ENTERING THE RNA WONDERLAND: OPPORTUNITIES AND CHALLENGES FOR RNA THERAPEUTICS IN THE CARDIOVASCULAR SYSTEM

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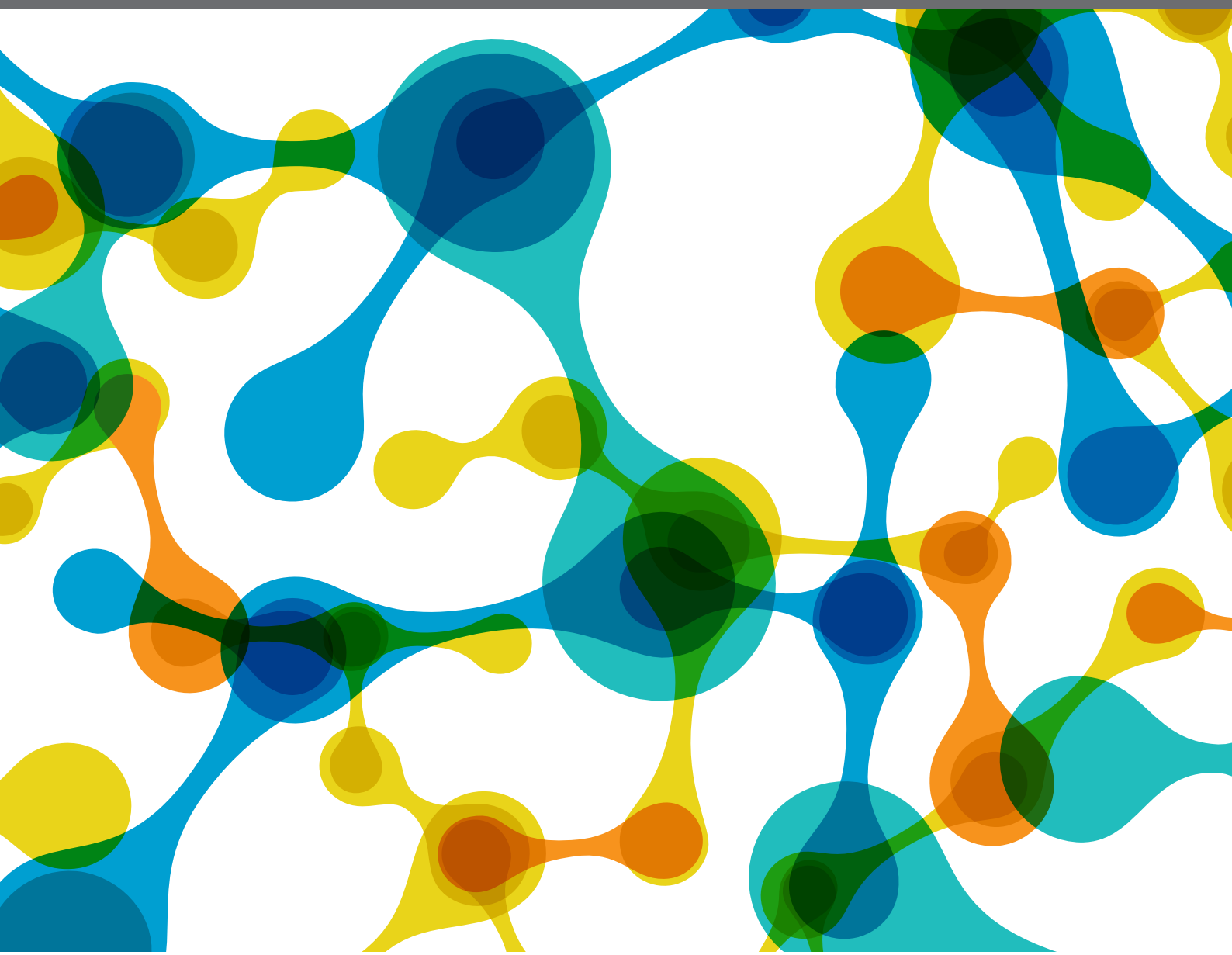

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ENTERING THE RNA WONDERLAND: OPPORTUNITIES AND CHALLENGES FOR RNA THERAPEUTICS IN THE CARDIOVASCULAR SYSTEM

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Editorial: Entering the RNA Wonderland: Opportunities and Challenges for RNA Therapeutics in the Cardiovascular System

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Editorial on the Research Topic

Entering the RNA Wonderland: Opportunities and Challenges for RNA Therapeutics in the Cardiovascular System

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The discovery that transcription is pervasive with the vast majority of the genome encoding transcripts not translated into proteins, has transformed our understanding of the basic unit of genetic information (Ulitsky and Bartel, 2013). Non-coding RNAs (ncRNAs) display distinct expression profiles in different pathologies and have sparked interest for their potential role in tissue homeostasis and disease (Zampetaki and Mayr, 2012; Thum and Condorelli, 2015). This Research Topic describes the key bioinformatic and experimental tools to identify ncRNA expression and elucidate their mode of function and focuses on the recent findings on ncRNAs in cardiovascular diseases (CVDs) and the exciting developments in RNA therapeutics.

Advances in high-throughput sequencing platforms have facilitated the identification of ncRNAs. Technologies such as RNA sequencing (RNA-seq) are powerful tools for gene expression profiling and can sequence large numbers of DNA fragments in parallel producing millions of short reads in a single run (Metzker, 2010). These massive datasets are then processed in a number of computational steps to identify the transcripts in the RNA sample and provide an estimate of their abundance in the dataset. Weirick et al. review the computational tools that can be employed with a particular emphasis on datasets derived from endothelial cells. They discuss the methods to characterize lncRNAs that are not annotated and the bioinformatic programs to detect RNA editing within RNA-seq data.

Bioinformatic analysis of RNA-seq data also revealed a novel class of lncRNA transcripts, the circular RNAs (circRNAs) that emerge by RNA "backsplicing," whereby the spliceosome fuses a splice donor site in a downstream exon to a splice acceptor site in an upstream exon (Memczak et al., 2013). They are produced in a cell-type specific manner, are stable against exonucleolytic decay and recent evidence suggests that they can exert biologically meaningful functions. Holdt et al. report on the latest findings on circRNA in CVDs, the potential therapeutic approaches based on either modulation of native circRNAs by therapeutic knockdown or by ectopic expression and the prospect of engineering non-native (artificial) circRNAs. The major hurdles for therapeutic strategies targeting circRNA in terms of design, delivery, and side effects are considered.

Once the transcripts are correctly annotated, understanding the molecular mechanism of action is a prerequisite for the development of RNA based therapeutic interventions. Intriguingly, lncRNAs demonstrate poor nucleotide sequence conservation (Hezroni et al., 2015). However,

they tend to fold into thermodynamically stable secondary structures, such as double helixes and hairpins and conservation of the secondary structure rather than the sequence was proposed to define lncRNA function. Zampetaki et al. and Zampetaki et al. elaborate on the implications and challenges in linking function and lncRNA structure to design novel RNA therapeutic approaches and the experimental tools to determine the RNA structure.

LncRNAs exert their function through interaction with DNA, RNA and proteins to form ribonucleoprotein complexes (RNP). Their interaction with RNA binding proteins (RBPs) is thought to be crucial for very diverse cellular functions (Guttman and Rinn, 2012). High throughput screening techniques and *in silico* analysis have enabled us to interrogate protein-RNA binding, identify and predict binding motifs and sequence patterns in RBP-lncRNA interactions. These platforms can provide useful insights into RNP perturbations in disease. The review of Yang et al. reports on the RBP-regulated RNA networks in diabetes. Proper function of this intricate post-transcriptional RNA network is essential for the vascular endothelium and its disruption is associated with endothelial dysfunction under diabetic conditions. The clinical implication of their manipulation and the prospect of targeting RBPs or RBP-RNA interactions as a therapeutic strategy against diabetic vasculopathy are discussed in detail.

Apart from diabetic vasculopathy, ncRNAs have a profound effect in the vasculature at baseline and in disease. Hung et al. report on the distinct mechanisms of function for microRNAs (18–23 nt) and lncRNAs and the large body of evidence demonstrating their pleiotropic effects in pathological processes in vascular diseases. The potential of ncRNAs as effectors and biomarkers in vascular pathology is critically evaluated and insights into the technical limitations in establishing a standard protocol to ensure robust reproducibility for circulating ncRNAs as biomarkers in vascular diseases are provided.

In the heart, an increasing number of studies highlight the critical regulation of lncRNAs in cardiac disorders. Hobuß et al. summarize the function of lncRNAs in the development and progression of cardiac diseases with a particular emphasis on their molecular mode of action in pathological tissue remodeling. They also examine the challenges that have to be overcome to establish lncRNA based therapies and effective intervention strategies in the heart. In addition, the prognostic and diagnostic value of lncRNAs in biological fluids as a novel class of circulating biomarkers for heart diseases and the prospect of using these molecular fingerprints to replace protein-based indicators of disease is discussed.

Extending beyond ncRNA, RNA therapeutics focus on RNA as a prime target for therapeutic applications. Laina et al. highlight the two main designs to target RNA and modulate gene expression the double-stranded small interfering RNAs (siRNAs) and single stranded antisense oligonucleotides (ASOs) their advantages and limitations. The review also summarizes results from the clinical trials of RNA-targeting interventions and elaborates on the advances and hurdles for RNA based therapeutic applications. The future prospect of RNA therapeutics to empower precision medicine implementation and fulfill the promise of patient specific therapeutics is also evaluated.

In conclusion, this Research Topic elucidates the current understanding about the mechanisms of function of ncRNA and its role in CVDs and highlights the major advancements and promising developments in RNA therapeutics. Despite the significant progress this is a new field of research and several challenges remain. Better understanding of the mechanisms of function of ncRNAs and integration of innovative approaches to enhance target binding affinity, cellular uptake and efficient *in vivo* delivery of targeting agents are required to bring RNA based therapeutics closer to the clinic.

AUTHOR CONTRIBUTIONS

AZ and LM wrote and revised the manuscript.

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MINI REVIEW
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Long Non-coding RNAs in Endothelial Biology

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In recent years, the role of RNA has expanded to the extent that protein-coding RNAs are now the minority with a variety of non-coding RNAs (ncRNAs) now comprising the majority of RNAs in higher organisms. A major contributor to this shift in understanding is RNA sequencing (RNA-seq), which allows a largely unconstrained method for monitoring the status of RNA from whole organisms down to a single cell. This observational power presents both challenges and new opportunities, which require specialized bioinformatics tools to extract knowledge from the data and the ability to reuse data for multiple studies. In this review, we summarize the current status of long non-coding RNA (lncRNA) research in endothelial biology. Then, we will cover computational methods for identifying, annotating, and characterizing lncRNAs in the heart, especially endothelial cells.

Keywords: bioinformatics, databases, lncRNAs, miRNAs, RNA-seq, RNA editing, RNA modifications

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INTRODUCTION

The development of next generation sequencing (NGS) and RNA sequencing (RNA-seq) has significantly improved the understanding of transcriptomes. For example, we now know that most of the human genome is transcribed (Lander et al., 2001), yet only a small percent of these RNAs code for protein (Weirick et al., 2016a). When the human genome was annotated (i.e., giving the definition to the genome by naming a particular gene and its corresponding exons), it was originally thought that the number of protein-coding genes in humans should be more than those of lower organisms (e.g., yeast, plants, fishes, amphibians) (Mercer et al., 2011; Ezkurdia et al., 2014). However, when the numbers of protein-coding genes are compared among species, the number of human genes is not more than those of lower organisms (**Figure 1A**). Given that humans are able to carry out more complex tasks than lower organisms, the question remains in the field: What aspect of our genome allows for the increased complexity? One school of thoughts is that proteins can be modified for various biological processes (e.g., phosphorylation of a protein for its activation). Another school suggests for the increased variety of isoforms resulting from one gene due to the alternative splicing events. In both schools, the ultimate final products are proteins as we know more about proteins than RNAs. Last school postulates that the increased number of ncRNAs (especially, lncRNAs) is at the base of the highest complexity in human, although it is highly subjective as the number of ncRNAs depends on how well the organism is studied as *C. elegans* has more lncRNAs than any other organisms (**Figure 1B**). At the moment, it is most likely that the combination of these schools of thoughts may yield important answers to the question.

In recent years, many review articles about lncRNAs in the heart are published (Geisler and Coller, 2013; Archer et al., 2015; Devaux et al., 2015; Iyer et al., 2015; Ounzain et al., 2015a; Philippen et al., 2015; Rizki and Boyer, 2015; Uchida and Dimmeler, 2015; Ballantyne et al., 2016; Busch et al., 2016; Lorenzen and Thum, 2016; Uchida and Bolli, 2017; Viereck and Thum, 2017; Sallam et al., 2018). Furthermore, there are large amounts of screening data available for lncRNAs

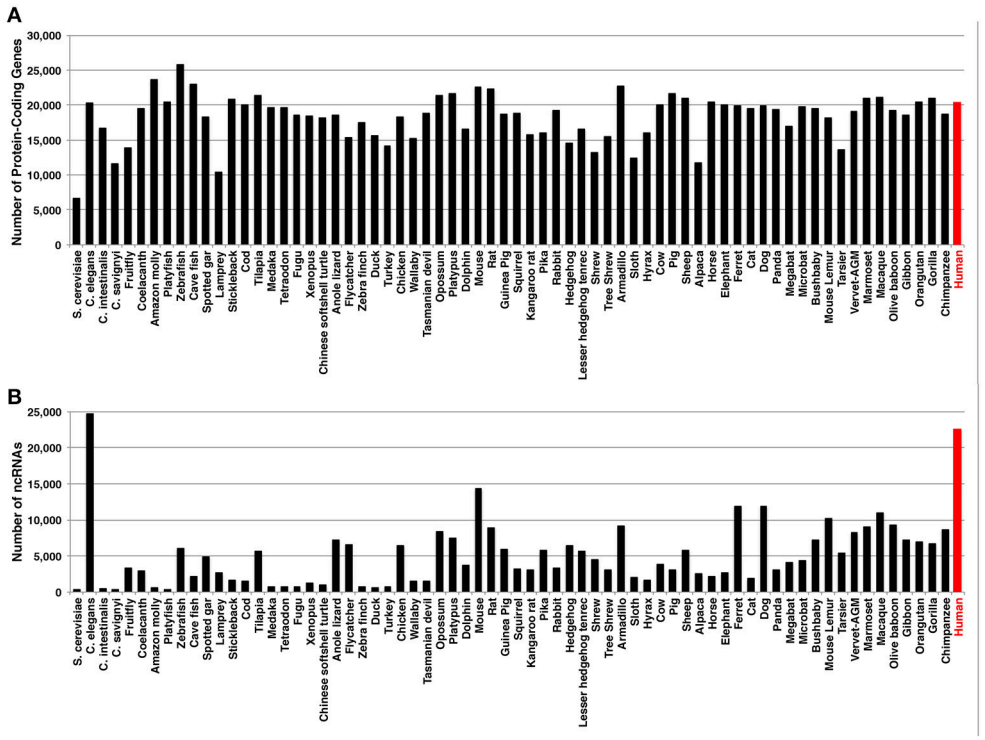

FIGURE 1 | Numbers of **(A)** protein-coding genes and **(B)** ncRNAs, including miRNAs and lncRNAs. The information is based on the Ensembl database (Accessed on May 5, 2017).

expressed in the heart (Ounzain et al., 2014, 2015b,c; Kurian et al., 2015) even single-cell RNA-seq data (Chan et al., 2016; Delaughter et al., 2016; King et al., 2017; Lescroart et al., 2018; Skelly et al., 2018). However, these data sets were mostly generated for specific purposes and have largely not been analyzed for lncRNAs. Here, we will focus on endothelial cells (ECs), an important cell type in cardiovascular medicine. Furthermore, we will cover computational methods for identifying, annotating, and characterizing lncRNAs in the heart, especially ECs.

lncRNAs IN ENDOTHELIAL CELLS

Vessels deliver metabolites and oxygen to the tissue and export waste products to sustain the well-being of an organism (Asahara et al., 2011). After tissue injury [e.g., myocardial infarction (MI)], ECs migrate to the site of injury to re-establish the capillary network through a process called "angiogenesis" (Jakobsson et al., 2010; Oka et al., 2014). Furthermore, ECs contribute to the multicellular communications that maintain the balance between the regeneration and dysfunctional or maladaptive healing (Cines et al., 1998; Libby, 2012; Kluge et al., 2013; Eelen et al., 2018). Although advances have been made to understand angiogenesis, the recent emergence of lncRNAs has added another layer of complexity to the genetic network of angiogenesis. To date, a number of lncRNAs are identified and characterized (**Table 1**) as ECs can be found throughout the human body. For example, *MALAT1* regulates endothelial cell function and vessel growth via cell cycle control (Michalik et al., 2014); the histone demethylase JARID1B controls the lncRNA *MANTIS*, which regulates EC function and vessel growth by binding to the chromatin modifying enzyme BRG1 (Leisegang et al., 2017); and several lncRNAs bind miRNAs to function as miRNA sponges (He et al., 2015, 2017; Huang et al., 2015; Yan et al., 2015; Lu et al., 2016; Ming et al., 2016; Ma Y. et al., 2017; Sun et al., 2017; Zhang B. Y. et al., 2017; Bao et al., 2018).

In addition to the above lncRNAs, we recently reported the presence of an emerging class of lncRNAs called "circular RNAs (circRNAs)" in ECs (Boeckel et al., 2015). CircRNAs are byproducts of splicing events (more specifically, "backsplicing") of mostly protein-coding genes (Jeck et al., 2013; Jeck and Sharpless, 2014; Boeckel et al., 2015), are stable and localized predominantly in the cytoplasm (Nigro et al., 1991; Cocquerelle et al., 1993). When some circRNAs are knocked down, there are phenotypes observed, which may not be observed when the parental transcripts of circRNAs are knocked down (Boeckel et al., 2015; Gerstner et al., 2016). For example, the hypoxia-regulated circRNA *cZNF292*, which is derived by backsplicing of *ZNF292* protein-coding gene, exhibits proangiogenic activities (Boeckel et al., 2015). Some studies suggest that circRNAs function as miRNA sponges (Hansen et al., 2013; Memczak et al., 2013; Geng et al., 2016; Liu Q. et al., 2016; Zheng et al., 2016). However, recent comprehensive bioinformatics analysis (Guo et al., 2014) and our biological validation experiments

TABLE 1 | List of lncRNAs in endothelial cells.

lncRNA	Organism	Tissue	Cell type	Function	References
ALT1	Human	N/A	HUVEC	Interacts with ACE2 and CUL1 to control the expression of Cyclin D1 possibly via ubiquitinatiion and degradation.	Li et al., 2017b
ASncmtRNA-2	Human; mouse	Aortas of old mice	HUVEC	Might be involved in the RS establishment by participating in the cell cycle arrest in G2/M phase, possibly through the production of *miR-4485* and *-1973*.	Bianchessi et al., 2015
GAS5	Human	Atherosclerotic plaques	HUVEC	Can be transferred from macrophages to EC in exosomes to induce apoptosis of ECs.	Chen et al., 2017
GATA6-AS	Human; mouse	Cell-based Xenograft model	HUVEC	Binds the lysyl oxidase LOXL2 to impair its function as H3K4me3 deaminase.	Neumann et al., 2018
H19	Human	Brain; Glioma tissue specimens	HBMVEC	Knockdown of *H19* suppressed glioma induced angiogenesis by inhibiting *miR-29a*, which may modulate the onset of glioma by regulating biological behaviors of glioma vascular ECs.	Jia et al., 2016
H19	Human	N/A	HUVEC	Is contained in exosomes released by CD90+ cancer cells to promote angiogenic phenotype and cell-to-cell adhesion in ECs.	Conigliaro et al., 2015
HIF1A-AS2	Human; rat	Permanent middle cerebral artery occlusion model	HUVEC	Facilitates the up-regulation of *HIF-1α* by sponging *miR-153-3p*, thereby promoting angiogenesis in hypoxia.	Li et al., 2017a
HOTAIR	Human	N/A	HBMVEC	Is contained in the glioma cell-derived extracellular vesicles and transmitted into ECs.	Ma X. et al., 2017
HOTAIR	Human	Atherosclerotic plaques	HUVEC; HAEC	Positively regulates proliferation and migration of ECs.	Peng et al., 2017
HOTTIP	Human	CAD and normal arterial tissues	HUVEC	Its overexpression induces β-catenin expression and enhances the downstream protein c-Myc expression in ECs to affect cell proliferation and migration.	Liao et al., 2018
IGF2AS	Rat	Heart	mMVE	Reciprocal regulation *IGF2AS* and *IGF2* is critical in modulating angiogenic development in myocardial tissues in type 2 diabetes.	Zhao et al., 2017
LEENE	Human; mouse	Thoracic aorta and aortic arch	HUVECs; HAoEC	Serves as a guide to facilitate RNA Pol II binding to the promoter of *eNOS*.	Miao et al., 2018
LINC00305	Human	N/A	HUVEC	Binds *miR-136* to control apoptosis.	Zhang B. Y. et al., 2017
LINC00341	Human	N/A	HUVEC	Guides EZH2 [the catalytic subunit of polycomb repressive complex 2 (PRC2)] to the promoter region of the *VCAM1* gene to suppress VCAM1.	Huang et al., 2017
LINC00657	Human	N/A	HUVEC	Binds *miR-590-3p* to attenuate the suppression of *miR-590-3p* on *HIF-1a*, and to promote angiogenesis through VEGF, MMP-2, and MMP-9.	Bao et al., 2018
lincRNA-p21	Mouse	N/A	mouse lymphoid endothelial cell line SVEC4	Binds *miR-130b* to promote cell apoptosis and induce cell cycle progression.	He et al., 2015
LISPR1	Human	N/A	HUVEC; HAoEC; HMEC	Acts as a novel regulatory unit important for S1PR1 expression and EC function.	Josipovic et al., 2018

(Continued)

TABLE 1 | Continued

lncRNA	Organism	Tissue	Cell type	Function	References
LOC100129973	Human	N/A	HUVEC	Binds *miR-4707-5p* and *-4767*, which promote apoptosis by targeting and downregulating two apoptosis inhibitors, API5 and BCL2L12, respectively.	Lu et al., 2016
MALAT1	Human	Peripheral blood from patients diagnosed with unstable angina	HUVEC	Protects the endothelium from ox-LDL-induced endothelial dysfunction partly through competing with *miR-22-3p* for endogenous RNA.	Tang et al., 2015
MALAT1	Human; mouse	Mouse retinal angiogenesis model	HUVEC	Regulates EC function and vessel growth via cell cycle control.	Michalik et al., 2014
MALAT1	Human	N/A	HUVEC	Binds *miR-320a*, which targets the pro-proliferative gene *FOXM1* for ECs.	Sun et al., 2017
MALAT1	Rat	Retina of diabetic rats	Monkey choroid, retina cell line RF/6A	Regulates EC function via p38 MAPK signaling pathway.	Liu et al., 2014
MALAT1	Human; mouse	Kidneys of diabetic mice	HUVEC	Regulates glucose-induced up-regulation of inflammatory mediators IL-6 and TNF-α through activation of SAA3.	Puthanveetil et al., 2015
MANTIS	Human	Brain microvessel isolation from glioblastoma patients	HUVEC; HAoEC; HDLEC; PAEC	Regulates EC function and vessel growth by binding to the chromatin modifying enzyme BRG1.	Leisegang et al., 2017
Meg3	Mouse; monkey	Retina of diabetic mice	Monkey choroid, retina cell line RF/6A	Activates PI3k/Akt signaling.	Qiu et al., 2016
Meg3	Rat	Brain	RBMVEC	Physically interacts with p53, which binds to the promoter of *Nox4* to regulate cell growth and the blood vessel growth factor expression.	Zhan et al., 2017
MEG3	Human	N/A	HUVEC	Is regulated by HIF-1α to maintain VEGFR2 expression in ECs and plays a vital role for VEGFA-mediated endothelial angiogenesis.	Ruan et al., 2018
MEG3	Human; mouse	Hind-limb ischemia in aged mice	HUVEC	Its silencing prevents aging-mediated inhibition of sprouting activity.	Boon et al., 2016
MEG3	Human; mouse	Circulating ECs from metabolic syndrome (MetS) patients	EPC	Protects ECs via decreasing *miR-140-5p* expression and increasing HDAC7 expression in MetS.	Liu H. Z. et al., 2016
MEG3	Human	N/A	HUVEC	Binds *miR-9* to control the proliferation and angiogenesis of ECs.	He et al., 2017
MIAT	Human; rat	Diabetes mellitus	HUVEC; HMVEC	Binds *miR-150-5p* to regulate EC function by forming a feedback loop with VEGF.	Yan et al., 2015
PUNISHER	Human; zebrafish	Heart	HUVEC	Its inhibition results in severe vascular defects in zebrafish embryos and reduced cell proliferation in HUVEC.	Kurian et al., 2015
PVT1	Human	N/A	Human cerebral microvascular endothelial cell line hCMEC/D3	Binds *miR-186*, which targets *Atg7* and *Beclin1* mRNAs.	Ma Y. et al., 2017
RNCR3/LINC00599	Human; mouse	Aortic atherosclerotic lesions	HUVEC	Forms a feedback loop with KLF2 and *miR-185-5p* to regulate EC function.	Shan et al., 2016
SENCR	Human	N/A	HUVEC	Induces proliferation, migration, and angiogenesis.	Boulberdaa et al., 2016
SIRT1 AS lncRNA	Mouse	N/A	EPC	Relieves *miR-22*-induced SIRT1 downregulation by competitively sponging *miR-22*.	Ming et al., 2016

(Continued)

TABLE 1 | Continued

lncRNA	Organism	Tissue	Cell type	Function	References
STEEL	Human	N/A	HUVEC; HMVEC	Binds the chromatin-associated enzyme PARP1 to assist its binding to the *KLF2* and *eNOS* promoters.	Man et al., 2018
TGFB2-OT1	Human	N/A	HUVEC	Binds *miR-3960*, *-4488*, *and -44459*, which target *CERS1*, *NAT8L*, and *LARP1*, respectively, the key proteins involved in autophagy and inflammation.	Huang et al., 2015
tie-1AS lncRNA	Human; mouse; Zebrafish	Zebrafish Tg(flk:EGFP)	HUVEC	Binds *tie-1* mRNA and regulates *tie-1* transcript levels, resulting in specific defects in endothelial cell contact junctions.	Li et al., 2010
uc001pwg.1	Human	Stenosed and nonstenotic uremic veins	HUVEC; EC derived from human-induced pluripotent stem cells	Its overexpression increases eNOS phosphorylation and NO production by affecting the expression level of nearby protein-coding gene MCAM.	Lv et al., 2017

Each lncRNA is listed with organism(s), tissue(s), cell type(s), and function(s) along with the corresponding reference. The abbreviations used are as follows: "coronary artery disease (CVD)"; "endothelial progenitor cells (EPCs)"; "human aortic endothelial cell (HAEC)"; "human aortic endothelial cells (HAoEC)"; "human brain microvascular endothelial cells (HBMVEC)"; "human dermal lymphatic endothelial cells (HDLEC)"; "human microvascular endothelial cells (HMEC)"; "human microvascular endothelial cells (HMVEC)"; "myocardial microvascular endothelial cells (mMVE)"; "human pulmonary artery endothelial cells (PAEC)"; "rat brain microvascular endothelial cells (RBMVEC)"; and "not applicable (N/A)."

(Boeckel et al., 2015; Weirick et al., 2016b) indicate that circRNAs functioning as miRNA sponges are extremely rare. Along with lncRNAs, more studies are necessary to uncover the functions of circRNAs in ECs.

RNA-seq DATA ANALYSIS USING BIOINFORMATICS

There are two major methods of generating libraries for RNA-seq, which are based on poly-A selection and ribosomal RNA (rRNA)-depletion. Both methods are aimed at removing rRNAs, which constitute ∼80% of total RNA followed by 15% transfer RNAs (tRNAs) and only 5% for all other RNAs, including protein-coding genes and lncRNAs (Lodish et al., 2000). The poly-A selection will result in the identification of protein-coding genes and lncRNAs with poly A tails (∼60% of total lncRNAs; Cheng et al., 2005), while the rRNA-depletion can identify the rest of lncRNAs and circRNAs—in addition to those identified in the former method. The presence of circRNAs is detected only with the latter method as circRNAs arise from exons and/or introns that are spliced out, which are devoid of poly A tails.

Analysis of RNA-seq data usually involves a number of common computational steps to obtain the expression profiles of the RNA in a set of samples. At the start of a typical analysis pipeline, reads are trimmed to remove primers and low-quality regions of reads. Next, the reads are aligned to a genome in a "guided alignment." In the case of the organism with no reference genome, a "*de novo* assembly" of the transcriptome is performed. However, *de novo* assembly is more error-prone and difficult to operate, thus we will simply focus on guided alignments. Traditionally, Tophat (Trapnell et al., 2012) has been the most popular aligner, but it is now being supplanted by newer programs (e.g., STAR, HISAT2), which offer greater speed and alignment accuracy (Engström et al., 2013; Conesa et al., 2016; Costa-Silva et al., 2017; Zhang C. et al., 2017).

Similar to protein-coding genes, lncRNAs undergo alternative splicing (AS) to produce isoforms (Deveson et al., 2017; White et al., 2017). The current understanding of AS is mainly based on EST-cDNA sequencing and short-read RNA-seq data. In the second-generation sequencing (e.g., Illumina-based short RNA-seq), long strands of cDNA must be broken into small segments to infer nucleotide sequences by amplification and synthesis (Metzker, 2010), which fall short of detecting intact full-length transcripts. To address this shortcoming, third-generation sequencing (also known as "long-read sequencing") may be a solution. PacBio RS II (Pacific Biosciences, CA, U.S.A.) is the first commercialized third-generation sequencer, which utilizes a novel single molecule real-time (SMRT) technology (Schadt et al., 2010). Compared to second-generation sequencing, SMRT technology offers long read lengths (up to 92 kb), high consensus accuracy (free of systematic sequencing errors), and low degree of bias (even coverage across G+C content) (Nakano et al., 2017). When this technology is applied to any transcriptome (cDNA) sequencing (e.g., RNA-seq), it is called "Iso-Seq," which can monitor AS (Abdel-Ghany et al., 2016). With Iso-Seq, the need for transcriptome assembly is eliminated as "one read = one transcript" with each transcript can be read from its 5′-end to poly A tail. Iso-Seq has been applied to various species and tissues (Singh et al., 2016; Cheng et al., 2017; Hoang et al., 2017a,b; Jiang et al., 2017; Jo et al., 2017; Kim et al., 2017; Kuo et al., 2017; Wang et al., 2017a,b, 2018; Xue et al., 2017; Zhang S. J. et al., 2017; Zulkapli et al., 2017; Filichkin et al., 2018) but not yet to ECs.

The largely unbiased manner in which RNA-seq captures information is another interesting aspect of the technology, which enables new findings via re-analysis of published data. For example, most of the RNA-seq studies have been focused on analyzing expression of protein-coding genes. As lncRNA are

also present in the data sets, these data offer a rich resource for studying lncRNA expression patterns. We have developed a number of bioinformatics tools to exploit these resources (Gellert et al., 2013; Weirick et al., 2015, 2016b, 2017), including some specifically designed to identify lncRNAs and to associate their expressions in various tissues and cell types, including ECs (e.g., our database ANGIOGENES; Müller et al., 2016). Although ECs can be found throughout the human body, there are only few databases available that contain the expression profiles for genes expressed in ECs (e.g., Causal Biological Network database Boué et al., 2015, dbANGIO4 Savas, 2012, and PubAngioGen Li et al., 2015). Our ANGIOGENE is one of the few that contain the expression profiles of both protein-coding genes and lncRNAs in various ECs based on RNA-seq data. Furthermore, ANGIOGENES covers humans, mice, and zebrafish to allow for the screening of lncRNAs in the positional conserved regions (not necessary sequence-conserved) (Weirick et al., 2015).

There are many transcripts whose sequencing reads are present in RNA-seq data but are not annotated in the public databases, including NONCODE (Zhao et al., 2016), which is one of the hallmark databases for lncRNAs. Our previous study (Weirick et al., 2016a) shows that 77,656 novel isoforms of annotated reference transcripts and 102,848 intergenic transcripts are identified with 58,789 (75.70%) and 101,993 (99.17%) being predicted as non-coding, respectively, from 12 human tissues (Nielsen et al., 2014), while there are 181,434 annotated transcripts (87.13% out of 208,244 transcripts in Ensembl version 77) are expressed in at least one of 12 tissues analyzed. Although we could validate the presence of novel lncRNAs by RT-PCR experiments, many novel lncRNAs contain repetitive elements, such as microsatellites (Bidichandani et al., 1998) and short interspersed nuclear elements (SINE), including ALU elements (Häsler and Strub, 2006). Thus, it is highly recommended to consult the available methods to characterize lncRNAs (Li et al., 2014; Liu et al., 2017), including CAGE-seq to annotate the 5′-end of lncRNAs (Hon et al., 2017) and ribo-seq/ribosomal footprinting RNA-seq technology to understand the coding potential (Ruiz-Orera et al., 2014; Ji et al., 2015; Alvarez-Dominguez and Lodish, 2017) before proceeding to more functional experiments.

It is well-known that ECs are heterogeneous populations of cells as their activities and functions differ based on their physiological locations (Aird, 2012; Regan and Aird, 2012; Yuan et al., 2016). In order to understand such heterogeneity of ECs, it is important to perform single-cell RNA-seq (scRNA-seq) instead of bulk RNA-seq by using a piece of tissue or those in a culture dish. As the technique for scRNA-seq matures, the immediate problem is the data analysis, especially positioning each cell to a particular cell type in order to organize their molecular signatures matching to the anatomical location in which each cell was isolated from. For example, hearts contain multiple cell types (e.g., cardiomyocytes, ECs, fibroblasts, pericytes, and smooth muscle cells). In regards to ECs, their expression profiles may differ for those contained in the artery and vein. When such profiles are compared to ECs from other tissues (e.g., kidneys, lungs), there are some genes that are expressed at the similar level in all tissues while others are expressed specifically in ECs isolated from a particular tissue. In order to understand such hierarchical organization of cells, their corresponding cell types, and tissues, it is utmost importance that the ontology of each cell must be organized in relation to its corresponding cell type and tissue. To achieve this hierarchical and ontological organization, we recently introduced the usage of logic programing (Weirick et al., 2016b), which was applied to kidneys. Logic programming is a programming paradigm based on formal logic, using a set of logical sentences consisting of facts, rules, and queries (Eklund and Klawonn, 1992). For example, consider a transcript expressed in the renal cortex. The renal cortex is located within kidneys. When sequencing whole kidney under the same condition, the same transcript should be expressed. One could even descend to the level of cell types (e.g., ECs isolated from interlobular arteries, which are located within the kidney cortex). Similarly, all sequences expressed within these ECs are expressed in the kidney. Furthermore, it is well-known that high abundance sequences can overwhelm lower abundance sequences. Thus, logic programming can be useful for integrating RNA-seq data at different hierarchical levels and beyond. This can be accomplished by: (1) modeling the anatomical and experimental relationships; (2) creating rules to define various types of expression characteristics; and (3) using queries to determine expression characteristics of a given RNA. The analysis of RNA-seq data of ECs in the heart for lncRNAs, coupled with logic programming, should help to facilitate the further usage of the available RNA-seq data (e.g., single cell RNA-seq data from the heart) to test various hypotheses that were not originally intended when the data were generated. Such an approach should yield the identification of lncRNAs in a variety of conditions (e.g., expressed in atherosclerotic plaques but not in the healthy artery), which can be further validated in functional studies.

DETECTION OF RNA EDITING PATTERNS FROM RNA-seq DATA

In addition to studying lncRNAs, re-analysis of publicly-available RNA-seq data is also useful for studying RNA editing. RNA editing is a post-transcriptional modification to alter the sequence of RNA molecules (Keegan et al., 2001; Hideyama and Kwak, 2011). The full extent and reasons for RNA editing is largely unknown. However, recent studies show that the editing in exons leads to an amino acid substitutions from altered codons (Alon et al., 2015; Liscovitch-Brauer et al., 2017), whereas editing in 3′-untranslated regions (UTRs) may affect binding of RNA binding proteins (RBPs) or microRNAs (miRNAs) thereby modulating RNA stability and/or translation (Keegan et al., 2001). There are two types of RNA editing: adenosine to inosine (A-to-I) and cytidine to uridine (C-to-U). A-to-I is the most common form and occurs through RNA editing enzymes called "adenosine deaminases acting on RNA (ADARs)," which convert adenosine in double-stranded RNA into inosine (Savva et al., 2012). When reverse transcribed to complementary DNA (cDNA), an inosine is converted to guanine ("G"), which can be identified by comparison to the reference genome. A number

of studies have been conducted to detect RNA editing events from RNA-seq data (Bahn et al., 2012; Park et al., 2012; Peng et al., 2012; Ramaswami et al., 2012, 2013; Solomon et al., 2013), including our recent study in ECs (Stellos et al., 2016). Because of the detection from RNA-seq data, several databases for RNA editing events have been constructed to provide evidence for the frequency of RNA editing in various conditions (Kiran and Baranov, 2010; Picardi et al., 2011, 2017; Laganà et al., 2012; Ramaswami and Li, 2014; Solomon et al., 2016; Gong et al., 2017). We recently reported that cathepsin S (*CTSS*), which encodes a cysteine protease associated with angiogenesis and atherosclerosis, is highly edited (Stellos et al., 2016). Such RNA editing enables the recruitment of stabilizing RBP human antigen R (HuR) to the 3′-UTR of *CTSS* transcript, thereby controlling *CTSS* mRNA stability and expression. The RNA editing enzyme ADAR1 levels and the extent of *CTSS* RNA editing are associated with changes in CTSS levels in patients with coronary artery diseases. Our study highlights the involvement of RNA editing in cardiovascular diseases, which has not yet been investigated (Uchida and Jones, 2018). Our finding was further supported by the recent large-scale, multi-center study analyzing RNA-seq data from the NIH Common Fund's Genotype-Tissue Expression (GTEx) program, which reported that the aorta, coronary, and tibial arteries were the most highly edited tissue type among 53 body sites from 552 individuals analyzed (Tan et al., 2017).

In humans, RNA editing occurs mostly in repetitive Alu regions (Levanon et al., 2004; Peng et al., 2012), which can be found in lncRNAs as lncRNAs can also be edited (Picardi et al., 2014; Szczesniak and Makalowska, 2016; Gong et al., 2017). Although proposed but not tested extensively, the functions of lncRNAs may depend on their conformation (e.g., 3D structures), which can be affected by their primary sequences. This folding process can be influenced by a variety of factors, including (but not limited to) RNA modifications on lncRNAs, such as RNA editing. Given that RNA editing can be readily detected from RNA-seq data, more systematic analysis of RNA editing patterns is necessary, especially targeting lncRNAs in the heart (Uchida and Jones, 2018). For this purpose, several bioinformatics tools are available to detect editing within RNA-seq data, including GIREMI (Zhang and Xiao, 2015), JACUSA (Piechotta et al., 2017), RED (Sun et al., 2016), RED-ML (Xiong et al., 2017), REDItools (Picardi and Pesole, 2013), RES-Scanner (Wang et al., 2016), and our RNAEditor (John et al., 2017).

HOW COULD WE TRANSLATE THE CONCEPT OF lncRNAs INTO RNA THERAPEUTICS

The one obvious usage of lncRNAs in medicine is using lncRNAs as diagnostic biomarkers as lncRNAs are more cell-type specifically expressed than protein-coding genes (Thurman et al., 2012; Gellert et al., 2013; Necsulea et al., 2014; Weirick et al., 2015). Although some progresses have been made, most of RNA-seq data analyzed so far does not consider lncRNAs due to the reasons mentioned above. Thus, without performing further RNA-seq experiments, it should be feasible to discover lncRNAs that capable of differentiating between diseased and healthy individuals by re-analyzing publicly-available RNA-seq data. For this purpose, bioinformatics tools mentioned above should be useful.

AUTHOR CONTRIBUTIONS

All authors made contributions to survey the current status of lncRNA research. All authors approved the final version of this manuscript.

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REVIEW
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RNA Therapeutics in Cardiovascular Precision Medicine

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Since our knowledge on structure and function of messenger RNA (mRNA) has expanded from merely being an intermediate molecule between DNA and proteins to the notion that RNA is a dynamic gene regulator that can be modified and edited, RNA has become a focus of interest into developing novel therapeutic schemes. Therapeutic modulation of RNA molecules by DNA- and RNA-based therapies has broadened the scope of therapeutic targets in infectious diseases, cancer, neurodegenerative diseases and most recently in cardiovascular diseases as well. Currently, antisense oligonucleotides (ASO), small interfering RNAs (siRNAs), and microRNAs are the most widely applied therapeutic strategies to target RNA molecules and regulate gene expression and protein production. However, a number of barriers have to be overcome including instability, inadequate binding affinity and delivery to the tissues, immunogenicity, and off-target toxicity in order for these agents to evolve into efficient drugs. As cardiovascular diseases remain the leading cause of mortality worldwide, a large number of clinical trials are under development investigating the safety and efficacy of RNA therapeutics in clinical conditions such as familial hypercholesterolemia, diabetes mellitus, hypertriglyceridemia, cardiac amyloidosis, and atrial fibrillation. In this review, we summarize the clinical trials of RNA-targeting therapies in cardiovascular disease and critically discuss the advances, the outcomes, the limitations and the future directions of RNA therapeutics in precision transcriptomic medicine.

Keywords: cardiovascular precision medicine, RNA therapy, antisense oligonucleotides, ASO, silence interfering RNA, siRNA, aptamer, microRNA

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death and disability in developed countries despite advances in risk stratification strategies and treatment (Benjamin et al., 2017). Thus, the need for developing novel therapeutic strategies remains a major challenge in cardiovascular medicine. Several lines of evidence have expanded our understanding of RNA function beyond its role as an intermediate molecule between DNA and proteins to a dynamic and versatile regulator of gene expression (Kapranov et al., 2007; Mercer et al., 2009). Today, we know that RNA is

edited (Stellos et al., 2016), modified (Stellos, 2017b), forms secondary and tertiary structures (Cate, 2016) and undergoes a tight, dynamic and in some cases reversible post-transcriptional regulation by a plethora of RNA-binding proteins (Cate, 2016; Stellos et al., 2016; Stellos, 2017b). To this end, RNA-targeting therapies are currently under clinical development by biotechnology companies expanding the range of "drug-able" targets. Small interfering RNAs (siRNAs) and microRNAs (miRNAs), the endogenous regulators of gene silencing, have been investigated as potential therapeutic agents. Synthetic siRNAs are used to inhibit the expression of the mRNA target, while miRNA-based therapeutics comprise miRNA inhibitors and miRNA mimics that antagonize and mimic the function of an endogenous miRNA, respectively. Synthetic antisense oligonucleotides and aptamers, a new class of either short DNA or RNA oligonucleotides, are also used to target the RNA. To date, RNA-targeting therapies are already being applied in various diseases including cancer (Moreno and Pego, 2014), infectious (Schluep et al., 2017), and neurodegenerative diseases (Scoles et al., 2017) as well. Nevertheless, RNA-targeting therapeutic modalities merit various chemical modifications prior to achieving greater stability and specificity, improved potency, and decreased toxicity (Kole et al., 2012; Wittrup and Lieberman, 2015).

The therapeutic potential of RNA-targeting therapies in the context of cardiovascular disease therapeutics is currently explored in multiple clinical trials. This review focuses on two approaches used to therapeutically target RNA, that is siRNA and ASOs, and summarizes the clinical trials of RNA-targeting therapies in cardiovascular diseases. Further, we critically discuss the advances, the outcomes, the limitations and the future directions of RNA therapeutics in precision transcriptomic medicine.

MECHANISMS OF ACTION AND CHEMICAL MODIFICATIONS OF DNA- AND RNA-BASED THERAPIES TARGETING RNA MOLECULES

Therapeutic targeting of RNA ("transcriptomic medicine") is currently based on two main approaches: single-stranded antisense oligonucleotides (ASO) and double-stranded RNA-mediated interference (RNAi). Below, we discuss the mechanisms of action of RNA-targeting therapies and chemical modifications introduced to improve drug design.

Antisense Oligonucleotides (ASOs)

Antisense oligonucleotides comprise a promising class of synthetic agents designed to modulate gene expression (Shen and Corey, 2018). They are short, typically 20 base pairs (bp) in length, single-stranded DNA based oligonucleotides which inhibit protein translation by binding to the target mRNA in a sequence-specific manner via Watson-Crick base-pairing (Shen and Corey, 2018). Antisense oligonucleotides target various classes of nucleic acids inside the cell (pre-mRNA, mRNA, non-coding RNA). ASOs have been developed to exert various mechanisms of action depending on the location of hybridization and ASOs' chemical properties (Chan et al., 2006). ASOs inhibit protein production mainly through stimulation of RNAase H activity, which in turn results in target mRNA degradation (ASO "Gapmers") (Crooke, 1999). ASOs can also induce alternative splicing by preventing binding of splicing factors (Dominski and Kole, 1993; Havens and Hastings, 2016), resulting in translational arrest through ribosome attachment blocking (steric hindrance) (Crooke, 1999; **Figure 1**).

Antisense oligonucleotides are subject to chemical modifications that can be utilized to improve their pharmacodynamic and pharmacokinetic properties (Crooke et al., 2017). Obstacles that have to be overcome are: (i) instability and degradation by nucleases, (ii) low cellular uptake and poor delivery to the tissues, (iii) inadequate binding affinity to target mRNA, and (iv) off-target effects and toxicity (Kole et al., 2012). To this purpose, phosphorothioate (PS) linkages between the nucleosides that form the backbone were introduced in replacement to phosphodiester bond generating the first generation ASOs (Crooke et al., 2017). Phoshodiester linkages are hydrophilic and highly charged, thus vulnerable to rapid degradation by nucleases. In contrast, PS linkages confer increased stability against nucleases and improve serum protein binding, thus facilitating tissue distribution and increasing ASOs pharmacokinetic profile (Dowdy, 2017). Sub-optimal affinity of the target mRNA leading to low potency was addressed by second and third generation ASOs, where 2′-O-Methyl (2′-OMe), 2′-O-methoxyethyl (2′-OMOE), and Locked Nucleic Acids (LNAs) are the leading types of chemical modifications, respectively (Wahlestedt et al., 2000). Collectively, chemical modifications result in improved stability and selective binding, thus enabling efficient delivery (Freier and Altmann, 1997).

Small Interfering RNAs (siRNAs)

RNA interference (RNAi) is a highly conserved natural process present in most eukaryotic cells in which double-stranded (ds) RNA molecules silence the post-transcriptional expression of specific genes (Siomi and Siomi, 2009). Small interfering RNAs (siRNAs) and microRNAs are small non-coding RNAs consisting major mediators of the RNAi process. siRNAs have been used as synthetic mediators of RNAi specifically designed to silence the expression of target genes (Siomi and Siomi, 2009). Unlike ASOs which are single-stranded oligodeoxynucleotides, siRNAs are double-stranded RNA molecules ranging in length from 19 to 25 bp. After they are exogenously transfected into the cell, either in a short form or in the form of long dsRNA molecules, they are further incorporated into the RNAi machinery (Siomi and Siomi, 2009). Long dsRNAs, transfected in low concentrations to prevent immune response through activation of the interferon pathway are cleaved by Dicer, a dsRNA-specific ribonuclease, into 21–25 nucleotide-long double-strand siRNAs with 2 nucleotides in their 3′ overhang and 5′ phosphate groups. siRNAs are then recognized by the Argonaute 2 (AGO2) and RNA-induced silencing complex (RISC) and unwind into their single strand components (Sledz et al., 2003; Liu et al., 2004; Meister, 2013). The sense strand is degraded and its complement (antisense) strand binds with perfect complementarity to the

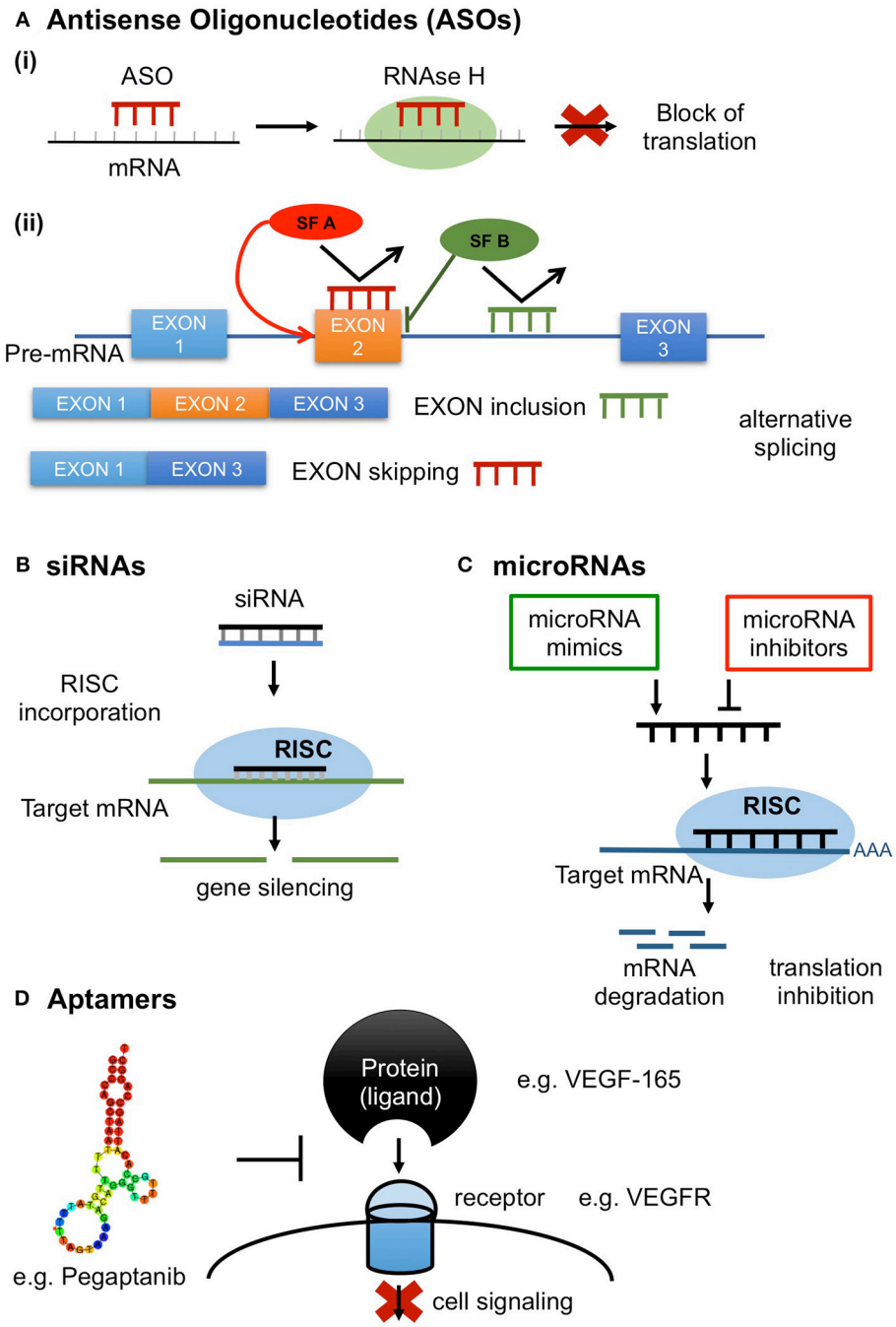

FIGURE 1 | RNA therapeutics in action. **(A)** Antisense oligonucleotides (ASOs); short, synthetic, single-stranded oligodeoxynucleotides that modify protein expression through the following mechanisms **(Ai)** Inhibition of protein production by antisense gapmers through activation of the ribonuclease RNAase H resulting in target mRNA degradation; **(Aii)** Control of splicing by ASOs in alternative splicing. ASOs can modulate alternative splicing by preventing the binding of splicing factors (SF) resulting in translational arrest through ribosome attachment blocking; **(B)** siRNAs. Double stranded (ds) RNA is processed by Dicer, a dsRNA-specific ribonuclease, into 21–25 nucleotide-long ds siRNAs with 2 nucleotides in their 3′ overhang and 5′ phosphate groups. siRNAs are then recognized by the Argonaute 2 (AGO2) and loaded into the RNA-induced silencing complex (RISC) and unwind into their single strand components. AGO2, which is a component of RISC, cleaves the sense strand of siRNA and the antisense strand binds with perfect complementarity to the target mRNA resulting in target mRNA cleavage; **(C)** microRNAs. Induction or inhibition of gene expression by microRNA mimics or inhibitors. **(D)** Aptamers. Aptamers are single-stranded DNA or RNA molecules selected through a large oligonucleotide library, called SELEX, to bind a specific target with high selectivity and specificity. Common targets include small metal ion and organic molecules, proteins, viruses, bacteria and whole cells. Target recognition and binding involve three dimensional, shape-dependent interactions as well as hydrophobic interactions. Here is a schematic illustration of the aptamer Pegaptanib inhibiting the action of the target protein VEGF-165 by binding to its receptor VEGFR.

target mRNA sequence which is cleaved by AGO2 and degraded by exonucleases (Rand et al., 2005; Ozcan et al., 2015; **Figure 1**).

Specificity and off-target effects depend on the complementarity between an siRNA and the target gene (Jackson and Linsley, 2010). In fact, the off-target effect along with efficacy, delivery issues, immune system activation, and toxicity are challenges in siRNA therapeutic approach that have hampered their development into drug agents. Despite the fact that siRNAs are designed to silence specific targets, they could also knock down unintended genes, either through imperfect complementarity to non-targeted mRNAs or by entering the endogenous miRNA machinery (Jackson and Linsley, 2010). Poor delivery of siRNAs due to rapid degradation by circulating nucleases or rapid renal excretion has been addressed by introducing chemical modifications including PS modifications, hydrophobic ligands and encapsulation in nano carriers, in order to increase both protection and half-life enabling systemic delivery (Sledz et al., 2003; Kaczmarek et al., 2017). Chemical modifications have also been employed to inhibit innate immune system activation and release of pro-inflammatory cytokines induced by siRNAs through toll-like receptor signaling pathways (Shen and Corey, 2018).

microRNAs (miRNAs)

MicroRNAs are endogenous small non-coding RNAs which similarly to siRNAs regulate gene expression at a post-transcriptional level, with the exception that are capable of silencing multiple mRNAs and not one target like siRNAs (Lam et al., 2015). miRNA maturation is a stepwise process during which miRNA is first transcribed as primary miRNA (pri-miRNA), which in turn is processed to a loop-structured precursor miRNA (pre-miRNA) of ∼60- to 70 nucleotides (nt) by Drosha enzyme. Dicer, another RNase, cleaves the pre-miRNA yielding a miRNA duplex of ∼22-nt which forms with the RISC a complex called miRISC. The complex is then unwinding and the sense strand is discarded leaving the single-stranded miRNA to bind to the mRNA target through partial complementary base pairing, resulting in gene silencing through translation suppression (**Figure 1**). Oligonucleotides that target miRNAs are known as antagomiRs, which bind with high complementarity to miRISC preventing the binding of the complex to the mRNA target. Oligonucleotides can also be synthezised to mimic miRNA function, providing another strategy for drug development (Li and Rana, 2014). ASOs targeting microRNA undergo chemical modifications in order to improve their pharmacokinetic profile, binding and resistance from nuclease cleavage (Rupaimoole and Slack, 2017). To date only one miRNA therapeutic agent, miravirsen which is an LNA-modified DNA phosphorothioate ASO that inhibits miR-122, has been evaluated in a clinical trial (NCT01200420) in patients with chronic hepatitis C, showing prolonged dose-dependent decreased HCV RNA levels without serious adverse events (SAE) (Janssen et al., 2013). Several experimental studies are investigating the use of miRNAs as therapeutic targets in atherosclerosis, coronary artery disease and myocardial infarction and clinical trials in humans are expected to test microRNAs either as antagomiRs or microRNA mimics in cardiovascular disease (Obad et al., 2011; Rayner et al., 2011; Bernardo et al., 2012).

Aptamers

Aptamers are a new class of agents used both for diagnostic and therapeutic purposes. They are synthetic single-stranded DNA or RNA molecules selected for binding to a specific target through an iterative process called SELEX (systematic evolution of ligands by exponential enrichment) (Zhou and Rossi, 2017). Numerous aptamers have been generated to target a wide range of molecules, including small metal ion and organic molecules, proteins, viruses, bacteria and whole cells. The ability to form three dimensional interactions with their targets renders them comparable to monoclonal antibodies. In fact, they are often termed as "chemical antibodies" as they share functional capacities with monoclonal antibodies. On the other hand, aptamers have smaller size, higher affinity and selectivity as compared to monoclonal antibodies and importantly they possess a more flexible structure being able to bind to inaccessible targets for larger antibodies. Taken together, given these advantages as well as that aptamers can be synthesized *in vitro* and lack immunogenicity, these agents consist an attractive alternative therapeutic strategy to monoclonal antibodies (Zhou and Rossi, 2017). Moreover, besides binding their cognate protein, aptamers also act as antagonists efficiently inhibiting the function of a specific target. The first aptamer approved by the FDA, Macugen, is a typical aptamer antagonist (**Figure 1**). Macugen was designed to target vascular endothelial growth factor (VEGF) for the treatment of age-related macular degeneration (AMD), however did not show superior therapeutic effect than VEGF-specific monoclonal antibodies (Ng et al., 2006; Mousa and Mousa, 2010; Ferrara and Adamis, 2016).

CLINICALLY APPLICABLE TARGETS IN CARDIOVASCULAR DISEASE

Apolipoprotein B is an essential structural component of all atherogenic lipoproteins, including low-density lipoprotein cholesterol (LDL-C), responsible for LDL-C transport and removal and a major determinant of cardiovascular risk (Crooke and Geary, 2013). **Mipomersen** is a 20 nucleotide-long antisense oligonucleotide targeting apolipoprotein B (ApoB) mRNA indicated in homozygous familial hypercholesterolemia (HoFH) exerting its action by binding to ApoB mRNA and inhibiting the subsequent synthesis of the protein through RNase H activation.

The approval of mipomersen [Kynamro, Kastle Therapeutics] by FDA has paved the way towards investigating other possible targets using antisense technology in the field of CVD. One of the most promising targets is **proprotein convertase subtilisin/kexin-9 (PCSK9)**, which is responsible for reduced LDL receptor expression and uptake of LDL-C and transport into hepatocytes, resulting in elevated LDL-C circulating levels. Inclisiran is a long-acting RNAi therapeutic agent that inhibits the protein synthesis of PCSK9 and has been evaluated in a phase 2 randomized clinical trial showing promising results

(Ray et al., 2017; **Table 2**). **Apolipoprotein C3 (APOC-III)** is another target currently being evaluated in patients with familial chylomicronemia syndrome. APOC-III exerts its atherogenic action by attenuating lipolysis of triglyceride-rich lipoproteins through lipoprotein lipase (LPL) inhibition that results in increased circulating levels of very low density lipoproteins and chylomicrons (Huff and Hegele, 2013). Increased levels of APOC-III are found in patients with hypertriglyceridemia and have been causally associated with metabolic syndrome and insulin resistance (Baldi et al., 2013). On the contrary, carriers of mutations disrupting APOC-III function presented 40% lower risk for coronary heart disease compared to non-carriers (TG and HDL Working Group of the Exome Sequencing Project et al., 2014). To date, Volanesorsen is a second generation antisense oligonucleotide that has been designed to target *APOC-III* mRNA and is currently being evaluated in phase 3 clinical trials in patients with familial chylomicronemia syndrome (The APPROACH Study, The COMPASS Study) (**Table 4**).

Apolipoprotein(a) is an essential component of lipoprotein(a) which has been identified as an independent risk factor for cardiovascular disease and calcific aortic valve stenosis (Danesh et al., 2000; Capoulade et al., 2015). The atherogenic properties of Lp(a) are attributed to the LDL-like particle and apo(a) components and to the latter's homology to plasminogen, as well as to its content of pro-inflammatory oxidized phospholipids (OxPL) (Wiesner et al., 2013).

Genome-wide association studies have identified **angiopoietin-like 3 (ANGPT3) and angiopoetin-like 4 (ANGPT4)** as potential cardiometabolic therapeutic targets to reduce cardiovascular burden. Several lines of evidence support a beneficial metabolic profile in patients with loss-of-function genetic variants in the gene encoding ANGPTL3 and ANGPTL4 expressed as low levels of plasma LDL cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and reduced insulin resistance (Robciuc et al., 2013; Dewey et al., 2016). An ASO to ANGPTL3 has been developed and recently investigated in human volunteers in a phase 1 clinical trial (Graham et al., 2017; **Table 1**).

Coagulation factors are currently being investigated as potential therapeutic targets and anti-coagulant aptamers have been designed and tested in patients with coronary artery disease. **REG1** is a two-component system: RB006 is a single-stranded, nucleic acid aptamer and comprises the anticoagulant component inhibiting **IX factor** (Rusconi et al., 2002). Its action is reversed by the antidote component of the REG1, RB007 that binds and neutralizes RB006. Phase 2 clinical trials have evaluated the efficacy of REG1 in patients with stable CAD or ACS undergoing percutaneous coronary intervention (PCI) compared to unfractionate heparin, reporting a beneficial bleeding profile along with reduced thrombotic complications in patients receiving the aptamer nucleic acid agent (Cohen et al., 2010; Povsic et al., 2014; **Table 2**). However, a subsequent study comparing the efficacy between REG1 anticoagulation system and bivalirudin in patients undergoing PCI in terms of periprocedural ischemic complications and major bleeding was terminated due to severe allergic reactions reported in subjects receiving the RNA aptamer (Ganson et al., 2016; Lincoff et al., 2016). Of note, REG1 was not associated with reduced bleeding and ischemic events compared to bivalirudin (Lincoff et al., 2016). **ARC-1779** is a 39-nucleotide modified DNA aptamer designed to target **von Willebrand factor** (Gilbert et al., 2007) assessed in phase 2 studies in patients, undergoing carotid endarterectomy and patients with acute myocardial infarction undergoing PCI, respectively. However, both trials have been terminated (NCT00742612 and NCT00507338) due to slower enrolment than expected and unfeasible mode of drug administration, respectively**.** Similarly, **NU172** is a 26-nucleotide unmodified DNA aptamer targeting **thrombin.** Results from a phase 1 study provide initial evidence that this agent could achieve reversal of anticoagulation without the need of an antidote. An open-label phase 2 clinical trial has been conducted to evaluate the therapeutic efficacy of NU172 in patients undergoing CABG (NCT00808964). Its outcome is currently unknown.

Chemokine C-C motif-ligand 2 (CCL2) is a pro-inflammatory cytokine involved in the development of insulin resistance and macrophage infiltration and recent evidence supports a role of CCL2 in diabetic nephropathy (Carr et al., 1994). A CCL2 antagonizing L-RNA aptamer (Spiegelmer) was found to improve renal function in experimental studies and after confirming a safety profile in a phase 1 clinical trial in humans, emapticappegol (NOX-E36) was evaluated in a phase 2a study in patients with type 2 diabetes mellitus and albuminuria (**Table 2**). Patients treated with emapticappegol presented a trend toward reduced urinary albumin excretion and HbA1c, suggesting a promising role of this CCL2 inhibitor in both kidney disease and diabetes mellitus (Menne et al., 2017).

Elevated levels of **C-reactive protein** (CRP) are associated with high cardiovascular risk (Strandberg and Tilvis, 2000) and could consist a potential target in RNA precision medicine. ISIS-CRPRx is an ASO complementary to the coding region of the human CRP mRNA and in a phase 1 double-blind placebo-controlled study was administered in healthy volunteers during the acute-phase response to endotoxin challenge (Noveck et al., 2014). Pre-treatment with ISIS-CRPRx attenuated the expected endotoxin induced increase in CRP levels in a dose-dependent manner. The antisense agent was well-tolerated in all doses tested (Noveck et al., 2014). Subsequently, this second generation ASO was assessed in a phase 2 clinical trial in patients with paroxysmal atrial fibrillation and an implanted dual chamber permanent pacemaker. The rationale of the clinical trial was based on the association between atrial fibrillation (AF) and inflammation and evidence supporting increased CRP levels as a risk for AF development and perpetuation (Dernellis and Panaretou, 2004; Marcus et al., 2010; Liu et al., 2011; Pena et al., 2012). However, no reduction in AF burden was observed in patients treated with ISIS-CRPRx despite substantial decrease in CRP levels (Sugihara et al., 2015). The potential anti-inflammatory effect of ISIS-CRPRx has also been explored in rheumatoid arthritis in a phase 2 clinical trial showing a dose-dependent reduction of high sensitivity CPR (hs-CRP) at 36 days. In specific, the group receiving 400 mg ISI-CRPRx demonstrated a decrease equal to 76.7% in hs-CRP compared with a 14.4% decrease in the placebo group at 36 days which was lost by day 92 (Warren et al., 2015).

TABLE 1 | Phase 1 randomized clinical trials of RNA therapeutics in cardiovascular disease.

Study registration number (ref)	Condition	Intervention arm	Comparator arm	Primary endpoint	Outcomes: % change from baseline (95% CI)		Adverse events
					Placebo	Study drug	
APOC-III mRNA							
(Graham et al., 2013)	Healthy volunteers	Volanesorsen ISIS 304801 200 mg/weekly ($n = 3$) **doses 50, 100 and 400 mg/weekly tested	Placebo ($n = 4$)	% change from baseline in apoC-III and TG 1 week post-treatment	ApoC-III: −11* TG:28.5*	ApoC-III: −70.5* TG: −43.1*	**Injection site reaction** Plc: 0% Volanesorsen: 52% **Elevated CRP** Plc: not reported Volanosersen: 28%
PCSK9 mRNA							
(Fitzgerald et al., 2014) NCT01437059	Healthy adults with LDL>115 mg/dl	ALN-PCS 0.4 mg/kg single dose ($n = 6$) **doses 0.015, 0.045, 0.09, 0.150, 0.250 mg/kg tested ($n = 24$)	Placebo ($n = 8$)	Safety and tolerability	PCSK9: −8.7* LDL-C: −24*	PCSK9: −58.6*,† LDL-C: −36.1*,†	**Rash** Plc: 50% ALN-PCS: 50% **Headache** Plc:25% ALN-PCS: 20.8%
(Fitzgerald et al., 2016) NCT02314442	Healthy adults with LDL>100 mg/dl TG<400 mg/dl or statin therapy	ALN-PCS 300 mg/month ($n = 12$) **doses 125, 250, 500 mg at single or multiple doses infusion tested	Placebo ($n = 17$)	Safety, pharmacokinetics and lipid parameters 4 weeks post-treatment	PCSK9: −0.6 (−24.2 to 30.4) (sd, $n = 6$) LDL-C: −10.9 (−26.0 to 7.1) (sd, $n = 6$) PCSK9: 16.9 (−2.4 to 40.0) (dd, $n = 11$) LDL-C: −14.2 (−30.2 to 5.5) (dd, $n = 11$)	PCSK9: −74.5 (−82.1 to −63.6) (sd, $n = 3$) LDL-C: −50.0 (−60.7 to −36.3) (sd, $n = 3$) PCSK9: −71.8 (−77.4 to −64.8) (dd no statin, $n = 6$) LDL-C: −59.7 (−68.7 to −48.1) (dd no statin, $n = 6$) PCSK9: −79.9 (−85.4 to −72.5) (dd with statin, $n = 6$) LDL-C: −45.1 (−61.6 to −21.4) (dd with statin, $n = 6$)	**Cough, Musculoskeletal pain, Nasopharyngitis** Plc: 0 ALN-PCS: 2 (11.1%) **Headache** Plc: 2 (16.7%) ALN-PCS: 6 (18.2%) **Backpain** Plc: 2 (16.7%) ALN-PCS: 5 (15.2%) **Diarrhea** Plc: 3 (25.0%) ALN-PCS:4 (12.1%) **Nasopharyngitis** Plc: 1 (8.3%) ALN-PCS: 5 (15.2%)
(van Poelgeest et al., 2015) NCT01350960	Healthy adults with LDL-C>100 mg/dl	SPC5001 siRNA 5 mg/kg ($n = 6$) **doses 0.5 and 1.5 mg/kg tested	Placebo ($n = 6$)	Safety, pharmacokinetics and lipid parameters 2 (PSCK9) or 3 weeks (LDL-C) post-treatment	PCSK9: −49.0 (−58.5, −37.2)¥ LDL-C: −0.36 (−0.75 to 0.03)¥		**Injection site reaction** Plc: not reported SPC5001:44% **Headache** Plc: 33% SPC5001: 61% **Tiredness** Plc: 17% SPC5001: 56% Renal tubular toxicity (4/6 highest dose)

(Continued)

TABLE 1 | Continued

Study registration number (ref)	Condition	Intervention arm	Comparator arm	Primary endpoint	Outcomes: % change from baseline (95% CI)		Adverse events
					Placebo	Study drug	
APO(a) mRNA							
(Tsimikas et al., 2015) 2012-004909-27	Healthy adults With Lp(a)>100 mg/L	ISIS-APO(a)Rx 200 mg ($n = 12$) **doses 50, 100, 300, and 400 mg at single or multiple doses tested	Placebo ($n = 4$) (sd) ($n = 6$) (md)	% change in Lp(a) 4 weeks (single) or 12(md) post treatment	Lp(a): # (single dose) Lp(a): −59*,†,¥ (multiple doses)		2 discontinuations due to injection site adverse event and flu-like syndrome
(Viney et al., 2016) NCT02414594	Healthy adults with Lp(a)>75 nmol/L	APO(a)-LRx 120 mg single dose ($n = 6$) and 40 mg ($n = 8$) for multiple doses **doses 10, 20, 40 and 80 mg for single dose and 10 and 20 mg at multiple doses tested	($n = 13$)	%change in Lp(a), safety and tolerability One (single dose) or two (multiple doses) weeks post-treatment	Lp(a): −84.5 (−112.6 to −65.2) (single dose)¥ Lp(a): − 82·4% (−99.8 to −67.6) (multiple dose)¥		No SAE, local injection-site reactions, influenza-like symptoms, or other safety issues
ANGPTL3 mRNA							
(Graham et al., 2017) NCT02709850	Healthy adults with LDL-C>70 mg/dl and TG>90 mg/dl	ANGPTL3-LRx 80 mg (single dose) ($n = 3$) and 60 mg (multiple doses) ($n = 6$) **doses 10, 20 and 40 mg at single or multiple doses tested	Placebo ($n = 4$) (single dose) ($n = 8$) (multi doses)	Safety, pharmacokinetics pharmacodynamics 1 week post- treatment	ANGPTL3: 0.3±17.4* LDL-C: −0.3±24.5* (single dose) ANGPTL3: −1.6± 15.4* LDL-C: 13.6±12.1* (multiple doses)	ANGPTL3: −61.7±1.1* LDL-C: −24.0±5.9* (single dose) ANGPTL3: −84.5± 5.1*,† LDL-C: −32.9± 10.4*,† (multiple doses)	**Headache** Plc: 1 ANGPTL3-LRx:2 **Dizziness** Plc: 2 ANGPTL3-LRx: 1 No SAE

LDL-C, low-density lipoprotein cholesterol; apoB, apolipoprotein B; TC, total cholesterol; Lp(a), lipoprotein a; CI, confidence intervals; PCSK9, proprotein convertase subtilisin/kexin type 9; ANGPTL3, angiopoietin-like 3; sd, single dose; dd, double dose; md, multi dose; Plc, placebo; SAE, serious adverse events.

**95% CIs for % change are not provided. Standard deviation or interquartile range (25 to 75th percentile) are shown where available.*

*** Indicate different dosage scheme.*

† Indicates observed statistical significance <0.05.

¥Indicates relative changes in treatment group as compared to placebo.

#No numeric estimates are provided.

TABLE 2 | Phase 2 randomized clinical trials of RNA therapeutics in cardiovascular disease.

Study registration number (ref)	Condition	Intervention arm	Comparator arm	Primary endpoint	Outcomes: % change from baseline (95% CI)	
					Placebo	**Study drug**
APOC-III mRNA						
(Gaudet et al., 2015) NCT01529424	Hypertriglyceridemia (>350 mg/dl or >225 mg/dl added to fibrate)	Volanosersen (ISIS 304801) 300 mg/weekly ($n = 21$) ** doses 100 and 200 mg/weekly have been tested	Placebo ($n = 24$)	% change in apoC-III levels from baseline to end of treatment	ApoC-III: 4.2 ± 41.7* TG: 20.1 ± 72.0* ApoC-III: 2.2 ± 25.2* TG: −7.7 ± 33.8*	ApoC-III: 79.6 ± 9.3*,† (volanosersen only, $n = 11$) TG: −70.9 ± 14.1*,† ApoC-III: 70.9 ± 13.0*,† (+fibrate, $n = 10$) TG: −64.0 ± 8.9*,†
(Digenio et al., 2016) NCT01647308	Type 2 DM and Hypertriglyceridemia	Volanesorsen 300 mg/weekly ($n = 10$)	Placebo ($n = 5$)	% change in apoC-III from baseline to end of treatment	ApoC-III:−7.3 ± 14* TG:−9.9 ± 19.9* LDL-C: −5.5 ± 7.2* ApoB: −10.4 ± 8.5* HbA1c: −0.78 ± 0.71*	ApoC-III:−87.5 ± 5.4*,† TG: −69.1 ± 10.1*,† LDL-C: 0 ± 26.3* ApoB: −20.8 ± 15.9* HbA1c: −0.44 ± 0.39*,†
(Yang X. et al., 2016)	FCS Hypertriglyceridemia	Volanosersen 300 mg/weekly ($n = 24$) **doses 100 and 200 mg/weekly tested	Placebo ($n = 24$)	% changes in apoCIII-apoB, apoCIII-apoAI, and apoCIII-Lp(a)	apoCIII-apoB: −82.3 ± 11.7*,†,¥ apoCIII- Lp(a): −81.3 ± 15.7*,†,¥ apoCIII-apoA1: −80.8 ± 13.6*,†,¥ (baseline to end of treatment)	
PCSK9 mRNA						
(Ray et al., 2017) ORION-1 NCT02597127	Hypelipidemia	Inclisiran 300 mg per quarterly ($n = 119$) ** doses 200 and 500 mg, single or double quarterly dose tested	Placebo ($n = 125$)	% change from baseline in LDL cholesterol 3 months post-treatment	LDL-C: 2.1 (−2.9 to 7.2) ($n = 64$, single dose) PCSK9: 2.2 ± 23.4* ($n = 64$, single dose) LDL-C: 1.8 (−2.6 to 6.3) ($n = 61$, double dosage) PCSK9: −1.2 ± 20.7* ($n = 61$, double dose)	LDL-C: −38.4 (−43.6 to −33.2) ($n = 60$, single dose) PCSK9: −56.0 ± 19.2*,† ($n = 60$, single dose) LDL-C: −52.6 (−57.1. to −48.1) ($n = 59$, double dose) PCSK9: −69.1 ± 12.1*,† ($n = 59$, double dose)
APO(a) mRNA						
(Viney et al., 2016) NCT02160899	Healthy adults with Lp(a)>125 nmol/L (cohort A) or Lp(a)>437 nmol/L (cohortB)	ISIS-APO(a)Rx 100–300 mg Cohort A($n = 24$) Cohort B ($n = 8$)	Placebo ($n = 29$)	%change in Lp(a), safety and tolerability end of or 2 weeks post-treatment	Lp(a): −62.8 (−71.9 to −53.8) (cohort A)¥ Lp(a): −67.7% (−80.8 to −54.5) (cohort B) ¥	
IX FACTOR						
(Povsic et al., 2014) (RADAR-PCI) NCT00932100	ACS patients undergoing PCI	Pegnivacogin 1 mg/kg with 25%, 50%, 75%, or 100% anivamersen reversal ($n = 277$)	UFH ($n = 111$)	$Composite ischemic endpoint and bleeding through 30 days	Bleeding: 7% (100% reversal) vs. 11% Ischemic events: 4.4 vs. 7.3% TVR: 1.1 vs. 0.9% MI: 4 vs. 6.4% Angiographic complications: 11.2 vs. 10.8%	
CHEMOKINE C-C MOTIF-LIGAND 2						
(Menne et al., 2017)	Type 2 DM	Emapticap ($n = 50$)	Placebo ($n = 25$)	Change in urinary ACR at the end of treatment	−15%*	−29%*,†

FH, familial hypercholesterolemia; CHD, coronary heart disease; CVD, cardiovascular disease; HCL, hypercholesterolemia; LDL-C, low-density lipoprotein cholesterol; apoB, apolipoprotein B; TC, total cholesterol; Lp(a), lipoprotein a; CI, confidence intervals; PCSK9, proprotein convertase subtilisin/kexin type 9; FCS, familial chylomicronemia syndrome; ACS, acute coronary syndrome; PCI, percutaneous coronary intervention; DM, diabetes mellitus; TVR, target vessel revascularization; MI, myocardial infarction; ACR, albumin/creatinine ratio.

**95% CIs for % change are not provided. Standard deviation or interquartile range (25 to 75th percentile) are shown where available.*

*** Indicate different dosage scheme.*

† Indicates observed statistical significance <0.05.

¥Indicates relative changes in treatment group as compared to placebo.

$Death, non-fatal MI, urgent TVR, or recurrent ischemia.

Small interfering RNAs and ASOs have been developed for the treatment of **transthyretin** (TTR) amyloidosis, a progressive heart disease causing severe congestive heart failure. After siRNAs encapsulated in lipid nanoparticles were shown to successfully induce transthyretin knockdown in patients with TTR amyloidosis in a phase 1 study (Coelho et al., 2013), a subsequent phase 2 study evaluated patisiran as a potential therapeutic strategy in TTR mediated familial amyloidotic polyneuropathy (Suhr et al., 2015). The ENDEAVOUR study was a phase 3 double-blind placebo-controlled clinical trial that evaluated the safety and efficacy of revusiran in patients with TTR mediated familial amyloidotic cardiomyopathy but was withdrawn due to safety concerns (NCT02319005) (**Table 4**). Furthermore, a specific TTR antisense oligonucleotide (IONIS-TTRRx) was evaluated in an open label study examining functional and structural cardiac parameters in patients with either hereditary or wild type TTR amyloidosis. Overall, the ASO was well-tolerated slowing down the progression of disease, as expressed by reduced left ventricular wall thickness and left ventricular mass, improved global systolic strain, 6-min walk test and NYHA class (Benson et al., 2017).

RNA-TARGETING THERAPEUTICS IN RANDOMIZED CLINICAL TRIALS

Apolipoprotein B

Mipomersen

The efficacy of subcutaneous administration of mipomersen at 200 mg/weekly dosage has been explored in phase 3 clinical trials. In particular, Raal et al., reported a mean percentage reduction in LDL-C levels, of ∼25% (95% CI −31.6 to −17.7) in patients older than 12 years old with homozygous FH already receiving the maximum tolerated dose of a lipid-lowering drug, compared with a ∼3% decrease in the placebo group. Twenty-six out of thirty-four treated patients experienced injection-site reactions, and four presented a significant increase in alanine aminotransferase (Raal et al., 2010; **Table 3**).

Similar results were reported by McGowan et al., who randomly assigned 58 patients with heterozygous FH and/or coronary heart disease (CHD) to either placebo or s.c. mipomersen 200 mg/weekly on top of the maximally tolerated dose of lipid-lowering drugs. The mipomersen group presented a 36% reduction of LDL-C from a baseline of 278 mg/dL in comparison to the placebo group which presented a 13% reduction from a baseline of 250 mg/dL. Conventional hypolipidemic drugs have limited effect on Lp(a). Surprisingly, in this study mipomersen induced a significant reduction in Lp(a) compared to placebo (−33 vs. −1.5%). Adverse events included injection site reactions, alanine transaminase increase and flu like symptoms (McGowan et al., 2012). Another study of similar design and population, that is patients with heterozygous FH and coronary artery disease, reported similar results concerning percentage change of LDL-C up to week 28. In specific, mipomersen decreased mean LDL-C by 28.0% compared with 5.2% increase with placebo. Moreover, mipomersen significantly reduced apolipoprotein B (−26.3%), total cholesterol (−19.4%), and Lp(a) (−21.1%) compared with placebo. No significant change occurred in HDL-C. Adverse events included injection site reactions and flu-like symptoms (Stein et al., 2012; **Table 3**).

The efficacy of mipomersen was also investigated in hypercholesterolemic subjects of high cardiovascular risk. In specific, 158 patients on maximally tolerated lipid lowering agents statin and LDL-C>100 mg/dL were randomized into receiving placebo ($n = 53$) or mipomersen ($n = 105$) for a 26-week period. Mean % change in LDL-C was −37% with mipomersen vs. −5% with placebo. No changes in HDL-C were observed, while on the contrary significant reductions were established in apoB (38%), total cholesterol (26%) and Lp(a) (24%) (Cromwell et al., 2012). Along this line, another study evaluated the efficacy of mipomersen in patients with baseline LDL cholesterol levels>100 mg/dL with or at high risk for CHD already receiving maximally tolerated lipid-lowering therapy. Mipomersen reduced LDL cholesterol by 36.9% compared to placebo's effect of 4.5%. Target LDL cholesterol <100 mg/dl was achieved in 76% of mipomersen and 38% of placebo patients. Mipomersen conferred significant reductions in other lipid parameters as well. As in previous mipomersen studies, most common adverse events included injection site reactions (78% with mipomersen vs. 31% with placebo) and flu-like symptoms (34% with mipomersen vs. 21% with placebo) (Thomas et al., 2013; **Table 3**).

Finally, mipomersen was evaluated in high-risk patients with statin intolerance. LDL cholesterol decreased by 47.3%, with a parallel decrease in apoB by 46.2% and Lp(a) by 27.1%. Injection site reactions and flu-like symptoms were the most common adverse events resulting in 18% of the mipomersen-treated patients and 17% of the placebo-treated patients discontinuation of therapy. Among mipomersen treated patients, 33% of them presented liver function tests above three times the upper limit of normal (Visser et al., 2012; **Table 3**).

Interestingly, Duell et al. (2016) sought to assess the rate of major adverse cardiovascular events (MACE) across a follow-up period of 24 months in patients with FH having received mipomersen for at least 12 months in a *post-hoc* analysis of three RCTs and one open-label study (Santos et al., 2015). Patients after mipomersen initiation treatment experienced 13 MACE in comparison to 146 MACE identified in the 2-year period previous to the mipomersen therapy. In fact, the authors report that FH patients after mipomersen treatment initiation present 94.7% lower odds of experiencing MACE compared with the pre-treatment period.

Apolipoprotein C-III

Volanesorsen

Volanesorsen is a second-generation 2′-O-methoxyethyl chimeric ASO that is designed specifically to reduce levels of *APOC-III* messenger RNA (mRNA). Through ribonuclease H1, volanesorsen induces the degradation of the target mRNA and inhibits the production of the APOC-III protein. This glycoprotein plays a regulative role on lipoprotein metabolism and single nucleotide polymorphisms (SNPs) in the APOC-III gene are emerging as a cause of severe hypertriglyceridemia. A recent meta-analysis found evidence that two SNPs in

TABLE 3 | Phase 3 randomized clinical trials of RNA therapeutics in cardiovascular disease.

Study registration number (ref)	Phase	Condition	Intervention arm	Comparator arm	Primary endpoint	Outcomes: % change from baseline (95% CI)	
						Placebo	Study drug
APOB mRNA							
(Raal et al., 2010) NCT00607373	3	Homozygous FH	Mipomersen 200 mg/weekly ($n = 34$)	Placebo ($n = 17$)	% change in LDL-C 2 weeks post-treatment	LDL-C: −3.3 (−12.1 to 5.5) apoB: −2.5 (−9.0 to 3.9) TC: −2.0 (−9.6 to 5.6) Lp(a): −7.9 (−19.1 to 3.4)	LDL-C: −24.7 (−31.6 to −17.7) apoB: −26.8 (−32.7 to −20.8) TC: −21.2 (−27.4 to −15.0) Lp(a):−31.1 (−39.1 to −23.1)
(McGowan et al., 2012) NCT00794664	3	Heterozygous FH ± CHD	Mipomersen 200 mg/weekly ($n = 39$)	Placebo ($n = 18$)	% change in LDL-C 2 weeks post-treatment	LDL-C: 12.5 (−10.7 to 35.8) apoB: 11.4 (−6.9 to 29.7) TC: 11.2 (−6.2 to 28.5) Lp(a): −1.5 (−14.2 to 11.3)	LDL-C: −35.9 (−51.3 to −15.3) apoB: −35.9 (−43.3 to −28.4) TC: −28.3 (−34.9 to −21.7) Lp(a): −32.7 (−43.3 to −22.0)
(Stein et al., 2012) NCT00706849	3	Heterozygous FH + stable CAD	Mipomersen 200 mg/weekly ($n = 83$)	Placebo ($n = 41$)	% change in LDL-C 2 weeks post-treatment	LDL-C: 5.2 (−0.5 to 10.9) apoB: 7.02 (1.8 to12.2) TC: 3.85 (−0.2 to 7.9) Lp(a): 0.0 (−8.0 to 13.0)	LDL-C: −28.0 (−34.0 to −22.1) apoB: −26.3 (−31.2 to −21.4) TC: −19.4 (−23.7 to −15.2) Lp(a): −21.1 (−37.9 to 0.0)
(Visser et al., 2012) (ASSIST) (75) NCT00707746	3	High CVD risk Statin intolerance	Mipomersen 200 mg/weekly ($n = 21$)	Placebo ($n = 12$)	% reduction in LDL-C 2 weeks post-treatment	LDL-C: −2.0 ± 8.4* apoB: −4.3 ± 7.5* TC: −1.8 ± 6.5* Lp(a): 0.0 ± 8.6*	LDL-C: −47.3 ± 18.5,*,† apoB: −46.2 ± 19.5*,† TC: −36.9 ± 14.7*,† Lp(a): −27.1 ± 31.2*,†
(Cromwell et al., 2012) (abstract)	3	HCL and High CHD risk	Mipomersen 200 mg/weekly ($n = 105$)	Placebo ($n = 53$)	% change in LDL-C 2 weeks post-treatment	LDL-C: −5% (−11, 2)	LDL-C: −37% (−42% to −32%) apoB: −38%*,† TC: −26% Lp(a): −24% ‡
(Thomas et al., 2013) NCT00770146	3	HCL and High CHD risk ± CHD	Mipomersen 200 mg/weekly ($n = 105$)	Placebo ($n = 52$)	% reduction in LDL-C 2 weeks post-treatment	LDL-C: −4.5 ± 24.22* apoB: −4.1 ± 18.09* TC: −2.7 ± 14.58* Lp(a): 0.0 (−16.0, 17.6)*	LDL-C: −36.9 ± 26.85*,† apoB: −37.5 ± 23.59*,† TC: −26.4 ± 18.65*,† Lp(a): −25.6 (−40.0, −7.8)*,†

FH, familial hypercholesterolemia; CHD, coronary heart disease; CAD, coronary artery disease; CVD, cardiovascular disease; HCL, hypercholesterolemia; LDL-C, low-density lipoprotein cholesterol; apoB, apolipoprotein B; TC, total cholesterol; Lp(a), lipoprotein a.

**95% CIs for % change are not provided. Standard deviation or interquartile range (25 to 75th percentile) are shown where available.*

† Indicates observed statistical significance <0.05.

‡ Relative changes from baseline are not provided. Baseline and post-treatment absolute values are shown.

TABLE 4 | Ongoing clinical trials on RNA therapeutics in cardiovascular disease.

Trial registration number	Status	Study design	Phase	Condition (n)	Intervention arm	Comparator	Estimated completion date
PCSK9							
NCT03060577 (ORION-3)	Active-not recruiting	Open label	2	FH ($n = 490$)	Inclisiran	Evolocumab	January 2022
NCT02963311 (ORION 2)	Recruiting	Open-label	2	Homozygous FH ($n = 10$)	ALN-PCS	SOC	December 2018
NCT03159416 (ORION-7)	Active, not recruiting	Open-label	1	HCL and renal impairment ($n = 24$)	Inclisiran		September 2018
APOC-III							
NCT02527343 (BROADEN Study)	Active, not recruiting	RDBPC	2/3	Familial Partial Lipodystrophy ($n = 60$)	Volanesorsen	Placebo	September 2021
NCT02900027	Recruiting	RDBPC	1	Hypertriglyceridemia ($n = 56$)	APOC-III-L-Rx	Placebo	September 2017
ANGPT3							
NCT03371355	Recruiting	RDBPC	2	Hypertriglyceridemia, Type 2 DM and NAFLD ($n = 144$)	ISIS 703802		May 2019
NCT03455777	Not yet recruiting	Open-Label	2	Homozygous FH ($n = 3$)	ISIS 703802		December 2018
NCT03360747	Recruiting	Open-Label	2	FCH ($n = 3$)	ISIS 703802		September 2018
APO(a)							
NCT03070782	Active, not recruiting	RDBPC	2	Hyperlipoproteinemia(a) and CVD ($n = 270$)	ISIS 681257	Placebo	November 2018
GCGR							
NCT02824003	Active, not recruiting	RDBPC	2	Type 2 DM ($n = 15$)	ISIS-GCGRRx	Placebo	May 2017
NCT02583919	Active, not recruiting	RDBPC	2	Type 2 DM ($n = 80$)	ISIS-GCGRRx	Placebo	March 2017
Completed trials with unpublished data							
VEGF-A							
NCT02935712	Completed	RSBPC	1	CVD ($n = 44$)	AZD8601	Placebo	January 2018
APOC-III							
NCT02211209 (APPROACH Study)	Completed	RDBPC	3	FCS ($n = 67$)	Volanosersen	Placebo	March 2017
NCT02300233 (COMPASS Study)	Completed	RDBPC	3	Hypertriglyceridemia ($n = 114$)	Volanosersen	Placebo	January 2017
TTR							
NCT02319005 (ENDEAVOR)	*Terminated	RDBPC	3	Cardiac Amyloidosis ($n = 206$)	ALN-TTRSC (revusiran)	Placebo	December 2017

**Due to an imbalance of mortality in the revusiran arm as compared to placebo. RDBPC, randomized double-blind placebo controlled; RSBPC, randomized single-blind placebo controlled; PCSK9, proprotein convertase subtilisin/kexin type 9; FH, familial hypercholesterolemia; HCL, hypercholesterolemia; DM, diabetes mellitus; NFLD, non-alcoholic fatty liver disease; CVD, cardiovascular disease; Apo(a), apolipoprotein a; ApoC-III, apolipoprotein C-III; ANGPTL3, angiopoietin-like 3; GCGR, glucagon receptor; FCS, familial chylomicronemia syndrome; VEGF, vascular endothelial growth factor; TTR, transthyretin.*

APOC-III are associated with increased CHD risk. In specific two polymorphisms, SstI and T-455C, increased the odds for CHD development by up to 48 and 77%, respectively (Li et al., 2016).

In a phase 1 study, healthy volunteers received either placebo ($n = 8$) or volanesorsen (ISIS 304801) ($n = 25$) and presented a deep dose-dependent reduction up to ∼90% and up to ∼80% of APOC-III and triglyceride levels, respectively 4 weeks post-treatment (Graham et al., 2013; **Table 1**). This led to a number of phase 2 clinical trials investigating the effect of APOC-III inhibition in subjects with hypertriglyceridemia.

Volanosersen was tested both as monotherapy and as an adjunct to fibrates in a placebo-controlled RCT. When administered as a single agent in fifty-seven subjects, volanosersen resulted in a dose-dependent and prolonged reduction in both plasma apoC-III (percentage decrease of 63.8 in the 200-mg group vs. an increase of 4.2% in the placebo group) and triglyceride levels (−57.7% in the 200-mg group vs. 20.1%). Similar results were observed when it was administered as an add-on treatment to fibrates (Digenio et al., 2016; **Table 2**). In addition, a phase 2 study explored the effect of volanesorsen in subjects with hypertriglyceridemia and poorly controlled type 2 diabetes. Volanosersen apart

from significantly reducing apoC-III (−87.5% vs. −7.3%) and triglyceride levels (−69 vs. −9.9%) compared to placebo, also improved glycemic control, expressed as −0.44% reduction of HbA1c at the end of follow-up, and increased insulin sensitivity. Both findings were associated with suppression of apoC-III and triglyceride levels (Bennet et al., 2008; **Table 2**). Yang et al., using high-throughput ELISA to capture apoB, Lp(a) and apoA-I in plasma, identified significantly reduced apoC-III levels on these individual lipoproteins as apoCIII-apoB, apoCIII-Lp(a), and apoCIII- apoAI complexes in subjects who received volanosersen compared to those who received placebo (Yang X. et al., 2016; **Table 2**).

Ongoing studies with volanesorsen include the APPROACH [The APPROACH Study: A Study of Volanesorsen (Formerly ISIS-APOCIIIRx) in Patients with Familial Chylomicronemia Syndrome, NCT02211209] trial, the COMPASS [The COMPASS Study: A Study of Volanesorsen (Formally ISIS-APOCIIIRx) in Patients with Hypertriglyceridemia, NCT02300233] trial, and the BROADEN [The BROADEN Study: A Study of Volanesorsen (Formerly ISIS-APO-CIIIRx) in Patients with Partial Lipodystrophy, NCT02527343] trial (**Table 4**).

PCSK9

Treatment with PCSK9 inhibitors, evolocumab and alirocumab, reduce LDL-C levels by ∼60% (Desai et al., 2017). Interestingly, the FOURIER (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk) study has shown additional cardiovascular benefit exerted by PCSK9 inhibition besides lipid lowering effect. Evolocumab significantly reduced the risk of the composite endpoint of cardiovascular death, myocardial infarction, stroke, hospitalization for unstable angina, or coronary revascularization by 15% as compared to placebo matched patients (HR 0.85, 95% CI 0.79–0.92) (Sabatine et al., 2017). According to recently released results from meta-analysis of 35 randomized controlled trials PCK9 inhibition is not associated with improved all-cause (OR 0.71, 95% CI 0.47–1.09) or cardiovascular mortality (OR 1.01, 95% CI 0.85–1.19). However, a metaregression analysis revealed an association between higher baseline LDL-C and an all-cause mortality benefit (Karatasakis et al., 2017).

Inclisiran

Inclisiran (formerly known as ALN-PCS) is an investigational GalNAc-conjugated siRNA targeting PCSK9, designed for the treatment of hypercholesterolemia. Based on promising preliminary results from phase 1 studies (Fitzgerald et al., 2014, 2016) in healthy volunteers (**Table 1**), the efficacy of inclisiran was tested in a phase 2 double-blind, placebo-controlled RCT in patients with history of CVD or CVD risk equivalents and LDL levels over 70 or 100 mg/dl, respectively (Ray et al., 2017). Patients were treated with the maximum tolerated dose of statins before entering the study and were assigned to receive either a single dose of placebo or inclisiran at doses 200, 300, or 500 mg or two doses of placebo/inclisiran at doses 100, 200, or 300 mg with a 3-month interval. The greatest reduction in LDL-C was detected in the two-dose 300 mg inclisiran group with nearly half of the patients having an LDL cholesterol level below 50 mg/dL 6 months after treatment initiation. Importantly, both PCSK9 and LDL cholesterol levels remained below the baseline across a 8 months follow-up period (**Table 2**). The most common adverse events (occurring in >2% of patients) included myalgia, headache, fatigue, nasopharyngitis, back pain, hypertension, diarrhea, and dizziness occurred among 11% of inclisiran treated patients compared to 8% receiving placebo. Two deaths were reported, one in a patient with CVD history assigned to the single-dose 500-mg inclisiran group who experienced cardiac arrest and the second in the single-dose 200-mg inclisiran group who died from sepsis. Of note, injection site reaction and transaminasemia were uncommon in inclisiran treated patients, in contrast to high rates of these adverse events reported in studies of ASOs described above.

Ongoing clinical trials are currently assessing the safety, efficacy and tolerability of inclisiran under various different clinical conditions. ORION-2 (NCT02963311) is a phase 2 open-label clinical trial in patients with homozygous FH and ORION-3 (NCT03060577) a phase 2, open-label, non-randomized, extension trial designed to compare inclisiran to evolocumab in high CVD risk patients (history of atherosclerotic CVD, symptomatic atherosclerosis, type 2 DM or FH) with elevated LDL cholesterol levels (**Table 4**).

SPC5001

Inhibition of PCSK9 was also assessed in a phase 1 double-blind, placebo-controlled clinical trial using a 14-mer oligonucleotide with locked nucleic acid (LNA) modifications, SPC5001 (van Poelgeest et al., 2015). In total, twenty-three adult volunteers with mild hypercholesterolemia (fasting LDL-C≥100mg/dl) were enrolled, of whom 17 were exposed to Apo-B SNALP (Apo-B siNA in an LNP formulation) and the rest to placebo. In this first in-human study, SPC5001 reducedPCSK-9 by 49%. Regarding lipid parameters, SPC5001 decreased LDL-C at the end of therapy, but this effect was attenuated 3 weeks post treatment. Importantly, dose-dependent injection site reactions developed in 44% of the SPC5001-treated subjects and transient serum creatinine increases of ≥20 μmol/L (15%) were observed. Four out of six subjects receiving SPC5001 at the highest dose developed renal tubular toxicity and one subject was diagnosed with biopsy-proven acute tubular necrosis resulting in termination of the clinical development of the study drug (**Table 1**).

Lipoprotein(a)

Lipoprotein(a) is an independent risk factor for CVD events, especially myocardial infarction (Waldeyer et al., 2017), potentially through accelerated atherosclerosis as a result of intimal deposition and/or prothrombotic or anti-fibrinolytic effect as apolipoprotein (a) possesses structural homology with plasminogen and plasmin. In a large prospective study investigating the association between Lp(a) excess and incident CHD reported an odds ratio of 1.60 (95% CI 1.38–1.85) between the upper and lower thirds of baseline Lp(a) levels after adjustment for traditional cardiovascular risk factors (Bennet et al., 2008). Despite convincing data linking Lp(a) with CVD, there is no definite clinical trial evaluating the effect

of lowering Lp(a) on prevention of CHD. Currently, plasma Lp(a) measurement, while not recommended for risk screening in the general population, should be considered in people with high CVD risk or a strong family history of premature atherothrombotic disease (Nordestgaard et al., 2010).

An ASO targeting Lp(a) has been developed and tried in a dose escalating phase 1 study conducted in healthy volunteers with baseline levels of Lp(a) >250 nmol/L (100 mg/dL) (**Table 1**). Patients assigned to treatment with ISIS-APO(a)$_{Rx}$,received single or multiple (six) subcutaneous injections ranging from 100 to 300 mg over 4 weeks. No decrease in Lp(a) concentration was observed in the single dose group, whereas patients receiving the multiple dose scheme experienced a dose-dependent reduction in Lp(a) levels (−39.6% in the 100 mg group, −59% in the 200 mg group, and −77.8% in the 300 mg group). The most common adverse event was site injection reaction leading to treatment discontinuation in one participant (Tsimikas et al., 2015). Two randomized, placebo-controlled, dose ranging clinical trials were subsequently conducted in order to investigate the efficacy, safety, and tolerability of two unique ASOs -IONIS-APO(a)-LRx and IONIS-APO(a)Rx- designed to lower Lp(a) concentrations (**Tables 1**, **2**). Significant reductions of Lp(a) between 62.8 and 84.5%were observed in the intervention arm compared to the placebo group in both clinical trials. Concerning safety, both ASOs were well-tolerated, although two episodes of myocardial infarction were reported in the IONIS-APO(a)Rx phase 2 trial that were deemed unrelated to the study drug (Viney et al., 2016).

Angiopoetin-Like Protein-3

Angiopoetin-like protein-3 has been established as a central regulator of lipoprotein metabolism and loss-of-function variants have been associated with increased insulin sensitivity, reduced free fatty acid circulating levels and decreased plasma lipid levels. A study examining the relationship between ANGPTL3 loss-of-function variants and coronary artery disease in 58,355 adults reported that the presence of an ANGPTL3 loss-of-function variant was associated with a 41% lower odds of CAD (OR 0.59; 95% CI 0.41–0.85) (Dewey et al., 2016). In a phase 1 RCT fourty-four volunteers (with triglyceride levels of either 90–150 mg per deciliter or >150 mg per deciliter, depending on the dose group) were randomly assigned to receive subcutaneous injections of placebo or an ASO targeting *ANGPTL3* mRNA in a single- or multiple doses scheme. Participants receiving the ASO, presented dose-dependent reduction in ANGPT3 levels and both lipids and lipoproteins. In specific, 6 weeks post-treatment, the multiple-dose group presented reduced levels in reduced ANGPTL3 protein (reduction of 46.6–84.5%) triglyceride levels (reduction of 33.2–63.1%), LDL cholesterol (reduction between 1.3 and 32.9%), apolipoprotein B (reduction of 3.4–25.7%) and apolipoprotein C-III (reduction of 18.9–58.8%) and these reductions were significantly higher than those in the placebo group. The agent was well tolerated with no serious adverse events reported, or discontinuations of treatment (Graham et al., 2017; **Table 1**). Safety and efficacy of ANGPTL3 is now being explored in phase 2 clinical trials in subjects with hypertriglyceridemia, type 2 diabetes mellitus and non-alcoholic fatty liver disease (NCT03371355) and in patients with familial chylomicronemia syndrome (NCT03360747) (**Table 4**).

FDA-APPROVED RNA THERAPEUTICS

Besides mipomersen, specific ASOs have received FDA approval for use in non-cardiovascular diseases (**Table 5**). **Fomiversen** [Vitravene, Novartis] is the first ASO to be approved for clinical use in 1998 indicated for cytomegalovirus (CMV) retinitis. This 21-mer phosphorothioate oligodeoxynucleotide targets the mRNA encoding the CMV immediate-early (IE)-2 protein, which is required for viral replication (Vitravene Study Group, 2002). **Pegaptanib** [Macugen, OSI pharmaceuticals, Pfizer] is an aptamer targeting vascular endothelial growth factor (VEGF165) and was approved by the FDA for the treatment of AMD of the retina. This is the leading cause of blindness in people older than 50 years of age and is attributed to VEGF165-stimulated neovascularization of the choroid (Gragoudas et al., 2004). Recently, approval was granted for **eteplirsen** [Exondys 51, Sarepta Therapeutics] to be used in Duchenne muscular dystrophy, a fatal neuromuscular disorder characterized by a mutation in the dystrophin gene. Eteplirsen exerts its action by restoring the translational reading frame of dystrophin mRNA through specific skipping of exon 51 in the defective gene variants, thus promoting dystrophin production (Mendell et al., 2013). Another FDA-approved ASO is **nusinersen** [Spinraza, Biogen] a 18-mer phosphorothioate 2′-O-methoxyethoxy antisense oligonucleotide which modulates alternative splicing of the survival motor neuron (SMN) gene and is indicated for spinal muscular atrophy (Finkel et al., 2017).

CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, we are witnessing tremendous advances in RNA therapeutics field and a rapid translation of experimental studies to human clinical trials paving the way toward precision medicine. There are challenges though to be overcome before RNA-based therapeutic agents could efficiently evolve into drugs. A number of chemical modifications have been introduced to enhance target binding affinity, cellular uptake, pharmacokinetics and drug potency along with the development of natural or synthetic carriers to achieve efficient *in vivo* delivery (Wei et al., 2017; Yin et al., 2017). Minimising off-target effects and immunogenicity remains the most challenging setback and significant efforts are being made in order to mitigate unwanted toxicity, before this exciting novel technology could be largely implemented in clinical practice.

To this end, a sophisticated genome editing tool consisting of RNA-guided DNA endonucleases such as Cas9 and CRISPR (clustered regularly interspaced short palindromic repeats) was recently introduced to the scientific community. This versatile tool, in contrast to its "predecessors" RNAi/genome delivery systems, allows suppression (knock out) and/or overexpression (knock in) of a target's expression by introducing a double-stranded break on the site of interest within the genome, which

TABLE 5 | FDA-approved oligonucleotide therapies.

Brand name [Generic name]	Type of treatment	Target	Disease	Year of approval	Relevant studies
Vitravene [Fomivirsen]	ASO	mRNA encoding IE2	CMV retinitis	1998	The Vitravene Study Group, 2002
Macugen, [Pegaptanib]	Aptamer	VEGF165	AMD of the retina	2004	Gragoudas et al., 2004
Kynamro [Mipomersen]	ASO	ApoB-100 mRNA	Homozygous familial hypercholesterolemia	2013	Raal et al., 2010; McGowan et al., 2012; Stein et al., 2012; Thomas et al., 2013
Exondys 51 [Eteplirsen]	SSO	DMD 001-gene (exon 51 target site)	Duchenne muscular dystrophy	2016	Mendell et al., 2013
Spinraza [Nusinersen]	ASO	SMN2 mRNA	Type 1, 2, and 3 spinal muscular atrophy	2016	Finkel et al., 2017

ASO, antisense oligonucelotides; IE2, immediate early region 2; CMV, cytomegalovirus; VEGF, vascular endothelial growth factor; AMD, age-related macular degeneration; ApoB-100, apolipoprotein B-100; SSO, splice-switching oligo; DMD, Duchenne muscular dystrophy; SMN, survival motor neuron.

is guided upon RNA oligonucleotides, of 20–21 nt length, complementary to genomic regions of the targeted segment (Cong et al., 2013; Mali et al., 2013). Given that the cellular machinery relies on two known mechanisms by which repairs double-stranded nicks; the non-homologous end joining, which bridges the two newly formed ends, and the homology-directed repair, which utilizes a neighboring template to replace the affected area through homologous recombination; the latter may be exploited in order to introduce site-specific mutations in the genome, supporting further the versatility of this method. Importantly, this system empowers the simultaneous targeting of multiple sites by simply providing more than one different RNA guides that are directed toward different genomic regions later subjected to Cas9 "nicking." Since the endonuclease, Cas9, is not endogenously expressed in human or mice systems, but only in bacteria, like Streptococcus pyogenes (Sp) from which has been originally isolated (Sapranauskas et al., 2011; Jinek et al., 2012), a forced expression of Cas9 to the studied system is a prerequisite that also features a common laboratory practice hurdle when it comes to transfection-resistant systems, e.g primary cells and thus the delivery strategy of Cas9 shall be extensively considered in advance and tailored accordingly. This innovative technology has already been employed to "correct" disease contexts like Duchenne muscular dystrophy (Long et al., 2016) hereditary tyrosynemia type I (Yin et al., 2016) and lethal metabolic liver disease (Yang Y. et al., 2016) in animal models. However, despite these promising outcomes there are several limitations regarding the CRISPR–Cas9 system that have to be acknowledged: (a) off-target activity resulting in unwanted mutations, (b) low efficiency of genome editing using homology-directed repair (HDR) and (c) challenging delivery of CRISPR–Cas9 components into desired tissues using both viral and non-viral methods (Long et al., 2016; Tycko et al., 2016; Komor et al., 2017). Of particular interest, similar strategies have been developed for rendering feasible the editing of the transcriptome (Abudayyeh et al., 2016) instead of the genome. We, and others, have previously underpinned the importance of adenosine (A)-to-inosine (I) RNA editing, a widespread RNA modification (Stellos et al., 2016) in human transcriptome, in RNA metabolism thus modulating the context of several diseases (Choudhury et al., 2012; Chen et al., 2013; Yamashita et al., 2013; Shoshan et al., 2015) including atherosclerotic heart disease (Stellos et al., 2016), as we have rigorously reviewed (Gatsiou et al., 2017). Recently, a breakthrough proof-of-principle study, documented that the use of Cas system in conjunction with the catalytic activity of an A-to-I RNA editing enzyme, namely ADAR2, is able to edit and correct disease-relevant mutations of full-length transcripts, previously introduced into immortalized human cells (Cox et al., 2017). In a different approach, another group fused the catalytic domain of ADAR2 with an RNA binding domain directed to a specific stem loop sequence embedded within the RNA guide, restoring in this way the function of a neurodevelopmental disorder-associated protein in primary neurons (Sinnamon et al., 2017). Whether these concepts can be adopted first in animal models at a preclinical stage manipulating endogenous A-to-I RNA editing within specific transcripts, of which A-to-I RNA editing levels have been previously linked with a particular disease context, in order to attenuate the progression of a disease remains yet to be investigated. Nevertheless, these "trail blazer" findings provide convincing argumentation prompting us to contemplate that genome and mainly transcriptome editing feature as dynamic new additions to the "quiver" of RNA therapeutics landmarking the dawn of a new era in RNA therapeutics (Stellos, 2017a).

Without doubt, we are heading toward the new era of precision medicine based on the discipline of an evidence-based approach customized to patient-specific unique characteristics (MacRae et al., 2016). Precision medicine is gaining ground over the traditional "one-size-fits-all" medical treatment approach (Houser, 2016; MacRae and Seidman, 2017) by enabling the identification of the exact underlying molecular mechanism of the disease and the design of therapeutic interventions deployed specifically for this mechanism (Benjamin et al., 2017). A bold, innovative research effort is now emerging based on the fundamental concept of individualized treatment taking into account individual variability in genes, environment

and lifestyle (PMI Working Group report to the Advisory Committee to the Director, NIH, 2015[1]) in order to identify which prevention strategy and treatment is effective in each patient (MacRae et al., 2016; Shah et al., 2016). Advances in precision medicine will soon be applicable in cardiology and medicine.

AUTHOR CONTRIBUTIONS

KoS initiated this review study, designed its structure, provided conceptual advice to all coauthors and revised critical parts of it. AL wrote the first draft of the manuscript. AG provided conceptual advice on the basic science part of the manuscript and wrote the future perspectives part and revised the whole manuscript. GG organized data collected from clinical trials into tables and revised the manuscript. KiS provided conceptual advice on the organization of the information into the tables. All authors contributed to manuscript revision, read and approved the submitted version.

[1]http://www.nih.gov/precisionmedicine/09172015-pmi-working-group-report.pdf

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MINI REVIEW
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Long Non-coding RNA Structure and Function: Is There a Link?

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RNA has emerged as the prime target for diagnostics, therapeutics and the development of personalized medicine. In particular, the non-coding RNAs (ncRNAs) that do not encode proteins, display remarkable biochemical versatility. They can fold into complex structures and interact with proteins, DNA and other RNAs, modulating the activity, DNA targets or partners of multiprotein complexes. Thus, ncRNAs confer regulatory plasticity and represent a new layer of epigenetic control that is dysregulated in disease. Intriguingly, for long non-coding RNAs (lncRNAs, >200 nucleotides length) structural conservation rather than nucleotide sequence conservation seems to be crucial for maintaining their function. LncRNAs tend to acquire complex secondary and tertiary structures and their functions only impose very subtle sequence constraints. In the present review we will discuss the biochemical assays that can be employed to determine the lncRNA structural configurations. The implications and challenges of linking function and lncRNA structure to design novel RNA therapeutic approaches will also be analyzed.

Keywords: non-coding RNA, lncRNA, RNA structure, gene editing, cardiovascular diseases

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INTRODUCTION

The HUMAN GENOME project has transformed our understanding of the basic unit of genetic information with RNA emerging as a versatile regulator of central cellular processes (Thum and Condorelli, 2015). The non-coding RNAs (ncRNAs), transcripts that do not encode proteins comprise the biggest class and are arbitrarily divided into small (<200 nucleotides) and long non-coding RNAs (lncRNA (>200 nucleotides). MicroRNAs (miRNAs) are the best studied small ncRNAs, representing an additional layer of posttranscriptional regulators that absorb perturbations and ensure the robustness of biological systems (Liu and Olson, 2010; Ebert and Sharp, 2012; Rotllan et al., 2016).

Substantial effort has now been directed toward dissecting the function of lncRNAs. In the cardiovascular system, lncRNAs were reported to play key roles in physiology and disease and targeting lncRNAs for novel therapeutic interventions has been explored (Uchida and Dimmeler, 2015; Boon et al., 2016; Buhrke et al., 2018). Here we will discuss the experimental tools to determine the RNA structure that can offer unique insights into the lncRNA function in the cardiovascular system.

CHALLENGES IN ASSESSING lncRNA FUNCTIONALITY

The unique features of lncRNA have been extensive investigated (Guttman and Rinn, 2012; Ulitsky and Bartel, 2013; Bar et al., 2016; Ulitsky, 2016). Several characteristics of lncRNAs make functional evaluation challenging. Typically, lncRNAs display poor conservation across species showing only "patches" of conserved bases surrounded by large seemingly unconstrained sequences (Ponjavic et al., 2007; Guttman et al., 2009; Necsulea et al., 2014; Washietl et al., 2014). Additionally, lncRNAs exhibit low abundance that restricts their mode and sites of action (Mercer et al., 2008; Cabili et al., 2011, 2015; Washietl et al., 2014; Ulitsky, 2016; Wilk et al., 2016; Jandura and Krause, 2017). In terms of the modes of function, both *cis*- and *trans*-regulatory activity have been described (Mercer and Mattick, 2013). As *cis*-regulators, lncRNAs exert their function on neighboring genes on the same allele from which they are transcribed, displaying expression correlation and perturbation in an allele-specific manner. CARMEN, an enhancer associated lncRNA and a crucial regulator of cardiac specification in human cardiac progenitor cells was shown to act in *cis* to control the expression of miR-143/145 (Ounzain et al., 2015). On the other hand, acting in *trans*-lncRNAs can control gene expression at a distance from their transcription site, by altering the chromatin state, influencing the nuclear structure or regulating protein function (Vance and Ponting, 2014; Kopp and Mendell, 2018).

Intriguingly, for some low abundance lncRNAs the act of transcription seems to be more important than the transcript itself. In a seminal study, Engreitz et al. (2016) genetically manipulated 12 genomic loci that produce lncRNAs to find that 5 loci influenced the expression of a neighboring gene in *cis*. The expression of the lncRNAs transcripts themselves was not required but instead processes associated with their transcription were critical (Engreitz et al., 2016).

THE RNA INTERACTOME

The above functional versatilities of lncRNAs stem from their ability to conform to different structures and molecular interactions with proteins, RNA and DNA (Guttman and Rinn, 2012; Marchese et al., 2017). In ribonucleoprotein complexes (RNPs), lncRNAs may act as scaffolds to stabilize the complexes, directing them to specific subcellular loci or the DNA. In endothelial cells, interaction of the lncRNA MANTIS with the ATPase catalytic subunits confers specificity to the switch/sucrose non-ferentable (SWI/SNF) chromatin remodeling complex directing it to a subset of angiogenic genes and facilitating nucleosome remodeling and transcription initiation (Leisegang et al., 2017; Zampetaki and Mayr, 2017). In fact binding of lncRNA to specific ATPase subunits of the SWI/SNF complex is a common regulatory mechanism (Cajigas et al., 2015; Zhu et al., 2016).

Interaction of lncRNAs with chromatin complexes is particularly important as these lncRNA-RNPs can trigger chromatin modifications through interference with the chromatin-modifying machinery (Tsai et al., 2010; Brockdorff, 2013; Simon et al., 2013). In the heart, Chaer a cardiac enriched lncRNA acts as an epigenetic switch by interfering with the polycomb repressive complex 2 (PRC2) and inhibiting H3K27m3 at genes involved in cardiac hypertrophy (Wang et al., 2016), while mesoderm faith determining lncRNA Fendrr can bind to both PRC2 and Trithorax group/MLL (TrxG/MLL) complexes acting as a fine tuner (Grote et al., 2013).

Apart from proteins, interaction of lncRNAs with DNA has also been described. This can lead to the formation of RNA-DNA triplex, a structure that is widespread *in vivo* and facilitates target gene recognition by lncRNAs (Mondal et al., 2015). This interaction was elegantly demonstrated in MEG3, a cardiac fibroblast enriched lncRNA that promotes fibrosis (Piccoli et al., 2017). MEG3 interacts with the PRC2 complex and forms RNA-DNA triplex structures through GA-rich sequence binding sites. Chromatin RNA immunoprecipitation revealed that MEG3 modulates the activity of TGF-b pathway genes and target recognition occurs via the triplex structures (Mondal et al., 2015).

Long non-coding RNA regulatory functions also rely on RNA–RNA interactions. Crosstalk with miRNAs creates an intricate network that exerts post-transcriptional regulation of gene expression. LncRNAs can harbor miRNA binding sites and act as molecular decoys or sponges that sequester miRNAs away from other transcripts. Noteworthy, competition between lncRNAs and miRNAs for binding to target mRNAs has been reported and leads to de-repression of gene expression (Yoon et al., 2014; Ballantyne et al., 2016). Finally, lncRNAs may contain embedded miRNA sequences and serve as a source of miRNAs (Piccoli et al., 2017).

LINKING RNA STRUCTURE TO FUNCTION

RNA molecules adopt higher order tertiary interactions (Staple and Butcher, 2005; Wan et al., 2011). Although links between structure and function are emerging, the structural domains that dominate the RNA interactome are still not well defined. The functional implications of transcript structure are better understood in the processing the primary miRNAs (primiRNAs) to mature miRNAs. Using multiple mutagenesis assays, the secondary structures such as stem length, hairpin pairing, bulge size and position, and apical loop size that contribute to effective miRNA biogenesis were defined (Auyeung et al., 2013; Fang and Bartel, 2015; Nguyen et al., 2015; Roden et al., 2017). In clustered miRNAs that consist of multiple miRNA genes, the tertiary structure was also proposed to contribute to the processing to individual mature miRNAs. An autoregulatory role for the tertiary structure of miR-17∼92 cluster in its maturation and binding of auxiliary factors to conserved terminal loops was shown (Chakraborty et al., 2012). Recently, in the miR-497∼195 cluster, mutations in miR-195a hairpin were reported to affect the processing of miR-497a that resides in the same

cluster. Computational analysis highlighted differences in the tertiary structure of the primiRNA in mutants that may affect the maturation process (Lataniotis et al., 2017). On a different note, in primiR-30c-1 the tertiary structure promotes the interaction with SRSF3, an SR protein family member that facilitates primiRNA recognition and processing. A single G/A sequence variation leads to a structural rearrangement of the apical region of the primiRNA affecting the conserved residues placed at the basal part of the stem and mature miRNA generation (Fernandez et al., 2017).

In lncRNAs, selection acting on structure rather than primary sequence may explain the rapid rate of evolution, that led to the "RNA modular code" hypothesis based on the view that selection acts on structural domains (Wutz et al., 2002; Tsai et al., 2010; Guttman and Rinn, 2012). Some experimental evidence supports this concept. The *MEG3* lncRNA gene contains three distinct structure modules M1, M2, and M3. Deletion analysis showed that motifs M2 and M3 are important for p53 activation. Intriguingly, a hybrid MEG3 transcript in which half of the primary sequence in the M2 motif was replaced by an entirely unrelated artificial sequence that displayed a similar secondary structure was fully functional in stimulating p53-mediated transcription (Zhang et al., 2010).

RNA STRUCTURE DETERMINATION METHODS

Chemical and enzymatic probing methods can provide an understanding of the secondary structure of RNA (Ehresmann et al., 1987). Enzymatic probing relies on nucleases that bind to paired and unpaired RNA and digest it to generate RNA fragments that can be analyzed. On the other hand, in chemical probing small size chemicals that react and covalently modify solvent accessible nucleotides are used. Following modification or cleavage, positions are typically mapped by reverse transcription, which either stops or introduces a mutation into the cDNA (Wilkinson et al., 2006). An analysis of the resulting cDNA is then used to determine the nucleotide position and modification frequency. Next generation sequencing (NGS) can be applied to directly sequence the cDNA products. This allows RNA structural characterization at a transcriptome-wide level in a single experiment (Lucks et al., 2011; Incarnato et al., 2014; Loughrey et al., 2014; Rouskin et al., 2014). Although initially the technologies were established to analyse RNA structure *in vitro*, structural characterization *in vivo* mainly through the use of probes that can diffuse quickly across membranes has also been reported (Spitale et al., 2013; Ding et al., 2014; Spitale et al., 2015; Flynn et al., 2016).

ENZYMATIC PROBING

PARS

PARS (parallel analysis of RNA structure) is a high-throughput enzymatic probing method that measures the structural properties of isolated polyadenylated transcript pools that are renatured *in vitro* and treated with RNase V1 or S1. RNase V1 and RNase S1 cleave the 3′ phosphodiester bonds of double-stranded and single-stranded RNA, respectively, allowing evaluation of the double- or single-stranded conformation (Kertesz et al., 2010).

Frag-Seq

Frag-Seq (fragmentation sequencing) is an enzymatic method that uses a nuclease P1 to specifically cleave single-stranded RNA. High-throughput sequencing then analyses the fragments generated. This workflow provides an "RNA accessibility profile" that is likened to the DNase hypersensitivity assays on chromatin (Underwood et al., 2010). Noteworthy, Frag-seq isolates fragments <200 bases after RNase P1 cleavage, hence large RNAs maybe underrepresented. As Frag-seq and PARS can provide complementary data a combined approach could improve the accuracy of genome-wide RNA structure measurements (Wan et al., 2011).

CHEMICAL PROBING

DMS Probing

The dimethyl sulfate (DMS) is a base specific reagent that can bind and alter the methylation state of unpaired adenosine and cytosine nucleotides (Tijerina et al., 2007; Rouskin et al., 2014). DMS footprinting is optimized for structural analysis of RNA. Protein binding to RNA generates a "footprint" that can be traced due to alterations in the RNA structure. The transcript size that can be evaluated is rather small (<500 nt) but this method can be performed both *in vitro* and *in vivo* as DMS can easily penetrate the cell membrane. DMS-seq that combines DMS methylation with NGS was recently performed *in vivo* (Ding et al., 2014; Rouskin et al., 2014).

Targeted Structure-Seq

Targeted Structure-Seq relies on RNA methylation by DMS being performed *in vivo*. Subsequently, RNA is isolated from cells and the methylation sites are determined by employing gene specific primers for the reverse transcription reaction. Sequencing of the DMS derived fragments can be used to assess the cellular conformation of the RNA. Based on this method, structural models of elements within Xist were developed (Fang et al., 2015). Although initially reported using DMS this workflow can be adapted for other probing reagents.

SHAPE

SHAPE (selective 2′-hydroxyl acylation by primer extension) can interrogate the RNA structure both *in vitro* and *in vivo* using the chemical NMIA and its derivatives to detect flexible regions in RNA secondary structure (Wilkinson et al., 2006; Weeks and Mauger, 2011). Several SHAPE reagents have been tested in order to improve the signal to background ratio (Lee et al., 2017). In SHAPE, the 2′-hydroxyl groups of all four nucleotides are selectively acylated when flexible and unpaired. This results in the formation of covalent SHAPE adducts that block the reverse transcription leading to truncated cDNA fragments. SHAPE

reactivities can then be used to model secondary structures and quantify any process that modulates RNA dynamics.

SHAPE-MaP

SHAPE-MaP (SHAPE and mutational profiling) was the first to combine the SHAPE protocol with NGS. Initially performed and reported to define the HIV-1 RNA genome, SHAPE-MaP is a highly sensitive technique that allowed rapid, *de novo* discovery and direct validation of new functional motifs (Siegfried et al., 2014; Mustoe et al., 2018).

In-cell SHAPE-Seq

In-cell SHAPE-Seq is a modification of the SHAPE-Seq technique that combines the SHAPE-seq with gene expression measurements to elucidate the association of RNA structure and function *in vivo*. It revealed translational regulatory mechanisms in *E. coli in vivo* (Watters et al., 2016).

icSHAPE-seq

icSHAPE-seq (*in vivo* click SHAPE sequencing) uses the in-cell SHAPE chemical NAI-N_3 followed by selective chemical enrichment of NAI-N_3-modified RNA that provides an improved signal-to-noise ratio (Flynn et al., 2016). Follow-up NGS allows accurate identification at single-nucleotide resolution. In mouse embryonic stem cells it was shown that *in vitro* RNA folding is programmed entirely by the sequence, whereas *in vivo*, the RNA structure depends on the context of intracellular environment and interaction with RNA binding proteins that may lead to focal structural rearrangements (Spitale et al., 2015). Hence, this assay offers the exciting possibility of viewing the RNA structurome *in vivo* in the presence or absence of stimulation.

RNA STRUCTUROME AND INTERACTOME DETERMINATION

PARIS

PARIS (psoralen analysis of RNA interactions and structures) was recently developed to determine both RNA structure and interactions *in vivo*. It uses the highly specific and reversible nucleic acid crosslinker psoralen-derivative 4′-aminomethyltrioxsalen to fix base pairs in living cells. Subsequently, partial RNase and complete proteinase digestion lead to purification of a set of small crosslinked and directly base-paired RNA fragments. Purification of the crosslinked fragments using 2D electrophoresis, followed by proximity ligation of duplex RNA fragments, reversal of crosslinks, and high throughput sequencing reveals the direct base pairing between fragments. Based on these reads, models of RNA structures and interactions can be generated with high specificity and sensitivity (Lu et al., 2016). Using this approach a model for the higher order structure of Xist was interrogated (Lu et al., 2016). Encouragingly, these findings are in agreement with crystallographic studies of the defined domains *in vitro* (Arieti et al., 2014).

lncRNA STRUCTURE DETERMINATION

Structure determination of lncRNA *in vivo* is extremely challenging as they are highly heterogenic with regions with well-defined base-pairing, others without base-pairing and regions with multiple structures. Additionally, lncRNAs may stretch across thousands of nucleotides, they are expressed in low abundance and tend to be part of multicomponent complexes (Busan and Weeks, 2017). Nevertheless, the structure of several lncRNAs has been experimentally determined (**Table 1**).

Xist

This is a very long lncRNA (17,000 nucleotides) controlling X chromosome inactivation. It spreads across the entire chromosome while triggering stable epigenetic modifications through recruitment of the PRC2 complex and enrichment for the H3K27me3 repressive chromatin modification (Simon et al., 2013; Fang et al., 2015; Smola et al., 2016). *In vivo* SHAPE data identified 33 regions in Xist that form well-defined secondary structures linked by structurally variable and dynamic regions.

RepA

This is a 1,600 nucleotides mouse lncRNA encoded by an internal promoter on the Xist-gene sense strand. Applying SHAPE and DMS chemical probing *in vitro*, an intricate structure of three independently folding modules was revealed. Phylogenetic analysis and computational 3D modeling demonstrated a defined tertiary architecture that can form autonomously in the absence of protein partners (Liu et al., 2017a).

Rox1/Rox2

In Drosophila dosage compensation is achieved using two lncRNAs that are transcribed from the X chromosome. RNA on the X 1 and 2 (roX1 and roX2) are 3,700 and 1,200 nucleotides in length, respectively. *In vitro* SHAPE probing and PARS analysis revealed common, conserved and distinct structural motifs that may function as targeting sites and assembly platforms for the male specific lethal complex (Ilik et al., 2013).

SRA

The human steroid receptor RNA activator (SRA) is an 870 nucleotide lncRNA that is derived from a gene encoding both lncRNA and protein coding transcripts. The structure of SRA was experimentally interrogated using SHAPE and DMS chemical probing *in vitro*. In parallel, RNase V1 enzymatic probing was performed. It was shown that SRA consists of four distinct domains with a variety of secondary structures (Novikova et al., 2012). More importantly, comparative structural analysis between mouse and human strongly suggested that a large number of evolutionary changes had minimal mutational effect on the protein derived from the locus while stabilizing the RNA structural core (Novikova et al., 2012).

HOTAIR

Associated with Sporadic Thoracic Aortic Aneurysm through regulation of extracellular matrix deposition and human

TABLE 1 | Structural Determination of lncRNAs.

LncRNA (size)	Mode of action	Function	Structure	Probing techniques	References
Xist (17,000 nucleotides)	*cis*	X-chromosome inactivation.	Regions A-F with distinct repeat sequences.	*In vivo* and *in vitro* SHAPE-MaP. Targeted structure Seq.PARIS	Simon et al., 2013; Fang et al., 2015; Lu et al., 2016; Smola et al., 2016
RepA (1,600 nucleotides)	*cis*	Encoded by an internal promoter on the Xist gene sense strand.	Three folding modules.	*In vitro* using chemical probing with SHAPE and DMS reagents.	Liu et al., 2017a
Rox1 (3,700 nucleotides) Rox2 (1,200 nucleotides)	*cis* and *trans*	Male specific nuclear RNAs. Dosage compensation.	Rox1: three stable helices connected by flexible linker regions. Rox2: two clusters of tandem stem-loops.	*In vitro* using chemical probing with SHAPE and PARS analysis. Both methods independently support the rox2 structure model.	Ilik et al., 2013
SRA (870 nucleotides)	*trans*	Interacts with SRA protein to regulate cardiac muscle differentiation.	Four distinct domains.	*In vitro* SHAPE and DMS chemical probing. Good agreement with RNase V1 enzymatic probing.	Novikova et al., 2012
HOTAIR (2,148 nucleotides)	*trans*	Associated with sporadic thoracic aortic aneurysm and non-end stage heart failure. Circulating biomarker for acute myocardial infarction and congenital heart diseases.	Four structural modules.	*In vitro* using chemical probing with SHAPE and DMS reagents.	Somarowthu et al., 2015; Greco et al., 2016; Gao et al., 2017; Guo et al., 2017; Jiang et al., 2018
Braveheart (590 nucleotides)	*trans*	Cardiovascular lineage commitment.	Three domains. Critical structure: a 5′ asymmetric G-rich internal loop (AGIL).	*In vitro* using chemical probing with SHAPE and DMS reagents.	Xue et al., 2016

aortic smooth muscle cells apoptosis, this lncRNA plays a key role in the cardiovascular system (Guo et al., 2017). In non-end stage heart failure patients HOTAIR was among a panel of lncRNAs that were significantly modulated (Greco et al., 2016). A protective role of HOTAIR in cardiomyocytes (Gao et al., 2017) and as a circulating biomarker for acute myocardial infarction and congenital heart diseases were also proposed (Jiang et al., 2018). Hotair is 2,148 nucleotides long making the structural determination extremely challenging. To address this issue a non-denaturing purification protocol to obtain a homogeneous and monodisperse form was established. Structural modules and distinct evolutionary conserved elements were determined *in vitro* using chemical probing with SHAPE and DMS reagents (Somarowthu et al., 2015).

Braveheart

Braveheart is a 590 nucleotide lncRNA that acts in *trans* to regulate cardiovascular lineage commitment. Its secondary structure was experimentally assessed using SHAPE and DMS probing *in vitro*. It emerged that *Braveheart* is organized into a highly intricate modular structure comprising of three domains, consisting of 12 helices, 8 terminal loops, 5 sizeable internal loops, and a five-way junction. Intriguingly, it includes a 5′ asymmetric G-rich internal loop (AGIL) and a 55 nucleotide stretch at the 3′ end exhibiting high reactivity suggesting low probability of structure. Genetic deletion of this specific 11 nucleotide fragment demonstrated that the AGIL motif is essential for mouse embryonic stem cell differentiation to cardiomyocytes through binding of the zing-finger protein CNBP/ZNF9 (Xue et al., 2016).

FUTURE DIRECTIONS

RNA structure determination combined with genetic manipulations can elucidate the important functional domains of lncRNAs. To this end, advanced experimental tools, bioinformatics and genome engineering should be integrated. The CRISPR/Cas9 gene editing system emerged as a robust technology that can be used to generate targeted modifications at precise genomic loci. Cas9, a nuclease that can induce double-stranded breaks (DSBs) to the DNA, can be guided in the immediate vicinity of the proto-adjacent motif NGG by an RNA molecule (sgRNA) consisting of a small 20 nucleotide long variable sequence and an adaptor transactivating RNA. Precise insertions, deletions, or base substitutions can be introduced at a DSB site (Lin et al., 2014) in primary cells and *in vivo* in mouse models of disease (Platt et al., 2014; Abrahimi et al., 2015). A modified version of the CRISPR/Cas9 system has recently been employed for genome scale screenings of functional lncRNAs. This CRISPR interference approach uses a nuclease dead Cas9 (dCas9) that is not capable of inducing DSB to the DNA. Fused to a repressor domain (e.g., KRAB) (Liu et al., 2017b) or an activation domain (e.g., VP64) (Konermann et al., 2015; Bester et al., 2018) dCas9 and can be guided by sgRNAs to specific loci in the upstream regulatory region to trigger repression or activation of lncRNA transcription, respectively. Such approaches are extremely useful to test the functionality of lncRNAs in a high throughput manner.

Once specific lncRNAs are identified, technologies that can define the lncRNA structure *in vivo* are critical to determine lncRNA modules and structural domains. RNA structure determination can be coupled with comparative genomics analysis that will take into consideration the positional

conservation and the fact that lncRNAs may rely on short elements rather than long stretches of conserved sequences. Genetic studies that can target precisely these structural domains while maintaining the expression of the lncRNA (Matsumoto et al., 2017) will delineate the functional impact of these motifs. The use of CRISPR/Cas9 gene editing in induced pluripotent stem cells that can be clonally expanded, engineered to harbor defined deletions of the structural motifs and differentiated to other cell types (Cochrane et al., 2017; Granata et al., 2017) can provide conclusive evidence for the functional impact of these domains in the cardiovascular system. The potential of these elements as novel targets could be explored further for precise interventions suitable for therapeutic applications.

AUTHOR CONTRIBUTIONS

AZ initiated the study, designed its structure, and wrote the manuscript. AA provided conceptual advice and revised the manuscript. KS designed the review structure, provided conceptual advice, and revised the manuscript. All authors read and approved the submitted version.

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Corrigendum: Long Non-coding RNA Structure and Function: Is There a Link?

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In the original article, there was a mistake in **Table 1** as published. A previous version of the Table was published that was not revised and did not include updated references. The corrected **Table 1** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

TABLE 1 | Structural Determination of lncRNAs.

LncRNA (size)	Mode of action	Function	Structure	Probing techniques	References
Xist (17,000 nucleotides)	*cis*	X-chromosome inactivation.	Regions A-F with distinct repeat sequences.	*In vivo* and *in vitro* SHAPE-MaP. Targeted structure Seq. PARIS	Simon et al., 2013; Fang et al., 2015; Lu et al., 2016; Smola et al., 2016
RepA (1,600 nucleotides)	*cis*	Encoded by an internal promoter on the Xist gene sense strand.	Three folding modules.	*In vitro* using chemical probing with SHAPE and DMS reagents.	Liu et al., 2017a
Rox1 (3,700 nucleotides) Rox2 (1,200 nucleotides)	*cis* and *trans*	Male specific nuclear RNAs. Dosage compensation.	Rox1: three stable helices connected by flexible linker regions. Rox2: two clusters of tandem stem-loops.	*In vitro* using chemical probing with SHAPE and PARS analysis. Both methods independently support the rox2 structure model.	Ilik et al., 2013
SRA (870 nucleotides)	*trans*	Interacts with SRA protein to regulate cardiac muscle differentiation.	Four distinct domains.	*In vitro* SHAPE and DMS chemical probing. Good agreement with RNase V1 enzymatic probing.	Novikova et al., 2012
HOTAIR (2,148 nucleotides)	*trans*	Associated with sporadic thoracic aortic aneurysm and non-end stage heart failure. Circulating biomarker for acute myocardial infarction and congenital heart diseases.	Four structural modules.	*In vitro* using chemical probing with SHAPE and DMS reagents.	Somarowthu et al., 2015; Greco et al., 2016; Gao et al., 2017; Guo et al., 2017; Jiang et al., 2018
Braveheart (590 nucleotides)	*trans*	Cardiovascular lineage commitment.	Three domains. Critical structure: a 5′ asymmetric G-rich internal loop (AGIL).	*In vitro* using chemical probing with SHAPE and DMS reagents.	Xue et al., 2016

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REVIEW
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RBPs Play Important Roles in Vascular Endothelial Dysfunction Under Diabetic Conditions

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Diabetes is one of the major health care problems worldwide leading to huge suffering and burden to patients and society. Diabetes is also considered as a cardiovascular disorder because of the correlation between diabetes and an increased incidence of cardiovascular disease. Vascular endothelial cell dysfunction is a major mediator of diabetic vascular complications. It has been established that diabetes contributes to significant alteration of the gene expression profile of vascular endothelial cells. Post-transcriptional regulation by RNA binding proteins (RBPs) plays an important role in the alteration of gene expression profile under diabetic conditions. The review focuses on the roles and mechanisms of critical RBPs toward diabetic vascular endothelial dysfunction. Deeper understanding of the post- transcriptional regulation by RBPs could lead to new therapeutic strategies against diabetic manifestation in the future.

Keywords: RNA binding protein, diabetes, vascular endothelia, dysfunction, therapy

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INTRODUCTION

Diabetes mellitus (DM) is a chronic, metabolic disorder characterized by hyperglycaemia due to impaired glucose homeostasis, reduced insulin activity and insulin resistance (Rother, 2007). Because of the multisystem manifestations, DM is one of the primary health care problems affecting 435 million people in 2015 around the world (Ingelfinger and Jarcho, 2017). Diabetes related complications are the 8th leading cause of death worldwide. Half of people who die from diabetic complications are under the age of 60, while the rate of incidence is equal in both sexes (Shi and Hu, 2014). There are two types of diabetes, namely, type 1 and 2. Type 1 diabetes (T1D) is caused by an autoimmune attack on the β-cells of the pancreas, which lead to pancreatic islet inflammation (insulitis), β-cell apoptosis and subsequent hyperglycemia due to low insulin production. T1D patients need daily administration of insulin and are likely to suffer ketoacidosis, coma and death (Størling and Pociot, 2017). T1D accounts for 10–15% of diabetes incidences, while Type 2 diabetes (T2D) contributes to a majority of 85–90%. T2D is characterized by insulin resistance, defects in insulin secretion, β-cell apoptosis and islet amyloid deposits. The glucose cannot be utilized by target tissues such as liver and skeletal muscle, hence the blood glucose level increased (Rawshani et al., 2017). The etiology of T2D is unknown, however, physiological, genetic, and environmental factors such as obesity, family history and pollution are known to be risk factors (Stumvoll et al., 2005). Diabetes triggers multisystemic complications such as cardiovascular diseases, neuropathy, nephropathy etc. and the patients have higher predisposition of infection, cancer, and Alzheimer's disease (Harcourt et al., 2013; Schneider et al., 2016). Nowadays people see diabetes not only as a metabolic disorder but also a cardiovascular disease because of the parallel occurrence of cardiovascular complications along with diabetes. The intimate correlation between diabetes and

the predisposition of cardiovascular disease has been well-reported (Leon and Maddox, 2015). Diabetes is characterized by a two- to four- fold increased risk of cardiovascular disease while endothelial cell dysfunction is the initiating and perpetuating factor in the development of vascular complications (Brownlee, 2001; Shi and Vanhoutte, 2017).

The endothelium is the monolayer of endothelial cells (ECs) covering the lumen of blood vessels. In addition to providing a physical barrier between tissues and the circulating blood, vascular ECs play important roles in the maintenance of vascular homeostasis under physiological conditions. The endothelium is critical for the regulation of vasodilation, prevention of platelet adhesion, aggregation and thrombogenesis, as well as behavior of the underlying smooth muscle cells. The endothelium secretes various factors, regulating vessel integrity, blood vessel development, metabolism, inflammation, cell adhesion, angiogenesis, haemostasis, and vascular permeability (Sena et al., 2013).

In diabetes, endothelial functions are compromised, including increased permeability, disturbed vascular tone, aberrant angiogenesis, enhanced adhesion, and deposition of monocytes and platelets, leading to thrombogenesis. The most prevailing mechanism of endothelial dysfunction is an increase in oxidative stress and reactive oxygen species (ROS), which inactivates nitric oxide (NO) and ablates its role in regulating vascular tone as well as prevention of adhesion and aggregation of leukocytes and platelets, smooth muscle cell proliferation, inflammation and apoptosis (Vallance and Chan, 2001; Giacco and Brownlee, 2010). In addition, NO bioavailability is reduced due to down-regulation of nitric oxide synthase (eNOS), the critical enzyme in catalyzing the generation of NO from L-arginine (Sena et al., 2008).

It has been established that hyperglycaemia contributes to a significant alteration of gene expression profile in vascular ECs. High throughput assays of the transcriptome have revealed a plethora of candidate genes involved in extracellular matrix (ECM) reorganization, angiogenesis, vascular tone, inflammatory response, apoptosis, cell cycle, cell adhesion, coagulation, platelet activation etc. (**Table 1**) (Stenina, 2005; Ambra et al., 2014; Moradipoor et al., 2016).

The gene expression profile in vascular ECs is finely regulated by both transcriptional and post- transcriptional regulation systems. Post-transcriptional regulation includes processing of the pre-mRNAs toward mature mRNAs as well as mRNA transportation, quality control, mRNA decay and translational regulation etc. (Whelan et al., 2012).

There are around 424 RNA-binding proteins (RBPs) encoded by the human genome. Many of the RBPs are reportedly dysregulated in diabetes (Keene, 2007; Vanderweyde et al., 2013). For instance, HuR, hnRNP K, hnRNP F, IGF2BP2, and LIN28 are dysregulated in diabetic nephropathy, QKI, TTP and hnRNP C are related to atherosclerosis, CUGBP1, RBFOX1 and eIF4E are associated with diabetic skeletal muscle myopathy and LIN28, HuR and QKI are linked to diabetic cardiomyopathy as well (Nutter and Kuyumcu-Martinez, 2018) RBPs bind to a specific RNA sequence and/or RNA structure to form ribonucleoprotein (RNP) complexes dynamically (Gerstberger et al., 2014; Heinrich et al., 2017). RNA- binding domains (RBDs) encompassed in RBPs act as key modules for RNA recognition (Lunde et al., 2007). The most common RBDs exist in RBP of varying functions, including the hnRNPK homology (KH) domain, RNA recognition motif (RRM), double-stranded RNA-binding domain (dsRBD), zinc finger (ZnF) motif, cold shock domain (CSD) etc. (Gerstberger et al., 2014).

RBPs interact with target (pre)mRNAs at the 5′- and 3′-untranslated regions (UTRs), as well as at non-coding (intronic) and coding (exonic) regions and function in every aspect of RNA processing to produce mature mRNA and regulate mRNA localization, stability and translation (Gerstberger et al., 2014). 5′cap and 3′ poly(A) tail structures can be removed by decapping enzymes and deadenylases to cause mRNA degradation (Roy and Jacobson, 2013). Binding to cis-acting elements in the mRNA, RBPs interact with decapping or deadenylation enzymes to affect mRNA stability (Feigerlová and Battaglia-Hsu, 2017). For instance, RBPs HuR and TTP bind to the AU-rich elements (AREs) in the 3′UTRs of mRNA to promote stability or trigger decay of mRNA (Brennan and Steitz, 2001) (**Figure 1**). Proper function of these intricate post-transcriptional manipulations of the RNA network is essential for the vascular endothelial system. Many RBPs and RBP-regulated RNA network disruptions have been implicated in the development of diabetic dysfunction of

TABLE 1 | Dysregulated genes and vascular dysfunctions under diabetic conditions.

Vascular functions	Dysregulated genes under diabetic conditions	Related vascular dysfunctions
Angiogenesis and cell junction	VEGF KDR FGF2 VE-Cadherin	Neovascularization and vascular leakage
Vascular tone	Cox-2 EDN-1 EDN-2	Enhancement of vascular contractility
Inflammation	IL3 IL8 MCP1 CX3CL1	Inflammatory infiltration
Apoptosis	CASP1 CASP3 BAX BCL2	Apoptosis of ECs
Intercellular adhesion	ICAM-1 SELP SELE ITGB3	Adhesion of monocytes and activated platelet
Cell matrix	FN1 COL3A LAMB1 MMP-1 MMP-9	Promoted matrix degradation and accelerated atherogenesis and reduced plaque stability

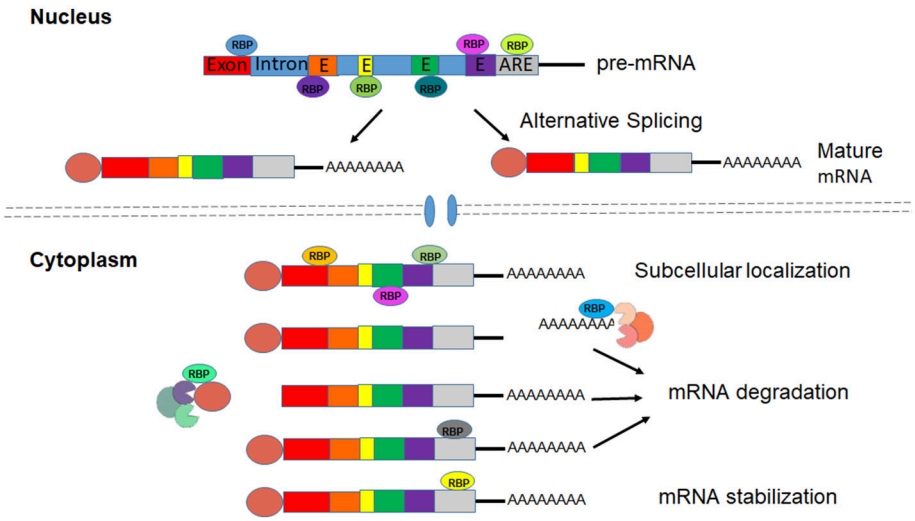

FIGURE 1 | RBPs regulate processing, Localization and stabilization of mRNAs.

the vascular endothelium (Scott et al., 2007). The current review focuses on five critical RNA binding proteins HuR, TTP, SRSF1, SRSF6, and QKI (**Table 2**).

HUR

HuR is a member of the Drosophila embryonic lethal abnormal visual (ELAV) protein family that binds to mRNA PolyU- and AU- rich elements and prevents RNase mediated degradation (Chang and Hla, 2014; Pullmann and Rabb, 2014). HuR is up-regulated and activated in diabetes by various mechanisms including MiRNA regulators (MiR23 and MiR9) and protein kinase C (PKC)-mediated phosphorylation (Amadio et al., 2010; Jeyabal et al., 2016). Upon activation, HuR translocates from nucleus to the cytoplasm to bind and affect the stability and translation of target mRNAs (Govindaraju and Lee, 2013).

Vascular endothelial growth factor (VEGF) acts as a key regulator in the process of neovascularization and angiogenesis. In ECs from patients with diabetic retinopathy, HuR binds and stabilizes VEGF which triggers endothelial proliferation, migration and tube formation leading to pathological angiogenesis (Amadio et al., 2010). When streptozotocin (STZ)-induced diabetic rats were treated with intravitreal injection of lipoplexes, a Nanosystem loaded with siRNA silencing HuR expression, retinal HuR and VEGF are significantly decreased and diabetic retinal damage is alleviated (Amadio et al., 2016). Zhang et al. reported that when murine macrophages adhered to the β2 integrin ligand intercellular adhesion molecule-1 (ICAM-1), VEGF and matrix metalloproteinase-9 (MMP9) mRNAs were stabilized. Whereas, in tissue-specific HuR knockout mice, this mRNA stabilization effect was lost in bone marrow-derived macrophages. Further functional study verified the impaired recovery of blood flow and muscle neovascularization post femoral artery ligation (Zhang et al., 2012). Therefore, in contrary to its pathological angiogenic effects in diabetic retinopathy, HuR contributes to promoting the repair of damaged vascular endothelium via stabilization of VEGF.

Activation of vascular endothelial cells results in vascular diseases such as sepsis and atherosclerosis. In human pulmonary microvascular endothelial cells, HuR stabilized, and up-regulated mRNA levels of tumor necrosis factor (TNF)-induced interleukin-6 (IL-6) (Shi et al., 2012). Tiedje et al. reported that HuR binding to ARE was mandatory to stabilize and initiate translation of TNF at the endoplasmic reticulum (ER) (Tiedje et al., 2012). Cheng et al. demonstrated that HuR promoted endothelial activation by suppressing eNOS expression (Cheng et al., 2013). HuR knockdown by MiR-146a in vascular ECs negatively regulated inflammation via suppression of pro-inflammatory NF-κB, MAPK signaling pathways and downstream EGR transcription factors as well as decreases in ICAM-1, VCAM-1, and adhesion of monocytes (Rhee et al., 2010).

Sirtuin 1 (SIRT1) is the leading deacetylase in the SIRT family that serves as a protector against environmental stresses to promote cell survival (Chen et al., 2012). Vascular ECs under hyperglycaemia showed decreased SIRT1 expression. Abdelmohsen et al. reported that HuR bound to the 3′UTR of SIRT1 mRNA, stabilizing and increasing SIRT1 expression levels. Under oxidative stress, HuR was phosphorylated by ChK2 at residue Ser-100 leading to segregation of the HuR-SIRT1 mRNA complex. The degradation of separated SIRT1 mRNA coincided with the decreased SIRT1 abundance and compromised cell viability (Abdelmohsen et al., 2007).

TTP

Tristetraprolin (TTP), also known as zinc finger protein 36 homolog (ZFP36), belongs to the TIS11 family commonly containing tandem CCCH zinc fingers. TTP binds to 3′ UTR ARE region to induce destabilization and decay of mRNA by

TABLE 2 | RNA binding proteins associated with endothelial dysfunctions under diabetic conditions.

Gene	Description	Mechanisms	Target genes	Clinical implications	References
HuR	ELAV (Embryonic Lethal, Abnormal Vision, Drosophila)- Like 1	Binding to 3' UTR to stabilize mRNA	VEGF MMP9 TN Fα IL6 SIRT1	Knockdown of HuR alleviated diabetic retinal damages and suppressed monocyte adhesion	Abdelmohsen et al., 2007; Rhee et al., 2010; Zhang et al., 2012; Amadio et al., 2016
TTP	Tristetraprolin	Binding to 3' UTR to Induce mRNA decay by recruitment of deadenylation and decapping complexes	VEGF TNFα IL6 P65 HDAC1,3,7 CD36 HIF1α	TTP inhibited inflammation through TNFa/NFKB and suppressed atherosclerosis through regulation of CD36	Carballo and Blackshear, 2001; Ciais et al., 2004; Liang et al., 2009; Dai et al., 2014
SRSF1	Serine And Arginine Rich Splicing Factor 1	Alternative splicing of VEGF pre-mRNA adopting PSS in exon 8 to favor pro-angiogenic VEGF165	VEGF	Inhibition of SRSF1 activity switched VEGF splicing from pro- to anti-anglogenic and reduced neovascularization	Nowak et al., 2008; Peiris-Pagès, 2012; Mavrou et al., 2015; Batson et al., 2017
SRSF6	Serine And Arginine Rich Splicing Factor 6	Alternative splicing of VEGF pre mRNA adopting DSS in exon 8 to favor anti-angiogenic VEGF165b	VEGF	intravitreal application of recombinant VEGF165b reduced the neovascularization of the retina and the normally vascularized area was increased	Nowak et al., 2008; Peiris-Pagès, 2012
QKI	Quaking Homolog, KH Domain RNA Binding	Binding to 3′ UTR of STAT3 to stabilize mRNA or regulated translation.	STAT3 VE-cadherin VEGFR2 Fox01 β-catenin	Neovascularization and blood flow recovery were improved by transplantation of QKI5 over expressing IPS-ECs in the hind limb ischemia model; *in vivo* reduction of QKI increased vascular leakage; GKI-haploinsufficient patient showed suppressed foam cell formation implicating the suppression of atherosclerosis.	Puthanveetil et al., 2012; de Bruin et al., 2016a,b; Cochrane et al., 2017

recruiting deadenylation and decapping complexes (Lai et al., 2000; Lykke-Andersen and Wagner, 2005). mRNA- decapping enzymes DCP1A and DCP2, CCR4-NOT deadenylase, the 5′-3′ exoribonuclease 1 (XRN1), exosome complex endonuclease PM-SCl75 and argonaute 2 (AGO2) are important components of the mRNA decay machinery that directly binds to TTP (Fabian et al., 2013).

Both TTP and HuR bind to ARE elements, whereas studies have shown that ARE-containing mRNAs are stabilized by HuR but destabilized by TTP. There are three members in the TTP family, TTP, TTPL1 and TTPL2 who share the activity to destabilize mRNA. TTPL1 interacts with the two ARE elements in the 3′ UTR of VEGF mRNA to trigger degradation (Ciais et al., 2004), which was opposite to the effect of HuR. In another case, HuR stabilized and up-regulated IL-6 mRNA in human pulmonary vascular ECs, whereas TTP promoted IL- 6 mRNA degradation (Sauer et al., 2006).

Apart from IL-6, a plethora of mRNAs of inflammatory modulators have been reported to be destabilized by TTP, such as interleukin, interferon and chemokine ligand family members (Xin et al., 2014). TTP was discovered to be readily stimulated by insulin (Lai et al., 1990) and was well-characterized in immune functions (Sanduja et al., 2011). TTP-deficient mice showed severe, complex inflammatory phenotype such as cachexia, arthritis and autoimmunity. Such phenotypes were ameliorated by treatment with anti-TNFα antibody or backcrossing with TNFR1 knockout mice, suggesting that TTP suppressed inflammation through inhibition of TNFα production (Taylor et al., 1996; Carballo and Blackshear, 2001).

NF-κB mediates the major inflammatory signal pathways and aberrant NF-κB activation is related to tissue damage and inflammatory disorders such as atherosclerosis and arthritis (Pfitzner et al., 2004; O'Neill, 2006). TTP physically interacts with the p65 subunit of NF-κB and functions as a corepressor of p65/NF-κB. Overexpression of TTP inhibited NF-κB- dependent transcription. TTP is also associated with histone deacetylases HDAC1, −3, and −7 *in vivo*. HDAC1 or HDAC3 knockdown by histone deacetylase inhibitors or small interfering RNA completely or partly ablated the repression of TTP on NF-κB reporter activation (Liang et al., 2009). TTP repressed the expression of inflammatory cytokines in target cells via inhibition of NF-κB transcriptional activation and destabilization of the bound cytokine mRNAs (**Figure 1**).

Vascular endothelial dysfunction was also observed in TTP-deficient mice (Bollmann et al., 2014). $TTP^{-/-}$ mice showed stabilized and up-regulated NADPH oxidase 2 mRNA, which was associated with enhanced levels of ROS and nitrogen species (RNS). The alteration of ROS and RNS level was highly related to the disruption of acetylcholine-induced NO-mediated vasorelaxation.

Zhang et al. investigated the regulation of TTP on inflammation in vascular ECs and its direct binding to target cytokine mRNAs (Zhang et al., 2013). Healthy aorta showed minimal expression of TTP which was significantly increased in ECs overlying atherosclerotic lesions. TTP upregulation was also observed in macrophage foam cells of atherosclerosis. After migration into the subendothelial arterial space, the monocytes readily differentiate into macrophages and take in modified low-density lipoprotein (LDL) to form foam cells which are essential for atherosclerosis. The uptake of oxidized low-density lipoprotein (oxLDL) by macrophages is mediated by the scavenger receptor CD36. Dai et al (Dai et al., 2014) revealed that TTP, which bound to ARE in the 3′ UTR, promoted CD36 mRNA degradation. Therefore, TTP may act as an important inhibitor of macrophage foam-cell formation to deter atherosclerosis.

Hypoxia-inducible factor 1 (HIF-1) is an important regulator of vascular ECs to direct their response to changes of environmental oxygenation. A variety of genes related to glucose metabolism and angiogenesis are regulated by HIF-1(Dewhirst et al., 2008). Chamboredon et al. analyzed the regulation of HIF-1α mRNA expression in ECs under hypoxic conditions and revealed that hypoxia-induced down-regulation of HIF-1α mRNA in ECs was mediated by TTP, which bound specifically to HIF-1α 3′ UTR. The decrease in the half-life of luciferase HIF-1α-3'UTR reporter transcript with prolonged hypoxia was mediated by TTP. While knockdown of TTP in ECs reversed the decrease of HIF-1α mRNA induced by hypoxia (Chamboredon et al., 2011).

SRSF1/6

Splicing is one of the key steps of RNA processing toward mature mRNA including intron removal from the pre-mRNA and subsequent exon ligation. By alternative splicing, selection of various subsets of exons results in different isoforms of transcripts generated from the same gene. Alternative splicing is a major origination of proteins with different functions, but in some cases abnormal RNA splicing can lead to disorders (Feero et al., 2010).

The VEGF gene plays a big part in the processes of vascularization and angiogenesis. The VEGF mRNAs are derived from eight exons to encode at least six protein isoforms. These products are termed VEGF121, VEGF165, VEGF189, etc. based on the number of amino acids, among which VEGF165 is the main isoform. When the distal splice site (DSS) in exon 8 is selected, the last exon turns to exon 8b resulting in a novel family of isoforms of VEGF, termed VEGF121b, VEGF165b, VEGF189b, etc. The first identified member of this family was VEGF165b, which is the only one whose effect on EC functions has been investigated (Bates et al., 2002). The produced proteins of the two families have different C-terminal amino acid sequences. During alternative splicing, when the proximal splice site (PSS) is selected, the C-terminal codes for CDKPRR to form VEGF165, while the selection of distal splice site (DSS) results in the C-terminal coding of SLTRKD to form VEGF165b isoform. In spite of the encompassment of receptor-binding domains, the VEGF165b isoform is not able to activate VEGF-R2. What's more, it acts as a competitor to inhibit the normal functions of VEGF165 on the regulation of EC proliferation, migration and vasodilation (Ladomery et al., 2007). Therefore, the alternative splicing products of VEGF165 and VEGF165b function as pro- or anti-angiogenic regulators separately (Peiris-Pagès, 2012). In the normal vitreous VEGF165b isoforms account for nearly two thirds of the total VEGF. In the diabetic vitreous, however, VEGF165 is significantly increased and becomes the dominant form Perrin et al. (2005). In the circumstance of diabetic retinopathy, the splicing of VEGF switches to the pro-angiogenic isoform (VEGF165) to favor vascularization in the retina.

The serine/arginine-rich (SR) proteins play critical roles in the alternative splicing of the VEGF RNA transcript. Computational sequence analysis of the VEGF gene revealed a predicted binding site for Serine/Arginine Rich Splicing Factor 1 (SRSF1) before the DSS, and a predicted SRSF6 binding site behind the DSS (Peiris-Pagès, 2012). When SRSF1 binds to the pre-mRNA of VEGF, the PSS in exon 8 is preferred to generate VEGF165 isoform. When SRSF6 binds to the pre-mRNA, the DSS dominates to form VEGF165b (Nowak et al., 2008). SRSF1 is modulated by upstream regulators SR protein kinases 1 and 2 (SRPK1/2). SPRK1 can be inhibited by small molecule inhibitors or down-regulated by RNA interference (RNAi) to block activation and nuclear transportation of SRSF1. SRSF1 inhibition consequently switches the selection of PSS to DSS and benefits the generation of anti-angiogenic VEGF165b isoform (Amin et al., 2011; Mavrou et al., 2015).

Through PKC-induced activation of SRPK1, IGF1 and TNFα support the selection of PSS in exon 8, whilst TGFβ activates p38 mitogen-activated kinases (p38 MAPK) through Clk1 and phosphorylates SRSF6, which favor the usage of DSS element (Harper and Bates, 2008; Nowak et al., 2008). Small molecule inhibitors of SRPK1, such as SRPIN340, MVRL09, and SPHINX31 are potent regulators to inhibit the binding of PSS in exon 8 by SRSF1 to elevate the level of VEGF165b through alternative splicing. In an animal model of retinal angiogenesis, application of SRPK1 inhibitor greatly inhibited activation of SRSF1, switching VEGF splicing from the pro- to anti-angiogenic form to block neovascularization (Gammons et al., 2013a,b; Batson et al., 2017). Injection of SRPK1/2 inhibitor SRPIN340 into the retina of an oxygen-induced retinopathy mouse model significantly reduced the neovascular area of the retina and the normally vascularized area was increased, which was equivalent to the effect of intravitreal application of recombinant VEGF165b. Based on these findings, modulation of VEGF165/VEGF165b balance by regulation of alternative splicing machinery may be a novel therapeutic strategy for diabetic retinopathy and other vascular disorders (Nowak et al., 2010).

QKI

Quaking (QKI) is an RNA binding protein belonging to the Signal Transduction and Activation of RNA (STAR) protein family which contain SH2 and SH3 domains, an RNA-binding motif (e.g. a KH domain) and phosphorylation sites. This implies they may play a role in the splicing of pre-mRNAs (Vernet and Artzt, 1997), mRNA nuclear exportation, mRNA stability and translation into the subsequent protein. They may also be involved in some signal transduction pathways (Justice and Hirschi, 2010). There are several QKI isoforms, three of which (QKI5, QKI6 and QKI7) have been linked to vascular development. Each of these isoforms holds different carboxy-terminal ends but matching RNA binding domains (Chénard and Richard, 2008). QKI is generally required in endothelial barrier function maintenance as it increases VE-cadherin and β-catenin expression in epithelial intercellular junctions. These three QKI isoforms are present in ECs, with QKI5 being the most abundant (de Bruin et al., 2016b). QKI gene has been discovered in functional studies to be critical in the formation and remodeling of embryonic blood vessels (Noveroske et al., 2002). The expression of QKI was observed in the yolk sac endoderm and homozygous QK^{k2} allele was found to be lethal to the embryos due to disrupted vasculature development.

Forkhead box O1 (FoxO1) belongs to the forkhead family of transcription factors that participates in a variety of important biological events including cell proliferation, cell death, immunologic reaction, regulation of metabolism in response to oxidative stress etc. (Puthanveetil et al., 2013). Studies have revealed the involvement of FoxO1 in the development of cardiovascular diseases and diabetes (Puthanveetil et al., 2012). In diabetes, the activated FoxO1 was associated with the dysregulation of metabolic homeostasis and activation of cell death signaling. Studies have revealed the decreased expression of QKI5 in the myocardium of ob/ob diabetic mice. When QKI5 expression was up-regulated, FoxO1 expression was repressed and the NS and ER stresses as well as ischemia/reperfusion injury alleviated. By RNA co-immunoprecipitation the interaction between QKI5 and FoxO1 mRNA was verified. When QKI5 was overexpressed the half-life of FoxO1 mRNA was shortened, suggesting the negative effect of QKI5 on FoxO1 mRNA stability.

Previous work from our lab demonstrated that QKI5 was suppressed in heart vessels isolated from diabetic mice compared to healthy controls, suggesting a role of QKI5 in vascular dysfunction under diabetic conditions. Using the model of induced pluripotent stem (iPS) cell differentiation toward ECs, our recent paper (Cochrane et al., 2017) reported a critical role of QKI5 in the generation of ECs from iPS cells via stabilization of CD144 and activation of VEGFR2 through STAT3 signaling. RNA immunoprecipitation confirmed the direct binding of QKI5 to the 3' UTR of STAT3 to promote mRNA stability. Remarkably, angiogenesis, neovascularization, and blood flow recovery were significantly improved by transplantation of QKI5 overexpressing iPS-ECs in the animal model of experimental hind limb ischemia. These findings suggest that QKI5 down-regulation in diabetes may contribute to vascular complications resulting from EC dysfunction and probably become a novel target for therapeutic treatment of diabetes. Meanwhile, QKI5 induced the splicing factor SF3B1 during EC differentiation in a time dependent manner, implying that QKI-5 contributed to EC induction from iPSCs as an important splicing regulator.

QKI was also reported to participate in the regulation of macrophage differentiation from monocytes (de Bruin et al., 2016a). QKI showed low levels of expression in monocytes and early human atherosclerotic lesions. When naïve human monocytes were converted to macrophages with GM-CSF or M-CSF, the expression of QKI protein isoforms was significantly increased. Consistently, the examination of $CD68^+$ macrophages in advanced atherosclerotic lesions revealed high abundance of QKI. In the QKI-haploinsufficient patient, foam cell formation was suppressed due to limited differentiation of macrophages from monocytes and subsequent reduced uptake of oxLDL along with decreased QKI expression. These studies illustrated the key role for QKI as a post-transcriptional regulator in the determination of macrophage fate and development of atherosclerosis (de Bruin et al., 2016b).

Given the observation that QKI isoforms were highly expressed in the healthy vascular endothelium in comparison with smooth muscle (van der Veer et al., 2013), de Bruin et al. studied the relevance for QKI and endothelial functions and underlying mechanisms. Both VE-cadherin and β-catenin were predicted to harbor high-affinity Quaking Response Element (QRE) (NACUAAY-N1–20-UAAY) in the 3′ UTRs of their mRNAs (Galarneau and Richard, 2005), suggesting their possible target identity of QKI which was confirmed by RNA immunoprecipitation of both mRNAs by QKI antibody. With QKI overexpression, luciferase- reporter assay with the 3′ UTRs of both genes achieved significant enhancement of luciferase activity, which was diminished when QKI was repressed. When QKI was knockdown by shRNA in the ECs, neither VE-cadherin nor β-catenin mRNA was significantly reduced, but their protein levels were decreased. For functional study, ECs with QKI knockdown failed to form a proper monolayer of high resistance, although their adherence and spread capacities were not affected. *In vivo* reduction of QKI resulted in a significant 40% increase of vascular leakage. Therefore, the translation of VE-cadherin and β-catenin was regulated by QKI through direct binding to 3′ UTRs. Reduced QKI expression resulted in the decrease of VE-cadherin and β-catenin proteins and subsequent impaired endothelial barrier function leading to vascular leakage.

LncRNAs AND RBPS IN ENDOTHELIAL CELLS UNDER DIABETIC CONDITIONS

Most of current research is centered on protein-coding RNAs, however, noncoding RNAs (ncRNAs) including long noncoding RNAs (LncRNAs) account for the majority of genome transcripts. Cooperating with RNA-binding proteins, LncRNAs participate in all aspects of biological processes and their importance is widely recognized. LncRNAs are >200 nucleotide long RNA transcripts which lack protein-coding potential (Yang et al., 2014). LncRNAs regulate gene expression at all levels through modulation of epigenetic machineries, recruitment of RNA-binding proteins, function as decoys and interact with miRNAs etc. Growing evidence has revealed the correlation of disrupted lncRNA

levels and a variety of human diseases, including diabetes. A number of LncRNAs have been reported to be dysregulated under diabetic conditions, such as MALAT1, MEG3, MIAT, RNCR3, and ANRIL.

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was significantly increased in retinal endothelial cells of STZ-induced diabetic rats and db/db mice or high glucose treated human umbilical vein endothelial cells (HUVEC) (Liu et al., 2014; Yan et al., 2014; Puthanveetil et al., 2015) indicating its involvement in the development of diabetic retinopathy and endothelial dysfunction. Knockdown of MALAT1 by intraocular injection of shRNA ameliorated retinal vascular dysfunctions of cell barrier defects, pericyte loss, capillary degeneration, and retinal inflammation. *In vitro* assays revealed the decreased retinal endothelial cell proliferation, migration and tube formation with MALAT1 downregulation. It is also reported that MALAT1 knockdown decreased the level of ROS in endothelial cells under hyperglycaemic conditions (Puthanveetil et al., 2015).

MALAT1 binds to the splicing factor SRSF1 directly and influences its phosphorylation, distribution in nuclear speckle domains and alternative splicing function (Tripathi et al., 2010). Depletion of MALAT1 or SRSF1 led to the increase of anti-angiogenic VEGF isoform VEGF165b. Tube formation assay of endothelial cells in the presence of conditioned medium from modified SKBR3 cells showed that in SRSF1 and MALAT1 interfered scenarios the angiogenic capacity of ECs was significantly decreased (Pruszko et al., 2017). Liu et al. reported that MALAT1 regulated the function of endothelial cells through the p38/MAPK signaling pathway (Liu et al., 2014). The cell proliferation induced by MALAT1 was blocked by p38/MAPK pathway inhibitor SB203580 or p38 siRNA and p38 stimulated phosphorylation was blunted by MALAT1 knockdown. The crosstalk between MALAT1 and p38/MAPK pathway may become a novel strategy for the therapy of diabetes-related microvascular complications. With MALAT1 silenced, the S-phage endothelial cyclins CCNA2, CCNB1, and CCNB2 were significantly downregulated, while cell cycle inhibitory genes p21 and p27Kip1 increased (Michalik et al., 2014). More study is still required to elucidate the mechanisms of MALAT1 effects on cell signaling and cell cycle regulation.

Myocardial infarction associated transcript (MIAT) is another LncRNA identified to be upregulated under diabetic conditions in retinal endothelial cells and fibrovascular membrane of diabetic patients (Strawbridge et al., 2011; Yan et al., 2015). *In vitro*, MIAT knockdown inhibited EC proliferation under hyperglycaemic conditiona and *in vivo* downregulation of MIAT in STZ diabetic rats alleviated the retinal vascular dysfunctions of pericyte loss, vascular degeneration and inflammation. MIAT possesses a tandem UACUAAC repeat motif and binds directly to the splicing factors SRSF1, QKI, Cef3 to form nuclear bodies (Tsuiji et al., 2011; Barry et al., 2014). The UACUAAC repeat motif binds to SRSF1 with a higher affinity than the divergent branch point sequence in mammals and therefore may modulate the alternative splicing of VEGF in favor of angiogenesis. MIAT also competes with miR-150 and miR-29b in retinal endothelial cells to regulate VEGFA level and apoptosis (Yan et al., 2015; Zhang J. et al., 2017).

Antisense Noncoding RNA in the INK4 Locus (ANRIL) is a 3.8 kb antisense RNA to INK4 locus. ANRIL has been found to be associated with vascular dysfunction in diabetes (Congrains et al., 2013). ANRIL level elevation was observed in human retinal endothelial cells exposed to high glucose and also the retina of diabetic animals, while overexpression of ANRIL upregulated VEGF expression (Thomas et al., 2017; Zhang B. et al., 2017). The increased retinal microvascular permeability in diabetic mice was alleviated by ANRIL knockout, which was in consistence with the dynamics of VEGF level. In human retinal endothelial cells exposed to high glucose, the key components of PRC complex EZH2 and p300 were significantly increased, while with ANRIL knockdown the dysregulation was corrected. VEGF was found to be regulated by miR200b through PRC complex and p300 and RNA-IP assay verified the direct binding of ANRIL to EZH2 and p300, indicating that upregulated ANRIL induced VEGF generation in high glucose treated ECs through interaction with PRC complex and p300. Moreover, by recruitment of PRC complexes, ANRIL epigenetically suppressed the expression of cell cycle regulators p15 and p16 which contain an overlapping sequence with ANRIL in the promoter region (Yap et al., 2010; Kotake et al., 2011).

It was reported that ectopic expression of ANRIL promoted angiogenesis by stimulation of NFκB signaling (Zhang B. et al., 2017). Moreover, in vascular ECs, TNFα induced ANRIL expression through NFκB and ANRIL interacted with PRC-associated transcriptional factor YY1 to regulate gene expression of IL6 and IL8 (Holdt et al., 2013; Zhou et al., 2016). Thus, the ANRIL- NFκB feedback loop may serve as a target to protect endothelial cells against dysfunction and atherosclerosis.

Retinal non-coding RNA3 (RNCR3) was first identified during mouse retinal development with dynamic expression (Blackshaw et al., 2004). RNCR3 was significantly up- regulated in retinas of diabetic animals and endothelial cells upon high glucose exposure. *In vivo* knockdown of RNCR3 ameliorated the diabetes-induced vascular dysfunctions of acellular capillaries, vascular leakage and inflammation. *In vitro* knockdown of RNCR3 suppressed EC proliferation, viability, migration and tube formation (Shan et al., 2016, 2017). It was revealed that RNCR3 functioned as a competing endogenous RNA (ceRNA) to regulate KLF2 levels by sponging miR-185-5p in endothelial cells.

Opposite to the above LncRNAs, maternally expressed gene 3 (MEG3) was reported to be decreased in retinal ECs of STZ diabetic mice (Qiu et al., 2016). *In vivo* knockdown of MEG3 led to retinal vascular dysfunctions of capillary degeneration, microvascular leakage and inflammation. *In vitro* knockdown of MEG3 in retinal vascular endothelial cells compromised EC angiogenic potential which was mediated by activation of PI3k/Akt signaling pathway.

Zhou et al. found that MEG3 interacted with p53 and overexpression of MEG3 enhanced p53 level and stimulated p53-dependent transcription implicating the involvement of p53 in the functioning of MEG3 (Zhou et al., 2007). As it is known that p53 binds to the VEGFA promoter and negatively regulates its transcription (Qin et al., 2006). Therefore, downregulation of MEG3 may contribute to the neovascularization and leakage

of diabetic retinopathy through p53 suppression and subsequent induction of VEGF.

RBP-BASED THERAPIES AND FUTURE PERSPECTIVE

As RBP-regulated RNA networks play a critical role in the development of diabetic vascular manifestations, targeting the candidate RBPs or RBP-RNA interactions could be a promising therapeutic strategy against diabetic vascular endothelial dysfunction.

Some RBPs *per se* are promising treatments for diabetic disorders. For instance, tristetraprolin (TTP) acts against diabetic inflammation and atherosclerosis through degradation of pro-inflammatory cytokines. TTP knockout mice showed overexpression of the potent pro-inflammatory cytokine TNFα and severe inflammatory phenotypes (Carballo et al., 1998). Inversely, TTP overexpression exerted profound suppression effect on inflammatory disease models (Patial et al., 2016). Kirkwood et al. reported the study on a rat model of periodontitis induced by intra-oral injection of LPS. When TTP overexpression was achieved by local application of an adenovirus expression vector, the complications of bone loss and inflammatory infiltration were significantly alleviated and the level of local cytokines was markedly reduced (Patil et al., 2008). Diabetic vascular complications feature inflammatory events and TNFα is implicated as an important mediator cytokine of inflammation in diabetes. Therefore, the discoveries from animal studies implies the possible beneficial effects of TTP application on diabetic inflammation conditions.

Under various pathologic conditions such as age-related macular degeneration (AMD) and diabetic retinopathy (DR), VEGF serves as a key mitogen to stimulate angiogenesis. Anti-VEGF antibodies have emerged as clinical treatment against AMD and DR. However, intravitreal injection of VEGF antibodies may harbor the risks of various complications, such as infection, inflammation and vitreous hemorrhage (Ventrice et al., 2013). Therefore, interest has grown to invent more effective drugs (e.g., small molecules) and safer methods (e.g., eye drop, ointment) of drug delivery to deal with neovascularization complications. Inhibition of serine-arginine protein kinase 1 (SRPK1) promotes the switch from the pro-angiogenic isoform VEGF165 to the anti-angiogenic isoform VEGF165b and suppresses pathologic angiogenesis (Dong et al., 2013). Using a computational protocol combined with a pharmacophore-based database search, Morooka et al. identified a new small molecule, SRPIN803, that inhibits both casein kinase 2 (CK2) and SRPK1 to suppress VEGF generation synergistically. In a laser-induced choroidal neovascularization mouse model, topical administration of SRPIN803 substantially suppressed intraocular neovascularization, suggesting SRPIN803 as a promising therapeutic drug for pathologic angiogenesis. (Morooka et al., 2015).

In diabetic conditions, highly expressed HuR translocates from nucleus to cytoplasm to bind and stabilize VEGF which triggers pathological angiogenesis. Amadio et al. carried out intravitreal injection of nanosystems loaded with siRNA against HuR (lipoplexes) to treat streptozotocin (STZ)-induced diabetic retinopathy in rats. The results showed that retinal HuR and VEGF were significantly silenced by HuR siRNA treatment and diabetic retinal damage was rescued (Amadio et al., 2016).

Anti-sense oligos (ASOs) are short single-stranded deoxyribonucleotides complementary to sense strand nucleic acid sequences. ASOs bind to target RNA sites and regulate the expression of genes by several mechanisms, including modulation of RNA stability, modification of RBP binding to RNA, regulation of RNA splicing, and mRNA translation (Lundin et al., 2015; Bishop, 2017). Some ASOs have progressed to human clinical trials for disease treatment, including cancer, diabetes, neurodegenerative disorders, and muscular dystrophy. Alternative polyadenylation of the KDR gene gives rise to two protein products with different functions, membrane-bound KDR (mbKDR) and soluble KDR (sKDR). sKDR functions as antagonist of lymphangiogenesis due to the lack of a tyrosine kinase domain. Accordingly, an antisense morpholino oligomer was designed complementary to the exon 13-intron 13 junction sequence to increase sKDR at the expense of mbKDR, thereby suppressing both haemangiogenesis and lymphangiogenesis. Uehara et al. demonstrated the suppression of laser choroidal neovascularization by intravitreal morpholino injection. Furthermore, subconjunctival application of the morpholino significantly inhibited corneal angiogenesis, lymphangiogenesis as well as graft rejection after transplantation in the mouse cornea. (Uehara et al., 2013). In conclusion, the post-transcriptional dysregulation of gene expression by RBPs plays important roles in the development and progression of diabetic endothelial dysfunction. The emergence and advancement of high throughput technologies enables the identification of RNA targets of RBPs, which offers novel insight into the mechanisms of diabetic disorders. Our deepened understanding of the mechanisms of RNA transcripts, as well as the critical regulatory roles played by RBPs enables the potential to provide novel therapeutic options for diabetic patients. In terms of the designation and application of RBP-RNA based therapeutic strategies, caution is necessary to ensure the requirement of safety and accuracy is fulfilled. The advances on specificity and efficacy of RBP-RNA treatments are currently being investigated. The future will see the possible inclusion of these smart therapeutic modalities in the therapeutic field to combat diabetic complications.

AUTHOR CONTRIBUTIONS

CY conception and design, manuscript writing. SK and RC revision and approval of manuscript. AM conception and design, financial support, final approval of manuscript.

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Circular RNAs as Therapeutic Agents and Targets

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It has recently been reported that thousands of covalently linked circular RNAs (circRNAs) are expressed from human genomes. circRNAs emerge during RNA splicing. circRNAs are circularized in a reaction termed "backsplicing," whereby the spliceosome fuses a splice donor site in a downstream exon to a splice acceptor site in an upstream exon. Although a young field of research, first studies indicate that backsplicing is not an erroneous reaction of the spliceosome. Instead, circRNAs are produced in cells with high cell-type specificity and can exert biologically meaningful and specific functions. These observations and the finding that circRNAs are stable against exonucleolytic decay are raising the question whether circRNAs may be relevant as therapeutic agents and targets. In this review, we start out with a short introduction into classification, biogenesis and general molecular mechanisms of circRNAs. We then describe reports, where manipulating circRNA abundance has been shown to have therapeutic value in animal disease models *in vivo*, with a focus on cardiovascular disease (CVD). Starting from existing approaches, we outline particular challenges and opportunities for future circRNA-based therapeutic approaches that exploit stability and molecular effector functions of native circRNAs. We end with considerations which designer functions could be engineered into artificial therapeutic circular RNAs.

Keywords: circRNA, transcription, splicing, microRNA sponges, aptamer, innate immnuity

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INTRODUCTION

High-throughput RNA-sequencing has shown that at least 20% of presently active genes express circRNAs (Salzman et al., 2012; Jeck et al., 2013; Memczak et al., 2013; Guo et al., 2014; Jeck and Sharpless, 2014; Wang et al., 2014). CircRNAs are 3′-5′ covalently closed RNA rings, and circRNAs do not display 5′Cap or 3′poly(A) tails. 3–10 different circRNA isoforms are formed per host gene, resulting in tens of thousands of distinct circRNAs per cell type (Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2013; Guo et al., 2014; Westholm et al., 2014; Zhang et al., 2014; Ivanov et al., 2015; Rybak-Wolf et al., 2015). circRNAs are produced by the process of splicing, and circularization occurs using conventional splice sites mostly at annotated exon boundaries (Starke et al., 2015; Szabo et al., 2015). For circularization, splice sites are used in reverse: downstream splice donors are "backspliced" to upstream splice acceptors (see Jeck and Sharpless, 2014; Barrett and Salzman, 2016; Szabo and Salzman, 2016; Holdt et al., 2018 for review). The majority of circRNAs are 3′-5′-linked circles containing exons without intervening introns. On average, circRNAs contain 1–5 exons and are 500 ribonucleotides (nts) long (Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2013). Most circRNAs tend to be enriched in the cytoplasm, but notable exceptions exist (Salzman et al., 2012; Hansen et al., 2013; Jeck et al., 2013; Memczak et al., 2013;

Liang and Wilusz, 2014; Zheng et al., 2016; Legnini et al., 2017). In 20% of all circularization events, introns do remain in the mature circRNA, constituting the class of Exon-Intron-containing-circular-RNAs (EIciRNAs) (Li et al., 2015). An even smaller fraction of circRNAs contains only introns and no exons, termed circular intronic RNAs (ciRNAs), which do not stem from backsplicing, but from conventionally spliced-out intronic lariats (Zhang et al., 2013). EIciRNAs and ciRNAs are defined by their presence and function in the nucleus (see chapter on molecular effector mechanisms below) (Zhang et al., 2013; Li et al., 2015). Given their circular nature, circRNAs of all classes are particularly stable compared to many linear RNAs because they are resistant to exonucleolytic decay by the cellular exosome ribonuclease complex (Szabo and Salzman, 2016; Holdt et al., 2018). The average half-life of endogenously produced 3′-5′-linked circRNA was found to amount to 19–24 h (Enuka et al., 2016) and can be up to 48 h (Jeck et al., 2013). In contrast, linear mRNAs show an average lifetime of only 4–9 h (Schwanhausser et al., 2011). High stability in biological systems is a major criterion for why circRNAs are becoming interesting for RNA-centered medical applications. Simultaneously, circularity is associated also with some other molecular properties that may be useful for therapeutic purposes, as will be discussed in the following.

BIOGENESIS OF ENDOGENOUS circRNAs

Details on circRNA biogenesis are described in recent reviews (Jeck and Sharpless, 2014; Barrett and Salzman, 2016; Szabo and Salzman, 2016; Holdt et al., 2018). In this chapter, we only highlight major points that are relevant for the subsequent considerations regarding therapeutic potential.

Splicing not only makes the linear transcriptome more diverse, but splicing also creates a very diverse set of circular RNAs. There is a tendency toward higher circRNA formation in more highly expressed genes, but overall, circRNAs are produced cell-type specifically (Salzman et al., 2013) and independent from changes in expression of their linear cognate transcripts (Salzman et al., 2013; Conn et al., 2015; Rybak-Wolf et al., 2015; Kristensen et al., 2018). To what extent circRNAs are regulated by global effects on backsplicing or circRNA stability during cellular transitions or due to gene-specific effects will have to be determined (Bachmayr-Heyda et al., 2015; Rybak-Wolf et al., 2015; Kristensen et al., 2018).

Analyses in different animal models have consistently shown that forward splicing is dominant, but backsplicing frequency is favored depending on context and availability of splice sites (Salzman et al., 2012; Ashwal-Fluss et al., 2014; Guo et al., 2014; Liang and Wilusz, 2014; Starke et al., 2015; Zhang et al., 2016; Liang et al., 2017): Two fundamentally different modes of circRNA biogenesis have been described: (1) Cotranscriptional backsplicing within the linear pre-mRNA and (2) Posttranscriptional backsplicing from within already excised exon(s)- and intron(s)-containing lariats (Barrett et al., 2015; Zhang et al., 2016). Intra-lariat backsplicing occurs physically separated from the maturing linear mRNA molecule. This distinction is of importance in functional terms, because only the execution of co-transcriptional backsplicing has been found to affect linear host mRNA from which the circRNA derives: After more than half of co-transcriptional backsplice events, the linear mRNA lacking the circularizing exon(s) cannot be detected anymore (Jeck et al., 2013). Also, there is a global anti-correlation of mRNA levels and circRNA formation frequency, supporting the view of mutual competition between linear splicing and backsplicing (Ashwal-Fluss et al., 2014; Zhang et al., 2014; Koh et al., 2016).

The following parameters determine circRNA biogenesis (relating to both co- and post-transcriptional modes):

(A) Reverse complementary repeats in the flanking introns, for example of the family of Alu repeats in the human genome, favor the backsplice reaction by hybridizing with each other and bringing splice sites in close proximity (Dubin et al., 1995; Liang and Wilusz, 2014; Zhang et al., 2014; Ivanov et al., 2015).

(B) A rather specialized compendium of splicing factor and RNA-binding proteins partakes in backsplicing through positioning splice sites in 3-dimensional space in the nucleus (Fujita et al., 1988; Ho et al., 2004; Ashwal-Fluss et al., 2014; Conn et al., 2015; Kramer et al., 2015; Errichelli et al., 2017; Li X. et al., 2017). This includes SR-family and hnRNP-family splicing regulators (Kramer et al., 2015; Li X. et al., 2017), and factors like Quaking (Conn et al., 2015), Muscleblind (Ashwal-Fluss et al., 2014) and FUS (Errichelli et al., 2017).

(C) The efficiency of backsplicing increases when forward splicing was kinetically slowed-down, such as after linear mRNA polyadenylation was impaired and read-through into adjacent genes occurred, or after core-spliceosomal components, such as the SF3a/b complexes, were limited in promoting the forward splicing reaction (Liang et al., 2017).

How circRNA biogenesis occurs in detail is a matter of ongoing research, and also bioinformatic circRNA-mapping algorithms are still improving (Szabo and Salzman, 2016; Hansen, 2018). Most DNA constructs that have been successfully employed for circRNA overexpression in cells used parts of native introns with inverted repeats (IRs) therein, or employ sequences artificially cloned in reverse complementary orientation adjacent to circularizing exons (**Figure 1A**) (Zhang et al., 2014; Kramer et al., 2015; Starke et al., 2015).

MOLECULAR EFFECTOR MECHANISMS OF circRNAs

Details of known circRNA-effector mechanisms and functions in physiology and disease are described in recent reviews (Jeck and Sharpless, 2014; Barrett and Salzman, 2016; Szabo and Salzman, 2016; Holdt et al., 2018; Patop and Kadener, 2018). Briefly, circular RNAs have been found to be biologically active agents in two conceptually distinct ways: either as freely diffusible molecules in nucleus and cytoplasm or because the circRNA

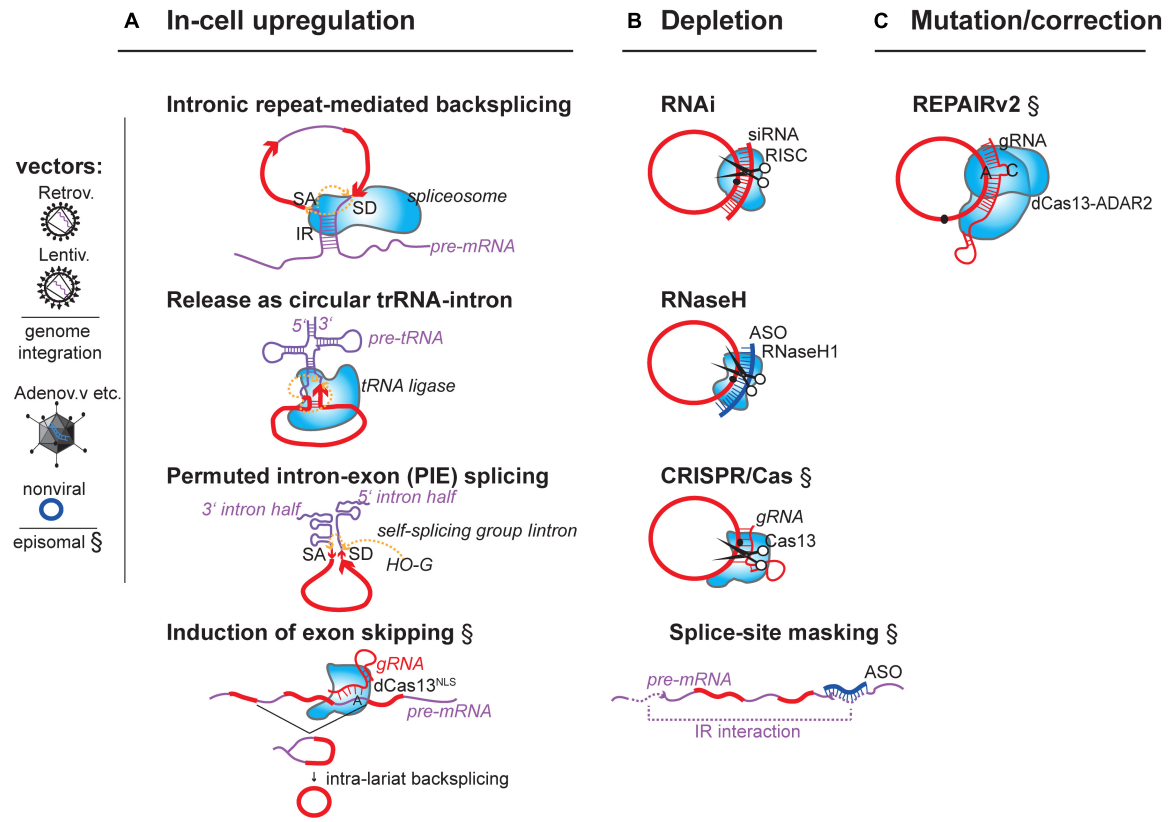

FIGURE 1 | Summary of concepts for modulation of endogenous circRNAs in mammalian cells. Strategies that have experimentally not yet been achieved are marked (§). **(A)** circRNA-overexpression using mini-gene constructs on RNA-/DNA vectors or affecting the endogenous circRNA-generating locus. (i) DNA mini-gene constructs contain RNA polymerase II (RNAP II) transcription start and terminator sites, splice acceptor (SA) and splice donor sites (SD). Exon(s) in the mini-gene constructs are >300 nucleotides (nts) long (Barrett et al., 2015; Kramer et al., 2015) and are flanked by intronic inverted repeats (IR) (Zhang et al., 2014) that are >30–40nts (Liang and Wilusz, 2014) and up to 300–500 nts long (Hansen et al., 2013; Kramer et al., 2015). (ii) RNA circularization through tRNA intron splicing. Expression by U6 promoter and capping signal (Lu et al., 2015). (iii) RNA circularization through autocatalytic intron-group I self-splicing. Catalysis still occurs when the catalytic intron is split into two halves, and when the order of introns and exons is inverted (3′ intron and SA upstream of 5′ intron and SD), but in such (permutated) cases, different from normal group I splicing, the intervening exonic sequence (arrow) in the PIE construct becomes circularized (Puttaraju and Been, 1992). G-OH (free guanosine) serving as hydrophile that initiates transesterifications. (iv) Enhancing circRNA levels by increasing the frequency of circRNA biogenesis. When exon-skipping is increased, more circRNAs can be produced that derive from skipped exons through post-transcriptional intra-lariat splicing. Targeting the catalytically inactive nuclear dCas13 variant via guide RNAs to intronic branchpoint adenosines and/or splice donor sites is known to increase skipping (Konermann et al., 2018). **(B)** circRNA depletion strategies: Depletion of mature circRNAs by (i) the shRNA-/siRNA loaded RISC complex (top), (ii) by RNase H endonuclease-dependent degradation. RNase H can be recruitment to circRNAs via transfected DNA-like antisense oligonucleotides (ASO, middle), or (iii) by single-stranded-RNA-targeting CRISPR/Cas nuclease versions (Class 2 CRISPR/ Cas13a, b, d). An alternative is to (iv) block circRNA biogenesis through masking intronic inverted repeats (IRs) via complementary ASOs (bottom). **(C)** Mutating/Correcting circRNA sequence: Expressing a fusion protein consisting of the ADAR2 adenosine deaminase linked to the catalytically inactive dCas13 nuclease allows deamination of A > I (inosine) at sites specified by a mismatch (C)-containing guide RNA that locates dCas13 to a specific circRNA. Inosine will base-pair with cytidine, potentially changing the properties of the circRNA at this site (changing binding affinities to other RNAs, DNAs or protein). Fusion of dCAs13 to different RNA-editing enzymes will expand the range of possible ribonucleotide conversions. RNA (red), introns (purple), antisense oligos (dark blue), RNA-binding proteins (light blue shapes).

biogenesis process itself has functional implications. Five major functions are known altogether:

(1) Circular RNAs may influence RNA polymerase II (RNAP II) initiation and elongation of their host gene. As best understood cases, subgroups of EIciRNAs (Li et al., 2015) and ciRNAs (Zhang et al., 2013), amounting to approximately 100 circular RNAs in each class, have been found to bind to RNAP II at promoters and to stimulate transcription by molecularly mostly unclear effector pathways. For example, EIciRNAs *circEIF3J* and *circPAIP2* associated with promoters depending on the U1 non-coding RNA (Li et al., 2015), which is known from separate work to instruct and activate general transcription initiation and elongation factors TFIIH and P-TEFb in the context of RNAP II transcription initiation and elongation.

(2) The co-transcriptional biogenesis of 3′-5′-linked circRNAs can reduce linear host mRNA levels and change downstream splice-site choice in mRNAs by still unknown mechanisms (Jeck et al., 2013; Ashwal-Fluss et al., 2014; Zhang et al., 2014; Koh et al., 2016): This has been described for endogenous candidate circRNAs,

such as *circMbl* (Ashwal-Fluss et al., 2014) or *ASXL1* (Koh et al., 2016), but evidence suggests this mechanism is of genome-wide significance. One possible determinant for competition with linear splicing is that backsplicing inflicts a 2′-5′ knot in the linear mRNA (Jeck and Sharpless, 2014; Barrett et al., 2015), possibly leading to termination of downstream transcription or serving as an entry point for exonuclease-mediated mRNA degradation (Liang et al., 2017). A second point is that backsplicing correlates positively with faster RNAP II transcription speed on gene bodies (Zhang et al., 2016), which *per se* affects splice site choice, an effect known as kinetic coupling between transcription and splicing apparatus (Caceres and Kornblihtt, 2002; de la Mata et al., 2003; Ip et al., 2011; Dujardin et al., 2014; Fong et al., 2014). Overall, backsplicing is thought to impair linear mRNAs not so much when circRNAs are posttranscriptionally produced, that is when they arise by backsplicing from within already excised exon-containing lariats (Barrett et al., 2015). Yet, also circRNAs are known that can affect linear mRNAs splicing choice as *trans*-acting factors, as shown for *circSEP3* in the plant *Arabidopsis thaliana* (Conn et al., 2017a).

(3) Few circRNAs, including *CDR1as* and Sry, carry a higher density of microRNA binding sites. These sequester and functionally inactivate microRNAs, an effect termed "sponging" (Hansen et al., 2013; Memczak et al., 2013). While *CDR1as* and *Sry* encode 74 and 16 of microRNA seeds, and are sufficiently highly expressed compared to the number of microRNA targets in a cell, as well as compared to the absolute number of microRNA copies per cell, few other endogenous circRNAs are expected to fulfill the stoichiometric requirements to be functional circRNAs at endogenous level (Mullokandov et al., 2012; Bosson et al., 2014; Denzler et al., 2014, 2016; Karreth et al., 2015; Thomson and Dinger, 2016). Also, compared to exons in mRNAs that do not circularize, circRNA-included exons are not overrepresented in Argonaute 2 (AGO2) pull-downs, nor are microRNA seed-matched sites overrepresented near backsplice junctions (Guo et al., 2014; You et al., 2015). Thus, the classical sponging concept seems inflated in currently published studies of endogenous circRNAs. The situation may be different for circRNAs whose therapeutic usefulness resides in their overexpression, as discussed below.

(4) Some mature circRNAs can bind to proteins to sequester them or affect their activity, like *circMBL* binding to MBL protein, *circPOLR2A, circDHX34* binding to NF90 and NF110, *circANRIL* binding to PES1 and *circPAPBN1* binding to HuR (Ashwal-Fluss et al., 2014; Holdt et al., 2016; Abdelmohsen et al., 2017; Li X. et al., 2017). CircRNAs can also serve as a scaffold for protein complexes, such as *circFOXO3* binding to CDK2/p21 (Du et al., 2016).

(5) At the moment, the view prevails that the vast majority of the thousands of circRNAs is not found in an active translation state (Jeck et al., 2013; Bazzini et al., 2014; You et al., 2015). Most circRNAs do also not stem from the very 5′ regions of genes (Guo et al., 2014), and, therefore, besides the fact that they do not carry a linear 5′Cap structure, do not encode features that enable protein translation from an endogenous start codon. A tiny fraction (<1%) of circRNAs does happen to contain the start AUG codon and to be associated with active ribosomes. In these cases circularization had led to the inclusion of 5′ untranslated region (5′UTR) sequences. For a handful of tested circRNAs, like *Drosophila circMbl, circPde8*, *circTai, circCdi* and mouse *circZNF609*, these 5′UTRs structurally folded into internal ribosome entry sites (IRES). This allowed the circRNAs to associate with the small subunit of ribosomes and become templates for protein translation despite being circular (Legnini et al., 2017; Pamudurti et al., 2017). Besides IRES-mediated translation, N(6)-adenosine methylation (m6A) has recently been shown to promote 5′Cap-independent translation in linear mRNAs (Zhou et al., 2015). This modification is also observed on circRNAs (Legnini et al., 2017; Zhou et al., 2017). But any specific role of m6A for circRNA translation, among many other possible influences of this modification on RNA half-life, sorting, and structure, remains to be decisively determined.

circRNA MANIPULATION IN CVD DISEASE MODELS *IN VIVO*

The molecular analysis of circRNAs is still in its infancy, but it is becoming clear that circRNAs can be functionally relevant, and that functions can be quite diverse. In the following chapter, we will focus on those circRNAs whose therapeutic potential can be assessed from knockdown or circRNA overexpression in mouse disease models of CVD *in vivo* (**Table 1**).

Myocardial Infarction

The mouse circRNA *MFACR* (acronym for *mitochondrial fission and apoptosis-related circRNA*) was identified to be upregulated in a mouse model of heart ischemia/reperfusion (Wang et al., 2017). *MFACR* was implicated as a sponge for *miR-652-3p*, a microRNA that targets MTP18, a nuclear-encoded mitochondrial protein important for balanced mitochondrial fission and cell viability. *MFACR* has 15 seed-matched sites for *miR-652-3p*. In a heart injury model *in vivo*, systemic inhibition of *MFACR* reduced apoptosis and heart dysfunction (Wang et al., 2017). Thus, a future therapeutic intervention would be to deplete *MFACR* after injury or to protect *miR-652-3p* from being sequestered by using conventional linear DNA/RNA oligonucleotides. Metabolically active cardiomyocytes may be particularly susceptible to this mechanism (**Table 1**).

Heart Failure

The mouse circRNA *HRCR* (acronym for *heart-related circRNA*) was found to be downregulated during experimentally-induced cardiac hypertrophy (Wang et al., 2016). Having six seed-matched binding sites for *miR-223*, *HRCR* was implicated as *miR-223* sponge. This microRNA targets the mRNA transcribed from the *ARC* gene (*Apoptosis repressor with CARD domain*)

TABLE 1 | Therapeutic potential of circRNAs in CVD.

Name (Species)	Investigated disease	Expression in disease	Endogenous function	Target cells (indirect evidence)	Protection from disease through	Mouse models *in vivo*	Dose; route	Reference
MFACR	Myocardial infarction	Up	Deleterious	Cardiomyocytes?	circRNA KD	Injection of adenovirus expressing shRNAs against *MFACR* into the aortic root rescues from heart dysfunction (I/R-induced heart injury mouse model).	2×10^{11} Pfu; once per 14 days	Wang et al., 2016, 2017
HRCR	Heart failure (cardiac hypertrophy)	Down	Protective	Cardiomyocytes?	circRNA OE	Injection of *HRCR*-generating adenovirus into jugular vein rescues from cardiac hypertrophy (ISO-induced heart injury mouse model).	2×10^{11} Pfu; once per 14 days	Wang et al., 2016
circAmotl1	Heart failure (Cardiomyopathy)	n.d.	Protective	Cardiomyocytes?	circRNA OE	Intraperitoneally injection of nanoparticles with mPEG-SH-formulated DNA plasmid (pBS) expressing human *circAmotl1* ameliorates ventricle dilation and stress-induced cell death (dox-induced mouse cardiomyopathy model).	100 mg plasmid; 2-3x per week	Zeng et al., 2017
circFoxo3	Heart failure (senescence, cardiomyopathy)	Up	Deleterious	Cardiomyocytes, fibroblasts?	circRNA KD	Injection of PEG-(10-nm) gold nanoparticle with siRNAs against *circFoxo3* ameliorates doxorubicin-induced cardiomyopathy.	6 μg, 3 times/week	Du et al., 2017b
circHipk3	Diabetic retinopathy	Up	Deleterious	Retinal vascular endothelial cells?	circRNA KD	Intravitreal injection of adenovirus-encoded shRNAs targeting *circHIPK3* in a mouse model for diabetic retinopathy is protective.	1.5×10^{9} Pfu; once per month	Shan et al., 2017
cZnf609	Ischemic retinopathy	Up	Deleterious	Retinal vascular endothelial cells?	circRNA KD	Intravitreal injection of adenovirus-encoded shRNAs against *cZNF609* ameliorates retinopathy in diabetic and hyperoxia-induced mouse retinopathy models.	1×10^{7} Pfu; once-twice per week	Liu et al., 2017
circDlgap4	Stroke	Down	Protective	Brain endothelial cells?	circRNA OE	Injection of *circDLGAP4*-generating lentivirus ameliorates brain damage in a transient middle cerebral artery occlusion mouse model.	5×10^{8} Pfu; once per 2 weeks	Bai et al., 2018

List of studies where levels of circRNAs have been manipulated with a therapeutic success in animal CVD models in vivo. CVD, cardiovascular disease; I/R, ischemia/reperfusion; ISO, isoproterenol; KD, knockdown; mPEG-SH, thiol-functionalized methoxyl polyethylene glycol; OE, overexpression; Pfu, plaque forming unit; shRNA, short hairpin interfering RNA.

and protects from hypertrophy in injured hearts. Systemic *HRCR* overexpression in a mouse heart failure model inactivated *miR-223,* hence, activating its target ARC. This also curbed cardiac hypertrophy. Since human *miR-223* levels were increased and ARC levels decreased in failing human hearts, a therapeutic option would be to deliver *HRCR* or to inhibit its downregulation (**Table 1**).

circ-Amotl1 was enriched in the neonatal (regenerative) heart compared with mature hearts in humans (Zeng et al., 2017). Systemic overexpression of human *circ-Amotl1* ameliorated left ventricle dilation and reduced apoptosis in a mouse cardiomyopathy model (Zeng et al., 2017). As an effector mechanism, the authors suggested that *circ-Amotl1* physically bound to well-known pro-growth and pro-survival AKT-PDK1 protein complexes in the phosphoinositide-3-kinase–protein kinase signaling pathway and activated them. *circ-Amotl1* would have to be therapeutically delivered with caution, however, because it has been independently described to be tumorigenic (Zeng et al., 2017) (**Table 1**). Whether the therapeutic effect in the rodent disease model is due to the physical interaction of human *circ-Amotl1* with the mouse AKT-PDK1 complex is still not fully clear. It should also be noted in this context, that human *circ-Amotl1* is not conserved in mouse or rat, although triggering consistent cellular effects. At least theoretically one can expect that the structural conservation of the AKT and PDK1 proteins are sufficiently high in mammals to allow *trans*-species interaction of circRNA with these proteins, but this remains to be shown.

Chemotherapeutic drugs and other stressors trigger senescence-associated pathological changes in hearts, and *circFoxo3* was studied as aging-induced circRNA (Du et al., 2017b). When siRNAs against *circFoxo3* were administered, the pathological left ventricular dilation in a doxorubicin-induced cardiomyopathy mouse model decreased, cardiac fibrosis was ameliorated and apoptosis and senescence-associated β-Galactosidase accumulation decreased (Du et al., 2017b). Conversely, overexpression of *circFOXO3* from mini-gene constructs on injected plasmids worsened heart function parameters. Determining the underlying molecular pathway will be complex, not last, because the described function cannot easily be tied to the known role of *circFoxo3* as more general cell cycle inhibitor (Du et al., 2016). Also, Foxo3 protein produced from linear *Foxo3* mRNA is implicated in cell growth suppression in the heart and other organs, and in senescence onset and longevity control (**Table 1**).

Neoangiogenesis

circHIPK3 is induced in diabetic human retinas (Shan et al., 2017). When systemically depleting *circHIPK3* with shRNAs targeting the backsplice junction in a mouse model for diabetic retinopathy, the disease phenotype was ameliorated. Complicating a direct therapeutic use, *circHIPK3* activity confers a general pro-proliferative function in a number of different cell types (Zheng et al., 2016). Additionally, *circHIPK3* downregulation increases migration, invasion, and angiogenesis of cancer cells (Li Y. et al., 2017), and impairs β-cell function (Stoll et al., 2018). Therefore, a system-wide and long-term downregulation of *circHIPK3* may carry an increased risk for cancer development and metabolic syndrome (**Table 1**).

circZNF609, a proposed *miR-615-5p* sponge, was shown to be induced in diabetic human retinas. *circZNF609* contains one seed-matched binding site. The mouse orthologous circRNA exerted deleterious cell overproliferation and a pro-angiogenic role while expressing shRNAs against *cZNF609* reduced capillary degeneration and pathological angiogenesis in a mouse oxygen-induced retinopathy model (Liu et al., 2017). The accessibility of the eye for topically administering interfering oligonucleotides might make *cZNF609* an accessible candidate for therapy. At the same time, *cZNF609* carries separate functions in myoblast differentiation (Legnini et al., 2017), raising issues on the specificity of effect (**Table 1**).

Stroke

circDLGAP4, a proposed *miR-143* sponge, was less abundant in brain tissue in a rodent cerebral stroke model, and in blood plasma of human acute ischemic stroke patients (Bai et al., 2018). The circRNAs has one seed-matched binding site for *miR-143*. Injecting *circDLGAP4*-generating lentivirus into the lateral brain ventricle reduced the ischemic infarct volume and improved neurological function in the mouse stroke model (**Table 1**).

The studies described here are representative of how circRNAs are currently used as therapeutic agents and targets in different pathologies. It should be understood that the presented cases are published studies related to CVD, and not necessarily those with the highest expectable therapeutic potential. In all cases, circRNAs and circRNA-knockdown construct are delivered systemically, although lesions are local, and although the candidate circRNAs are known to be endogenously expressed in more than one organ. It is, therefore, rather astonishing, but also promising, that such interventions were able to ameliorate specific CVD endpoints at all. It must be stated that before clinical trials could be thought of, the expected negative effects on proliferation and differentiation of non-target cells and organs have to be addressed.

STABILITY OF CIRCULAR RNAs IN BIOLOGICAL SYSTEMS

Stability against exonucleases is an overarching feature of circRNAs. Highly stable therapeutic RNAs could be valuable in cases where the therapeutic agent must be administered less frequently, or in smaller doses, which would also minimize non-specific side effects. Endogenously produced circRNAs are 2-5 times more stable than linear RNAs (Schwanhausser et al., 2011; Jeck et al., 2013; Enuka et al., 2016). What these numbers mean for ectopically delivered therapeutic ribonucleic acids is not so clear, yet. No data exist that report on the stability of circRNAs *in vivo*. A single study, so far, reported on the stability of synthetic circRNAs in cultured cells *in vitro* (Jost et al., 2018): Their half-life was 8–20 h, depending on the delivery regime, which can be considered short compared to endogenous circRNAs and to the times of effectiveness of modern linear RNA therapeutic agents. For example, antagomiRs, modified antisense DNA- or

RNA-like oligonucleotides (ASOs) and double-stranded siRNAs all show *in vivo* effects for 10–15 days, and up to several weeks, after a single injection into the body (Bennett and Swayze, 2010; Crooke et al., 2018; Jost et al., 2018). In analogy to ASOs, and with the aim to improve stability especially in the extracellular space, circRNAs could in the future be engineered to carry, for example, 2′-*O*-methyl-, -fluoro- or –*O*-methoxyethyl conjugates, phosphorothioate backbones, or 2′,4′-cyclic 2′-*O*-ethyl modifications (Krutzfeldt et al., 2005; Crooke et al., 2018). However, some types of modifications are thought to trigger liver and renal toxicity or local cutaneous reactions (Swayze et al., 2007; Burdick et al., 2014; Burel et al., 2016). On the other hand, chemical modification of circRNAs may theoretically optimize also other pharmacokinetic properties, binding to targets and escape from immune sensors.

FUTURE THERAPEUTIC AVENUES USING circRNAs

A future therapeutic use of circRNAs could be envisioned in two ways. One is the modulation of native disease-linked circRNAs by therapeutic knockdown or by ectopic expression. A second is the engineering of non-native (artificial) circRNAs with designer molecular effects (**Figure 1A**).

Modulation of Native Disease-Linked circRNAs

At least two concepts for therapeutic modulation of endogenous disease-linked circRNAs are conceivable: (1) Modulation of native circRNAs (**Figure 1**) and (2) Engineering of *in vitro*-produced circRNAs (**Figure 2**). To modulate native circRNAs, one can overexpress native protective circRNAs and reconstitute missing circRNAs from genetic vectors (**Figure 1A**), deplete endogenous disease-promoting circRNAs (**Figure 1B**), or correct aberrantly produced circRNA-isoforms (**Figure 1C**). On the other hand, *in vitro*-produced RNA circles can be transferred into cells as unmodified RNA (**Figure 2A**) or as modified RNA (**Figure 2B**).

Overexpression of native protective circRNAs has been achieved from RNAP II-driven constructs on standard DNA expression vectors in cell culture (Hansen et al., 2013; Memczak et al., 2013; Ashwal-Fluss et al., 2014; Li et al., 2015; Du et al., 2016; Holdt et al., 2016; Legnini et al., 2017), and from lentiviral or adenoviral vectors *in vivo* (Wang et al., 2017; Bai et al., 2018; Xia et al., 2018) (**Figure 1A**, see also **Table 1**). These encode a mini-gene cassette with exon(s), endogenous splice donor and acceptor sites, and flanking intronic inverted repeats that support RNA backfolding (**Figure 1A**). Alternatively, and independently of the cellular spliceosome, circRNAs are successfully expressed also from within intron boundaries encoded from the anticodon stem-loop on an engineered tRNA construct (Salgia et al., 2003; Lu et al., 2015; Noto et al., 2017). Processing, in this case, occurs by the evolutionarily conserved tRNA ligase complex (RTCB/HSPC117 ortholog in humans (Popow et al., 2011)) (**Figure 1A**). In-cell production of human circRNAs can be engineered to involve RNA ribozymes that mediate circularization by RNA self-processing (Zaug et al., 1983; Sullivan and Cech, 1985; Puttaraju and Been, 1992; Wesselhoeft et al., 2018) (**Figure 1A**). Another tangible approach for post-transcriptional production of circRNAs would be to use ASOs that bind to splice sites or splice enhancers and increase the frequency of co-transcriptional backsplicing. This would enhance the primary alternative splicing event so that more circularization can occur when more exon-containing lariats are present (**Figure 1A**). Given the competition with linear splicing (Jeck et al., 2013; Ashwal-Fluss et al., 2014; Jeck and Sharpless, 2014; Zhang et al., 2014, 2016; Barrett et al., 2015; Koh et al., 2016; Liang et al., 2017), the same strategy would indirectly also decrease the levels of cognate linear host mRNAs. Since the underlying competition is thought to occur *in cis*, at the level of pre-mRNA splicing, and not via *trans*-acting circRNAs, such a re-programming of mRNA levels would not be possible by delivering circRNAs via plasmids or transfecting synthetic circRNAs. Since backsplicing amounts, however, to only a low percentage (>10%) of the total mRNA output of any protein-coding gene (Chen et al., 2017), the potency of such trans-interference will be accordingly low. This can, however, also be an advantage and allow fine-tuning of the expression landscape.

Depletion of endogenous disease-promoting circRNAs has already been achieved using standard genetic tools, RNA interference (RNAi) or ASO-mediated RNase H-dependent degradation (see Swarts et al., 2014; Khvorova and Watts, 2017 for recent reviews) (**Figure 1B**). In the future, modern ssRNA-targeting by CRISPR-Cas9/13 variants (O'Connell et al., 2014; Abudayyeh et al., 2016, 2017; Konermann et al., 2018) will likely be applied (**Figure 1B**). To achieve specificity in circRNA-knockdown, all these nucleases must be guided selectively to the circRNA-specific backsplice-junction (Barrett et al., 2017; Piwecka et al., 2017). In the literature, *MFACR*, *circHIPK3*, *cZNF609,* and *circFoxo3* (**Table 1**) have been depleted *in vivo* using short hairpin (shRNAs) and small interfering siRNAs to trigger RNAi. An already implemented and very specific therapeutic approach for circRNA knockdown relates to depletion of fusion-circRNAs (f-circRNAs): These arise when chromosomal translocations bring introns from two unrelated genes in close genomic vicinity, which undergo backsplicing (Guarnerio et al., 2016). It was recently shown that knockdown of these f-circRNAs was sufficient to trigger apoptosis in leukemic cells while no toxic effects on normal cells could be measured (Guarnerio et al., 2016). Thus, f-circRNAs are interesting and potentially rather selective targets in treating certain cancers and, maybe, also other translocation-triggered pathologies. A parallel option to decrease circRNA levels would be to administer ASOs that bind and mask regulatory splice enhancers or silencers, or inverted intronic repeats in a specific pre-mRNA before circRNA biogenesis can take place (**Figure 1B**). This has not yet been experimentally tested. As mentioned before, inhibition of backfolding should in theory block circularization, and in some instances even favor linear mRNA splicing, given the competition between co-transcriptional backsplicing and mRNA maturation.

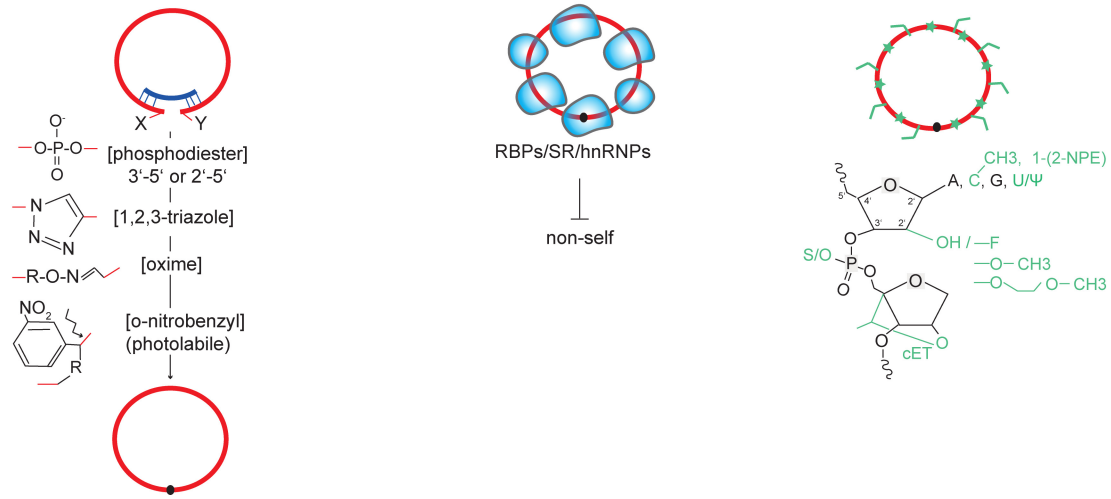

FIGURE 2 | Delivery of *in vitro* circularized circRNAs for therapeutic use. Strategies that have experimentally not yet been achieved are marked (§). **(A)** Chemical structures of key *cis*-reactive chemical moieties mediating chemical or enzymatic circularization (by RNA ligases or ribozymes). As one example, introducing photolabile linkers into the backbone of circular RNAs can be employed to acutely inactivate circRNAs through linearization (Richards et al., 2010; Griepenburg et al., 2013; Wu et al., 2013) (bottom). **(B)** Strategy for coating synthetically produced circRNAs with RNA-binding proteins and splicing factors. Coating could be used to avoid recognition as non-self and inhibit innate-immune activation after transfection. **(C)** Possible chemical modification of synthetic circRNAs at RNA bases, 2′ position in the ribose, and in the phosphodiester backbone. Modifications can have effects on improving stability, binding affinity, and pharmacokinetics (see text). ψ (Pseudouridine), cET (2′,4′-constrained ethyl), 1-[2-NPE] (1-[2-nitrophenyl]ethyl). NPE-like modifications of the RNA nucleobases, or other photolabile cages in RNA, can be conditionally cleaved-off by light, allowing acute conversion to an active state (Schafer et al., 2013). RNA (red), antisense oligos (dark blue), RNA-binding proteins (light blue shapes).

Not last, being able to sequence-specifically correct ribonucleic acid sequence inside living cells will, likely, become another interesting option also for circRNA-based therapeutics (**Figure 1C**): Fusion of catalytically-inactive Cas13 enzyme with an ADAR deaminase has already been proven successful for manipulating linear RNA sequence at the level of the transcript (Cox et al., 2017).

Delivering synthetic RNA circles into cells is another therapeutic approach. Such synthetic circRNAs are produced *in vitro* and must then be transfected into target cells (**Figure 2**). Different methods exist to achieve RNA circularization (see Muller and Appel, 2017 for review) (**Figure 2A**) and to formulate such circRNAs, such as by coating them with native RNA-binding proteins (**Figure 2B**), or by adding chemical moieties that change the physicochemical properties of the RNA (**Figure 2C**). While the consequences of artificial circularizing linkers have to be addressed functionally in each case, first estimation allow to predict that their structural and chemical alterations tend to be minor (e.g., regarding circularization by 2′-5′ bonds (Sheng et al., 2014) or by triazole linkers (El-Sagheer and Brown, 2012)] (**Figure 2A**). Among different circularization strategies, methods using ribozymes and click-chemistry-based linkage seem to be particularly efficient and cost-effective alternatives to commercial recombinant RNA ligases.

Engineered Non-native (Artificial) Circular RNAs With Designer Molecular Effects

Aside from modulating the expression of native circRNAs, circRNAs can also be engineered to carry specific properties (**Figure 3**). Here, we will discuss several major concepts for engineering designer circRNAs, with the most feasible ones ranked first: (A) MicroRNA-sponging by circRNAs; (B) Circularization of known linear therapeutic RNAs; (C) Protein translation from circRNAs; (D) Modulation of the immune system by circRNAs; (E) Protein activity control by circular RNA aptamers; (F) Transcription/splicing control by circRNAs; and (G) Engineering autonomous RNA replication of therapeutically delivered circRNAs *in vivo*.

(A) MicroRNA sponging by hybridization to 8 nts-long seed motifs in competing-endogenous RNAs, including circRNAs, prevents homologous target mRNAs from degradation or allows their translation (Franco-Zorrilla et al., 2007; Poliseno et al., 2010; Cesana et al., 2011; Salmena et al., 2011; Tay et al., 2011) (**Figure 3A**). Considering recently published experiments, only a few endogenous circRNAs like *circCDR1as* or *circSry* (Hansen et al., 2013; Memczak et al., 2013) would meet the very

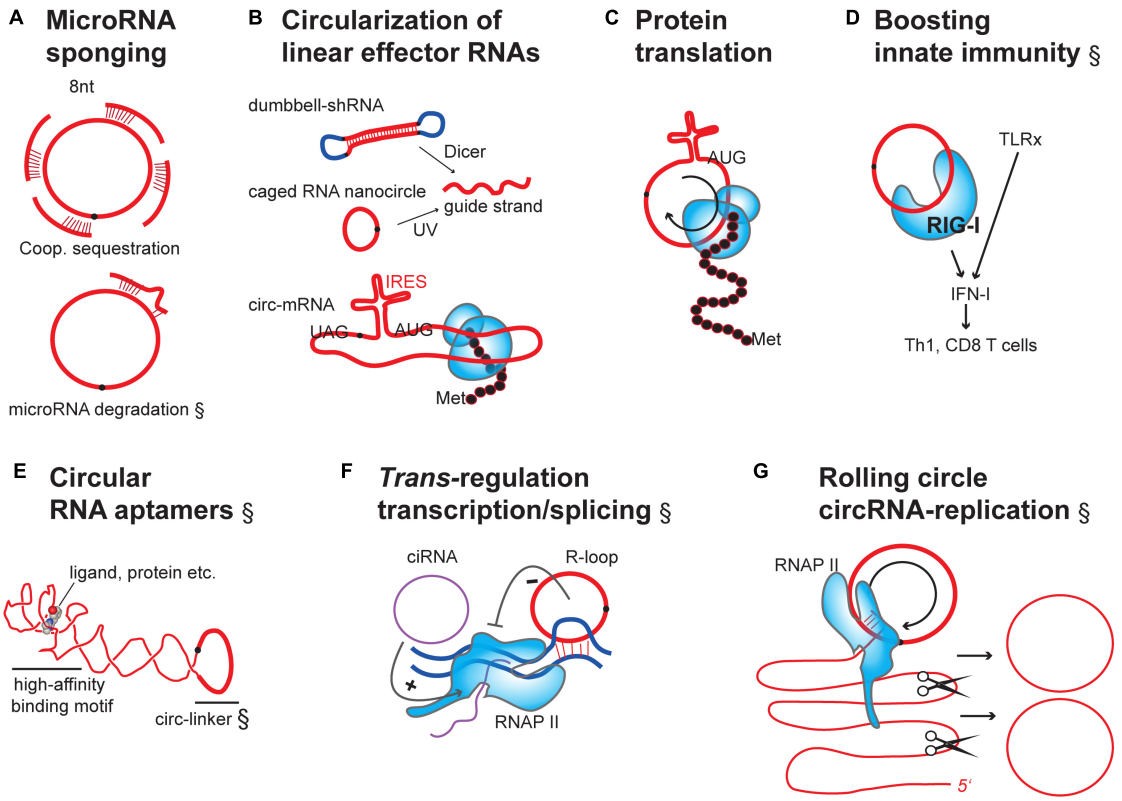

FIGURE 3 | Engineering designer effector functions in overexpressed or transfected circRNAs. **(A)** circRNAs as microRNA sponges: Binding of microRNAs to circRNAs containing multiple, cooperatively-acting seed-matched sites leads to inactivation by sequestration (top). Additional bonding via the 3′end of microRNAs can lead to target-induced decay of the microRNA (bottom). **(B)** Artificial circularization of linear effector RNAs: linear effector RNAs can be circularized to increase the potency of effect (e.g., shRNAs, siRNAs or mRNA). For siRNAs to become active, their precursors need to be processed (top). mRNAs must be engineered to contain an internal ribosome entry site (IRES) for protein translation (bottom). **(C)** Rolling-circle translation from circRNAs: In the absence of stop codons continuous translation can occur, leading to repetitive polypeptides. These can have potential structural and functional use, or be toxic. **(D)** Naked circRNAs can be delivered to purposefully boost the innate immune system and, indirectly, influence quality and quantity of effector and memory lymphocyte adaptive immunity. TLRx, Toll-like receptors; IFN, interferon; Th1, T helper cells; CD8 T cells, cytotoxic T cells. **(E)** Use of circular RNAs as stable aptamers: schematic of circular RNA aptamer binding to a ligand/protein by adopting a high-affinity structure (G-quadruplex motif) (Huang et al., 2014). Redrawn from the crystal structure of the *Spinach* RNA aptamer bound to its ligand (PDB (Berman et al., 2002): 4Q9Q). Note that the circularizing linker was not in the original structure. **(F)** Stimulation of transcription initiation and elongation by different circular RNAs species binding to RNAP II: Intron-only circular RNAs (ciRNAs) bind to promoter regions: exon-only 3′-5′ circRNAs can bind to gene loci through triple-helix formation (R-loop). circRNAs of this class would hypothetically be useful for fine-tuning of transcription, epigenetic engineering, or encoding synthetic transcriptional circuits. **(G)** Hypothetical model for self-replication of synthetically produced and transfected circRNA: Permanent circRNA maintenance in dividing cells involves host cell RNAP II-dependent rolling-circle RNA transcription,self-splicing of the long linear multimeric RNA precursor into single linear RNA units and circularization by host cell RNA ligases. RNA (red), introns (purple), DNA or DNA linkers (dark blue), RNA-binding proteins (light blue shapes).

specific conditions and stoichiometric requirements to be endogenous microRNA sponges (Mullokandov et al., 2012; Bosson et al., 2014; Denzler et al., 2014, 2016; Karreth et al., 2015; Thomson and Dinger, 2016). Yet, it can be expected that the act of overexpressing a therapeutic circRNAs changes stoichiometry and allows these circRNAs to function as microRNA sponges. Such overexpressed circRNAs could even be designed to be better sponges by closely spacing microRNA seeds, which is expected to trigger cooperative microRNA-inhibition (Grimson et al., 2007; Saetrom et al., 2007; Schirle and MacRae, 2012; Pfaff et al., 2013). However, to be efficient, it has been calculated that circRNA abundance would have to be sufficiently high relative to the number of microRNA seed-targets in mRNAs (Mullokandov et al., 2012; Bosson et al., 2014; Denzler et al., 2014, 2016; Karreth et al., 2015; Thomson and Dinger, 2016) and close to the target mRNA abundance (Yuan et al., 2015). Another consideration has to be taken into account: Recently, 6 nts-long seeds have been found to trigger microRNA-degradation instead of mere sequestration (Denzler et al., 2016). This opens another therapeutic avenue for circRNAs. Already, one study has reported that an artificial circRNA had been successfully overexpressed that was able to sponge the liver-specific *microRNA-122* (Jost et al., 2018). The engineered circRNA sponge contained 8 seed-matched sites for *miR-122*, and these were separated by 4 nts spacers (Jost et al., 2018). This circular RNA construct inhibited *miR-122* in its known role as stimulator of translation of the human *Hepatitis C RNA-Virus* in a cell culture

model (Jost et al., 2018). The inhibitory efficiency *in vitro* was significant and comparable to the effect of the drug Miravirsen, a short linear locked-nucleic-acid (LNA) that targets the virus by complementary binding to *miR-122* (Jost et al., 2018).

(B) Given their stability against exonucleases, one tempting option would be to circularize classically employed linear RNAs, such as the small siRNA precursors or the sense strand of siRNAs (**Figure 3B**). This is possible, because compared to the rigid dsDNA helix (Egli et al., 1990), the flexibility of RNA molecules allows circularization of even very small RNAs. But also longer linear mRNAs and long non-coding RNAs (lncRNAs) could hypothetically be circularized (Kool, 1998; Abe et al., 2007, 2008, 2011). Still, one would have to test whether basic interaction parameters, such as binding to other nucleic acids and proteins, would still be intact in such artificial circles.

A promising case is the circularization of mRNA and their use for stable protein translation in target cells (**Figure 3B**, bottom). As described above, more than 99% of endogenous circRNAs are never productively associating with ribosomes, and in no case, so far, has the function of a circRNA-templated polypeptide been documented. Therapeutically it is, however conceivable, that exons encoded in engineered circRNAs could be translated to proteins when the ribosome is capture via an IRES, and when a complete open reading frame with stop and start codon were included through engineering (Chen and Sarnow, 1995). Therefore, mRNA circularization could be useful for therapeutic consideration since it has been shown that circularity is no principle obstacle for translation through the ribosome, once the ribosome has been recruited through an engineered IRES (Chen and Sarnow, 1995; Wang and Wang, 2015) (**Figures 3B,C**). Such a strategy could be interesting for many linear RNAs/mRNAs that are already therapeutically used, for gene expression control, vaccination, or as aptamers (see existing reviews for example of linear therapeutic RNAs (Sullenger and Nair, 2016; Matsui and Corey, 2017; Crooke et al., 2018; Lieberman, 2018). A recent proof-of-concept study has shown that translation from circularized mRNA sequences is a viable option for therapeutic protein expression and is even superior to translation from linear transcripts (Wesselhoeft et al., 2018): mRNAs of up to 5 kilobases were shown to be efficiently circularized when transcribed in mammalian cells from rationally-designed DNA vectors encoding a self-splicing group I intron from a cyanobacterial (*Anabaena*) pre-tRNA as ribo-enzymatic factor. Translation was particularly strong when using unconventional IRES elements, such as from the Coxsackievirus B3, and when adding structural spacing elements between IRES and splice sites. Comparing to state-of-the-art translation from transfected linear mRNAs with 5′Cap, 3′polyA tail and internal 5′methoxyuridine modifications for stabilized expressed, translation from unmodified circRNAs drove 50% more protein and provided protein over a two-times longer time period (80–120 h after a single RNA transfection) (Wesselhoeft et al., 2018).

(C) Care has to be taken about the specificity of translation from circRNAs. In the absence of in-frame stop codons and termination signals, or after read-through, rolling-circle-translation can occur on circular RNAs. Rolling-circle translation can lead to translation of multimeric repetitive protein motifs, which could be useful for cell engineering, but which can become toxic for cells if happening in an uncontrolled fashion (Jeck and Sharpless, 2014; Abe et al., 2015) (**Figure 3C**).

(D) In another consideration, linear RNAs have been shown to be therapeutically useful as adjuvants for boosting immune therapy (Le Bon et al., 2003; Hochheiser et al., 2016) (**Figure 3D**). In this pathway, RNA-sensing in the cytoplasm triggers innate immunity, and innate immune signaling is known to contribute to adaptive immunity by diverse routes. One option would, thus, be to use circRNAs as tools to purposefully boost innate immune signaling to counteract disease-induced immunosuppression, or as adjuvants for boosting adaptive immunity during vaccination. This is not far-fetched as circularity of RNA has recently been shown to be sensed by the RNA-binding RIG-I molecules, which trigger downstream innate immune signaling (Chen et al., 2017). One way ahead would be to introduce generic circRNAs (not targeting any host mRNA) which carry RNA-sequence or dsRNA-forming motifs known from independent work to activate the RIG-I (Hwang et al., 2012; Schnell et al., 2012) and RIG-I-like receptors (Pippig et al., 2009), which might increase circRNA-dependent immunity even further.

(E) Another therapeutic option would be to employ circRNA-based RNA aptamers that bind with high specificity, for example, to proteins (even those without RNA-binding domains) (**Figure 3E**). Among many other options, aptamers are useful for selective drug delivery or protein activity control. Circularizing them might make them more potent. circRNAs have already before been suggested to be potentially useful to affect the availability and subcellular sorting of RNA-binding proteins RBPs (Hentze and Preiss, 2013), many of which are already known to function in physiology and disease, not last through impacting linear mRNA splicing (see Holdt et al., 2018 for review). Technically, SELEX ("*Systematic Evolution of Ligands by EXponential Enrichment*") has been devised as a powerful unbiased PCR-based method for molecularly improving binding or effector mechanism *in vitro* for linear aptamer nucleic acids (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Many modern variations of this technique exist (see Darmostuk et al., 2015 for review), and SELEX would be adaptable, in principle, for circular RNa aptamer evolution *in vitro*. In fact, a recent attempt has succeeded to produce circular RNA aptamers (Umekage and Kikuchi, 2009). Circular RNA aptamers can be screened totally *de novo*, but in might also base on native sequences known to confer binding to a range of proteins (Ashwal-Fluss et al., 2014;

Kramer et al., 2015; Li et al., 2015, Du et al., 2016, 2017a,b; Holdt et al., 2016; Schneider et al., 2016; Abdelmohsen et al., 2017; Chen et al., 2017; Li X. et al., 2017). To date, no specific protein-interaction motif in a circRNA has been analyzed in any detail. But the fact that circular RNAs are constraint in secondary structure compared to linear RNAs, has let to speculations that circular aptamers may perform even better in terms of specificity and binding affinity (Lasda and Parker, 2014).

(F) Many endogenously expressed 3′-5′-linked circRNAs are thought to be modulators of transcription and splicing of linear mRNA, mostly of cognate linear transcripts produced from the circRNA host gene. The modulation happens when backsplicing occurs cotranscriptionally within pre-mRNA (Zaphiropoulos, 1996; Lewis et al., 2003; Suzuki et al., 2006; Jeck et al., 2013). Specific cases of *trans*-regulatory circular RNAs exist, and these spur the hope to be able to exploit this mechanism for therapeutic purposes (**Figure 3F**): For example, the class of intron-only ciRNA is thought to influence genomic loci in *trans,* whereby ciRNAs bind (RNAP II) and boost transcription (Zhang et al., 2013). Independently, at least one classical 3′-5′-linked exonic circRNA has been shown to affect linear splicing when provided posttranscriptionally *in trans* (Conn et al., 2017b). It was found to hybridize to complementary sequences in the host DNA locus, forming a triple helix (termed R-loop), which might be causal for impairing RNAP II elongation and RNAP II-coupled splicing. It has so far not been shown whether either of these *trans*-regulatory effects on transcription can be engineered into artificial circRNAs in therapeutic applications (**Figure 3F**).

(G) To date, no tools exist that would allow circRNA replication after transfection of synthetic RNA into mammalian cells. RNA replication mechanisms that do not rely on the integration of DNA intermediates into the genome exist in nature (**Figure 3G**). For example circular RNA viroids in plants are replicated as RNAs *in vivo*. This occurs by an RNA-rolling-circle mechanism, whereby host DNA-dependent RNA-polymerase is repurposed for transcription of the RNA circle (Muhlbach and Sanger, 1979; Navarro et al., 2000). After transcription, the linear transcripts are cleaved into monomers by host RNase II-type enzymes (Gas et al., 2008) or self-encoded hammerhead-ribozymes (Hutchins et al., 1986). Finally, unit-length RNAs are circularized by host RNA-/DNA-/tRNA ligases (Gas et al., 2008; Nohales et al., 2012). Importantly, also the single-stranded RNA genome of the human hepatitis-delta-virus replicates as RNA *in vivo*, speculatively by an RNA-rolling-circle-mechanism (Taylor, 2015). For RNA-therapeutics, it will be paramount to explore whether RNA-replication could be artificially transferred from any of the known physiological systems onto an engineered synthetic circRNA (of minus-polarity) and exploited for long-term maintenance of effector RNA circles (plus-polarity). This would be particularly relevant for maintaining circRNA that are to be transfected into dividing cell types, where circRNA levels quickly drop by continued dilution.

THERAPEUTIC DELIVERY OF circRNAs

A longstanding problem inherent to the therapeutic use of nucleic acids has been its route of application. The delivery of circRNAs follows, in principle, existing methods for delivering therapeutic RNAs, as no specialized possibilities or physicochemical obstructions have yet been found associated with circularity *per se*. Delivery strategies involve systemic injection into the vasculature, subcutaneous injection or depots, or local application. Obviously, strategies to achieve delivery to receptors on specific target organs through chemically functionalizing therapeutic RNAs (e.g., with *N*-acetylgalactosamine), cannot use end-modification in a circular RNA. Instead such modifications would have to be introduced, for example, by functionalizing the ribose 2′ hydroxyl group in the circRNA backbone. A lot of work has been dedicated to achieve tropism in atherosclerosis therapy by using homing peptides or fatty acids, or functionalized RNAs and lipid carriers that target only atherosclerotic plaques (Hamzah et al., 2011; Hofmeister et al., 2015; Deshpande et al., 2016; Huang et al., 2016; Zhang J. et al., 2017). For CVD therapy, local delivery at sites of atherosclerotic lesions includes stent-coating with polymers and hydrogels containing and releasing therapeutic RNAs (Koenig et al., 2017). Also, modern transfection methods could be adaptable to deliver circRNAs during cardiovascular operations, including photo-/optoacoustic approaches, which allow defining target cells and delivering cargo via nanostructures (Zhang Y. et al., 2017).

Typically, individual nucleotides can freely enter cells, while circRNAs and circRNA-generating vectors are insufficiently hydrophobic and too large (>1000 Daltons) to passively pass the cell membrane's phospholipid bilayer (Lipinski et al., 2001; Mansy et al., 2008). Instead endogenously expressed circRNAs, as other linear RNAs, are thought to exit the circulation and enter target cells via endocytosis (Juliano et al., 2014). Developing strategies for therapeutic circRNA delivery to cells via lipid carriers and for exiting from the endosomal membrane after cellular uptake will, therefore, directly benefit from the existing experience with transfecting nucleic/ribonucleic acids, naked or complexed with proteins, via traditional cationic and lipidic transfection routes, ionizable lipids and modern lipid, synthetic and functionalized nanoparticles (see Crooke et al., 2017, 2018; Dowdy, 2017 for review). In the end, despite emerging insight into the nuclear export mechanism for circRNAs (Huang et al., 2018), is not yet known how circular RNAs could be delivered to the nucleus, or how the nuclear exit to the cytoplasm could be controllable, which restrains some of the therapeutic considerations.

POTENTIAL SIDE EFFECTS OF circRNAs MODULATION

As with any therapy, it is anticipated that modulation of circRNAs might also have side effects. The knockdown of

circRNAs suffers from the same potential off-targeting effects as for linear mRNA interference, aggravated by the fact, that knockdown must specifically target the unique circRNA back-splice junction and is, thus, even more, constrained (Barrett et al., 2017; Piwecka et al., 2017). Also, the ectopic expression of circRNAs from DNA vectors is potentially problematic: circRNAs biogenesis has been found not to be uniform in all cell types, and, besides, running-circle transcription from the DNA and production of linear RNA concatemers can occur. Concatemers can be toxic, and be substrates for linear splicing, which can lead to uncontrollable biological effects (Barrett and Salzman, 2016). While synthetic circRNAs might be preferred, for this reason, their mass synthesis and delivery are more problematic. Concerning delivery, synthetic circRNAs, unless coated through RBPs, are sensed as "non-self" and trigger RIG-I-dependent innate immune signaling upon cell entry (Umekage and Kikuchi, 2009). This can confound analysis and therapy. Immunodetection might, in the future, be circumvented by masking circRNAs with recombinant proteins that would usually be loaded on circRNAs by endogenous backsplicing (Umekage and Kikuchi, 2009), or by chemical RNA pseudo-uridination, as has already described for linear ribonucleotides (Kariko et al., 2005; Durbin et al., 2016).

It should also be noted that for all cases of therapeutic circRNAs described in **Table 1**, it has not yet been explored, whether the therapeutic effect was due to the change in circRNA level in the diseased target tissue. Especially for CVD, disease parameters might change as a consequence of the systemic effects of circRNAs on liver function, metabolism or the immune system. In this case, superior methods may deliver the therapeutic circRNAs more directly to those primary effector organs and increase the therapeutic potency.

OUTLOOK

Form today's perspective, it is well conceivable that circRNAs may serve as therapeutic agents and targets in the near future. CircRNAs might either be modulated intracellularly or administered as *in vitro* synthesized formulations. As of now, it is still difficult to synthesize circRNAs at a sufficient scale in a cost-effective manner. *In vitro* synthesis of circRNAs is currently still performed with recombinant enzymes, which is costly when substantial amounts are required for therapy. Thus, alternatives for routine high-scale synthesis are being developed, such as allosterically regulated ribozymes, which perform circularization but are unable to re-cleave the circular RNA end-product and, thus, are envisioned to increase circRNA yield (Muller and Appel, 2017). Different hybrid approaches are conceivable, where large amounts of mammalian circRNA could be produced in yeast, which is cultivatable in industrial scale and genetically easy to program (Hossain and Johnson, 2014; Wang et al., 2014). This is an issue, as genetically impairing the RNA-processing machinery has recently been shown to be a valid option to increase backsplicing (Liang et al., 2017). If and how this can be achieved without compromising cell growth is still unknown.

Overall, it has taken 4 decades to overcome some of the major hurdles for using linear RNA as therapeutics. Since technological insight is in many cases directly transferrable from linear therapeutic RNAs to circRNAs, the use of circRNAs as therapeutic agents and targets in human diseases is only a question of time and might occur earlier than expected.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Targeting Non-coding RNA in Vascular Biology and Disease

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Only recently have we begun to appreciate the importance and complexity of the non-coding genome, owing in some part to truly significant advances in genomic technology such as RNA sequencing and genome-wide profiling studies. Previously thought to be non-functional transcriptional "noise," non-coding RNAs (ncRNAs) are now known to play important roles in many diverse biological pathways, not least in vascular disease. While microRNAs (miRNA) are known to regulate protein-coding gene expression principally through mRNA degradation, long non-coding RNAs (lncRNAs) can activate and repress genes by a variety of mechanisms at both transcriptional and translational levels. These versatile molecules, with complex secondary structures, may interact with chromatin, proteins, and other RNA to form complexes with an array of functional consequences. A body of emerging evidence indicates that both classes of ncRNAs regulate multiple physiological and pathological processes in vascular physiology and disease. While dozens of miRNAs are now implicated and described in relative mechanistic depth, relatively fewer lncRNAs are well described. However, notable examples include *ANRIL*, *SMILR*, and *SENCR* in vascular smooth muscle cells; *MALAT1 and GATA-6S* in endothelial cells; and mitochondrial lncRNA *LIPCAR* as a powerful biomarker. Due to such ubiquitous involvement in pathology and well-known biogenesis and functional genetics, novel miRNA-based therapies and delivery methods are now in development, including some early stage clinical trials. Although lncRNAs may hold similar potential, much more needs to be understood about their relatively complex molecular behaviours before realistic translation into novel therapies. Here, we review the current understanding of the mechanism and function of ncRNA, focusing on miRNAs and lncRNAs in vascular disease and atherosclerosis. We discuss existing therapies and current delivery methods, emphasising the importance of miRNAs and lncRNAs as effectors and biomarkers in vascular pathology.

Keywords: vascular disease, atherosclerosis, ncRNA, microRNA, lncRNA

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INTRODUCTION

Vascular disease in its various forms remains a global health epidemic, despite decades of medical and scientific endeavour to prevent it. Cardiovascular disease alone accounts for over 17 million deaths each year, more than double what is attributable to cancer (Libby et al., 2016), and the prevalence is only expected to rise (World Health Organization [WHO], 2017). Although major

advances in the fields of cardiothoracic and vascular surgery and percutaneous coronary intervention continue to improve outcomes in patients who have already suffered cardiovascular events, the key battle lies upstream in prevention. Strategies to address risk factors, such as aggressive lipid-lowering, anti-hypertensive, and anti-platelet therapies, have conferred some benefit, but vascular disease rates still continue to rise (Bhatnagar et al., 2016). Novel approaches to prevent onset and reduce progression of vascular disease are desperately needed, but progress is slow, and molecular mechanisms underpinning the pathology remain poorly understood.

Atherosclerosis is the principal driver of cardiovascular disease world-wide. It progresses over decades in the arterial walls of susceptible individuals, in response to numerous well-known risk factors, including inflammation and high circulating cholesterol. The clinical effect of atherosclerosis varies widely dependent on site, severity, and co-morbidity, and can range from completely asymptomatic to clinically catastrophic in the case of myocardial infarction (MI), stroke, and the acutely ischaemic limb. In its early stages, atherosclerosis is sub-clinical with endothelial dysfunction, intimal thickening, and plaque formation occurring silently and insidiously, only becoming clinically apparent when the plaque becomes large enough to impinge on luminal blood flow or becomes unstable and ruptures. In the coronary arteries, luminal stenosis results in angina, manifest most commonly as chest pain on exertion, and the equivalent in the peripheral vasculature, "intermittent claudication," can be completely disabling to those affected. These chronic diseases, while not in themselves immediately life-threatening, reduce quality of life and consume large amounts of healthcare resources annually. Other sites of atherosclerosis result in chronic kidney disease and hypertension, cognitive impairment in cerebrovascular disease, and abdominal angina in mesenteric disease.

Besides lifestyle interventions, attempts at managing atherosclerosis have to date largely focused around the now well-accepted dogma that high levels of circulating lipid lead to accumulation of an expanding lipid-rich necrotic core in the sub-intimal space of the arterial wall forming predominantly in areas of low shear stress and endothelial dysfunction. Consequently, lipid lowering strategies have predominated with no less than seven different available statins, other drugs like ezetimibe, fibrates, and most recently the development of monoclonal antibodies against the PCSK9 receptor, such as evolocumab (Sabatine et al., 2017) and alirocumab (Schwartz et al., 2014). However, despite initially impressive results in the statin trials, some now doubt their efficacy in primary prevention, and staggering levels of LDL reduction as in the FOURIER trial (Sabatine et al., 2017) do not seem to translate into dramatically improved clinical outcomes. This suggests that lipid lowering may not be the only mechanism of action at play (Sabatine et al., 2017). The anti-inflammatory effect of statin drugs may be a major factor, and new drugs targeted at reducing inflammation such as canakinumab look promising. In the recently published CANTOS trial, canakinumab which targets interleukin-1β reduced the relative risk of MI in ischaemic heart disease patients by 16%, without affecting lipid levels (Ridker et al., 2017).

In the search for new therapeutically exploitable pathways, much is now known about endothelial cell (EC), smooth muscle cell (SMC), adventitial cell, and immune cell behaviour in atherosclerosis and vascular biology. The plaque is a complex environment though, and mechanistic unravelling has long been in process with notable recent additions, such as adventitial-derived mesenchymal stem cell (MSC)-like $GLI1^{+}$ cells shown to modulate calcification in murine models (Kramann et al., 2016). EC dysfunction is the earliest known detectable abnormality in plaque formation, occurring at damage prone sites, such as vessel bifurcations. ECs entrap circulating monocytes and lipoproteins and facilitate their permeation into the sub-endothelial space (Ross and Glomset, 1976; Gimbrone and García-Cardeña, 2016). SMCs then proliferate and migrate into the intima in response to secreted chemokines and growth factors, synthesising extra-cellular matrix and sometimes undergoing phenotypic switch to macrophage-like cells, upregulating inflammation. The recruited monocytes therein become phagocytic and ingest the modified lipoproteins until they are over-laden with lipid (foam cells), and contribute to the accumulating necrotic core by undergoing apoptosis and necrosis (Ley et al., 2011; Moore et al., 2013). Although these pathways are now well investigated, there are still novel angles to interrogate, and a wealth of protein coding gene interactions still to explore. New genomic techniques such as single cell RNA sequencing (RNA-Seq) in atherosclerotic plaque promise to provide deeper insight in this respect (Tang et al., 2009; Wills et al., 2013; Cochain et al., 2018) and small interfering RNA (siRNA) therapies like inclisiran, which targets PCSK9 mRNA hold real promise (Ray et al., 2017).

This review focuses on another such avenue in the form of non-coding RNA (ncRNA). The non-coding genome represents an exciting yet complex layer in human physiology and pathology. Although some function had been previously ascribed to ncRNAs as early as the 1950s (transfer RNA and ribosomal RNA), its only in the last few decades upon the discovery that approximately 98% of the human genome is non-protein-coding (Mattick, 2001), that the potential biological significance of ncRNA has been even partly appreciated (Taft et al., 2007). MicroRNA (miRNA) is the best studied family of ncRNA, known to regulate thousands of protein-coding genes through messenger RNA (mRNA) degradation (Jonas and Izaurralde, 2015). MiRNAs are 20–25 nucleotides in length and form a characteristic "hairpin" structure. They are inherent in multiple pathologies, and due to their stability in plasma, miRNAs have also been proposed as biomarkers of MI (Bostjancic et al., 2010), cancers (Hayes et al., 2014), rheumatological, and other diseases. In terms of therapies, clinical trials are currently ongoing to determine the efficacy and safety of "antimiRs" in diseases such as cancer, hepatitis (Christopher et al., 2016), and other conditions (see later). Long non-coding RNAs (lncRNAs) are much less well characterised to date, but their importance in gene expression is increasingly being recognised. In contrast to miRNAs, lncRNAs are much longer at >200 nucleotides, with more complex secondary structures. They may act both to activate and to repress genes, exerting their effects by a variety of mechanisms at both transcriptional and translational levels (Mercer et al., 2009; Ponting et al., 2009). Evidence is limited

but growing, and lncRNAs appear to be critical regulators of many processes inherent to atherosclerosis, including endothelial dysfunction, SMC behaviour, immune response, and glucose and lipid metabolism. In this review, we intend to discuss ncRNA biology and mechanisms, new and existing evidence for ncRNA in vascular disease, and the potential for delivery of novel ncRNA therapies in human vascular disease.

NON-CODING RNA BIOSYNTHESIS AND MECHANISM OF ACTION

Since their discovery in 1993 in *Caenorhabditis elegans* (Lee et al., 1993), miRNAs have now taken up an important niche in genomics with almost 2000 known sequences in the human genome. Usually, transcription of intronic miRNA is regulated by the same promoter as the host gene. However, some miRNAs, are shown to have multiple transcription start sites (Ozsolak et al., 2008) and may be transcribed by a promoter distant from the gene they occupy (Monteys et al., 2010). MiRNA transcription is controlled by RNA polymerase II and RNA Pol II-related transcription factors (Lee et al., 2004). The first product in the process is a primary miRNA (pri-miRNA) molecule, with a characteristic stem–loop structure, and lengths reaching up to 1 kilobase (kb). The next product in the sequence is a smaller precursor miRNA (pre-miRNA) molecule, which is generated by RNase III enzyme Drosha that cleaves the stem-loop of pri-miRNA (Lee et al., 2003). The small, hairpin-shaped pre-miRNA is then transported into the cytoplasm by exportin 5, where the Dicer enzyme further processes it to generate a double-stranded molecule, ready for assembly with the Argonaute (Ago) protein (Bohnsack et al., 2004; Lund et al., 2004). The final product in the miRNA biogenesis pathway is formation of the RNA-induced silencing complex (RISC), consisting of a mature, single-stranded miRNA molecule and Ago protein, which induces post-transcriptional gene silencing (Huntzinger and Izaurralde, 2011). Importantly, human miRNA lacks Ago specificity and can bind to all Ago variants (Huntzinger and Izaurralde, 2011). Recent evidence demonstrates that the Ago-unbound miRNA strand isn't always cleaved and both the 5p and 3p strands are found in different sorts of tissues (Meijer et al., 2014). The mature miRNA then facilitates gene silencing, whereby mRNA degradation occurs in 66–90% of cases (Jonas and Izaurralde, 2015) and mechanistically depends on mRNA deadenylation, decapping, and cleavage by XRN1 nuclease (Fabian and Sonenberg, 2012). On the contrary, miRNA-induced repression of translation targets only 6–26% of genes (Eichhorn et al., 2014) using primarily RNA helicases eIF4A and DDX6, which repress cap-dependent translation (Jonas and Izaurralde, 2015).

A less simple but equally fascinating class of ncRNA is lncRNA. Remarkably, lncRNAs outnumber not only miRNAs, but also protein-coding genes. In fact, the number of lncRNAs annotated continues to rise, due to recent advances and new strategies in RNA sequencing and bioinformatic techniques, and hence a much greater depth of sequencing (Clark et al., 2015). Despite this, there is still no strict classification method, and the most accurate definition of lncRNA at present is "long RNA transcripts that do not encode proteins" (Quinn and Chang, 2016). It is generally accepted that lncRNAs should be longer than 200 nucleotides, separating them from other shorter ncRNA molecules such as miRNAs, snoRNAs, an piRNAs among others, but confusingly, some lncRNAs do actually contain cryptic open-reading frames (ORFs) (Gascoigne et al., 2012), and short open-reading frames (sORFs) encoding micropeptides. These micropeptides can act independently from large proteins, regulating essential biological processes (Makarewich and Olson, 2017). For example, a skeletal muscle-specific RNA, annotated as lncRNA LINC00948, encodes a cryptic micropeptide "myoregulin" (MLN). MLN interacts with sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA), preventing Ca^{2+} uptake into the SR in skeletal muscle, and accordingly MLN silencing improves Ca^{2+} handling (Anderson et al., 2015). Similarly, it was reported that lncRNA LINC00961 also encodes a polypeptide termed "small regulatory polypeptide of amino acid response" (SPAR). Mechanistically, SPAR prevents mTORC1 activation via interaction with lysosomal v-ATPase while SPAR deletion induces mTORC1 leading to muscle regeneration (Matsumoto et al., 2016). Given these clear mechanistic roles for encoded micropeptides, one might consider then that the parent lncRNA transcripts are in fact "misannotated." How can a transcript that is experimentally proven to contain genetic code for a protein be referred to as "non-coding"? Nomenclature naturally evolves with new knowledge over time, and perhaps this will change. Clearly though there are differences between a simple mRNA which has one function only, to produce a peptide, and the complex lncRNA molecule, with its myriad of interactions and functions, which remain largely undiscovered.

Within the class of lncRNA, there are different sub-classes of lncRNAs which include long intervening/intergenic ncRNAs (lincRNAs), promoter upstream transcripts (PROMPTs), enhancer RNAs (eRNAs), and natural antisense transcripts (NATs). These are transcribed from intergenic and promoter upstream regions, enhancers, and reverse strand of protein-coding genes, respectively (Wu et al., 2017). Interestingly, lncRNAs do not require polyadenylation to be functional and upon transcription a significant proportion of lncRNA remains non-polyadenylated. In fact, many lncRNAs are bi-morphic and can exist in both polyadenylated and non-polyadenylated states (Hangauer et al., 2013). Furthermore, lncRNAs can exist in different forms and structures: some lncRNAs are capped by snoRNAs at 5′ (Wu et al., 2016) or both ends (Yin et al., 2012); others can occur in a circular form as circular intronic RNAs (ciRNAs) and circular RNA from back-splicing of exons (circRNAs) (Memczak et al., 2013; Zhang et al., 2013; Zhang X. O. et al., 2014). *ANRIL* (discussed in more detail later) is the most well-known ncRNA that can take a circular form. Currently considered to be a form of ncRNA, these transcripts exist in loops with a bond between the 3′- and 5′-ends. Much like other lncRNA, they are thought to regulate gene transcription and expression, acting as sponges for miRNA, and are extremely abundant in the circulation.

Its now accepted that lncRNAs can modulate gene expression on transcriptional, post-transcriptional, and translational levels.

The function of a specific lncRNA depends much on its cellular localization and context of the cell (i.e., basal/stressed). In particular, nuclear lncRNAs mainly act on transcription, while cytoplasmic lncRNAs modulate expression of gene post-transcriptionally. In the nucleus, lncRNAs regulate the epigenome, facilitate transcriptional control, and participate in alternative splicing (Zhang K. et al., 2014). A good example of lncRNA-mediated epigenetic control is *ANRIL*, which facilitates the recruitment of the chromatin-modifying complex PRC2 and promotes silencing of $p15^{INK4B}$ tumour suppressor gene (Kotake et al., 2010). Interestingly, after the discovery of eRNAs (Kim et al., 2010), it has been further suggested that eRNAs are able to modulate chromatin and facilitate its assembly (Mousavi et al., 2013) and interact directly with DNA structure to guide the enhancer to its promoter (Li et al., 2013). A similar type of lncRNA, termed activating ncRNAs (ncRNA-as), can also regulate transcription and are expressed from independent genes (Orom et al., 2010). Further, lncRNAs modulate different aspects of transcriptional control ranging from modulating the expression of transcription factors, such as *ncRNA-a7* (Orom et al., 2010) and *OCT4 pseudogene 5* (Bai et al., 2015), to acting as co-activators and repressors independently, such as Alu RNA inhibiting RNA polymerase Pol II directly (Mariner et al., 2008). *MALAT1* is an example of lncRNA modulation of alternative splicing. Specifically, *MALAT1* is enriched in nuclear speckles and upon interaction with SR splicing factors promotes alternative splicing (Tripathi et al., 2010). Within the cytoplasm of a cell, lncRNAs can stabilise or lead to mRNA decay and promote/inhibit translation. They can also act as miRNA precursors or sponges, mimicking mRNA for miRNA binding (Zhang K. et al., 2014). In the main lncRNA-induced mRNA degradation occurs via Staufen1 (STAU1)-mediated mRNA decay (SMD), where STAU1 binds to the pairing of mRNA 3′-UTR sequences with lncRNA due to complementary Alu elements (Park and Maquat, 2013). *BACE1-AS*, a natural antisense lncRNA forms a bond with mRNA of *BACE1* and protects it from miR-485-5p-induced degradation by masking the miRNA-binding site (Faghihi et al., 2010). LncRNA such as *Uchl1-AS* can recruit ribosomes to *Uchl1* mRNA and thus facilitate protein translation (Carrieri et al., 2012). An interesting subset of lncRNAs termed translational regulatory lncRNA (treRNA) can even repress translation. TreRNA facilitates the assembly of a new ribonucleoprotein (RNP) complex, which interacts with translation initiation factor eIF4G1 resulting in translational repression of E-cadherin (Gumireddy et al., 2013). Further, a well-studied lncRNA in cancers, *H19*, has been recently demonstrated to be a precursor for miR-675, which targets a tumour suppressor *RB* (Tsang et al., 2010). H19 is considered to be an effectual molecule itself, rather than just the primary transcript giving rise to miR-675. In the setting of keratinocyte differentiation H19 acts as a sponge to miR-130b-3p, its target (Li et al., 2017; **Figure 1**). Finally, it has been reported that circRNA *CDR1-as* contains 63 binding sites for miR-7 and can act a "sponge" or decoy for miR-7, thus reactivating the expression of its target genes in neuronal tissues (Hansen et al., 2013; Memczak et al., 2013).

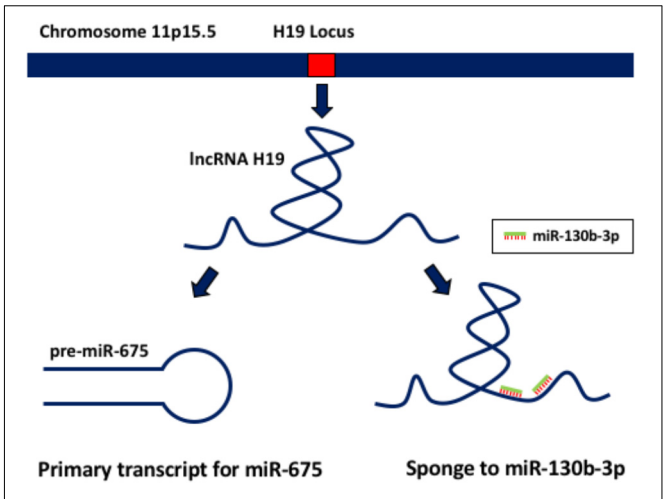

FIGURE 1 | lncRNA H19 functions. This lncRNA acts both as a primary transcript giving rise to miR-675, and also as a sponge to miR-130b-3p.

Overall, it is clear that small ncRNAs such as miRNAs and different subsets of lncRNAs form complex molecular networks within the cell, where both classes interact closely to regulate vital cellular processes.

NON-CODING RNA IN VASCULAR DISEASE

Discovery of novel ncRNAs in disease-specific context continues to increase, largely due to the widespread application of high-throughput gene expression arrays and development of next-generation RNA sequencing. By far the most studied field in this area is cancer, but already a significant number of miRNAs are implicated in vascular disease and biology, and although much less studied, several lncRNAs have been discovered and are currently being investigated. Development, cellular differentiation, and commitment are a logical starting point to identify novel RNA candidates, and evidence of EC growth and phenotype regulation in the case of *MALAT1* increases the likelihood of an important function in pathology for this lncRNA. The scope of this review, however, focuses on ncRNAs in pathology. Here we describe some of the most important discoveries to date, adding weight to the proposition that miRNA and lncRNA transcripts represent worthwhile and plausible targets for novel gene therapies in vascular disease (see **Table 1** for summary of key ncRNA in vascular diseases).

MiRNAs

There is a large body of evidence demonstrating miRNA involvement in many of the pathological processes that occur in vascular disease, and atherosclerosis. Hundreds of miRNAs are now reported as key regulators of lipid handling, inflammation, and cellular behaviours, such as SMC and EC proliferation,

TABLE 1 | Non-coding RNA in vascular disease.

Type	Vascular examples	Biological context	Reference
miRNA	miR-21	Biomarker of coronary artery disease, upregulated in vein grafts	(Raitoharju et al., 2011; McDonald et al., 2013; Han et al., 2015)
	miR-126	Promotes EC proliferation, atheroprotective	(Wang et al., 2008; Schober et al., 2014)
	miR-92a	Endothelial inflammation, atherogenic	(Loyer et al., 2014)
	miR-33a/b	Inhibits *ABCA1* translation, atherogenic	(Rayner et al., 2010; Horie et al., 2012)
	miR-143/145	Complex interaction in atherosclerosis and pulmonary hypertension: upregulated in human unstable carotid plaque, knockout blocks pulmonary hypertension in murine model	(Cipollone et al., 2011; Deng et al., 2015)
	miR-221/222	Dysregulated in acute plaque	(Cipollone et al., 2011; Maitrias et al., 2015)
	miR-1	Biomarkers for MI	(Wang et al., 2010; McManus and Ambros, 2011)
	miR-133a		
	miR-499		
	miR-208a		
	miR-192	Predictive of heart failure post-MI	(Matsumoto et al., 2013)
	miR-194		
	miR-34a		
lncRNA	ANRIL	Transcribed from 9p21 locus, associated with pathogenic changes in atherosclerotic plaques	(Holdt et al., 2010)
	MIAT	Biomarkers for MI	(Ishii et al., 2006; Vausort et al., 2014; Zangrando et al., 2014)
	MIRT1/2		
	HIF1-AS2		
	KCNQ1OT1		
	SENCR	Downregulated in human critical limb ischaemia, and in premature coronary artery disease	(Boulberdaa et al., 2016)
	SMILR	Induces SMC proliferation, upregulated in human carotid plaques	(Ballantyne et al., 2016)
	meXis	Improves cholesterol efflux, atheroprotective	(Sallam et al., 2018)
	MALAT1	Downregulated in plaque, endothelial phenotypic switch	(Arslan et al., 2017)
	LIPCAR	Predictive of heart failure post-MI	(Kumarswamy et al., 2014)

migration, and phenotypic switch. Translation of these important mechanistic findings into novel therapies in humans involves a long and expensive process, and the vast majority of mechanistic investigation is undertaken pre-clinically, *in vitro*, and in animal models. Human experimental evidence usually amounts to correlative findings in excised tissue samples, such as carotid plaque, or in readily available plasma or cellular blood components like peripheral blood mononuclear cells (PBMCs).

As a readily accessible source of human tissue, carotid plaque profiling has led to discovery and validation of several dysregulated ncRNAs in this way. MiR-21 for example is an important miRNA, which was found to be increased more than sevenfold in human peripheral arteries with severe atherosclerotic disease compared with controls. MiR-21 was further shown to be increased in the circulation of *apoE* knockout mice and humans with coronary artery disease (Raitoharju et al., 2011; Han et al., 2015). In clinical application, expression of miR-21 was shown to rise after engraftment of veins into murine and porcine models, and in cultured human saphenous vein explants. Subsequent knockdown in *ex vivo* human saphenous vein explants using anti-miRs was then >95% efficacious, and neointima formation was attenuated (McDonald et al., 2013). MiRNAs can be tissue and disease specific, but as in the case of miR-21, some transcripts are enriched across multiple tissues, and regulate multiple processes (Ludwig et al., 2016). While known to be an important factor in fibrosis and neointima formation in vascular disease as above, miR-21 is also thought to be part of an inflammatory positive feedback loop in vascular ECs. It promotes adherence of monocytes via activator protein AP-1, as well as negatively regulating LPS-induced lipid accumulation and inflammatory responses in macrophages by the TLR4–NF-κB pathway (Feng et al., 2014). The well-expressed and widespread miR-21 has in fact now been targeted in several other diseases

too, including kidney disease, and cancer and trials of therapy are well underway (Denby et al., 2014; Nedaeinia et al., 2017).

Endothelial cell dysfunction is a critical and probable triggering event in vascular disease mediated at least in part by disturbed laminar flow in predisposed locations such as vessel bifurcations. MiR-126 is an EC-enriched miRNA (Wang et al., 2008) and is critical for EC proliferative reserve in such areas, effective by suppression of the Notch1 inhibitor delta-like 1 homolog (Dlk1). When denuded, recovery of the endothelial layer was compromised when miR-126-5p deficient, and treatment with miR-126-5p could rescue proliferation and thereafter protect against atherosclerosis, suggesting a possible therapeutic angle for this miR (Schober et al., 2014). Investigating the effect of blood flow on miR expression, pulsatile flow was shown to increase expression of the well-studied miR-92a (while laminar flow reduces it) (Wu et al., 2011; Fang and Davies, 2012) via the regulation of Kruppel-like factor 2 (KLF2), which is a positive regulator of nitric oxide synthesis. MiR-92a inhibition then reduced endothelial inflammation, and hence plaque size (Loyer et al., 2014). Clinical development of an "antagomir" designed to inhibit miR-92a is now in process, and multiple other animal models have demonstrated significant vascular benefit in downregulating this important transcript. Roles have since been defined beyond endothelial dysfunction too, in angiogenesis and proliferation, translating into enhanced cardiac recovery and cardiac protection (see later).

Lipid handing is also central to evolution of atherosclerotic plaques and appears to be regulated by a number of different miRNAs. The adenosine triphosphate-binding cassette (ABC) transporter, *ABCA*1 specifically is subjected to complex miRNA regulation, and ABCA1 is known to be critical to cholesterol transport and high-density lipoprotein (HDL) biogenesis in the liver (Rotllan et al., 2016). This is particularly important because HDL is recognised as one of the few reliably validated risk factors in atherosclerotic disease. There have been multiple unsuccessful pharmacological attempts to increase HDL. Trials using niacin and *CEPTB* inhibitors, such as torcetrapib, have shown no benefit and in fact, the ILLUMINATE trial was stopped early due an increase in all cause mortality in the treatment group (Barter et al., 2007; Boden et al., 2011). MiRNA manipulation may still be possible though, as we know that *ABCA1* translation is directly inhibited by miR-33 (Rayner et al., 2010) which results in attenuation of cholesterol efflux, and when miR-33 is genetically deleted in atherogenic murine models, plaque volume and lipid content are reduced (Horie et al., 2012). Correspondingly, circulating HDL is increased in miR-33 knockout mice, and reduced when it is overexpressed. This has been further validated in non-human primates, perhaps more reassuring of significance in humans, particularly as the mouse locus does not encode miR-33b, whereas in primates the local genomic profile is much more similar (Rayner et al., 2011). MiR-33a and miR-33b remain exciting targets in vascular prevention, but recent evidence suggests that while loss of these miRNA may be beneficial in terms of macrophage behaviour within the plaque (reduced size and volume), full knockout actually results in obesity, insulin resistance, and hyperlipidaemia overall, albeit in a mouse model (Price et al., 2017). Obviously the environment is complex, and the *ABCA1* locus is regulated by a number of other miRNAs, and interestingly a recently discovered lncRNA *meXis* (Rottiers and Näär, 2012; Sun et al., 2012; Zhang M. et al., 2014; Sallam et al., 2018) (described later).

In vascular SMCs, the miR-143/145 cluster is now a well-recognised regulator of cell function. Generally, the literature describes downregulation in the disease state, and an improvement when replaced or over-expressed. For example, in murine aortic constriction, miR-143/145 expression is reduced, and knockout mice have abnormal arterial structures and evidence of de-differentiation on histological analysis (Elia et al., 2009). Further, the knock-out of *miR-143/145* resulted in loss of SMC contractility and favoured neointimal formation (Boettger et al., 2009). Whereas in lentiviral overexpression of miR-145, plaque appearances were overall improved with a reduction in plaque size, and amelioration of features associated with instability such as size of lipid core and thickness of fibrous cap (Lovren et al., 2012). Findings in human samples then largely corroborate the hypothesis that the cluster is downregulated in disease, consistent in aortic aneurysm tissue versus control, and in the PBMCs of hypertensive patients (Elia et al., 2009; Kontaraki et al., 2014). It has been demonstrated that the process of actin depolymerisation in mild aortic dilation leads to downregulation of miR-143/145 regulated by myocardin-related transcription factors (MRTFs) (Alajbegovic et al., 2017). However, another level of complexity is suggested by conflicting evidence in apparent upregulation in carotid artery plaques of patients with stroke (Cipollone et al., 2011), and similarly higher expression in the carotid plaques of patients with hypertension (Santovito et al., 2013), and pulmonary artery SMCs of patients with pulmonary hypertension (Caruso et al., 2012; Deng et al., 2015). Clearly much is yet to be learned about their significance, and complex interactions before precise therapeutic options are defined in this case.

The miR-221/222 cluster is another important regulator of vascular cell function in plaque rupture although it too has a complicated, sometimes conflicting representation in the literature. Prevention of plaque rupture is of course an important aim of treatment in vascular disease, whether carotid, coronary, or peripheral. The miR-221/222 cluster has been implicated in this acute process, and while consistently identified as a dysregulated miRNA in acute plaque (Cipollone et al., 2011; Maitrias et al., 2015) was shown to have apparently opposing effects dependent on cell type in VSMCs and ECs (pro-proliferative/migratory in VSMCs, yet anti-proliferative/migratory in ECs) (Liu et al., 2012). Critically, miR-221/222 levels appear to reduce at the time of plaque rupture, and then normalise around 2 weeks after the event (Bazan et al., 2015). Precision targeting of this cluster in the plaque to prevent fibrous cap degradation, hence, seems beneficial. However, the widespread effects of manipulating circulating levels of miR-221/222 need to be thoroughly studied, because this very well-conserved miRNA appears to be enriched in patients with diabetes (Coleman et al., 2013) and plays important roles in various types of cancer, too (Song J. et al., 2017). As with any ncRNA therapy targeted at a particular disease process in specific

cells or tissues, the specificity of action is hence absolutely critical. Similarly, in the development of an anti-miR to miR-21, off-target effects of systemic dissemination were observed as expected, including a rise in serum creatinine, but some success was had with a drug-eluting stent (DES) delivery which reduced in-stent restenosis, without off-target problems (Wang et al., 2015) (see later).

LncRNAs

Increasingly, lncRNAs emanating from multiple sources are shown to be implicated in cardiovascular disease. The lncRNA anti-sense noncoding RNA in the *INK4* locus (*ANRIL*) is transcribed from the now well-known 9p21 locus, which has been strongly implicated in vascular disease (Holdt et al., 2010; Congrains et al., 2012; Cheng et al., 2017). It was initially discovered through genome-wide association studies detecting several polymorphisms, which were predictive of atherosclerosis (Wellcome Trust Case Control Consortium, 2007; Samani et al., 2007). Notably, important tumour suppressor genes *CDKN2A* and *CDKN2B* are also found near this region, but are not found to be dysregulated in atherosclerosis models and patients, unlike *ANRIL* (Chen et al., 2014). *ANRIL* was one of the first lncRNAs described in atherosclerosis, and multiple interactions have now been demonstrated, although the full extent of its regulation in vascular disease remains unclear. As is the case with most lncRNAs, *ANRIL* is alternatively spliced, and expressed as multiple "isoforms." Ensembl currently reports 21 splice variants, each of which may interact differently to the others. Further adding to its complexity, *ANRIL* is found in multiple cell types including SMCs, ECs, and inflammatory cells. Initially, *ANRIL* appeared to be grossly associated with pathogenic changes; reduced cell viability and proliferation in SMCs (Congrains et al., 2012), upregulation of inflammation and apoptosis in ECs (Song C.L. et al., 2017), and a consistent correlation with atherosclerotic burden in human PBMCs and plaque samples (Holdt et al., 2010). More recently though a circular variant has been characterised, *circANRIL*, which seems to be atheroprotective in its behaviour; by controlling ribosomal RNA biogenesis and modulating some atherogenic processes in VSMCs and macrophages (Holdt et al., 2016). Unless otherwise stated, *ANRIL* would hence usually refer to the linear transcript (*linANRIL*). This alternatively structured RNA from the same locus exemplifies the added layers of complexity in lncRNA biology compared with smaller micro and other ncRNAs.

Until now, *in vitro* work has led to limited characterisation of some other vascular lncRNAs in relevant cell types, but translation into definite therapeutic targets in humans requires complex mechanistic unravelling, which in most cases is in the early stages. LncRNAs identified in circulatory samples from atherosclerosis patients, or from GWAS, may provide a starting point for vascular targets, but confounding processes such as myocardial injury and repair or the general upregulation of inflammation are equally likely to account for these correlative findings. Notable examples include MI-associated transcript (*MIAT*) (Ishii et al., 2006), another transcript discovered by large case–control genome association study in patients with MI, and MI-related transcripts 1 and 2 (*MIRT1*, *MIRT2*) in a murine MI model (Zangrando et al., 2014). While dysregulation in the disease state is apparent, mechanistic work to show why this is remains to be seen.

As with miRNAs, a certain insight has been achieved from *in vitro* cellular models. Although cultured cell lines representing one cell type are not truly analogous to the multicellular *in vivo* environment, it is possible to investigate candidate novel transcripts, and to identify at least if these are important in cellular function in the first instance. In coronary artery SMCs, the smooth muscle and EC-enriched migration/differentiation-associated lncRNA (*SENCR*) is an example of a transcript discovered by RNA sequencing, and functionally characterised in relevant cell types. *SENCR* was shown to be anti-migratory in SMCs, and subsequent RNA-Seq after *SENCR* knockdown then demonstrated a reduced expression of myocardin and numerous contractile genes, consistent with the observed phenotype (Bell et al., 2014). Subsequently, lower levels of *SENCR* in human critical limb ischemia and in the ECs of patients with premature coronary artery disease were observed (Boulberdaa et al., 2016), again supporting that hypothesis that *SENCR* is downregulated in pathology. One might propose then that targeted delivery of *SENCR* in areas of deficiency might then ameliorate maladaptive SMC behaviours. Another vascular lncRNA, smooth-muscle-induced lncRNA (*SMILR*), was investigated in a similar manner, but in this case upregulation of *SMILR* was associated with vascular SMC proliferation, and in human atherosclerotic plaque *SMILR* is enriched. Mechanistic investigation showed that *SMILR* may exert its effects by interaction with *cis* protein *HAS2*, which codes for hyaluronic acid. *HAS2* was also reduced on *SMILR* knockdown, and the SMC phenotype was of reduced proliferation (Ballantyne et al., 2016). Therapeutic targeting of *SMILR* with *in vivo* locked nucleic acids (LNAs) might prove useful in preventing neointima formation in clinical settings.

Aside from the cells which are inherent to the vascular wall, inflammatory cells like monocytes and macrophages are the key regulators of lipid handling within the plaque, and recently it was shown that the lncRNA *meXis* is an amplifier of the *ABCA1* gene, via the sterol activated liver X receptors (LXRs). LXRs are sterol-activated nuclear transcription factors, and may be important in the pathology of atherosclerosis, as key regulators of genes involved in cholesterol transport. *MeXis* interacts with and guides promoter binding of the transcriptional coactivator *DDX17*, so loss of the gene resulted in impaired cholesterol efflux, and accelerated atherosclerosis in murine models. As discussed, any novel target for improving cholesterol efflux (which is beneficial in plaque disease) certainly warrants further investigation (Sallam et al., 2018).

Utilising human samples excised at coronary endarterectomy a recent study compared lncRNA expression from diseased left anterior descending (LAD) artery plaque with inferior mammary artery control tissue. This is a rarely performed procedure, and coronary artery samples from live patients are not usually readily available. The inferior mammary artery used as a control here is preferentially used for LAD coronary artery bypass and tends to be quite resistant to atherosclerosis with low failure

rates (Etienne et al., 2013). Differential lncRNA expression was observed in three of the five plaques chosen for analysis, with upregulation of *ANRIL* and *MIAT* while metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) was downregulated (Arslan et al., 2017). This reinforces the initial premise that these lncRNAs which had been previously proposed as atherosclerosis-related lncRNAs, and had been found in the circulation of patients with acute MI (Vausort et al., 2014), are in fact reproducibly found in human tissue.

MALAT1 briefly described above is frequently cited in vascular disease and widely in multiple pathologies (primarily cancer) is a very well-conserved and highly expressed lncRNA (Gutschner et al., 2013). Having initially been demonstrated as a prognostic marker in non-small cell lung cancer (Ji et al., 2003), *MALAT1* appears to be also relevant in vascular disease, and was shown to control phenotypic switch in ECs, as well as impair vessel recovery in a hind-limb ischaemia model when knocked down using *in vivo* GapmeRs (Liu et al., 2014; Michalik et al., 2014). The use of *in vivo* GapmeRs to knock down chosen ncRNAs will be discussed later.

NON-CODING RNA AS BIOMARKERS

Peripheral blood samples are easy to access, as are large-scale array systems for identification of differentially regulated genes, so it is unsurprising that there are now many miRNAs described as biomarkers of various diseases. In addition, miRNAs are quite well expressed in the circulation, and stable in stored samples of blood and plasma, throughout freeze/thaw cycles, making them reasonable to handle (Chen et al., 2008; Mitchell et al., 2008).

Examples in vascular disease include striated muscle-enriched miRNAs such as miR-1, miR-133a, and miR-499, which were upregulated in a population of acute MI patients and were highly sensitive for MI by receiver operating characteristic (ROC) curve analysis (Wang et al., 2010) compared with patients with no MI. Similarly, miR-197 and miR-223 were shown to be predictive of future cardiovascular death over a 4-year follow up in a large sample of patients with documented coronary artery disease, some with and some without acute infarction (Schulte et al., 2015). These are two miRNAs which have been serially implicated in this context, and in a different study were shown to predict future cardiovascular events from baseline. This time there was a negative correlation between circulating levels of miR-197 and miR-223, and another miRNA miR-126 was shown to be positively correlated. More in depth investigation showed that miR-223 and 197 were highly expressed in platelets, whereas miR-126 was more EC enriched (Zampetaki et al., 2012). The origin of circulating transcripts is usually difficult to ascribe, but as the authors speculate in this case, it seems in-keeping with previous knowledge that the lower levels of miR-197 and miR-223 may reflect platelet dysfunction, and the higher levels of miR-126 could be indicative of endothelial dysfunction.

In heart failure post-MI, miR-192, miR-194, and miR-34a are predictive of heart failure up to 1 year, and correlate with left ventricular dilatation on echocardiography (Matsumoto et al., 2013). Centrally acquired samples from the coronary circulation are potentially more valuable though, and in a more intricate biomarker study the localisation of miRNA release was characterised in a similar panel of candidates. Using coronary catheters to access the aorta and coronary venous sinus, simultaneous EDTA blood samples were taken from patients with stable coronary disease, troponin positive acute coronary syndrome (ACS), and normal controls. Not only were miR-133a, miR-499, and miR-208a elevated in patients with ACS, but a *trans*-coronary gradient was noted in miR-133 and miR-499, strongly suggestive of myocardial or coronary release due to injury (McManus and Ambros, 2011). As well as validating the novel biomarkers in ACS, this study suggests that localised cardiac sampling may be preferable to peripheral methods, especially in lowly expressed targets. Peripheral blood sampling remains the easiest and most accessible route, however, and caution must be exercised when undertaking PCR in centrally acquired samples, because heparin which is frequently used to anticoagulated cardiac patients undergoing cardiac catheterisation is known to inhibit PCR downstream due to binding of proteins required for the reaction (Beutler et al., 1990).

Although comparatively fewer lncRNAs are described as biomarkers of vascular disease thus far, these versatile molecules have been reliably detected in the circulation in cancer (Bolha et al., 2017) and their tissue specificity like miRNA makes them likely candidates. In a cohort of 414 patients with acute MI, the expression levels of five known lncRNAs in circulating cells were measured, demonstrating higher levels of hypoxia inducible factor 1A antisense RNA 2 (*HIF1A-AS2*), *KCNQ1OT1*, and *MALAT1* compared with controls, while *ANRIL* went down. In addition, four of the five were predictive of future left ventricular systolic function. Similarly, long intergenic ncRNA predicting cardiac remodelling (*LIPCAR*) is readily detectable in human plasma and is uncharacteristically well expressed for a lncRNA. In a global transcriptomic analysis of patients post-MI, *LIPCAR* independently predicted cardiovascular death, despite being initially lower expressed immediately after MI, levels subsequently rose (Kumarswamy et al., 2014).

Already it is clear that many ncRNA species are deeply involved in the pathology of vascular diseases. These manipulable molecules represent feasible targets for therapeutic modification, but also as diagnostic biomarkers or predictors of risk. It seems unlikely that ncRNA could ever replace cardiac troponins in the evaluation of sudden onset chest pain and diagnosis of MI, which are tremendously well validated, but due to their potential specificity to individual processes within the pathology, such as plaque instability, they may still find a role (Chapman et al., 2017).

NON-CODING RNA LIMITATIONS AS BIOMARKERS

While ncRNA hence have the required specificity to pathology to be suitable biomarkers, a current limitation is in their extraction and quantification. Unlike highly abundant proteins such as troponin, which can be easily measured using an ELISA assay for example, RNA is more difficult to isolate in reasonable

quantities and reproducibly so from acellular bodily fluids such as plasma or serum. Very small amounts of RNA are generally exported in extracellular vesicles, as in the case of miRNA, and some likely escapes from damaged circulating or vascular cells and may be bound to proteins. However, the exact origins of circulating lncRNA in particular are not at all understood, and the vast majority of RNA in the body is intracellular. However, traditional methods, such as Nanodrop™ spectrophotometry, fluorometric measurement using Qbit™, and even automated electrophoresis with Agilent Bioanalyzer which are commonplace for cellular RNA applications, are not sensitive enough in realistically sized sample volumes of cell-free bodily fluids. Not only does this hamper scientific study and development, but inherent biases may also be introduced. With the expected low expression of ncRNA molecules, cycle threshold (CT) values on qPCR will be very high, towards the upper limit of detection for the machinery, and become less reliable. Of course, accuracy is always compromised throughout the entire work stream when the absolute quantity is reduced. Furthermore, in the discovery phase, the tendency to study only the highest expressed of the ncRNA species leads to neglect of the less well-expressed transcripts. Lower abundance does not necessarily mean lower importance. As a result, although multiple studies report differential expression of novel lncRNA biomarkers in human plasma and serum, others have found difficulty in replicating (Schlosser et al., 2016). A noteable exception is LIPCAR, which is highly expressed and consistently detectable in plasma samples (Kumarswamy et al., 2014).

NON-CODING RNA THERAPEUTICS AND DELIVERY METHODS

Here we discuss current knowledge on new and emerging therapies using ncRNA technologies. Proposed methods of delivery are shown in **Figure 2**.

MiRNA AS THERAPEUTIC AGENTS

In the large class of ncRNAs, miRNAs currently possess the strongest therapeutic potential due to our greater knowledge of them, clearer mode of action, and ability to regulate multiple genes in multiple molecular pathways. Moreover, due to their pleiotropic mechanism of action, a manipulation of a single miRNA could potentially induce a therapeutic effect within several cells and tissues. With re-emerging interest in RNAi therapeutics (Zeliadt, 2014), it is possible that miRNA-based therapies will progress further in the coming years.

Ultimately, efficacy depends on both modification of the RNA molecule, and its vehicle for delivery. The two principle strategies for miRNA therapeutics include overexpression of miRNA by synthetic oligoribonucleotide (ORN) delivery or targeted miRNA inhibition using single-stranded antisense oligonucleotides (anti-miRs). In order to obtain better delivery efficiency, the ORNs can be subjected to chemical modifications, such as 2′-*O*-methoxyethyl (2′-MOE) substitutions and LNA bases, which stabilise the ORNs (Hutvagner et al., 2004; Orom et al., 2006; Elmen et al., 2008a). In particular, the 2′-MOE modification is

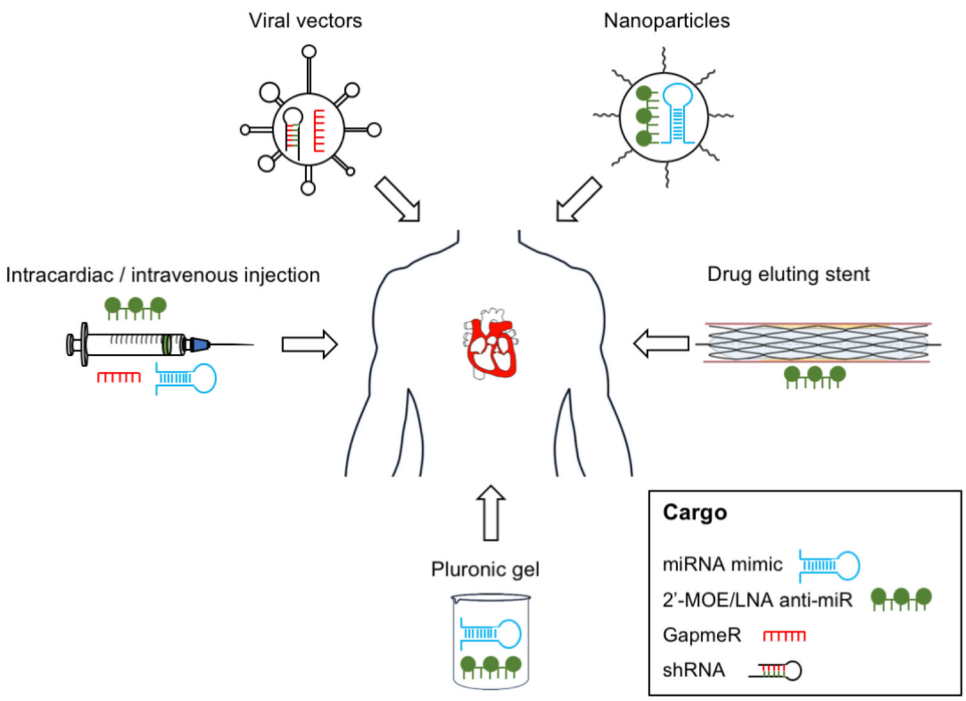

FIGURE 2 | Non-coding RNA therapy delivery – proposed delivery methods of ncRNA-based therapeutics in vascular disease. RNA therapeutics can be delivered to the vascular system using vehicles such as viral vectors, nanoparticles, and pluronic gel; intravascular devices such as drug-eluting stents; or by direct injection.

generally used to prevent degradation of ORN as well as to mask it from the immune response, whereas LNA enhances the affinity of miRNA and stabilises the ORN further (Davis et al., 2006; Henry et al., 2011). The use of such modifications to improve expression modulation of certain miRNA has been validated numerously in the vascular disease setting, including examples such as delivery of 2′-OMe-miR-21 to rat carotid artery (Ji et al., 2007), systemic downregulation of miR-92a with LNA-anti-miR to induce re-endothelialisation (Daniel et al., 2014) as well as intravenous delivery of LNA-anti-miR-15b to reduce cardiac remodelling in mice post-MI (Hullinger et al., 2012).

In addition to ORN modifications, efficient RNA delivery vehicles are required to facilitate their uptake. There are several studied and trialled thus far, as discussed below. The most common delivery method to date is the use of lipid-based nanocarriers, which package the RNA, allowing it to cross the cellular membrane (Pirollo et al., 2008; Trang et al., 2011). Notably, in a study carried out by Weber's group it was shown that miR-126-5p mimics packaged in this way were effectively and efficiently transfected *in vivo*. The cargo was delivered intravenously to high cholesterol diet mice, resulting in a marginal decrease in atherosclerotic lesion formation, and an increase in luminal EC proliferation. They reported in the same study that using pluronic gel-based delivery was also effective, in this case for local delivery of anti-miRs injected around the mouse carotid artery (Schober et al., 2014). Pluronic gel can be used internally or topically, and was also used effectively in a recent study by Miscianinov et al., where miR-148b mimics were topically administered to efficiently enhance angiogenesis and wound healing *in vivo*. It was noted in this case, however, that the delivery of anti-miR-148b within the pluronic gel had impaired wound closure and induced EndMT in the wound vasculature, possibly a result of the target manipulation more-so than the mode of delivery (Miscianinov et al., 2018). Alternative methods to package and deliver RNA-based therapies involve polymer- and peptide-based systems, such as polylactic-co-glycolic acid (PLGA) and polyamine-co-ester terpolymer (PACE) nanoparticles. The latter are able to release the ORNs steadily over time (Woodrow et al., 2009; Zhou et al., 2011). PLGA was used in the vascular setting when an anti-miR to miR-92a encapsulated by PLGA microspheres was delivered directly to pig coronary arteries. MiR-92a expression was downregulated locally, preventing left ventricular remodelling in a model of reperfused MI (Bellera et al., 2014).

Of course, viral vectors such as adenovirus (AV) and adeno-associated virus (AAV) are among the best known vehicles for gene therapies due to their natural ability to infect, and transfer genetic information. Viruses have been used to efficiently deliver many different cargoes *in vivo* previously like the tissue inhibitor of metalloproteinase-3 (TIMP3) in vein graft failure, which reduced neointimal formation consistently (George et al., 2000). The commonly used vector AAV has been equally well-studied, and delivery of hsa-miR-590 and hsa-miR-199a pre-miRs to the neonatal rat heart was shown to significantly reduce infarct size and improve cardiac function post-MI (Eulalio et al., 2012). Despite concerted efforts to optimise this process however, viral delivery can be hampered by several drawbacks, namely the triggering of immune response (especially in the case of AV), off target effects, and the sometimes undesirable long-term incorporation of the virus's genes into those of the host.

Specificity of site of action hence represents one of the major barriers to developing successful miRNA therapies, as in both of the above classes of examples. However, some success has been seen with attempts to deliver miRNA therapies locally. In the vasculature in particular, drug eluting stents were already widely used in percutaenous coronary intervention, releasing anti-proliferative and immunosuppressive drugs to prevent re-stenosis. In a similar approach, it was shown that delivery of LNA anti-miR-21 by DES significantly attenuated in-stent restenosis in the humanised rat myointimal hyperplasia model (Wang et al., 2015). Photoactivatable antimiRs, which have photolabile cages attached to the oligonucleotide structure are only activated by light, therefore generating an inducible model (Zheng et al., 2011; Connelly et al., 2012). This ensures that the miR treatment is only efficiently released at the intended site. Clearly this is useful in skin, but perhaps less so internally. Efficacy of a miR-92a light-inducible compound was demonstrated in superficial mice wounds, and was able to enhance proliferation and angiogenesis (Lucas et al., 2017). Finally, incorporation of miRNA therapy with a thioaptamer, specifically interacting with a chosen ligand, E-selectin in this example, may be useful to ensure specificity. The aptamer binds to the chosen molecule and guides the miR therapy, miR-146a and miR-181b to inflamed endothelium, reducing atherosclerosis in mice (Ma et al., 2016).

As there are currently no clinical trials focused on ncRNAs in vascular disease, we will describe studies in liver and heart and translational relevance for vascular disease. The most successful example of miRNA-based therapeutics to date is anti-miR-122 compound for hepatitis C. It has been demonstrated in Jopling et al. study that miR-122 is highly and specifically expressed in human liver. Moreover, it has been shown that inhibition of miR-122 was able to strongly decrease viral RNA of hepatitis C, introducing the novel idea for a potential miR-122-modulating therapy (Jopling et al., 2005). Since then a lot of work has been done to achieve an efficient miR-122 inhibition method *in vivo*, primarily using 2′-MOE-modified anti-miR (Krutzfeldt et al., 2005; Esau et al., 2006). Furthermore, anti-miR-122 LNA modification approach was efficient in downregulating miR-122 expression in mice, which was delivered intravenously (Elmen et al., 2008b). Notably, preclinical trials on chimpanzees have confirmed that LNA-anti-miR-122, now known as SPC3649 or Miravirsen, leads to significant reduction in hepatitis C viral load with no side-effects (Lanford et al., 2010). Currently, Miravirsen is being developed by Santaris Pharma and since 2017 is in Phase II clinical trials (Titze-de-Almeida et al., 2017).

In other translational studies, direct intra-cardiac injection of miR-21, miR-1, and miR-24 reduced infarct size in mice, with LAD ligation, at 24 h (Yin et al., 2009). The mice underwent ischaemic preconditioning, forcing an altered miRNA expression profile, with the hypothesis that these miRNA would be protective in the case of infarct. The miRNAs were then extracted and injected into the infarct model, resulting in an increase of eNOS, HSF-1, and HSP70. Direct injection of miRNA seems to be effective therefore in altering tissue expression of important

mRNAs and proteins. In a mesenchymal stem cell vector, miR-1 was overexpressed and injected into infarcted myocardium again in a mouse model of infarct, resulting in enhanced cell survival and improved cardiac function (Huang et al., 2013). In a similar way but with a different mode of delivery, polyketal (PK3) nanoparticles (a solid polymer) were used to deliver miRNA mimics miR-106b, miR-148b, and miR-204 to macrophages in mice hearts, all targeting Nox2 expression. Infarct size was significantly reduced, and function improved (Yang et al., 2017). The alternative approach of miRNA inhibition has also been shown to be effective in the cardiovascular setting, and in some cases may be technically easier to achieve. Anti-miRs can be quickly designed and developed, and *in vivo* delivery of the anti-miR-143 is protective in the development of pulmonary arterial hypertension in mice (Deng et al., 2015). MiR-143-3p is selectively upregulated in cell migration, and its modulation significantly reduces cell migration and apoptosis.

Vein graft failure in surgical coronary artery bypass has been a longstanding problem, and the setting for some significant advances in ncRNA therapy. MiR-21 expression is elevated in mice, porcine, and human *ex vivo* models of vein graft failure, and localises to the SMC layers of the forming neointima in the failing grafts. Delivery of anti-miR-21 to inhibit miR-21 was effective to reduce expression and attenuate the pathological neointima formation in a model of vein graft failure (McDonald et al., 2013), and now further clinical investigation is needed before this can be applied clinically. In a completely different disease system its also been reported that inhibition of the same miR-21 using anti-miR treatment can prevent Alport syndrome in mice. This disease which is characterised by glomerulonephritis and progressive renal failure also results in sensorineural deafness in the human form (Gomez et al., 2015). As a result of these findings, 'Regulus Therapeutics' are now carrying out a Phase II clinical study (NCT02855268) of the safety and efficacy of RG-012 drug (anti-miR-21) in the treatment of patients with Alport syndrome.

In the targeting of another miRNA, miR-92a, it was shown that downregulation of miR-92a expression using 2′-*O*-methyl anti-miR ORNs enhanced *in vivo* angiogenesis, neovascularisation as well as enhanced post-MI recovery in mice (Bonauer et al., 2009). This apparent pro-angiogenic and cardioprotective effect of anti-miR-92a therapy was further investigated by the same group. Specifically, they demonstrated that catheter-based delivery of anti-miR-92a with LNA modification (LNA-92a) led to a decrease in the infarct size in pig hearts and improved cardiac function (Hinkel et al., 2013).

With the advances of CRISPR/Cas9 technology and the ability to modify the genome at the base pair level, this method has become a very attractive and promising approach to generate both gain- and loss-of-function miRNA phenotypes. Interestingly, an *in vitro* transfection of CRISPR/Cas9 vectors, which contain sgRNAs, targeting the biogenesis processing sites of miR-17, miR-200c, and miR-141, decrease the expression of these miRNAs up to 96% (Chang et al., 2016), an impressive knockdown. Moreover, subcutaneous injection of HT-29 cells with CRISPR/Cas9-mediated *miR-17* knockdown into nude mice resulted in almost complete knockout of miR-17 expression in the *in vivo* environment after 28 days (Chang et al., 2016). Despite the clear benefits of the CRISPR/Cas9 approach in modulating miRNAs *in vivo*, the usual obstacles, such as off-target effects and lack of delivery vehicles with tissue specificity are yet to be overcome.

LncRNA-BASED INTERVENTION STRATEGIES

Given the fact that lncRNAs guide gene expression from start of transcription to protein translation, this class of molecules possess a promising therapeutic potential. The first study involving modulation of lncRNA expression for therapeutic purposes described the oncogenic lncRNA *H19*. In particular, it has been reported that *H19* is specifically expressed in over 30 tumours. Based on those findings, a plasmid expressing diphtheria toxin under control of the *H19* promoter (BC-819) has been intratumoraly injected into bladder tumour leading to a significant reduction in the tumour size in mice (Smaldone and Davies, 2010). This led to intiation of phases I and II human clinical trials, in which *H19* promoter-based plasmid is used to treat patients with different types of malignancies such as bladder, pancreatic, and ovarian cancers (Smaldone and Davies, 2010). Recently reported results seem promising, including in the treatment of early stage bladder cancer, BioCanCell report that BC-819 treatment resulted in 54% of patients recurrence-free at 24 months (Halachmi et al., 2018).

Currently, there are two main approaches to silence lncRNA expression, which are employed in pre-clinical models: the use of RNAi-based methods, such as siRNA, and LNA-GapmeR antisense oligonucleotides (ASOs), which induce RNase cleavage. Generally, the RNAi approach, including siRNA and short hairpin RNA (shRNA), which can be delivered via viral vector, is used predominanly for lncRNA that are localised in the cytoplasm. In particular, it has been demonstrated that siRNA targeting cytoplasmic lncRNA *SMILR* can reduce *SMILR* expression and attenuate pathological human saphenous vein SMC proliferation (Ballantyne et al., 2016). On the other hand, GapmeRs can be used for nuclear-localised lncRNA due to the fact that it induces degradation by RNase-H and is RISC-independent (Haemmig and Feinberg, 2017). Furthermore, GapmeRs can be used to modulate lncRNA expression for *in vivo*. In particular, intraperitoneal injection of GapmeRs in mice model of hindlimb ischaemia was able to significantly reduce *MALAT1* expression in the muscle tissue leading to poor blood flow recovery and diminished capillary density (Michalik et al., 2014). In another study, the GapmeR was used to inhibit the lncRNA *Chast*, which is upregulated in hypertrophic heart tissue from aortic stenosis patients. Notably, the *in vivo* delivery of GapmeR targeting *Chast* resulted in decrease in pathological cardiac remodelling with no side effects (Viereck et al., 2016). Finally, CRISPR/Cas9 genome editing method is an attractive emerging tool for modulation of gene expression including manipulation of lncRNAs. To date, the CRISPR-inhibition (CRISPRi) approach has been shown to knockdown the expression of six lncRNA including *GAS5*, *H19*, *MALAT1*, *NEAT1*, *TERC*, and *XIST* (Gilbert et al., 2014). Despite the fact that CRISPR/Cas9 method

is at its nascent stages, coupled with viral-based delivery systems, it holds much potential in terms of ncRNA-based therapeutics.

CONCLUSION

There remains little doubt that ncRNAs are important players in the pathology of vascular disease, and have been repeatedly demonstrated as key regulators of vascular biology in cell culture models, animal models, and in human samples. Aggressive lifestyle and risk factor management has had only a modest incremental effect on the trajectory of atherosclerotic disease in the last 70 years, even including the introduction of statins, and the huge improvements in surgical and interventional medicine. A new approach is now needed, and with the advent of CRISPR technology and the ongoing advances in delivery by viral vectors, non-coding gene therapy to up or downregulate culprit loci and transcripts has become a reality. With ever deeper understanding of molecular disease mechanisms and the refinement of safe and efficacious delivery methods the targeting of miRNA and lncRNA holds much promise for vascular disease treatment in future.

AUTHOR CONTRIBUTIONS

VM and JH were primary authors. JS, DN, and AB were involved in editing and reviewing of the manuscript.

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Long Non-coding RNAs: At the Heart of Cardiac Dysfunction?

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During the past decade numerous studies highlighted the importance of long non-coding RNAs (lncRNAs) in orchestrating cardiovascular cell signaling. Classified only by a transcript size of more than 200 nucleotides and their inability to code for proteins, lncRNAs constitute a heterogeneous group of RNA molecules with versatile functions and interaction partners, thus interfering with numerous endogenous signaling pathways. Intrinsic transcriptional regulation of lncRNAs is not only specific for different cell types or developmental stages, but may also change in response to stress factors or under pathological conditions. Regarding the heart, an increasing number of studies described the critical regulation of lncRNAs in multiple cardiac disorders, underlining their key role in the development and progression of cardiac diseases. In this review article, we will summarize functional cardiac lncRNAs with a detailed view on their molecular mode of action in pathological cardiac remodeling and myocardial infarction. In addition, we will discuss the use of circulating lncRNAs as biomarkers for prognostic and diagnostic purposes and highlight the potential of lncRNAs as a novel class of therapeutic targets for therapeutic purpose in heart diseases.

Keywords: non-coding RNA, cardiac hypertrophy, myocardial infarction, cardiovascular disease, therapy

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LONG NON-CODING RNAs

Although the majority of the human genome is transcribed into RNA molecules, only ∼2% of these transcripts code for proteins, thus, scientists have started to explore the wide universe of non-coding RNAs (ncRNAs) as crucial regulators of physiological and pathological cell function. Numerous studies demonstrated the involvement of small ncRNAs like microRNAs (miRNAs) in cardiovascular disease (Quiat and Olson, 2013; Thum, 2014), whereas the importance of long non-coding RNAs (lncRNAs) as key regulators in the development and progression of cardiac diseases is only beginning to be understood. As all ncRNAs longer than 200 nucleotides in length are arbitrarily classified as lncRNAs, this group of molecules is very heterogeneous and exhibit multifaceted biological functions and interact with a variety of other RNAs or proteins. Depending on their subcellular localization in the nucleus or cytoplasm, lncRNAs can interfere with transcriptional and post-transcriptional gene regulation, as well as mRNA translation, respectively. Nuclear transcripts, for example, can mediate epigenetic gene modifications or transcriptional activation and silencing, whereas cytoplasmic lncRNA often interact with miRNAs to post-transcriptionally regulate gene expression or act as molecular scaffolds for RNA-protein complexes. For further details on lncRNA modes of action we refer to the current literature available (Sun et al., 2017; Noh et al., 2018). Here, we discuss the growing body of evidence depicting a central role of lncRNAs in cellular responses

during cardiac disease focusing on pathological hypertrophic cardiac growth and myocardial infarction (MI), and their potential to serve as novel therapeutic targets and diagnostic biomarkers.

LncRNAs IN CARDIOVASCULAR DISEASE

The term cardiovascular disease (CVD) comprises a wide range of pathologies. However, in order to adequately address the importance of lncRNAs in distinct diseases and to highlight specific molecular modes of action, we will focus on a selection of lncRNAs associated with cardiac hypertrophy and myocardial infarction. In addition, **Table 1** provides an exhaustive list of relevant lncRNAs in CVD. Importantly, lncRNAs have also been shown to play essential roles in embryonic development and cardiac lineage commitment [such as Fendrr and Braveheart (Grote et al., 2013; Klattenhoff et al., 2013)] and are important regulators of accurate heart functionality. More details on lncRNAs in organogenesis and heart development have been extensively reviewed elsewhere; (Devaux et al., 2015; Grote and Herrmann, 2015).

Cardiac Hypertrophy

One of the first studies on functionally relevant lncRNAs in cardiac hypertrophy discovered a cluster of lncRNAs partially overlapping the Myh7 gene locus which, accordingly, were called *myosin heavy-chain-associated RNA transcripts* (Mhrt) (Han et al., 2014). Mhrt was permanently downregulated after induction of cardiac hypertrophy by transverse aortic construction (TAC) surgery in mice. In addition, the dynamic regulation of this conserved, cardiac-specific lncRNA was accompanied by the TAC-induced isoform switch from Myh6 to Mhy7, a hallmark of developing cardiomyopathy (Miyata et al., 2000; Krenz and Robbins, 2004). Inducible transgenic overexpression of Mhrt resulted in reduced cardiac hypertrophy and fibrosis and improved cardiac function compared to TAC operated mice without reactivated Mhrt (Han et al., 2014). Importantly, this effect was observed when Mhrt expression was induced before TAC surgery as well as 2 weeks after pressure overload initiation, indicating that downregulation of Mhrt is important for the progression of pressure overload induced cardiac remodeling. Mechanistically, Mhrt directly interacts with the chromatin-remodeling factor Brg1 in order to inhibit its own transcriptional silencing at the shared Mhrt/Myh6 bidirectional promoter region under physiological conditions. In contrast, during cardiac stress Brg1 expression exceeds Mhrt abundance, resulting in active Brg1-mediated chromatin remodeling than leads to Mhy6 to Mhy7 isoform switch. This thereby represents an important regulatory circuit in the development and progression of cardiac hypertrophy.

Using a microarray approach to compare the lncRNA transcriptome of TAC versus sham operated mice, Viereck and colleagues identified the conserved lncRNA *cardiac hypertrophy-associated transcript* (Chast) to be upregulated in hypertrophic cardiomyocytes (Viereck et al., 2016). Chast expression is, at least partially, induced via the pro-hypertrophic transcription factor nuclear factor of activated T cells (NFAT) and acts in cis to regulate Pleckstrin homology domain–containing protein family M member 1 (Plekhm1), resulting in impaired autophagy. Remarkably, adeno-associated virus (AAV)-overexpression of Chast was sufficient to induce hypertrophic growth *in vitro* and *in vivo* in the absence of additional hypertrophic stress factors. In contrast, silencing of Chast using GapmeR antisense chemistries (for further details see section below 'LncRNAs as potential therapeutic targets in CVD') prevented hypertrophic cardiac growth and preserved cardiac function in TAC operated animals. Of note, silencing Chast was cardio-protective in a preventive approach, as well as in a clinically more relevant therapeutic approach with repeated GapmeR injection starting 4 weeks after induction of pressure overload in mice. Strikingly, the human CHAST transcript was able to induce hypertrophic cell growth in murine cardiomyocytes *in vitro*, suggesting functional conservation. Furthermore, CHAST was upregulated in hearts of patients with aortic stenosis, where cardiac hypertrophy occurs as a compensatory response to increased afterload, highlighting the therapeutic potential of CHAST for the treatment of cardiac hypertrophy in humans.

Another example of a pro-hypertrophic lncRNA, which emphasizes the importance of lncRNAs to precisely regulate a switch in gene expression upon cardiac stress, is *cardiac-hypertrophy-associated epigenetic regulator* (Chaer). Following TAC surgery Chaer-knockout mice showed less hypertrophic cardiac growth, reduced fibrosis and preserved cardiac function in comparison to wildtype control animals (Wang et al., 2016). In contrast, overexpression of Chaer induced hypertrophic cell growth in both phenylephrine and vehicle treated cardiomyocytes. The mainly nuclear located Chaer directly interacts with the EZH2 subunit of polycomb repressive complex 2 (PRC2), resulting in reduced H3K27 trimethylation at promoter regions and thereby enhanced expression of the pro-hypertrophic genes Anf, Myh7, and Acta. Furthermore, the authors highlighted the pivotal role of Chaer-PCR2 interaction at the onset of pathological cardiac stress by knocking down Chaer expression either 2 days before or 1 day after TAC surgery. Loss of Chaer at the very beginning of pathological pressure overload reduced hypertrophic heart growth and marker gene expression and improved cardiac function compared to control animals, while Chaer knockdown 24 h post TAC showed no protective effect. This early interaction between PRC2 and Chaer seems to be required for the onset of cardiac epigenetic reprogramming but not progression of hypertrophic remodeling.

Besides hypertrophic growth of cardiomyocytes, pressure overload induced pathological remodeling is accompanied by cardiac fibroblast (CF) activation and rearrangement of the extra cellular matrix (ECM), resulting in fibrosis and impaired cardiac function. By performing lncRNA array analysis in CFs of mice undergoing 13 weeks of TAC, Piccoli et al. identified over 1400 deregulated lncRNAs in CF fractions of hypertrophic mouse hearts (Piccoli et al., 2017). The most abundant and CF-specific lncRNA *Maternally expressed gene 3* (Meg3) appeared to be upregulated during the first 4 weeks after TAC

followed by a long term repression. Meg3 interaction with p53 facilitates transcriptional activation of matrix metalloproteinase-2 (MMP-2) and subsequent remodeling of ECM composition (**Figure 1A**). Consecutive injection of GapmeRs targeting Meg3 reduced cardiac fibrosis and ameliorated cardiac performance and diastolic function in TAC operated mice compared to control GapmeR treated animals. Interestingly, knockdown of Meg3 also diminished hypertrophic cardiomyocyte growth, although

TABLE 1 | Overview of lncRNAs in different CVDs.

lncRNA	Associated disease	Reported function	Reference
Anril	Coronary artery disease	Biomarker (in diabetic type II patients)	Rahimi et al., 2018
	In-stent restenosis	Biomarker	Wang et al., 2017
	Left ventricular dysfunction	Biomarker	Vausort et al., 2014
Braveheart	Cardiac lineage development	Regulation of chromatin modifications via PRC2	Klattenhoff et al., 2013
Carl	Myocardial infarction	Inhibition of mitochondrial fission and cardiomyocyte apoptosis by sponging miR-539	Wang et al., 2014b
Chaer	Cardiac hypertrophy	Epigenetic modulation of hypertrophic gene expression	Wang et al., 2016
Chast	Cardiac hypertrophy	Induction of hypertrophic cell growth and gene expression	Viereck et al., 2016
Chrf	Cardiac hypertrophy	Sponging of miR-489	Wang et al., 2014a
	Doxorubicin-induced heart failure	Regulation of TGF-β signaling	Chen et al., 2018
Fendrr	Organ development derived from lateral mesoderm	Regulating chromatin modifications via PRC2 and TrxG/MLL	Grote et al., 2013
Ftx	Myocardial infarction	Regulation of cardiomyocyte apoptosis by targeting miR-29b-1-5p	Long et al., 2018
H19	Cardiac hypertrophy	Targeting Ca/calmodulin-dependent protein kinase IIδ (CaMKIIδ)	Liu et al., 2016
	Coronary artery disease	Biomarker	Zhang et al., 2017
	Diabetic cardiomyopathy	Regulation of cardiomyocyte apoptosis by targeting VDAC1	Li et al., 2016b
	Ischemia reperfusion injury	Regulation of necrosis by targeting miR-103/107	Wang et al., 2015
	Myocardial infarction	Activation of autophagy	Zhou et al., 2018
Hotair	Cardiac hypertrophy	Interaction with miR-19	Lai et al., 2017
Lipcar	Cardiac remodeling and heart failure	Biomarker	Kumarswamy et al., 2014
	Coronary artery disease	Biomarker	Zhang et al., 2017
	Left ventricular diastolic function	Biomarker	De Gonzalo-Calvo et al., 2016
Malat1	Atherosclerosis	Regulation of inflammation	Gast et al., 2018
	Cardiac fibrosis after myocardial infarction	Regulation of TGF-β signaling via miR-145	Huang et al., 2018
Mdrl	Ischemia-reperfusion injury	Inhibition of mitochondrial fission and cardiomyocyte apoptosis by sponging miR-361	Wang et al., 2014c
Meg3	Cardiac fibrosis and diastolic dysfunction	Regulation of TGF-β I induced p53 signaling	Piccoli et al., 2017
	Myocardial infarction	Regulation of cardiomyocyte apoptosis	Wu et al., 2018
Mhrt	Cardiac hypertrophy	Regulation of isoform switch Myh6 to Myh7	Han et al., 2014
	Doxorubicin-induced cardiomyopathy	Inhibition of cardiomyocyte apoptosis	Li et al., 2016a
	Heart failure	Biomarker	Xuan et al., 2017
Miat	Cardiac fibrosis after myocardial infarction	Regulation of cardiac fibrosis by interaction with several miRNAs	Qu et al., 2017
	Cardiac hypertrophy	Sponging miR-93	Li et al., 2018
		Sponging miR-150	Zhu et al., 2016
	Left ventricular diastolic function	Biomarker	De Gonzalo-Calvo et al., 2016
	Diabetic cardiomyopathy	Regulation of myocardial hypertrophy and apoptosis	Zhou et al., 2017
	Myocardial infarction	SNP in exon 5 as risk allele for MI	Ishii et al., 2006
Mirt1	Myocardial infarction	Suppression of NF-κB signaling	Li et al., 2017
Nron	Heart failure	Biomarker	Xuan et al., 2017
Sencr	Left ventricular diastolic function	Biomarker	De Gonzalo-Calvo et al., 2016
Wisper	Cardiac fibrosis after myocardial infarction	Alternative splicing of Plod2 mRNA by enabling nuclear localization of TIAR	Micheletti et al., 2017

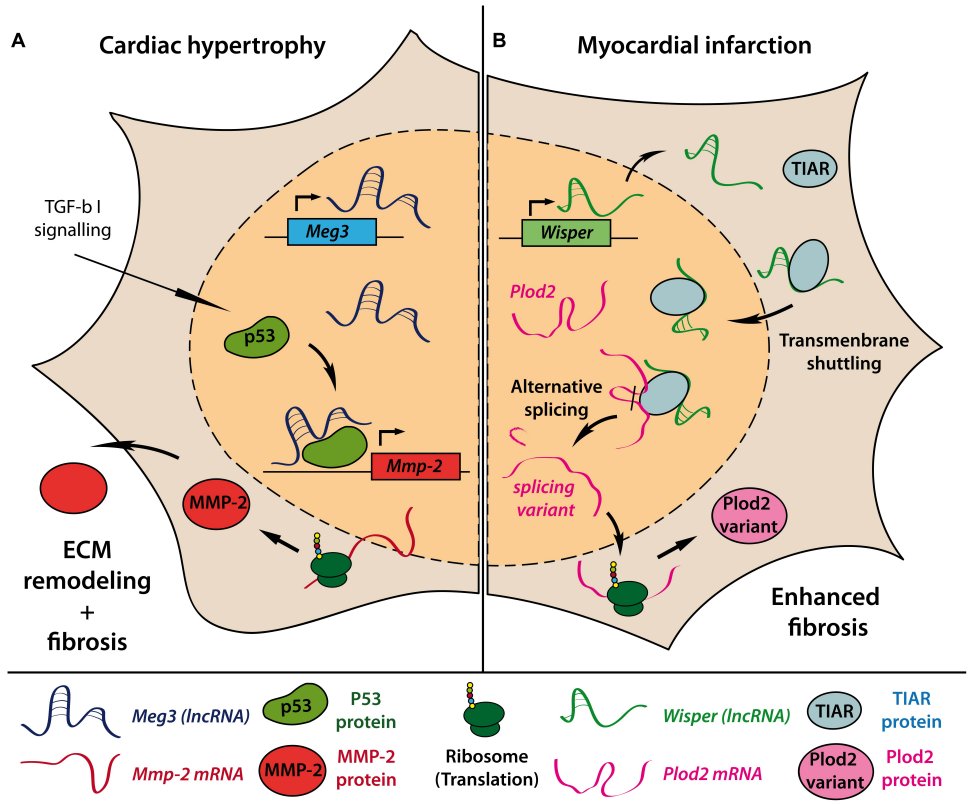

FIGURE 1 | Molecular mechanisms of lncRNAs involved in cardiac fibrosis. Introduction of pressure-overload-induced cardiac hypertrophy in mice causes upregulation of the lncRNA Meg3 in cardiac fibroblasts. Interaction of Meg3 with the transcription factor p53 is required for TGF-β–induced transcription of *Mmp-2* gene. Increased extracellular MMP-2 levels subsequently drive enhanced extracellular matrix (ECM) remodeling and cardiac fibrosis **(A)**. Myocardial infarction causes upregulation of lncRNA Wisper in cardiac fibroblasts. Only in the presence of Wisper can TIAR shuttle from the cytoplasm to the nucleus to interfere with RNA processing and induce alternative splicing of Plod2 mRNA. The resulting alternative Plod2 protein variant causes enhanced fibrosis **(B)**.

Meg3 showed no TAC-induced regulation in cardiomyocytes in the initial screen suggesting further paracrine effects of Meg3 silencing in CFs on other cardiac cell types.

Myocardial Infarction

The lncRNA *myocardial infarction associated transcript* (MIAT) was first described in a large scale single nucleotide polymorphism (SNP) association study from a Japanese population in 2006. In this study the presence of six SNPs in the MIAT locus was associated with an increased risk for MI (Ishii et al., 2006). *In 2017,* Qu and colleagues also identified MIAT as a pro-fibrotic lncRNA after MI *in vivo* (Qu et al., 2017). CF activation and differentiation, cardiac fibrosis and scar formation represent key events after MI. On the one hand, fibrotic remodeling of the infarct region is crucial for sustaining myocardial integrity and preventing cardiac wall rupture during wound healing processes. On the other hand, sustained fibrosis throughout subsequent cardiac remodeling contributes to increased cardiac stiffness, impaired cardiac function and development of heart failure (Van Den Borne et al., 2010). In regards to MIAT, permanent occlusion of the left anterior descending artery (LAD) in mice resulted in it's continuously upregulated expression in peri-infarcted tissue (Qu et al., 2017). By targeting several anti-fibrotic miRNAs (including miR-24, miR-29, miR-30, and miR-133), MIAT was shown to promote cardiac fibrosis and adverse remodeling in the infarcted mouse hearts. In contrast, lentiviral-mediated knockdown of MIAT prior to MI reduced infarct size and interstitial fibrosis contributing to preserved cardiac function, via the control of CF proliferation and collagen production. Additionally, two other publications highlighted further potential of MIAT to act as a pro-hypertrophic lncRNA in cardiomyocytes by sponging the anti-hypertrophic miR-150 (Zhu et al., 2016) and miR-93 (Li et al., 2018). Altogether, these findings emphasize the complex regulatory network of MIAT during cardiac disease and strengthen its potential to serve as a therapeutic target.

Recently, the conserved lncRNA *Wisp2 super-enhancer–associated RNA* (Wisper) has been described to control CF functions *in vitro* and *in vivo*. Wisper is transcribed from a cardiac-specific super-enhancer region and was found to be highly upregulated in the border zone after MI in mice (Micheletti et al., 2017). Wisper regulated proliferation, migration, and apoptosis as well as gene expression of pro-fibrotic factors like Col3a1, Fn1, and Tgfb2 in CFs but not fibroblasts from other origin. *In vivo*, GapmeR-induced knockdown of Wisper after MI resulted in a smaller infarct size, reduced

fibrosis and preserved cardiac structure and function. Of note, pre-operative GapmeR treatment also impaired CF function in the acute wound healing process leading to increased mortality due to left ventricular wall rupture. The authors demonstrated that Wisper directly interacts with TIA1-related protein (TIAR) to regulate alternative splicing of lysyl hydroxylase 2 (LH2 or Plod2), which has been associated with fibrosis-related disorders (Yeowell et al., 2009; **Figure 1B**). Knockdown of Wisper suppressed transmembrane shuttling of TIAR protein to the nucleus and prevented TIAR-Plod2-mRNA interaction. Consequently, reduced Plod2 expression coincided with the described phenotypical changes in CF function *in vitro* and *in vivo*.

During MI, oxygen-deficiency primarily serves to induce massive loss in viable cardiomyocytes by apoptotic and necrotic cell death. Several studies have highlighted the regulatory involvement of lncRNAs in apoptotic cell death during MI. For example, the lncRNAs *cardiac apoptosis-related lncRNA* (Carl) (Wang et al., 2014b) and *mitochondrial dynamic related lncRNA* (Mdrl) (Wang et al., 2014c) were downregulated after MI. Adenoviral overexpression of Carl or Mdrl was able to inhibit mitochondrial fission and cardiomyocyte apoptosis by inhibiting pro-apoptotic miRNAs miR-539 or miR-361, respectively, and resulted in smaller infarct sizes *in vivo*. Similar to Carl and Mdrl, the lncRNA *five prime to Xist* (Ftx) is transcriptionally repressed after ischemia reperfusion injury and showed anti-apoptotic potential by regulating the Bcl2l2 repressor miRNA miR-29b-1-5p (Long et al., 2018) *in vitro*.

As highlighted above lncRNAs are crucial regulators of pathological cardiomyocyte growth, fibrosis and cell survival in hypertrophic and infarcted hearts (**Figure 2**). Through interfering with epigenetic gene regulation, transcriptional activation or repression and post-transcriptional modifications by directly interacting with proteins or other ncRNAs such as miRNAs, lncRNAs orchestrate miscellaneous intracellular signaling pathways in a number of cardiac diseases.

Besides intracellular signaling, changes in lncRNA expression levels also have the potential to influence intercellular communication by modulating paracrine signaling. The lncRNA *myocardial infarction-associated transcript 1* (Mirt1) for example was shown to be involved in the regulation of the acute inflammatory response after MI (Li et al., 2017). In their study Li and colleagues demonstrated that hypoxia-induced upregulation of Mirt1 in neonatal mouse CFs facilitated nuclear transport of NF-κB and expression of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α. Secretion of these pro-inflammatory cytokines in turn mediated enhanced cardiomyocyte apoptosis and macrophage infiltration into the infarcted tissue.

In addition to the aforementioned modulation of paracrine signaling, first studies have appeared describing that secreted lncRNA display a novel layer of intercellular communication themselves by mediating cell proliferation (Zhang et al., 2018) or cancer progression (Pan et al., 2017). Furthermore, circulating lncRNAs might also have the potential to act in an endocrine manner and provoke systemic responses in CVD. Regardless, further studies need to be performed to reveal the consequences of altered lncRNA levels in the systemic circulation.

LncRNA AS BIOMARKERS

As outlined above, lncRNAs have multifaceted intracellular regulatory functions and the capability to directly or indirectly alter intercellular communication. Furthermore, it has been shown that lncRNAs can be detected in extracellular body fluids such as plasma or urine and display a dynamic alteration upon diseases (Zhou et al., 2015; Martignano et al., 2017; Terracciano et al., 2017). LncRNAs can enter the blood stream encapsulated in exosomes (Li et al., 2015) and extracellular vesicles or inside of apoptotic bodies released from dying cells (Viereck and Thum, 2017). Beside this, association with RNA-binding proteins might also explain the enhanced stability of circulating lncRNAs conferred by their resistance to rapid degradation by RNases. Long term stability of lncRNAs in easily accessible body fluids, in combination with disease specific abundancy patterns, make lncRNAs of particular interest as a novel class of non-invasive prognostic and diagnostic biomarker.

One prominent example of lncRNAs as a potential biomarker is the mitochondria-derived lncRNA *long intergenic non-coding RNA predicting cardiac remodeling* (LIPCAR), whose plasma levels are associated with left ventricular (LV) remodeling after MI and increased risk of developing heart failure (Kumarswamy et al., 2014). Kumarswamy et al. reported a higher abundance of LIPCAR in the plasma of patients with consecutive heart failure after MI compared to MI patients without LV remodeling. Furthermore, the magnitude of circulating LIPCAR was also associated with an increased risk of cardiovascular death in chronic heart failure patients. In a 2-year follow-up, circulating LIPCAR was further identified as an independent predictor for diastolic dysfunction in well-controlled type 2 diabetes patients (De Gonzalo-Calvo et al., 2016). In addition, two other circulating lncRNAs, MIAT and *smooth muscle and endothelial cell-enriched migration/differentiation-associated long non-coding RNA* (SENCR), were associated with LV cardiac remodeling in these patients from the same study. Moreover, Zhang et al. reported increased plasma levels of LIPCAR and the paternally-imprinted lncRNA H19 in patients with coronary artery disease (CAD), especially in those subjects with concomitant chronic heart failure (Zhang et al., 2017).

Other recent studies identified the circulating lncRNAs *non-coding repressor of NFAT* (NRON) and MHRT as further independent predictors for heart failure (Xuan et al., 2017) and associated increased plasma levels of ANRIL with a higher risk for in-stent restenosis (Wang et al., 2017).

The possibility of detecting lncRNAs in extracellular body fluids discloses an enormous pool of molecules to extend the current catalog of prognostic and diagnostic biomarkers for cardiovascular and other diseases (Gutschner et al., 2017; Viereck and Thum, 2017).

LncRNAs AS POTENTIAL THERAPEUTIC TARGETS IN CVD

CVDs still represent the number one cause of death worldwide (World Health Organization, 2017). As highlighted above,

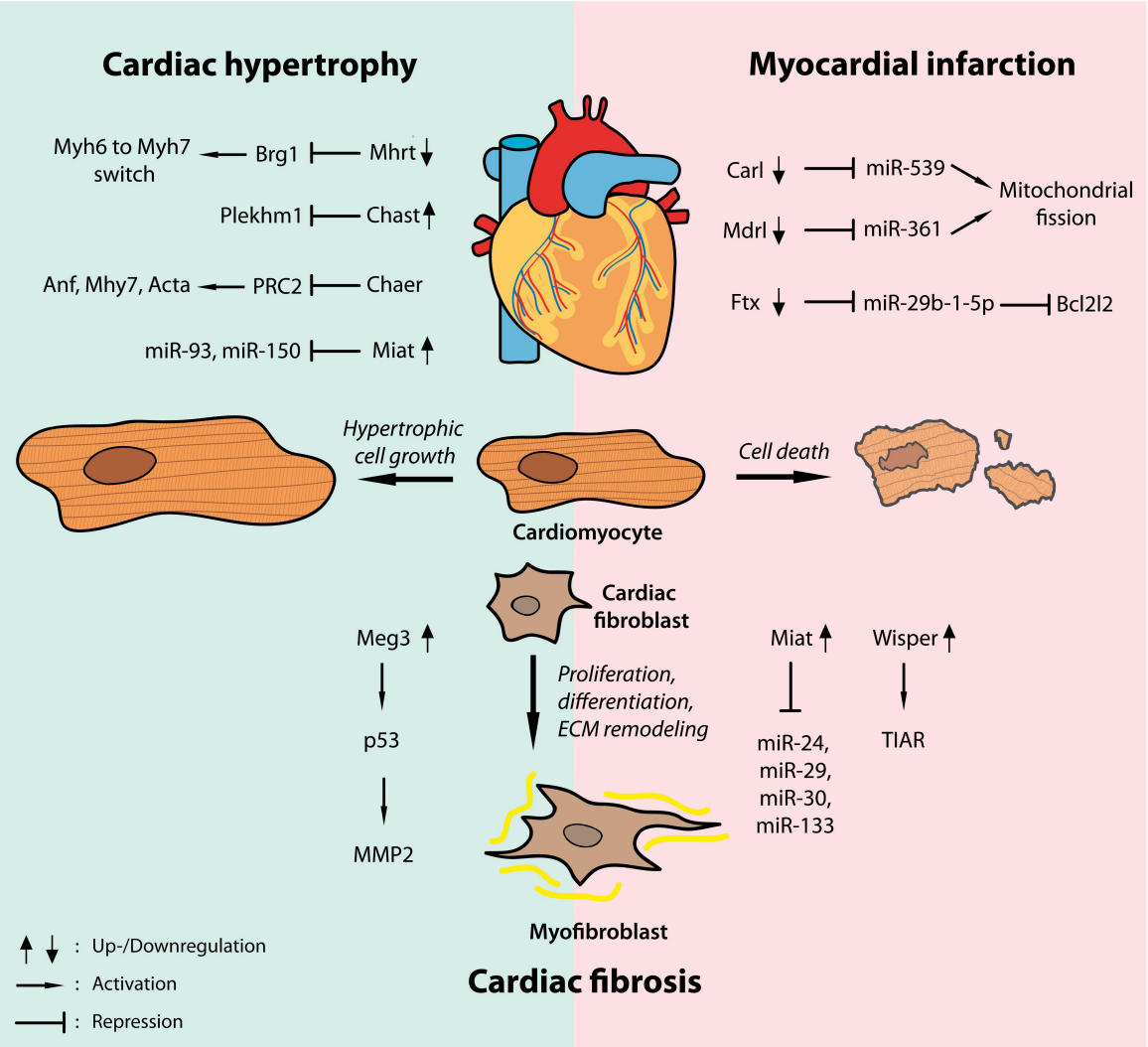

FIGURE 2 | Widespread regulatory functions of lncRNAs in cardiac disease. Dynamic regulation of a number of lncRNAs has been shown in models of cardiac hypertrophy (left, blue shade) as well as myocardial infarction (right, pink shade) driving hypertrophic cardiomyocyte growth and cardiac fibrosis or regulating cardiac cell death by interfering with transcription factors, epigenetic modifiers or different microRNAs.

cardiac-specific *in vivo* modulation of lncRNAs exhibit the potential to ameliorate cardiac dysfunction or diminish pathological progression in the diseased heart (Han et al., 2014; Viereck et al., 2016; Wang et al., 2016; Micheletti et al., 2017; Piccoli et al., 2017; Qu et al., 2017), potentially making them new targets for the treatment of CVDs. LncRNAs may represent therapeutic targets, provided their expression can be modulated *in vivo*.

Silencing of RNA molecules can be achieved by the use of sequence-specific antisense oligonucleotides (ASO) or RNA interference (RNAi) methods. Antisense drug therapies have already been applied in clinical trials targeting protein-coding mRNAs with one compound already on the market for the treatment of familial hypercholesterolemia [Mipomersen (Rader and Kastelein, 2014; Geary et al., 2015; Santos et al., 2015)] and another approved by the Food and Drug Administration to treat Duchenne muscular dystrophy [Eteplirsen (Mendell et al., 2013, 2016)]. In contrast to protein-coding mRNAs, lncRNAs may exert different functions with respect to their subcellular localization (nucleus or cytoplasm). This needs to be considered for the general targeting strategy. SiRNAs, for example, mainly function in the cytoplasm, therefore may be less effective against nuclear localized lncRNAs (Lennox and Behlke, 2016). In addition to subcellular localization, tissue and/or cell type-specific delivery of antisense therapeutics is crucial for targeted lncRNA modulation in different CVDs. Different delivery strategies to improve targeting of the heart have been reviewed by Lucas and colleagues (Lucas et al., 2018).

Currently, GapmeRs are the most promising class of ASOs used for pharmacological silencing of lncRNAs *in vivo*, as they are able to enter the nucleus, thus, enable targeting of nuclear transcripts as well. GapmeRs consist of a DNA core flanked by

two locked nucleic acids (LNA) sequences complementary to the target mRNA or lncRNA sequence. By chemically 'locking' the ribose backbone of the nucleotide structure, LNAs display a higher stability, target specificity and RNase H activation potential resulting in enhanced knockdown efficiency (Swayze et al., 2007). To date, no clinical trials targeting lncRNAs have been performed. This might be due to the relative novelty of lncRNAs been regarded as potential therapeutic targets compared to proteins. However, therapeutic GapmeR injections have successfully been used to modulate lncRNAs in animal models of pressure overload [Chast (Viereck et al., 2016) and Meg3 (Piccoli et al., 2017)] and MI [Wisper (Micheletti et al., 2017)]. In all of the mentioned studies the authors presented remarkably improved cardiac function upon therapeutic intervention, stressing the great potential of antisense drugs therapeutically targeting lncRNAs. Nevertheless, the use of LNA oligonucleotides may be associated with hepatotoxicity (Swayze et al., 2007; Burdick et al., 2014), highlighting the need for further chemical refinement of this novel class of drugs. For detailed information on the pharmacology of antisense drugs, different modification strategies, and current clinical trials in general and CVDs we refer to further literature specifically discussing these topics in depth (Bennett et al., 2017; Lucas et al., 2018; Smith and Zain, 2019).

In contrast to silencing approaches, therapeutic overexpression of lncRNAs *in vivo* appears to be more challenging and requires the use of viral-mediated gene delivery, nanoparticles, or RNA mimics. Overexpression of lncRNAs using viral gene delivery poses several obstacles including the efficiency of lncRNA upregulation itself. The second challenge is to overexpress the target lncRNA in its endogenous subcellular localization – in other words, to enhance the function of cis regulatory lncRNAs via ectopic overexpression. AAV vectors that are commonly used for gene therapy approaches have relatively low packaging limit. Hence, they cannot be used for lncRNA transcripts longer than 3–4 kb. Furthermore, depending on the pathological context, a transient or stable overexpression may be needed for the therapeutic treatment. Of note, the targeted lncRNA should be overexpressed in a cell type specific manner, as lncRNAs may have varied functions in different cells or organs (Raveh et al., 2015). Despite the aforementioned shortcomings, two independent studies provided proof of principle for cardio protective viral-based overexpression strategies in a preventive therapy approach using the MI mouse model (Wang et al., 2014b,c). Although a pre-MI treatment does not represent a clinically relevant scenario, these two studies provide first evidence for the prospective potential of lncRNA overexpression as a promising therapeutic intervention in cardiovascular diseases.

In summary, a vast number of lncRNAs are dynamically regulated upon initiation and progression of CVDs. Many have important biological functions and/or have the potential to serve as a novel class of circulating biomarkers. Several *in vivo* experiments have revealed that modulation of lncRNAs offers a promising new therapeutic approach to treat cardiovascular diseases, albeit the silencing or overexpression approaches still require further refinements. Nevertheless, large screening approaches are often performed in animal models of CVD and lncRNAs are not always conserved among species but this is a prerequisite for clinical translation. However, as the field of lncRNAs as potential therapeutic targets is still in its infancy it is not unlikely that in the near future lncRNAs will emerge as valuable new tools for the treatment of numerous diseases, including CVDs.

AUTHOR CONTRIBUTIONS

LH wrote the manuscript and prepared the display items. CB and TT guided the manuscript preparation and critically revised it.

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Conflict of Interest Statement: TT has filed patents about the diagnostic and therapeutic use of several cardiovascular lncRNAs and is a founder of and holds shares in Cardior Pharmaceuticals.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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