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SPOTLIGHT REVIEW

Non-coding RNAs: key regulators of smooth muscle cell fate in vascular disease

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Abstract

The vascular smooth muscle cell (SMC) is one of the most plastic cells in the body. Understanding how non-coding RNAs (ncRNAs) regulate SMC cell-fate decision making in the vasculature has significantly enhanced our understanding of disease development, and opened up exciting new avenues for potential therapeutic applications. Recent studies on SMC physiology have in addition challenged our traditional view on their role and contribution to vascular disease, mainly in the setting of atherosclerosis as well as aneurysm disease, and restenosis after angioplasties. The impact of SMC behaviour on vascular disease is now recognized to be context dependent; SMC proliferation and migration can be harmful or beneficial, whereas their apoptosis, senescence, and switching into a more macrophage-like phenotype can promote inflammation and disease progression. This is in particular true for atherosclerosis-related diseases, where proliferation of SMCs was believed to promote lesion formation, but may also prevent plaque rupture by stabilizing the fibrous cap. Based on newer findings of genetic lineage tracing studies, it was revealed that SMC phenotypic switching can result in less-differentiated forms that lack classical SMC markers while exhibiting functions more related to macrophage-like cells. This switching can directly promote atherogenesis. The aim of this current review is to summarize and discuss how ncRNAs (mainly microRNAs and long ncRNAs) are involved in SMC plasticity, and how they directly affect vascular disease development and progression. Finally, we want to critically assess where potential future therapies could be useful to influence the burden of vascular diseases.

Keywords Non-coding RNAs • MicroRNAs • Long non-coding RNAs • Smooth muscle cells • Vascular disease • Atherosclerosis • Aortic aneurysms • Restenosis

This article is part of the Spotlight Issue on Novel concepts for the role of smooth muscle cells in vascular disease.

1. Non-coding RNAs

New technologies such as next-generation deep sequencing have revealed that the vast majority of our genome is transcribed into RNAs. Interestingly, <2% of the human genome codes for proteins,[1] leaving many RNAs without coding potential. These RNAs are referred to as non-coding RNAs (ncRNAs). Historically DNA being transcribed into ncRNA was considered 'junk DNA'. This view has shifted quite dramatically with increasing evidence that suggests a crucial mediating role for ncRNA on the molecular level. Furthermore, the amount of ncRNAs in an organism correlates with its complexity, which lets scientists assume a key influence of ncRNAs on the development and organization of higher developed animals.[2]

NcRNAs are divided into two subclasses according to a relatively broad size threshold. NcRNAs longer than 200 nucleotides (nt) are termed long non-coding RNAs (lncRNAs), while shorter ncRNAs (<200 nt) are called small or short ncRNAs. Small ncRNAs can range from very few to 200 nt, while lncRNAs have a size up to several kilobases.[3] MicroRNAs (miRNAs) with a size of ~20 nt have certainly received most of the attention over the last two decades, in particular for their role in tempering gene expression. Several clinical trials have been initiated that utilize miRNA inhibitors in various diseases, mainly targeting kidney and liver pathologies, but also cancer.[4] Currently no miRNA-based trial in the cardiovascular field has been initiated, but several candidates are being pre-clinically explored.[5]

MiRNAs are defined as single-stranded, endogenously expressed ncRNAs that regulate gene expression on the post-transcriptional level. Genes encoding for miRNAs are located throughout the genome, with a large proportion being transcribed within clusters.[2] miRNA biogenesis is comprised of a multistep process that involves transcription, nuclear

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processing, export into the cytoplasm, and maturation before reaching the stage of a functional molecule.[6]

Unlike miRNAs, the mechanisms of action for lncRNAs thus far are only poorly understood. Importantly, lncRNAs can regulate gene expression at multiple levels in the cell, either in the nucleus or the cytoplasm, through complex molecular mechanisms (summarized in *Figure 1*). Furthermore, lncRNAs are unlike miRNAs less well-conserved across different species when it comes to the nucleotide sequence, even though lncRNA transcripts can be found in almost all living cells.[7] As one can imagine, this substantially limits experimental *in vitro* and *in vivo* models for investigation of lncRNA functionality, even though a difference in nucleotide composition can still lead to the same 3D geometrical pattern and genomic or epigenetic function.[8,9] LncRNAs appear as crucial epigenetic regulators of almost every cellular process, and expression of these molecules is distinctly mediated under physiological and pathological conditions, including cardiovascular disease (CVD). Intriguingly, the vast majority of disease-associated single nucleotide polymorphisms (SNPs) derived from recent genome wide association studies appear in non-coding regions of the human genome.[9–11]

Based on the large number of existing lncRNAs, it becomes evident that only a minority have already been described and investigated thoroughly. However, the ones that have been functionally characterized have proven to regulate gene expression at the transcriptional and post-transcriptional level through structural and sequence-specific manners.[12,13] One important feature regarding their true classification as a non-coding transcript is obviously the exclusion of their potential to code for protein. This becomes increasingly evident, as it has indeed been recently reported that some previously considered lncRNAs actually do code for (micro)-peptides.[14] Whether these peptides are really functional and have any relevance for the molecular signalling control of lncRNAs remains to be determined.

In addition to these classic linear RNA transcripts, it appears that there is also a back-splicing variant of expression which gives rise to circular RNAs (circRNAs). CircRNAs lack a 5′ cap and 3′ tail, and they are processed as covalently closed continuous loops.[15] Interestingly, circRNAs are stably conserved across species and appear relatively tissue-specific compared with other ncRNAs.[16] Due to their stability in circulation and tissue specificity, circRNAs might in particular become interesting as future biomarkers in CVD.[17,18]

2. SMCs and vascular disease development

As this current article is part of a series of reviews for a *Spotlight Issue* on 'Novel concepts for the role of smooth muscle cells (SMCs) in vascular

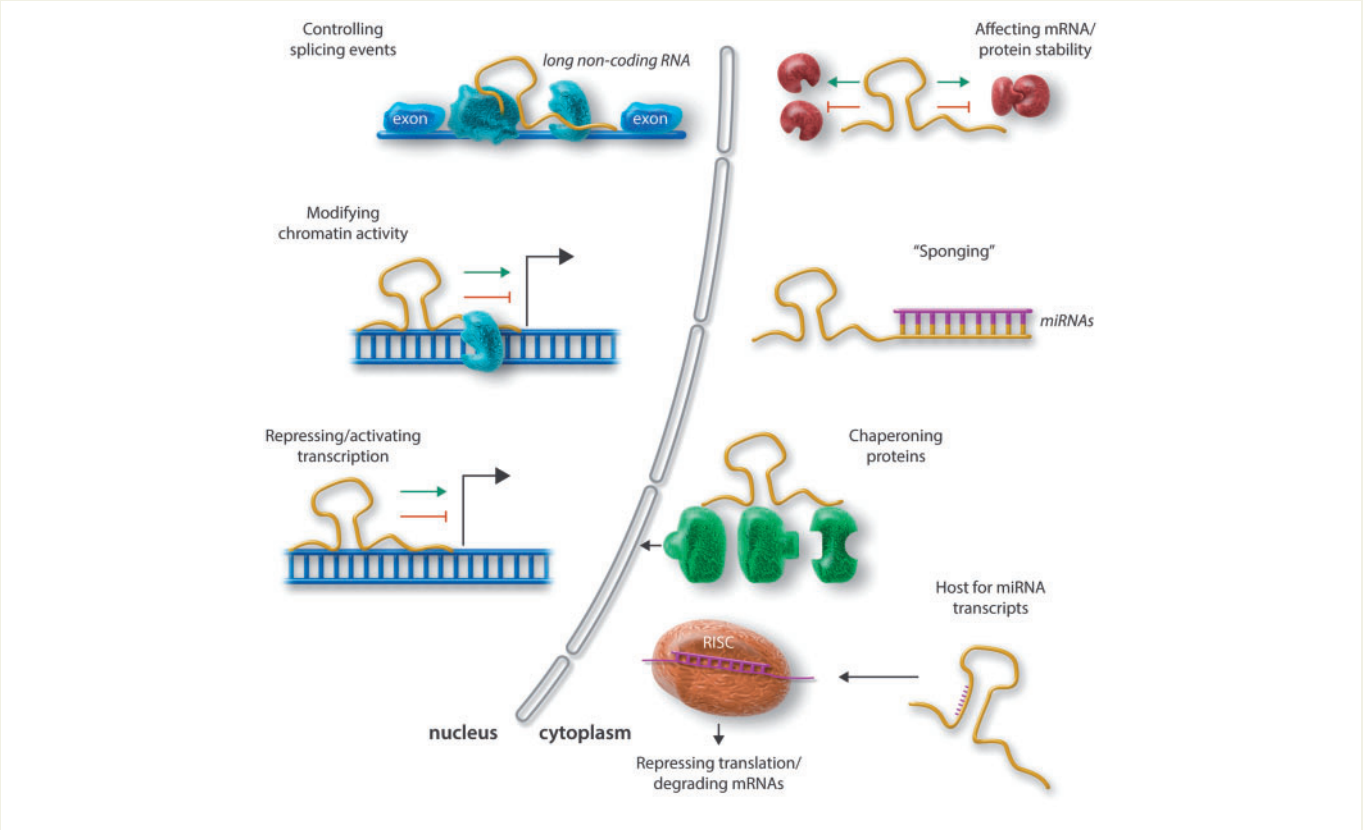

Figure 1 Schematic overview of prominent mechanisms of action for ncRNAs (miRNAs and lncRNAs). lncRNAs can act within the nucleus, where they exert regulation on the transcriptional level, as well as in the cytoplasm (mainly affecting mRNA/miRNA and protein stability). Within *the nucleus* lncRNAs have been shown to be important regulators for splicing events, to modify chromatin activity, and to repress or activate transcription of genes. Within *the cytoplasm*, lncRNAs mediate mRNA and protein stability and thus translational activity; they can act as sponges for miRNAs, meaning that they capture them before they get uploaded into the RISC where they suppress target gene expression. Furthermore, lncRNAs can serve as chaperons (scaffolds) for proteins (e.g. transcription factors) trying to enter the nucleus. Mature, single-stranded miRNAs can originate from lncRNA transcripts and act upon loading into the RISC *via* repressing translation or induction of mRNA degradation.

disease', the physiology and contribution to pathology surrounding SMCs in the vasculature will only be briefly discussed. Under physiological—somewhat un-diseased conditions in healthy arteries—SMCs express a range of SMC markers, including SMC-α actin (ACTA2), smoothelin, SMC myosin heavy chain (MYH11), and transgelin (TGLN), among others. SMCs in culture—as well as during disease development and progression—reduce expression of these markers, and at least *in vitro* become prone for mechanisms that involve proliferation, migration, calcification, and production of various extracellular matrix (ECM) proteins and cytokines.[19] In the following paragraphs, several SMC-related mechanisms, their contribution to vascular disease, and the regulatory role specific ncRNAs play in this context, will be discussed (summarized in *Tables 1* and *2*).

3. SMC de- and trans-differentiation

Several ncRNAs have been shown to control the varying mechanisms, which govern SMC plasticity and fate (*Figure 2*). A strong indicator for the importance of miRNAs in this context, are studies being performed in mice with a genetic deletion of Dicer.[20–22] The enzyme Dicer plays a central role in miRNA biogenesis, as it has the ability to cleave pre-miRNA (as well as other double-stranded RNAs) into short double-stranded RNA fragments (of about 18–25 base pairs), facilitating the activation of the RNA-induced silencing complex (RISC), in which mature miRNAs act upon their target mRNAs.[6] The consequences of deficiency in SMCs are impaired vascular development and embryonic lethality.[23,24] Recent data from Zahedi *et al.*[25] revealed that conditional deletion of Dicer in SMCs of *Apoe*$^{-/-}$ mice reduces their proliferation rate. The SMC-specific deletion mediated a network of anti-proliferative miRNAs and targets in wire-injured mouse carotid arteries. Albinsson *et al.*[23,26] further revealed that Dicer-deficient mice displayed significant blood pressure reductions with limited SMC contractile functionality. Interestingly, the same researchers were able to show that miR-145 can to some extent rescue the contractile phenotype of Dicer-deficient SMCs.

Phenotypic switching of SMCs has long been considered as an important contributing factor to vascular disease development. Recent studies have discovered that SMCs that undergo phenotypic switching resemble a more macrophage-like phenotype, which is mainly based on the markers being expressed on their surface.[27] The stem cell and differentiation mediator Kruppel-like factor 4 (KLF4) has been shown previously to be required for phenotypic switching of cultured SMCs in response to platelet-derived growth factor BB (PDGF-BB),[28,29] oxidized phospholipids,[30,31] and interleukin (IL)-1β,[32] while repressing SMC marker genes to limit the activation of myocardin (MYOCD)-responsive genes.[29,33] One of the first studies investigating the role of miRNAs in SMC differentiation led to the discovery of the miR-1/miR-133a family in this context. In particular miR-1, which becomes activated through MYOCD, destabilizes the cytoskeleton in contractile state-bound SMCs.[34] miR-24 and miR-29a are additional miRNAs that get induced through MYOCD. Both have been investigated for their regulatory role in migrative and proliferative processes in SMCs through facilitating the expression of PDGFRβ levels.[35] miR-24 has further been shown to mediate Tribbles-like protein 3 upon PDGF-BB stimulation, which through SMURF1 and reduced SMAD1 enhances SMC differentiation.[36,37]

Regarding the specific role of KLF4 in SMCs *in vivo*, it could be shown that this transcription factor is strongly associated with an augmented phenotypic transitioning in response to carotid ligation injury.[38] More

Table 1 miRNAs in SMC fate and functionality

miRNA (cluster)	Regulation	Target(s)	Role and function in SMC dynamics	References
miR-1/-133a	↑	KLF4, SRF, CCND, RUNX2	Differentiation, migration, proliferation	33,42–45,84
miR-21	↑	PTEN, PDCD4, BCL2	Proliferation, apoptosis	86–90
miR-23b/-24/-27b	↑↓	TLP3, CHI3L1, FOXO4,	Proliferation, differentiation, cytokine production & release	34–35,91,92
miR-26a	↓	SMAD1, SMAD4	Differentiation	93
miR-29a/b/c	↑↓	COL1A1, COL3A1, COL5A1, ELN, MMP2, MMP9, PTEN	ECM production, proliferation	34,116–120
miR-34a	↑↓	SIRT1, NOTCH1	Proliferation, migration	97
miR-130a	↑	MEOX1	Proliferation, migration	99
miR-138	↑↓	SIRT1	Proliferation, migration	98
miR-143/-145	↑↓	KLF4/5, MYOCD, ELK1, SRF	Differentiation, proliferation	23,48–56
miR-146a	↑	KLF4/5	Differentiation, proliferation	57
miR-155	↑	SMAD2, BCL6, CTLA4	Differentiation, inflammation	58–60
miR-195	↓	ELN, MMP2	ECM production, remodelling	118
miR-204	↓	PTPN11	Proliferation	100
miR-205	↓	SMAD1, RUNX2	Proliferation, calcification	95
miR-206	↓	ARF6, SLC8A1	Differentiation	94
miR-210	↓	APC	Apoptosis	125
miR-221/-222	↑	CDKN1B, CDKN1C	Proliferation, migration, and anti-apoptotic effects	101,102
miR-424/-322	↓	CCND1, CALU, STIM1	Proliferation	103
miR-663	↓	JUNB	Proliferation	104

miRNAs and their regulation (up- or down) under vascular disease relevant conditions and stimuli. Further depicted in the table are the direct downstream targets of specific miRNAs, their main effect in SMC dynamics, and the respective references. Abbreviations of target genes are explained in the running text.

Table 2 lncRNAs in SMC fate and functionality

lncRNA	Regulation	Related targets and factors	Role and function in SMC dynamics	References
ANRIL	↓↑	CDKN2A, CDKN2B, DAB2IP, LRP1, LRPR, CNTN3	Proliferation	10, 109–112
RNCR3	↑	KLF2, miR-185	Proliferation	108
H19	↑	miR-675, IGF2, let7, p53, NOTCH1, miR-106a, MYC, miR-19b, SOX6	Differentiation and proliferation	64–76
lnc-362	↑	miR-221/222	Host-gene for miRNAs, proliferation	105
SENCR	↑	FLI1	Migration and proliferation	77,78
Lnc-GAS5	↓	ANXA2	Differentiation, proliferation, and migration	106,107
Lnc-MEG3	–	p53, MMP2, IFN-γ	Proliferation, migration, and apoptosis	137,138
MYOSLID	–	MYOCD, MRTF-A, TGF-β (SMAD)	Differentiation and proliferation	79
HIF1α-AS1	↑	HIF1A, BRG1	Apoptosis and proliferation	45,135,136
HAS-AS1	↑	HAS2	ECM remodelling and deposition	122
lincRNA-p21	↑	p53, p300	Apoptosis	133,134

lncRNAs and their regulation (up- or down; if applicable) under vascular disease relevant conditions and stimuli. Further depicted in the table are the associated up- and downstream targets, their main effect in SMC dynamics, and the respective references. Abbreviations of lncRNAs and associated mediators/genes are explained in the running text.

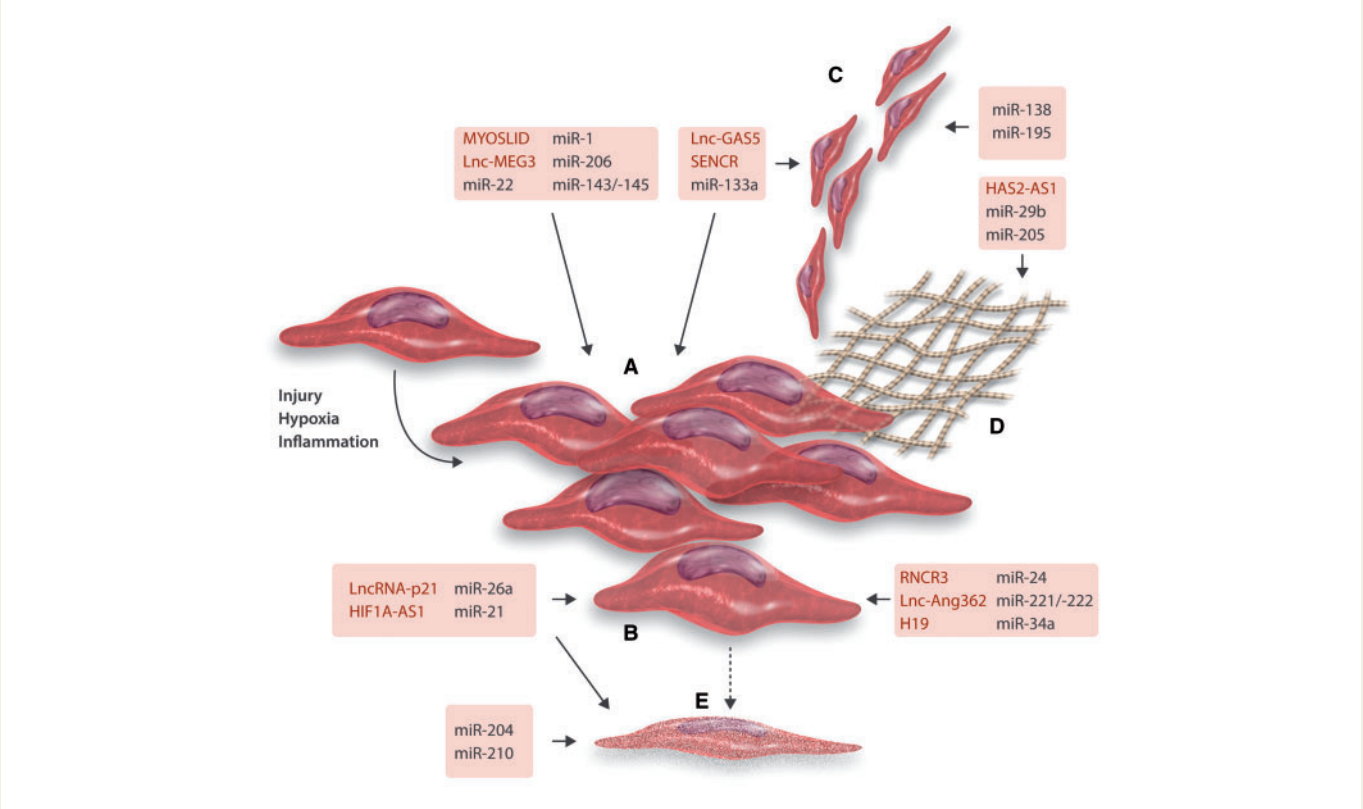

Figure 2 Schematic overview of miRNAs and lncRNAs involved in SMC fate decisions. Contractile SMCs upon different stimuli (injury, hypoxia, inflammation, etc.) undergo phenotypic switching (*A*) to a more synthetic, proliferative cellular subtype. Proliferation (*B*), migration (*C*), ECM production (*D*), and cell death (apoptosis) (*E*) of SMCs has been shown to be regulated by several miRNAs as well as more recently lncRNAs. For details on specific mechanisms of action and targets of all displayed ncRNAs, please refer to the main text.

recent studies from the Owens laboratory have shown that conditional SMC-specific genetic deletion of KLF4 does not necessarily prevent SMC phenotypic switching, but substantially limits the size of atherosclerotic lesions in parallel with increased fibrous cap and plaque stability.[27] Interestingly, overall SMC numbers were not affected by KLF4 deletion, but a substantial reduction in SMC-derived macrophage-like and mesenchymal stem cell-like cells was discovered, indicating a crucial and novel role for KLF4 regulating SMC-macrophage transition. The gene expression of SMC-derived macrophages was importantly different from classical macrophages, dendritic cells, or monocytes.[39] The SMC-derived macrophages showed a reduction in phagocytic capacity when being compared with activated peritoneal macrophages. This study

challenges our more classical view on the role of SMCs, being considered as purely protective in fibrous caps of advanced atherosclerotic lesions.[40,41] Certainly SMC function might vary quite drastically depending on the stimulus and outcome of their phenotypic switching and transformation.

The aforementioned miR-1 directly targets KLF4, guiding the differentiation process of embryonic stem cells into SMC progenitors,[42] while miR-1 depleted mice display abnormal SMC differentiation.[43] Two human miR-1 genes exist (miR-1-1 and miR-1-2), and both are co-transcribed with the respective miR-133a genes (miR-1-2 and miR-133a-1 on chromosome 18; miR-1-1 and miR-133a-2 on chromosome 20).[44] miR-1-1 and miR-1-2 become stimulated by MYOCD and myocyte enhancer factor-2 in cardiac and skeletal muscle precursor cells.[45] Mice with genetic deletions of miR-133a display abnormal SMC gene expression in the heart and limited cardiomyocyte proliferation during development, which the authors at least partially attribute to increased expression of its direct targets serum response factor (SRF) and cyclin D2 (CCND2).[46] An additional study was able to indicate a role for miR-133a in SMC-driven arterial calcification processes.[47] In this study, miR-133a overexpression limited the trans-differentiation of SMCs into osteoblast-like cells, whereas inhibition of miR-133a was able to promote a pro-osteogenic response by inducing the expression of its direct target RUNX2.

The miR-143/-145 cluster originates from a bi-cistronic transcript sharing a common promoter,[48] harbouring various binding sites for transcription factors known to be involved in SMC differentiation.[49–51] Both miRNAs are augmented in SMC progenitors in vascular development,[52] and influence SMC dynamics by directly targeting KLF4 and KLF5, which then induces MYOCD.[49,53] In most studies, induction of miR-145 limits neointima formation upon experimental vascular injury.[48,53] Contrary results were reported for cultured SMCs originating from clinical tissue specimen and experimental animal models of pulmonary arterial hypertension (PAH). Here, miR-145 appeared initially up-regulated, and Caruso *et al.*[54] revealed that vector-based repression of miR-145 halted SMC differentiation and switching towards a pro-proliferative phenotype.

Two interesting studies have assessed the intercellular communication and potential differentiation of SMCs and endothelial cells (ECs) being triggered through miR-143/-145. Climent *et al.*[55] discovered that SMCs can at least partially control EC functionality *via* sending miR-143/-145 through tunnelling nanotubes, enhancing the pro-angiogenic capabilities of ECs in a transforming growth factor-β (TGF-β) dependent manner. Hergenreider *et al.*[56] have demonstrated the exchange of miRNAs (as well as other RNAs) from ECs to SMCs, discovering a key role for travelling miR-143-/-145 in SMC differentiation and vascular functionality. In this study, laminar shear stress was able to KLF2-dependently increase expression, exosomal release, and intercellular transfer of miR-143/145, exerting atheroprotective effects.

Another interesting KLF-4 related mechanism in SMC differentiation is exerted by miR-146a. This miRNA directly targets KLF4, but at the same time has binding sites for both KLF4 and KLF5 in its own promoter region. The two KLF transcription factors have opposing effects on SMC phenotypic switching and proliferation depending on active binding to the miRNA gene.[57]

Finally, miR-155 has been indicated to revoke SMC differentiation by limiting SM-MHC levels.[58,59] Apart from its role in differentiating SMCs, miR-155 is well known in vascular disease-related mechanisms for its repression of B-cell CCL, lymphoma 6 (BCL6) in macrophages.[60]

Apart from miRNAs, several lncRNAs have been suggested to play a role in SMC differentiation. H19 was one of the first RNAs discovered to act as a transcript without protein-coding ability.[61] Initially, H19 was discovered as a paternally imprinted ncRNA, which remains highly expressed throughout embryonic and foetal development, with its expression being shut down in most tissues (including the vasculature) shortly after birth.[62] This onco-fetal behaviour pattern and its uniparental monoallelic expression are considered principal characteristics of imprinted genes, with many of these genes becoming altered in various malignancies.[63]

The multiple functions of H19 in vascular disease progression in relation to pathological processes involving SMCs are under current investigation by several labs. Of interest to SMC dynamics, H19 is a known regulator of p53,[64] which mediates cellular differentiation and apoptosis. Other important functions of H19 include its hosting of the pri-miR-675 gene that suppresses growth and migration,[65,66] as a modulator of miRNA expression (including let-7 and miR-106a),[67–69] or mediating RNA: protein interactions.[70,71] One SMC-specific study revealed that let-7a attenuates migration and proliferation *in vitro* and *in vivo* by targeting V-myc avian myelocytomatosis viral oncogene homologue (MYC).[72] Additional effects in the cardiovascular system of H19 include increasing proliferation, while blocking apoptosis during late-stage cardiac differentiation *via* regulation of miR-19b and SOX6.[73] H19 inhibition was further detected to limit human umbilical vein endothelial cell (HUVEC) growth and capillary formation,[74] while H19-bourne miR-675 was shown to magnify restenosis development by targeting phosphatase and tensin homologue (PTEN) in SMCs.[75] Recently, altered DNA methylation in the promoter region of H19 in calcified aortic valve disease was shown to accelerate mineralization by silencing NOTCH1.[76]

The Miano Lab has recently shown the first lncRNA being predominantly expressed in ECs and SMCs.[77] The investigators assessed human aortic SMCs *via* RNA-sequencing and revealed an antisense RNA overlapping the Friend Leukaemia Integration virus 1 (FLI1) gene, an established regulator of endothelial development.[78] They termed the transcript *smooth muscle and endothelial cell enriched migration/differentiation-associated lncRNA* (SENCR), which has two variants with enhanced expression in arterial ECs and SMCs, as well as other tissues (e.g. lung, skeletal muscle). SENCR depletion led to SMC de-differentiation and induction of migration, mainly being indicated through increased expression of midkine and pleiotrophin. Repression of both pro-migratory genes upon SENCR inhibition halted this effect. Interestingly, SENCR appears to be mainly expressed and active cytoplasmaticly, and thus not regulating FLI1 on the transcriptional level.[77] Additional studies looking at the mesodermal and endothelial lineage commitment upon SENCR modulation discovered that its overexpression promotes proliferation, migration, and angiogenesis in HUVECs.[78]

A novel transcript termed myocardin-induced smooth muscle lncRNA (MYOSLID)[79] has been shown to increase SMC phenotypic switching while reducing SMC proliferation rates. MYOSLID gets induced *via* TGF-β/SMAD pathways, as well as MYOCD and SRF. Inhibition of MYOSLID unsettles actin formation by blocking nuclear translocation of the MYOCD-related transcription factor A (MRTF-A), while limiting the TGF-β1-induced phosphorylation of SMAD2.

4. SMC migration, proliferation, and ECM production

Absolute or relative quantification of SMC migration in human arteries is considered very difficult—if not impossible to perform—due to the lack of solid and specific markers. Thus, *in vitro* studies are providing us with

the only evidence that human SMCs can migrate in response to varying stimuli.[39] As a result, the contribution of SMC migration to aneurysm development (or its limitation), as well as restenosis, or the maturation of atherosclerotic plaques in patients appears largely unclear. Also, until now it could not be clarified whether migration occurs independently—or is dependent on SMC proliferation.[80] Classically, the presence of a large number of intimal SMCs in aortic aneurysms as well as in fibrous cap formation in advanced atherosclerosis has been taken as evidence that SMC migration from the media plays an important role in vascular disease progression. Unlike humans, rodents have no SMCs in the intima of a healthy artery, which leads to the assumption that neointimal SMCs must have migrated from the intima—or originate from invading myeloid cells from the lumen that underwent trans-differentiation.[39,81]

Accelerated SMC proliferation rates can be observed in response to vascular injury as well as during early atherogenesis and upon vascular injury.[82] On the contrary, SMCs derived from either aged arteries, aneurysmal disease, or advanced atherosclerotic plaques display augmented proliferation rates and extended population doubling times.[83,84] The aforementioned miR-1 has been reported as an important regulator to limit SMC proliferation *via* mediating the expression of PIM1.[85] A more prominent and well-studied miRNA in determining SMC fate in aortic aneurysm development as well as after vascular injury and neointima formation is miR-21, which provides mainly pro-proliferative and anti-apoptotic effects in SMCs.[86,87] Inhibition of miR-21 diminishes the neointimal response *via* induction of PTEN expression (*Figure 3*). In studies investigating the development of in-stent restenosis (ISR), miR-21-depleted mice appeared protected from this complication by limiting the burden of neointima formation in response to vascular injury. The authors of the study associated this to an increase of anti-inflammatory M2 macrophage signalling in conjunction with a compromised proliferative reaction in *miR-21*$^{-/-}$ SMCs.[88] Wang *et al.*[89] were able to show that local delivery using anti-miR-21 coated drug eluting stents limited SMC-driven myointimal hyperplasia, and thus effectively blocked ISR development. One of the early studies on miR-21 by Davis *et al.*[90] was able to discover an opposing effect for this miRNA in SMC differentiation and proliferation. Here, miR-21 down-regulated programmed cell death 4 (PDCD4), which acts as a negative regulator of SMC contractile genes. Interestingly, enhanced signalling of TGF-β and bone morphogenic protein pathways led to increased expression of mature miR-21 through a post-transcriptional regulatory circuit, which triggered the processing of the pri-miR-21 gene into pre-miR-21.

Another cluster with implication for SMC dynamics and cell-fate decision making is the miR-23b/-24/-27b family.[91] miR-23b limits SMC proliferation and migration, effecting neointimal hyperplasia in an arterial injury model through targeting of FOXO4.[92] miR-24 has further been shown to limit aortic aneurysm progression by targeting cytokine release and survival rates in SMCs (and macrophages) by targeting chitinase 3-like 1 (CHI3L1).[91]

miR-26a has been shown to limit serum starvation-induced differentiation, while enhancing proliferation of SMCs. Leeper *et al.*[93] identified SMAD1 and SMAD4 as direct targets of deregulated miR-26a with direct implications for angiotensin II (ANGII)-induced aortic aneurysms in *ApoE*$^{-/-}$ mice. Interestingly, another study discovered that miR-206 in HUVECs helps to maintain the contractile phenotype of SMCs by suppressing exosome release of miR-26a-enriched particles *via* the adenosine diphosphate (ADP)-rybosilation factor 6 (ARF6) and soluble carrier family 8 member A1 (SLC8A1, previously known as NCX1).[94] Another miRNA targeting SMAD1 (as well as RUNX2) expression levels is miR-205, which adversely regulates β-glycerophosphate-induced calcification of human SMCs.[95]

Another prominent miRNA regulating migration and proliferation is miR-34a, which modulates Sirtuin1 (SIRT1),[96] as well as NOTCH1 expression levels.[97] Induction of miR-34a led to limited neointima formation in murine models of arterial injury. Another miRNA targeting SIRT1 is miR-138, which has been indicated to enhance SMC migration and proliferation in a diabetic model utilizing *db/db* mice.[98] A similar mechanism aiming at the proliferative response in SMCs could be reported for miR-130a, which regulates Mesenchyme Homeobox 1 (MEOX1) in this process.[99]

Interesting observations on the regulation of miRNAs in SMC proliferation and contribution to vascular disease progression stem from studies investigating PAH. Here, it was discovered that miR-204 was repressed in human PAH disease specimens and murine models. Upon down-regulation of miR-204, Protein tyrosine phosphatase, non-receptor type 11 (PTPN11, also known as SHP2) increases, enhancing STAT signalling, which mediates SMC proliferation and intimal hyperplasia in pulmonary arteries.[100]

miR-221 is another miRNA that has been shown to stimulate SMC proliferation downstream of PDGF. Induction of this miRNA decreased the expression of c-Kit and p27Kip1, which are both critical mediators of cell proliferation.[101] A similar mechanism was observed for miR-221's cluster member miR-222.[102]

MiRNA profiling studies using microarray technology led to the discovery of miR-424 and its rat ortholog miR-322 in studies investigating myointimal hyperplasia and SMC responsiveness. Functional *in vitro* and *in vivo* studies identified CCND, as well as $Ca^{(2+)}$-regulating proteins calumenin (CALU) and stromal-interacting molecule 1 (STIM1) as direct targets.[103] Adenoviral overexpression of miR-424/-322 limited the pro-proliferative response in an arterial injury model in rats.

Through direct targeting of the oncogene and transcription factor JUNB, miR-663 was identified as a crucial inducer of SMC proliferation.[104] Again, utilization of adenoviral overexpression limited SMC proliferation in a mouse model of carotid ligation injury.

Similar to the molecular mechanisms regulating SMC differentiation, several lncRNAs were identified in recent years to contribute to SMC proliferation and migration. One of the first studies using RNA deep sequencing in rat SMCs discovered that ANGII stimulation deregulates several lncRNAs. Interestingly one of them, lncRNA-362 functions as a host for the aforementioned miR-221/-222 cluster. Inhibition of lncRNA-362 limited SMC proliferation rates in response to ANGII treatment.[105]

Further profiling approaches using microarrays identified several deregulated transcripts in varicose great saphenous veins compared with un-diseased controls. Co-expression analysis revealed their potential importance in metabolic pathways; however, none of the lncRNAs was experimentally or functionally analysed.[106] A second study performed in saphenous veins and SMCs originating from these vessels identified lncRNA-GAS5A as a novel mediator in SMC proliferation. RNA pull-down experiments indicated a direct binding for this lncRNA to the RNA-binding protein Annexin A2 (ANXA2). Inhibition of lncRNA-GAS5 was able to limit ANXA2 expression and SMC proliferation, while ANXA2 overexpression increased proliferation rates.[107]

The retinal lncRNA 3 (lncRNA–RNCR3) has been linked to atherosclerosis-related vascular dysfunction with a potential mechanism relating to SMC dynamics.[108] RNCR3 levels are augmented in human and mouse atherosclerotic lesions, in which the lncRNA co-locates with ECs and SMCs. Inhibition of RNCR3 sufficiently enhances pro-inflammatory signalling during atherogenesis and hypercholesterolaemia, while blocking proliferation in SMCs. Cell-fate decisions in SMCs are

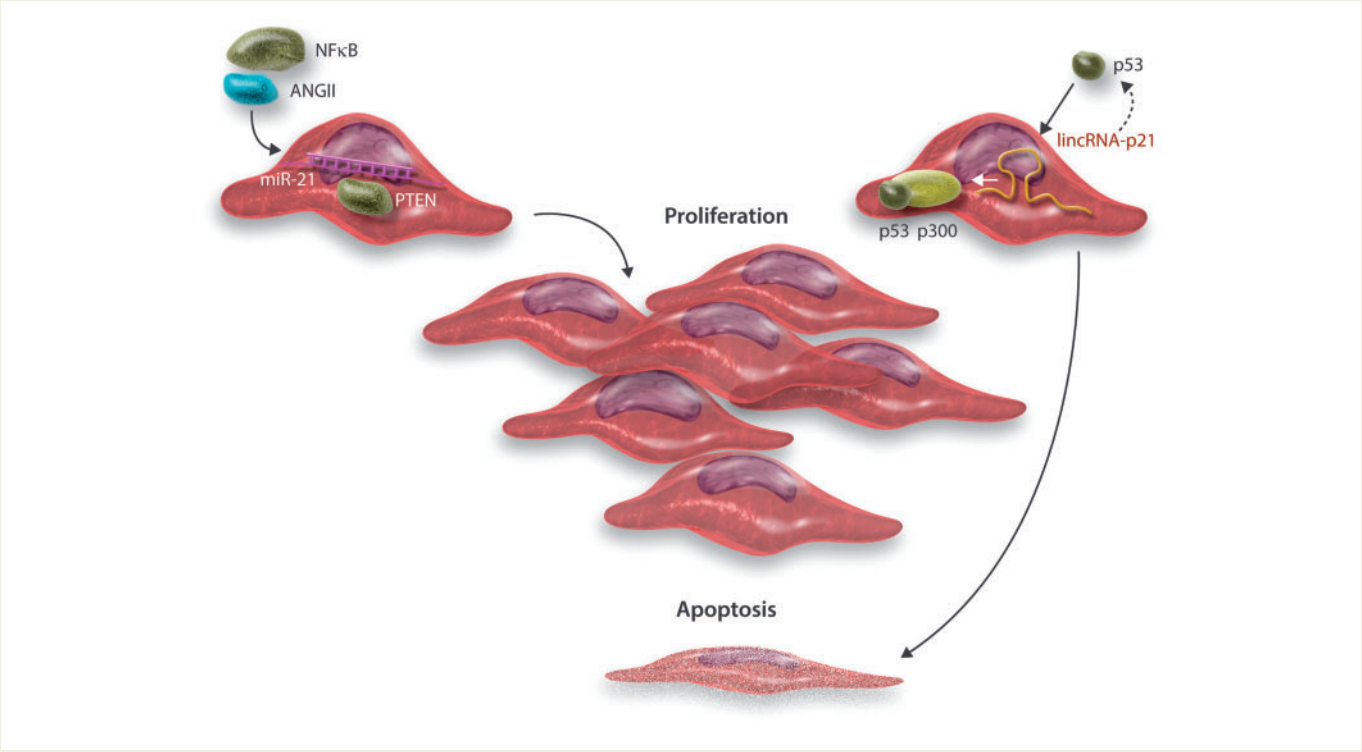

Figure 3 miR-21 regulates SMC proliferation, while lincRNA-p21 mediates apoptosis. miR-21 has been reported to get induced by different factors involved in vascular disease evolvement and progression, such as nuclear factor kappa-b and ANGII.[86] SMC-enriched miR-21 targets and down-regulates expression of PTEN, activating proliferation.[86,87] lincRNA-p21 is a transcriptional target of p53, which is enabled to feed back to accelerate p53 activity through binding to other factors (mouse double minute 2 = MDM2, an E3 ubiquitin-protein ligase not shown in the scheme).[133] Increased levels of lincRNA-p21 allow p53 to interact with p300, which leads to SMC apoptosis.

regulated by RNCR3 through the formation of a feedback loop with KLF2 and miR-185.

The anti-sense RNA in the INK4 locus (ANRIL) is localized in the CVD-associated 9p21.3 region of the human genome.[9,109] ANRIL is a crucial regulator of cell cycle genes, which it mediates in *cis* through polycomb repressive complexes.[110] SMCs stemming from humans with a SNP variant in the ANRIL locus exhibit increased proliferation rates.[111] Holdt *et al.*[112] reported that the molecular mechanism controlled by ANRIL affects target-genes in *trans*, which leads to cellular proliferation, adhesion, and concomitantly a reduction in apoptosis. Importantly, the reported *trans* regulatory mechanisms relied on Alu motifs, which were responsible for identifying the promoters of the respective ANRIL targets.

SMCs synthesize components of the ECM, in which they are embedded. Earlier studies revealed that the ECM suppresses phenotypic switching, keeping SMCs in a contractile state while being less responsive to varying stimuli (e.g. cytokines). On the contrary, ECM breakdown and anti-fibrotic factors (like matrix-metalloproteinases released from macrophages and SMCs) were shown to promote phenotypic switching and to induce a pro-migratory and -proliferative response in the arterial wall as well as the plaque.[113,114] Interestingly, no study has been conducted until now that uses knockout of a certain ECM gene exclusively in SMCs to prove that this affects disease progression by de-stabilizing atherosclerotic lesions.[115]

Regarding miRNA involvement into ECM production and release mechanism, it was shown that miR-29b and miR-195 are targeting several pro-fibrotic components (collagen isoforms) and mediators of inflammation,[116–118] and that their inhibition can limit aneurysm progression, as well as stabilize experimental atherosclerotic lesions.[119] miR-29b is again part of a cluster, together with miR-29a and miR-29c. Interestingly, miR-29b appears lower expressed in SMCs compared with the other two, underlining differences in miRNA processing between the cluster members in SMC-related cell-fate decisions. In this study by Bretschneider *et al.*,[120] aldosterone was labelled as a distinct regulator being capable of adjusting miR-29b expression levels.

Accumulation of hyaluronan into the matrix of arteries increases wall thickening, and thus contributes to atherogenesis and vascular disease progression.[121] The natural antisense transcript (NAT) hyaluronan synthase 2 gene (HAS2)-AS1 is transcribed opposite to the HAS2, and appears generally required to induce the transcription of the protein-coding gene, and its depository and remodelling effects in the vascular matrix.[122]

5. SMC apoptosis

Cell death of any kind plays a crucial role in vascular disease development.[123] Aortic aneurysms continue to expand rapidly if SMC survival is augmented,[86] and the importance of apoptosis in advanced atherosclerotic lesions has been indicated in numerous studies.[82,83,124,125] In the setting of atherosclerosis, the level of apoptosis seems low in early lesions (Stary[126] Grades I–III), but substantially increases as lesions progress.

Apoptosis appears predominantly in SMCs, but can also be detected in macrophages.[127] However, in general all cellular subtypes of the vasculature can potentially undergo apoptosis. As with other mechanisms driving SMC functionality and fate; interpretation of the apoptosis effect in actual human patients is limited due to the lack of specific markers.

Although apoptosis is seen in all kinds of different vascular diseases, conclusions about the absolute rates of cell death are almost impossible to be estimated, as our understanding of the initiation and duration of the process *in vivo* is limited.[115,128] Chronic SMC apoptosis accelerates vascular disease progression (in particular aneurysm disease as well as atherosclerosis-related pathologies), promotes calcification, and induces features of medial degeneration, like atrophy, elastin fragmentation, as well as enhanced glycosaminoglycan deposition.[129] Cystic medial degeneration, which becomes visible for example in Erdheim-Chester disease, Marfan syndrome, and to a lesser extent in normal aging, are good examples of SMC loss being connected with apoptosis.[130–132]

A recent study from our lab by Eken *et al.*[125] has shown that miR-210 can stabilize fibrous caps of advanced atherosclerotic lesions by blocking SMC apoptosis *via* inhibition of the tumour suppressor Adenomatous polyposis coli (APC) and canonical Wnt-pathway signalling. p53 was identified as a negative upstream regulator of miR-210.

Another molecular process involving p53-mediated apoptosis signalling in atherosclerotic lesions relates to lincRNA-p21, which was recently identified as a crucial mediator of SMC and macrophage survival.[133] lincRNA-p21 is decreased in lesions of *ApoE*-deficient mice and plaque specimens from individuals suffering from coronary artery disease. Inhibition of lincRNA-p21 enhanced neointimal hyperplasia in a murine model of carotid artery injury. The overall effect of this lncRNA could be linked to p53:p300 interactions, allowing these factors to bind to their promoters and enhancers in apoptotic signalling pathways (*Figure 3*). In addition, p53 and lincRNA-p21 form a feedback loop, in which p53 regulates the transcriptional activity of the lncRNA.[134]

The NAT to the transcription factor HIF1α (HIF1α-AS1) has been discovered to be elevated in circulation of patients with aortic aneurysms.[135] siRNA-guided inhibition of this NAT in aortic SMCs limited the apoptotic response as indicated by different markers of cell death (e.g. Caspases 3 and 8).[45]

Another study linking HIF1α-AS1 to thoracic aneurysms was able to identify Brahma-related gene 1 (BRG1) as a potential target. Overexpression of BRG1 in human aortic SMCs could increase apoptosis, while inhibition of HIF1α-AS1 not only reduced BRG1 levels, but also promoted cellular proliferation.[136] The direct molecular mechanism behind this regulation remains to be determined.

The lncRNA MEG3 is another recently unravelled transcript with important implications in vascular disease. Boon *et al.*[137] have shown that MEG3 regulates endothelial aging while mediating angiogenesis. For SMC plasticity, MEG3 seems of potential interest, as it has been shown to be under the control of dNK-derived interferon γ (IFN-γ), negatively affecting SMC survival and migration in uterine spiral arteries during vascular transformation.[138]

6. Conclusion and perspectives

The emerging links between ncRNAs and diseases have opened up a new field of therapeutic and diagnostic opportunities. Many miRNAs have already successfully been shown to serve as biomarkers or therapeutic targets for many different pathologies. There is also evidence that the same holds true for lncRNAs (and maybe circRNAs). It is evident that RNA molecules exhibit many more functions beyond their classic role as templates for protein synthesis. Considering the ability of RNA to form 3D structures and interact with DNA, proteins, and other RNA molecules, non-coding transcripts are assumed to be as versatile as proteins, allowing them to mediate all major cellular processes. The classical view on SMCs and how they are involved in vascular disease development and progression has been challenged in recent times. Many studies in the field have moved SMCs, with their plastic and dynamic features, into the centre of attention. Potential new therapies should be considered for being directed towards manipulating SMC fate decisions, enabling us to limit the burden of SMC apoptosis and transformation into macrophage-like cells with enhanced pro-inflammatory activity. Strategies that modulate the expression of ncRNAs, like miRNAs and lncRNAs, could play an important role in this regard, as they have been shown to be master regulators of SMC plasticity in vascular disease evolvement.

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