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Long intergenic noncoding RNAs in cardiovascular diseases: Challenges and strategies for physiological studies and translation

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Abstract

Long intergenic noncoding RNAs (lincRNAs) are increasingly recognized as important mediators of many biological processes relevant to human pathophysiologies, including cardiovascular diseases. *In vitro* studies have provided important knowledge of cellular functions and mechanisms for an increasing number of lincRNAs. Dysregulated lncRNAs have been associated with cell fate programming and development, vascular diseases, atherosclerosis, dyslipidemia and metabolic syndrome, and cardiac pathological hypertrophy. However, functional interrogation of individual lincRNAs in physiological and disease states is largely limited. The complex nature of lincRNA actions and poor species conservation of human lincRNAs pose substantial challenges to physiological studies in animal model systems and in clinical translation. This review summarizes recent findings of specific lincRNA physiological studies, including *MALAT1, MeXis, Lnc-DC* and others, in the context of cardiovascular diseases, examines complex mechanisms of lincRNA actions, reviews *in vivo* research strategies to delineate lincRNA functions and highlights challenges and approaches for physiological studies of primate-specific lincRNAs.

Keywords

Long intergenic noncoding RNAs; Cardiovascular diseases; Animal models

1. Introduction

Pervasive transcription in mammalian genome generate tens of thousands RNA transcripts that do not encode proteins. Long intergenic noncoding RNAs (lincRNAs) are defined as over 200 nucleotides in length and, like protein-coding mRNAs, most are spliced and 3' polyadenylated [2]. Compared to mRNAs, lincRNAs are poorly conserved across species, and their expression is lower and more tissue specific [2–4]. Although the biological roles of

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most lincRNAs remain to be elucidated, they are emerging as key mediators in a vast variety of cellular functions, such as cell proliferation and differentiation, and have been implicated in many human pathologies, including cancer [5,6], cardiovascular diseases (CVD) [7–9] and metabolic disorders [10,11]. In addition, recent functional genomic studies suggest that long noncoding RNAs, not the nearby protein-coding genes, are the likely causal element driving human disease association at some genome wide association study (GWAS) loci. These include candidates like *ANRIL* at the 9p21 locus for CVD [12–15], *CCAT2* for colon cancer [16], and *lnc13* for celiac disease association [17]. Indeed, multiple functional variants at lncRNA loci have been identified for CVD traits (Table 1) [18–22] including *ANRIL* variants that regulate the expression of *CDKN2A/B* and atherogenic pathways [18,19], *in cis* SNPs related to the expression level of *Myocardial Infarction Associated Transcript (MIAT)* and corresponding susceptibility of myocardial infarction [20], and H19 variants associated with increased IGF2 expression increased CVD risk [21]. These findings suggest that studying functional roles of lincRNAs in pathophysiology may provide new insights into causal pathogenic mechanism and opportunities for novel diagnostic and therapeutic strategies in human diseases, including CVD.

LincRNAs mediate gene expression or function through transcriptional and post-transcription regulation. Through their primary sequence motifs or secondary structure, lncRNAs can interact with specific proteins or RNA partners, such as transcriptional regulators and histone modifying enzymes in the nucleus [23,24], or RNA-binding proteins and microRNA in the cytoplasm [25,26]. Some lincRNAs encode microRNAs within their gene loci and are precursors for functional microRNAs [27]. Independent of RNA-dependent mechanisms, lincRNAs can act *in cis* through regulatory DNA elements or transcription activities at lincRNA genomic loci [28,29]. Individual lincRNAs can regulate diverse gene pathways or distinct cellular processes through multiple distinct molecular mechanisms. For example, our group has shown that adipose *linc-ADAL* binds with distinct protein partners in adipocyte nucleus and cytoplasm to regulate adipocyte differentiation and *de novo* lipogenesis respectively [30]. LincRNA-p21 regulates the expression of nearby gene CDKN1A through *in cis*-acting DNA elements while also modulating a larger set of genes *in trans* through RNA-dependent mechanism [31–33]. The complex nature of lncRNA actions underscores the importance of studying their physiological roles in tissues and whole organisms. This review summarizes examples of CVD-related lincRNAs (Fig. 1) and their physiological impacts in animal models (Table 2), discusses *in vivo* research strategies for interrogating lincRNA function (Table 3) and highlights challenges and approaches for *in vivo* studies of primate-specific lincRNAs (Fig. 2). The primary focus of this review is on lincRNAs i.e., lncRNAs that are intergenic, the genomic location of the vast majority of lncRNAs and GWAS loci for CVD, and for that reason we do not discuss in detail lncRNAs that are antisense or overlap protein coding genes, including *ANRIL* the well-studied lncRNA at the 9p21 locus for CVD [12–15].

2. Importance of physiological studies in lincRNA research

Over the past decade, lincRNA studies have flourished in many research fields, from systematic identification and categorization of lincRNAs in an increasing number of species to in-depth functional and mechanistic interrogation of individual lincRNAs. Although the

functional significance of most lincRNAs remain unclear, *in vitro* loss-of-function and gain-of-function studies have provided valuable insights into the potential biological roles of lincRNAs, at least in cellular context. However, emerging evidence has suggested that knowledge of lincRNA functions gleaned from *in vitro* systems may poorly inform or match the physiological impact of these lincRNAs in animal studies. The limitation of *in vitro* findings has been observed for both well-characterized lincRNAs (e.g. *NEAT1*) and newly identified lincRNAs. For example, *NEAT1* knockdown in cell lines markedly impaired the formation of papaspeckles [34,35], nuclear bodies important for gene regulation and mRNA processing. Mice with *NEAT1* genetic deficiency also had impaired papaspeckles in specific tissues yet exhibited no apparent physiological abnormalities [36]. *LincRNA-EPS*, an erythroid lincRNA, was recently found to regulate mouse erythroid differentiation [37] yet genetic ablation of this lincRNA showed no effects on erythropoiesis or hemoglobin levels in mice [38]. Conflicting findings from cell and animal studies are not specific to lincRNA research and can be attributed to various factors, such as biological difference between cellular and physiological conditions, the interactions of target genes with other genetic and physiological factors in animal models. For example, lincRNA *HOTAIR* regulates gene silencing of homeotic genes in HoxD cluster [39] and *Hotair*$^{-/-}$ mice on C57B6 background demonstrated impaired skeleton development consistent across independent studies [40,41]. However, *Hotair*$^{-/-}$ mice on a mixed ground showed minimal impairment [42], suggesting that mouse genetic background may have a significant impact on lincRNA knockout phenotypes *in vivo*.

3. Physiological roles of lincRNAs in cardiovascular diseases

3.1. LincRNAs in vascular function

3.1.1. MALAT1—MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) is a conserved lincRNA ubiquitously expressed in a number of cells and tissues. Its expression is frequently elevated in many types of cancers [43,44] and other pathological conditions, such as hypoxia and hyperglycemia [45–47]. As a nuclear lincRNA, MALAT1 plays important roles in alternative splicing by modulating the distribution and activities of splicing factors in nuclear speckles [48,49]. In addition, MALAT1 is involved in epigenetic gene regulation by interacting with polycomb protein CBX4 [50]. Transcriptome analyses from MALAT1 knockdown studies suggest MALAT1 regulates gene expression of cell cycle regulators such as CCNA2, CCNB1, and CCNB2 in endothelial cells [51]. Consistent with these findings, mouse xenograft studies demonstrate that both genetic deficiency and knockdown of MALAT1 significantly reduce tumor development and metastasis [52–54]. Notably, recently studies support an emerging regulatory role of MALAT1 in the pathogenesis of cardiovascular diseases. Michalik et al. demonstrated that *MALATs1*$^{-/-}$ mice showed a delayed vessel extension in the retina revascularization of neonatal mice while *in vivo* inhibition of MALAT1 by antisense oligonucleotides suppressed blood flow recovery in the ischemic hindlimb muscle of adult mice, consistent with impaired proliferation of MALAT1-knockdown endothelial cells *in vitro* [51]. Similarly, *MALAT1*$^{-/-}$ mice exhibit higher expression of pro-apoptotic and pro-inflammatory genes in the cerebral cortex, larger brain infarct size, worsened neurological scores, and reduced sensorimotor functions after transient focal cerebral ischemia [55]. Together, these *in vivo* studies support an important

role of MALAT1 in promoting endothelial cell proliferation and blood vessel growth [51,55]. Interestingly, Cardenas et al. recently revealed that MALAT1 form a ternary complex with histone deacetylase HDAC9 and the chromatin-remodeling enzyme BRG1 to recruit polycomb repressive complex 2 (PRC2) and inhibit gene expression of contractile proteins in vascular smooth muscle cells [56]. Importantly, *in vivo* genetic ablation of MALAT1 restored contractile protein expression in mouse aortas and reduced the development of thoracic aortic aneurysms [56]. The distinct regulatory roles of MALAT1 in different vascular cells highlight the complexity of lincRNA action in diverse pathophysiological conditions and the challenge in therapeutic targeting of this ubiquitously expressed lincRNA.

3.1.2. H19—H19 (H19 imprinted maternally expressed transcript) is one of the first identified imprinted lncRNAs and is only transcribed from the maternal allele [57]. Both the primary sequences and the secondary structures of H19 are well conserved across many mammals [58,59]. H19 expression is highly induced during embryogenesis, reduced after birth and only retained in a few adult tissues, such as skeleton muscles, adipose and adrenals [60,61]. H19 is often abnormally activated in many types of cancers and has been increasingly recognized as an oncogene [62,63]. Besides serving as a precursor of microRNA miR-675 [64], H19 has been shown to interact with a variety of proteins (such as RNA-binding proteins HuR [65], KSRP [66] and hnRNPU [67]) and microRNAs (such as Let-7 [68] and miR-106 [69]) to regulate genes involved in cell proliferation, migration, differentiation and tumor-igenesis.

For endothelial cells, increased H19 expression in microvessels has been associated with increased angiogenesis in glioma tissues [70]. Hyperglycemia-induced reduction of H19 expression in endothelial cells was linked to impaired angiogenesis in diabetes [71] and Tao et al. showed that *in vivo* delivery of exogenous H19 by extracellular vesicle-mimetic nano-vesicles markedly improve wound healing in diabetic rats, supporting a pro-angiogenic role of H19 [71]. For SMCs, recent conflicting studies reported opposing effects of H19 on *in vitro* proliferation and apoptosis: Li et al. showed H19 knockdown significantly inhibited apoptosis and increased proliferation of human aortic SMCs [72] while Zhang el al. observed the opposite phenotype in human aortic SMCs stimulated by oxidized LDL (ox-LDL) [73]. Importantly, Li et al. demonstrated that H19 expression in aortic media SMCs was elevated in mouse models of abdominal aortic aneurysms (AAAs) and *in vivo* H19 knockdown limited AAA growth, consistent with the pro-apoptotic effect of H19 in their *in vitro* SMC studies [72]. The exact regulatory roles of H19 in SMC function and vascular disorders may dependent on different H19-interacting partners in distinct pathophysiological and SMC phenotype contexts. For example, Li et al. demonstrated H19 promote SMC apoptosis via HIF1α [73] while Zhang et al. showed H19 inhibited apoptosis of ox-LDL stimulated SMCs via miR-148b/WNT/β-catenin [73]. Further studies are required to resolve these apparently conflicting data.

3.1.3. lincRNA-p21—lincRNA-p21 is a conserved and p53-regulated lincRNAs. It has been shown to act *in trans* by forming a repressive complex with hnRNPK to inhibit many gene in p53 transcriptional network [31] or binding directing with target mRNAs to suppress

their translation [32]. In addition, lincRNA-p21 appears to regulate its nearby protein-coding gene Cdkn1a (p21) through *cis*-regulatory DNA elements in the locus [29,33]. As a p53-target lincRNA, the expression of lincRNA-p21 was decreased in many cancer types [74]. Many studies on lincRNA-p21 focused on p53-dependent apoptosis, cell cycle regulation and cancer research [74]. Wu et al. demonstrated that the expression level of lincRNA-p21 was reduced in mouse aortas with atherosclerotic plaques and in human coronary artery tissues of CAD patients [75]. Consistent with previous finding on the effects of lincRNA-p21 in cell cycle, *in vitro* knockdown of lincRNA-p21 promoted proliferation and reduced apoptosis of vascular SMCs [75]. *In vivo* lincRNA-p21 knockdown in mouse carotid arteries by lentiviral-based shRNA markedly increased neointima formation in the carotid artery injury model [75]. Furthermore, Wu et al. present a novel mechanism of lincRNA-p21 to promote p53 activity in SMCs. LincRNA-p21 modulates the dynamic interaction between p53 and its post-transcriptional regulators p300 and MDM2 by directly binding MDM2, reducing p53-MDM2 complex while promoting p53-p300 interaction. This results in a marked increase in P53 activities [75].

3.1.4. RNCR3—RNCR3 (Retinal non-coding RNA 3), is a conserved lincRNAs highly expressed in brain tissues [27]. Its expression is actively regulated during mouse retina development [76] and the differentiation of neurons and oligodendrocytes [77]. Similar to H19, RNCR3 also serves as the precursor for a microRNA, miR-124a. Genetic ablation of RNCR3 resulted in several defects in mouse brain development, including smaller brain size and aberrant growth of dentate granule cell axons [27]. These phenotypes were rescued by overexpressing miR-124a [27], suggesting RNCR3 act through miR-124a to regulate central nervous system development. Recently Shan et al. demonstrated that RNCR3 was expressed in vascular smooth muscle and endothelial cells and was increased by hypercholesterolemia stress (oxidized LDL) *in vitro* [78]. RNCR3 expression was marked increased in mouse aortas with atherosclerotic lesions [78]. Importantly, *in vivo* knockdown of RNCR3 by viral-based short hairpin RNA (shRNA) accelerated development of atherosclerosis, supporting a protective role of RNCR3 in atherosclerosis [78].

3.1.5. AK098656—AK098656 is a human lincRNA that are highly expressed in human testis [2] and appears not conserved in rodents [79]. Its expression is also predominantly detected in smooth muscle cells among 15 human cells derived from various tissues [79]. Jin et al. found that the level of AK098656 was significantly elevated in the plasma of hypertensive patients [79]. *In vitro* knockdown of AK098656 inhibited, whereas overexpression increased, SMC proliferation, suggesting a regulatory role of AK098656 in promoting SMC synthetic phenotype [79]. A transgenic rat model overexpressing AK098656 had spontaneous hypertension as well as narrowed diameter and reduced contractile protein expression of resistant arteries [79]. Mechanistically, AK098656 binds with SMC contractile protein myosin heavy chain-11 and fibronectin-1 to promote their degradation, possibly by interacting with 26S proteasome non-ATPase regulatory subunit 11 [79].

3.2. lincRNAs in lipid metabolism

3.2.1. lncLSTR—lncLSTR (liver-specific triglyceride regulator) is a mouse lincRNA predominantly expressed in liver [80]. Its liver expression fluctuates during fasting and refeeding, suggesting a regulatory role in energy metabolism [80]. *In vivo* lncLSTR knockdown in mouse liver significantly reduced plasma triglyceride levels in normal and hyperlipidemic mice by increasing expression of liver-secreted lipoprotein ApoC2, a potent activator of lipoprotein lipase (LPL) and enhancing LPL-mediated triglyceride clearance in peripheral tissues [80]. Interestingly, *in vitro* lncLSTR knockdown in hepatocytes showed no effects on ApoC2 expression, suggesting lncLSTR does not directly regulate apoC2 through a cell-autonomous mechanism [80]. *In vivo* lncLSTR knockdown reduced liver expression of Cyp8b1, a key enzyme determining the ratio of two most abundant bile acids in mouse, cholic acid and muricholic acid, thus resulting in increased muricholic acid in bile pool composition [80]. Muricholic acid exhibited greater induction of apoC2 genes after binding with bile acid receptor FXR in liver [80]. In summary, lncLSTR modulates hepatic and plasma apoC2 levels through liver Cyp8b1-FXR pathway to regulate LPL-mediated triglyceride clearance in peripheral tissues, highlighting a novel lincRNA mechanism that operates *in vivo* in both physiological and pathophysiological contexts. Notably, Li et al. failed to detect a human ortholog for lncLSTR in annotated human lincRNA catalogs [80]. Further studies are required to determine if lncLSRT is encoded in human or has any functional impact on lipid metabolism in primates.

3.2.2. LeXis—LeXis (liver-expressed LXR induced sequence) is a conserved, liver-enriched lincRNA that is robustly induced by high cholesterol diet feeding and activation of liver x receptor (LXR) [81]. Recent studies demonstrated that *in vivo* LeXis overexpression in mouse liver significantly reduced expression of hepatic cholesterol biosynthesis genes, total serum cholesterol and triglyceride levels and atherosclerotic lesion in aortas [81,82]. In addition, genetic deficiency and *in vivo* knockdown of LeXis increased hepatic lipid accumulation and elevated gene expression of lipogenesis genes [81].

3.2.3. MeXis—MeXis (macrophage-expressed LXR-induced sequence) is a conserved and macrophage-enriched lincRNAs that is encoded near protein-coding gene ABCA1, an important regulator of cholesterol efflux. MeXis expression in mouse macrophage was markedly induced by physiologic lipid signals, such as oxidized or acetylated LDL, and LXR activation [83]. Sallam et al. demonstrated that MeXis regulated the expression of nearby gene ABCA1 by interacting with transcriptional coactivator DDX17 in macrophages [83]. In addition, overexpressing MeXis in liver by adenovirus significantly increased hepatic ABCA1 expression and serum cholesterol level [83]. *MeXis*−/− mice exhibited decreased ApoA-I-dependent cholesterol efflux capacity and increased cholesterol content in macrophages after Western diet feeding [83]. Importantly, atherosclerotic plaque in aortas was significantly increased in hyperlipidemic mice transplanted with *MeXis*−/− bone marrows [83], supporting macrophage MeXis upregulation of ABCA1-mediated cholesterol efflux and protection from atherosclerosis development in mice.

3.3. lincRNAs in immune response

3.3.1. LincRNA-EPS—lincRNA-EPS is a conserved lincRNAs, whose expression is high in resting macrophages and markedly reduced by toll like receptor (TLR) activation [38]. Atianand et al. recently demonstrated that lincRNA-EPS suppresses transcription of immune response genes in resting macrophages by associating with chromatin at regulatory regions of these genes through hnRNPL nuclear protein and controlling nucleosome positioning [38]. LincRNA-*EPS*−/− mice show enhanced basal and TLR4 induced expression of immune response genes in macrophages while the numbers of macrophages, dendritic cells, lymphocytes, natural killer cells and red blood cells were unaffected [38]. Furthermore, lincRNA-*EPS*−/− showed elevated levels of inflammatory cytokines in serum and peritoneal fluid as well as decreased survival rate following LPS challenges [38]. These findings support that lincRNA-EPS plays an important role in controlling both the homeostatic and TLR-inducible immune gene responses *in vivo*.

3.3.2. Lnc-DC—Lnc-DC is a conserved lincRNA that is detected in many immune cells, including macrophages, monocytes and lymphocytes [84]. Lnc-DC expression is highly enriched in dendritic cells and actively regulated by PU.1 transcription factor [84]. Wang et al. recently demonstrated that lnc-DC regulates dentritic cell differentiation by interacting with STAT3 [84], a transcription factor important to dendritic cell function. Lnc-DC bound directly to STAT3 in the cytoplasm and promoted STAT3 phosphorylation on tyrosine-705 by preventing STAT3 dephosphorylation by SHP1 [84]. Knockdown of lnc-DC in mouse bone marrow cells resulted in impaired mouse dendritic cell differentiation after bone marrow transplantation [84], supporting a regulatory role of lnc-DC in dendritic cell function *in vivo*.

4. Application and limitation of animal models in lincRNA research

Loss-of-function and gain-of-function approaches are widely used to study physiologies of protein-coding and non-coding genes in animal models. Unlike coding genes exerting their biological functions through proteins, a lincRNA gene can act through multiple elements, including diverse functions of the mature lincRNA transcript itself, the DNA sequences within the lincRNA gene loci, and transcription activity of the lincRNA. For example, lincRNA-p21 has been identified to regulate its neighboring protein-coding gene Cdkn1a *in cis* [33]. Recent studies demonstrate that deletion of lincRNA-p21 locus markedly reduced Cdkn1a gene expression even in mouse tissues not expressing lincRNA-p21, suggesting lincRNA-p21 gene acts through functional enhancer DNA elements at the locus, i.e., in a RNA-independent manner [29]. Indeed, many *in cis*-acting lincRNAs appear to share the same promoter or upstream enhancer regions with nearby protein coding genes. Engreitz et al. recently found that multiple lincRNAs (e.g. *Blustr* and *Bendr)* regulated nearby gene expression by the transcriptional activities at these lincRNA loci, not by lincRNA transcripts themselves [28]. Genetic ablation of lincRNA transcription, by deleting promoter regions, inserting premature transcription termination signals or perturbing promoter-proximal splice sites, significantly reduced expression of the lincRNA neighboring protein genes [28]. Whereas sequential deletions of *Blustr* exons and introns had no effect on nearby gene expression, suggesting that *Blustr in cis* regulation is independent of specific sequences [28].

Similarly, Anderson et al. demonstrate that inhibiting transcription of *Upperhand*, a lincRNA sharing a bi-directional promoter with nearby gene *Hand2*, markedly reduced *Hand2* expression while knockdown of the mature *Upperhand* transcript by antisense oligonucleotides (ASOs) had no effect on Hand2 level [85]. Since RNA molecules, genomic regulatory DNA and gene transcription can all contribute to lincRNA molecular mechanisms, it is critical to carefully design experimental strategies for animal models that address each possible mechanism and perturb these individual potential actions of lincRNA. Interpreting *in vivo* phenotypes using complementary approaches, including recently developed CRISPRi or CRISPR activation, which allow robust transcriptional modulation without altering genetic sequences (Table 3), are often necessary to dissect regulatory mechanisms and physiological functions of lincRNA.

In vivo loss-of-function approaches targeting lincRNA transcript molecules include RNA interference (RNAi, e.g. siRNA and shRNA) and antisense RNA analogues (e.g. locked nucleic acids ASO). ASOs bind with RNA transcripts and activate RNase H mediated RNA degradation while siRNA and shRNA activate RNA-induced silencing complex to degrade targets [86]. Systemic administration of siRNA or ASO by tail vein injection or osmotic pump delivery can achieve sustained knockdown of lincRNA abundance in many tissues in rodents [87,88]. Viral-based shRNAs (e.g. adeno-associated virus) can be applied locally or systemically, providing opportunities to examine lincRNA functions in tissue-specific manner. However, there are still concerns regarding efficiency and off-target effects of *in vivo* lincRNA knockdown by RNAi. RNAi methods often results in inefficient knockdown of nuclear RNAs as RNAi-mediated RNA destabilization predominantly occurs in the cytoplasm. Importantly, these RNA-targeting strategies preclude interrogation of lincRNAs acting through the transcription or DNA elements at their genomic loci, but can be a useful complement when applied with other genetic strategies (Table 3).

For lincRNA knockout animal models, several strategies can be used to target lincRNA genetic loci and prevent lincRNA transcription, including deleting promoter regions or whole gene bodies of lincRNAs and inserting transcription termination signals (e.g. polyadenylation). While these approaches are very effective to ablate lincRNA expression *in vivo*, there are several caveats to take into account. For the lincRNAs sharing bi-directional promoters or upstream DNA elements with nearly protein-coding genes, genetic ablation of transcription activities at these loci would likely inhibit the expression of both lincRNAs and local genes [28]. While transcription-mediated *in cis* regulation of nearby genes might be the mechanism for some lincRNAs, it is possible that they also may act through lincRNA transcripts to regulate genes *in trans*. In these cases, the unintended knockdown of nearby protein coding genes would confound interrogation of the function of an active transcribed lincRNA molecule. For lincRNAs acting through regulatory DNA element at lincRNA loci, inserting polyadenylation signals after the lincRNA transcription start sites may cause no significant perturbation of regulatory DNA sequences and this potential lincRNA function, even if lincRNA transcription is ablated. In addition, it should be noted that any one lincRNA can function through multiple mechanisms, e.g. DNA-dependent *in cis* regulation and RNA-dependent *in trans* action. For example, genetic deletion of lincRNA *Fendrr* locus impaired lung maturation and mesenchymal differentiation [89] while polyadenylation signal insertion resulted in heart and body wall defects in mouse models [90], suggesting

distinct physiological roles of *Fendrr* in development through independent mechanisms. These findings highlight that lincRNA transgenic animal models and rescue studies are important to delineate specific RNA-dependent lincRNA actions.

5. Challenges to and strategies for the study of non-conserved lincRNAs *in vivo*

LincRNAs have evolved more recently and more rapidly than protein coding genes and microRNAs [91]. It has been estimated that the majority of primate lincRNAs (~80%) are not conserved in mouse [92,93]. Conservation has to be considered at several levels and requires a systematic comparison of lincRNAs across species in terms of genomic (DNA) positional conservation, primary sequence, transcription status and splicing patterns, as well as secondary structure [94]. For example, most primate and human lincRNAs are not "syntenic" (positionally conserved) in rodents, i.e., at the genomic level the 5′ and/or 3′ neighboring protein coding genes are not the same. In this context, conservation of *cis*-regulatory functions cannot be considered or studied in mice models. Furthermore, a large percentage of human lincRNAs that are positionally syntenic between human and mice have not been found to be expressed (transcribed) in mouse tissues suggesting that these molecules and functions evolved later and only in primate species. Importantly, sequence similarity does strongly hint at functional conservation but there haven't been enough studies to suggest that lack of sequence similarity implies lack of functional conservation. For example, FENDRR (FOXF1 adjacent non-coding developmental regulatory RNA) represents one example of a functional lncRNA with a conserved position yet very limited sequence conservation between human and mouse [95].

An increasing amount of data support the concept that many primate-specific lincRNAs, not found in rodents or other organisms, play important regulatory roles in cellular processes, such as pluripotency and differentiation, and have been implicated in human cancer and cardiometabolic disorders [96–98]. While *in vitro* studies provide useful insights on biological roles of primate-specific lincRNAs in cells, it is crucial to study the physiological impacts of these non-conserved lincRNAs. Yet, interrogation of these non-conserved lincRNAs in animal models is particularly challenging as most model system, such as rodents, often do not encode them. Perturbation of protein coding genes and microRNAs by RNAi or transgene have been applied to non-human primates in translational or pre-clinical studies. However, RNA-based strategies may not always capture lincRNA actions as discussed above. Additionally, non-human primates are scarce and far more costly than other animal models, thus limiting their use and feasible application for most investigators. Recently, however, non-primate models have been adapted and can be very useful tools for examination of these nonconserved lincRNAs *In vivo* (Fig. 2).

Transgene approach can be used to express primate-specific lincRNAs in non-primate animal models. It is possible, although not widely demonstrated, that the protein or RNA partners of these lincRNAs are conserved and can interact with primate-specific lincRNAs in non-primate animal models. For example, transient overexpression of a primate-specific lincRNA *LncND* in mouse brains elicited a phenotype on neuron development consistent

with that found *in vitro* in human cell studies [99], supporting the plausibility of the transgene approach for some non-conserved lincRNAs. And bacterial artificial chromosome (BAC) transgene mouse models can utilize the gene body and genomic regulatory DNAs of non-conserved lincRNA loci to drive lincRNA expression at physiological levels and with tissue expression patterns as endogenous lincRNAs *in vivo* [100]. For lincRNAs that require for function a significant portion of the transcript to be produced, the transgene approach is important to examine non-conserved lincRNAs with some RNA-dependent mechanisms.

Another *in vivo* approach is to engraft human cells expressing primate-specific lincRNAs in rodent models with immune deficiency. Combined with RNA-based or genetic perturbation of lincRNAs in human cells, these "humanized" animal models permit study of primate-specific lincRNAs acting in their native cellular context and the examination of their functional impacts under physiological conditions. As an example, Xenograft model of human cancer cells in immunodeficient rodents (e.g. nude and NSG mice) have been widely used to study the roles of human lincRNA in tumor development and metastasis [101,102]. Similarly, implantation of human hepatocytes and adipocytes derived from primary cells or IPSC (induced pluripotent stem cells) in immunodeficient mice has been used to study protein-coding genes in hepatic and adipose function *in vivo* [103,104]. Such models can be adapted to interrogate primate-specific lincRNAs in lipid metabolism homeostasis and cardiometabolic disorders. Indeed, our group is pursuing such strategies for the study of several non-conserved, human adipose and macrophage lincRNAs for which we have already demonstrated functions in *in vitro* models [30,105,106].

Although not an *in vivo* approach per se, it's worth noting that recent technical advances in tissue engineering have established stem cell-based organoids as a powerful experimental method to study human physiology and diseases [107,108]. Such organoid models are *in vitro* 3D cell clusters derived from embryonic cells or IPSs. In the presences of artificial extracellular matrices and suitable induction exogenous factors, these pluripotent cells form organized cell clusters that exhibit similar functionalities as many *in vivo* tissues, such as liver, kidney, brain and intestine [107,108]. These organoid systems recapitulate important biological processes involved in tissue physiological functions, including cellular mechanisms, cell-cell interactions and cell-matrix interactions. Organoids are more physiologically relevant than *in vitro* 2D cell systems and more easily adaptable to biological or genetic manipulation than *in vivo* animal models [109]. As a near-physiological model system, organoids can serve a complementary approach to study physiological roles of non-conserved lincRNAs.

For clinical translation and study designs to study human lincRNAs, we face unavoidable challenges and uncertainties, including that (a) RNA sequencing (RNA-seq) of human material is required to identify novel non-coding RNA that are not conserved outside primates (i.e., most human lincRNAs), (b) lincRNAs have lower expression and greater tissue specificity than protein coding mRNAs thus, relative to mRNAs, they require more complete tissue profiling and deeper RNA-seq for comprehensive and reproducible annotation in humans, and (c) low expression and RNA degradation pose difficulties for detection and measurement of lincRNA levels in body fluids such as blood and plasma and thus for querying the potential roles of secreted lincRNAs in physiology and disease.

6. Concluding remark

LincRNAs have been increasingly identified as key mediators in many CVD-related cellular and biological processes. Recent advances in gene editing technologies, such as CRISPR/Cas, are greatly facilitating research on novel genes and will enhance efforts to reveal new knowledge of lincRNA in normal physiology and disease. While different animal models of lincRNA perturbation have provided solid evidence to support important physiological roles of lincRNAs in cardiovascular health, their complex biological functions and regulatory mechanisms as well as the marked primate-specificity (> 80%) of most human lincRNAs pose great challenges yet even greater opportunities to advance lincRNA research and to reveal novel mechanisms of cardiometabolic pathophysiology and disease. Determining the physiological impact of lincRNA using *in vivo* models is crucial to understanding basic lincRNA functions and translating lincRNAs into novel diagnostic and therapeutic strategies in CVD.

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HIGHLIGHTS

- Long intergenic noncoding RNAs (lincRNA) play regulatory roles in cardiovascular health.
- Physiological interrogation of lincRNAs is challenging.
- Complementary approaches are often needed in the examination of lincRNA functions in cardiovascular diseases.

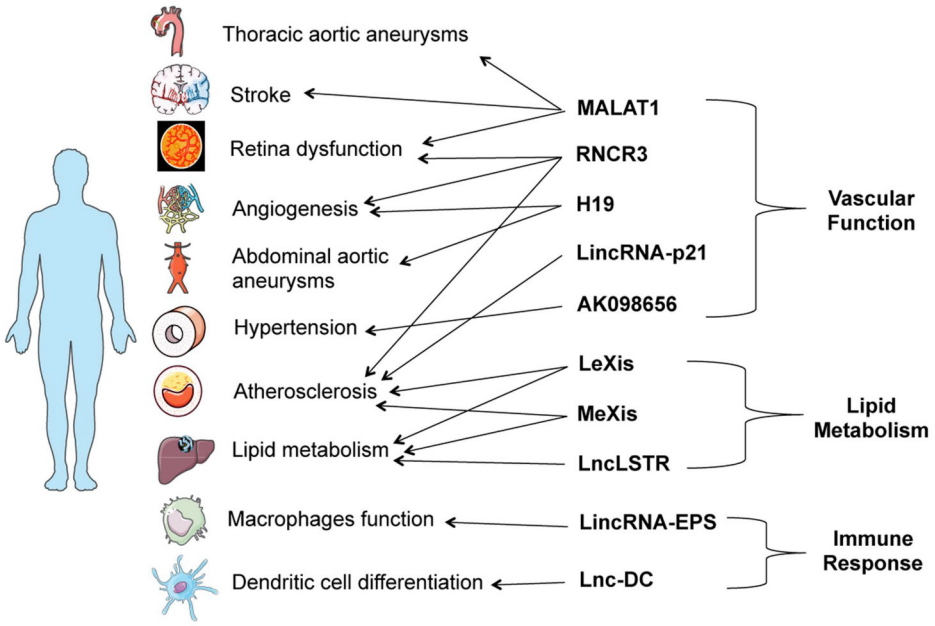

Fig. 1.

Established physiological and pathophysiological roles of lincRNAs in cardiovascular diseases.

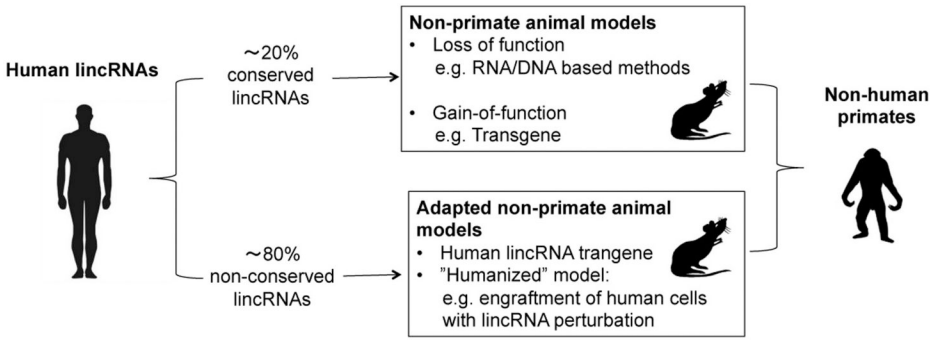

Fig. 2.

Strategies for interrogation of conserved and non-conserved human lincRNAs *in vivo*.

Table 1

Examples of lncRNA loci that harbor SNPs associated with cardiovascular diseases.[a].

lncRNA	SNPs	Functional effects	Related disease	References
ANRIL	rs1333049, rs10757278, rs2383206	Suppress expression of CDKN2A/B and regulate atherogenic pathways	Atherosclerosis	[18,19]
MIAT	Intron 1: 5338C > T (rs2331291), exon 3: 8813G > A, exon 3: 9186G > A (rs2301523), exon5: 11093 G > A, exon5: 11741 G > A, exon5: 12311 C > T	Regulate expression level of MIAT	Myocardial infarction	[20]
H19	rs217727, rs2067051	Increase expression level of IGF2	Coronary artery disease	[21]
Linc-VWF	rs1558324	Regulate expression level of VWF	Inflammation	[22]

lncRNA, long noncoding RNA; ANRIL, antisense noncoding RNA in the INK4 locus; CDKN2A/B, cyclin-dependent kinase Inhibitor 2A/B; IGF2, insulin-like growth factor 2; MIAT, myocardial infarction associated transcript; SNP, single-nucleotide polymorphism; VWF, Von Willebrand factor.

[a]Modified from Dechamethakun, J Hum Genet. 2017 [110].

Table 2

List of CVD-related lincRNA examples and their physiological impacts in animal models. Human (H) and mouse (M) genomic locations of these lincRNAs are based on human hg19 and mouse MM10 genome build.

Biological process	LincRNA	Conservation	Genomic location in human and mouse	*In vivo* perturbation	Physiological impact	Ref.
Vascular function	MALAT1	Yes	H-chr11 (+): 65265224–65273940	Genetic deletion of gene locus	Reduced endothelial cell proliferation and angiogenesis	[51]
			M-chr19(−): 5795690–5802671		Enhanced SMC contractile function and reduced thoracic aneurysm growth	[56]
	H19	Yes	H-chr11 (−): 2016406–2019065	RNA delivery by nano-vesicles	Improved wound healing in diabetic rats	[71]
			M-chr7(−): 142575530–142578146	Genetic deletion of gene locus	Reduced SMC apoptosis and development of abdominal aneurysms	[72]
	lincRNA-p21	Yes	H-chr6(−): 36631169–36635073 M-chr17(−): 29057474–29079126	RNAi-mediated knockdown	Increased neointima formation in the carotid artery injury model	[75]
	RNCR3	Yes	H-chr8(−): 9757574–9760839 M-chr14(+): 64588115–64593961	RNAi-mediated knockdown	Increased formation of atherosclerotic lesion in mouse aortas	[78]
	AK098656	Unknown	H-chr16(−):80601000–80606705	Transgenic rat model	Spontaneous hypertension, smaller diameters and reduced contractile protein expression of resistant arteries	[79]
Lipid metabolism	IncLSTR LeXis	No	M-chr1(+):151138034 −151144095	RNAi-mediated knockdown in liver	Reduced plasma triglyceride levels, increased hepatic expression of lipoprotein ApoC2	[80]
		Yes	H-chr9(−):107752367–107754061	Virus-mediated RNA overexpression	Reduced hepatic cholesterol biosynthesis, serum cholesterol and atherosclerotic lesion	[81,82]
			M-chr4(−): 53201519–53220013	Genetic deletion and lincRNA knockdown in liver	Increased lipid accumulation and expression of lipogenesis is genes in the liver	[81]
	MeXis	Yes	M-chr4(−):53261356–53265492	RNAi-mediated knockdown in liver	Increased hepatic ABCA1 expression and serum cholesterol level	[83]
			H-chr9(+):107854176–107881755	Genetic deletion of gene locus	Decreased cholesterol efflux in macrophages; increased aortic atherosclerotic lesions	[83]
Immune response	lincRNA-EPS	unknown	M-chr4(−): 109402279–109406257	Genetic deletion of gene locus	Enhanced basal and TLR4 induced expression of immune response genes in macrophages; decreased survival rate following endotoxemia challenge	[38]
	Lnc-DC	Yes	H-chr17(−): 58160927–58165828 M-chr11(+)83746940–83752646	RNAi-mediated knockdown in bone marrow	Impaired mouse dendritic cell differentiation	[84]

Table 3

Different strategies to perturb lincRNAs *in vivo* and their impacts on the endogenous lincRNA genetic loci.

Method		Perturbation of endogenous lincRNA gene		
		RNA	**DNA**	**Transcription**
RNA-based approach	RNAi or ASO	+	−	−
	Transgene for overexpression	+	−	−
DNA-based approach	Promoter deletion	+	(−)[a]	+
	Gene body deletion	+	+	+
	Transcription termination signal insertion	+	(−)[a]	+
	CRISPRi or CRISPR activation	+	−	+

+ = disrupts; − = no effect.

[a]No major disruption of DNA sequences in the lincRNA gene body.