MicroRNA and vascular remodelling in acute vascular injury and pulmonary vascular remodelling

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Abstract  
Vascular remodelling is an integral pathological process central to a number of cardiovascular diseases. The complex interplay between distinct cell populations in the vessel wall following vascular injury leads to inflammation, cellular dysfunction, pro-growth signals in the smooth muscle cell (SMC) compartment, and the acquisition of a synthetic phenotype. Although the signals for vascular remodelling are diverse in different pathological contexts, SMC proliferation and migration are consistently observed. It is therefore critical to elucidate key mechanisms central to these processes. MicroRNAs (miRNAs) are small non-coding sequences of RNA that have the capacity to regulate many genes, pathways, and complex biological networks within cells, acting either alone or in concert with one another. In diseases such as cancer and cardiac disease, the role of miRNA in disease pathogenesis has been documented in detail. In contrast, despite a great deal of interest in miRNA, relatively few studies have directly assessed the role of miRNA in vascular remodelling. The potential for modulation of miRNA to achieve therapeutic benefits in this setting is attractive. Here, we focus on the role of miRNA in vascular inflammation and remodelling associated with acute vascular injury (vein graft disease, angioplasty restenosis, and in-stent restenosis) as well as in vascular remodelling associated with the development of pulmonary arterial hypertension.  

Keywords  
MicroRNA • Vascular pathology • Vascular remodelling • Smooth muscle cell • Pulmonary arterial hypertension  

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1. Introduction  
The kinetics of neointimal formation following vascular intervention have been studied in extensive detail, e.g. pivotal by Clowes et al.1 and subsequently by a series of eminent reviews by Ross.2–4 In brief, vascular injury following angioplasty or surgical preparation of a vein graft leads to endothelial injury and dysfunction resulting in platelet aggregation and activation. Activated platelets release growth factors and cytokines such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), interleukin (IL)-1, IL-6, IL-8, and thrombin. These molecules subsequently up-regulate selectin molecules resulting in inflammatory cell infiltration which, in turn, leads to key changes in underlying smooth muscle cell (SMC) behaviour. The up-regulation and secretion of selectin molecules results in recruitment of inflammatory leukocytes (monocytes and neutrophils) to the site of vascular injury,5 where they subsequently migrate into the subendothelial layer and release growth factors, cytokines, and proteases, the latter of which lead to degradation of the basement membrane surrounding SMC.6 Following degradation of the basement membrane, SMC become responsive to the chemokines and growth factors released following platelet activation and mechanical injury of SMC. These processes initiate SMC migration and proliferation resulting in vessel remodelling of the medial and adventitial layers (a summary is shown in Figure 1). Vein graft disease and pulmonary arterial hypertension (PAH) are initially characterized by excessive mechanical forces during vascular intervention. Although the aetiology and pathogenesis of each of these diseases is quite distinct, these pathologies do share a number of important molecular and pathological characteristics. This review will focus on the role of microRNA (miRNA) in these pathological changes.
2. Introduction to miRNA

Much recent interest has focused on the role of miRNAs as regulators of gene and protein expression in the vessel wall in homeostasis and pathology. miRNAs are a new class of small (~22 nucleotides (nt)) non-coding RNAs. In most cases, miRNAs negatively regulate expression of protein-coding genes by promoting degradation or suppressing translation of target miRNAs and modulate various biological functions in animals, plants, and unicellular eukaryotes. miRNAs are tissue-specific and developmentally regulated and abnormal expression of miRNAs is known to cause developmental abnormalities and human diseases, such as cancer and cardiovascular disorders.

The mechanism of miRNA biosynthesis is evolutionarily conserved and involves sequential endonucleolytic cleavages mediated by two RNase III enzymes, Drosha and Dicer (Figure 2). Following transcription by RNA Polymerase II (Pol II), Drosha processes the primary miRNA transcript (pri-miRNA) into a ~60–100 nt hairpin structure termed the precursor-miRNA (pre-miRNA) in the nucleus. Following cleavage by Drosha, the pre-miRNA is transported out of the nucleus through the interaction with Exportin-5 and Ran-GTP. The pre-miRNA then undergoes further processing catalysed by Dicer. This cleavage event gives rise to a ~22 nt double-stranded (ds) RNA product containing the mature miRNA guide strand and the passenger (miRNA*) strand. The mature miRNA promotes the association of a large protein complex, termed the RNA-induced silencing complex (RISC), with specific regions in the 3′-untranslated region (3′UTR) of target genes. Selection of miRNA targets is mediated by imperfect base pairing between the miRNA and miRNA-binding site present in the 3′UTR of the target mRNA. The imperfect nature of the miRNA:mRNA interaction means that a single miRNA can target tens to hundreds of mRNAs. Association of the miRNA-RISC results in the repression of the target gene by promoting mRNA degradation and/or translational inhibition. Through the repression of targets, miRNAs elicit critical changes in gene expression programmes which have been reported to underlie diverse aspects of biology, including developmental timing, differentiation, proliferation, cell death, and metabolism.

3. miRNA and vascular SMC integrity

Studies in mice with global ablation of DICER have unveiled the vital role of miRNA in development and vascular integrity. A number of important studies utilized lineage-specific DICER knockout mice to demonstrate that SMC-specific deficiency in DICER resulted in...
embryonic lethality due to defective blood vessel formation.\textsuperscript{13–16} Albinsson et al.\textsuperscript{14} published elegant work in a tamoxifen-inducible DICER knockout mouse. Expression was controlled by the SMC-specific promoter SMC myosin heavy chain (SM-MHC) resulting in cytoskeletal defects that lead to a significant reduction in systemic blood pressure. This phenotype bares a number of similarities to mice genetically deficient in the SMC-associated miR-143/145 cluster. In addition, DICER knockdown has been associated with reduced blood pressure.\textsuperscript{13} Studies by a number of groups have reported a reduction in systemic blood pressure secondary to dysfunctional actin filament assembly and reduced medial wall thickness.\textsuperscript{17–19} Two of these studies provide compelling evidence that these vascular abnormalities are the result of malfunction in vascular SMC (VSMC) differentiation concomitant with a broad range of structural and functional changes.\textsuperscript{17,18} These changes include elevated rates of proliferation, decreased expression of a panel of contractile proteins (calponin, SM22, SM-MHC, and α-actin), and changes in ultrastructures, including a general loss of myofilaments. Previous studies by Albinsson et al.\textsuperscript{14} support the roles of miR-145 in phenotypic modulation of SMC, since they demonstrate that reintroduction of miR-145, but not miR-143, into VSMC rescues the reduction in SMC marker expression in Dicer KO VSMCs. These effects were mimicked following overexpressing miR-145 in injured carotid arteries of rats and mice.\textsuperscript{20,21} Cheng et al.\textsuperscript{20} also reported that overexpression of miR-145 attenuated intimal lesion formation compared with vehicle infused animals. Similar effects were also reported by Elia et al.\textsuperscript{18} Vascular lesions isolated from mouse models of pressure overload, atherosclerosis and clinical samples from aortic aneurysm also contain reduced levels of miR-143 and miR-145.\textsuperscript{18} Furthermore, Boettger et al.\textsuperscript{17} reported that aged miR-143/145-deficient mice develop spontaneous intimal lesions in the femoral arteries. However, Xin et al.\textsuperscript{19} reported that intimal lesion formation is almost abolished in miR-143/145-deficient mice subjected to ligation of the carotid artery. Taken together, these studies highlight the fundamental importance of miRNA in vascular cell development and in the maintenance of vascular compliance and integrity.

4. Role of miRNA in endothelial cell biology, activation, and inflammation

Endothelial cells (ECs) play a pivotal role in maintaining the homeostatic balance of the vasculature by producing factors that regulate vessel tone, coagulation, and leucocyte trafficking. Under pathological conditions such as pulmonary hypertension or acute vascular injury, the endothelium becomes dysfunctional, resulting in impaired relaxation and increased leucocyte adhesion. An emerging body of evidence indicates that miRNA plays an essential role in EC homeostasis under physiological and pathological conditions. Despite the extensive list of miRNA implicated in the control of endothelial biology, here we discuss those miRNAs that have been implicated in pathologies related to induction of vessel inflammation.

Initial studies investigating the role of miR-126 in ECs demonstrated that a reduction in miR-126 levels enhanced TNF-α-stimulated expression of vascular cell adhesion molecule 1 (VCAM-1) resulting in increased leucocyte adhesion.\textsuperscript{22} Targeted deletion or disruption of miR-126 caused a loss of vascular integrity resulting in a phenotype containing leaky vessels and haemorrhaging, resulting in \(-50\%\) embryonic lethality in two independent studies in mice.\textsuperscript{23,24} These authors also utilized loss- and gain-of-function approaches to demonstrate that depletion of miR-126 decreases wound healing and angiogenesis in models of vessel sprouting in cultured aortic rings and ear angiogenesis, respectively. These effects were attributed to miR-126 targets Sprey-1 and PIK3R2. Furthermore, miRNA profiling in plasma from type 2 diabetic patients demonstrate a down-regulation in miR-126.\textsuperscript{25} Subsequent studies involving the miR-221/222 cluster indicate that these miRNAs have an opposing effect on angiogenesis compared with miR-126. Overexpression of miR-221/222 resulted in an inhibition of angiogenesis by blocking tube formation and wound healing.\textsuperscript{26} These authors report that miR-221/222 mediates such
effects by decreasing c-kit protein expression following inhibition of translation. Subsequent studies in ECs rendered Dicer-deficient also utilized gain-of-function experiment with miR-221/222 to demonstrate that these miRNAs indirectly reduce expression of endothelial nitric oxide synthase (eNOS). Further studies have implicated endothelial miR-221 in endothelial dysfunction following exposure to elevated glucose levels. In these studies, the authors report that elevated glucose levels increase endogenous miR-221 levels resulting in a reduction in EC migration and c-kit expression effects which are prevented following pre-treatment with antagonors to miR-221. This study hints at a role for miRNAs in hyperglycaemia. However, a recent paper by Trajkovski et al. places a spotlight on miRNAs and diabetes. These authors conducted profiling experiments in genetic and dietary mouse models of obesity and demonstrated that these mice contain substantially elevated levels of miR-103 and miR-107, effects which were verified in clinical samples isolated from patients with alcoholic and non-alcoholic fatty liver disease. Silencing of miR-103/107 following treatment with antagonors to miR-103 improved glucose homeostasis in obese and diabetic mice, while gain-of-function experiments with adenoviruses overexpressing miR-107 caused impaired glucose homeostasis in the liver and adipose tissue. In contrast, adenoviral mediated overexpression of miR-107 resulted in an increase in fasting blood–glucose and insulin levels in wild-type mice. Direct targets of miR-103/107 identified include caveolin-1, a critical regulator of the insulin receptor. Induction of miR-103/107 causes an up-regulation of caveolin-1, resulting in stabilization of the insulin receptor.

Furthermore, a previous paper by Jordan et al. also conducted miRNA profiling in livers isolated from mouse models of diabetes and demonstrated that the miR-143/145 cluster was up-regulated. These authors utilized an inducible mouse model to demonstrate that overexpression of miR-143 results in impaired glucose tolerance and insulin resistance without affecting β-cell function. The authors established that miR-143 overexpression impairs insulin-stimulated AKT activation in the liver. Further analysis in miR-143/145-deficient mice demonstrates that these mice have improved insulin sensitivity and glucose tolerance following high-fat feeding. Scrutiny of miR-143 overexpressing mice via SILAC analysis indicated that oxysterol-binding protein-like 8 (ORP8) was a target of miR-143 and experiments in cultured liver cells demonstrated that depletion of ORP8 significantly reduced insulin-stimulated activation of AKT activity. A recent study has also demonstrated a role for miR-503 in diabetes-induced vascular complications using a series of in vitro and in vivo studies as well as analysis of circulating levels in patients. These studies illustrate that miRNAs can regulate glucose homeostasis. Modulation of this condition by manipulation of specific miRNA would potentially be beneficial in the setting of cardiovascular disease, since patients with diabetes are predisposed to cardiovascular pathologies.

Effects of additional miRNAs on EC biology and pathology are continuously being identified. In particular, a recent study by Fang et al. has implicated miR-10a in the so-called athero-susceptible endothelium (isolated from the aortic arch and renal branches). In these studies, the authors report that miR-10a is down-regulated in inflamed ECs. Phosphorylation of IkBx, resulting in degradation of this inhibitory subunit, results in NF-kB activation following knockdown of miR-10a via targets mitogen-activated kinase kinase and the β-transduction repeat-containing gene. Furthermore, knockdown of endogenous miR-10a elevated the levels of the inflammatory biomarkers: monocyte chemotactic protein 1, IL-6, IL-8, and VCAM-1, and E-selectin. In addition, subsequent studies with miRNA mimics and antagonors to miR-21 demonstrated that miR-21 levels are negatively correlated with endothelial migration and angiogenesis. ECs enhance recruitment of leucocytes to sites of vascular inflammation. Microarrays have been used to investigate whether the potent atherogenic stimuli ox-LDL induced changes in miRNAs levels following activation of human peripheral blood monocytes. These authors describe an up-regulation of five miRNAs: miR-9, miR-125a-5p, miR-146a/b, and miR-155 following exposure to ox-LDL. The authors focused on miR-125a-5p, since this miRNA was up-regulated 11-fold following exposure to ox-LDL, although basal levels of miR-125a-5p are relatively low in monocytes. In these studies, the authors report that antagonors to miR-125a-5p enhanced cholesterol uptake via up-regulation of scavenger receptors. They also report an important observation in terms of vascular inflammation by documenting that antagonors elevated the levels of inflammatory cytokines (IL-2, IL-6 and TNF-α and TGF-β) secreted from PMA-differentiated THP-1 cells. The authors also identified and verified ORP9 as a target for miR-125a-5p which mediates these effects.

The expression of this particular miRNA signature is interesting in the context of inflammation, since previous reports have documented an up-regulation of miR-146a/b family and miR-155 following exposure to a variety of pathogens and cytokines. Indeed, one of the first reports linking miRNA with immune responses came from profiling on mononuclear cell lines treated with lipopolysaccharide, a Toll-like receptor ligand. These authors utilized databases to identify IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) as targets and validated them using luciferase report plasmids. Subsequent studies in alveolar epithelial cells demonstrate that miR-146a negatively regulates IL-1β-induced IL-8 and RANTES. However, they provide evidence that these effects are not mediated through IRAK1 or TRAF6, but some undefined target. Furthermore, overexpression of miR-146a by miR-mimics results in a reduction in inflammatory cytokine section (IL-6 and TNF-α) from dendritic cells stimulated with ox-LDL and antagonors to miR-146a increase inflammatory cytokine section. In this study, the authors identify and confirm CD40 ligand as a target for miR-146a via 3′UTR analysis. These results are particularly interesting in the setting of chronic obstructive pulmonary disease since miR-146a levels are reduced following stimulation of fibroblast with inflammatory cytokines IL-1β and TNF-α. Taken together, these studies suggest that miR-146a acts to suppress inflammation, but the induction of chronic inflammation down-regulates miR-146a.

An expanding number of clinical reports have started to document modulation of these so-called inflammatory miRNAs in disease pathophysiology which contains a substantial inflammatory component. The fact that these miRNAs have been implicated in leucocyte activation is particularly interesting in the setting of vascular inflammation, since a recent report by Raithoharju et al. compared the miRNA profile of control arteries to carotid and femoral arteries containing atherosclerotic lesions and found a substantial up-regulation of miR-21, miR-34a, and miR-146a/b. Moreover, previous reports have documented an up-regulation of miR-132, miR-146a, and miR-155 in mononuclear cells isolated from patients with rheumatoid arthritis. However, Li et al. reported that the expression of miR-146a in peripheral blood mononuclear cells from patients with stable angina
5. miRNA and the response to acute vascular injury

As we have discussed above, pathological remodelling of the vessel wall following vascular injury involves a switch in SMC phenotype, from a differentiated and contractile phenotype to a proliferative, synthetic state. Although this phenotype switch is believed to be essential for repair following injury, dysregulation of this process also plays a role in the pathological remodelling in a variety of human vascular diseases, including in-stent restenosis, vein graft disease, and vascular remodelling associated with pulmonary hypertension. Knowledge of the molecular mechanisms which control phenotypic modulation of SMC is a key requirement for the development of intervention strategies. Although numerous growth factors and cytokines have been implicated in vascular remodelling in response to acute and chronic injury (see earlier), few factors/pathways have been identified that selectively promote phenotypic modulation of SMC. The exceptions are PDGF and TGF-β (reviewed in more detail by Owens et al.27).

A schematic figure representing some of the following text is shown in Figure 3. A number of independent groups have demonstrated that overexpression of myocardin, a transcription factor for CArG box proteins, was sufficient to activate SMC differentiation markers in a number of SMC and non-SMC cell lines (NIH 3T3 or 10T1/2 fibroblasts),43–45 suggesting that myocardin is sufficient to activate the SMC lineage programme in SMC and fibroblasts. In a recent study by Madonna et al.,46,47 it has been demonstrated that myocardin can interact with telomerase, a key enzyme involved in senescence and anti-apoptosis in somatic cells. The co-expression of the two molecules in SMC and their progenitors may therefore have regulatory effects. MiR-145 was shown to be necessary for myocardin-induced reprogramming of adult fibroblast into SMC.21 The authors utilized pre-miRs and antagonirs to demonstrate that miR-145, but not miR-143, is able to lower the threshold for myocardin-induced differentiation. However, these results were obtained in an in vitro culture system. Luciferase reporter assays and site-directed mutagenesis were used to validate Klf-4 and CaMKII-β as molecular targets of miR-145.21 It was also suggested that miR-143 represses Elk-1 (Ets Like gene 1), resulting in an inhibitory effect on SMC differentiation21; details of Ets-like genes binding to myocardin can be found from studies by Wang et al.48 Analysis by Cheng et al.20 indicated that KLF-5 is also a target for miR-145 in the setting of vascular injury. Independent studies by Boettger et al.17 utilized novel SILAC-based mass spectrometry technique and gene chip technology to identify a number of mRNA and protein targets for miR-143/145. Tropomyosin 4 (Tpm4) and a down-regulation of angiotensin receptor 1 (Agtr 1b) were identified as targets which were hypothesized to play a role in the reduced blood pressure observed, since the levels of cGMP were reduced and AngII is a known stimulator of cGMP.17 Taken together, the results from these studies suggest that the miR-143/145 cluster can induce a profound effect on SMC differentiation following modulation of two critical transcription factors directly linked to SMC differentiation and molecular targets.

Although the miR-143/145 cluster has received a great deal of attention in the setting of vascular differentiation, it was not the first miRNA to be implicated in pathological vascular injury. Ji et al.49 utilized microarray technology to profile miRNA signatures following balloon injury in the rat carotid artery. The authors utilized modified pre-miRs and antagonirs to demonstrate that miR-21 promotes proliferation and inhibits apoptosis of cultured VSMC following down-regulation of phosphatase and tensin homologue. A subsequent study in the pulmonary vascular setting delineated a role for miR-21 in VSMC differentiation in response to TGF-β superfamily agonists [TGF-β and bone morphogenetic protein (BMP)] following inhibition of programmed cell death protein-4 (PDCD4) expression50 (see later section on pulmonary vascular remodelling for further details). A subsequent study also documented a role for miR-221 in SMC dedifferentiation and proliferation in response to PDGF, where miR-221 decreased protein levels of c-kit and p27kip1 resulting in SMC proliferation.51 These results were confirmed when miR-221 and miR-222 expression were found to be up-regulated following balloon injury or stimulation of primary SMC with serum or PDGF.52 Although this study confirmed that these miRNAs modulate p27kip1, the study also reported that miR-221 and miR-222 reduce p57kip2 protein levels.53 In both of these studies, the authors utilized antagonirs to miR-221 to demonstrate that the proliferative effect of miR-221 was mediated via down-regulation of miR-221 targets p27kip1 and p57kip2, both of which are established inhibitors of SMC proliferation in vivo.51,52 Recently, two independent studies demonstrated that the signalling pathway of the TGF-β superfamily of agonists transcriptionally activate the miR-143/145 gene cluster in the Smad-dependent manner, which leads to down-regulation of KLF4 and promotes SMC differentiation (Figure 3).53,54 These studies illustrate an intricate mechanism of regulation of miRNAs by different growth factors.

Further studies have investigated the role of miRNAs in airway SMC dedifferentiation in response to a cocktail of cytokines (IL-1β, TNF-α, and INF-γ) using microarray technology.55 One of the most intriguing miRNAs to be identified in this study was miR-25 which was down-regulated following exposure to cytokines leading to elevated expression of SMC differentiation markers. It was also documented that antagonirs to miR-25 inhibited up-regulation of SMC differentiation markers causing a concomitant increase in KLF-4. However, these effects were modest and antagonirs to miR-25 did not affect basal levels of SMC differentiation markers or KLF-4 expression.55

A recent study has implicated a role for miR-10a in the vascular system. Huang et al. utilized screening of embryonic stem cells subjected to a vascular differentiation protocol in order to identify miRNAs importance in this process. Gain-of-function experiments demonstrate that miR-10a played a role in up-regulation of SMC differentiation markers via its targets histone desethylase 4 (HDAC4).56 Overexpression of myocardin increases miR-1 levels resulting in inhibition of SMC proliferation and decreases KLF-4 expression via a Pim-1-dependent mechanism, but not HDAC4.57 miR-1 is enriched in sarcomeric muscle with very low-level expression in VSMC. This study also documented that miR-1 is down-regulated in vascular lesions induced by ligation of the mouse carotid artery.58 However, subsequent studies confirm that miR-1 levels in VSMC are extremely
miRNA in vascular remodelling

Figure 3 Vascular injury and miRNA regulation. A summary illustrating some of the mechanisms whereby specific miRNA molecules interact with known transcription factors to activate or repress SMC-specific marker genes and pathways of proliferation. Expression of virtually all SMC marker genes is dependent on one or more CArG elements within their promoter. Serum response factor (SRF) activates genes involved in SMC differentiation and proliferation by recruiting a number of co-activators such as myocardin and a number of co-repressors such as Kruppel transcription factors (KLF-4 and -5) and the est-1 domain containing proteins to CArG elements in the promoter. MiR-24 and miR-221/222 up-regulate KLF-4 and -5, which results in a down-regulation of myocardin resulting in inhibition of signalling. Note that miR-145 antagonizes these effects. The middle boxed (hatched region) demonstrates that KLF-4 can inhibit myocardin signalling following binding to both the SRF and directly binding to G/C rich region next to the CArG box preventing binding of SRF to the CArG elements resulting in reduced SMC gene expression. Activation of the PDGF receptor increases miR-24 level and inhibits up-regulation of miR-21 in response to TGF-β and BMP, preventing subsequent up-regulation of SMC differentiation marker gene expression. The far left panel demonstrates that activation of est-1 proteins (such as Elk-1, SAP-1, and -2) following phosphorylation by ERK-1/2 increases their affinity for SRF leading to displacement of myocardin resulting in decreased SMC gene expression. Note that miR-143 can inhibit Elk-1 activation which would prevent down-regulation of SMC marker genes. The circular hatched region illustrates changes in actin treadmilling following growth factor stimulation or injury. Growth factor activation inhibits F-actin polymerization, resulting in increased free G-actin. This signalling event is transduced to the nuclease resulting in nuclear export of MRTF-A (and/or MRTF-B) and disruption of SRF/MRTF complexes, leading to down-regulation of genes encoding SMC contractile proteins. Pathways where miRNAs induce a positive effect are depicted with filled arrows and negative effects are depicted with hashed arrows.
low, but levels of miR-133 which is co-transcribed with miR-1 in SMC was relatively abundant.58 These authors go on to demonstrate that modulation of miR-133, but not miR-1, can inhibit SMC proliferation in vitro and in vivo via its target Sp-1. In these detailed studies, the authors utilize adenoviruses and antagonors to modulate miR-1 and miR-133 levels following filament injury in rats and demonstrate that overexpression of miR-133, but not miR-1, inhibits neointimal formation. Further analysis by these authors also demonstrated that silencing of miR-133 following treatment with antagonors augmented intimar lesion formation.59

A recent paper by Sun et al.59 has implicated miR-146a in SMC proliferation following balloon injury and demonstrated that inhibition of miR-146a reduces neointimal formation. It was shown that KLF-4 and KLF-5 bind and regulate the promoter of miR-146a to modulate miRNA expression levels. Furthermore, overexpression of KLF-4 down-regulates miR-146a expression.59 It is interesting to note that this is one of the miRNAs which was implicated by profiling miRNA in acute vascular injury.59 Taken together, these recent studies highlight the increasing relevant and fundamental role of miRNAs in vascular remodelling and alterations in a VSMC phenotype in response to injury.

6. miRNA and vascular remodelling associated with PAH

PAH is caused by narrowing and obliteration of small pulmonary arteries in the lung periphery.60,61 The resulting increase in pulmonary vascular resistance increases pulmonary arterial pressure. Initially, the right ventricle may undergo adaptive hypertrophy to cope with the increased pressure, but ultimately dilates and fails. Patients die from worsening right ventricular failure.62 Despite modern treatments, the 3-year survival remains less than 60%.63 Although the underlying cause of PAH in many idiopathic cases remains obscure, most familial forms of the disease are caused by mutations in the BMP type II receptor (BMPR-II), a receptor for the TGF-β superfamily.54,64 An important feature of families with heritable PAH is that the disease gene penetrance is less than 50%, and often as low as 15–20%.64 This implies that additional genetic or environmental triggers are required for disease manifestation. The genetic modifiers of disease penetrance remain obscure. The pathology of PAH is characterized by thickening of the walls of pulmonary arteries.65 Disease may be initiated by a combination of endothelial dysfunction, increased endothelial permeability, and endothelial apoptosis.66,67 Clonal expansion of apoptosis-resistant ECs contribute to the formation of plexiform lesions.68 Loss of endothelial integrity results in exposure of the underlying vascular matrix and intimial cells to serum-derived factors leading to activation of growth factors and expression of elastases that degrade the elastic laminae.70 There is an expansion of the adventitia, smooth muscle media, and the formation of a neointima. Other important factors thought to contribute to disease are inflammation and hypoxic signalling.71–73 Dysfunctional BMP signalling contributes to many of the cellular and metabolic disturbances described above. Pulmonary artery SMCs from patients with BMPR-II mutations are hyperproliferative and are insensitive to the growth suppressive effects of BMPs.74,75 Loss of BMPR-II signalling is associated with an abnormal response of pulmonary artery SMCs (PASMCs) to TGF-β.74,76 BMPR-II mutation and dysfunction are associated with a loss of signalling via downstream Smad proteins, specifically Smads 1, 5, and 8.75 There may be increased activation of TGF-β-regulated Smads, Smad 2 and 3.77 Among the many genes regulated by BMP/Smad signalling a family of transcription factors, the inhibitors of differentiation family of proteins (Id proteins, Id1–4) have been shown to be important transcriptional targets.78 Id proteins have wide-ranging effects on gene transcription and consequently cell growth and differentiation.79

Not surprisingly, given their roles in numerous cellular responses during normal development and disease, evidence is emerging for a key role for miRNAs regulating the cellular processes involved in pulmonary vascular remodelling. A range of miRNAs are dysregulated in the lungs of rats exposed to chronic hypoxic and the monocrotaline model of PAH.80 In hypoxia, this is in part due to reduced expression of Dicer, involved in miRNA processing, Common to both the hypoxic and monocrotaline models, miR-22, miR-30, and let-7f were down-regulated, whereas miR-322 and miR-451 were up-regulated. There were additional changes specific to each model. In human pulmonary hypertensive lung tissue and cells, miR-21 was down-regulated and this was also observed in the monocrotaline model. A further study found that miR-204 was consistently down-regulated in PASMCs from patients with PAH and in cells from mice with PAH.81 These authors showed that miR-204 directly influenced PASMC function, favouring a proliferative and anti-apoptotic phenotype. Delivery of miR-204 to the lungs of mice with PAH significantly reduced disease severity. In a study designed to identify miRNAs that could inhibit the translation of BMPR-II, members of the miRNA cluster 17/92 were identified as potential regulators.82 This cluster is up-regulated by the IL-6/STAT3 pathway and exposure of cell lines to IL-6 led to STAT3 activation and reduced BMPR-II expression. These observations could provide a mechanism by which BMPR-II expression is reduced in many forms of PAH, though they require confirmation in vascular cells. Taken together, these studies have identified specific miRNA species that could be targeted as potential therapeutic applications in PAH.

It has also been demonstrated that treatment of PASMCs with different growth factors modulated expression of various miRNAs and affected a PASMC phenotype, which subsequently alters cell growth, migration, and differentiation. For example, the TGF-β-superfamily of growth factors (TGF-β and BMP4) rapidly elevates expression of miR-21.50 Knockdown of the R-Smads prevents processing of mature and pre-miR-21 by BMP4. However, no change in pri-miR-21 transcription can be observed. This implies that R-Smads affect the level of miR-21 post-transcriptionally.50 BMP4 elevates the expression of pre- and mature miR-21 when expression is from a cytomegalovirus promoter. This implies that miR-21 is regulated at the Drosha post-transcriptional processing point. The identification of R-Smads as binding partners of p68 implies that R-Smads can link with the Drosha complex.83 Detailed experiments have indicated that R-Smad is present in a complex with Drosha and p68 on the pri-miR-21 hairpin in response to BMP4 or TGF-β exposure.50 Concomitantly, binding of Drosha to the pri-miR-21 sequence is increased after BMP4 or TGF-β exposure, indicating that R-Smads enhance Drosha association with the ds hairpin region of pri-miR-21 and enhance cropping.50 Interestingly, several other miRNAs are found to be post-transcriptionally induced by BMP and TGF-β. This implies that the post-transcriptional mechanism of modulation of miRNA biosynthesis is important for a number of cellular processes (Figure 4). R-Smads recognize and directly associate with ~20 pri-miRNAs which contain a conserved sequence (5′-CAGAC-3′) within the stem
These authors reported that a direct association of R-Smads to pri-miRNA facilitates recruitment of Drosha and DGCR8 and leads to efficient cropping of the pri-miRNA and elevated expression of mature miR-21. Induction of miR-21 results in repression of PDCD4 which leads to activation of VSMC-specific genes, such as α-smooth muscle actin, calponin1, and SM22α. Knockdown of PDCD4 using small interference RNA (siRNA) mimics the effect of BMP4 or TGF-β and activates transcription of VSMC-specific genes. In PASMCs derived from PAH patients who carry mutations in the BMPR-II gene, it is speculated that the miR-21 level is reduced due to decreased BMP signalling pathway. Consistently, a recent study demonstrated that lung tissues from idiopathic PAH patients also exhibit lower expression of miR-21 in comparison with normal tissues. These observations support the contention that TGF-β or BMP-dependent regulation of miR-21 is critical for the homeostasis of pulmonary vasculature.

In addition to miR-221, PDGF also induces expression of miR-24. One important target of miR-24 is Tribbles-like protein 3 (Trb3). In the absence of BMP signalling, Trb3 interacts with BMPR-II. However, upon receptor activation, Trb3 dissociates and promotes the degradation of Smurf1. By decreasing the expression of Smurf1, Trb3 increases the expression of Smad1 and therefore potentiates BMP signalling. Increased expression of Trb3 promotes expression of SMC contractile markers, while decrease in Trb3, as occurs in response to PDGF due to induction of miR-24, results in reduced expression of contractile genes. Induction of miR-24 by PDGF thus represents an interesting example of the crosstalk between the pro-synthetic pathways mediated by PDGF and the pro-contractile pathways mediated by BMP.

Taken together, it is clear that miRNA are modulated in the molecular and cellular responses central to the pathophysiology of PAH.

### 7. Therapeutic opportunities

One of the most important aspects of therapeutic intervention for manipulation of miRNA, as with other therapeutic regimens aimed at clinical translation, is the choice of strategy and the delivery modality. In the discipline of prevention/regression of pathological vascular remodelling, this is a very important aspect for translation of basic science to the clinic. We have focused our review on the vascular remodelling associated with acute vascular injury and vascular remodelling associated with the development of PAH. There are a number of important factors that will affect translation in this setting. First, we anticipate that while it may be possible to achieve wide distribution of miRNA interventions via systemic approaches (e.g. intravenous, subcutaneous, intraperitoneal routes), it is unlikely that this would be optimal since off-target effects of miRNA modulation may be apparent. However, extensive analysis of this point in experimental studies is critical and will be dependent on the individual miRNA in question. Thus, miRNA interventions in each of these vascular targets would optimally require precise strategies for localized delivery of the therapeutic entity. A second issue is the requirement for short- vs. longer-term manipulation required for therapeutic gain. Thirdly, strategies may require either reduction or augmentation of miRNA levels and the mode of each is clearly going to be very
different (e.g. viral or non-viral vector-mediated augmentation vs. antiMir/antagomir approaches for miRNA reduction). Much can be learned from already-tested nucleic acid delivery strategies (e.g. siRNA) and a broad range of gene therapy systems. Many of these systems have additional regulatory issues but many have previous safety data already from clinical studies, including adenovirus, adeno-associated virus, and lentivirus (http://www.wiley.com/legacy/ wileychi/genmed/clinical/). With these issues in mind, we envisage unique opportunities for innovative therapies. Exemplars in the acute vascular setting include ex vivo manipulation of miRNA in vein grafts and in vivo delivery of miRNA therapeutics via catheters and stents. In the setting of pulmonary vascular remodelling, there is only one study to date that has manipulated miRNA in experimental PAH. This was discussed above and concerns the manipulation of miR-204 via a lung-selective delivery approach. We envisage a substantial opportunity to efficiently manipulate miRNA in the lung due to advances in vector technology and improved knowledge of delivery procedures. These approaches are both via inhalation techniques and via intravascular approaches. Many of these systems have been developed for a broad range of lung pathologies. In the lung, a number of specific hurdles exist, including delivery to defined cell types within the lung. However, opportunities exist for a number of systems for efficient delivery to the lung, including adeno-associated virus and adenovirus. Furthermore, it is possible to create selectivity for the lung via engineering vector systems to ‘target’ the lung vasculature and achieve therapeutic effects.

8. Concluding remarks

Although it is relatively early for this research area, it is clear that miRNAs play a fundamental role in the development of the vascular system, maintenance of vascular homeostasis, and in the development of vascular pathologies. Environmental factors, such as hyperlipidaemia and hyperglycaemia, may also have an impact on miR expression in tissues and in the circulation. Defining the importance and contribution of individual miRNA, miRNA clusters, their miRNA targets, and miRNAs acting alone or in concert, together with the pathway(s) targeted, is pivotal to improve understanding of the complex aetiologies in vascular pathology. Concomitantly, it will be important to evaluate and refine delivery technologies to interfere with miRNA systems (individually or targeting multiple miRNA simultaneously) in an optimized manner—alternatively, small molecules that target individual genes or pathways that are critical to the effect of the miRNA could be developed. Direct modulation of miRNA, whether this is via systemic miRNA modulation or via localized delivery to afflicted vascular site of injury, will depend on the miRNA target, access to the vascular site (if required) and potential off-target effects. This intriguing research area remains vibrant and will lead to new discoveries relating to vascular pathophysiology and potentially novel therapeutic opportunities.

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