14q32 Noncoding RNAs in vascular remodelling

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Part I

Chapter 2

The multifactorial nature of microRNAs in vascular remodelling

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Abstract

Vascular remodelling is a multifactorial process that involves both adaptive and maladaptive changes of the vessel wall through, among others, cell proliferation and migration, but also apoptosis and necrosis of the various cell types in the vessel wall. Vascular remodelling can be beneficial, e.g. during neovascularization after ischaemia, as well as pathological, e.g. during atherosclerosis and aneurysm formation. In recent years, it has become clear that microRNAs are able to target many genes that are involved in vascular remodelling processes and either can promote or inhibit structural changes of the vessel wall. Since many different processes of vascular remodelling are regulated by similar mechanisms and factors, both positive and negative vascular remodelling can be affected by the same microRNAs. A large number of microRNAs has been linked to various aspects of vascular remodelling and indeed, several of these microRNAs regulate multiple vascular remodelling processes, including both the adaptive processes angiogenesis and arteriogenesis as well as maladaptive processes of atherosclerosis, restenosis and aneurysm formation. Here, we discuss the multifactorial role of microRNAs and microRNA clusters that were reported to play a role in multiple forms of vascular remodelling and are clearly linked to cardiovascular disease (CVD). The microRNAs reviewed are miR-126, miR-155 and the microRNA gene clusters 17-92, 23/24/27, 143/145 and 14q32. Understanding the contribution of these microRNAs to the entire spectrum of vascular remodelling processes is important, especially as these microRNAs may have great potential as therapeutic targets for treatment of various CVDs.

Introduction

MicroRNAs

MicroRNAs are a class of endogenous noncoding RNA molecules of approximately 22 nucleotides in length. MicroRNAs inhibit translation of mRNAs into proteins by binding to specific sites in the 3'-untranslated region (3'UTR) of their target mRNAs. Rather than completely silencing their target gene, binding of a microRNA leads to modest target downregulation. However, a single microRNA is able to downregulate the expression of numerous target genes, and by doing so, that single microRNA can regulate complex, multifactorial physiological processes[1]. MicroRNAs have been shown to play an important role in human diseases, including cardiovascular disease (CVD). In this review, we describe the multifactorial nature of microRNAs in the regulation of vascular remodelling, by discussing the different target genes and regulatory mechanisms that have been described for these microRNAs. Although many microRNAs play a role in some aspects of vascular remodelling, we focused on those microRNAs that play a role in multiple forms of vascular remodelling and are clearly linked to CVD. The individual microRNAs miR-126 and miR-155, the microRNA gene clusters 17-92, 23/24/27, 143/145, and the largest known microRNA gene cluster 14q32, all met these criteria (Figure 1). An overview of confirmed target genes for these microRNAs is given in Tables 1 and 2.

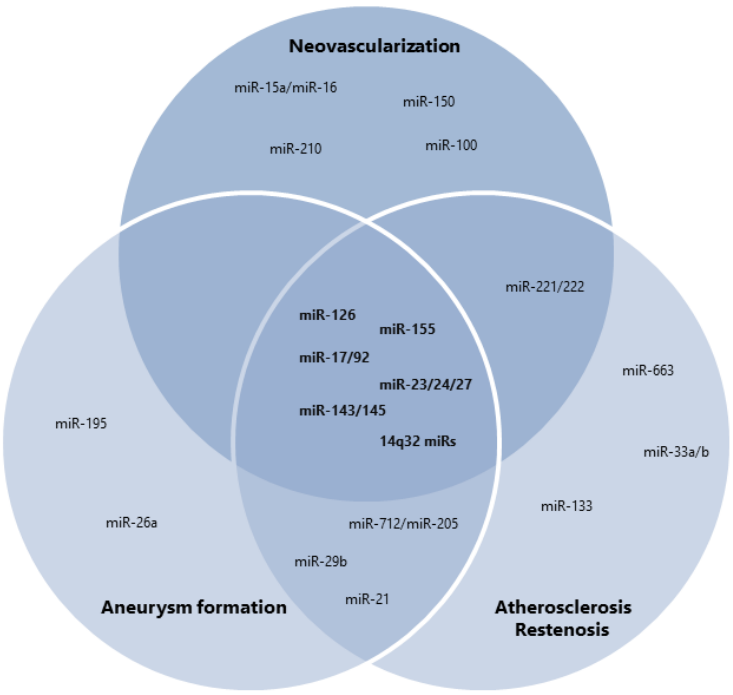

Figure 1 The top microRNAs reported to play a role in each of the following vascular remodelling processes, atherosclerosis and restenosis formation, aneurysm formation, and neovascularization are shown. MicroRNAs that were reported to play a role in multiple forms of these processes were selected for this review and shown here in bold.

Non-standard abbreviations and acronyms	
3'UTR	3'-untranslated region
AAA	Abdominal aortic aneurysm
AAV	Adeno-associated virus
AngII	Angiotensin II
CAD	Coronary artery disease
CVD	Cardiovascular disease
EC	Endothelial cell
HDL	High-density lipoprotein
IA	Intracranial aneurysm
LNA	Locked nucleic acid
miR	MicroRNA
MO	Morpholino
MP/MV	Microparticle/Microvesicle
MSC	Mesenchymal stem cell
oxLDL	Oxidized low-density lipoprotein
PBMC	Peripheral blood mononuclear cell
siRNA	Small-interfering RNA
TLR	Toll-like receptor
(V)LDL	(very) low-density lipoprotein
(V)SMC	(vascular) smooth muscle cell
Abbreviations of NCBI-annotated target genes	
ABCA1	ATP-binding cassette transporter A1
ACAT1	Acyl-CoA cholesterol acyltransferase-1
ACE	Angiotensin-converting enzyme
ANGPTL3	Angiopoietin-like 3
ARF6	ADP ribosylation factor 6
AT1R	Angiotensin II type 1 receptor
BCL2/6	B-cell lymphoma 2/6
bFGF	Basic fibroblast growth factor
BIC	B-cell integration cluster
BMP4	Bone morphogenetic protein 4
CCL2/MCP1	Monocyte chemoattractant protein 1
CD146	Cluster of differentiation 146 (melanoma cell adhesion molecule)
CDK4	Cyclin-dependent kinase 4
CPT1α	Carnitine palmitoyl transferase 1α
CXCL12/SDF1	Stromal derived factor-1
CXCR4	Chemokine (C-X-C motif) receptor 4
DGAT2	Diacylglycerol O-acyltransferase 2
DLK1	NOTCH1 inhibitor delta-like 1 homolog
E2F1	E2F transcription factor 1
EFNB2	Ephrin B2
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-related kinase 1/2
ETS-1	V-ets avian erythroblastosis virus E26 oncogene homolog 1
FGFGR2	Fibroblast growth factor receptor 2
FLT1	VEGF receptor fms-related tyrosine kinase 1
FOXO3/4	Forkhead box O 3/4
FSR2	Fibroblast growth factor receptor substrate 2
FZD4	Frizzled class receptor 4
GATA2	GATA binding protein 2
GPAM	Glycerol-3-phosphate acyltransferase 1 mitochondrial
HIF1α/HIF2α	Hypoxia inducible factor 1/2, alpha subunit
HKII	Hexokinase II

HMGB1	HMG box-transcription protein 1
HMOX1	Heme oxygenase 1
ICAM1	Intracellular cell adhesion molecule 1
IGF1	Insulin-like growth factor 1
IL33	Interleukin 33
INSIG1	Insulin-induced gene 1
IRS1/2	Insulin receptor substrate 1/2
ITGβ8	Integrin β 8
JAK1	Janus kinase 1
KLF2/4/5	Krüppel-like factor 2/4/5
LPL	Lipoprotein lipase
LRP6	LDL receptor-related protein 6
MCP1/CCL2	Monocyte chemoattractant protein 1
MEF2a	Myocyte enhancer factor 2
MEG3	Maternally expressed gene 3
MIF	Macrophage migration inhibitory factor
MKK4	Mitogen-activated protein kinase kinase 4
MMP1/3	Matrix metalloproteinase 1/3
MRTFA	Myocardin-related transcription factor A
Myd88	Myeloid differentiation primary response gene
PAK4	p21-activated kinase 4
PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 2
PPARγ	Proliferator-activator receptor gamma
PPP2R2A	Protein phosphatase 2 regulatory subunit B, alpha
RGS16	Regulator of G protein signalling 16
SDF1/CXCL12	Stromal derived factor-1
SEMA6A/6D/3B	Semaphorin 6A/6D/3B
SMAD3	SMAD family member 3
SOCS1/5	Suppressor of cytokine signalling 1/5
SPRED1	Sprouty-related, EVH1 domain containing 1
SREBPs	Sterol regulatory element-binding proteins
SRF	Serum response factor
TAB2	TGF-β activated kinase1/MAP3K7 binding protein 2
TGF-β(2)	Transforming growth factor β (2)
TGF-βR2	TGF-β receptor 2
TIMP3	Tissue inhibitor of metalloproteinase 3
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon-b
uPA	urokinase-type plasminogen activator
VCAM1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

Table 1 Non-standard abbreviations and acronyms

MicroRNA	Confirmed targets	Biological process affected
miR-126	VCAM1[2], SPRED1, and PIK3R2[3–5]	Angiogenesis, vascular integrity
	SDF1/CXCL12[6,7]	Migration of CD34+ progenitor cells
	RGS16[6]	Recruitment of Sca-1+ endothelial progenitor cells, atherosclerosis
	FOXO3, BCL2, and IRS1[8]	VSMC turnover
	DLK1 (miR-126-5p)[9]	Endothelial repair, atherosclerosis

miR-155		SOCS1[10]	Pro-inflammatory signalling
		TAB2[11]	Anti-inflammatory signalling
		PU.1[12]	Monocyte/macrophage infiltration, T lymphocyte activation
		AT1R[13], ETS-1[14]	HUVEC activation and migration
		AT1R[15], SOCS1[15]	Angiogenesis, Arteriogenesis
		BCL6, CCL2[16]	Atherosclerosis
		HMGB1[17]	Foam cell formation
		MMP1 and MMP3[18]	Matrix degradation
miR-23-24-27	miR-23 and miR-27	SEMA6A, SEMA6D, SPROUTY2[19]	EC sprouting, angiogenesis
	miR-23b	E2F1[20]	Rb phosphorylation, EC growth arrest
		uPA, SMAD3, FOXO4[21]	VSMC phenotypic switching
	miR-24	GATA2, PAK4[22]	Vascularity, cardiac function, and infarct size after myocardial infarction
		NDST1[23]	HSPG sulfation and affinity of HSPGs for VEGF, endothelial cell responsiveness to VEGFA
		HMOX1[24]	SMC apoptosis and proliferation
		INSIG1[25]	Lipid accumulation and plasma triglyceride levels
		CHI3L1[26]	Inflammation, AAA formation
	miR-27a	VE-cadherin[27]	Vascular leakage
	miR-27b	ABCA1, LPL, ACAT1[28]	Cholesterol efflux, lipid uptake and cholesteryl-ester formation
miR-17-92	miR-17-92	TSP1, CTGF[29]	Tumour angiogenesis
	miR-17/20	JAK1[30]	Angiogenesis
	miR-19a	CyclinD1[31]	EC proliferation
		FZD4 and LRP6[32]	WNT signalling, arteriogenesis
	miR-92a	KLF2, KLF4, SOCS5[33]	Endothelial homeostasis, atherosclerosis
		ITGA5[34]	Blood flow recovery after ischaemia and LV function after myocardial infarction
		KLF4 and MKK[35]	EC proliferation and migration
miR-143/145	miR-143/145	HKII, ITGβ8[36]	Angiogenesis, vessel stability
		ACE[37]	Atherosclerosis
	miR-143	ELK1[38]	VSMC proliferation
		AKT[39]	Angiogenesis, tumourigenesis
	miR-145	IGF-I, IRS1[40,41]	Tumour angiogenesis
		HIF2α[42]	Angiogenesis
		KLF5[43]	Transdifferentiation of fibroblasts to myofibroblasts, neointima formation
		KLF4[44]	VSMC differentiation
		JAMA1[45]	Leukocyte recruitment
		ABCA1[46]	Cholesterol efflux
14q32 miRs	miR-329	MEF2a[47], CD146[48]	Angiogenesis, arteriogenesis, EC proliferation
	miR-494	VEGFA, EFNB2, FGFR2[47]	Angiogenesis, arteriogenesis, myofibroblast proliferation
		TIMP3, TGFB2, IL33[49]	Atherosclerosis
	miR-376b-5p	HIF1α/VEGF signalling pathway[50]	Angiogenesis
	miR-377	VEGFA[51]	Angiogenesis
	miR-136	PPP2R2A[52]	VSMC proliferation
	miR-758	ABCA1[53]	Cholesterol efflux
	miR-370	CPT1α[54]	Fatty acid β oxidation
	miR-487b	IRS1[55]	Outward remodelling of the aorta

Table 2 Overview of confirmed target genes for microRNAs discussed

Vascular remodelling

Vascular remodelling comprises beneficial adaptive responses of the vessel wall to changes in haemodynamic forces, vasoactive stimuli or growth factors, but also maladaptive responses that can lead to CVD[56]. Thus, vascular remodelling can be divided into adaptive and maladaptive processes regarding vessel wall structure and blood supply towards downstream tissues[57]. For this review, we focused on neovascularization on the one hand and on atherosclerosis, postinterventional restenosis and aneurysm formation on the other. All of these processes are orchestrated by microRNAs[57].

When studying the role of microRNAs in these processes, there are several microRNAs that have been very well described. For example, one of the most promising microRNAs as therapeutic target for the treatment of atherosclerotic disease is miR-33a/b (discussed below), as it controls cholesterol metabolism, a crucial mechanism in CVD. The phenotype of smooth muscle cells (SMCs), either contractile or proliferative, is also imperative for vascular remodelling and neointima formation. Several microRNAs, including miR-133, miR-125b, miR-26a, miR-663, and miR-1, have been shown to control SMC phenotype and function[58–62]. In aneurysm formation, the miR-29 family has been shown to play a major role by targeting genes that are involved in extracellular matrix homeostasis. Inhibition of miR-29b in two murine abdominal aortic aneurysm (AAA) models increased expression of genes encoding for collagen and elastin and reduced expression of matrix metalloproteinases, resulting in decreased aneurysm progression in these mice[63]. Similarly, miR-21 regulated AAA expansion through targeting of PTEN[64].

Therapeutic potential of microRNAs

Several microRNAs that gave promising results as therapeutic targets in murine models of CVD are now being studied in larger animal models. A relevant example is the miR-33 family, consisting of miR-33a and miR-33b. Both miR-33a and miR-33b regulate the expression of cholesterol transporter ABCA1, which mediates the efflux of cholesterol[65]. Inhibition or deficiency of miR-33a reduced progression of plaques and raised HDL levels in atherosclerotic mouse models[65 – 67]. Since rodents lack miR-33b, extrapolation of these results to a human situation was not straightforward. Systemic inhibition of miR-33a/b in African green monkeys, which do express miR-33b, led to increased expression of ABCA1 in the liver of treated animals and increased plasma HDL levels[68]. The authors also observed the regulation of other genes involved in fatty acid oxidation and fatty acid synthesis, leading to a decrease in plasma VLDL levels, an effect that was not observed in mice[68]. Moreover, no overt toxicity was observed in animals treated with anti-miRs, supporting the development of anti-miR-33 therapeutics for treatment of atherosclerosis.

Janus phenomenon

However, caution is wanted when intervening in individual processes of vascular remodelling. This is best illustrated by the Janus phenomenon, named after the two-faced Roman deity Janus. The Janus phenomenon was first described by Epstein et al., who noticed that interventions used to stimulate arteriogenesis also increased atherosclerosis and vice versa[69]. The phenomenon is explained by the fact that there is a strong overlap in the mechanisms that underlie the various forms of vascular remodelling. One of the important mechanisms shared in vascular remodelling are the inflammatory responses. Since microRNAs can target numerous genes that may be involved in many processes, modulation of one microRNA could influence more than one form of vascular remodelling. This could be positive, for example when targeting a single microRNA inhibits various forms of maladaptive remodelling simultaneously. However, an unwanted effect could be that anti-atherogenic microRNAs also have anti-arteriogenic effects due to common pathways in atherosclerosis and arteriogenesis. The Janus phenomenon is a major drawback for many novel therapeutics designed to modulate vascular remodelling and must also be taken into account when exploring the therapeutic potential of microRNAs.

Therefore, we chose to discuss those microRNAs, miR-126, miR-155, and microRNA gene clusters 17-92, 23/24/27, 143/145, and 14q32, that play a confirmed role in multiple forms of vascular remodelling and are clearly linked to CVD.

miR-126

MiR-126 is one of the most abundantly expressed microRNAs in endothelial cells (ECs)[70]. The miR-126 gene is located on human chromosome 9 and gives rise to two mature microRNAs, miR-126-3p and miR-126-5p. Generally, the role of miR-126 in vascular remodelling as described in the literature corresponds to miR-126-3p (Figure 2). MiR-126 is also abundantly expressed in platelets, suggesting a role for miR-126 in vascular homeostasis and inflammation[71]. Platelets are a major source of circulating miR-126[72]. Consequently, levels of circulating miR-126 are influenced by the use of platelet inhibitors, such as aspirin[72]. The delivery of miR-126 by platelet microparticles (MPs) to primary human macrophages was reported recently, and miR-126 derived from these platelet MPs influences macrophage gene expression and function[69]. Levels of miR-126 are differentially expressed in plasma samples of patients with coronary artery disease (CAD)[74].

Neovascularization

The first studies from 2008 that investigated the role of miR-126 in EC function demonstrated that miR-126 targets VCAM1[2]. Increased expression of adhesion molecules such as VCAM1

and increased leukocyte adherence to ECs are necessary for the initiation of angiogenesis. Both mechanisms are stimulated by inhibition of miR-126[2]. In HUVECs, inhibition of miR-126 led to increased proliferation and migration[3]. Furthermore, injection of miR-126 inhibitors into zebrafish embryos affected blood vessel integrity, as was demonstrated by collapsed lumens and compromised endothelial tube organization[3]. Studies in mice showed that inhibition of miR-126 decreased recovery after myocardial infarction and impaired angiogenic capacity in a hindlimb ischaemia model[4,5,75]. These effects were partially mediated via inhibitors of VEGF signalling, namely SPRED1 and PIK3R2[3–5]. Both Spred1 and Pik3r2 are upregulated in the absence of miR-126, causing an increase in vascular permeability and leakage[3,5]. MiR-126 was also shown to target CXCL12[6]. Silencing miR-126 induced CXCL12 expression that enhanced migration of CD34+ progenitor cells *in vitro* and increased the number of circulating bone marrow-derived progenitor cells after hindlimb ischaemia *in vivo*[6,7].

In addition, exosomes from human CD34+ cells, which are rich in miR-126, have great angiogenic capacity both *in vitro* and *in vivo*[76]. Mocharla et al. showed that CD34+ peripheral blood mononuclear cells (PBMCs) secrete microvesicles and exosomes that are enriched with miR-126[77]. These microvesicles and exosomes are taken up by ECs and facilitate the pro-angiogenic effects of miR-126[77].

Atherosclerosis

Atherosclerotic plaque progression is often accompanied by apoptosis of (vascular) cells in the plaque[78]. During apoptosis, ECs release microvesicles that are enriched with miR-126[6]. Delivery of miR-126 to recipient vascular cells inhibits the progression of atherosclerosis, presumably via suppression of RGS16, which is a negative regulator of CXCR4. Subsequent upregulation of CXCR4 led to the production of CXCL12. This reduced lesion formation by decreasing the number of macrophages and apoptotic cells in the plaque and increasing the recruitment of endothelial progenitor cells for repair in a mouse model for atherosclerosis[6]. Vesicle-independent transfer of miR-126 from ECs to SMCs was also reported, increasing miR-126 levels in SMCs (Figure 2). Decreased expression of miR-126 target genes FOXO3, BCL2, and IRS1 led to increased proliferation of SMCs[8]. Subjecting ECs to laminar shear stress or miR-126 inhibition abolished these effects. In miR-126$^{-/-}$ mice, neointima formation was attenuated compared with wild-type mice after ligation of the left common carotid artery[8]. Recently, the contribution of miR-126-5p to atherosclerosis formation was demonstrated by Schober et al[9]. Hypercholesterolaemic miR-126$^{-/-}$ApoE$^{-/-}$ and miR-126$^{+/+}$ApoE$^{-/-}$ mice were subjected to endothelial denudation. After 14 and 28 days, lesion area was increased in miR-126$^{-/-}$ApoE$^{-/-}$ mice compared with control animals[9]. Moreover, endothelial recovery of the

carotid lumen was impaired in miR-126$^{-/-}$ApoE$^{-/-}$ animals due to reduced EC proliferation[9]. In these animals, expression of multiple miR-126-5p predicted target genes was increased, whereas expression of known miR-126-3p targets was not[9]. The authors confirmed targeting of DLK1 by miR-126-5p and demonstrated that inhibition of miR-126-5p increased Dlk1 expression and reduced EC proliferation[9]. To identify the specific role of miR-126-3p and miR-126-5p in endothelial repair, denuded arteries of ApoE$^{-/-}$ mice were treated with miR-126-3p-, miR-126-5p-, or control-miR inhibitors. Treatment with anti-miR-126-5p, but not anti-miR-126-3p, significantly increased the lesion area and impaired endothelial recovery and EC proliferation[9].

In untreated ApoE$^{-/-}$ mice, disturbed flow led to decreased miR-126-5p levels and increased Dlk1 mRNA and protein levels in the carotid artery, whereas miR-126-3p levels were unaltered[9]. The authors proposed that miR-126-5p plays a role in regulating EC proliferation at non-predilection sites, whereas miR-126-3p presumably regulates the replicative capacity of ECs at predilection sites[9]. Finally, in human atherosclerotic lesions, miR-126-5p levels were found to inversely correlate with DLK1 expression and the number of lesional macrophages and positively correlated with EC proliferation, suggesting an atheroprotective effect of increased miR-126-5p levels in humans[9].

Aneurysm

Although miR-126 is upregulated in AAA and upregulation correlated with decreased TNF-α expression, the exact function of miR-126 in AAA pathogenesis is still unknown[79]. In plasma of patients with AAA, miR-126 was significantly downregulated compared with plasma of healthy volunteers, but not compared to patients with CAD[79].

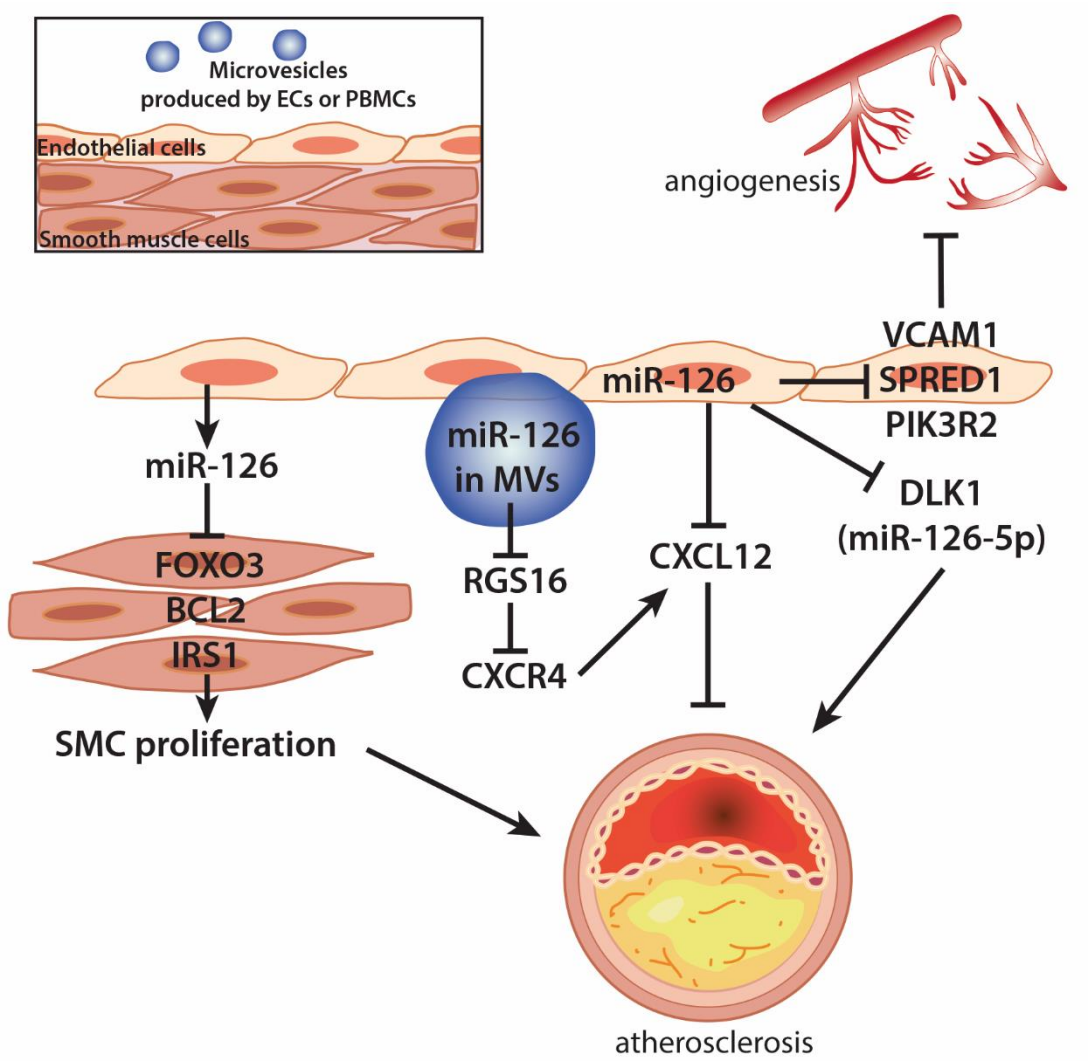

Figure 2 The role of endothelial miR-126 in vascular remodelling. MiR-126 regulates angiogenesis and vascular integrity via targeting of VCAM1 and targeting the inhibitors of VEGF signalling; SPRED1 and PIK3R2. Via microvesicle-mediated delivery from ECs to neighbouring vascular cells, miR-126 inhibits RGS16, an inhibitor of CXCR4, resulting in the expression of CXCL12 and reducing atherosclerosis. Paracrine secretion of miR-126 from ECs to SMCs leads to inhibition of FOXO3, BCL2, and IRS1 target genes and increases proliferation of SMCs, which contributes to the atherogenic actions of miR-126. In addition, miR-126-5p suppresses the NOTCH1 inhibitor DLK1, thereby limiting atherosclerosis. Arrows indicate upregulation. Capped lines indicate inhibition. MV, microvesicle; EC, endothelial cell; SMC, smooth muscle cell; PBMCs, peripheral blood mononuclear cells. For full target gene names, see Table 1.

miR-155

The miR-155 gene is located within an exon of the noncoding RNA BIC on human chromosome 21. MiR-155 is highly expressed by activated B and T cells, but also by monocytes and macrophages[80,81]. In addition, miR-155 is expressed in ECs and SMCs[14].

MiR-155 is upregulated in macrophages via TLR ligands, such as LPS[81]. MiR-155 exerts pro-inflammatory effects via targeting of the anti-inflammatory SOCS1[10]. In contrast, anti-

inflammatory effects of miR-155 signalling have also been described via targeting of TAB2[11] (Figure 3).

In 2012, Corsten et al.[12] described a role for miR-155 in CVD, demonstrating upregulation of miR-155 during the acute inflammatory phase of viral myocarditis. Systemic inhibition of miR-155 reduced cardiac monocyte/macrophage infiltration, decreased T-lymphocyte activation, and reduced myocardial damage in a mouse model of acute viral myocarditis[12].

Neovascularization

MiR-155 is co-expressed with AT1R in HUVECs and SMCs, where it represses AT1R expression[13]. Interestingly, a single nucleotide polymorphism (+1166 A/C), which is associated with CVD, was found to disrupt a miR-155 target site in the 3'UTR of AT1R[13]. Overexpression of miR-155 reduced migration of HUVECs in response to Angiotensin II (AngII) via targeting of the AT1R.

ETS-1 has two potential binding sites for miR-155 in its 3'UTR and is another target of miR-155 in HUVECs[14]. ETS-1 and its downstream target genes VCAM1, MCP1, and FLT1 were induced in HUVECs upon stimulation with AngII. Overexpression of miR-155 abrogated this effect[14].

Recent work by Pankratz et al.[15] demonstrated that miR-155 exerts both anti-angiogenic and pro-arteriogenic functions after induction of hindlimb ischaemia in mice[82]. MiR-155 was upregulated 7 days after femoral artery ligation in mice. Inhibition of miR-155 in HUVECs resulted in increased EC proliferation and tube formation[15]. These results were confirmed in aortic ring assays, as well as in *in vivo* Matrigel plug assays using miR-155$^{-/-}$ mice. In miR-155$^{-/-}$ ECs, expression of AT1R was increased. AT1R expression could be manipulated by overexpression or inhibition of miR-155 in both human and murine ECs. The authors concluded that the antiangiogenic properties of miR-155 are mediated via AT1R[15] (Figure 2). Despite the anti-angiogenic properties of miR-155, blood flow recovery after hindlimb ischaemia was impaired in miR-155$^{-/-}$ mice. MiR-155 deficiency decreased migration of bone marrow-derived macrophages. MiR-155$^{-/-}$ macrophages showed significantly reduced expression levels of pro-arteriogenic cytokines and chemokines upon LPS stimulation, compared with wild-type cells. SOCS1 was identified as a potential mediator, as this was the most upregulated target gene in miR-155$^{-/-}$ BMDMs. Knockdown of SOCS1 indeed reversed the effects of miR-155 deficiency on pro-arteriogenic cytokine production[15].

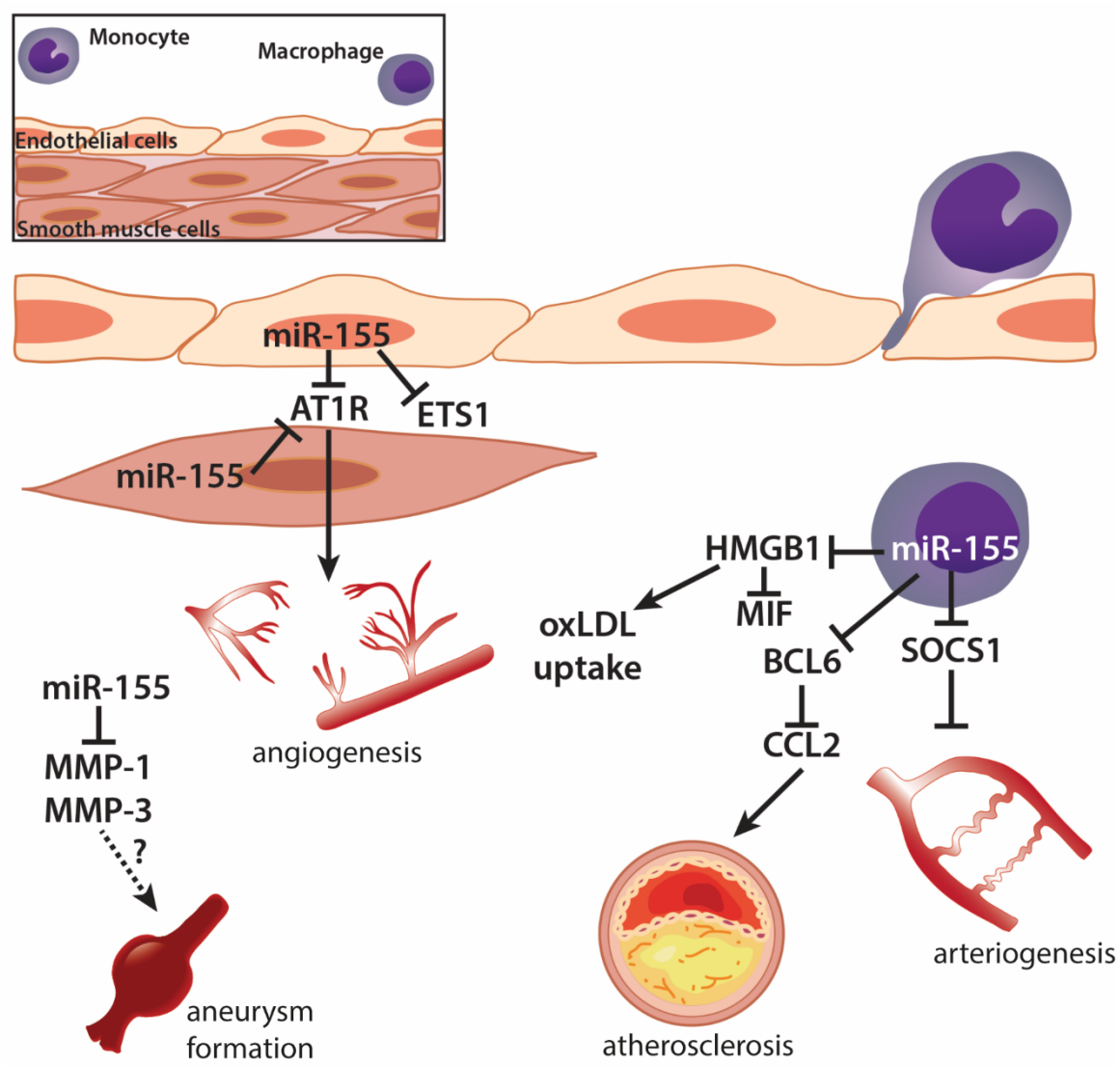

Figure 3 The inflammatory miR-155 in vascular remodelling. MiR-155 is co-expressed with AT1R in HUVECs and SMCs and inhibits expression of AT1R in these cells. ETS1 transcription factor is also targeted by miR-155 in HUVECs. Via these targets, miR-155 affects angiogenesis. In addition, miR-155 has been demonstrated to affect arteriogenesis. This effect is mediated by inhibition of SOCS1 in macrophages, resulting in upregulation of pro-arteriogenic cytokines. MiR-155 in atherosclerotic plaques is predominantly expressed in (pro-inflammatory) macrophages, where it suppresses the transcription factor BCL6. In addition, miR-155 targets HMGB1, increasing oxLDL uptake by macrophages. MiR-155 reduces matrix metalloproteinases MMP-1 and MMP-3, which could reduce matrix degradation and progression of aneurysm formation. Arrows indicate upregulation. Capped lines indicate inhibition. The dashed line indicates possible interactions that have not been confirmed yet. (HUV)EC, (human umbilical venous) endothelial cell; SMC, smooth muscle cell. For full target gene names, see Table 1.

Atherosclerosis

Expression of miR-155 was upregulated in human atherosclerotic plaques, predominantly in pro-inflammatory macrophages[16,83]. However, circulating levels of miR-155 were significantly lower in patients with CAD compared with healthy volunteers[74]. In several studies, treatment

of macrophages with oxidized LDL and IFN-γ led to upregulation of miR-155, whereas suppression of miR-155 by oxLDL treatment has also been reported[16,84 – 86]. Nazari-Jahantigh et al. demonstrated that miR-155 targets BCL6, a transcription factor that attenuates pro-inflammatory NF-kB signalling and directly represses CCL2. Leukocyte-specific deletion of miR-155 decreased Ccl2 signalling and reduced atherosclerotic plaque formation in ApoE$^{-/-}$ mice[16]. Recently, Tian et al.[17] showed that miR-155 targets HMGB1, which suppresses MIF and increases uptake of oxLDL by macrophages. Elevated miR-155 levels enhanced oxLDL-induced foam cell formation by targeting HMGB1. Systemic inhibition of miR-155 in ApoE$^{-/-}$ mice resulted in smaller atherosclerotic plaques that contained less lipid-laden macrophages[17]. However, opposite findings on the role of miR-155 in atherosclerosis have also been reported. LDL-R$^{-/-}$ mice transplanted with miR-155$^{-/-}$ bone marrow developed larger lesions compared with mice transplanted with wild-type bone marrow[87]. Increased numbers of macrophages and neutrophils were present in these lesions as well as increased numbers of granulocytes and inflammatory monocytes in the circulation[87]. Apparently, miR-155 can have opposite effects in macrophages, being either pro- or anti-inflammatory (Figure 3).

Aneurysm

MiR-155 is significantly upregulated in AAA tissue[79]. However, expression of miR-155 was lower in plasma of patients with AAA compared with plasma levels of healthy controls and of patients with CAD[79]. In models for rheumatoid arthritis, overexpression of miR-155 led to downregulation of MMP1 and MMP3[18]. This suggests that overexpression of miR-155 in AAA may function as an endogenous rescue mechanism that inhibits matrix degradation and progression of aneurysm formation[18].

miR-17-92 cluster

The miR-17-92 gene cluster is located within intron 3 of the C13orf25 gene on human chromosome 13 and encodes six individual microRNAs, namely miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a[88] (Figure 4). Recently, it was shown that expression of the miR-17-92 cluster in ECs is stimulated by VEGF, via activation of the Erk/Elk1 pathway[89]. Upon stimulation, expression of miR-17-92 contributed to endothelial proliferation and angiogenic sprouting *in vitro* and physiological angiogenesis *in vivo*[89].

Neovascularization

In 2009, Bonauer et al. showed that miR-92a is highly expressed in human ECs and overexpression of miR-92a in ECs blocked sprouting in a three-dimensional angiogenesis

model. *In vivo* inhibition of miR-92a increased the number of perfused vessels in Matrigel plugs and improved blood flow recovery after hindlimb ischaemia[34]. ITGA5 was identified as a direct target of miR-92a[34]. To elucidate the specific function of the other members of the miR-17-92 cluster in angiogenesis, Doebele et al.[30] overexpressed or blocked individual members of the cluster both *in vitro* and *in vivo*. *In vitro* inhibition of all miR-17-92 members, except miR-19, resulted in increased sprouting of EC spheroids[30]. Combined inhibition of miR-17 and miR-20a was shown to promote angiogenesis in Matrigel plugs *in vivo*, whereas inhibition of other members showed trends but no significant effects on angiogenesis[30]. Expression of JAK1 was reduced at mRNA and protein level upon miR-17 overexpression and inhibition of JAK1 using siRNAs was shown to reduce *in vitro* angiogenesis. Using luciferase assays, JAK1 was confirmed as a direct target of miR-17[30].

The contribution of the miR-17-92 cluster to physiological and pathological arteriogenesis was studied by Landskroner-Eiger et al[32]. Endothelial specific knockout of miR-17-92 in mice showed that these animals had more pre-existent collateral arterioles. Consequently, these animals showed improved blood flow recovery after ischaemia. MiR-19a targets components of WNT signalling, namely FZD4 and LRP6. Inhibition of miR-19a improved post-ischaemic blood flow recovery[32].

Expression of the miR-17-92 cluster is repressed by HDAC9 in ECs[90]. Inhibition of HDAC9 reduced neovascularization *in vitro* and *in vivo*. Inhibition of HDAC9, using either a broad spectrum HDAC inhibitor or siRNAs against HDAC9, increased expression of the miR-17-92 cluster, suggesting that the anti-angiogenic effects of HDAC9 inhibition are mediated through the miR-17-92 cluster. Indeed, inhibition of miR-17-20a combined, but not of miR-17 alone, completely rescued the reduced sprouting and network formation in HDAC9-deficient ECs[90].

Atherosclerosis

Several studies showed that miR-17-92 cluster members are regulated by changes in shear stress[20,31]. Upregulation of miR-19a by laminar shear stress has an anti-proliferative effect on ECs via targeting of Cyclin D1[31]. MiR-92a expression was reduced in HUVECs that were subjected to atheroprotective laminar shear stress, leading to upregulation of KLF2[91]. Expression of KLF2 targets eNOS and thrombomodulin were decreased upon miR-92a overexpression[91].

MicroRNA expression profiling in HUVECs revealed upregulation of miR-92a upon low shear stress conditions and the presence of oxLDL[33]. Accordingly, miR-92a expression was higher in the vasculature of both mice and humans in atheroprone regions with low shear stress[33]. MiR-92a inhibition reduced atherosclerosis formation in hypercholesterolaemic $LDLR^{-/-}$ mice. Expression of target genes Klf2 and Klf4 was increased upon anti-miR-92a treatment. The

authors identified SOCS5 as a novel target of miR-92a, which is involved in the regulation of endothelial inflammation[33]. Furthermore, circulating ICAM-1 levels were reduced in anti-miR-92a-treated animals. These results suggest that upregulation of miR-92a by oxLDL in atheroprone regions promotes endothelial dysfunction and atherosclerosis formation[33]. Interestingly, inhibition of miR-92a in rats reduced neointima formation in carotid arteries after vascular injury[35]. MiR-92a inhibition increased EC proliferation and migration, improving reendothelialization after balloon injury or arterial stenting. Expression of KLF4 and MKK was upregulated by miR-92a inhibition[35]. MiR-92a is a promising therapeutic target to reduce atherosclerosis development and postinterventional restenosis.

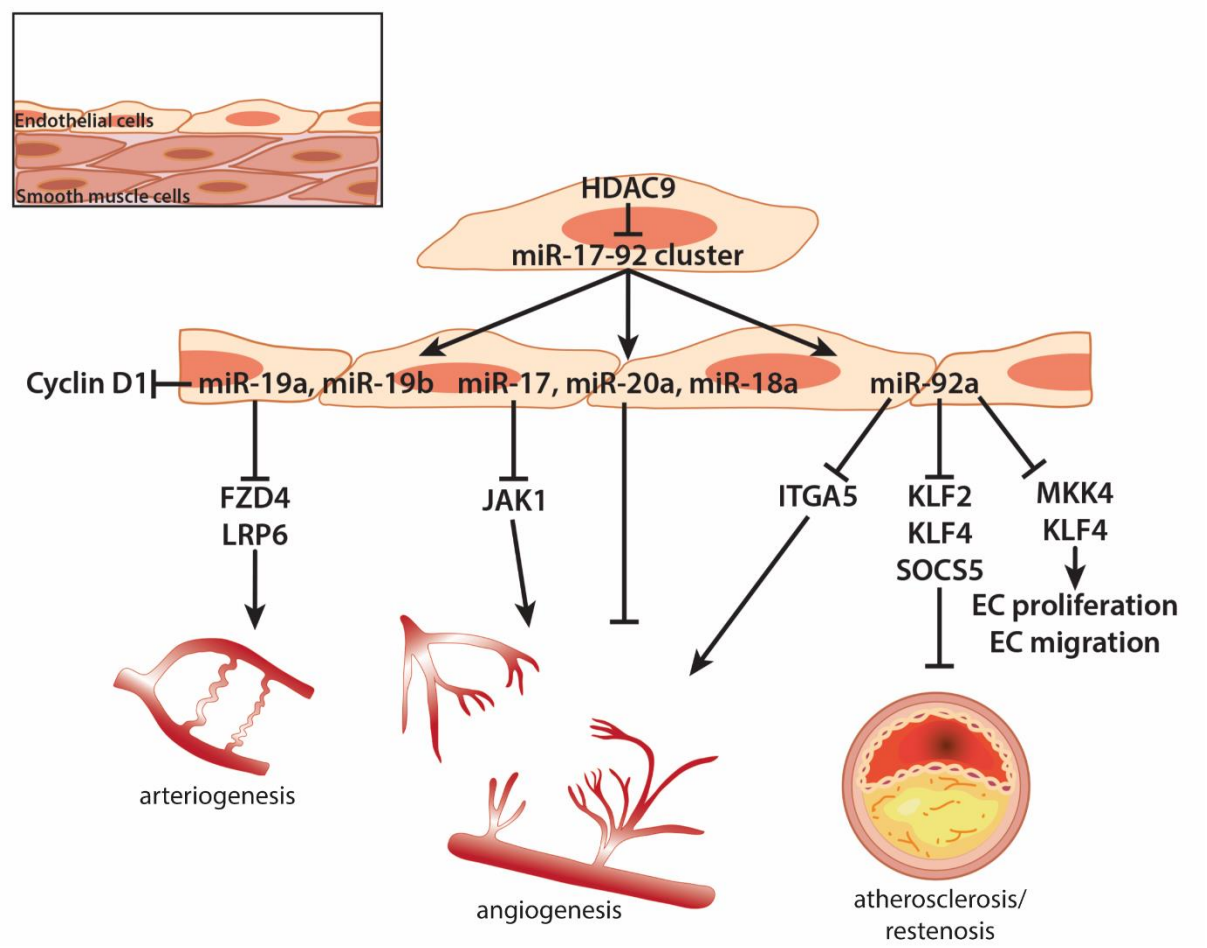

Figure 4 The role of the miR-17-92 cluster in vascular remodelling. MiR-17-92 cluster members regulate angiogenesis via suppression of several target genes. MiR-17 reduces expression of JAK1. ITGA5 is targeted by miR-92a. In addition, miR-92a targets KLF2, KLF4, and SOCS5, promoting atherosclerosis formation. Proliferation and migration of endothelial cells are regulated by targeting of MKK4 and KLF4 by miR-92a. MiR-19a has an anti-proliferative effect in ECs via suppression of Cyclin D1. Other target genes of miR-19a include FZD4 and LRP6, regulators of WNT signalling. Targeting of these genes by miR-19a affects collateral artery formation and blood flow recovery after ischaemia. Arrows indicate upregulation. Capped lines indicate inhibition. EC, endothelial cell. For full target gene names, see Table 1.

Aneurysm

Two members of the 17-92 cluster, miR-20a and miR-92a, were significantly upregulated in ECs of AAA tissue, but a causative role has yet to be confirmed[79].

miR-23/24/27 family

The miR-23/24/27 family consists of two separate microRNA gene clusters. The mouse intergenic miR-23a-27a-24-2 cluster lies on chromosome 8; in humans this cluster is located on chromosome 19. The miR-23b-27b-24-1 cluster has an intronic location on mouse chromosome 13, chromosome 9 in humans[19,92]. Members of the miR-23/24/27 family are highly expressed in vascularized tissues and ECs[93] (Figure 5). Laminar flow and unidirectional shear stress increase the expression of miR-23b, miR-27a/b, and miR-24 in ECs[20,94,95]. Increased expression of miR-23b and miR-27b by pulsatile shear flow was found to correlate with EC growth arrest. The expression of cell cycle gene E2F1 was downregulated by miR-23b and miR-27b[20]. Moreover, phosphorylation of the Rb protein was blocked by miR-23b. Decreased Rb-phosphorylation reduces EC proliferation and inhibits cell cycle progression[20]. Anti-miR-23b, but not antimiR-27, treatment of HUVECs resulted in partial reversal of shear stress-induced growth arrest[90].

Neovascularization

Knockdown of miR-23a/b and miR-27a/b decreased *in vitro* EC sprouting and *ex vivo* aortic ring sprouting[19]. Anti-angiogenic genes SEMA6A, SEMA6D, and SPROUTY2 are targeted by miR-23a/b and miR-27a/b, as shown by luciferase gene reporter assays[19]. Urbich et al. showed that *in vivo* angiogenesis was also affected upon inhibition of miR-27a/b. Anti-miR-27a/b treatment decreased the number of perfused vessels in Matrigel plugs[96]. Moreover, inhibition of miR-27a/b impaired vasculogenesis in zebrafish embryos. *In vitro* experiments showed additional targeting of SEMA3B but *in vivo*, only SEMA6A was a target of miR-27 a/b[96]. Young et al.[27] showed that miR-27a also targets VE-cadherin, both *in vitro* and *in vivo*.

MiR-24 is expressed in cardiac ECs[22]. The expression of miR-24 is induced upon hypoxia and miR-24 is enriched in cardiac ECs compared with other cardiac cells after cardiac ischaemia[22,24]. Overexpression of miR-24 in HUVECs increased apoptosis and impaired tube formation, sprouting, migration, and proliferation[22]. The endothelium-enriched transcription factors GATA2 and PAK4 were validated as targets of miR-24. Inhibition of miR-24 increased vascularity and decreased myocardial infarct size in mice[22]. Another confirmed target gene for miR-24 in ECs is NDST1. Inhibition of NDST1 by miR-24 decreased sulfation of HSPGs and subsequently the binding affinity of HSPGs for VEGFA. MiR-24-mediated suppression of NDST1 lowered VEGFR2 levels and reduced EC responsiveness to VEGFA[23]. Via these

mechanisms, miR-24 affects EC responsiveness to VEGFA. MiR-24 also affected apoptosis, proliferation and function of SMCs, partially through HMOX1[24].

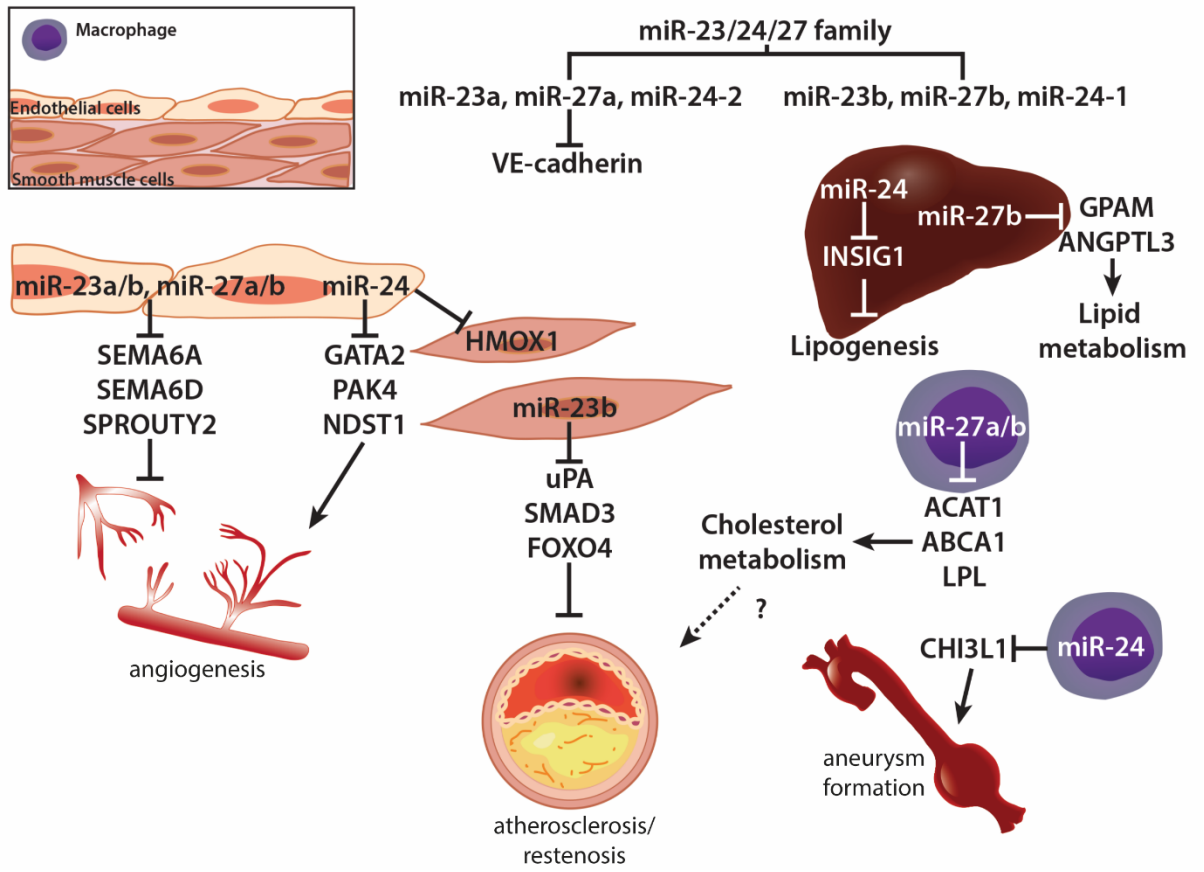

Figure 5 Different roles in vascular remodelling for microRNAs of the miR-23/24/27 family. The miR-23/24/27 family consists of two miR clusters, namely the miR-23a-27a-24-2 cluster and the miR-23b-27b-24-1 cluster. MiR-23a/b and miR-27a/b target the anti-angiogenic genes SEMA6A, SEMA6D, and SPROUTY2. In addition, miR-27a inhibits expression of VE-cadherin. MiR-24 inhibits proliferation, migration, and sprouting of HUVECs via the endothelium-enriched transcription factor GATA2, PAK4, and NDST1. Moreover, miR-24 affects SMC apoptosis, proliferation, and function via HMOX1. Expression of miR-23b in SMCs was found to target uPA, SMAD3, and FOXO4, which results in decreased proliferation and migration of SMCs. Overexpression of miR-23b decreases neointima formation upon balloon injury in rats. Members of the miR-23/24/27 family also play an important role in lipid metabolism. Cholesterol metabolism is affected by miR-27a/b through suppression of ACAT1, ABCA1, and LPL in macrophages. In the liver, miR-27b targets the lipogenic genes GPAM and ANGPTL3, whereas miR-24 suppresses expression of INSIG1. Of the miR-23/24/27 family members, only miR-24 has been described to affect AAA formation. MiR-24 is expressed in macrophages in the adventitia of murine aneurysmal tissue, where it is co-localized with and inhibits expression of the CHI3L1 gene. Arrows indicate up-regulation. Capped lines indicate inhibition. The dashed lines indicate an interaction that has not been confirmed yet. (HUV)EC, (human umbilical venous) endothelial cell; SMC, smooth muscle cell. For full target gene names, see Table 1.

Atherosclerosis, restenosis and lipid metabolism

The effects of miR-24 and 27b as described here are predominantly on lipid metabolism, which will ultimately also influence atherosclerosis[25,28,97]. MiR-24 and miR-27b are upregulated in livers of high-fat diet (HFD)-fed mice[25,97]. Inhibition of miR-24 in HFD-fed mice reduced plasma triglyceride levels and lipid accumulation in the liver, but did not affect plasma cholesterol levels. This effect was mediated via increased expression of INSIG1 in the liver and subsequent decreased expression of SREBPs and other lipogenic genes[25].

MiR-27b targets several additional lipogenic genes, including PPARγ, ANGPTL3, and GPAM. However, direct binding of miR-27b to the 3'UTR of the mRNAs of these genes was not demonstrated[97]. Upregulation of hepatic miR-27b was observed in HFD ApoE$^{-/-}$ mice, and expression of miR-27b target genes Angptl3 and Gpam was reduced[97]. Experiments performed in the THP-1 human monocyte cell line showed that miR-27a/b regulates cholesterol homeostasis[28]. MiR-27a/b targeting of ABCA1 affected apoA1-mediated cholesterol efflux in macrophages[28]. Lipid uptake was also affected by miR-27a/b, as was shown by reduced oxLDL binding to macrophages after miR-27a/b overexpression. This was mediated by miR-27a/b target gene LPL. Finally, cholesteryl-ester formation was reduced by miR-27a/b via targeting of ACAT1[28].

The contribution of miR-23b to SMC phenotypic switching upon vascular injury was recently reported by Iaconetti et al[21]. Expression of miR-23b was reduced after carotid injury in rats. Increased proliferation and migration of SMCs was observed upon miR-23b inhibition, whereas overexpression of miR-23b led to reduced proliferation and migration[21]. Overexpression of miR-23b resulted in decreased neointima formation in rat carotid arteries after balloon angioplasty and target genes uPA, SMAD3, and FOXO4 were downregulated in these animals[21].

Aneurysm

MicroRNA expression profiling revealed decreased expression of the miR-23b/miR-24-1 cluster, in human intracranial aneurysmal (IA) samples[98,99]. In murine AAA models, the miR-23b-27b-24 cluster is also downregulated. MiR-24 was most significantly downregulated, leading to upregulation of the inflammatory target gene CHI3L1[26]. In situ hybridization showed localization of miR-24 in adventitial macrophages of aneurysmal aortic mouse tissue. MiR-24 co-localized with CHI3L1 in activated macrophages, where CHI3L1 drives inflammatory gene expression[26]. Modulation of miR-24 levels in murine AAA models using either pre-miR-24 or anti-miR-24 led to reduced and increased AAA formation, respectively[26]. In summary, miR-23b-24-27b family members are downregulated in human IA samples and murine AAA models and modulation of miR-24 influences aortic inflammation, thereby

contributing to AAA development[26,98,99]. This renders the miR-23-24-27 family a potentially interesting therapeutic target for AAA treatment.

miR-143/145 cluster

The miR-143/145 gene cluster contains two highly conserved microRNAs, which are located on human chromosome 5. In 2007, these microRNAs were first described as downregulated in rat carotid arteries after induction of balloon injury[100]. Restoration of miR-143 and miR-145 expression levels using an adenoviral vector reduced neointima formation upon balloon injury in rat carotids[101,102]. MiR-145 is the most abundantly expressed microRNA in healthy rat carotid arteries, where it is predominantly localized in SMCs[103]. During SMC differentiation from multipotent stem cells, high transcript levels of miR-143 and miR-145 are observed[44]. Upregulation of these microRNAs allows for SMC differentiation, whereas their expression is downregulated upon proliferation[44]. Together, these microRNAs play an important role in the differentiation and proliferation of SMCs. Differentiation of SMCs is induced via SRF, myocardin and myocardin-related transcription factors, but can also be induced via the Jag-1/Notch signaling pathway[38,104]. These factors also regulate the transcription of the miR-143/145 cluster, further promoting differentiation of SMCs[38,104] (Figure 6, upper panel). Expression of contractile genes is mediated via (among other factors) KLF4, which is directly targeted by miR-145[44]. Inhibition of KLF4 by miR-145 increases expression of SMC markers[44]. MiR-143 can directly inhibit proliferation of SMCs via targeting of ELK1[38].

In accordance with these findings, Boettger et al.[37] described that miR-143/145-deficient mice have a thinner arterial medial layer and a decreased blood pressure. In general, the miR-143/145 cluster has proven essential for SMC function and controls the phenotypic switch of contractile SMCs towards synthetic VSMCs[37,44,101,103] (Figure 6, upper panel).

Neovascularization

MiR-145 inhibits tumour angiogenesis via targeting of IGF1, the IRS1 pathway, and its downstream genes N-RAS and VEGFA[40,41]. MiR-143 was found to inactivate AKT, which is a downstream signalling molecule in the IGF1 receptor pathway and thereby regulates angiogenesis and tumourigenesis[39]. Inactivation of AKT by miR-143 resulted in decreased protein levels of HIF-1α and reduced VEGFA expression[39]. In neuroblastoma samples, miR-145 expression was also downregulated, which was inversely correlated with HIF-2α expression[42]. The authors showed that miR-145 can directly target HIF-2α and suppress angiogenesis, which was demonstrated by tube formation of neuroblastoma cells[42].

Although these findings relate mainly to pathological angiogenesis, many fundamental mechanisms are shared with physiological angiogenesis, such as receptor signalling cascades

(e.g. HIF-1α), proliferation, and migration of vascular cells and tube formation[105]. Indeed, Wang et al.[43] found that miR-145 was transiently downregulated *in vivo* following coronary artery occlusion in mice and *in vitro* upon hypoxia treatment of cardiac fibroblasts. Inhibition of miR-145 increased infarct scar size at 7 and 28 days after myocardial infarction in mice. However, reduced differentiation of cardiac fibroblasts towards myofibroblasts, and not decreased angiogenesis, most likely mediated these effects[43]. The authors demonstrated that transfection with miR-145 increased the number of α-SMA positive cells in fibroblast cultures, thus inducing transdifferentiation of fibroblasts into myofibroblasts. KLF5 is a direct target of miR-145. Transfection with miR-145 decreased expression of KLF5 and increased myocardin expression. These data suggest that miR-145 mediates differentiation of cardiac fibroblasts to myofibroblasts through the KLF5-myocardin pathway[43].

Work by Climent et al.[36] suggests that miR-143 and miR-145 are transferred from SMCs to ECs via membrane protrusions. TGF-β induces the transfer of miR-143/145, as inhibition of either the TGF-β pathway or TGF-βR2 reduced miR-143/145 transfer towards ECs[36]. Overexpression of miR-143 and miR-145 in ECs reduced proliferation and the ability to form capillary-like structures on Matrigel[36]. The authors identified HKII and ITGβ8 as direct targets of miR-143/145 that modulate the angiogenic potential of ECs[36] (Figure 6).

Atherosclerosis and restenosis

MiR-143/145$^{-/-}$ mice develop spontaneous neointimal lesions in the femoral arteries at older age[37]. Angiotensin-converting enzyme (ACE) was identified as a target for miR-143/145. Increased expression of ACE in miR-143/145$^{-/-}$ mice resulted in increased AngII levels, which subsequently contributed to the synthetic phenotype of miR-143/145$^{-/-}$ SMCs[37]. ApoE$^{-/-}$ mice treated with SMC-specific lentiviral miR-145 showed a reduction in plaque size and an increase in atherosclerotic plaque stability[106]. This is in line with the finding that overexpression of miR-145 decreased neointima formation in balloon injured arteries by modulation of KLF5 expression[102]. However, reduced neointima formation after carotid artery ligation in miR-143/145$^{-/-}$ mice has also been reported[107]. The authors explained this by the fact that the SMCs in their knockout model were already deficient in miR-145 at the onset of injury, whereas in the overexpression model, miR-145 expression was normal at the onset of the experiment[107].

In humans, miR-145 levels were significantly lower in plaques than in atherosclerosis-free regions[106]. Cholesterol loading of mouse aortic SMCs resulted in downregulation of SMC markers, whereas expression of macrophage markers was increased[108]. Expression of miR-143 and miR-145, as well as the expression of SRF and myocardin, was downregulated[108]. Cholesterol loading, via downregulation of the miR-143/145/SRF/Myocardin axis, causes

reprogramming of SMCs towards a macrophage-like phenotype[108]. Moreover, statin treatment, which is the most common form of anti-atherosclerotic therapy today, increases expression of miR-143/145 in ECs[109].

In addition to intercellular transfer of miR-143/145 from SMCS to ECs, transport of miR-143/145 in the opposite direction has also been described[36]. Increases in laminar shear stress lead to upregulation of KLF2, which subsequently induces transcription of the miR-143/145 cluster in ECs[109]. Upregulation of miR-145 in ECs was shown to repress JAM-A, which reduces leukocyte recruitment and infiltration and thus atherosclerosis formation[45]. KLF2 also triggered release of EC-derived MVs, which transfer miR-143/145 from ECs to SMCs. Injection of MVs that are rich in miR-143/145 in HFD-fed mice led to a reduction of plaque formation[109]. In contrast, miR-143/145$^{-/-}$ mice developed smaller atherosclerotic lesions compared with LDLR$^{-/-}$ controls[46]. Plaques of miR-143/145$^{-/-}$ mice contained less macrophages and analysis of plasma cholesterol levels revealed decreased VLDL and LDL fractions. ABCA1 was confirmed as miR-145 target in this study, but this was not reflected by increased HDL levels in miR-143/145$^{-/-}$ mice[46].

Aneurysm

MiR-145 was downregulated in IA tissues[98]. Elia et al.[101] demonstrated that both miR-143 and miR-145 were reduced in human thoracic aorta aneurysms, which correlated with SMC function[110]. In AAA however, expression levels of miR-145 and miR-143 were similar to those in normal abdominal aortic tissues[79]. Homozygous miR-143/145$^{-/-}$ mice showed structural defects in the SMC layer of the aorta[21]. Additionally, the SMCs in the media of aortas from miR-143/145$^{-/-}$ mice had a dedifferentiated phenotype, demonstrated by increased migration and proliferation and an increased protein synthesis[21].

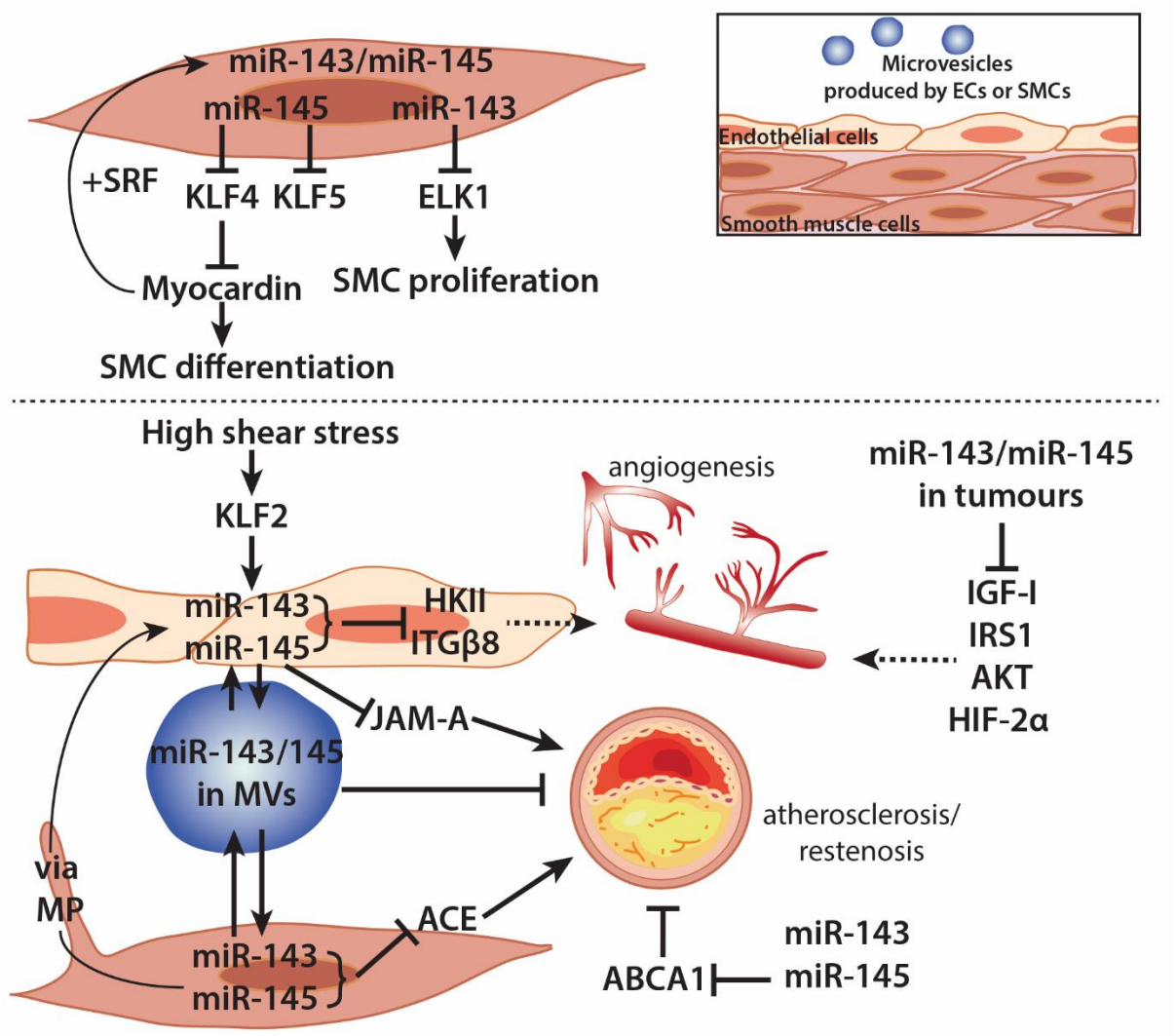

Figure 6 The miR-143/145 cluster in vascular remodelling. MiR-143 and miR-145 control SMC phenotype. Differentiation of SMCs is regulated by SRF, Myocardin and myocardin-related transcription factors (MRTFs). Via a feedback loop, these factors also regulate expression of the miR-143/miR-145 cluster itself (upper panel). Inhibition of KLF4 and KLF5 by miR-145 results, via Myocardin, in SMC differentiation and myofibroblast transdifferentiation, whereas targeting of ELK1 by miR-143 inhibits proliferation of SMCs. TGF-β and BMP4 are also able to activate expression of the miR-143/miR-145 cluster (not shown here). In addition, high shear stress, via KLF2, increases expression of miR-143/miR-145 in ECs (Figure 6, lower panel). In tumours, miR-145 suppresses IGF1 and the IRS1 pathway, affecting angiogenesis. In addition, miR-145 targets HIF2α and suppresses angiogenesis. MiR-143 targets AKT, thereby decreasing HIF1α and VEGFA expression. Transfer of miR-143/145 from VSMCs to ECs via membrane protrusions (MP) decreases the expression of HKII and ITGβ8 and via this mechanism presumably modulates angiogenesis. Intercellular transfer of miR-143/145 via MVs has also been described. MVs rich in miR-143/145 inhibit atherosclerotic plaque formation. ACE is another target of miR-143/145 which affects atherosclerosis. Arrows indicate upregulation. Capped lines indicate inhibition. The dashed lines indicate interactions that have not been confirmed yet. MP, membrane protrusion; MV, microvesicle; EC, endothelial cell; SMC, smooth muscle cell. For full target gene names, see Table 1.

14q32 microRNA gene cluster

The 14q32 microRNA cluster is the largest known mammalian microRNA gene cluster, located on human chromosome 14 and mouse chromosome 12. The cluster consists of 54 microRNAs in humans and 61 in mice[47,111] (Figure 7). It is assumed that transcription of the 14q32 microRNA gene cluster is controlled by the long noncoding RNA MEG3, also located on human chromosome 14, as deletion of MEG3 leads to downregulation of 14q32 microRNAs, as was shown in MEG3$^{-/-}$ mice[112]. MEG3$^{-/-}$ embryos have increased expression of VEGF pathway genes and increased cortical microvessel density[112] .

Neovascularization

Using a reverse target prediction analysis, where we looked for putative microRNA binding sites in the 3'UTRs of a set of nearly 200 neovascularization genes, our research group observed enrichment of binding sites for 14q32 microRNAs in the 3'UTRs of these genes. Microarray analyses performed on adductor muscle tissue of mice that underwent single ligation of the femoral artery as a model for effective neovascularization showed upregulation of 14q32 microRNAs following three different expression patterns[47]. We observed so-called early responders, microRNAs whose expression was upregulated 24 h after induction of ischaemia, late responders whose expression was upregulated from 72 h after ischaemia induction and non-responders. Inhibition of early responders miR-487b, miR-494, late responder miR-329, and non-responder miR-495 led to increased neovascularization and an improved blood flow recovery after hindlimb ischaemia in mice. Inhibition of miR-329, miR-487b, and miR-495 increased proliferation of human umbilical arterial ECs[47]. *In vivo*, inhibition of miR-329 led to upregulation of target genes TLR4, VEGFA, FGFR2, and MEF2A, whereas TLR4, VEGFA, ARF6, EFNB2, and FGFR2 were upregulated upon inhibition of miR-494. Using dual luciferase reporter gene assays, direct binding of miR-494 to the 3'UTRs of VEGFA, EFNB2, and FGFR2 was demonstrated. MiR-329 directly targets MEF2a and although VEGFA was regulated by miR-329, this was an indirect effect[47]. MiR-495 directly targets the 3'UTR of CCL2 and via this mechanism, proliferation, and apoptosis of HUVECs is affected[113]. Inhibition of miR-329, miR-487b, miR-494, and miR-495 also increased sprouting in aortic ring assays[47]. Wang et al. showed that miR-329 is a negative regulator of angiogenesis by targeting CD146, which functions as co-receptor for VEGFR2. Inhibition of miR-329 increased angiogenesis in this study, both *in vitro* and *in vivo*[48].

In a model for cerebral ischaemia, 14q32 miR-376b-5p also regulates angiogenesis. Expression of miR-376b-5p was decreased following middle cerebral artery occlusion (MCAO) in rats and miR-376b-5p inhibited angiogenesis *in vivo*, as well as in HUVEC cultures, via targeting of the HIF-1α-mediated VEGFA/Notch-1 signalling pathway[50]. In another study, 14q32 microRNA

miR-377 was identified as the most significantly downregulated microRNA in hypoxia-treated mesenchymal stem cells (MSCs) in rats[51]. Knockdown of miR-377 in HUVECs promoted angiogenesis *in vitro*, via direct targeting of VEGFA. To elucidate whether hypoxia-associated miR-377-regulated MSC induced myocardial angiogenesis in ischaemic hearts, the authors transduced rat MSCs with lentiviral vectors to overexpress or suppress miR-377 expression. MSCs with lentiviral miR-377, anti-miR-377, or empty vector were then injected into ischaemic rat hearts after ligation of the left anterior descending coronary artery. Inhibition of miR-377 in MSCs enhanced angiogenesis, decreased the area of fibrosis, and improved cardiac function of these animals[51].

Atherosclerosis

MicroRNA expression profiling in symptomatic vs. asymptomatic human atherosclerotic plaques showed upregulated expression of 14q32 microRNA miR-127[114]. Our group also investigated expression of 14q32 microRNAs in stable vs. unstable plaques of patients who underwent carotid endarterectomy surgery. We observed upregulation of 14q32 miR-494 in unstable atherosclerotic plaques. Inhibition of miR-494 led to reduced plaque formation in mice, while plaque stability was increased[49]. Moreover, total plasma cholesterol and VLDL fractions were decreased in these animals. Inhibition of miR-494 led to upregulation of target genes TGFB2, TIMP3, and IL33.

In addition, 14q32 microRNA miR-136 was upregulated in human atherosclerotic plaques. This microRNA is also highly expressed in synthetic SMCs *in vitro*[52]. MiR-136 targets PPP2R2A, resulting in increased ERK1/2 phosphorylation and increased proliferation of SMCs. The authors proposed that via this mechanism, miR-136 contributes to abnormal proliferation of SMCs, which is often observed in atherosclerosis[52].

In a study performed by Ramirez et al., the 14q32 microRNA miR-758 regulated ABCA1 in macrophages. Transfection of J774-macrophages with miR-758 reduced cholesterol efflux[53]. MiR-758 levels were furthermore regulated by dietary cholesterol *in vivo*. High dietary fat repressed miR-758 expression in the liver as well as in peritoneal macrophages, whereas ABCA1 levels were increased[53]. In another study, miR-758 levels were upregulated in hypercholesterolaemic human plaques compared with normocholesterolaemic plaques[115]. ABCA1 mRNA levels were also increased in hypercholesterolaemic patients, whereas protein levels were similar to normocholesterolaemic patients, suggesting strong posttranscriptional regulation of ABCA1 by miR-758. These human data suggest a role for miR-758 as ABCA1 modulator in human atherosclerotis[115].

A role for the 14q32 microRNA miR-370 in lipid metabolism and atherosclerosis was first described by Iloupoulos et al., mainly via direct targeting of CPT1α, an important enzyme in

fatty acid β-oxidation[54]. Other lipogenic genes such as SREBP-1c and DGAT2 were also regulated by miR-370. Using transfection experiments with sense and antisense miR-370, this regulation was mediated indirectly via miR-122[54].

Finally, extensive hypomethylation of the 14q32 locus was observed in human atherosclerotic plaques, which resulted in upregulation of several 14q32 microRNAs[116]. These findings suggest a role for epigenetic modulation of the 14q32 microRNA cluster in atherosclerosis[116].

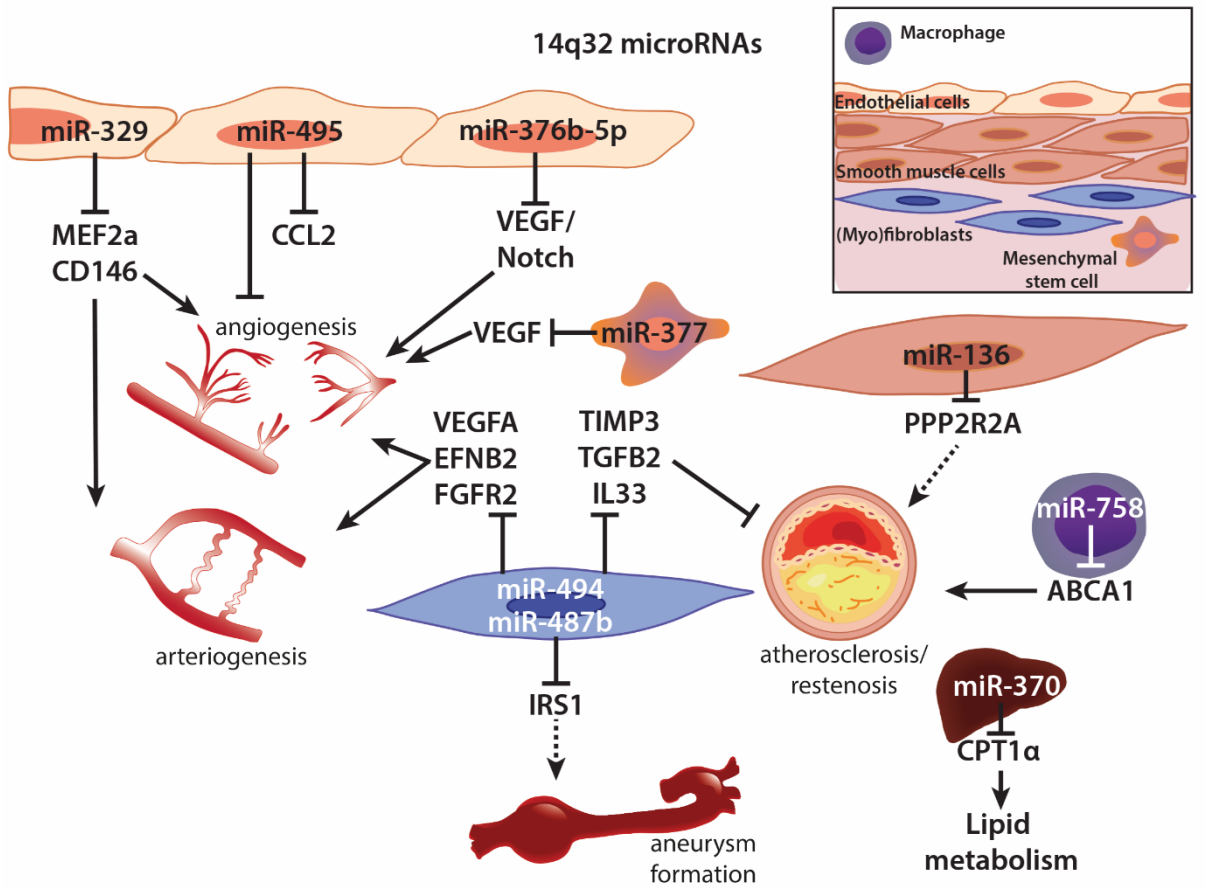

Figure 7 Roles for 14q32 miRs in vascular remodelling. MiR-329 inhibits angiogenesis and arteriogenesis via targeting of the co-receptor for VEGFR2, CD136, and MEF2a. Arteriogenesis and angiogenesis are also inhibited by miR-494, which suppresses VEGFA, EFNB2, and FGFR2. MiR-495 inhibits both arteriogenesis and angiogenesis. Proliferation and migration of HUVECs are affected by miR-495, via targeting of CCL2. VEGF signalling is further influenced through targeting of the HIF1α-mediated VEGF/Notch signalling pathway by miR-376-5p and via direct targeting of VEGF by miR-377. MiR-494 also influences atherosclerosis, through the inhibition of several target genes namely TIMP3, TGF-β2, and IL33. MiR-136 is up-regulated in human atherosclerotic plaques, where it targets PPP2R2A. Furthermore, cholesterol metabolism is affected by miR-758 through suppression of ABCA1 and by miR-370 via direct targeting of CPT1α. MiR-487b is highly expressed in the adventitia of rat aortae during chronic hypertension. Here, miR-487b targets IRS1, where it is thought to contribute to outward remodelling of the aorta. Arrows indicate up-regulation. Capped lines indicate inhibition. (HUV)EC, (human umbilical venous) endothelial cell. For full target names, see Table 1.

Aneurysm

MiR-487b is involved in hypertension-induced outward remodelling of the aorta. Chronic hypertension induced via AngII infusion led to significant upregulation of miR-487b in the aortae of rats[55]. MiR-487b was predominantly expressed in the adventitia and co-localized with the vasoactive IRS1. Using luciferase reporter gene assays, miR-487b was shown to directly target the IRS1 3'UTR, both in rats and in humans. MiR-487b downregulated expression of IRS1 in aortae of hypertensive rats, both at mRNA and at protein level[55].

Although further research into this extraordinarily large microRNA cluster is necessary, it is clear that the 14q32 microRNAs play important but diverse roles in the multiple processes of vascular remodelling, opening up new possibilities for prevention, detection, and treatment of CVD[117] (Figure 7).

Circulating microRNAs

Several of the microRNAs discussed in this review are also expressed in the circulation and could be used as biomarkers for vascular remodelling and CVD. For example, circulating miR-155 was expressed at significantly lower levels in CAD patients compared with healthy volunteers[73]. Likewise, circulating levels of miR-126, miR-17, and miR-92a were decreased in these patients[73,117]. Several miRs, including miR-126, were significantly increased in plasma of patients with insufficient collateral artery function[118]. Furthermore, circulating levels of several 14q32 microRNAs, including miR-134, miR-328, miR-370, miR-487a ,and miR-480 may have a diagnostic value for acute myocardial infarction, CAD, and cardiac death[119–123]. 14q32 miR-487b was increased in circulating leukocytes of patients with acute ischaemic stroke[124]. Other 14q32 microRNAs, including miR-665 and miR-541, have been reported to play a role in heart failure and cardiac hypertrophy, respectively[125,126]. Nevertheless, these findings need to be confirmed in large prospective cohort studies to determine the potential use of these microRNAs as biomarkers for CVD.

Future perspectives

In this review, we have described the multifactorial nature of microRNAs in vascular remodelling, as demonstrated by their role in multiple remodelling processes. Adaptive remodelling, such as arteriogenesis and angiogenesis, is stimulated by miR-126 and by the miR-23/24/27 family. Correspondingly, these microRNAs inhibit pathological remodelling, including atherosclerosis and restenosis. MiR-17/92, the 14q32 miRs, and miR-143/145 induce pathological remodelling, while they inhibit adaptive remodelling (except miR-143/145, for which different effects on pathological remodelling have been described).

MiR-155 was found to inhibit angiogenesis but stimulated arteriogenesis and was also reported to play contradicting roles in atherosclerosis formation.

The role of these microRNAs in the molecular mechanisms leading to aneurysm formation however is still poorly described; except for miR-24 and miR-487b, the microRNAs discussed here were only described to be differentially regulated in aneurysms, and a causative role has yet to be elucidated (Figure 8). As mentioned in the introduction, a single microRNA is able to target numerous genes and can regulate complex (patho)physiological processes. Often in literature, a single target gene is validated to explain the observed *in vivo* and *in vitro* effects upon microRNA modulation. However, it is far more plausible that the observed effects are caused by modulation of many genes involved in these (patho)physiological processes rather than one gene. Since microRNAs only modestly downregulate the expression of their target genes, it can be difficult to confirm significant changes in target gene expression. Nevertheless, the multifactorial nature of microRNAs in both adaptive and maladaptive vascular remodelling offers great opportunities for development of future therapeutics for treatment and prevention of CVD. In conclusion, the multifactorial effects on vascular remodelling observed for the microRNAs discussed here will hopefully stimulate the continued efforts to explore the potential of microRNAs as therapeutic target.

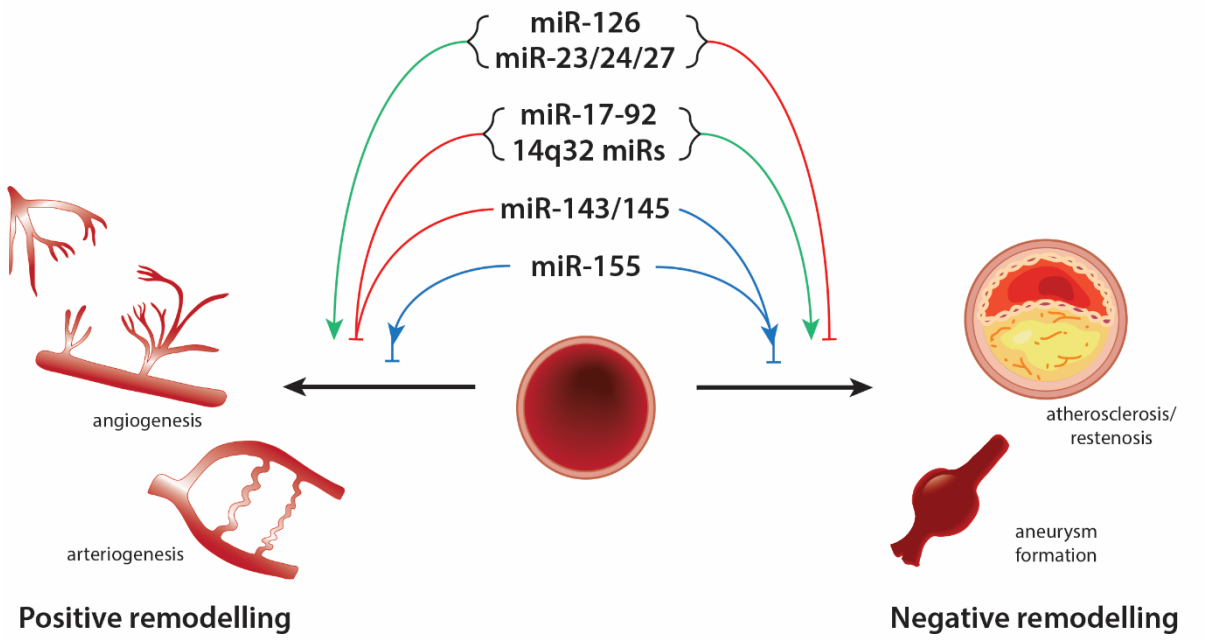

Figure 8 Graphical abstract. Several vascular microRNAs can influence multiple processes of vascular remodelling. MiR-126 is both pro-angiogenic as well as anti-atherosclerotic, like the miR-23-24-27 family. On the other hand, the 17-92 microRNA cluster and 14q32 miRs are anti-angiogenic, but pro-atherosclerotic. For miR-155 and miR-143/145, both pro- and anti-angiogenic functions have been reported, as well as pro- and anti-atherosclerotic roles. Green arrows indicate a stimulatory function, whereas red arrows represent an inhibitory function. Blue arrows are used when both stimulatory as well as inhibitory roles have been reported.

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