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REVIEW

The short and long of noncoding sequences in the control of vascular cell phenotypes

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Abstract The two principal cell types of importance for normal vessel wall physiology are smooth muscle cells and endothelial cells. Much progress has been made over the past 20 years in the discovery and function of transcription factors that coordinate proper differentiation of these cells and the maintenance of vascular homeostasis. More recently, the converging fields of bioinformatics, genomics, and next generation sequencing have accelerated discoveries in a number of classes of noncoding sequences, including transcription factor binding sites (TFBS), microRNA genes, and long noncoding RNA genes, each of which mediates vascular cell differentiation through a variety of mechanisms. Alterations in the nucleotide sequence of key TFBS or deviations in transcription of noncoding RNA genes likely have adverse effects on normal vascular cell phenotype and function. Here, the subject of noncoding sequences that influence smooth muscle cell or endothelial cell phenotype will be summarized as will future directions to further advance our understanding of the increasingly complex molecular circuitry governing normal vascular cell differentiation and how such information might be harnessed to combat vascular diseases.

Keywords microRNA · Long noncoding RNA · Transcription factor binding site · Smooth muscle cell · Endothelial cell · Differentiation

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Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
EC	Endothelial cell
ENCODE	Encyclopedia of DNA Elements
ETS	E26 transformation specific
LncRNA	Long noncoding RNA
LSS	Laminar shear stress
Mir	MicroRNA
MYOCD	Myocardin
NAT	Natural antisense transcript
NICD	Notch intracellular domain
RACE	Rapid amplification of cDNA ends
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SRF	Serum response factor
TFBS	Transcription factor binding site

Introduction

The establishment and maintenance of a fully differentiated program of gene expression in endothelial cells (ECs) and subjacent smooth muscle cells (SMCs) are indispensable for vascular homeostasis. The unique gene expression signature of ECs and SMCs is attained through DNA-binding transcription factors, associated cofactors, and the TFBS to which such transcriptional complexes bind. In addition to protein-coding transcription factors, a growing number of noncoding RNA genes have been discovered that modify normal and stress-induced conditions of gene expression in ECs and SMCs. These molecular rheostats provide an important layer of regulatory control over a cell's transcriptome or proteome, often times by adjusting

the steady-state expression of key transcription factors. Variations in nucleotide sequence or altered expression or activity of transcription factors and/or noncoding RNA genes have unfavorable effects on normal EC and SMC differentiation and, hence, vascular homeostasis. For example, acute and chronic diseases of the vessel wall (e.g., restenosis and atherosclerosis) may be manifest by reduced expression of a critical SMC cofactor called myocardin leading to SMC phenotypic modulation, wherein the normal contractile program of gene expression is subverted to a less differentiated status and either adopts a synthetic state typified by excessive cell proliferation, migration, and matrix hyper-secretion or transdifferentiates into other cell types that can perturb normal vascular function [1–3]. Although there have been challenges to the notion of SMC phenotypic modulation [4, 5], strong evidence for such a phenomenon is emerging from studies using rigorous confocal microscopy and in vivo lineage tracing [2, 3, 6–8].

Do ECs similarly exhibit phenotypic modulation? It is well documented that pulsatile blood flow and attending laminar shear stress ($\sim$15 dynes/cm^2) promote a quiescent EC differentiated phenotype characterized by a gene expression program that enables normal vasoactivity, selective permeability, anti-thrombogenicity, and anti-inflammation. However, changes in laminar blood flow, such as the disturbed blood flow ECs encounter at critical branch points and curvatures of the vasculature, promote a less differentiated, pro-inflammatory EC phenotypic state, presumably through mechanosensory signals that destabilize normal EC gene expression [9]. In both cases of SMC and EC phenotypic modulation, there are antecedent changes in the molecular determinants governing normal patterns of gene expression such as altered levels or activity of DNA-binding transcription factors or associated cofactors (e.g., myocardin). Elucidating the transcriptional circuitry of SMC and EC gene expression and illuminating the basis for deviations in the homeostatic settings of such phenotypic states hold promise for the development of new diagnostic and treatment modalities for many vascular diseases.

Thanks in large part to efforts of the ENCODE (Encyclopedia of DNA Elements) Project Consortium [10–13], we have a broad understanding of functional sequences in the human genome as well as variations in nucleotide sequences that may disrupt normal cellular activity. Accordingly, the concept of "junk DNA" [14] has been challenged with the reality of "pervasive transcription" [15] as well as the prodigious number of functional regulatory elements throughout the human genome [16, 17]. Indeed, advances in genome science have yielded a bewildering number of noncoding sequences, including millions of regulatory elements and tens of thousands of noncoding RNA genes defined broadly as either short noncoding RNA (processed transcript length of less than 200 base pairs) or long noncoding RNA (processed transcript length of more than 200 base pairs). This review will highlight the biology and interplay of several noncoding sequences among three major classes (TFBS, microRNAs, and long noncoding RNAs) that control SMC and EC phenotype. Also discussed will be challenges that must be overcome to gain further insight into the function of vascular cell-associated noncoding sequences and existing opportunities for diagnostic or therapeutic intervention.

Transcription factor binding sites in vascular cells

The human genome is punctuated with millions of transcription factor binding sites (TFBS) that act as molecular zip codes for some 1400 transcription factors [18]. The combinatorial permutations for gene regulation at this level are staggering. Nevertheless, tools in computational biology and genomics have greatly advanced the historical and pain-staking reductionist approach of defining functional TFBS in vascular cells. Considered below are the major TFBS and associated transcription factors involved in the establishment and maintenance of vascular SMC and EC differentiation.

Smooth muscle cell TFBS

SMCs are derived from numerous anatomical sites during embryonic development and exhibit variations in both signaling inputs as well as functional outputs [19]. Despite such diversity in origin and function, there exists a gene expression signature that distinguishes differentiated SMCs from the remaining 250 cell types making up the mammalian body plan. The majority (25/42) of these genes harbor one or more functional CArG box, the consensus sequence of which is CC[A/T]$_6$GG (Table 1). The CArG box is the core sequence of the serum response element, initially described in the 5′ promoter region of the *Fos* proto-oncogene [20]. Subsequent work revealed conserved CArG boxes in the regulatory region of several contractile genes in sarcomeric muscle [21]. The CArG box binds the widely expressed serum response factor (SRF) [22]. Alterations in SRF expression or activity have been associated with a number of diseases across many organ systems, including the cardiovascular system [23].

There are more than 1200 permutations of the CArG box [24], and previous computational analyses have revealed thousands of conserved CArG boxes in the human genome [25, 26]. Validating the function of SRF-binding CArG boxes has been an important research objective. Historically, transgenic reporter mouse studies were done to

Table 1 SMC transcriptome and functional TFBS (number)

Gene symbol	Alias	CArG boxes	RBPJ sites	MEF2 sites	SMAD sites
MYH11	*SM-MHC*	Yes (5)	Yes (1)	Yes (1)	No
MYLKv7	*Telokin*	Yes (1)	No	No	No
SMTNB	***Smoothelin B***	**No**	**No**	**No**	**No**
HDAC8	***HDAC8***	**No**	**No**	**No**	**No**
KCNMB1	*Maxi-K-β1 subunit*	Yes (2)	No	No	No
ACTG2	*SM γ-actin*	Yes (5)	No	No	No
NOTCH3	***Notch3***	**No**	**No**	**No**	**No**
ITGA8	***α8-integrin***	**No**	**No**	**No**	**No**
PTK2v	***FRNK***	**No**	**No**	**No**	**No**
GLMN	***Glomulin***	**No**	**No**	**No**	**No**
LPP	*Lipoma preferred partner*	Yes (3)	No	No	No
CNN1	*SM-Calponin*	Yes (5)	No	No	No
LMOD1	*SM-Leiomodin*	Yes (2)	No	No	No
MIRN143/145	*miR-143/145*	Yes (1)	Yes (3)	No	Yes (1)
FHL2	*4.5 LIM domain 2*	Yes (1)	No	No	No
TGFB1I1	*HIC-5*	Yes (1)	No	No	No
SPEG	***APEG1***	**No**	**No**	**No**	**No**
SMTNA	*Smoothelin-A*	Yes (2)	No	No	No
ACTA2	*SM α-actin*	Yes (3)	Yes (1)	No	Yes (1)
ITGA1	*α1-integrin*	Yes (1)	No	No	No
EFNB2	***Ephrin B2***	**No**	**No**	**No**	**No**
TAGLN	*SM22α*	Yes (2)	Yes (1)	No	Yes (1)
HEY2	*CHF-1*	No	Yes (2)	No	No
CSRP1	*CRP1*	Yes (1)	No	No	No
MYOCD	*Myocardin*	No	No	Yes (1)	No
TPM2	*β-tropomyosin*	Yes (1)	No	No	No
MRF2A	***MRF2α***	**No**	**No**	**No**	**No**
BARX2	*Barx-2b*	Yes (1)	No	No	No
MEOX2	***Gax***	**No**	**No**	**No**	**No**
ELN	*Elastin*	No	No	No	Yes (2)
DES	*Desmin*	Yes (1)	No	Yes (1)	No
DMD	*Dystrophin*	Yes (1)	No	Yes (1)	No
HRC	*Histidine-rich calcium BP*	No	No	Yes (1)	No
PGM5	***Aciculin***	**No**	**No**	**No**	**No**
SRF	*SRF*	Yes (2)	No	No	No
MYLKv6	*smMLCK*	Yes (2)	No	No	No
CALD1	*Heavy caldesmon*	Yes (1)	No	No	No
VCL	*meta-vinculin*	Yes (1)	No	No	No
ACTN1	*a-actinin*	Yes (2)	No	No	No
AEBP1	***ACLP***	**No**	**No**	**No**	**No**
TPM1	*α-tropomyosin*	Yes (1)	No	No	No
ARHGAP42	***GRAF3***	**No**	**No**	**No**	**No**

assess the functional importance of CArG boxes in such SMC-restricted genes as *Tagln* [27], *Acta2* [28], *Telokin* [29], *Myh11* [30], *Csrp1* [31], *Kcnmb1* [32], *Cnn1* [33], and *Lmod1* [34]. These genetic studies offered strong support for the in vivo functionality of CArG boxes and in some cases resulted in the development of novel mouse strains that could direct transgene expression (e.g., Cre recombinase) in a SMC-restrictive manner [35, 36].

More recently, genome-wide studies have been carried out to demonstrate global SRF-binding to CArG elements,

albeit studies have been limited to only a few cell types (mostly immortalized cancer cell lines) analyzed under specific cell culture conditions. Thus, ChIP-seq experiments have established SRF-binding to thousands of CArG boxes, including those in proximity to non-contractile genes [37–39]. Many of these CArG boxes were computationally predicted based on the plasticity of this TFBS in what has come to be known as the CArGome [25, 26]; however, there are a number of ChIP-seq-derived SRF binding sites that do not conform to any of the >1200 permutations of the CArG box suggesting we still have much to learn about the binding rules for SRF to this class of TFBS [37, 40].

An important outgrowth of the CArGome has been the computational identification of CArG sequence variants, such as single nucleotide polymorphisms (SNPs). These CArG-SNPs may have consequences for target gene expression in disease states, including vascular disorders. For example, there is a CArG-SNP in the first intron of *KLF6* (rs10795076) that severely reduces SRF binding [26]. KLF6 is known to stimulate the pro-angiogenic factor, ALK1, in vascular cells following vascular injury [41]. Therefore, it would be of interest to know whether patients with poor angiogenic responses following myocardial infarction have reduced KLF6 due to the aforementioned CArG-SNP. To date, there are no annotated CArG-SNPs surrounding SMC contractile genes. Rare CArG-SNPs around SMC contractile genes probably do exist but their identification will require extensive sequencing across thousands of families. This "clan genomics" line of inquiry represents a powerful approach to personalized genomics because while the existence of private CArG-SNPs likely is rare, they would probably have a large effect on a phenotype [42]. Finally, it is possible that SNPs create functional CArG boxes in sequences that otherwise would not support SRF binding.

Several challenges and opportunities exist for the next generation of studies on the CArGome. First, we need to define CArG box function under various SMC phenotypic states using ChIP-seq coupled to RNA-seq following SRF knockdown. Second, the function of CArG boxes in pericytes, which have some attributes of SMC, is virtually unchartered territory as we are naïve to the gene expression profile of these cells. Third, there is a need to identify the SRF cofactor (among more than 60) facilitating CArG-dependent target gene expression under various conditions, including those related to perturbations in the SMC differentiated phenotype. Elegant ChIP-seq experiments from the Treisman lab revealed an interaction between SRF and the myocardin-related transcription factors in the serum-induced response of murine fibroblasts [43]. These and other comprehensive genomic studies will provide new and perhaps unexpected findings that will require more reductionist approaches to address such matters as linking SRF-bound CArG boxes to their respective target gene and defining whether a CArG box is functionally important for target gene expression in live animals. The advent of chromatin conformation capture assays [44] and precision-guided genome editing with clustered regularly interspaced short palindromic repeats (CRISPR) technology [45] offer innovative and powerful approaches to study the functionality of CArG boxes in their native genomic milieu. For example, CRISPR-mediated genome editing of a consensus intronic CArG box in the SMC-restricted *Cnn1* gene nearly abolished this gene's mRNA and protein expression in SMCs of the vessel wall [46]. Although other SMC differentiated markers were unaffected by the near abrogation of CNN1 expression, a moderate increase in SMC DNA synthesis was noted suggesting an important, yet poorly understood, role for CNN1 in the maintenance of a SMC quiescent state. The marked reduction of CNN1 upon mutation of an intronic CArG box is also seen in visceral SMCs and subjacent vascular SMCs (Fig. 1). These results, representing the first ever application of precision-guided genome editing of a regulatory element in an animal

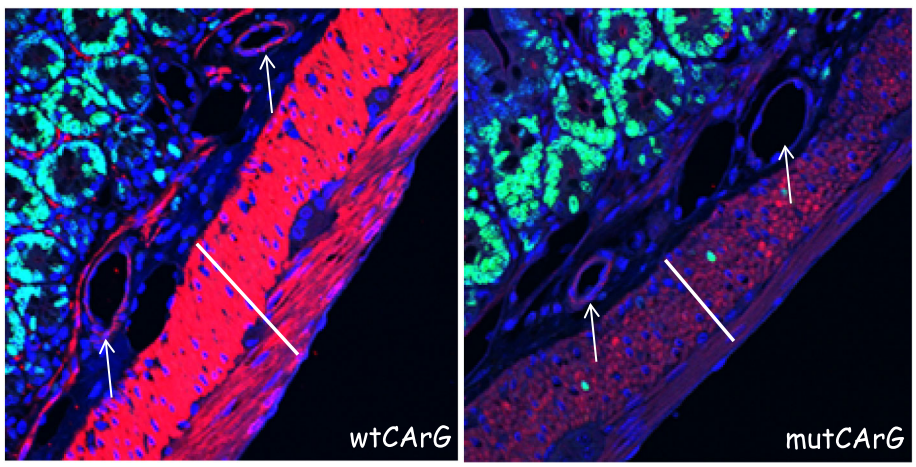


Fig. 1 CRISPR editing of a TFBS. Immunofluorescence staining for CNN1 protein (*red*) in vascular SMCs (*white arrows*) and visceral SMCs (*white lines*) of small intestine in mice carrying a wildtype CArG box (*left*) or a mutant CArG box (*right*). Note the presence of proliferating visceral SMCs at right (*green* nuclei)

model, offer definitive genetic proof for the importance of a CArG box in the control of an SRF target gene [46]. CRISPR will likely be the method of choice over traditional approaches (e.g., lacZ reporter, Cre-lox excision) to the functional study of enhancers and TFBS in living animals.

CRISPR technology will also inform us as to the in vivo function of CArG boxes shown previously to be dominant over other, neighboring CArG boxes adjacent to such SMC-restricted genes as *Acta2* [28], *Tagln* [27], and *Actg2* [47]. Moreover, CRISPR will be instrumental in modeling CArG-SNPs such as the intronic CArG-SNP in the *KLF6* gene in animal models [26]. The latter approach represents an exciting new chapter in genetics because traditional genome wide association studies have defined hundreds of non-coding regulatory variants, but rarely define the functional importance of the sequence variant.

The manner in which CArG-SRF directs SMC gene expression was solved with the seminal discovery of myocardin (MYOCD) in Eric Olson's laboratory [48]. MYOCD is one of nature's most powerful coactivators of gene expression [48]. Notably, whereas the conversion of fibroblasts to specific differentiated cell types (e.g., cardiomyocytes) required a cocktail of several gene products [49], conversion of similar cells to SMCs necessitates the ectopic expression of only MYOCD [50–52]. MYOCD can also program human embryonic stem cells to adopt a functional SMC state [53]. Thus, the CArG-SRF-MYOCD triad is the primary transcriptional switch directing the SMC differentiated state. Of note, not all genes expressed in differentiated SMCs contain functional CArG boxes (bold genes in Table 1), and MYOCD is not sufficient to activate some of these [53, 54], although at least one SMC-restricted gene (*ITGA8*) appears to be activated by MYOCD in a CArG-SRF-independent manner [55]. Understanding how MYOCD performs such SRF-independent actions of transcription is an open area for future investigation. One of the challenges in studying MYOCD is the lack of reliable antibodies to assess endogenous protein expression under various experimental conditions such as vascular injury responses. CRISPR may be of value in this context for epitope tag targeting of the endogenous *Myocd* locus.

Although the CArG-SRF-MYOCD triad is the dominant driver of SMC differentiation [56], parallel signaling pathways exist that also converge upon the genome to facilitate SMC differentiation associated gene expression. For example, the NOTCH intracellular domain (NICD) is a cofactor that interacts with the DNA binding transcription factor, Recombination signal Binding Protein for immunoglobulin kappa J region (RBPJ), to control the SMC contractile gene program [57, 58]. The core consensus TFBS for RBPJ is TGG/AGAA and recent ChIP-seq experiments revealed the presence of both NICD-dependent and NICD-independent RBPJ binding sites [59]. Key NICD-RBPJ target genes in SMC include *ACTA2* [60], *MYH11* [61], and the microRNA143/145 gene [62]. The latter genes also contain CArG boxes (Table 1) so there may be synergistic pathways of SMC target gene activation at these gene loci. The *SMTNB* isoform in the *Smoothelin* locus is highly specific to vascular SMCs and lacks functional CArG boxes [63], but is activated by NOTCH signaling [61]. Formal genetic proof for a functional RBPJ binding site near the *SMTNB* locus awaits further study. Recently, the NOTCH ligand, JAG1, was shown to mediate SMC differentiation in the developing mouse embryo by repressing a chondrogenic program of cell differentiation within progenitor cells of the sclerotome; inactivating JAG1 resulted in reduced SMC contractile gene expression and the emergence of several chondrocytic markers [64]. MYOCD can repress the skeletal muscle program of gene expression and promote a SMC contractile phenotype in progenitors of the same sclerotomal compartment of the somite [65]. Together, these studies underscore the existence of parallel transcriptional circuits for the establishment of differentiated SMCs derived from a specific developmental niche.

In addition to CArG and RBPJ sites, several SMC genes contain functional SMAD-binding elements (consensus site is GTCTAGAC) that are the convergence points of TGFβ1 signaling [66] or MEF2 binding sites (consensus element is CTA[A/T]$_4$TAG) which regulate genes in all three muscle lineages [67] (Table 1). Gene inactivation studies have implicated SMAD4 and MEF2C in the proper differentiation of SMCs [68, 69]. Although the CArG and MEF2 TFBS have related sequences, and the associated DNA-binding transcription factors harbor similar functional domains [70], SRF and MEF2 do not exhibit reciprocal DNA-binding activities across CArG and MEF2 elements, nor do they physically interact. Collectively, there are, in addition to CArG-SRF-MYOCD, additional TFBS and associated complexes that can direct at least some aspects of the SMC differentiated state. Important future experiments include elucidating all RBPJ, SMAD, and MEF2 binding sites and associated cofactors during SMC differentiation through ChIP-seq. One important way to filter potentially functional TFBS is through comparative genomics against other species. This has been particularly powerful in defining functional CArG boxes across different animal species [25, 26]. There will also likely be SNPs within MEF2, SMAD, and RBPJ binding sites that could alter SMC gene expression. In this context, a refined comparative genomics tool called phylogenetic module complexity analysis was used to elucidate the function of a TFBS variant in the *PPARG* gene that appears to contribute to Type II diabetes [71]. This and other emerging technologies will be

important to ascertain the clinical relevance of TFBS variants associated with vascular SMC-related disorders.

Endothelial cell TFBS

Like SMCs, ECs have diverse embryonic origins and functionality [72]. However, whereas conversion of cells to a SMC-like phenotype can occur through transient ectopic expression of just one factor (MYOCD), several transcription factors and signaling pathways appear necessary for conversion of cells to an EC-like phenotype, possibly because of the common developmental origin EC progenitors share with those of hematopoietic precursors [73]. Moreover, in vitro specification of some ECs occurs only after protracted periods of time under defined culture conditions. For example, induced pluripotency stem cell factors (e.g., KLF4 and OCT4) can convert human fibroblasts into ECs when cells are primed with various EC growth factors such as VEGF or basic FGF over many weeks [74]. Another report found that an abbreviated treatment protocol resulted in the induction of a protein known as SETSIP, which translocates into the nucleus and binds to and activates the *CDH5* promoter within 1 week of conversion of cells to an EC phenotype [75]. Further, several members of the E26 transformation specific (ETS) family of DNA-binding transcription factors (e.g., ETV2, ERG1, and FLI1) could convert amniotic cells into ECs without traversing a pluripotency state if TGFβ signaling was suppressed [76]. More recently, Morita et al. [77] screened 18 transcription factors in a human fibroblast cell line (HFL-1) and discovered ectopic ETV2, in coordination with endogenous FOXC2, was sufficient for EC conversion; addition of VEGF and basic FGF to the culture medium augmented expansion of functional ECs. These latter findings will need to be replicated but from this discussion, it is clear that specification of the EC lineage is complex and not quite as straightforward as that of the MYOCD-directed program of SMC gene expression. Nevertheless, the same approaches used to define TFBS governing SMC differentiation have been employed to define the function of TFBS in ECs and this line of work will be summarized next.

Much of what we have learned about transcriptional control of EC-restricted gene expression originates from transgenic mouse studies using the lacZ reporter gene under control of promoter/enhancer sequences of candidate EC-specific genes. Hence, EC-restricted promoter/enhancer activity has been demonstrated in mice for sequences adjacent to *Tek* [78], *VWF* [79], *Kdr* [80], *Cdh5* [81], *Tie1* [82], *Acvrl1* [83], *PROCR* [84], *ROBO4* [85], *FLT1* [86], and *Dll4* [87]. As in the SMC field, these transgenic reporter mouse studies paved the way for the development of new and improved mice that can inducibly express transgenes in ECs [88]. Experimental characterization of sequences around EC regulatory regions revealed functional TFBS such as GC-rich binding sites for SP1 [83, 85] and EGR1/3 [86], a site for HIF2A [80], and a composite GATA/TAL1 element [84]. Recently, a functional CArG box was suggested to direct myocardin-related transcription factor binding to and activation of the *CDH5* gene in human ECs [89]. The latter result, which will require validation in mice, is consistent with the established role of SRF in maintaining normal EC homeostasis in vivo [90].

Several reports have defined functional ETS elements that control EC-restricted gene expression [83–87]. The ETS family of transcription factors, with more than 25 members, is, like SRF, widely expressed and controls disparate programs of gene expression. However, a subset of ETS factors (ETS1, ETS2, FLI1, ERG, ETV6, ELK3, and ETV2) is of particular importance for EC differentiation and embryonic vascular formation [91]. These family members recognize a core DNA consensus sequence of GGAA/T, a regulatory element that is nearly ten times more frequent in the human genome than the CArG or MEF2 elements. How such a prevalent TFBS confers EC-specific gene expression is complex, but likely relates to a combination of binding partners, their post-translational modification, and flanking sequence content [91]. The latter is relevant to this discussion as an elegant computational study discovered a disproportionately large number of EC-restricted genes harboring a composite ETS-FOXC2 binding site [92]. More than 1500 composite ETS-FOXC2 sites exist in the reference human genome [92]. Recently, a composite ETS-FOXC2 element was characterized in the *ECE1* gene and shown to be critical for enhancer activity in transgenic mice [93]. The *ECE1* gene encodes for endothelin-converting enzyme, a protein that catalyzes the formation of one of nature's most powerful vasoconstrictors [94]. Interestingly, the *ECE1* enhancer drives arterial EC-specific activity and is co-dependent on a conserved SOX17 TFBS [93]. This highlights the subtle differences that can exist between genes similarly regulated by a common TFBS as a means to direct appropriate levels of expression of a target gene in sub-types of a specific cell lineage, such as arterial EC. Other ETS-containing EC-restricted promoters that exhibit arterial EC-specific activity include *Acvrl1* [83] and *Dll4* [87]. Whether a specific TFBS exists that directs venular EC-specific promoter/enhancer activity is presently unknown.

Just as CArG-SRF regulates a large number of SMC-restricted genes, ETS factors bound to ETS elements are dominant in controlling EC-specific genes (26/39 in Table 2). Conversely, not all EC-restricted genes harbor ETS or composite ETS-FOXC2 elements implying there

Table 2 EC transcriptome and functional TFBS (number)

Gene symbol	Alias	ETS-FOXC2 site	ETS site	KLF2 site	MEF2 site	CArG box
KDR	*VEGFR-2*	Yes (3)	Yes (3)	Yes (1)	No	No
CDH5	*VE-Cadherin*	Yes (1)	Yes (2)	No	No	Yes (1)
DLL4	*Delta-like 4*	Yes (1)	Yes (1)	No	Yes (1)	No
TEK	*TIE2*	No	Yes (2)	No	No	No
TAL1	*SCL*	Yes (1)	Yes (1)	No	No	No
NFATC1	***NFAT2***	**No**	**No**	**No**	**No**	**No**
TIE1	*TIE1*	No	Yes (1)	No	No	No
VWF	*Von Willebrand factor*	No	Yes (1)	No	No	No
TAL1	*SCL*	Yes (1)	Yes (1)	No	No	No
PECAM1	***CD31***	**No**	**No**	**No**	**No**	**No**
PDGFRB	*PDGFR-1*	Yes (1)	Yes (1)	No	No	No
NOS3	*eNOS*	No	Yes (1)	Yes (1)	No	No
NFATC4	***NFAT3***	**No**	**No**	**No**	**No**	**No**
MMRN1	*Emilin-4*	Yes (1)	Yes (1)	No	No	No
MEF2C	*MEF2C*	Yes (1)	Yes (1)	No	No	No
LMO2	*RHMO2*	No	Yes (3)	No	No	No
KLF2	*LKLF*	No	No	No	Yes (1)	No
FLT1	*VEGFR-1*	No	Yes (1)	No	No	No
ESM1	*Endocan*	Yes (2)	Yes (2)	No	No	No
FLI1	*EWSR2*	No	Yes (3)	No	No	No
EPHB4	***Ephrin Receptor B4***	**No**	**No**	**No**	**No**	**No**
ENG	*Endoglin*	Yes (1)	Yes (3)	No	No	No
EDN1	***Endothelin-1***	**No**	**No**	**No**	**No**	**No**
S1PR1	*EDG1*	No	No	Yes (1)	No	No
S1PR3	***EDG3***	**No**	**No**	**No**	**No**	**No**
CD34	*CD34*	Yes (1)	Yes (2)	No	No	No
ANGPT1	*Angiopoietin-1*	No	Yes (3)	No	No	No
ANGPT2	*Angiopoietin-2*	No	Yes (3)	No	No	No
ROBO4	*MRB*	No	Yes (1)	No	No	No
EGFL7	*VE-Statin*	No	Yes (2)	Yes (1)	No	No
THBS1	***Thrombospondin-1***	**No**	**No**	**No**	**No**	**No**
NRP1	*Neuropilin-1*	Yes (1)	Yes (1)	No	No	No
THBD	*Thrombomodulin*	No	Yes (3)	No	No	No
PODXL	***PCLP-1***	**No**	**No**	**No**	**No**	**No**
CLDN5	***Claudin-5***	**No**	**No**	**No**	**No**	**No**
ECE1	*Endothelin converting enzyme*	Yes (1)	No	No	No	No
PROCR	*EPCR*	No	Yes (2)	No	No	No
ACVRL1	***ALK1***	**No**	**No**	**No**	**No**	**No**
NOTCH4	*NOTCH4*	No	Yes (4)	No	No	No

exists parallel transcriptional circuits for the differentiation of committed cells to the EC lineage [92] (Table 2). For example, laminar shear stress (LSS) is an essential biomechanical signaling mediator for EC gene expression that antagonizes diseases of the vessel wall such as atherosclerosis [95]. One of the EC-restricted genes lacking functional ETS sites and induced with LSS is KLF2 [96]. KLF2 promoter analyses in vitro support a proximal MEF2 binding site as the LSS-responsive element [97]. KLF2 binding sites (GCCGGG) are similar to those of SP1 and EGR family members; the latter can also be induced by LSS in ECs to direct specific patterns of gene expression [98]. The existence of multiple GC-binding factors complicates the distinction between those factors binding one GC-rich TFBS over another. For example, early in vitro experiments provided evidence for a role of SP1 in the

transcriptional regulation of *NOS3* [99]. Subsequent work, however, revealed *NOS3* to be a direct transcriptional target of LSS-induced KLF2 [100]. This emphasizes the need for deeper analyses of all TFBS in ECs and their binding partners in the regulation of EC gene expression under normal and pathological states. ChIP-seq coupled to RNA-seq following knockdown of a specific ETS factor and determining whether these or other transcription factors synergistically activate EC gene expression will be informative. Further, it will be important to pinpoint regulatory SNPs that fall within EC-restricted genes and ascertain whether such regulatory variants are associated with alterations in EC phenotype. Finally, several EC-restricted genes do not contain any of the major EC-associated TFBS (bold genes in Table 2) and have not been rigorously analyzed in an in vivo setting. Thus, it will be imperative to delineate control elements governing expression of these less characterized EC-restricted genes and determine functional regulatory variants therein.

Summary

Vascular cell identity and function is conferred by distinct TFBS and associated protein complexes that drive unique signatures of gene expression in SMCs and ECs. Traditional means of analyzing vascular cell TFBS such as CArG boxes and ETS elements have been rather arduous and artificial. The revolutionary CRISPR system of genome editing will soon be commonplace in most laboratories to test the in vivo functionality of vascular cell TFBS in their native genomic milieu as well as clinically significant regulatory variants that may disturb normal SMC/EC-restricted gene expression. Ultimately, it may be instructive to screen patients for the presence of TFBS-SNPs as a means of sub-classifying disease states or responsiveness to drug therapies. Where specific vascular TFBS-SNPs contribute to a disease process, genome editing with CRISPR in patient-derived induced pluripotent stem cells may hold therapeutic promise.

Short noncoding RNA genes in vascular cells

Several classes of short noncoding RNA genes exist in mammalian genomes, including small interfering RNAs, small nuclear RNAs, small nucleolar RNAs, and microRNAs. As very little published work exists with respect to most short noncoding RNA classes in vascular cells, this review will focus on the microRNA class only. MicroRNA (miR) genes reside within introns or exons of protein coding or long noncoding RNA genes or between two gene loci, either as single or clustered, co-transcribed miRs. The biogenesis of miR genes is increasingly understood and involves step-wise trimming from a primary transcript to fully mature 20–23 nucleotide guide and passenger strand RNAs through the sequential action of Drosha/DGCR8 and Dicer [101]. The importance of these miR processing proteins for normal SMC [102–104] and EC [105, 106] phenotype has been firmly established. Mature miR transcripts almost always exert their action on target genes through either mRNA destabilization (increasingly understood) or translational repression (less understood) via Watson-Crick base pairing between the 5′ seed sequence of the miR plus a variable number of 3′ nucleotides and the target mRNA sequence found predominantly in the 3′ untranslated region of coding genes. The bias for functional miR binding sites in the 3′ untranslated region appears to relate to the interference of miR activity by active translation [107]. The net effect of most miR gene products is attenuated vascular cell gene/protein expression, though notable exceptions exist. Here, we provide an up-to-date review of several SMC- and EC-associated miR genes that are of significance in regulating the phenotype of each cell type.

Smooth muscle cell miR genes

The initial foray into SMC miR gene discovery was done in the laboratory of Chunxiang Zhang who used microarray to define differentially expressed miR genes in normal versus balloon-injured rat carotid artery [108]. This screen revealed that some miR genes are down-regulated (e.g., miR-145) and others up-regulated (e.g., miR-21) following arterial injury. Inhibiting expression of miR-21 with antisense oligonucleotides blunted the neointimal response in vivo and reduced proliferation of cultured SMCs through elevations in programmed cell death or the de-repression of PTEN [108]. In contrast, another group showed miR-21 to promote human pulmonary artery SMC differentiation via BMP4 or TGFβ1 signaling pathways [109]. These disparate findings are commonplace in biology and underscore the context-dependent effects that occur across different species and types of SMC, particularly those grown in vitro. The miR-221/222 gene cluster was then shown to be induced by growth factors and to mediate SMC proliferation in vitro and in vivo, presumably through the targeting of key negative growth regulators [110, 111]. Another miR gene that promotes SMC proliferation is miR-146a, and reducing this miR gene's expression could attenuate the neointimal response to injury [112]. The miR-130a gene stimulated vascular SMC proliferation in the context of hypertension, and the growth-arrest homeobox gene product, MEOX1, was shown to be a target of miR-130a [113]. In addition to proliferation, miR genes have been demonstrated to regulate SMC inflammatory phenotypes [114, 115] or help mediate transitions to another cell

type [116]. It is relevant to point out that many of the aforementioned miR genes have yet to be inactivated in vascular SMCs, which is necessary to gain definitive proof for their importance in controlling vascular SMC phenotypes. Nevertheless, there is much interest in using miRs as biomarkers of vascular disease and/or developing therapeutic strategies to blunt their action [117]. One of the challenges in blocking the action of a miR is the unanticipated consequence related to the broad spectrum of mRNA targets that any one miR can bind and negatively regulate.

There are a number of miR genes that counteract the growth promoting effects of miR-21, miR-221/222, and miR-146 in SMCs as well as the miR-mediated SMC pro-inflammatory and transdifferentiating phenotypes. For example, MYOCD induces miR-1, a weakly expressed miR gene in SMCs that appears to inhibit SMC proliferation possibly through its repression of the *Pim1* target mRNA [118]. There is also some evidence to support miR-1 directing the differentiation of ES cells into a SMC-like phenotype by targeting KLF4 [119]. On the other hand, miR-1 knockout mice exhibit aberrant SMC differentiation marker expression in the developing heart because of sustained expression of a miR-1 target mRNA, *Myocd* [120, 121]. These results provide insight into how the expression of several CArG-SRF-MYOCD-dependent SMC markers in the heart is attenuated during embryonic development and point to the in vivo interplay that exists between TFBS, miR genes, and the targets of mature miR transcripts.

There are two virtually identical miR-1 genes located on separate chromosomes and each is co-transcribed with highly homologous miR-133a genes. In humans, the miR1-1/miR-133a-2 and miR-1-2/miR133a-1 gene pairs are contained within introns of longer "host genes" (http://genome.ucsc.edu/). Though each miR-133a and miR-1 pair of paralogs is highly similar in sequence, miR-1 and miR-133a exhibit low sequence homology indicating non-overlapping mRNA targets. The induction of both miR-1 genes by MYOCD occurs through conserved SRF-binding CArG boxes located in enhancers that drive expression primarily in cardiac and skeletal muscle cells [122].

As for miR-133a, there are conflicting reports on its role in SMC phenotypic control. An early report from the Olson lab [123] showed that suppression of miR-133a resulted in aberrant expression of SMC markers in the developing heart, much like the aforementioned results seen with miR-1 inactivation. On the other hand, levels of miR-133a have been demonstrated to correlate with the differentiation of adult vascular SMCs, and miR-133a appears to target the SP1 transcription factor [124], a known repressor of the gold standard marker for SMC lineages, *Myh11* [125]. Accordingly, the down-regulation of SP1 upon miR-133a overexpression had the effect of increasing *Myh11*; however, several other CArG-SRF-dependent SMC genes such as *Cnn1* and *Acta2* were reduced [124]. This highlights rare uncoupling of the expression of markers for the SMC differentiated state. Gain-of-function experiments showed that miR-133a suppressed SMC proliferation and migration, two key processes in the development of vascular lesions [124]. Further studies showed that neointimal formation could be attenuated with miR-133a and exacerbated with anti-miR-133a [124]. While miR-133a expression was noted in vascular SMCs and a clear effect on phenotype was seen upon manipulating its expression, levels of miR-1 were not detected. This raises the challenge of elucidating the differential stability of co-transcribed miRs and the variation in stability of a mature miR transcript under different biological conditions.

Another MYOCD-induced miR defined in the context of SMC phenotypic modulation is miR-29a [126]. This miR transcript reduces SMC migration, an effect proposed as a mechanism for lower neointimal formation seen in the injured arterial wall of mice transduced with adenovirus carrying MYOCD. Evidence was provided to support the *Pdgfrb* mRNA as a probable target of miR-29a suggesting the observed sessile SMC phenotype stems from miR-29a targeting this growth factor receptor [126]. Inhibition of neointimal formation with MYOCD overexpression provides support for harnessing this cofactor as a therapeutic intervention for the treatment of vascular disorders, and recent experimental data support this concept [127].

One of the most studied SMC miR genes is the bicistronic cluster comprising miR-143 and miR-145. This gene cluster, as with an increasing number of other miR genes, lies within a long noncoding host gene (http://genome.ucsc.edu/, see below). Both miR-143 and miR-145 are co-transcribed in an SRF-dependent manner through a conserved upstream CArG box [128, 129] as well as binding sites for SMAD3/4 [130] and RBPJ [62] (Table 1). The miR-143/145 gene cluster is highly specific for SMCs [129, 131] and promotes SMC contractile gene expression through the repression of several transcription factors that antagonize the SMC contractile gene program [128, 132]. An important aspect of the SMC contractile phenotype is the expression of numerous ion channels and the establishment of proper currents across the SMC membrane. Evidence shows that miR-145 controls key ion channel genes in SMCs [104, 133]. Levels of miR-143/145 are reduced under conditions of SMC phenotypic modulation as seen in acute vascular injury or atherosclerosis [128, 132] and this likely contributes to the attending decrease in SMC contractile markers. On the other hand, levels of miR-145 are elevated in both experimental and clinical pulmonary arterial hypertension. In the experimental setting, disease progression can be attenuated when levels of

miR-145 are reduced either genetically or pharmacologically [134]. The disparate direction of miR-145 expression under conditions of SMC phenotypic modulation highlight important differences in the pathogenesis of arterial disease and suggest unique pathways of activation or transcript stability worthy of future study.

It is interesting to note that miR-145 may directly bind and activate *Myocd* mRNA through a site in the 3′ untranslated region [128]. Similarly, miR-34a appears to bind and activate one of its target mRNAs, SIRT1, and this deacetylase goes on to bind and activate SMC promoters in a CArG-dependent fashion [135]. The miR-34a transcript is associated with SMC differentiation of both mouse and human embryonic stem cells and appears to function upstream of the SRF-MYOCD molecular switch [135]. MicroRNA-mediated activation of target mRNAs is unusual given the near universal repressive mode of action of miRs on target mRNA sequences. The precise mechanism by which miR-145 and miR-34a promote activation of their respective target mRNAs is unknown; possibilities include mRNA stabilization through displacement of mRNA destabilization factors or enhanced translation. There are likely other miR genes with direct activation properties similar to those of miR-145 and miR-34a, particularly given the recent surge in miR gene identification [136].

Ectopic expression of miR-145 reduces neointimal burden in vivo [132, 137, 138] and partially rescues the reduction in SMC contractile genes seen with loss of Dicer in vitro [104]. Several independent knockouts of miR-143/145 have been carried out with varying results [129, 131, 137]. In general, loss in miR-143/145 compromised expression of SMC contractile genes and caused a mild hypotensive state. On the other hand, there were observed differences in lesion formation; two studies reported mild increases in neointimal formation [131, 137] whereas another study showed the lack of a neointima upon acute vascular injury [129]. Variations in the phenotype of miR-143/145 null mice likely relates to genetic background, the mode of vascular injury, and perhaps subtle differences in the loss of genetic information around the locus following Cre-mediated excision. The effect of miR-34a on the neointimal response to injury is unknown; however, there may be no effect since the influence of miR-34a on SMC differentiation is only seen in the context of embryonic stem cell differentiation to the SMC lineage [135]. Given the effects of miR-145, particularly, on SMC phenotype and vascular lesion formation, there may someday be a direct test of its efficacy in limiting lesion formation [138]. Challenges exist, however, in fully elucidating the targets of miR-145 (or any miR brought to the clinic) in order to gauge potential off targets and attending toxicity issues. Other miR genes that effect changes in the SMC differentiated state include miR10a [139], miR-1 [140], and miR-26a [141]. Table 3 summarizes many miR genes implicated in SMC differentiation and phenotypic changes associated with vascular disease.

Endothelial cell miR genes

The initial reporting of EC-associated miR genes was from the laboratory of Giuseppe Rainaldi who used a microarray to define 15 abundantly expressed miR genes in human umbilical vein ECs, including miR-221/222 which appeared to be necessary for normal EC migration and tube formation through the repressive action on KIT [142]. Since laminar shear stress (LSS) is central to a normal EC phenotype, there has been much work devoted to the definition of miR genes responsive to changes in blood flow [143, 144]. For example, the miR-101 and miR-19a genes are induced by LSS and promote a less proliferative phenotype in ECs by repressing expression of MTOR and CCND1, respectively [145, 146]. The SMC enriched miR-143/145 cluster is induced by LSS in a KLF2-dependent manner and is transported via exosomes from ECs to SMCs to confer an atheroprotective SMC phenotype [147]. AMP activated protein kinase alpha 2 subunit phosphorylates p53 which appears to up-regulate miR143/145 in ECs via post-transcriptional processing [148]. Elevated EC miR-143/145 targets angiotensin-converting enzyme, thus, maintaining normal vascular homeostasis [131, 148]. Remarkably, TGFβ stimulates the transfer of miR-143/145 from SMCs to ECs through "tunneling nanotubes" and this has the effect of reducing EC proliferation and tubulogenesis thereby implicating the miR-143/145 cluster in the control of angiogenesis [149]. MicroRNA-10a, which is also active in SMCs (Table 3), is induced by LSS and exerts an anti-inflammatory phenotype in ECs by targeting regulators of the IκBα gene. Accordingly, miR-10a levels are low in atherosusceptible regions of the vasculature where disturbed flow patterns exist [150]. Recently, miR-10a was shown to be transferred from quiescent ECs to monocytes where the NF-kB pathway of inflammation could be suppressed [151]. Thus, the movement of mature miRs (and other RNA products) from one vascular cell type to another cell type represents a juxtacrine/endocrine signaling mechanism of fine-tuning local or remote programs of gene expression that may function to maintain or perturb vascular homeostasis.

There are some miR genes whose expression is reduced with LSS leading to EC phenotypic modulation. For example, LSS-mediated attenuation of miR-663 is linked to increased monocyte adhesion to ECs, and blocking miR-663 with a locked nucleic acid antagonist reversed this phenotype [152]. The EC enriched miR-92a gene can also be reduced with LSS leading to elevations in KLF2, a

Table 3 SMC miR function and targets

miR	Function	Target mRNA	Species	References
1	↓ SMC proliferation	*PIM1*	*Hsa*	[118]
1	↑ SMC differentiation in ES cells	*Klf4*	*Mmu*	[119]
10a	↑ SMC differentiation in ES cells	*Hdac4*	*Mmu*	[139]
124	↓ SMC proliferation/↑ SMC differentiation	*Nfatc1*	*Hsa*	[235]
125b	↑ SMC inflammation	*Suv39h1*	*Mmu*	[236]
130a	↑ SMC proliferation	*Meox1*	*Rno*	[113]
132	↓ SMC proliferation/↓ neointima	*Lrrfip1*	*Rno*	[237]
133a	↓ SMC proliferation/↓ SMC migration	*Sp1*	*Rno*	[124]
138	↓ SMC apoptosis	*Mst1*	*Rno*	[238]
143/145	↓ SMC proliferation/↑ SMC differentiation	*Kfl4/Elk1/Camk2d*	*Mmu*	[128]
143/145	↑ SMC differentiation/↓ neointima	*Kfl5*	*Rno*	[132]
143/145	Cytoskeletal dynamics	*Klf4/Klf5/Add3*	*Mmu*	[129]
146a	↑ SMC proliferation/↑ neointima	*Kfl4*	*Rno*	[112]
195	↓ SMC proliferation/↓ SMC migration	*CDC42/CCND1*	*Hsa*	[239]
200	↑ SMC inflammation in diabetes	*Zeb1*	*Mmu*	[114]
204	↓ SMC proliferation/↑ SMC apoptosis	*SHP2*	*Hsa*	[240]
205	↓ SMC calcification	*Runx2*	*Hsa*	[241]
206	↓ SMC proliferation/↑ SMC differentiation	*Notch3*	*Hsa*	[242]
21	↑ SMC differentiation	*PDCD4*	*Hsa*	[109]
21	↓ SMC differentiation/↑ neointima	*Pten*	*Rno*	[108]
210	↓ SMC apoptosis	*E2F3*	*Hsa*	[243]
221/222	↑ SMC proliferation/↓ SMC differentiation	*KIT/CDKN1B*	*Hsa*	[110]
221/222	↑ SMC proliferation/↑ neointima	*Cdkn1b/Cdkn1c*	*Rno*	[111]
22	↑ SMC differentiation	*Mecp2*	*Mmu*	[244]
224	?	*TCF21*	*Hsa*	[168]
24	↓ SMC inflammation/↓ SMC migration	*CHI3L1*	*Hsa*	[115]
26a	↑ SMC proliferation/↓ SMC differentiation	*SMAD1*	*Hsa*	[141]
29a	↓ SMC migration	*Pdgfrb*	*Mmu*	[126]
30b/30c	↓ SMC calcification	*RUNX2*	*Hsa*	[116]
31	↑ SMC proliferation	*Lats2*	*Rno*	[245]
31	↓ SMC differentiation	*CREG*	*Hsa*	[246]
322	↓ SMC proliferation/↓ SMC migration	*Ccnd1/Ppp3 cc*	*Rno*	[247]
34a	↑ SMC differentiation	*Sirt1*	*Mmu*	[135]
365	↓ SMC proliferation	*Ccnd1*	*Rno*	[248]
638	↓ SMC proliferation/↓ SMC migration	*NR4A3*	*Hsa*	[249]
663	↓ SMC proliferation/↑ SMC differentiation	*JUNB*	*Hsa*	[250]
96	↓ SMC differentiation	*TRB3*	*Hsa*	[251]
Let7a	↓ SMC proliferation/↓ SMC migration	*Myc/Kras*	*Rno*	[252]

transcription factor critical for mediating an EC differentiated state [153]. Another miR that is suppressed in ECs by LSS is miR-34a [154]. Here, low miR-34a promotes a normal EC differentiated phenotype (opposite to what it does in SMC precursor cells, above), presumably through reduced degradation of its target, SIRT1, thereby inhibiting adhesion molecule expression (VCAM-1) and subsequent monocyte binding and attending inflammatory events on the surface of ECs [154]. Taken together, these few examples highlight the important role LSS has on miR gene expression in ECs and the regulation of EC differentiation or phenotypic modulation.

The most abundant miR gene in ECs is miR-126 [155, 156]. This miR transcript is co-expressed with the EC-restricted gene *EGFL7* (Table 2) through two ETS elements [157] and effects several biological processes through context-dependent transcript targeting. An early study showed that miR-126 repressed the vascular cell

adhesion molecule, thus maintaining an anti-inflammatory phenotype in mature ECs [155]. Gene knockout experiments demonstrated miR-126 to play an important role during developmental angiogenesis via negative regulation of SPRED1, an anti-angiogenic factor [156, 158]. Of note, the vascular defect seen with miR-126 inactivation is very similar to the defective sprouting angiogenesis phenotype observed with initial knockout studies of *Egfl7* [159]. It was subsequently discovered, however, that the *Egfl7* null phenotype was a consequence of inadvertent disruption of the miR-126 gene [160]. This highlights the importance of carefully thought out gene inactivation strategies and revisiting previously defined phenotypes from the conditional knocking out of protein coding genes where Cre-mediated deletion of exons and introns may also have disrupted noncoding genes or key TFBS.

Important phenotypic responses of ECs during development and disease processes include their proliferation and migration, which lead to the augmentation of vascular channels from pre-existing vessels, a process known as angiogenesis. Not surprisingly, the majority of miR genes studied in ECs exerts some effect on the establishment of this critical biological process (Table 4). Accordingly, the miR-17-92 cluster is expressed abundantly in ECs, and miR-92a targets pro-angiogenic genes, including *Itga5* [161]. Over-expressing miR-92a inhibits angiogenic responses both in cultured ECs and an animal model of neovascularization [161]. Interestingly, miR-34a attenuates an angiogenic response through the induction of EC senescence, which is linked to miR-34a-mediated repression of SIRT1 [162]. A major driver for the angiogenic response is hypoxia and some miR genes are induced by this powerful stimulus. For example, miR-424 (ortholog is miR-322 in mice) is induced by hypoxia in human ECs and stabilizes HIF1A through the targeting of CUL2, a component of the ubiquitin ligase system [163]. Transactivation of miR-424 was also shown to occur through the ETS factor, SPI1 (aka PU.1), which in turn was activated by RUNX1 [163]. Since both SPI1 and RUNX1 are transcription factors found in hemogenic endothelium, the phenotypic modulation of differentiated ECs in this context may involve their transition through this specialized type of EC during the angiogenic response. There is a major effort towards developing therapeutic interventions targeting specific miR genes for measured angiogenic responses under ischemic conditions, most notably peripheral artery disease associated with type II diabetes [164]. A challenge in the development of miR-based therapy for the promotion of angiogenesis is limiting the response to the desired tissue and avoiding the activation of dormant tumors.

Several additional miR genes play a role in the maintenance of the normal EC phenotype. The miR-223 gene, which is abundant in freshly dispersed ECs but then is transcriptionally down-regulated upon further growth in vitro, was shown to promote EC quiescence through the targeting of integrin beta 1, thus minimizing the action of growth factor signaling [165]. In human embryonic stem cells induced to an EC lineage, several miR genes (miR-99b, miR-181a/b) were induced and promoted the expression of EC restricted genes (*CDH5* and *PECAM1*) as well as increases in nitric oxide production and the potentiation of revascularization in a hind limb ischemia model [166]. Conversely, knockdown of Dicer blocked EC gene expression and normal morphology [166], consistent with pioneering Dicer knockdown studies in cultured adult ECs [105, 106]. Finally, the miR-21 gene was shown to facilitate EC differentiation and angiogenic potential in an induced pluripotent cell model of EC differentiation [167]. Table 4 summarizes many miR genes expressed in ECs, their associated function, and validated targets.

Summary

A problematic task in studying the biological significance and potential therapeutic efficacy of miR genes in EC/SMC biology continues to be accurate target gene identification. This is further complicated by the recent surge in novel, human miRs showing Dicer-dependency and bonafide target RNA binding [136]. Thus, reductionist studies will be needed to assess new human-specific miR genes in vascular cells and their modulated expression during vascular cell differentiation and phenotypic modulation. The fact that more than 6500 miRs now exist in the human genome (a likely underestimate of the true total) [136] compels investigators to be more mindful of the increasingly complex interplay between multiple miR genes, including those modulated by LSS and hypoxia, over individual target transcripts as well as the ever-present need to elucidate the mechanisms underlying miR-mediated target transcript destabilization or translational arrest. As with TFBS, elucidating nucleotide variants in either miR genes or their target sequences, and the functional importance therein, represent potentially fruitful areas of future research. For example, a cardiovascular disease-associated SNP (rs12190287 at 6q23.2) located in the 3′ untranslated region of the *TCF21* gene creates a favorable seed binding sequence for miR-224 facilitating repression of the target mRNA and perturbed expression of this important transcription factor in human coronary artery SMC [168].

Long noncoding RNA genes in vascular cells

The emergence of high-throughput transcriptomics heralded the concept of pervasive transcription and the expansive class of long noncoding RNA (lncRNA) genes

Table 4 EC miR function and targets

miR	Function	Target mRNA	Species	References
10	↑ Angiogenesis	*FLT1*	*Hsa*	[253]
10a	↓ EC inflammation	*MAP3K7/BTRC*	*Hsa*	[150]
100	↓ Angiogenesis	*Mtor*	*Mmu*	[254]
101	↑ With LSS/↓ EC proliferation	*MTOR*	*Hsa*	[145]
107	↑ With hypoxia/↓ EPC differentiation	*Arnt*	*Rno*	[255]
126	↓ EC inflammation	*VCAM*	*Hsa*	[155]
126	↑ Angiogenesis	*Spred1*	*Mmu*	[156]
126-5p	↑ EC proliferation	*Dlk1*	*Mmu*	[256]
130a	↑ Angiogenesis	*MEOX1/HOXA5*	*Hsa*	[257]
132	↑ Angiogenesis	*RASA1*	*Hsa*	[258]
146	↓ EC inflammation	*ELAV1*	*Hsa*	[259]
147b	↓ EC permeability	*ADAM15*	*Hsa*	[260]
149	↓ Angiogenesis	*GPC1/FGFR1*	*Hsa*	[261]
15a	↓ Angiogenesis	*FGF2/VEGF*	*Mmu/Hsa*	[262]
152	↓ EC proliferation/↓ EC migration	*ADAM17*	*Hsa*	[263]
155	↓ Vasodilation	*NOS3*	*Hsa*	[264]
155	↓ EC inflammation	*BACH1*	*Hsa*	[265]
17-3p	↓ Angiogenesis	*KDR*	*Hsa*	[266]
181a	↑ EC differentiation	*PROX1*	*Hsa*	[267]
181b	↑ EC inflammation	*KPNA4*	*Hsa*	[268]
19a	↑ With LSS/↓ EC proliferation	*CCND1*	*Hsa*	[146]
200b	↓ Angiogenesis	*ETS1*	*Hsa*	[269]
200c	↓ EC proliferation/↑ EC apoptosis	*ZEB1*	*Hsa*	[270]
200c	↑ EC differentiation	*ZEB1*	*Hsa*	[271]
21	↑ EC differentiation from iPSC	*Pten*	*Mmu*	[167]
21	↑ With LSS/↓ EC apoptosis/↑ EC NO	*PTEN*	*Hsa*	[272]
210	↑ With hypoxia/↑ EC migration	*EFNA3*	*Hsa*	[273]
214	↓ Angiogenesis	*QKI*	*Hsa*	[274]
217	↓ Angiogenesis/↑ EC senescence	*SIRT1*	*Hsa*	[275]
218	↓ Vasculogenesis	*ROBO1/2/GLCE*	*Hsa*	[276]
221	↑ Angiogenesis	*ZEB2*	*Hsa*	[277]
221	↑ Angiogenesis	*Cdkn1b/Pik3r1*	*Dre*	[278]
223	↓ Angiogenesis/↓ EC proliferation	*ITGB1*	*Hsa*	[165]
23/27	↑ Angiogenesis	*Spry2/Sema6a*	*Mmu*	[279]
29a	↑ Angiogenesis	*PTEN*	*Hsa*	[280]
30b	↑ Angiogenesis	*Dll4*	*Dre*	[281]
34a	↑ EC inflammation	*SIRT1*	*Hsa*	[154]
424	↑ With hypoxia/↑ angiogenesis	*CUL2*	*Hsa*	[163]
492	↓ EC migration	*RETN*	*Hsa*	[282]
503	↓ EC proliferation/↓ EC migration	*Ccne1/Cdc25a*	*Mmu*	[283]
6086/6087	↓ EC differentiation	*CDH5/ENG*	*Hsa*	[284]
663	↓ With LSS/↑ EC inflammation	*?*	*Hsa*	[152]
92a	↓ Angiogenesis	*Itga5*	*Mmu*	[161]
92a	↓ With LSS/↑ EC differentiation	*KLF2*	*Hsa*	[153]

[169–171]. Indeed, the number of lncRNA genes in the human genome is soaring with recent totals surpassing all known protein-coding and miR genes [172]. LncRNA genes are transcribed by RNA polymerase II, exist as single or multi-exonic transcripts, undergo 5′ capping and splicing, and can either be polyadenylated or

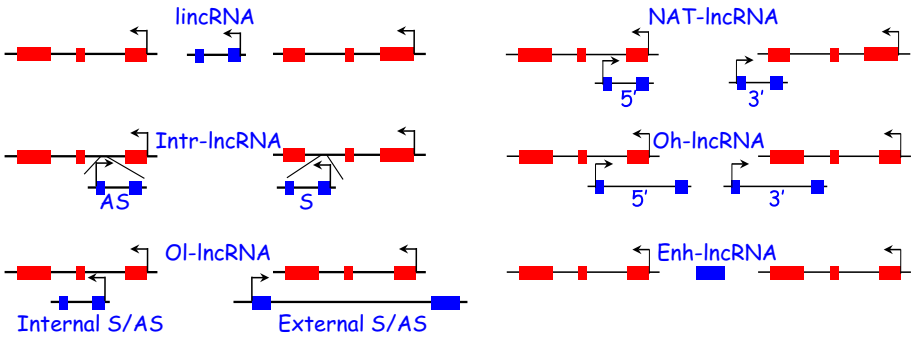


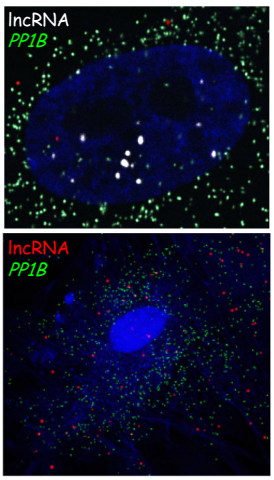
Fig. 2 LncRNA classification based on orientation. In each example, exons of the lncRNA gene are *blue* while those of neighboring genes are *red*. *Bent arrows* indicate the direction of transcription. See text for further details. *S* sense, *AS* antisense

non-polyadenylated [173]. In general, compared to protein-coding genes, lncRNA genes are poorly conserved and weakly expressed. Nevertheless, lncRNA genes are regulated by external stimuli such as hypoxia [174] and inflammatory agonists [175] and exert important functions even at low copy number [176]. In fact, recent knockout studies have clearly demonstrated a biologically relevant role for many lncRNAs [177]. In this context, whereas miR genes almost always function to down-regulate mRNA and/or protein expression, lncRNA genes exhibit unpredictable and increasingly diverse functions related to the induction or suppression of transcription, translation, and signaling as well as the modification of chromosome structure and function and the formation of nuclear microdomains [178, 179]. It is possible that the varied functions associated with lncRNAs, while still in the embryonic stages of our understanding, may someday rival those of protein coding genes.

Several sub-classes of lncRNAs exist based on their genomic position relative to other gene loci. These include promoter-containing long intervening noncoding RNAs (lincRNAs), which do not overlap with other genes and are often found in what was once referred to as gene deserts [180]; natural antisense transcript lncRNAs (NAT-lncRNAs) whose one or more exons overlap with exons of an opposing transcribed gene [181]; intronic lncRNAs (Intr-lncRNAs) [182]; and the less numerous 5′ or 3′ overhanging lncRNAs (oh-lncRNAs) and internally or externally overlapping lncRNAs (ol-lncRNAs) (Fig. 2). Other subclasses of lncRNA genes include enhancer RNAs, which play an ill-defined role in directly mediating gene transcription [183], some pseudogenes, whose RNA functions are only beginning to be realized [184], and circular RNAs [185].

One method of functionally defining lncRNA genes is based on their directionality of transcription. For example, a large number of lncRNAs are transcribed in the antisense (or opposite strand, os) orientation to the nearest transcription unit; these may be NAT-lncRNAs (e.g., *MEF2C-AS1* and *GHRL-OS*) or non-NAT-lncRNAs (e.g., *DIAPH2-AS1* and *DNM3-OS*). Expression of antisense-sense gene pairs may be concordant or discordant. Concordantly expressed gene pairs may share similar regulatory regions (such as promoters). Discordant expression of antisense lncRNA-sense RNA gene pairs may reflect the negative regulation of the former on the latter. For example, the NAT-lncRNA, APOA1-AS, recruits the demethylase, LSD1, over the *APOA1* gene and represses its expression both in vitro and in vivo [186]. Thus, a first approximation of the function of an antisense lncRNA may be through an examination of its expression with respect to the overlapping mRNA. It is important to stress that while such correlative data have often been used to advance the function of an antisense lncRNA, deeper analyses are required to formally elucidate the function of any lncRNA (see "Summary" below).

Another method of functionally categorizing lncRNA genes relates to their localization within a cell as demonstrated by RNA-fluorescence in situ hybridization (RNA-FISH, Fig. 3). Accordingly, nuclear localized lncRNAs function primarily in the regulation of gene transcription either in *cis* (local) or *trans* (distal) by acting as guides or scaffolds that localize chromatin remodeling factors such as the polycomb repressor complex to specific gene loci. Nuclear lncRNAs may also function as decoys for key transcription factors or TFBS or through the very act of being transcribed [179]. Cytoplasmic lncRNAs can mediate changes in the level of gene/protein expression by acting as decoys (or competing endogenous RNAs) for microRNAs [187] as well as direct effectors of mRNA stability or translation [188, 189]. Cytoplasmic lncRNA genes have also been shown to regulate nuclear translocation of transcription factors and mediate critical signaling pathways via RNA–protein interactions [190–192]. While lncRNAs can suppress or stimulate transcription, translation and signaling, there are virtually no known features of lncRNAs that would be predictive of their function in a cell. One notable exception is a recent report that defined a sequence element (AGCCC plus A/T at -8 and G/C at -3) shown to be both necessary and sufficient for nuclear localization of a lncRNA [193].



Nuclear lncRNAs
- decoy (TFBS, TF)
- scaffold
- guide
- transcriptional interference
- chromatin remodeling
- nuclear architecture

Cytoplasmic lncRNAs
- decoy (miRs)
- mRNA stability
- translation
- signaling
- translocation
- cytoskeleton

Fig. 3 LncRNA classification based on spatial localization. Single molecule RNA-FISH of a nuclear lncRNA (*white dots* in the DAPI-stained nucleus, *upper panel*) versus a cytoplasmic lncRNA (*red dots* around DAPI-stained nucleus, *lower panel*). *Both panels* depict RNA-FISH in human coronary artery SMCs. The *green dots* represent RNA-FISH of a housekeeping gene which is used to demarcate cell borders for quantitative purposes

This result suggests there may be consensus nucleotide sequences (much like protein domains) that portend lncRNA function. Given the low sequence conservation of lncRNAs across species, the latter is likely to be the exception rather than the rule. Consequently, although it may now be convenient (even necessary) to sort lncRNAs based on genomic and cellular locale as well as their transcriptional directionality with respect to a neighboring gene locus, these categorizations will not be predictive of lncRNA function. It is possible that families of lncRNAs will emerge based on related structural characteristics that may serve as non-sequence specific interaction domains for particular RNA-binding proteins. In the end, the functions of lncRNAs may have more to do with the complex structures they assume in association with other macromolecules than their precise nucleotide sequence content.

In general, lncRNA genes have short open reading frames (<100 amino acids) and, by definition, do not encode for proteins, a concept that has support from ribosomal profiling studies [194]. However, recent studies have demonstrated the presence of small, conserved open reading frames in annotated lncRNAs that encode for previously unrecognized peptides [195, 196]. Thus, an important undertaking will be to carefully assess whether a mRNA is in fact masquerading as a lncRNA. The idea that human-specific lncRNAs encode for novel peptides is an intriguing concept that may elucidate subtle differences in vascular physiology between humans and other mammals. However, there are no known lncRNAs encoding for a human-specific SMC- or EC-associated peptide.

Whereas much is known about miR genes in vascular cells, there are only a handful of vascular lncRNA genes described at this time, though this will change dramatically in the coming years as more RNA-seq data accumulate. Here, we provide an up-to-date summary of experimentally defined lncRNAs in vascular cells and their putative function in regulating vascular cell phenotype.

Smooth muscle cell lncRNA genes

The first report of a lncRNA in vascular SMCs occurred more than 20 years ago with the cloning of *H19* [197]. *H19* is a conserved, imprinted NAT-lncRNA that serves as a host gene for miR-675 (http://genome.ucsc.edu/). *H19* is expressed in SMCs of developing arteries, but then levels are reduced in adult vessels until atherosclerotic lesions are manifest or following acute vascular injury [198, 199]. Although the precise function of *H19* in SMCs is unknown, recent data suggest a role as a sponge RNA for the let-7 family of miRs [200]. In this context, overexpression of let-7g in oxidized LDL-treated SMCs attenuated autophagy, apoptosis, and reactive oxygen species through the targeted repression of *LOX1* [201]. Reduced expression of *H19* in vascular lesions may therefore limit the local effects of oxidized LDL in intimal SMCs by permitting elevated expression of let-7g, though it remains to be determined whether let-7g levels are increased in intimal cells of SMC origin. Of note, *H19* overlaps in the antisense orientation with a tumor suppressor (*HOTS*); no studies have yet examined the interplay between these transcripts in vascular SMCs [202]. Another well-known lncRNA expressed in SMCs is *CDKN2B-AS1* (aka *ANRIL*), which resides on chromosome 9p21, a hotspot for variations linked to cardiovascular disease and cancer [203]. This nuclear antisense ol-lncRNA mitigates neighboring cell cycle gene expression through the recruitment of the polycomb repressor complex [204]. Human SMCs derived from patients carrying SNPs in the *ANRIL* locus exhibit elevated cell proliferation in vitro [205], a finding supported by genetic deletion of orthologous sequences in the mouse [206]. It will be important to determine whether correction of variants in *ANRIL*, using CRISPR, rescues the SMC growth phenotype. SNP variants in *ANRIL* highlight the critical importance of defining functional variations in other noncoding sequences in ECs and SMCs.

A conserved antisense transcript was discovered and found to have at least 3 untranslated exons overlapping exons at the 3′ end of the *NOS3* gene [207]. This antisense transcript may, therefore, be categorized as a NAT-lncRNA. Expression of the sense *NOS3*/NAT-lncRNA gene pair is discordant in SMCs with higher levels of the antisense lncRNA over *NOS3*. Similarly, in situ hybridization reveals little to no overlap in co-expression of these two

transcripts. Knockdown of the NAT-lncRNA results in increases in *NOS3* providing a post-transcriptional mechanism for low-level expression of *NOS3* in SMCs [207]. The *NOS3* NAT-lncRNA, known as *ATG9B*, is a rare example of a transcript annotated as both a protein-coding gene (involved in the regulation of autophagy) and a NAT-lncRNA gene (http://genome.ucsc.edu/). There are likely to be additional examples of such bifunctional RNA transcripts given the extensive number of sense-antisense gene pairs in the human genome.

A few lncRNAs have been defined in SMCs using next generation sequencing. The first reported use of RNA-seq for the discovery of SMC lncRNAs was in rat aortic SMCs stimulated with angiotensin II. This study revealed numerous lncRNA genes including one that is a host gene for the miR-221/222 cluster [208]. This lncRNA, provisionally named here as *MIR221HG*, appears to be conserved in the human genome. Knockdown of *MIR221HG* resulted in reduced miR221/222 expression and attenuated SMC growth responses in rat SMCs [208]. An increasing number of lncRNAs are found to be host genes for miRs (e.g., Fig. 4). Whether these host lncRNA genes have functions independent of the microRNA genes they harbor is unclear at this time, but should be a fruitful avenue for future investigation.

In an effort to define unannotated lncRNA genes in human SMCs, RNA-seq was done in human coronary artery SMCs and an antisense lncRNA, called *SENCR*, was found to overhang the 5′ end of the EC-restricted *FLI1* gene [209]. *SENCR* exons do not overlap those of *FLI1* and so *SENCR* is not a NAT-lncRNA and does not appear to regulate *FLI1* expression. The largely cytoplasmic localization of *SENCR* supports an extra-nuclear function. To gain insight into the function of *SENCR*, RNA-seq experiments were done following its knockdown in human coronary artery SMCs. This approach showed that *MYOCD* and its contractile target mRNAs were reduced with attenuated *SENCR* suggesting some loss in SMC differentiated properties. On the other hand, numerous genes involved in cellular migration were elevated upon *SENCR* knockdown. Accordingly, SMCs were shown to be hypermotile with knockdown of *SENCR*, a phenotype that could be completely rescued by simultaneous knockdown of motility genes that were up-regulated upon knockdown of *SENCR*. Thus, *SENCR* appears to play some role in the maintenance of the sessile, SMC contractile phenotype. There is a 61 amino acid open reading frame in *SENCR* that, if translated, would encode a novel small peptide. However, no evidence exists to support an encoded peptide within the *SENCR* transcript. Moreover, there are no known RNA-binding proteins associated with *SENCR*. The latter represents an important gap in understanding the function of this new vascular lncRNA.

A microarray revealed hundreds of lncRNAs differentially expressed between control and varicose saphenous veins, and several were validated by qRT-PCR. Many represent antisense lncRNAs and expression analysis revealed concordant expression with neighboring mRNAs [210]. In a follow-up report, reduced expression of the snoRNA-containing *GAS5* lncRNA in varicose veins correlated with increases in SMC proliferation and migration, two contributing factors to this pathology; overexpression of *GAS5* reduced SMC proliferation [211]. Importantly, RNA pulldown experiments showed an interaction between *GAS5* and a calcium-dependent RNA binding protein called ANXA2. The enhanced proliferation and migration of SMCs with attenuated *GAS5* could be rescued upon simultaneous knockdown of *ANXA2* suggesting that *GAS5* functions in some manner to regulate the expression and/or activity of ANXA2 [211]. Curiously, a single-exon, NAT-lncRNA (*GAS5-AS1*) exists at the 3′ end of *GAS5* though no investigation of this lncRNA was reported. Another NAT-lncRNA (*HAS2-AS1*), however, was shown to directly mediate the transcription of the overlapping *HAS2*

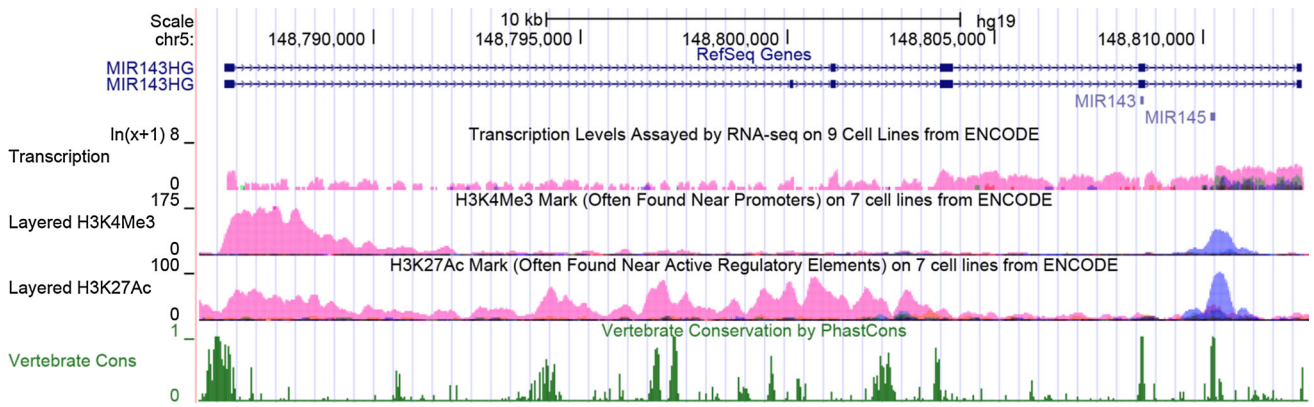


Fig. 4 UCSC Genome Browser screenshot of a host gene for the miR-143/145 cluster

gene in human SMCs by recruiting the p65 subunit of NFκB to the adjacent *HAS2* promoter [212]. HAS2-mediated increases in the glycosaminoglycan, hyaluronan, are found in atherosclerotic lesions, suggesting that therapeutic targeting of *HAS2-AS1* could have a desirable impact in models of atherosclerosis. Another atherosclerosis-related lncRNA is lincRNA-p21, which was reduced in the ApoE null model of atherogenesis as well as human atherosclerotic lesions [213]. Gain-of-function studies revealed that lincRNA-p21 represses SMC proliferation while loss-of-function in lincRNA-p21 exaggerated the neointimal response to acute injury. RNA pulldown experiments showed an interaction between lincRNA-p21 and the E3 ubiquitin-protein ligase, which had the effect of de-repressing p53-dependent target genes, including several apoptosis-related genes [213]. Thus, targeting SMCs with lincRNA-p21 could be of clinical value in chronic or acute vascular diseases. Another recent study showed that the NAT-lncRNA, *HIF1A-AS1*, was increased in the serum of patients with aortic aneurysms; targeted reduction of this lncRNA in SMCs reduced apoptotic related genes such as caspase 3 and caspase 8 while increasing *BCL2* [214]. Neither the mechanism of *HIF1A-AS1* in regulating SMC apoptosis nor its effects on the overlapping *HIF1A* mRNA was assessed. Table 5 lists the lncRNA genes that have been identified and studied in vascular SMCs.

Endothelial cell lncRNA genes

The first lncRNA to be described in ECs was an antisense transcript with several exons overlapping exons of the EC-restricted *NOS3* gene [207]. Unlike SMCs, expression of the overlapping transcript to this NAT-lncRNA is low in ECs whereas *NOS3* mRNA is high. Interestingly, overexpression of the overlapping NAT-lncRNA sequences in ECs attenuated levels of NOS3 protein with little effect on *NOS3* mRNA suggesting a block in translation of the processed *NOS3* transcript [207]. Subsequent studies showed that hypoxia augmented expression of the *NOS3* antisense lncRNA (now annotated as a noncoding isoform of *ATG9B*) in both cultured ECs and rat aortic tissue [215]. Hypoxia-induced decreases in NOS3 were blocked when the NAT-lncRNA, *ATG9B*, was down-regulated prior to the hypoxic stimulus in cultured ECs [215]. These results support a post-transcriptional mechanism for the known decrease in NOS3 under hypoxic conditions.

As ECs are the primary effector cells for angiogenesis, it should be no surprise that lncRNA genes, like miR genes, influence the angiogenic response whether developmental or pathological. Accordingly, the first angiogenesis-related lncRNA discovered in ECs was a NAT-lncRNA that overlaps the 3′ untranslated region of *TIE1* [216]. This lncRNA, called *TIE1-AS*, is conserved in mouse, human and zebrafish and its expression and spatial localization are concordant with that of the overlapping *TIE1* mRNA in developing zebrafish. Interestingly, bioinformatic and hybridization assays support a direct association between *TIE1-AS* and *TIE1* mRNA. Consistent with this interaction, forced expression of *TIE1-AS* could reduce levels of *TIE1* mRNA, and specific defects in EC junctional complexes and vascular patterning were seen in zebrafish injected with *TIE1-AS*. Interestingly, expression of *TIE1-AS* was reported to be elevated in human hemangiomas suggesting this lncRNA could be a viable target of therapy for vascular malformations [216].

More recently, RNA-seq analysis of human umbilical vein ECs revealed high level expression of *MALAT1* (aka *NEAT2*), a conserved nuclear lincRNA that could also be induced by hypoxia [217]. Knockdown of *MALAT1* in ECs evoked a pro-migratory phenotype in vitro and reduced capillary density and blood flow in a hind limb ischemia model. The latter phenotype was suggested to occur through reduced proliferation of stalk cells [217]. While gain-of-function studies were not conducted, an independent study revealed increases in EC viability with overexpression of *MALAT1* [218]. In this context, elevated levels of *MALAT1* were observed upon treatment of ECs with high levels of glucose, a stimulus that increases

Table 5 SMC lncRNAs

LncRNA	Type/localization	Putative function in SMC	References
H19	NAT-lncRNA/cytoplasmic	?	[197]
CDKN2B-AS1	Antisense external ol-lncRNA/nuclear	↑ SMC proliferation?	[205]
ATG9B	NAT-lncRNA/?	↓ *NOS3*	[207]
MIR221HG	LincRNA/?	↑ SMC proliferation	[208]
SENCR	5′ Antisense oh-lncRNA/cytoplasmic	↑ SMC differentiation/↓ migration	[209]
HIF1A-AS1	NAT-lncRNA/?	↑ SMC apoptosis	[214]
LincRNA-p21	LincRNA/nuclear	↓ SMC proliferation	[213]
HAS2-AS1	NAT-lncRNA/?	Matrix regulation	[212]
GAS5	LincRNA/cytoplasm	↓ SMC proliferation/↓ migration	[211]

inflammatory mediators associated with faulty angiogenic responses [218, 219]. Consistent with this observation, *MALAT1* RNA was increased in the defective retinal vasculature of diabetic or obese mice [218]. Both the diabetic retinopathy and the inflammatory phenotype of ECs could be reversed with targeted suppression of *MALAT1* [218, 219]. Perturbations in retinal angiogenesis were also noted in *MALAT1* knockout mice suggesting that a critical level of this lncRNA is needed for normal microvascular patterning [217]. Given the widely reported effects of *MALAT1* in regulating migration of various cancer cells and metastasis, it will be important to differentiate between effects of *MALAT1* suppression on cancer cells versus local tumor vasculature. It will also be important to assess the role of *SENCR* in EC phenotypes, since levels of this lncRNA are higher in ECs than SMCs [209].

A second conserved nuclear lncRNA implicated in pathological angiogenesis is *MIAT*. Like *MALAT1*, *MIAT* is induced in ECs treated with high glucose [220]. Knockdown of *MIAT* attenuated high glucose-mediated EC proliferation, migration, and tubulogenesis in vitro and improved diabetes-induced microvascular pathology in vivo. Importantly, multiple miR-150-5p binding sites were found in the VEGF transcript as well as *MIAT* suggesting this lncRNA could function as a sponge RNA and regulator of VEGF levels. Indeed, a series of studies provided direct support for this concept, thus offering new insight into pathological angiogenesis and a new potential target for the treatment of aberrant angiogenesis [220]. The results of the *MIAT* study also support the idea of RNA sponging of miRs within the nucleus. Another EC sponge RNA was recently reported to regulate autophagy in human ECs. This lncRNA (*TGFB2-ot1*) is a rare example of a sense lncRNA embedded within the 3′ untranslated region of a protein-coding gene. Consequently, loss-of-function studies are impossible to interpret since targeted knockdown of the lncRNA also reduces *TGFB2* [221]. Nevertheless, gain-of-function studies showed that *TGFB2-ot1* sponges miR-4459, which can target the autophagy-related 13 (*ATG13*) mRNA thereby increasing MTOR, a key regulator of autophagy [221]. There will, no doubt, be more examples of vascular lncRNAs that function as competing endogenous RNAs for miRs. Several databases are available to assist investigators in this context including StarBase (http://starbase.sysu.edu.cn/mirLncRNA.php) [222] and LNCipedia (http://www.lncipedia.org/) [172].

Two very recent reports have defined lncRNAs that effect changes in EC phenotype associated with angiogenesis. First, Kurian et al. [223] studied an annotated NAT-lncRNA located at the 3′ end of the *AGAP2* mRNA. The *AGAP2-AS1* lncRNA is elevated in human embryonic stem cells induced to differentiate to the EC lineage. Morpholino knockdown of *AGAP2-AS1* in zebrafish caused defective vascular branching leading to abnormal vascular patterning in the embryo. This phenotype could be rescued by introducing the human ortholog of *AGAP2-AS1* into mutant fish. In human ECs, knockdown of *AGAP2-AS1* resulted in reduced EC proliferation, tubulogenesis, and acetylated LDL uptake [223]. Though expression of EC-restricted markers was not reported, results suggest *AGAP2-AS1* functions downstream of EC lineage commitment. It will be important to ascertain the effects of loss or gain of *AGAP2-AS1* on *AGAP2* mRNA to determine whether effects of the lncRNA relate to changes in expression of the overlapping protein-coding gene. Second, Li et al. [224] discovered a multi-exonic NAT-lncRNA overlapping the EC-restricted *DLL4* gene. The *DLL4-AS* and *DLL4* gene pairs are concordantly expressed at baseline and under stimulatory conditions. Overexpression of two isoforms of *DLL4-AS* can increase *DLL4* protein levels whereas knockdown of *DLL4-AS* decreases *DLL4* mRNA. The precise mechanism of *DLL4-AS* regulation of DLL4 is unknown at this time. Intriguingly, there appears to be a shared promoter between the *DLL4* sense-antisense gene pair [224]. Formal testing of the function of this bi-directional promoter could be done with CRISPR-mediated editing and subsequent measuring of each transcript. Functional studies show that, like *SENCR* in SMCs and *MALAT1* in ECs, knockdown of *DLL4-AS* in ECs results in a hyper-motile state. Further, increases in EC proliferation and spouting were observed though the latter was considered to be non-productive [224]. These results indicate an important layer of regulation of *DLL4* expression and hence EC phenotype. Table 6 lists the known lncRNA genes and their putative functions in ECs.

Summary

Our understanding of lncRNA expression and function in vascular cells is rather primitive at this time. As more labs perform RNA-seq experiments under various experimental conditions in ECs and SMCs, a treasure trove of annotated lncRNAs will emerge for further study. Given the increasing number of non-polyadenylated lncRNA genes [225], and the fact that many deep sequencing experiments utilize polyA+ RNA, a vast number of non-polyadenylated lncRNA genes will need to be characterized. Depending on the manner in which the RNA-seq data are analyzed, final datasets will include new and already annotated transcripts with the latter designated as pseudogene, antisense RNA, opposite strand RNA, eRNA, and overlapping transcripts, to name a few [226]. Regarding the formal naming of lncRNA genes, there are established guidelines based on function or genomic position that investigators should follow [227]. Many lncRNA genes will likely have

Table 6 EC lncRNAs

LncRNA	Type/localization	Putative function in EC	References
ATG9B	NAT-lncRNA/nuclear+cytoplasmic	↓ *NOS3* following hypoxia	[215]
TIE1-AS	NAT-lncRNA/cytoplasmic	Regulation of EC contact junctions	[216]
NRON	Intr-lncRNA/cytoplasmic	↓ EC proliferation/↓ migration	[285]
SENCR	antisense oh-lncRNA/cytoplasmic	?	[209]
MALAT1	LincRNA/nuclear	Proper angiogenesis	[217]
TGFB2-ot1	sense ol-lncRNA/?	ceRNA regulating autophagy	[221]
MIAT	LincRNA/nuclear	ceRNA regulating EC function	[220]
ASncmtRNA-2	Stem loop/nucleus	Replicative senescence	[286]
AGAP2-AS1	NAT-lncRNA/cytoplasm	Proper angiogenesis	[223]
HIF1A-AS1	NAT-lncRNA/?	↑ EC apoptosis/↓ EC proliferation	[287]
DLL4-AS	NAT-lncRNA/cytoplasm	↑ EC proliferation/↑ EC migration	[224]

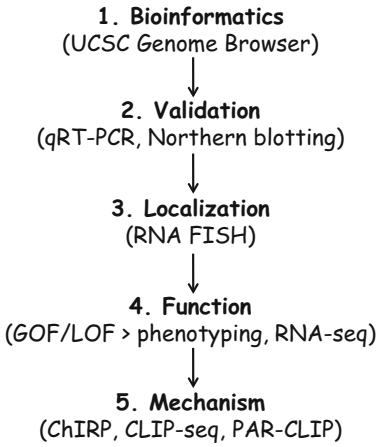


Fig. 5 Order of operations for the study of a lncRNA

functions related to phenotypic modulation or differentiation of vascular cells. Each putative lncRNA, whether novel or already identified and annotated in public databases, will require in depth analyses. Accordingly, we propose a logical order of operations for the systematic study of new and annotated transcripts of unknown expression and function in vascular cells (Fig. 5).

The first step in analyzing new lncRNA genes is to carefully examine the transcript on the UCSC Genome Browser and define such features as transcript abundance, conservation, and chromatin marks by exploiting the vast data from the ENCODE Consortium [228]. The presence of miR-binding sequences should be examined as an increasing number of lncRNAs function as sponge or competing endogenous RNAs [220]. Further bioinformatics might include the definition of open reading frames (e.g., NCBI, ORF Finder, http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and evaluating each ORF for the presence of known protein domains (e.g., Pfam databases, http://pfam.xfam.org). Much of this information can also be gleaned quickly by simply turning on tracks within the UCSC Genome Browser. An important question in this context is whether a transcript engages the ribosomal machinery and generates a translated peptide. This will require in vitro transcription/translation and/or epitope-tagging of the transcript followed by Western blotting. Whether or not a cytoplasmic lncRNA is translated, its association with ribosomes could have a regulatory role in fine-tuning translation through effects on the ribosomal machinery itself or mRNA stability. We have found several un-annotated transcripts (mostly single exon) of high abundance and conservation to be probable paralogs of known protein-coding genes (unpublished). This indicates there will be ongoing revision of the human genome as previously annotated lncRNAs emerge as mRNAs. Whether encoding a peptide or not, it seems that a noncoding function of any transcript can no longer be ignored [215, 229]. Finally, it will be important to determine whether any relevant SNPs exist in a putative lncRNA using LincSNP (http://210.46.85.180:8080/LincSNP/search.jsp) [230] or lncRNASNP (http://bioinfo.life.hust.edu.cn/lncRNASNP/) [231].

The second step in analyzing a lncRNA is to validate expression of the transcript in independent isolates of cells under conditions of the original RNA-seq experiment. Further confirmation of expression should include cell line and tissue profiling by qRT-PCR and Northern blotting, the latter representing a convenient method of ascertaining transcript length and alternative splice/polyadenylation variants. More often than not, rapid amplification of cDNA ends (RACE) will extend existing exons and reveal new exons with attending splice variants that could exhibit differential functions. The third step in lncRNA analysis should be to define the spatial localization of the transcript under baseline and stimulatory states. RNA fractionation

followed by qRT-PCR is a reasonable first approximation; however, single molecule RNA-FISH offers much more quantitative data. The incorporation of a second set of fluorescent probes in RNA-FISH and scoring coincident signals following knockdown of the lncRNA represents a powerful approach to the spatial localization of a lncRNA [209]. Understanding where a new transcript resides will assist greatly in defining how to proceed in the functional analysis of a lncRNA (Fig. 3). Of course, such assays only represent a snapshot of lncRNA localization and cannot predict dynamic nucleocytoplasmic shuttling. Thus, real-time imaging of lncRNAs will be an important development to further elucidate lncRNA biology in vascular cells.

The fourth step in analyzing a lncRNA gene relates to unveiling its function. Thus, loss-of-function assays should be done through appropriate means including dicer substrate siRNA or GapmeR knockdown approaches. Given the ease in which genes can now be inactivated using CRISPR, inserting a strong polyadenylation signal in the first exon of a lncRNA could be quite informative, particularly for conserved lncRNAs that can be inactivated in model organisms. CRISPR-mediated deletions are more difficult and potentially confounding with possible excision of other sequences independent of the lncRNA sequence. Thus, where possible, rescue experiments should be undertaken to address this possibility as well as distal off-targeting effects. Whether the goal is to delete an exon or entire transcript or insert a polyadenylation signal for premature transcriptional arrest, great care must be taken in fully elucidating the 5′ end of the lncRNA (using RACE) and strategizing in such a manner that other genes or regulatory elements are not disturbed [232]. Gain-of-function studies require complete definition of the full-length transcript by 5′ and 3′ RACE. Full-length lncRNAs may then be integrated in a suitable viral system (e.g., lentivirus) for delivery to cells or tissues. Whether the aim is to knockdown or overexpress the lncRNA, unambiguous phenotyping of the cells or animal will be of paramount importance towards understanding lncRNA function. One common approach is to knockdown the lncRNA and then conduct RNA-seq to assess the resultant transcriptome [209, 217, 223]. However phenotyping is done, the last step in the process of studying a lncRNA is elucidating its mechanism of action (Fig. 5). Several biochemical assays have been developed to understand the physical interaction between a lncRNA and a protein, a lncRNA and another RNA, or a lncRNA and DNA. These assays (e.g., CLIP-seq, PAR-CLIP, ChIRP, etc.) generally involve the crosslinking of a biotinylated lncRNA or tiled oligonucleotides that are antisense to an endogenous lncRNA followed by streptavidin bead capture of co-precipitating lncRNA and associated macromolecules. It is important to recognize that there likely will be issues of oligonucleotide accessibility to the lncRNA. Thus, it may be prudent to first demonstrate enrichment of the lncRNA following crosslinking and pulldown before proceeding with RNA binding protein or RNA/DNA sequence identification. Ultimately, interacting proteins, RNAs, or DNA can be identified by gel electrophoresis and mass spectrometry, RNA-seq, and qRT-PCR, respectively [233]. Obtaining such information will be critical in carrying forward additional experiments that would define interaction domains and 3-D structures with the goal of developing small molecule inhibitors that disrupt or stabilize lncRNA interactions with other macromolecules in the cell as well as constructing more sophisticated interactome maps under normal and pathological conditions. Finally, as additional assays emerge to detect macromolecules that bind lncRNA transcripts, a challenge will be interpreting the function and mechanism of action of the far more numerous ultra-low abundant lncRNA transcripts in vascular cells.

Perspective

The human genome represents a veritable digital library of information that includes a massive number of functional noncoding sequences, including TFBS that direct correct spatio-temporal and context-specific expression of protein coding mRNAs as well as the continually growing number of miR and lncRNA genes. The interplay among these three classes of noncoding sequences within and between ECs and SMCs is largely speculative at this time and will require integrated studies to build more complete interactome maps (Fig. 6). Methodological reductionism will continue to be of importance in understanding the basics of noncoding sequences (e.g., regulatory processes underlying miR stability and transcriptional control of miR/lncRNA expression), but the isolated study of any one mRNA/protein, TFBS, miR, or lncRNA can no longer proceed without addressing such issues as the existence of lncRNAs that act as TFBS decoys or whether a lncRNA is stabilized by a mRNA or some other lncRNA that sponges a miR. Accordingly, the next generation of scientists will have a rich resource of data and increasingly sophisticated tools to generate new hypotheses underlying vascular cell differentiation and phenotypic change in disease. The ever-present challenge will be supplementing our two-dimensional, reductionist approach to biology with higher dimensional thinking so as to make better sense of what clearly is a biological symphony of interactive processes necessary for vessel wall homeostasis. For example, one might imagine data derived from deep transcript profiling of individual vascular cells under various physiological, pathophysiological, or pharmacological conditions and the biophysical study of individual and interactive coding/noncoding genes being used to identify and

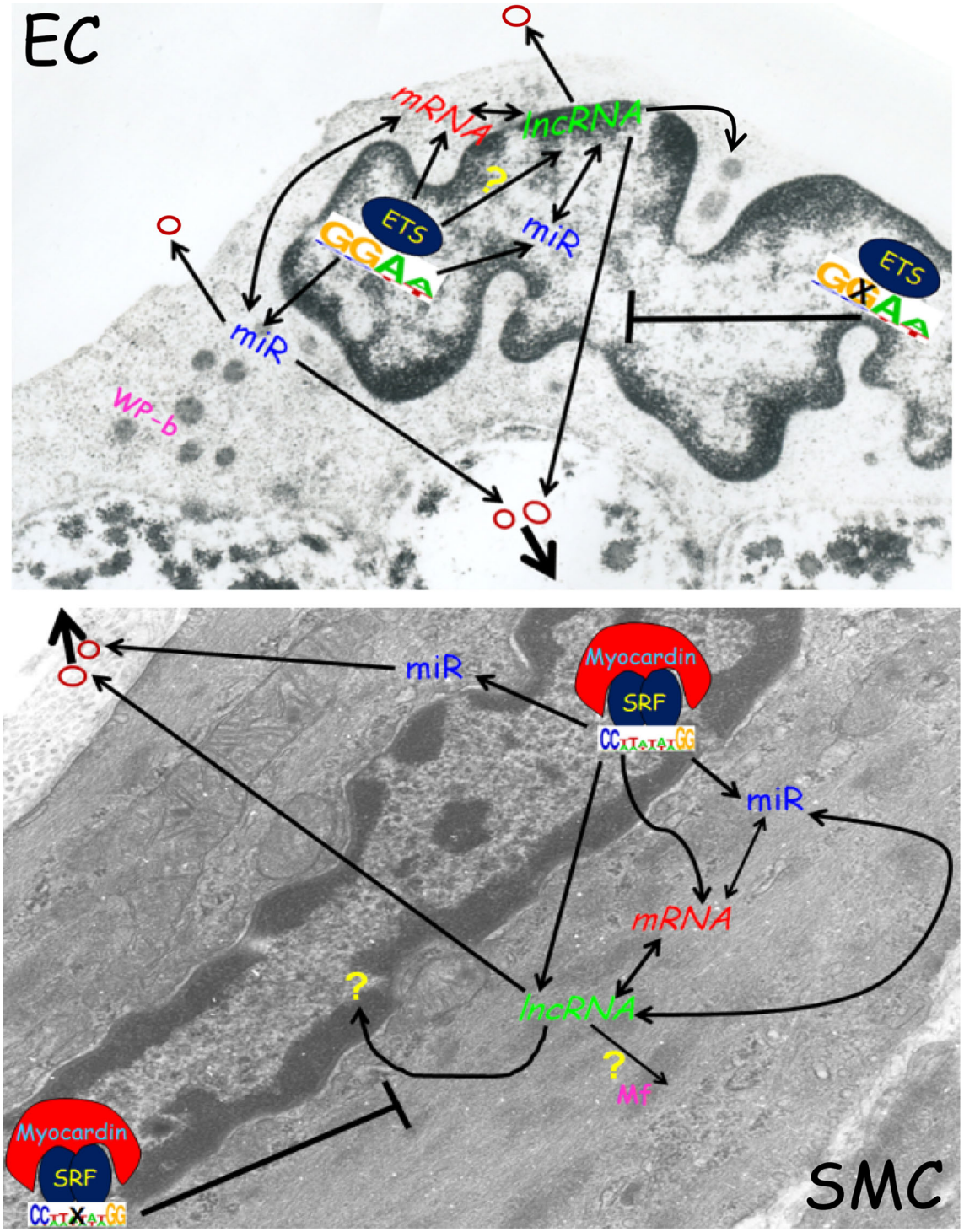


Fig. 6 Noncoding sequence control of vascular cell phenotypes. The differentiated phenotype of Weibel-Palade body (WP-b) containing ECs (top electron micrograph) and subjacent, Myofilament (Mf)-containing SMCs (bottom electron micrograph) is regulated through TFBS (such as ETS and CArG elements, respectively), directing expression of mRNA, miR, and lncRNA genes whose mature products serve structural and complex regulatory roles, the latter likely involving direct or indirect effects of each transcribed unit on other transcribed sequences in the nucleus or cytoplasm (*small arrows*). TFBS-SNPs (denoted with an *X* in each element) may interfere with normal target gene expression and hence perturb the differentiated vascular cell phenotype. Intercellular control of vascular cell phenotypes (*large arrows*) may be achieved through the action of RNA-containing extracellular vesicles (e.g., exosomes denoted as *small circles* released locally in the interstitial space or via circulation in the case of ECs)

integrate dominant mRNA-miR-lncRNA associations in vascular cells over time and developing models that will predict specific biological outcomes. Coupling these data with an individual's genomic sequence and the ~3 million sequence variants that confer individual genetic identity, should facilitate improved tailoring of an interventional approach (preventive or otherwise) that maintains vascular cell homeostasis or reverts a modulated, disease-dependent phenotypic state to one favoring health [234]. Achieving this and other goals will require a concerted effort by basic vascular biology labs that do cell, molecular, and animal modeling of disease (particularly CRISPR mice), clinical labs that furnish appropriately coded patient data, and computational labs that distill enormous wet (e.g., ChIP-seq) and dry (e.g., TFBS predictions) lab data to manageable datasets for interrogation and integration with the aforementioned information. Clearly, 21st century vascular biology has ushered in unprecedented challenges that demand cooperation along the lines of the human genome project in order to fully advance the concept of personalized cardiovascular medicine. In the meantime, much work lies ahead in the identification, functional analysis, and integration of noncoding sequences that control vascular cell phenotypes.

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