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Role of non-coding RNAs in the Pathogenesis of Abdominal Aortic Aneurysm: Possible Therapeutic Targets?

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Abstract

Abdominal aortic aneurysm (AAA) is a local dilatation of the abdominal aortic vessel wall and is among the most challenging cardiovascular diseases as without urgent surgical intervention, ruptured AAA has a mortality rate of >80%. Most patients present acutely after aneurysm rupture or dissection from a previous asymptomatic condition and are managed by either surgery or endovascular repair. Patients usually are old and have other concurrent diseases and conditions, such as, diabetes, obesity and hypercholesterolemia making surgical intervention more difficult. Collectively, these issues have driven the search for alternative methods of diagnosing, monitoring and treating AAA using therapeutics and/or less invasive approaches. Non-coding RNAs—small non-coding RNAs (microRNAs) and long-non-coding RNAs (lncRNAs) - are emerging as new fundamental regulators of gene expression. Researchers and clinicians are aiming at targeting these microRNAs and lncRNAs and exploit their potential as clinical biomarkers and new therapeutic targets for abdominal aortic aneurysms. While the role of miRNAs in AAA is established, studies on lncRNAs are only beginning to emerge, suggesting their important yet unexplored role in vascular physiology and disease. Here, we review the role of noncoding RNAs and their target genes focusing on their role in AAA. We also discuss the animal models used for mechanistic understanding of AAA. Furthermore, we discuss the potential role of microRNAs and lncRNAs as clinical biomarkers and therapeutics.

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Keywords

microRNAs; long-noncoding RNAs; abdominal aortic aneurysms (AAAs); aortic dissection; circulating biomarkers

1. Introduction: Pathophysiology of AAA

Abdominal aortic aneurysm (AAA) is defined as a permanent dilation or bulging of the aorta. AAA is relatively common and its rupture leads to a potentially life-threatening condition.¹ Ruptured AAA is the 13th-leading cause of death in the United States and is estimated to cause ~ 15,000 deaths per year. The frequency of rupture is 4.4 per 100,000 patients. Despite increased survival following diagnosis, incidence and crude mortality seem to be increasing.² Globally, the frequency rate of asymptomatic AAA ranges from 4–8% while the frequency of AAA rupture ranges from 4 to 13 cases per 100,000 persons.³ Aging and being male are two strong risk factors of AAA.¹ It is important to note that the frequency rate of AAA in males is much higher compared to the females early on which increases with aging. In addition, white men have the highest incidence of AAA (~3.5 times that in African American men).¹ Other risk factors include smoking, hypertension, and atherosclerosis in coronary artery or peripheral arteries.¹ Most AAAs occur in individuals with advanced atherosclerosis. Atherosclerosis may induce AAA formation by causing mechanical weakening of the aortic wall with loss of elastic recoil, along with degenerative ischemic changes occurring in the adventitial layer. However, it is unclear whether atherosclerosis is causing AAA or conspire with other pro-AAA factors. It is interesting, however, to note that the patients at greatest risk for AAA are men who are older than 65 years and have peripheral atherosclerotic disease.¹

The diameter of the aorta with AAA can increase by >50% increase over its normal size.⁴ Most AAAs begin below the renal arteries and terminate above the iliac arteries. The size, shape, and extent of AAAs vary considerably. Like aneurysms of the thoracic aorta, AAAs may be broadly described as either fusiform (circumferential) or saccular (more localized). AAA develops as the extracellular matrix proteins are degraded and remodeled in the aortic wall, resulting in the loss of the structural integrity and dilation of the wall.^{5–9} The pathogenic mechanisms underlying AAA include proteolytic degradation of aortic-wall connective tissue^{6, 10–13}, inflammation and immune responses^{14–18}, biomechanical wall stress^{19–24}, molecular genetics^{25–27}, and genetic predisposition^{5–8, 28–31}, as have been reviewed previously. Other less common causes of AAA include cystic medial necrosis, arteritis, trauma, inherited connective-tissue disorders, and anastomotic disruption.³²

Most AAAs are asymptomatic, progressive, and often are detected as an incidental finding on diagnostic imaging obtained for other reasons. At present, the only treatment option for AAA is surgical repair via laparotomy or less invasive endovascular stents.³² Unfortunately, there is no therapeutic treatment available to prevent, delay or reverse the pathology of AAA other than reducing the risk factors such as stop smoking, controlling blood pressure and lipid levels. This is in part due to our lack of understanding of the pathophysiological mechanism(s) and key regulators thereof. Efforts have been underway to identify the

genomic and epigenetic factors that regulate AAA pathogenesis as they may lead to better mechanistic understanding and therapeutic targets to prevent and treat the disease.

Through transcriptome studies using samples obtained from patients with AAA and animal models of AAA, various genes involved in extracellular matrix degradation, inflammation, and other critical pathophysiology observed in AAA formation have been identified.^{33–47} The combination of proteolytic degradation of aortic-wall connective tissue, inflammation and immune responses, biomechanical wall stress, and molecular genetics represents a dynamic process that leads to aneurysmal deterioration of aortic tissue. Matrix metalloproteinases (MMPs) and their inhibitors are present in normal aortic tissue and are responsible for vessel-wall remodeling. Aneurysmal tissue are associated with increased MMP activity and decreased inhibitor activity, which favor the degradation of the structural extracellular matrix (ECM) proteins, elastin and collagen. The mechanism that regulates degradation of elastin and collagen and remodeling of ECM in the aortic wall of AAAs is controlled by the balance between proteases including MMPs and protease inhibitors including tissue inhibitor of MMPs (TIMPs).^{8, 48} The detailed underlying mechanisms that regulate this key process still needs much more studies and involves non-coding RNAs. For these mechanistic studies human tissue samples and animal models, which will be further summarized below, have played crucial roles.

2. Animal models for AAA

Three different models of mouse AAA, elastase infusion, calcium chloride sponge, and Angiotensin II (AngII) infusion are widely used. While each model shows some features of human AAA, no single animal model has been shown to develop all clinical features of AAA. Therefore, many studies use all three mouse models for comprehensive understanding of human AAA.

Elastase Infusion

The infusion of elastase into the infrarenal segment of rat or mouse aortas has been a frequently used model of AAAs.^{26, 41, 49} Elastase infusion degrades elastin leading to the loss of structural integrity of the abdominal aorta. The procedure involves isolation of a segment of the abdominal aorta and incubation of porcine pancreatic elastase (PPE) for a few minutes before restoration of flow. This causes extensive destruction of the elastic lamellae and the infiltration of macrophages in the adventitial region within 2 weeks. Using this model, several microRNAs have been identified: miR-21, miR-24, and miR-29.^{50, 51}

Calcium Chloride model

Peri-aortic application of calcium chloride leads to structural disruption of the medial layer and inflammatory responses.^{48, 49} In this model, a gauze soaked in a calcium chloride solution is placed over the aorta. In this model, inflammation occurs on the luminal and medial layers of the aortas, leading to an increase in the aortic diameter >1.5 fold. This model induces luminal dilation without the mechanical effects that develops in the elastase-infused model.⁴⁸ MicroRNAs identified/studied using this model are miR-33 and miR-19a, miR-19b, miR-132, and miR-221.^{52, 53}

Angiotensin-II (AngII) infusion model

Infusion of AngII into low-density lipoprotein (LDL) receptor^{-/-} (LDLR^{-/-}), apolipoprotein E (ApoE)^{-/-} mice, or C57BL6 mice injected with adeno-associated virus (AAV) encoding for gain-of-function mutant of proprotein convertase subtilisin/kexin type 9 (PCSK9) (AAV-PCSK9) to overexpress PCSK9, which in turn knocks down LDLR in the liver, is another widely used approach to induce AAA or aortic dissection.^{6, 49, 54, 55} Delivery of a high-dose AngII at doses of 500 to 1000 ng/kg per minute, via subcutaneously implanted osmotic mini-pumps, leads to AAAs in the suprarenal region within the 28-day infusion period. Using this model, following miRNAs have been studied: miR-33, miR-145, miR-103a, miR-712, miR-205, miR-21, miR-24 and miR-29.^{50, 51, 56-58}

Studies from these animal models have provided key insights into the mechanisms of the initiation and progression of AAA.

3. Non-coding RNAs

It has been suggested that more than 97% of genome encodes for noncoding transcripts. It is likely that the differences in organism complexity may arise from the vast differences in noncoding transcripts between higher and lower organisms. Many of these noncoding transcripts are processed to generate small noncoding RNA such as miRNA or lncRNA. Although, microRNAs have gained widespread attention for their role in diverse physiological and pathophysiological processes, the knowledge about the functioning of lncRNAs still remains limited. Through their interaction with DNA, RNA and proteins, noncoding RNA have emerged as key regulators of gene expression under both physiological and pathological conditions. The table below summarizes the similarities and differences between miRNAs and lncRNAs.

3.1. MicroRNAs and AAA

MicroRNAs (miRs) are the best-characterized class of non-coding RNAs. These small single stranded RNAs are 18–21 nucleotides in length and inhibit gene expression by incorporation into a protein complex called RNA-induced silencing complex (RISC) and binding to mRNAs.⁶¹ One miR can bind to multiple mRNAs, frequently hundreds, so that miRs can broadly regulate gene expression patterns. Several miRs have been described over the last decade to regulate vascular remodeling.^{62, 63} Of interest, miRs can also be detected in the circulation and can serve as biomarkers for disease, including aneurysmal disease.⁶⁴ Multiple cell types including endothelial cells, smooth muscle cells, and immune cells in the vasculature play important role in development and progression of aneurysms. Many miRNAs have been shown to target these cell types, especially vascular smooth muscle cells, in the vasculature to alter the pathophysiology of AAA. Here we review these miRs associated with AAA for potential therapeutic translation based on pre-clinical evidence in animal models (Figure 1).

miR-29: One of the first described miRs that regulates aneurysm formation is miR-29.^{51, 65, 66} MiR-29 inhibits expression of several extracellular matrix proteins, as well as anti-apoptotic factors, thereby having an aneurysm-promoting effect on smooth muscle

cells. Inhibition of miR-29, using systemic application of anti-miRs in mouse models, blocks the formation and progression of aneurysms^{50, 65–68}, even on a long-term basis.⁶⁷ miR-29 in synergy with other members of miR-15 family have been implicated in regulating the vascular extracellular matrix.⁶⁸ Since systemic blockade of miR-29 could potentially induce fibrosis in other organs⁶⁹, suggesting a potential off-target effects of the anti-miRs as a long-term therapeutics. Therefore, a targeted, local delivery of anti-miRs seems to be worth considering for therapeutic development.

miR-21: Another very promising and one of the earliest described miRs to be involved in vascular remodeling is miR-21.⁵⁰ Overexpression of miR-21 leads to a decrease in PTEN and a subsequent survival of smooth muscle cells, which induces vessel wall stability in a mouse model of elastin degradation-induced aneurysms. Therapeutic overexpression or local delivery of functional miRs is challenging, since modifications that increase the lifetime or uptake by target cells limit functional incorporation of the miR in RISC.⁷⁰ Alternatively, viral vectors or nanoparticles might be used to deliver precursor microRNAs or mimics. Although some progress has been made with respect to endothelial or vascular targeting nanoparticles^{71–75}, so far no highly efficient and specific targeted delivery for AAA has been reported. This is the limiting factor for the development of a potential miR-21-based aneurysm therapy. Interestingly, Wang et al, showed that anti-miR-21 delivered as a drug-eluting stent successfully prevented in-stent myointimal hyperplasia in a humanized rat model.⁷⁶ While not tested in AAA, this result suggests the potential of stent-mediated local delivery of anti-miRs as a novel AAA therapy.

miR-24: miR-24 overexpression also shows promise as therapeutic strategy for aneurysmal disease.⁷⁷ The main effects that miR-24 seems to have is on limitation of macrophage survival, cytokine production and macrophage recruitment, thereby limiting vessel wall inflammation and extracellular matrix breakdown. Mechanistically, miR-24 limits the expression of CHI3L1, a secreted glycoprotein, which inhibits the production of the cytokines IL8 and CCL2 by smooth muscle cells and macrophages. For therapeutic development, the same limitations as described above for miR-21 delivery, also apply for miR-24.

miR-33: Another promising miR that has effects in both smooth muscle cells and macrophages is miR-33.⁵² This miR, known to inhibit the cholesterol transporter ABCA1, is a natural pro-inflammatory regulator in smooth muscle cells and macrophages.⁷⁸, which was recently described to inhibit smooth muscle cell proliferation and subsequent vein graft disease.⁷⁹ However, attenuation of miR-33 leads to a decrease of p38 and JNK signaling, as a result of increased ABCA1 expression, and inhibition of CaCl₂ and Angiotensin II-induced aneurysm formation in mice. Interestingly, miR-33 deletion also increases HDL levels as well as anti-inflammatory properties of HDL particles, attributed to effects in the liver. However, unexpectedly, genetic deletion of miR-33 or a systemic long-term treatment with anti-miR-33 changed metabolism leading to loss of anti-atherogenic effects, but macrophage-specific genetic deletion of miR-33 showed the desired anti-atherogenic effects.^{52, 80} These results demonstrate the importance of considering a tissue- or cell-type targeted delivery of anti-miR-33 as potential therapeutic approach.

miR-143/145: One of the first-described smooth muscle-enriched miR clusters⁸¹, miR-143/145, was also inversely associated with aneurysms in humans.⁸² Indeed, miR-143 and miR-145 induce a contractile, quiescent, and mature phenotype in smooth muscle cells and its local delivery may induce stabilization of aneurysms. Interestingly, both miR-143 and miR-145 have been described to be transported from one cell type to another. More specifically, miR-143/145 from endothelial cells can be taken up by smooth muscle cells⁸³, but also vice versa.⁸⁴ This mechanism may be exploited therapeutically, where one can envision that patient-derived vesicles or exosomes containing these miRs may be locally deposited to increase the stability of the vessel wall.

miR-181b: MiR-181b is increased in human aneurysms and targets TIMP3, an inhibitor of extracellular matrix breakdown, as well as elastin, and inhibition of miR-181b prevents aneurysm formation and even attenuates progression of existing aneurysmal disease.⁸⁵ The same study also reports a decrease in atherosclerosis after miR-181b inhibition. However, aging, one of the risk factors for aneurysms, reduces the expression of miR-181b and genetic deletion of miR-181b induces vascular stiffness and increases blood pressure.⁸⁶ Furthermore, miR-181b was also described to decrease inflammation and atherosclerosis (contradicting the study described above⁸⁵) by attenuating NFκB nuclear translocation⁸⁷, so that any strategy based on miR-181b inhibition to prevent aneurysm growth should carefully consider the risk of increasing atherosclerosis or vascular stiffness.

miR-195: Circulating levels of miR-195 in blood plasma inversely correlates with aortic diameter.⁶⁸ An independent study also suggested that plasma miR-195 measurements may be of prognostic value, as it described plasma miR-195 as differentially expressed in fast versus slow growth aneurysms value.⁸⁸ Another study found that miR-195 was also differentially expressed in human aneurysmal tissue.⁶⁴ Mechanistically, miR-195 targets extracellular matrix (ECM) proteins, but also matrix metalloproteinases (MMPs), so that therapeutic targeting of miR-195 is not likely to be straightforward.^{68, 89-92} Here, a more specific approach such as use of target-site blockers would be useful to inhibit the miRNA and a selected mRNA interaction.⁹³ For example, Target-site blockers could be designed to block the specific interaction between miR-195 and *elastin* without affecting the interaction of miR-195 with other mRNAs targets such as *MMP2/9*.

miR-205 and miR-712: Finally, miR-205 and its mouse-specific homolog miR-712, can be targeted to inhibit aneurysm formation in mice.⁵⁷ The mechanism by which miR-205 and miR-712 functions is similar to that described for miR-181 above, namely via the inhibition of TIMP3, so that miR-205 or miR-712 blockade leads to an increase in TIMP3 expression⁹⁴, a reduction in ECM degradation and subsequent stabilization of the vessel wall. Inhibition of these miRs also diminishes atherosclerosis, so that, in contrast to miR-181 inhibition strategies, therapeutic targeting of miR-205 in patients may be beneficial for both atherosclerosis and aneurysmal disease.^{57, 94}

3.2. lncRNAs and AAA

Long non-coding RNAs (lncRNAs) are RNAs that are more than 200 nucleotides in length that do not code for proteins. Based on their relative location in reference to the protein-coding genes, lncRNAs have been classified into five categories:⁹⁶ (Also see Table 1)

1. antisense RNA (overlaps a protein coding gene on the opposite strand),
2. lincRNA (long intergenic non-coding RNA),
3. sense overlapping RNA (contains a coding gene within an intronic sequence)
4. sense intronic RNA (located within intron of a coding/non-coding gene)
5. 3' overlapping ncRNA (located within the 3' UTR of larger gene).

Unlike the relatively straightforward mechanisms by which miRNAs regulate their target mRNAs through complimentary binding, much less is known about the role and the mechanism(s) by which lncRNAs regulate gene expression (Table 1). This is in part due to their diverse biogenesis⁹⁷, low abundance and poor conservation amongst species.^{98, 99} LncRNAs have been shown to be three-dimensional regulators of transcription and translation, for example by acting as molecular decoys and scaffolds, or through guiding ribonucleoprotein complexes to their targets *via* protein binding.^{100, 101} Furthermore, they can function as host genes for the transcription of miRNAs (in the nucleus) or miRNA sponges (in the cytoplasm).¹⁰² In addition, a mere act of lncRNA transcription process itself was known to regulate gene expression,¹⁰³ further adding complexity to the action mechanisms of lncRNAs. In contrast to miRNAs, lncRNA expression appears to be highly tissue-specific (approximately 80%)¹⁰⁴, which may become important in potential therapeutic and biomarker approaches as the risk for off-target effects and non-specific detection decreases.

To date, few studies have assessed the role of lncRNAs in the context of vascular disease development and progression. Examples include Meg3, which is a crucial regulator of endothelial aging and angiogenesis¹⁰⁵, as well as lincRNA-p21, which mediates SMC survival and macrophage activity in the atherosclerotic process.^{3, 106} At present, however, the only lncRNA specifically linked to AAA is H19.¹⁰⁷ Studies using other lncRNAs have only been observational and speculative for their potential role(s) in aneurysm development and progression. Some studies have revealed a crucial involvement for lncRNAs in mechanisms known to be of key importance to AAA development and expansion (*e.g.*, SMC proliferation and apoptosis, as well as aortic inflammation). However, only few reports cited here were able to provide *in vivo* evidence for a suspected molecular mechanism of action being mediated through lncRNAs. Future studies will have to answer the question whether other lncRNAs apart from H19 are as effective in mediating the fate of aortic aneurysms as miRNAs. Here, we briefly summarize recent lncRNA studies in the context of aortic aneurysms.

H19: Recently, the first lncRNA with a functional implication in AAA development and progression was reported. Li *et al.* identified the lncRNA H19 using RNA sequencing of AngII and PPE-induced AAAs in mice.¹⁰⁷ H19 was substantially increased in progressing

AAAs, and inhibition of H19 prevented aneurysm growth in both the AngII and PPE models of AAA in mice as well as in the LDLR-depleted Yucatan mini-pigs and human patient samples with AAAs. Increased H19 expression levels negatively correlated with SMC proliferation and migration, while positively correlating with apoptosis *in vivo*. Mechanistically, hypoxia-inducible factor 1-alpha (HIF1 α) was identified as the major downstream target of H19. Increased SMC apoptosis was mediated through cytoplasmic and nucleic interactions between H19 and HIF1 α . In the cytoplasm, H19-HIF1 α interaction increased p53 levels and activity. In the nucleus, H19 triggered transcription of HIF1 α *via* recruiting the SP1 transcription factor to the promoter region. These results suggest that H19 is a novel therapeutic target of AAA and that H19 LNA-GapmeR is a potential AAA therapy. This study further suggests the potential role of additional lncRNAs in AAA development and their application as diagnostic markers and therapeutics.

SENCR: Bell *et al.* used RNAseq of human coronary artery SMCs to discover the vascular cell-enriched lncRNA SENCER (Smooth muscle and endothelial cell-enriched migration/differentiation-associated long non-coding RNA).¹⁰⁸ SENCER is transcribed in antisense to the FLI1 gene, and active mainly in the cytoplasm. Inhibition of SENCER indicated only marginal cis-acting activity of SENCER on FLI1 as well as other nearby genes. However, RNA profiling in SMCs upon SENCER depletion resulted in decreased expression of Myocardin and various other SMC contractile genes and increases in pro-migratory marker genes. Functional assessment of SENCER inhibition utilizing scratch wound and Boyden chamber assays further showed SENCER as a powerful novel mediator of SMC proliferation and migration. However, its role in AAA is yet to be demonstrated.

SMILR: Another lncRNA with potential implications for AAA disease is the vascular remodeling associated transcript SMILR (Smooth Muscle Cell Enriched Long Non-Coding RNA).¹⁰⁹ SMILR was identified by RNAseq in SMCs upon interleukin-1 α (IL1 α) and platelet derived growth factor (PDGF) stimulation. Knockdown of SMILR significantly reduced cell proliferation rates. Importantly, its expression is increased in unstable atherosclerotic plaques and plasma from patients with elevated C-reactive protein levels.

Lnc-Ang362: The LncRNA362 was identified in rat aortic SMCs upon AngII stimulation, an important factor in human and experimental AAA development, as well as in atherosclerosis and hypertension.¹¹⁰ LncRNA362 was identified as a host transcript for miRs-221/-222, which are both well-established drivers of SMC proliferation in vascular diseases.¹¹¹ Its role in AAA is unknown yet.

HOTAIR: HOTAIR, a well-studied lncRNA in the cancer field, was shown to be decreased in sporadic thoracic abdominal aorta (TAA) specimens, and to negatively correlate with the size of aortic diameter.¹¹² Depletion of HOTAIR was not only capable of reducing proliferation and triggering apoptosis rates, but also to limit the expression of the collagen isoforms type I and III. However, its role in AAA in animal studies is yet to be demonstrated.

Pi15: Falak *et al*¹¹³ found the protease inhibitor 15 (Pi15), as well as another non-validated lncRNA, to be implicated in disruptions of elastic laminae in rat aneurysms. Whether these

lncRNAs play a role in human AAA development and mouse models of AAA needs to be studied.

lnc-HLTF-5: Li *et al.* carried out a microarray-based RNA profiling study using human TAA tissue samples and identified 147 lncRNAs that were differentially regulated compared to the controls.¹¹⁴ They further identified that lnc-HLTF-5 correlates with an increased ascending aortic diameter, AAA-relevant MMP9 expression, and arterial hypertension.¹¹⁴ Interestingly they found that more than half of these deregulated lncRNAs in TAA were sense-overlapping RNAs, containing protein-coding genes within their intronic sequences. These findings implicate the potential importance of these lncRNAs to their corresponding protein-coding genes in aneurysm development.

HIF1 α -AS1: Another study, using sera from patients with thoraco-abdominal aortic aneurysm (TAAA) patients.¹¹⁵, identified the lncRNA HIF1 α antisense RNA 1 (HIF1 α -AS1) to be increased. Inhibition of HIF1 α -AS1 by siRNA decreased the expression of the caspase-3 and -8, while upregulating the levels of Bcl2. Furthermore, inhibition of HIF1 α -AS1 limited palmitic acid-induced SMC apoptosis.¹¹⁶ However, the role of HIF1 α -AS1 in animal models of AAA needs to be established.

AK056155: The lncRNA AK056155 has been reported for being increased in subjects with a subform of TAA, the Loeys-Dietz syndrome (LDS). Interestingly, expression of AK056155 correlated with AKT/PI3K/TGF- β signaling, which are the known key factors contributing to aneurysm development and disease progression.¹¹⁷ They showed that AK056155 expression is stimulated by TGF- β 1 in HUVECs. Interestingly, in combination with TGF- β 1 stimulation, AK056155 expression can be augmented upon treatment with the PI3K inhibitor LY294002 or the AKT inhibitor GDC-0068. Their role in AAA development in human tissues and animal models needs to be established.

Linc00540: Finally, a recent meta-analysis of all available AAA-specific genome-wide association studies (GWAS) identified linc00540 as a novel risk locus for AAA.¹¹⁸ Expression data and functional studies in disease relevant cell subtypes as well as *in vivo* models are currently lacking for this lncRNA. This is further complicated by the fact that no mouse homologue for this lncRNA exists. Identifying its mouse homologues would be helpful in further studying the role of this lncRNA in AAA development.

4. Circular RNAs in aneurysms

Like lncRNAs, circular RNA transcripts (circRNAs) have been a recent addition to the functionally relevant non-coding RNAs in our genomic landscape. Upon their discovery in the 1990s, they have long been disregarded, due in part to a limitation in their detection method. Recently, this was changed through the support of novel bioinformatics in combination with deep RNA sequencing approaches.¹¹⁹ Recent studies suggest thousands of endogenous circRNAs in mammalian cells, with some of them being highly abundant and evolutionarily conserved. CircRNAs have been shown to mediate miRNA function (e.g., *via* sponging), and to control events important in transcription (e.g., RNA folding, endonuclease protection).¹²⁰ CircRNAs can be produced by the direct ligation of 5' and 3' ends of linear

RNAs, by “back-splicing”, in which a downstream 5’ splice site (donor) is linked to an upstream 3’ site (acceptor), or as intermediates in RNA processing reactions.¹¹⁹ Data on circRNAs in aneurysm disease are scarce, but emerging studies have shown evidence of abnormal expression of circRNAs in the disease context. However, the functional role(s) of these circRNAs in AAA development in animal models and their therapeutic potential is yet to be established.

circ000595:

Zheng and colleagues have screened circRNAs in tissue specimens from AAA patients and found increased expression of *circ000595* in diseased specimens. A similar pattern was found in hypoxic aortic SMCs, in which knockdown of *circ000595* reduced apoptosis. They further showed that miR-19a might serve as a potential downstream target of *circ000595*.¹²¹

circRNA-101238:

Another study utilized a targeted circRNA array to explore differentially expressed circRNAs in human tissue specimens from TAA patients undergoing surgery. Here, Zou *et al.* identified *circRNA-101238* not only as being deregulated with the disease, but also to potentially affect miR-320a expression as well as MMP9 levels.¹²²

circ0021001:

One recent effort identified the *circ0021001* in peripheral blood of patients with intracranial aneurysms without showing its potential contribution to aneurysmal expansion.¹²³

5. Summary and perspectives

Emerging studies have clearly established that non-coding RNAs such as microRNAs, lncRNAs and circRNAs are involved in AAA development and progression. While the roles of miRNAs and lncRNAs have been established, the functional role of circRNAs in AAA is yet to be elucidated. The role and therapeutic potential of miRNAs (miR-21, 24, 29, 33, 143/145, 181b, 195, 205 and 712) have been demonstrated in various animal models of AAA with some cross-validations in human aneurysmal tissue samples. In contrast, the only lncRNA that has clearly demonstrated its role in AAA is H19, although several lncRNAs (SENCR, SMILR, lncAng362, HOTAIR, Pi5, lnc-HLTF5, Hif1 α -AS1, AK056155 and lnc00540) have shown their potential roles in some pathophysiological processes of AAA development. Therefore, there is a tremendous need for additional studies for miRNAs, while the need to study the roles and mechanisms of lncRNAs and circRNAs in AAA are wide open. This information will lead to better understanding of mechanisms of AAA and development of therapies and diagnostic approaches.

To understand the biological and pathophysiological mechanisms by which these non-coding RNAs regulate the AAA development and progression, it is essential to identify and validate additional miRNAs, lncRNAs and circRNAs that are abnormally expressed in the diseased human tissues and relevant animal models. Given the tissue-specific expression of lncRNAs and potentially other non-coding RNAs, it is important to consider collecting cell-type specific RNA samples to determine the cell-specific expression of non-coding RNAs under

various disease conditions. For example, whenever possible, collecting RNAs from key cell types such as endothelial cells, smooth muscle cells and immune cells would be helpful to obtain specific insights into the cell-type specific changes in the noncoding RNAs in AAA development. A combination of cell sorting approaches and *in vitro* experiments would be further helpful.

Once their expression is validated, the molecular mechanisms of these lncRNAs should be tested in different cell types *in vitro* and *in vivo*. The most critical and difficult part of establishing the molecular mechanisms of noncoding RNAs is to identify the genes that are directly regulated by them. Although, several *in silico* miRNA target gene prediction programs¹²⁴ (TargetScan, miR-Walk, miR-TarBase, *etc.*) are available to help identifying their potential gene targets, it is nevertheless a daunting and challenging task to validate their gene targets in the specific cell types and tissues. Given the diverse and complex three-dimensional regulatory mechanisms of lncRNAs, there are no simple target gene prediction algorithms available. Initial efforts should be aimed at studying the genes which are either embedded, overlapping, or in close proximity to the genomic location the lncRNAs of interest. Although the computational methods for detection and downstream analysis of circRNAs are emerging, more sophisticated tools are needed to identify and study the circRNAs involved in AAAs.¹²⁵

To study the functional role of these noncoding RNAs in AAA, it is highly advisable to use both gain-of-function and loss-of-function approaches in both *in vitro* and *in vivo* models (Table 1). For miRNAs, anti-miRs and miR-mimics, are popular choices, while GapmeRs and CRISPR-Cas9 mediated genome editing are gaining popularity to study the role of lncRNAs in addition to conventional genetic engineering approaches. These tools may also be used for potential therapeutic approaches.

The critical challenges in using these tools for manipulating miRNA and lncRNA levels are specificity, safety and methods of delivery. Since miRNAs can target numerous genes simultaneously in various tissues and cells, it is important to minimize potential off-gene target effects and off-tissue effects by considering the use of targeted-delivery methods and more gene-specific miRNA inhibitors such as target site blockers (TSBs). Currently, these miRNA manipulators are delivered *in vivo* either systemically or locally. Systemic delivery of miRNA inhibitors or mimics are carried out using either native or modified oligomers such as locked nucleic acid or 2'-O-methyl nucleotide derivatives. Moreover, these oligomers are typically delivered systemically in saline, but can also be delivered using carriers such as lipid nanoparticles^{126–129}, gold nanoparticles^{130–132}, exosomes^{133–140} with or without additional surface coatings for targeted delivery. For example, naked anti-miRs in saline can be injected systemically to inhibit AAA in mouse models.⁵⁷ In contrast, lipid nanoparticles coated with VCAM1-targeting peptide carrying anti-miR-712 was used to deliver the payload to the inflamed endothelium to inhibit atherosclerosis in mice with minimum off-target effects.¹²⁸ Although this approach has not been tested in AAA, it would be worthwhile to test its efficacy and reduced off target effects in the murine AAA models. Another method to deliver anti-miRs is by using drug-eluting stents for local delivery to arteries.⁷⁶ This approach could be used to deliver similar inhibitors of miRNAs or lncRNAs using coated endovascular devices locally to the AAA regions to minimize off target effects

and enhanced therapeutic effects. Furthermore, using the local delivery approach with a combination strategy targeting multiple miRNAs could also be a promising therapeutic option. For example, treatment with miR-24 mimic and anti-miR-21 cocktail, or anti-miR-29 and anti-miR-181b cocktail may have more potent therapeutic effects on AAA than targeting single miRNA alone. However, it is important to determine whether the potential benefits of targeting multiple miRNAs using a cocktail of anti-miRs outweigh the potentially increased off target effects.

In addition to the therapeutic targeting of miRNAs and lncRNAs, they may serve as biomarkers to monitor progression of AAA in patients. Several studies have identified miRNAs for their diagnostic potential for existing aneurysms.^{17, 64, 77, 141} Since ultrasound imaging is widely available, economical, sensitive and effective in identifying aortic aneurysms, using miRNAs merely as a biomarker may not add additional significant prognostic value. However, a miRNA-based biomarker may be quite useful to predict aneurysm expansion rates and the risk of future ruptures. It may offer a significant benefit in the stratification and monitoring of patients with small, but growing AAAs. In this regard, Wanhainen and colleagues have identified a panel of 20 miRNAs that are expressed differently in patients with either fast- or slow-growing AAAs.⁸⁸ Although the number of individuals (169 AAA cases and 48 age-matched controls) included into this study was relatively small, the results from this explorative analysis encourage us to carry out much larger studies to identify ad classes of non-coding RNAs as prognostic biomarkers for AAA progression.

In summary, many noncoding RNAs have been identified as critical regulators of AAA and the list is growing, but more efforts are needed to identify additional players and demonstrate their action mechanisms and their diagnostic and therapeutic potential.

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Nonstandard Abbreviation and Acronyms:

AAA	Abdominal aortic aneurysm
AAV	adeno-associated virus
ABCA1	ATP Binding Cassette Subfamily A Member 1
AngII	Angiotensin II

AS	antisense
Cas9	CRISPR associated protein 9
CHI3L1	Chitinase 3 Like 1
circRNA	circular RNA transcript
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats
DGCR8	DiGeorge Syndrome Critical Region Gene 8
EC	Endothelial cells
ECM	extracellular matrix
FB	Fibroblasts
HC	Hepatocytes
HIF1α	hypoxia-inducible factor 1 alpha
HLTF5	Helicase Like Transcription Factor 5
HOTAIR	HOX Transcript Antisense RNA
IL8	Interleukin 8
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LDS	Loeys-Dietz syndrome
LincRNA	long intergenic non-coding RNA
Lnc-RNA	long-non-coding RNA
Meg3	Maternally Expressed 3
MMP	Matrix metalloproteinase
Mph	Macrophage
NFκB	Nuclear Factor Kappa B
PCSK9	proprotein convertase subtilisin/kexin type 9
PDGF	platelet derived growth factor
Pi15	protease inhibitor 15
PPE	porcine pancreatic elastase
PTEN	Phosphatase and tensin homolog
RISC	RNA-induced silencing complex

RNAseq	RNA sequencing
SENCR	Smooth muscle and endothelial cell-enriched migration/ differentiation-associated long non-coding RNA
SMC	Smooth muscle cells
SMILR	Smooth Muscle Cell Enriched Long Non-Coding RNA
TAA	Thoracic aortic aneurysms
TIMP	tissue inhibitor of matrix metalloproteinase
TSB	target site blocker
UTR	untranslated region

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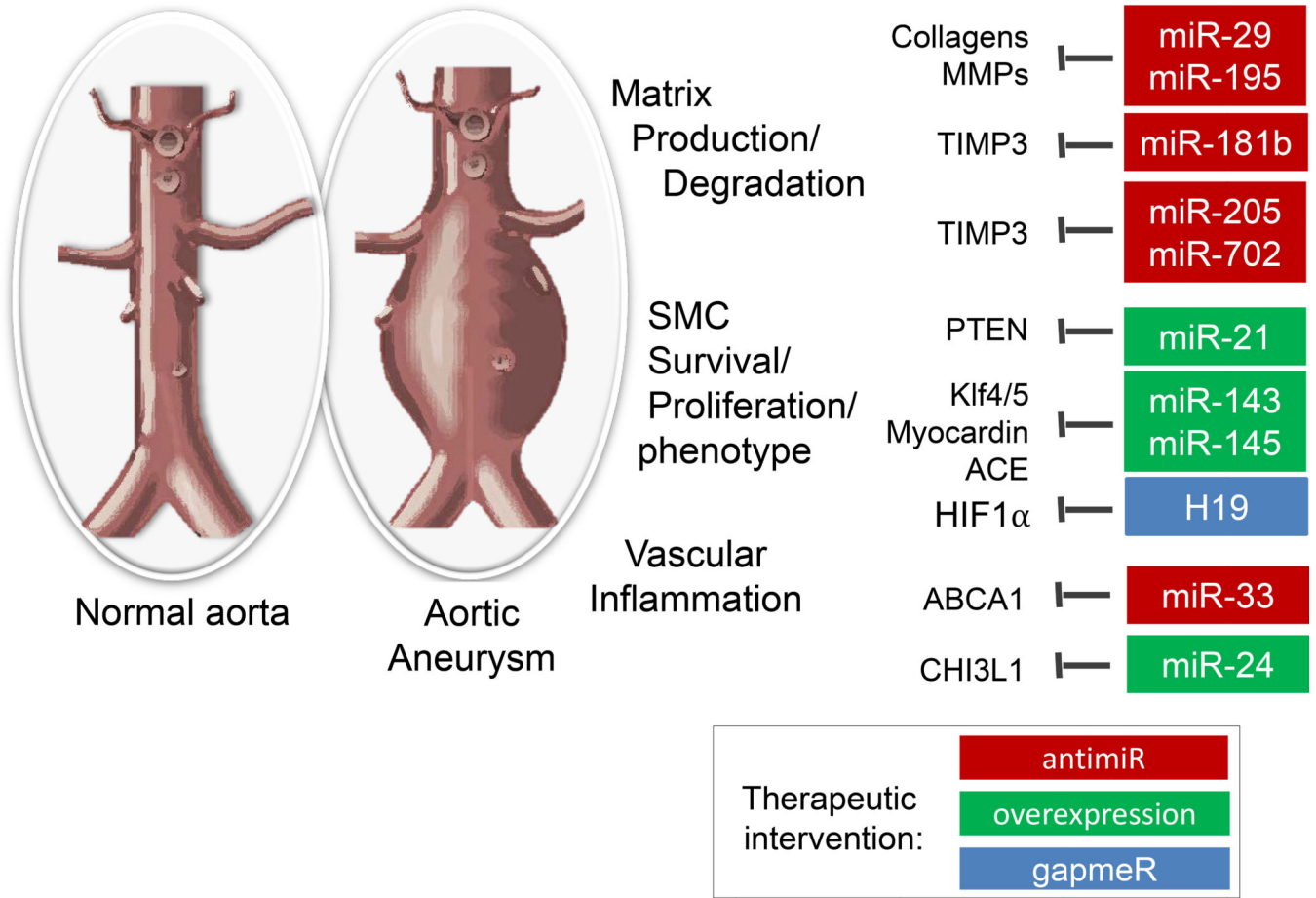


Figure 1: MicroRNAs regulating aortic aneurysm formation or progression. Selected targets that were shown to contribute to the effects of the miRs in aneurysm formation are shown. Red labelled miRs promote aneurysm formation and may be targets by antimicroRNAs for therapeutic interventions, whereas green labelled miRs inhibit aneurysm formation.

Table 1.

Similarities and differences between miRNAs and lncRNAs

Characteristics	miRNA	lncRNAs
Size	Short (18 to 21 nucleotides)	>200 nucleotides
Primary mode of action	Negatively regulating the expression by complementary binding to target mRNAs and causing their degradation or inhibiting translation	Highly diverse, regulate gene expression by various mechanisms. Lnc-RNAs may work as <ol style="list-style-type: none"> 1. <i>Signaling cues</i> 2. <i>Guides</i> 3. <i>Decoys</i> 4. <i>Scaffolds</i> 5. <i>miRNA sponges</i>
Genomic location	- <i>Exonic</i> - <i>Intergenic</i> (between genes) - <i>Intronic</i> (embedded in a gene)	- <i>Antisense RNA</i> - <i>LincRNA</i> - <i>Sense overlapping RNA</i> - <i>Sense intronic RNA</i> - <i>3' overlapping ncRNA</i>
Secondary structure	Usually the precursor miRNA forms a hairpin structure	Forms simple to complex secondary structures 59, 60
Post-transcriptional processing	Typically produced as pri-miRNAs, which are processed by miRNA-processing enzymes (Drosha, DGCR8) and Dicer to generate pre-miR and mature miRNAs	Undergo post-transcriptional processing like mRNAs, i.e. 5' capping, polyadenylation, and splicing
Computational gene target prediction	Relatively easy. Multiple bioinformatics tools/ prediction algorithms available that help to predict target genes	Difficult to predict. Not available.
Conservation between species	Relatively well conserved nucleotide sequences across species, although some species-specific miRNAs exist	Poorly conserved between species at the level of primary nucleotide sequences, but may have conserved secondary structures
Experimental gain-of-function strategies	miRNA mimics, over-expression plasmids, transgenic overexpression	over-expression plasmids, transgenic overexpression
Experimental loss-of-function strategies	Anti-miRs, antagomiRs, and morpholinos, genetic deletions	GapmeRs, genetic deletions or mutations

Table 2.

Summary of microRNAs regulating pathophysiology of AAAs

miRNA	Cell types	Potential biomarker	Animal model	miRNA modulation method	Results	Target genes	References
miR-29	SMC EC FB	No	Elastase, AngII, Marfan	Viral OE Anti-miR	Inhibition reduces AAA	collagens elastin	62, 65, 95
miR-21	SMC EC FB	No	Elastase, AngII	Viral OE Anti-miR	OE reduces AAA	PTEN	50
miR-24	SMC EC MPh	Yes	Elastase, AngII	Viral OE Inhibition	OE reduces AAA	CHI3L1	77
miR-33	SMC MPh HC	No	AngII, CCl2	Knockout	Inhibition reduces AAA	ABCA1	78,80
miR-143 miR-145	SMC FB EC	No*	AngII	Viral OE Knockout	OE induces SMC differentiation	KLF4 PHACTR4	83
miR-181b	MPh EC	No	AngII	Anti-miR	Conflicting results	TIMP3 elastin	85-87
miR-195	SMC	Yes	AngII	AntagomiR	Inhibition reduces AAA	collagen proteoglycan elastin SMAD3	68, 89-91
miR-205 miR-712	EC SMC	No	AngII	Anti-miR	Inhibition reduces AAA	TIMP3	57

SMC: Smooth muscle cells, EC: Endothelial cells, FB: Fibroblasts, MPh: Macrophages, HC: Hepatocytes, AngII: Angiotensin II, OE: Overexpression. *miR-143/145 may be useful biomarkers for vascular disease, but not specific for aneurysms.