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INVITED REVIEW

# Noncoding RNAs in smooth muscle cell homeostasis: implications in phenotypic switch and vascular disorders

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**Abstract** Vascular smooth muscle cells (SMC) are a highly specialized cell type that exhibit extraordinary plasticity in adult animals in response to a number of environmental cues. Upon vascular injury, SMC undergo phenotypic switch from a contractile-differentiated to a proliferative/migratory-dedifferentiated phenotype. This process plays a major role in vascular lesion formation and during the development of vascular remodeling. Vascular remodeling comprises the accumulation of dedifferentiated SMC in the intima of arteries and is central to a number of vascular diseases such as arteriosclerosis, chronic obstructive pulmonary disease or pulmonary hypertension. Therefore, it is critical to understand the molecular mechanisms that govern SMC phenotype. In the last decade, a number of new classes of noncoding RNAs have been described. These molecules have emerged as key factors controlling tissue homeostasis during physiological and pathological conditions. In this review, we will discuss the role of noncoding RNAs, including microRNAs and long noncoding RNAs, in the regulation of SMC plasticity.

**Keywords** Smooth muscle cells · Phenotypic change · Noncoding RNAs · miRNAs · lncRNAs

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**Abbreviations**

| | |
|---|---|
| SMC | smooth muscle cells |
| EC | endothelial cells |
| ncRNAs | noncoding RNAs |
| miRNAs | microRNAs |
| lncRNAs | long noncoding RNAs |
| SRF | serum response factor |
| SRE | serum response element |
| MYCD | myocardin |
| MRTF | myocardin-related transcription factor |
| TGFβ | transforming growth factor β |
| PDGF | platelet-derived growth factor |
| SBE | Smad-binding elements |
| bHLH | basic helix-loop-helix |
| KLF | Krüppel-like zinc finger |
| NAT | natural antisense ncRNA |
| ceRNA | competing endogenous RNAs |
| SENCR | smooth muscle and endothelial cell enriched migration/differentiation-associated |
| SNP | single nucleotide polymorphism |

## Introduction

Vascular smooth muscle cells (SMC) are a highly specialized cell type present within the medial region of arteries and arterioles. SMC express a repertoire of proteins that are important for contractility, ion channels and signaling cascades that allow them to regulate systemic and local pressure through the modulation of the vascular tone. In contrast to other terminally differentiated cells, SMC maintain high phenotypic plasticity throughout adulthood. In normal physiological conditions, these cells stay quiescent. However, under different environmental conditions, SMC are able to re-enter cell cycle and undergo phenotypic switch from a differentiated/contractile

phenotype to a dedifferentiated/proliferative phenotype [142, 144]. Dedifferentiated cells are characterized by high rates of migration and proliferation, increased expression of the extracellular matrix proteins (ECM) and low expression of contractile proteins. By contrast, contractile or differentiated SMC exhibit low levels of proliferation and migration and express a set of specific markers such as cytoskeleton and contractile proteins, which comprise smooth muscle actin-α (α-SMA), smooth muscle myosin heavy chain (SM-MHC), calponin, caldesmon and sm22-α [143]. When repairing vascular injury and after several rounds of unchecked proliferation, this dedifferentiated SMC participate in the intimal thickening and medial stiffening observed in vascular diseases like arteriosclerosis, chronic obstructive pulmonary disease (COPD) or pulmonary arterial hypertension (PAH) [71, 139, 144]. A recent report from Owens and colleagues, using cell-tracing system, shows that more than 80 % of SMC in the vascular lesion undergo phenotypic switch contributing to the intimal hyperplasia seen in vascular pathologies [158]. For this reason, much effort has been focused on identifying molecules that regulate both global SMC differentiation and specific SMC gene expression. Several observations have demonstrated that a complex network of both protein and noncoding RNAs governs the phenotypic switch process [4, 41, 98, 132, 143]. However, despite the importance of SMC changes during vascular diseases, the molecular mechanisms controlling them are not yet fully understood, in part due to the versatile characteristics of these cells. In this review, we briefly summarize the current understanding of the molecular regulation of SMC differentiation and phenotypic switch during physiological and pathological remodeling with special focus on recent discoveries concerning the contribution of the noncoding genome to this process.

## Regulation of SMC phenotypic switch by transcription factors

Figure 1 summarizes the main signaling cascades and transcription factors that maintain SMC phenotypic states. The study of SMC transcriptional regulation has been difficult due to the high variation of SMC marker expression across different tissues and the fact that SMC derive from multiple precursors throughout the embryo [98]. Contrary to the skeletal muscle, in which myoD is the master regulator of its differentiation, to date, there is no comparable transcription factor governing SMC differentiation. The role of the C-Fos serum response element-binding transcription factor (SRF) in SMC homeostasis has been demonstrated by the ability of dominant negative mutants of SRF to prevent differentiation [118, 123]. SRF regulates most SMC differentiation marker genes by binding as an homodimer to the highly conserved CArG cis-element ($CC(A/T)_6GG$) or serum response element (SRE) present within nearly all of the SMC-specific promoters [133]. Most of the SMC genes contain two or more CArG boxes, which act cooperatively to promote transcription [180, 181]. However, SRF cannot be considered a master regulator because it is an ubiquitously expressed protein that also regulates cardiac and skeletal muscle-specific gene expression, as well as the expression of a number of early response and structural genes across different cell types [163]. How SRF is able to regulate specific genes in precise environments is not yet fully understood. SRF activity is regulated by its association to different transcription factors, such as NkX and GATA family members and cofactors, predominantly myocardin (MYCD) and myocardin-related transcription factors A and B (MRTF-A and MRTF-B) [133]. Additionally, posttranscriptional modifications of SRF, variation of SRF-binding affinity among different CArG boxes as well as number, position and spacing of CArG boxes are now recognized as the main mechanisms regulating SRF activity [98]. MYCD, identified in 2001 by Olson and collaborators, form a ternary complex with SRF and acts as a transcriptional co-activator of almost all SMC-specific promoters including calponin, caldesmon, SM-MHC, α-SMA, sm22-α and specific cell-cycle-associated genes such as p21 [180]. Forced expression of MYCD in a skeletal muscle-related cell line is sufficient for the induction of the majority of SMC markers [116] but not to initiate the complete differentiation programme in multipotent stem cells [201]. MYCD is induced by angiotensin II, L-type voltage-gated Ca2 channels/Ras homology gene family A (RhoA) and transforming growth factor β (TGFβ) and inhibited by platelet-derived growth factor-BB (PDGF-BB) [152, 179, 199] (Fig. 1). This indicates that MYCD regulates changes in SMC contractile mass in response to functional demands. MYCD activity is subjected to regulation through multiple mechanisms, including alternative splice variants, and binding to regulatory proteins [74, 79]. For example, activity of MYCD is inhibited by the inflammatory-related factor NFkB [167] and the insulin-like growth factor-1/AKT-dependent phosphorylation of the transcription factor forkhead O4 (FoxO4) inducing its translocation from the nucleus, therefore reducing SMC marker genes [114]. MYCD can be phosphorylated by glycogen synthase kinase-3-β and extracellular signal-regulated kinase (ERK), resulting in decreased differentiation marker expression [6, 168]. A decreased expression of MYCD has been observed in several models of vascular injury, and its re-expression prevents the neointima formation in murine carotid arteries after injury [1, 166]. SRF/MRTF complexes also bind to consensus CArG elements within the promoters of contractile and SMC-specific target genes like α-SMA and sm22-α [175, 180, 181, 202]. RhoA-dependent actin polymerization has been shown to be required for nuclear localization of MRTF-A and for SMC-specific gene expression [120]. RhoA activity is mainly mediated by angiotensin II, sphingosine-1-phosphate, TGFβ, calcium, BMP2 and cell tension [73].

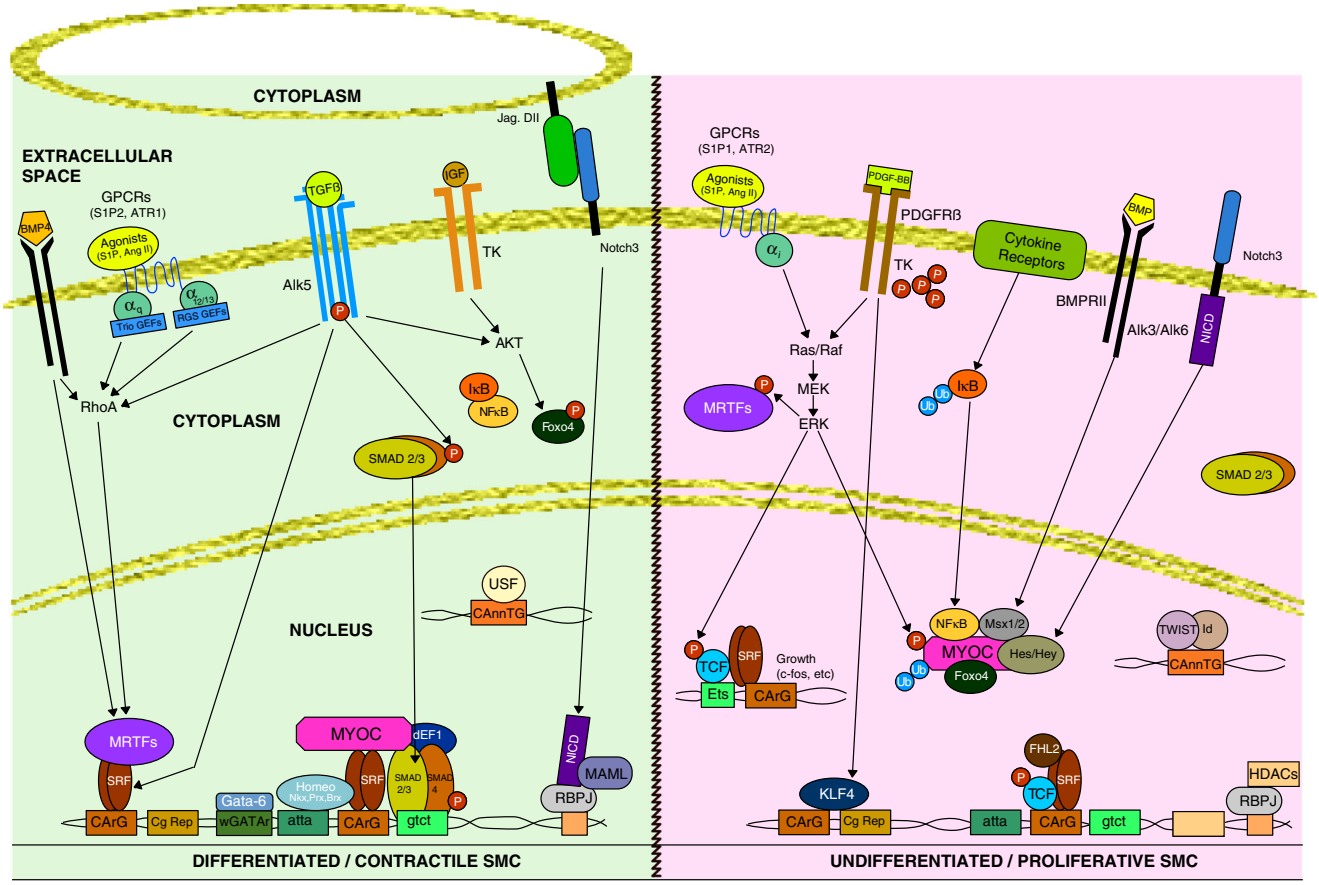


**Fig. 1** Signaling pathways controlling SMC phenotypic switch. In the *right panel*, the differentiated/contractile SMC state is displayed, and in the *left panel*, the proliferative/dedifferentiated SMC phenotype. *SRF* serum response factor, *MYCD* myocardin, *MRTF* myocardin-related transcription factor, *RBPJ* recombination signal binding protein for immunoglobin Kappa J, *NCID* Notch intercellular domain, *TGFβ* transforming growth factor β, *IGF* insulin growth factor, *TK* tirosin kinasa, *P* phosphorylation, *Jag* Jagged, *DII* delta-like II, *NFkB* nuclear factor kappa B, *IkB* inhibitor of kappa B protein, *AngII* angiotensin II, *ATR1/2* angiotensin receptor 1 and 2, *PDGF-BB* platelet-derived growth factor-BB, *PDGFRβ* platelet derived growth factor receptor β, *KLF4* Krüppel-like factor 4, *BMP* bone morphogenic protein, *HDAC* histone deacetylase, *TCF* ternary complex factor, *Ub* ubiquitin, *SP1* sphingosine-1-phosphate, *Id* inhibitor of DNA binding/differentiation proteins [7, 31, 33, 55, 56, 81, 87, 88, 91, 92, 103, 105, 107, 108, 110, 113, 122, 129, 134, 150, 153, 154, 177, 182, 183, 186, 195, 196, 206, 210]

TGFβ signals through the type II TGFβ receptor and the type I receptor ALK5. ALK5 activation results in the recruitment and phosphorylation of Smads 2 and 3 that complex with Smad4. The complex translocates to the nucleus and stimulates gene expression [126]. TGFβ control element (TCE; G(A/C)GT(T/G)GG(T/G)GA) has been found in several promoters of SMC genes, and its deletion in the sm22-α promoter blocks gene expression [66]. In concordance, TGFβ1 stimulates the expression of SMC genes and enhances SRF binding to CArG boxes [75] (Fig. 1), and several SMC genes contain functional Smad-binding elements (SBE) that are convergence points of TGFβ1 or myocyte enhancer factor 2 (MEF2) binding sites [44]. The related TGFβ family member bone morphogenetic 4 (BMP4) also promotes SMC contractile genes through MRTF [99].

Notch plays an important role in SMC homeostasis and vascular development [57]. In mammals, there are four transmembrane Notch receptors (1 to 4), being Notch 3 the most strongly expressed in SMC. The transmembrane ligands for Notch are Jagged 1 and 2 and delta-like 1, 3 and 5. Recombination signal-binding protein for immunoglobulin kappa J region (RBPJ) interacts with Notch intercellular domain (NICD), which is released upon Notch activation and stimulates SMC contractile genes [23] (Fig. 1). NICD-RBPJ target genes include α-SMA [140], SM-MHC [46] and microRNA 143/145 [18]. In the absence of NICD, RBPJ represses targets by recruiting histone deacetylases (HDACs). A number of studies have reported that NICD blocks SMC differentiation in part by regulating HEP/HEY family, which inhibits SMC marker gene through SRF/MYCD-dependent pathway [136, 149].

The GATA zinc transcription factors interact with the DNA regulatory elements with a consensus sequence A/T GATA A/G (WGATAR). The GATA-4, -5 and -6 are essential for cardiovascular system and endoderm-derived tissues [48], but only GATA-6 is expressed in the medial SMC of the

vasculature [135] and regulates SMC phenotype in vivo promoting the expression of SM-MHC, α-SMA and calponin [124]. In addition, GATA-6 regulates SMC proliferation by directly controlling cell cycle [148]. Preventing GATA-6 downregulation after vascular injury inhibits intimal hyperplasia [124]. These data demonstrate that the effects of both Notch and GATA signaling are cell-context dependent.

Many SM-specific promoters including SM-MHC [188], sm22-α [141] and α-SMA [159] contain enhancer-box (E-box)-binding sites (CAnnTG motifs). This element binds to homo- or heterodimers of basic helix-loop-helix (bHLH) proteins and to the snail family of transcription factors. Upstream stimulatory factor (USF) binds to two E-boxes present in the α-SMA promoter and activates its expression [85]. Overexpression of class I bHLH such as E2-2, E12 and HEB stimulates α-SMA, while the inhibitory bHLH proteins Id and Twist decreases α-SMA and sm22-α [151, 197]. The bHLH Msx1 and Msx2 as well as HERP1/HEY2 directly interact with MYCD and inhibit SMC marker genes [46, 67].

SMC phenotypic switching is also determined by factors that suppress SMC gene expression [144]. Although the factors mediating injury-induced phenotypic switching in vivo have not been clearly defined, PDGF-BB seems to play an important role. PDGFRβ triggers the Ras/Raf/MEK/ERK cascade leading to the SRF-dependent upregulation of early response growth genes as well as the phosphorylation of several SMC genes, including MYCD and MRTFs [187]. Furthermore, PDGF-BB induces suppression of SMC marker genes after vascular injury [9, 28] by promoting the expression of the Krüppel-like zinc finger family 4 (KLF4) or KLF5 [42, 125] (Fig. 1). KLF4 is normally absent in differentiated SMC in vivo but is rapidly induced in neointima after vascular injury [112, 158, 200] and promotes SMC phenotypic modulation by repressing multiple SMC marker genes like MYCD and sm22-α, as well as a group of genes that regulate pro-inflammatory responses [112, 158].

## Noncoding RNAs

In the last years, great advances in transcriptome sequencing and analysis have allowed the identification of many types of noncoding RNAs (ncRNAs) molecules. Interestingly, around 98 % of all RNA transcripts do not possess protein-coding capabilities [45, 64]. It is now clear that many (if not all) of them regulate individual steps of gene expression including transcription, RNA processing and translation [24]. In addition, ncRNAs are able to guide DNA synthesis or genome rearrangement among other functions [24] constituting the most versatile molecules that participate in the regulation of the genome. Broadly, ncRNAs can be classified according to their size. Small ncRNAs are conformed by less than 200 nucleotides (nt) while long ncRNAs (lncRNAs) are longer than 200 nt and can range up to tens or even hundreds of thousands of nucleotides in length [162].

### *Small noncoding RNAs*

The most prominent small ncRNAs are the "housekeeping" ncRNAs that are represented by ribosomal RNA (rRNA) and transfer (tRNA), which are required for protein translation [53, 137]. Small nuclear RNAs (snRNAs) are essential for mRNA splicing [14], small nucleolar RNAs (snoRNAs) for RNA modification [94] and YRNAs appear to be implicated in chromosome replication and cell proliferation [95]. Recently, much attention has arisen in a class of ncRNAs that posttranscriptionally regulates protein-coding genes. These include microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and PIWI-interacting RNAs (piRNAs), which interact with the RNA interference machinery [58]. Very little is known about small RNAs in vascular SMC homeostasis. The most studied small RNAs during SMC phenotype alterations are the miRNAs, which will be summarized here.

miRNAs are well-conserved 21-nt single-stranded RNA molecules. The miRNAs biosynthesis is initiated by the transcription of a long transcript by RNA polymerase II, which give rise to a primary miRNA capped and poly-adenylated [93]. In a sequential double step process, the RNA III enzymes Drosha and Dicer cleave the miRNA precursor, giving rise to a mature miRNA or guide strand and a passenger strand, which is removed by cellular nucleases [10]. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) where it binds to a member of the Argonaute (Ago) protein family and guides RISC to partially complementary target sites on mRNAs [47]. After the recruitment of downstream factors, the target mRNA is translational repressed and/or degraded by exonucleases [128]. miRNA gene regulation has been implicated in a number of cellular processes. They are expressed in a stage- or tissue-specific fashion and modulate cell differentiation, proliferation and apoptosis [51]. Consistent with their essential role in cellular functions, homozygous deletion of Dicer in mouse results in lethality at embryonic day 8.5 [15].

**The microRNAs in SMC homeostasis and phenotypic switch** Direct evidence of the importance of miRNAs mediating SMC differentiation in vivo is derived from conditional knockout mice studies. Defective blood vessel formation and loss of pluripotent cells have been observed in Dicer-knockout (KO) mice [13, 198]. Vascular SMC-specific deficiency of Dicer also induced defective blood vessel formation causing late embryonic lethality at E16–17 [3, 145]. Specifically, loss of Dicer in vascular SMC during development induced dilated, thin-walled blood vessels, as a consequence of the reduction in the proliferation state. The arteries of these mice exhibit

impaired contractility most likely due to a decreased expression of SMC contractile genes [3]. In accordance, conditional deletion of Dicer in adult mice shows a marked reduction in systemic blood pressure and an increase in vascular remodeling [2]. This phenotype shares a lot of similarities with the SMC-associated miR-143/145 cluster-deficient mice [16, 49]. However, the SMC-Dicer KO mice present a more complex and severe phenotype, indicating that additional miRNAs are involved in the regulation of postnatal SMC differentiation. miR-143 and miR-145 have been shown to be enriched in SMC progenitor cells during development [16, 36] and play a key role in SMC differentiation by targeting KLF4 and KLF5, with the subsequent increase of MYCD [32, 36]. In addition, miR-145 controls the establishment of key ion channels necessary for a proper contractile phenotype [173]. miRNA-143 and miR-145 are transcribed as a bicistronic transcript from a common promoter [49] that contains different binding sites for transcription factors involved in SMC differentiation, such as CArG [36, 194], Smad response element [117] and RBPJ [18]. Indeed, the SRF cofactors MYCD and MRTF-A/B activate the miR-143/145 in vivo and in vitro [36, 194]. Several studies associate the reduction of miR-143/145 with the decrease in SMC contractile markers, SMC phenotypic modulation and neointima formation [32, 36]. In contrast, increased levels of miR-145 have been described in PAH, both in cultured SMC from patients and in animal models. In this study, the authors show that adenoviral-mediated reduction of miR-145 attenuated the progression of the disease [22]. On the other hand, in vivo and in vitro ectopic expression of miR-145 reduces neointimal hyperplasia after injury [32, 49, 119] and partially rescues the SMC contractile gene reduction generated by the loss of Dicer, respectively [3]. Interestingly, TGFβ stimulates the transfer of miR-143/145 from SMC to endothelial cells (EC) through tunnelling nanotubes modulating the angiogenesis response through the decrease of both EC proliferation and tubulogenesis [34]. Analogously, laminar shear stress induces miR-143/145 expression in a KLF2-dependent fashion. These miRNAs are then transported through exosomes from EC to SMC conferring an SMC atheroprotective phenotype [70]. These data demonstrates that there is an exchange of miRNAs, and possibly other RNA products, from one vascular cell to another in response to cellular cues. These messengers trigger programmes of gene expression, locally or distantly, in order to modulate vascular homeostasis.

The miR-10a has been reported to mediate retinoic acid-induced SMC differentiation from ES cells by targeting the histone deacetylase HDAC4 [76]. The miR-1 and miR-133 family is another group that participates in SMC differentiation. Specifically, miR-1 is induced by MYCD and blocks contractile SMC expression impairing cytoskeletal organization of human aortic SMC in vitro [84]. In addition, miR-1 blocks proliferation by targeting Pim1 [29]. There is also evidence that miR-1 directs the differentiation of embryonic stem cells into SMC-like cells by targeting KLF4 [193] and miR-1 knockout mice show aberrant SMC differentiation [68]. These different outcomes in the action of miR-1 during SMC differentiation showed that the effects of specific miRNAs is cell-context dependent. There are two miR-1 genes located in different chromosomes both of which are stimulated by MYCD in a conserved SRF-CArG boxes-dependent fashion mostly in cardiac and skeletal muscle [208]. Both miR-1 genes are co-transcribed with homologous miR-133a genes. Similar to the response observed with miR-1, the suppression of miR-133a resulted in aberrant expression of SMC markers during heart development [109]. Later studies demonstrated a correlation between miR-133a levels and the differentiation state of vascular SMC. miR-133a targets the transcription factor SP1 [171], a well-known repressor of SM-MHC [121]. Accordingly, miR-133a overexpression experiments resulted in the upregulation of SM-MHC and the decrease of other SMC genes such as calponin and α-SMA, demonstrating that miR-133a does not fully direct SMC differentiation [171]. Further studies showed that neointimal formation can be attenuated with the overexpression of miR-133a, which blocks SMC proliferation and migration, whereas miR-133a inhibition exacerbates this condition [171]. Other miRNAs stimulated by MYCD, miR-24 and miR-29a, have shown to regulate cell proliferation and migration through regulating PDGFRβ levels indirectly and directly, respectively [166]. Another well-known target of miR-24 is the Tribbles-like protein 3 [26]. In response to PDGF-BB, miR-24 increases, promoting the downregulation of Tribbles-like protein 3, which in turn induces the Smurf1-mediated decrease of Smad1 and the consequent switch to a SMC proliferative phenotype [26, 27]. The miR-29b is also associated with the inhibition of SMC proliferation and migration, but its target is yet to be described [20]. Quantification analyses have determined that the level of miR-29b is much lower than miR-29a and miR-29c in SMC, highlighting differences in miRNA processing between family members [20]. In this study, aldosterone is described as a direct factor controlling the expression of the mature miR-29b, but not of the other mature miRNAs [20]. miR-24 is transcribed together with miR-23b and miR-27b [54]; therefore, they are likely to have related functions in SMC homeostasis. As expected, miR-23b inhibits SMC proliferation and migration and promotes SMC markers in vitro and neointimal hyperplasia in a model of balloon-injured arteries in vivo. miR-23b directly targets FoxO4 [78].

miR-34a is also associated with SMC differentiation of both mouse and human embryonic stem cells by activating SIRT1, which activates SMC genes in a CArG-dependent fashion [203]. How miR-34a activates SIRT1 needs further studies. The activation of target genes by miRNAs was also reported for miR-145, which directly binds and activates

MYCD [36]. More recently, miR-34a has been shown to inhibit proliferation and migration by regulating Notch 1 protein expression [30]. Overexpression of miR-34a resulted in inhibition of neointima formation in wire-injury femoral arteries [30].

Another important miRNA in determining the SMC fate after vascular injury and neointima formation is the miR-21, which has been shown to be upregulated in balloon injury and to be pro-proliferative and anti-apoptotic in SMC [83]. In vivo, miR-21 inhibition reduces the neointimal response through the de-repression of PTEN [83]. In contrast, Davis and collaborators demonstrated that miR-21 stimulates SMC contractile proteins by targeting PDCD4 [39]. In this study, the authors showed that BMP4 or TGFβ1-Smad signal transducers are recruited to the pri-miR-21 (*primary* miRNA) in association with a member of the DROSHA microprocessor complex promoting its processing into pre-miR-21 (*precurso*r miRNA) and the consequent increase of the mature sequence during SMC differentiation [39]. In a recent study, genetic ablation of the miR-21 stem loop attenuated neointimal formation in mice post-stenting probably via the enhancement of the anti-inflammatory M2 macrophage levels together with an impaired sensitivity to vascular responses of SMC [127].

The miR-221/222 cluster was shown to be induced by growth factors and to mediate SMC proliferation in vitro and in vivo, targeting key negative growth regulators [40, 111]. The miR-146a stimulates SMC proliferation via KLF4, which in turn regulates the expression of this miRNA [164]. Gain-of-function and loss-of-function experiments showed that miR-146a regulates SMC proliferation in vitro. Transfection of an antisense oligonucleotide against miR-146a into the balloon injury rat carotid arteries attenuates neointimal hyperplasia [164]. Another miRNA that regulates SMC proliferation is miR-130a. This gene targets MEOX1, a growth arrest-related gene [192].

miR-26a promotes SMC proliferation and attenuates serum starvation-induced SMC differentiation. The authors identified Smad1 and Smad4 as direct targets of miR-26a [100].

Courboulin et al. found that miR-204 was downregulated in PAH in humans and in rat models of PAH. Decreased miR-204 stimulates SHP2 expression and activates the STAT pathway contributing to SMC proliferation and pulmonary vessel wall thickening [37].

miR-155 has been shown to regulate genes required for differentiation of stem cells into smooth muscle cells, since exogenous overexpression of miR-155 inhibits expression of SM-MHC and abrogates SMC differentiation [38, 209].

miR-663 is associated with SMC marker expression, and the transcription factor JunB was identified as a target of this miRNA [104]. Transduction of an adenovirus anti-miR-663 partially suppresses the neointimal response to injury [104]. The hsa-miR-424 or its ortholog in rat miR-322 (miR-424/322) was found to inhibit SMC proliferation by targeting cyclin D1, while miR-424/322 overexpression in vivo protected against restenosis [131].

Table 1 summarizes the miRNAs genes implicated in the regulation of SMC plasticity and their validated targets.

*Long noncoding RNAs*

Currently, the faster growing area of noncoding RNAs research is the study of lncRNAs. The ENCODE project, together with the improvement of bioinformatics analysis and the new powerful RNA sequencing technologies, has revealed pervasive transcription of the majority of the human genome [35, 64]. The use of RNA capture followed of tilling arrays to target and sequence selected portions of the transcriptome has detected additional transcripts that are rare or transiently expressed [130]. lncRNAs are transcribed by RNA polymerase II, undergo 5′ capping and splicing, can be or not poliadenylated and, in general, are not well conserved across species [155]. In general, although the expression of these transcripts is very low, they exert important and different regulatory roles in a wide range of biological processes in health and disease [165, 174]. However, the number of lncRNAs with functional characterization is limited and the majority of the new annotated transcripts are derived from fibroblasts. Considering that specific transcripts or even alternative species of the same gene are expressed specifically in different cell types [130], the description and functional characterization of those transcripts in specific cells and states are necessary to better understand their function. The strongest support of lncRNAs as biologically relevant molecules that regulate in vivo functions comes from knockout studies in mice [157].

Classification of lncRNAs species has been difficult due to the high heterogeneity in their biogenesis, structure and function [162]. Based on the genomic position relative to other genes, they can be divided in several sub-classes including intronic ncRNA, circular RNA (cirRNAs), sense ncRNA, natural antisense ncRNA (NAT), chromatin-interlinking RNA (ciRNA) and others. Based on their association with other DNA elements of known function, they are classified into enhancer-associated RNA (eRNA), promoter-associated long RNA (PALR), upstream antisense RNA (uaRNA) and others (for a review, see Hangauer et al. [162]).

The current challenge in the study of lncRNAs is the elucidation of their mechanisms of action. These molecules exhibit unpredictable and diverse functions. They are able to influence gene expression in different manners, being able to stimulate or repress transcription, translation and signaling as well as to influence the structure and function of chromosomes [17, 24, 155, 190]. The problem with the understanding of lncRNA function is that there are not known features that correlate lncRNA sequence with specific function. The description of a novel RNA motif (AGCCC plus A/T at −8 and

**Table 1** Different known miRNAs and their functions in SMC homeostasis

| miRNA | Target | Functions | References |
|---|---|---|---|
| 1 | Pim1 | (−) SMC proliferation | Chen et al. [29] |
| 1 | Unknown | (−) SMC contractility | Jiang et al. [84] |
| 1 | KIf4 | (+) SMC differentiation in ES cells | Xie et al. [193] |
| 10a | Hdac4 | (+) SMC differentiation in ES cells | Huang et al. [76] |
| 15b/16 | Yap | (+) SMC differentiation | Xu et al. [195] |
| 21 | Pdcd4 | (+) SMC differentiation | Davis et al. [39] |
| 21 | Pten | (−) SMC differentiation/(+) neointima | Lin et al. [108]; Ji et al. [83] |
| 21 | Pten | (+) SMC proliferation | Green et al. [56]; Liu et al. [113] |
| 21 | Dock | (+) SMC proliferation | Kang et al. [87] |
| 22 | Mecp2 | (+) SMC differentiation | Zhao et al. [206] |
| 23b | Foxo4 | (−) SMC proliferation/(−) migration | Iaconetti et al. [78] |
| 24 | Chi3l1 | (−) SMC inflammation/(−) SMC differentiation | Maegdefessel et al. [122] |
| 24 | Trb3 | (−) SMC proliferation | Chan et al. [26] |
| 24 | Pdgfrb | (−) SMC migration | Talasila et al. [166] |
| 26a | Smad1 | (+) SMC proliferation/(−) SMC differentiation | Leeper et al. [100] |
| 26a | Smad4 | (−) SMC apoptosis | Leeper et al. [100] |
| 26a | Pdgfrb | (−) SMC migration | Talasila et al. [166] |
| 29a/29c | Cav1 | (+) insuline resistance/alterations in lipid metabolism | Chen et al. [31] |
| 29b | Unknown | (+) SMC proliferation/(+) SMC migration/(−) apoptosis necrosis ratio | Bretschneider et al. [20] |
| 30a | Ca(v)1.2 | (−) Vascular tone | Rhee et al. [154] |
| 30b/30c | Runx2 | (−) SMC calcification | Balderman et al. [7] |
| 31 | Lats2 | (+) SMC proliferation | Liu et al. [110] |
| 31 | Creg | (−) SMC differentiation | Wang et al. [183] |
| 34a | Sirt1 | (+) SMC differentiation | Yu et al. [203] |
| 34a | Notch1 | (−) SMC proliferation | Chen et al. [111] |
| 96 | Trb3 | (−) SMC differentiation | Kim et al. [92] |
| 124 | Nfatc1 | (−) SMC proliferation/(+) SMC differentiation | Kang et al. [88] |
| 125b | Suv39h1 | (+) SMC inflammation | Villeneuve et al. [177] |
| 126 | FoxO3/Bcl2/Irs1 | (+) SMC proliferation/(+) neointima | Zhou et al. [210] |
| 130a | Meox1 | (+) SMC proliferation | Wu et al. [192] |
| 132 | Lrrfip1 | (−) SMC proliferation/(−) neointima | Choe et al. [33] |
| 133a | Sp1 | (−) SMC proliferation/(−) SMC migration | Torella et al. [171] |
| 138 | Mst1 | (−) SMC apoptosis | Li et al. [105] |
| 138 | Sirt1 | (+) SMC proliferation/(+) SMC migration | Xu et al. [196] |
| 143/145 | Kfl4/Elk1/Camk2d | (−) SMC proliferation/(+) SMC differentiation | Cordes et al. [87] |
| 143/145 | Kfl5 | (+) SMC differentiation/(−) neointima | Cheng et al. [32] |
| 143/145 | KIf4/Kif5/Add3 | Cytoskeletal dynamics | Xin et al. [194] |
| 143/145 | KIf2 | (+) SMC differentiation | Hergenreider et al. [70] |
| 146a | KIf4 | (+) SMC proliferation/(+) neointima | Sun et al. [164] |
| 146b | Unknown | (+) SMC proliferation/(+) SMC migration | Wang et al. [182] |
| 195 | Cdc42/Ccnd1 | (−) SMC proliferation/(−) SMC migration | Wang et al. [186] |
| 200 | Zeb1 | (+) SMC inflammation in Diabetes | Reddy et al. [153] |
| 203 | c-Abl | (−) SMC proliferation | Liao et al. [107] |
| 204 | Shp2 | (−) SMC proliferation/(+) SMC apoptosis | Courboulin et al. [37] |
| 205 | Runx2 | (−) SMC calcification | Quiao and Zhang [150] |

**Table 1** (continued)

| miRNA | Target | Functions | References |
|---|---|---|---|
| 206 | Notch 3 | (−) SMC proliferation/(−) SMC differentiation | Jalali et al. [81] |
| 210 | E2f3 | (−) SMC apoptosis | Gou et al. [55] |
| 221/222 | Kit/Cdkn1b | (+) SMC proliferation/(−) SMC differentiation | Davis et al. [40] |

G/C at −3), which mediates nuclear localization signal, is one exception [204]. It is likely that our understanding about these versatile molecules is only beginning to emerge and new technologies are needed to unravel their mechanisms. Uncovered functions of lncRNAs are recapitulated briefly as follows:

Regulation of imprinting

The general definition of imprinting is "parental-specific gene expression in diploid cells" [8]. Most imprinted clusters contain protein coding genes and noncoding RNAs (microRNAs, snoRNAs and lncRNAs), which are essential for mechanisms of imprinting regulation. X-inactive specific transcript (XIST) was the first lncRNA described to have functionality, and it is currently known that regulates X-inactivation mainly by binding polycomb-repressive complex 2 (PRC2) [72, 160]. Other well-studied lncRNAs that regulate imprinting include H19 [11], Airn [161] and KCNQ1OT1 [170].

Regulation of transcription

Nuclear lncRNAs play important tasks in the nucleus-modulating transcriptional regulation either in *cis* or in *trans*. A function as a scaffold or guides of histone modification complexes was first described by Chang and collaborators and confirmed later by genome-wide studies [59, 172]. In the last, by using RNA immunoprecipitation (RIP), the authors concluded that lncRNAs function as a cell-context-specific scaffold to guide protein complexes, such as the polycomb repressor complex followed by regulation of transcription [155]. Another described function for nuclear lncRNAs is their role as enhancers. The eRNAs are smaller than 2000 kb and are transcribed in correlation with their related gene. The mechanisms of eRNA action are not well understood, and several mechanisms have been proposed, including the facilitation of enhancer-promoter communication by promoting loop formation, the promoter remodeling via nucleosome depletion or acting as decoys for key transcription factors or transcription factor binding sites [17].

Nuclear organization

The genomic organization at the three-dimensional level may facilitate, at a short scale, the formation of loops that bring distant regulatory regions, enhancers and their specific targets into contact. At larger scales, the compaction of higher-order chromosomal domains may affect the accessibility to the transcriptional machinery [52, 96]. Several lncRNAs have been connected with the regulation of nuclear organization, including MALAT1, NEAT1 [77], XIST [50] and Firre [60]. Studies using chromatin conformation capture analysis may contribute to find new transcripts that modulate either local structures or higher-order structures [43].

Molecular sponges

Cytoplasmic lncRNA transcripts can induce changes in protein expression by acting as competing endogenous RNAs (ceRNAs) for miRNAs. Recently, the characterization of endogenous cirRNAs, which harbour a number of miRNA-binding sites, promised their function as molecular sponges [65]. This type of lncRNA is difficult to detect, but the improvement in sequencing technologies with the use of better algorithms for mapping RNA has enabled the identification of more candidates [82]. A number of linear lncRNAs have also been proposed as sponges, such is the case of the linc-MD1, which captures miR-133 to regulate muscle differentiation [25]. Interestingly, noncoding function of known coding mRNAs has been also described for the regulation of the tumour suppressor PTEN [169].

Coding for micropeptides

Recently, two independent groups reported the presence of small, conserved, open-reading frames in annotated lncRNAs that encode for functional micropeptides [5, 147]. It is probable that many annotated lncRNAs are indeed concealed mRNAs and their precise function is yet to be described.

Other functions

Another proposed function of lncRNAs is the regulation of protein localization and translocation between the nucleus and cytoplasm [189] and the regulation of coding genes stability [97, 184].

## *Long noncoding RNAs in SMC function*

Early in the 1990s, H19 was identified as a molecule that acts as a RNA product [19]. Shortly after its discovery, H19 was reported to be expressed in SMC during blood vessel development reaching low levels in adult vessels [62]. Following acute vascular injury or in atherosclerotic lesions, this lncRNA is upregulated [61, 90]. H19 gene is located immediately

downstream of insulin-like growth factor II (IGF2) and comprises 2.3 kb. Both IGF2 and H19 are imprinted in a reciprocal manner where the paternal chromosome transcribes IGF2 but not H19 and the maternal chromosome transcribes H19 but not IGF2. Apart from its involvement in imprinted regulation of IGF2, the function of H19 is intriguing. A number of reports have described H19 as either an oncogene or a tumour suppressor in different cell systems. In addition, it has been shown that miR-675 is contained within the first exon of H19, which is expressed specifically in the placenta to promote growth [89]. Recently, a role as a sponge for let-7 family of miRNAs has been reported [86]. H19 depletion promotes precocious skeletal muscle differentiation in vitro by influencing let-7 targets expression [86]. The function of H19 in pathological SMC is unknown, but in this regard, it has been described that let-7a blocks proliferation and migration in vitro and in vivo by targeting myc [21]. It is possible that high levels of H19 following vascular injury sequester let-7 miRNAs. In consequence, proliferation and migration is augmented through modulation of its targets.

Another lncRNA described in SMC is an overlapping antisense to NOS3 gene (NOS3 NAT-lncRNA). This lncRNA has a discordant expression with its sense NOS3, with higher levels of the lncRNA over NOS3, and they do not display colocalization in assays of in situ hybridization. Knockdown of NO3 NAT-lncRNA results in an increase of NOS3 expression, suggesting a posttranslational mechanism of regulation [156].

The lncRNA termed antisense noncoding RNA in the INK4 locus (ANRIL) is localized in the 9p21.3 region. This locus has been associated with genetic susceptibility for coronary diseases, intracranial aneurysms and type II diabetes in genome-wide association studies (GWAS) [146]. It has been reported that human SMC carrying single nucleotide polymorphism (SNP) variants in the ANRIL locus displayed elevated cell proliferation in vitro [138], and its deletion in mice confirmed this result [178]. ANRIL is a nuclear antisense lncRNA that regulates cell cycle genes in *cis* by recruiting the polycomb repressor complex.

The lncRNA-p21 is downregulated in the ApoE null model of atherogenesis and in human atherosclerotic lesions. This lncRNA represses SMC proliferation while its downregulation exacerbates the neointimal hyperplasia following acute injury. Mechanistically, the lncRNA-p21 interacts with the E3 ubiquitin protein ligase, which acts de-repressing p53-dependent target genes [191].

The transcript natural antisense to HIF1α (HIF1A-AS1) was found to be increased in serum of patients with aortic aneurysms, and its downregulation in SMC in vitro reduces the apoptotic genes caspase 3 and caspase 8 and increases BCL2 [207]. Another research group, found that HIF1A-AS1 regulates the expression of Brahma-related gene 1 (BRG1), a gene that is increased in thoracic aortic aneurysm, by an unknown mechanism [185].

Leung and collaborators reported the first study using RNA-seq technology to evaluate lncRNA in SMC derived from rat. They found that treatment with angiotensin stimulates expression changes in a number of lncRNAs, including the lncRNA-362. This transcript contains the miR-221/222 cluster. lncRNA-362 downregulation results in a reduction of miR-221/222 expression and a decreased SMC proliferation [101].

More recently, the Miano Lab reported the first lncRNA that seems to be specific of SMC and EC [12]. They screened human aortic SMC by RNA-seq and found a lncRNA antisense to the EC-restricted FLI1 gene which they called smooth muscle and endothelial cell enriched migration/differentiation-associated (SENCR) lncRNA. They confirmed the expression of two variants highly enriched in EC, SMC and in tissues like arteries, lung and skeletal muscle. SENCR knockdown in SMC led to a dedifferentiated phenotype with the downregulation of SMC markers and the increase of MDK and PTN, two pro-migratory genes. Simultaneous inhibition of these genes prevented the migratory phenotype induced by SENCR silencing, suggesting that these genes mediate SENCR action [12]. The mechanism by which SENCR regulate its targets is unknown. SENCR is localized in the cytoplasm and does not control the expression of FLI1. The study of protein-RNA association using pull-down assays may help to discern its function.

Genome-wide studies using microarray revealed a number of lncRNAs differentially expressed in varicose saphenous with respect to control veins. Many of the identified transcripts are antisense lncRNAs and display a concordant expression with their associated gene [106]. More recently, the authors demonstrated a correlation between reduced expression of the snoRNA-containing GAS5 in varicose veins with an enhanced proliferation and migration of SMC [102]. Mechanistically, GAS5 interacts with ANXA2, a calcium-dependent RNA binding protein. Simultaneous knockdown of ANXA2 with GAS5 rescue the proliferative /migratory phenotype, suggesting that ANXA2 mediates the function of GAS5 in SMC [102]. The NAT HAS2-AS1 that is transcribed opposite to HAS2, a hyaluronan synthase, was reported to directly mediate the transcription of its overlapping gene. The authors showed that O-GlcNAcylation stimulates HAS2-AS1 promoter activity by recruiting p65. In turn, HAS2-AS1 activates HAS2 transcription by promoting an open chromatin structure specifically in the promoter region of HAS2 [176]. The exact mechanism by which HAS2-AS1 exerts its role is unclear.

Recently, a number of putative ceRNAs have been identified for MYCD using human vascular samples from patients with intracranial aneurysm [205]. The authors concluded that depletion of ARGHEF12, FGF12 and ADCY5 transcripts resulted in the reduction of MYCD levels, in a miRNA-dependent manner.

**Table 2** Different known lncRNAs and their functions in SMC homeostasis

| lncRNA | Localization | Functions | References |
|---|---|---|---|
| H19 | NAT-lncRNA/cytoplasmic | Unknown | Han et al. [62] |
| ATG9B | NAT-lncRNA/? | (−) NOS3 | Robb et al. [156] |
| CDKN2B-AS1 | Antisense lncRNA/nuclear | (+) SMC proliferation ? | Motterle et al. [138] |
| MIR221HG | lncRNA/? | (+) SMC proliferation | Leung et al. [101] |
| SENCR | 5′Antisense lncRNA/cytoplasmic | (+) SMC differentiation/(−) SMC migration | Bell et al. [12] |
| HIF1A-AS1 | NAT-lncRNA/? | (+) SMC apoptosis | Zhao et al. [207] |
| HIF1A-AS1 | BRG1 | (+) SMC apoptosis/(−) SMC proliferation | Wang et al. [184] |
| lncRNA-p21 | lncRNA/nuclear | (−) SMC proliferation | Wu et al. [191] |
| HAS2-AS1 | NAT-lncRNA/? | Matrix regulation | Vigetti et al. [176] |
| GAS5 | lncRNA/cytoplasmic | (−) SMC proliferation/(−) SMC migration | Li et al. [102] |

Table 2 summarizes the lncRNAs genes described in SMC, and their suggested mechanism.

## Perspectives

In the next years, many other miRNAs will probably be identified as important players in the regulation of SMC homeostasis, given the recent identification of new miRNA transcripts [115]. The next step will be to elucidate more accurately the identification of target genes. This is complicated due to the fact that many miRNAs bind to its target genes in a non-canonical manner [69], difficulting their identification through bioinformatics analysis. Another important topic for future research is the identification of nucleotide variations in non-coding regions, including promoters, miRNA sequence, their target 3′UTR and lncRNA sequences and their functional impact in vivo. For example, a SNP that has been associated to cardiovascular disease located in the 3′UTR region of TCF21 creates a binding site for miR-224 which in turn suppresses its expression in human coronary SMC [134]. Other examples include the SNP in a susceptible locus for myocardial infarction that encodes for MIAT, a lncRNA with unclear functions [80], and variants in the ANRIL locus [138]. The study of "master regulators" of noncoding RNAs is another poor explored area of research that needs more attention. Finally, the exploration of other small ncRNAs is an underdeveloped field in the study of SMC differentiation. For example, we have observed that the small ncRNA YRNA3 regulates a number of SMC marker expression (unpublished observations). Interestingly, EC and platelets produce and secrete microparticles full of this small ncRNA (unpublished observations) and other RNA products that may influence SMC fate. Therefore, the study of the noncoding genome in the understanding of SMC phenotype modulation is still in its infancy. Clustered regulatory interspaced short palindromic repeats (CRISPR) technologies of genome editing will surely help to discern the function of noncoding regions in vivo [63]. The major challenge in understanding SMC homoeostasis is being able to integrate the whole genome regulation under normal physiological states and during the development of vascular diseases.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no competing interests.

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