treated with methotrexate (MTX) had higher levels of lincRNA-p21 [88]. Mechanistically, MTX reduced NF-κB activity in TNFα–treated macrophages through a DNA-dependent protein kinase catalytic subunit (DNA PKcs)–dependent mechanism via induction of *lincRNA-p21*. Finally, *lincRNA-p21* can physically bind to RelA mRNA, thus regulating its translation and assembly in the NF-κB complex [88]. Taken together, current data highlight that *lincRNA-p21 may serve* as a potential therapeutic target for vascular injury, atherosclerosis, and potentially other inflammatory diseases such as RA.

Smooth muscle and endothelial cell–enriched migration/differentiation-associated long noncoding RNA (*SENCR)* is a lncRNA transcribed antisense from the 5′ end of the *FLI1* gene and exists as 2 splice variants, localized predominantly in the cytoplasm [89]. SENCR knockdown decreased expression of myocardin (MYOCD), a master regulator of numerous smooth muscle contractile genes, whereas several pro-migratory genes were increased. Loss-of-function studies indicated *SENCR* as an inhibitor of VSMC migration. SENCR-knockdown in human coronary aortic smooth muscle cells (HCASMCs) exhibited reorganization of the actin cytoskeleton with formation of lamellipodia, suggesting a role for SENCR in the regulation of VSMC differentiation and cellular motility [89]. In a different study, Zou et al. have reported decreased levels of *SENCR* in a db/db mouse model and in VSMCs exposed to high glucose, through a mechanism involving FoxO1 regulation [90]. However, this latter study requires further clarity since no mouse homologue of SENCR has been identified and the authors did not offer any details on how they identified the mouse transcript of SENCR or the exact transcript sequence used for silencing or overexpression studies, for either human or mouse SMCs employed in the study. Further studies will be needed to verify the existence and characterize the mouse isoform of SENCR in order to interpret the results from the diabetic mouse models. Interestingly, in human subjects with type 2 diabetes the expression of *SENCR* in the plasma was directly associated with left ventricular (LV) mass to LV end-diastolic volume ratio, a marker of cardiac remodeling [91]. This suggests that *SENCR* may serve as an independent predictor of diastolic function and remodeling in patients with type 2 diabetes. Since the *FLI1* gene that overlaps *SENCR* is a regulator of endothelial development, the expression of *SENCR* is also markedly regulated during endothelial commitment [65]. Although *SENCR* does not control the pluripotency of pluripotent cells, its overexpression significantly potentiated early mesodermal and endothelial commitment, and induced HUVEC proliferation, migration, and angiogenesis [65]. Collectively, these findings suggest SENCR may serve as a master regulator of VSMC and EC differentiation with potential implications in diabetes.

Growth arrest–specific 5 (*GAS5*) is a lncRNA that plays important roles in several biological processes including apoptosis, cell proliferation, and differentiation, cell growth arrest [92,93] and it was recently observed to regulate vascular remodeling in hypertension [94]. Silencing of *GAS5* accelerated the microvascular dysfunction in a hypertension rat model, as shown by increased capillary leakage and retinal neovascularization. In vitro, *GAS5* knockdown regulates VSMC dedifferentiation, accelerating VSMC proliferation and migration, and

Table 3
List of lncRNAs regulating leukocyte activation and lipid metabolism and their regulatory mechanisms. BMDMs: bone marrow derived macrophages; PBMCs: peripheral blood mononuclear cells.

lncRNA	Target cell type	Regulatory effect	Mechanism	Reference
GAS5	BMDM, THP-1	High expression in human atherosclerotic plaques, macrophage M2bM polarization, apoptosis	NMD pathway, HMBG1, miR-222	[120–123]
HOXC-AS1	THP-1	Low expression in human atherosclerotic plaques; suppresses Ox-LDL-Induced cholesterol accumulation	Unknown	[121]
IL7-AS	THP-1, RAW264.7, A549	Involved in inflammatory response	Unknown	[129]
LeXis	Hepa1–6	Regulates cholesterol synthesis	Binds to RALY	[124,125] [126]
Linc00305	THP-1	SNP rs2850711 for atherosclerosis; promotes monocyte activation	Binds to LIMR, which activates NF-kB through Ahr signaling	[114]
lincRNA-Cox2	THP-1, dendritic cells, BMDM	Mediates immune response; Promotes inflammation in macrophages;	Co-activator of NF-kB by forming a Complex with hnRNPA2/B1	[115,116,118,130]
lncRNA OTTHUMT00000387022	PBMC, plasma, THP-1	Biomarker for CAD; pro-inflammatory in macrophages	Unknown	[131]
lincRNA-TNFAIP3	RAW264.7, BV2	Likely regulating inflammatory genes	Involved in NF-kB/HMGB1 pathway	[132]
PACER	U937	Controls COX-2 mRNA transcription and monocyte Activation by LPS	Chromatin remodeling; regulation of NF-kB through p50 Component, binds p300	[133]
THRIL	THP-1	Kawasaki disease; transcriptional control of TNFa	Forms complex with hnRNPL	[119].
lncLSTR	Primary hepatocytes	Regulates apoC2 expression through FXR-mediated pathway. Modulates triglyceride levels in a hyperlipidemia mouse model;	It forms a molecular complex with TDP-43 to regulate expression of Cyp8b1, a key enzyme in the bile acid synthesis	[127]
Gm16551	Primary hepatocytes	Upregulated by SREBP1c in hepatocytes; downregulated in livers of obese mice; suppresses lipogenesis.	Unknown	[128]

decreasing the expression of contractile marker proteins including α-smooth muscle actin and calponin by the β-catenin signaling pathway [94]. *GAS5* knockdown also reversed apoptosis in response to hypoxia stress and partially reversed the H_2O_2-induced reduction of VSMC and EC viability. Similar results were observed by a different group, where *GAS5* regulated the VSMC proliferation and migration through AnnexinA2, a Ca2+-dependent RNA-binding protein [95]. *GAS5* expression was also decreased in cardiac fibroblasts treated with TGF-β1 and in rat cardiac fibrosis. *GAS5* overexpression inhibited the cardiac fibroblast proliferation by decreasing the expression of miR-21 and indirectly regulating one of its targets, PTEN, suggesting the importance of *GAS5* in cardiac and vascular remodeling [96]. *GAS5* was also observed to regulate SMC differentiation from mesenchymal progenitor cells, by modulating the TGF-β/Smad3 signaling pathway [97]. Overexpression of *GAS5* reduced, while knockdown of GAS5 increased, the expression of SMC contractile markers. Mechanistically, *GAS5* binds competitively to the TGF-β effector Smad3 via multiple RNA Smad–-binding elements (rSBEs), which prevents Smad3 from binding to the SBE in the promoter regions of TGF-β–responsive genes, resulting in suppression of SMC marker gene transcription and, consequently, inhibition of TGF-β/Smad3-mediated SMC differentiation.

Myocardin-induced Smooth muscle LncRNA, Inducer of Differentiation (*MYOSLID*) is another lncRNA recently discovered to regulate SMC differentiation in a (MYOCD)/serum response factor (SRF)-responsive manner [22]. *MYOSLID* is a direct transcriptional target of both TGF-β/SMAD and MYOCD/SRF pathways, regulating the HCASMC contractile phenotype. Although MYOSLID does not affect gene expression of MYOCD and SRF transcription factors, its depletion in VSMCs disrupted actin stress fiber formation and blocked nuclear translocation of MYOCD-related transcription factor A (MKL1). Functional studies revealed that *MYOSLID* promotes VSMC differentiation and inhibits VSMC proliferation. In human samples from patients with end-stage renal disease *MYOSLID* expression was reduced in failed human arteriovenous fistula samples compared with healthy veins, verifying its implication in vascular disease.

Smooth muscle–induced lncRNA enhanced replication (*SMILR*) is highly expressed in VSMCs after interleukin-1α and PDGF stimulation. *SMILR* expression increased in both the cytoplasm and nucleus after stimulation, and was also released in conditioned media. Knockdown of *SMILR* reduced VSMC proliferation and the expression of the nearby gene HAS2 [98], with no change in the expression of isoforms HAS1 and HAS3, HAS2-AS1 lncRNA, or the ZHX2 gene, indicating the specificity of *SMILR* silencing for HAS2. *SMILR* expression is increased in human samples from patients with unstable atherosclerotic plaques and in plasma from patients with high plasma C-reactive protein levels compared to control subjects. Taken together, these findings suggest that SMILR regulates VSMC proliferation with potential implications in vascular injury and atherogenesis, although future studies are required to verify a definitive role in relevant disease models.

4. LncRNAs in inflammatory cells and lipid metabolism

Accumulation of immune cells and low-density lipoproteins (LDL) in the intima occurs during the first stage of plaque formation. Products of oxidative modification of LDL (oxLDL) may activate endothelial cells (ECs) and VSMCs, and native LDL epitopes may instigate adaptive immune responses [109]. In response to both biochemical and biomechanical stimuli, EC activation triggers the expression of a number of adhesion molecules, mediating the recruitment of leukocytes to sites of inflammation. Monocytes and T cells may bind to these adhesion molecules and in combination with locally produced chemokines migrate into the arterial intima [110]. These mononuclear cells differentiate into macrophages induced by macrophage colony-stimulating factor secreted by ECs and VSMCs [111]. Scavenger receptor expression on macrophages enables the uptake of oxLDL particles, inducing the formation of foam cells, and intracellular cholesterol accumulation.

Furthermore macrophages and dendritic cells activate T cells to a T helper 1 (T_H1) cell response, promoting the production of IFN-γ, TNF-α, and expression of CD40 ligand. Mouse models and plaque analysis in humans revealed that T_H1-type cytokines as of IFN-γ, TNF-α, interleukin-12 (IL-12), IL-15, and IL-18 dominate over a few T_H2-type cytokines (e.g. IL-4), suggesting that atherosclerosis is a T_H1-cell-driven disease [112,113]. The non-coding RNA genome provides opportunities to identify new mediators involved in both innate and adaptive immunity in the multistep disease progression of atherosclerosis (Table 3).

In order to identify such new mediators, Zhang et al. [114] performed genome wide association studies (GWAS) in a database for atherosclerosis-associated SNPs. Among them, they identified the SNP rs2850711, which lies within the locus of the lncRNA *linc00305*. *linc00305* expression was significantly increased in human atherosclerotic plaques compared to normal artery samples based on RT-qPCR of whole tissue sections. In addition, *linc00305* was enriched in PBMCs from patients suffering from atherosclerosis compared to healthy individuals. Moreover, *linc00305* was highest expressed in monocyte-like THP-1 cells compared to ECs and VSMCs and in CD14-positive monocytes isolated from cord blood. LPS stimulation induced its expression [114]; however, it remains unknown whether *linc00305* is implicated in polarization of monocytes. Gain-of-function profiling studies revealed that biological pathways involving inflammation were induced. Because treatment with BAY11-7082, an inhibitor for NF-κB abolished the induction of pro-inflammatory genes, the authors suggest that *linc00305* mediates its function in a NF-κB-dependent manner. However, additional studies are required to fully understand whether alternative NF-κB signaling pathways may be involved in response to other pro-inflammatory stimuli. Functionally, THP-1 cells overexpressing *linc00305* lead to a phenotypic switch of VSMCs from the contractile to the synthetic phenotype in a co-culture experiment. Mechanistically, biotinylated *linc00305* bound to lipocalin-interacting membrane receptor (LIMR) in HeLa cells. LIMR itself was found to bind to the aryl hydrocarbon receptor repressor (AHRR), which is involved in Ahr signaling. Although LIMR and AHRR both increased NF-κB luciferase activity, *linc00305* alone had no significant effect on NF-κB activity, but combined with LIMR and AHRR it markedly increased its activity [114]. These findings suggest that although *linc00305* binding to LIMR is beneficial, it is not required for Ahr-mediated regulation of the NF-κB signaling pathway. Taken together, while these findings suggest a role of *linc00305* in the progression of atherosclerosis based on expression data and a SNP associated with atherosclerosis, further investigation is required to address causality and to decipher precisely whether *linc00305* observed effects may be due to regulation of overlapping antisense transcripts such as lincRNA01924 and AC100848.

After RNA-Seq profiling of macrophages stimulated with Pam_3CSK_4, a ligand for TLR2, Carpenter et al. identified the *lincRNA-Cox2,* which is in close proximity to the *Cox2* loci, among the top-induced lncRNAs candidates. TLR7/8 activation by LPS stimulation induced *lincRNA-Cox2* expression in both dendritic cells and BMDM in a similar pattern as Ptgs2 [115,116]. However, no regulation of *lincRNA-Cox2* could be observed by activation of TLR3 signaling using poly(I:C). In addition, *lincRNA-Cox2* expression was shown to be MyD88- and NF-κB–dependent. shRNA-mediated silencing of *lincRNA-Cox2* did not affect Cox2 expression, but significantly increased the expression of pro-inflammatory genes such as Irf7 and CCL5 in unstimulated BMDMs, while Pam_3CSK_4-induced Tlr1 and IL-6 expression was attenuated. Complementary *lincRNA-Cox2* gain-of-function experiments decreased expression levels of these genes in macrophages. Taken together, these results demonstrate that *lincRNA-Cox2* represses Ccl5, while simultaneously enhancing the expression of TLR-induced IL-6. Mechanistically, *lincRNA-Cox2* binds to hnRNP-A/B and hnRNPA2/B1 in cytoplasmic and nuclear compartments as well as affecting IKB-α and SWItch/Sucrose NonFermentable (SWI/SNF) complex stability in the cytosol, suggesting a regulatory role of *lincRNA-Cox2* as a co-activator of NF-κB or inducing SWI/SNF-associated chromatin remodeling [117,118]. In sum, these studies identified *lincRNA-Cox2* as a critical component of the inflammatory response. However, its causal role in CVD disease is not elucidated and requires further investigation.

In a similar experimental setup as described above [115], Li et al. [119] analyzed the expression of lncRNAs in PMA-activated THP-1 cells using the TLR2 ligand Pam_3CSK_4 by microarray profiling. They found a panel of 159 differentially expressed lincRNAs (i.e. 1.9-fold up/-down; p-value < 0.05). Out of the 159 lncRNA candidates they selected 20 candidates based on their genomic flanking genes within the range of 1 Mb. Those 20 candidates were further validated by RT-qPCR and their expression was compared across different tissues. Subsequently, loss-of-function studies for nine out of the 20 lncRNA candidates revealed that the *linc1992,* later named as TNFα and hnRNPL related immune-regulatory lincRNA (*THRIL*) was the lncRNA candidate that most significantly reduced TNFα cytokine expression [119]. This approach of stratifying microarray hits based on their proximity to the lncRNA locus assumes the lncRNA acts in a *cis* and not in *trans*. Moreover, TNFα expression as a read-out for systematical identification of lncRNAs involved in the activation of innate immune signaling in THP1 macrophages may be overstated. Mechanistically, *THRIL* was shown to form a complex with hnRNPL and by silencing either of those two components, binding to the TNFα promoter was compromised using ChIP. Those findings suggest that *THRIL* and hnRNPL form a RNP complex that regulates TNFα transcription by binding to its promoter. Clinically, *THRIL* expression correlated with the severity of symptoms in patients with Kawasaki disease, an acute inflammatory disease of childhood [119]. Future investigations will be of interest to solidify other top hit candidates from those screening platforms and to verify whether those lncRNAs may also have translational value in the context of chronic inflammation such as atherosclerosis or diabetes.

From expression analysis of human atherosclerotic plaques, *GAS5* and *HOXC-AS1* were found to be differentially expressed compared to healthy controls [120,121,122]. *HOXC-AS1* was found to be expressed lower in carotid atherosclerotic whole tissue sections compared to renal arterial intima tissue using microarray analysis. This lncRNA lies antisense to *HOXC6* loci. Both their expression was significantly reduced in THP-1 cells upon oxLDL treatment [121]; however, no further results were obtained to show any causal link of *HOXC-AS1* and HOX6 expression. The rationale for choosing this particular lncRNA was not clear. While microarray-based methods require less bioinformatics and data processing (which may be an advantage), they typically rely on one transcript or isoform per lncRNA of which annotation may not always be as accurate compared to deep-sequencing-based transcriptomic analysis. In contrast to *HOXAC-AS1*, *GAS5* expression was higher in human atherosclerotic plaques compared to healthy individuals [122]. An independent study showed that *GAS5* expression levels were reduced in mouse BMDMs polarized to M2bM (LPS + IC stimulation) compared to quiescent macrophages and other subpopulations of M2 and M1. Interestingly overexpression of *GAS5* abrogated LPS + IC-induced polarization to M2bM, suggesting that *GAS5* plays a role in macrophage polarization [123]. However, loss-of-function studies are missing to support this hypothesis. Other studies have showed that silencing of *GAS5* blocked oxLDL-induced apoptosis in THP-1 cells, which could be accelerated by overexpressing *GAS5* [120].

Disordered lipid metabolism is one of the pathological processes contributing to the onset and progression of atherosclerosis [124]. Sallam et al. identified a lncRNA named *LeXis* that regulates liver X receptor (LXR)-mediated cholesterol synthesis. *LeXis* promotes cholesterol efflux and inhibition of cholesterol biosynthesis by binding to a heterogeneous ribonucleoprotein named RALY. Mechanistically, *LeXis* binds to RALY, which affects its ability to interact with DNA, and in turn prevented cholesterol synthesis via transcriptional control of a subset of metabolic genes. As a consequence, total serum cholesterol was reduced in mice ectopically overexpressing *LeXis* [125]. Recently, adenoviral-mediated *LeXis* overexpression in the liver using a thyroxine-binding globulin promoter, significantly reduced aortic lesion

size determined by Oil-red O staining. In line with previous findings, hepatic sterol content and levels of serum cholesterol were significantly lower in these mice [126]. Another lncRNA involved in lipid metabolism is LncLSTR, which regulates apoC2 expression through an FXR-mediated pathway, to modulate triglyceride levels in a hyperlipidemia mouse model [127]. LncLSTR forms a molecular complex with TDP-43 to regulate expression of Cyp8b1, a key enzyme in the bile acid synthesis (XX add ref). Finally, the expression of the lncRNA Gm16551 suppresses lipogenesis and is induced by SREBP1c in hepatocytes, whereas its expression is reduced in livers of obese mice [128]. Collectively, these elegant studies raise the possibility for long-term lncRNA therapy in mice. Future studies that can overexpress lncRNAs in the liver or vessel wall may provide a novel therapeutic approach for regulating vascular inflammation in CVD.

5. Challenges and opportunities

Accumulating studies suggest that lncRNAs are important regulators of key biological processes vital for maintaining cellular homeostasis [134,135,136]. Disease-associated sequence variants exist in the non-coding genome where lncRNAs reside, raising the possibility that lncRNAs may provide genetic links to disease susceptibility that were initially dismissed as “junk” transcripts [134,137]. One example is the ANRIL lncRNA that resides in the chromosome 9p21 CAD susceptibility locus [138]. Recent studies provide incipient insights into lncRNA function and their regulatory effects in vascular-related diseases. As detailed above, lncRNAs have been identified as key regulators in various biological processes relevant to vascular homeostasis such as endothelial cells dysfunction, VSMC phenotypes, macrophage differentiation, and lipid metabolism (Fig. 2). Several lncRNAs have shown important regulation in the plasma or circulating cells, hence they hold promise as potential biomarkers and therapeutic targets for stage-specific vascular disease [139]. However, several challenges exist to the lncRNA field, including their relatively low level of cellular expression as compared to mRNAs. An important challenge here is the standardization of detection methods for reliable reporting. However, as the sensitivity of RNA-seq, microarray technologies, and bioinformatics has gradually increased over recent years, so too has the power to capture lncRNAs even in low-abundant cell types [140].

Although lncRNA regulation has been reported in human plasma and tissue samples and different disease models, their function and mechanism of action are only known for a few lncRNAs [28]. The poor conservation between species and the fact that most lncRNAs have various transcript variants challenges the identification of specific biological functions and mechanisms of action [141] and can limit their translational impact in vascular disease. However, often the neighboring genomic locus (the so-called “synteny”) is well-conserved [142], and recent bioinformatics approaches of comparative genomics including use of secondary and tertiary structures can identify lncRNA homologs in different species [143]. Future investigation into the precise expression of lncRNA in a cell-type or tissue-type specific manner in human subjects during disease progression and regression will inform lncRNA kinetics and potential use as biomarkers in diagnosis, prognostication, and response to therapies. Moreover, a broader perspective of how lncRNAs interact with RNA, DNA, and proteins to exact functional responses in vascular cells will be important for generating disease-specific networks or interactomes. Technical hurdles to identify such lncRNA interactors using ChiRP, RAP, and RIP pull-down approaches have already made substantial impact in the field [144].

While the translational potential of lncRNAs remains to be elucidated, RNA-based therapeutics have already been approved by the Food and Drug Administration (FDA) and may be successfully implemented for lncRNA regulation in vascular disease. RNA-based silencing strategies include antisense oligonucleotides (ASO), LNA (locked nucleic acid), aptamers or siRNA/shRNA, with great improvements in recent years in terms of stability, tolerability, reduced immunogenicity, and off-target effects [145]. One example is an ASO that targets a liver-specific ligand, the liver-specific asialoglycoprotein receptor (ASGPR)) that confers strong efficacy and reasonable safety [145,146]. Another example is Mipomersen, an FDA-approved ASO that targets apolipoprotein B, used for the treatment of homozygous familial hypercholesterolemia [147].

For therapeutic gain-of-function purposes, lncRNAs can be delivered by viral vectors such as lentivirus or by non-viral vectors such as polymeric or lipid nanoparticles. While viral vectors induce immunogenicity [148], there is great anticipation that non-viral vectors with different chemical modifications may be used successfully in clinical trials [149]. Important lessons can be learned from mRNA delivery and vaccination studies [149,150]. Challenges for lncRNA delivery remain with respect to their efficiency and tissue specificity. However, these challenges may be overcome using chemical modifications and/or nanoparticles targeted to specific ligands overexpressed by cells in the vessel wall in response to relevant stimuli [151]. Finally, new gene editing tools such as CRISPR can be successfully used to manipulate lncRNA expression by both loss- or gain-of function approaches with great specificity and efficiency, at least in vitro [130,45,152]. Similar delivery issues remain for use of gene editing tools in vivo in the vasculature.

Despite these challenges, accumulating findings from studies using gain- or loss-of function approaches suggest that lncRNAs indeed contribute to vascular dysfunction and their therapeutic regulation can prevent or repair specific pathological processes that lead to maladaptive vascular disease remodeling [126].

6. Conclusions

LncRNAs have been identified as key regulators in biological and pathological processes in a range of vascular disease states. Given the growing massive number of mammalian lncRNAs, the increasing correlation with GWAS hits, and their diverse mechanisms of action in the nucleus, cytosol, or exosomes, increased efforts are desperately needed to define their expression, function, and interactomes. Integrating knowledge of lncRNAs with other non-coding and protein-coding genes will be critical to our understanding of the biological orchestration necessary to finely-tune the vessel wall in health and disease.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgments

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