Diverse roles of microRNA-145 in regulating smooth muscle (dys)function in health and disease

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Abstract

MicroRNAs are short, non-coding RNAs that target messenger RNAs for degradation. miR-145 is a vascular-enriched microRNA that is important for smooth muscle cell (SMC) differentiation. Under healthy circumstances, SMC exist in a contractile, differentiated phenotype promoted by miR-145. In cases of disease or injury, SMC can undergo reversible dedifferentiation into a synthetic phenotype, accompanied by inhibition of miR-145 expression. Vascular disorders such as atherosclerosis and neointimal hyperplasia are characterised by aberrant phenotypic switching in SMC. This review will summarise the physiological roles of miR-145 in vascular SMC, including the molecular regulation of differentiation, proliferation and migration. Furthermore, it will discuss the different ways in which miR-145 can be dysregulated and the downstream impact this has on the progression of vascular pathologies. Finally, it will discuss whether miR-145 may be suitable for use as a biomarker of vascular disease.

Introduction

MicroRNAs (miRs) are short, non-coding RNAs that can act as master regulators of cellular function, switching entire pathways on or off through imperfect binding to the 3' UTR of target mRNAs. The biogenesis of mature miRs has been well characterised (recently reviewed in (1)) and they are now being examined as a class of therapeutic agents for cancers as well as age-related and vascular disorders (2-4). miR-145 is enriched in cardiac and vascular smooth muscle cells (SMC) and is highly conserved across multiple species (5). As well as critical roles in controlling SMC differentiation, miR-145 is also a tumour suppressor with loss of miR-145 implicated in the pathogenesis of cancer and metastasis (6).

Regulation of miR-145 expression

miR-145 is co-transcribed with miR-143 in a bicistronic unit named *MIR143HG* from human chromosome 5q32-33, in between *IL17B* and *CSNK1A1*. Promoters common to both miRs exist, as do those for each miR separately (7-9). Within these promoters lies a CArG box which interacts with serum response factor (SRF) to direct expression in SMC (10). Whilst this is the principal way that miR-145 expression is controlled in vascular SMC, it is promoted by other transcription factors including p53 (9), forkhead box factors O1 and O3 (FoxO1 and FoxO3; (11)), CCAAT enhancer binding protein beta (C/EPBβ; (12)) and transforming growth factor beta (TGFβ; (13)).

In addition to transcription factor control, miR-145 expression is regulated by DNA methylation. Here, data regarding the effect of methylation in different disease states versus control conditions is at variance. There are conflicting reports about miR-145 promoter DNA methylation in different cancers (6), and studies in human vascular cells have struggled to find evidence of substantive regulation by methylation in disease states such as type 2 diabetes (14) although hypermethylation has been observed in a murine model of atherosclerosis (15). Thus, whilst this is a rapidly evolving field of research, the current article will focus on

transcriptional regulation and the effect that this has on SMC function, and dysfunction in disease states.

Although miR-145 and miR-143 can be co-transcribed, the relative abundance of each miR within the cell is not identical, with miR-145 transcripts being approximately 10x higher than miR-143 transcripts (13, 16). This is due to a myriad of factors including differential transcription factor binding and miR turnover dynamics. Furthermore, the abundance of miR-145 also varies according to vascular bed with the highest expression levels seen within the saphenous vein and comparably less in the aorta (17). When studying the physiological and pathological impact of miR-145, it is therefore crucial to carefully consider the species and cell type that is being examined in order to critically evaluate its relevance.

miR-145 is a central regulator of smooth muscle cell function

miR-145 and miR-143 are co-transcribed from the same locus, MIR143HG. They have both shared and divergent mRNA targets, as does MIR143HG which has been identified as a long non-coding RNA (lncRNA; recently reviewed in (18)). This article will focus solely on miR-145 and its influence on SMC physiology and pathology.

Differentiation: The canonical role of miR-145 in controlling SMC phenotype was first revealed in 2009 in a series of elegant studies (5, 10, 19). SMC are plastic cells that can switch between differentiated (contractile) and dedifferentiated (synthetic) phenotypes. In healthy blood vessels, SMC are maintained in the contractile phenotype where they have a low turnover rate and abundant contractile machinery. However, in cases of disease or injury, SMC are able to reversibly switch to a synthetic phenotype characterised by proliferation, migration, and lack of contractile function. The SRF pathway is a master regulator of this switch (20).

SRF resides in the nucleus and utilises cofactors to bind to the CArG box motif in the promoters for SMC differentiation genes including alpha smooth muscle actin (α-SMA; encoded by *ACTA2*), calponin and smoothelin, as well as miR-145, to promote expression and favour the contractile phenotype (10, 20). These differentiation markers are intricately

involved with the actin cytoskeleton. The cofactors myocardin and myocardin-related transcription factors (MRTF) are ordinarily sequestered in the cytoplasm by G-actin. When actin polymerisation is induced (for example, through activation of RhoA), they are released and transport to the nucleus where they pair with SRF to further induce cytoskeletal transcription (21). This system is antagonised by Kruppel-like factor 4 (KLF4) which binds to myocardin, preventing its association with SRF and reducing SMC differentiation gene expression, thus promoting the synthetic phenotype (20, 22).

miR-145 is embedded within this transcriptional network. SRF, myocardin and MRTF-A have all been demonstrated to promote expression of miR-145 in vascular SMC (5, 10). miR-145 itself directly targets KLF4 in human pulmonary artery SMC (16) and mouse carotid artery (5), however the association was less clear in human venous SMC (13) which, given miR-145 is more highly expressed in vein than artery (17), is perhaps surprising. Through targeting KLF4 for degradation, miR-145 can maintain the contractile phenotype. It is thought that the inhibition of KLF4 by miR-145 is translated into an increase in the expression of α-SMA (5, 13), however again these are species and cell-type specific-events as knocking out miR-145 in a murine model reportedly has no impact on *ACTA2* expression (10). In contrast to these pro-contractile events, miR-145 is also involved in a potential negative feedback loop as it can target both myocardin (5) and MRTF-B (10) for degradation.

Cytoskeletal organisation: The cytoskeleton of SMC is intricately involved in its phenotype and function. Differentiated, contractile SMC possess a defined cytoskeleton characterised by extensive F-actin stress fibres traversing the length of the spindle-shaped cell. In contrast, dedifferentiated synthetic SMC lose this regimented organisation and the cytoskeleton becomes more disorganised and less defined, with the cell adopting a more rhomboid shape (20). miR-145 controls cytoskeletal organisation in part through its indirect up-regulation of α-SMA, but it also interacts with multiple other pathways to allow cytoskeletal remodelling in response to environmental cues.

Stress fibres are initiated by polymerisation of G-actin into F-actin fibres. In keeping with its pro-contractile role, miR-145 knock-out in murine cells resulted in a disorganised and weakened F-actin cytoskeleton (10). However, in human venous SMC, over-expression of miR-145 actually inhibited F-actin organisation (13), indicating that the effect of miR-145 on stress fibre formation is a species- and cell type-specific event.

RhoA is a major regulator of SMC cytoskeletal dynamics. Cellular exposure to cyclical stretch, changes in blood flow or increased levels of vasoactive substances rapidly activates the RhoA pathway which, in conjunction with the downstream regulator Rho kinase (ROCK), results in the formation of stress fibres and thus the contractile phenotype (23). ROCK1 was originally identified as a target of miR-145 in glioma (24) and has since been identified as a direct target in human SMC (25). Indeed, human venous SMC treated with Y27632 (a ROCK inhibitor) exhibit comparable morphological and F-actin cytoskeletal changes to SMC over-expressing miR-145 (13, 26) suggesting that any changes in F-actin organisation in response to miR-145 may be modulated through its effects on the RhoA/ROCK pathway. To add a further level of complexity, a very recent study has demonstrated that cell stretch induced miR-145 and contractile marker gene expression which was dependent on the actions of RhoA and ROCK. When either of these were inhibited in stretched cells, the induction of miR-145 was abolished and cells did not undergo differentiation (27). Thus, it is likely that a bidirectional relationship between miR-145 and RhoA/ROCK exists which allows the cell to alter its contractile state according a variety of extracellular cues.

Proliferation: One of the hallmarks of the phenotypic switch to a synthetic phenotype is an increase in cellular proliferation. Numerous studies have demonstrated that miR-145 reduces SMC proliferation both in human SMC (13, 25, 28) and in rat aortic SMC (5). This is likely through a variety of mechanisms including inhibiting autophagy (29) and inhibiting the expression of molecules directly involved in the proliferative response including phosphatidylinositol kinase (PI3K; (30)), Smad4 (31) and cluster of differentiation 40 (CD40; (28)). It is interesting to note that under disease conditions such as pulmonary arterial

hypertension (PAH), miR-145 can adopt a pro-proliferative role (32, 33). It can also adopt this role in airway SMC treated with inflammatory stimuli (34). Understanding whether this is unique to pathological SMC or may be apparent under other unidentified conditions requires further research.

DNA damage: In keeping with the tendency of miR-145 to inhibit proliferation, it was initially described in relation to cancer development where it acted as a tumour suppressor (35), interacting with p53 and the DNA damage response (DDR) pathways. However, very little is known about the potential protective influence of miR-145 on DNA repair in vascular SMC. Whilst one study has demonstrated increased miR-145 expression in DNA-damaged aneurysmal SMC, this was simply a correlative analysis and a relationship between the two observations was not explored (36). An examination of the role (if any) of miR-145 in DNA damage in SMC may shed new light on the pathogenesis of diseases in which miR-145 or DNA damage are dysregulated.

Given that potential negative feedback loops are apparent for miR-145 in the control of differentiation marker gene expression, cytoskeletal regulation and RhoA (Figure 1), it is possible that miR-145 is one of the key balancing factors for SMC differentiation, ensuring that SMC are maintained in the correct phenotype by responding to environmental cues (13). Thus, there is growing interest in miR-145 regulation as it could be manipulated in SMC to improve disease states (37).

miR-145 is dysregulated in cardiovascular diseases

Through its involvement in the p53-DNA damage signalling cascade, miR-145 has been extensively studied in the context of cancer as a tumour suppressor (recently reviewed in (6, 38)). However, given that it is a critical regulator of SMC phenotype and function, it would follow that dysregulation of miR-145 is implicated in the pathogenesis of multiple cardiovascular disorders.

Atherosclerosis: Atherosclerosis is an almost universal consequence of ageing. SMC dedifferentiate, proliferate and migrate within the vessel wall, attracting macrophages into the intimal layer. Macrophages and SMC take up cholesterol crystals and a fatty, necrotic core forms with a SMC fibrous cap covering it. Rupture can lead to myocardial infarction, stroke and peripheral occlusion. As miR-145 promotes SMC differentiation and contraction, one might expect miR-145 levels to be decreased in atherosclerotic lesions. However, while some studies have come to this conclusion, it is far from a consensus with multiple conflicting reports.

In human aortae, miR-145 expression was significantly decreased in atherosclerotic lesions compared to non-diseased regions of the same aortae, accompanied by reduced SMC differentiation markers myocardin and α-SMA (39). Similarly, in ApoE knockout mice, miR-145 expression reduced in aortae as atherosclerotic lesions progressed (15) suggesting that a loss of miR-145 was detrimental and increased atherosclerosis. Indeed, lentiviral delivery of miR-145 to ApoE knockout mice inhibited plaque development and promoted thick fibrous caps (40). However, in human carotid plaques, miR-145 was significantly increased up to 4-fold in symptomatic (vulnerable plaques prone to rupture) versus asymptomatic, stable plaques (41), suggesting that miR-145 itself has a detrimental impact on atherosclerosis progression. This highlights the interspecies variability in miR biology and is supported by studies in double LDL and miR-145 knockout mice, where the absence of miR-145 had a beneficial effect in reducing atherosclerotic lesion size. The authors determined that this was due, at least in part, to restoration of cholesterol transporter ABCA1 expression – a direct target of miR-145 (42). Similarly, in ApoE knockout mice, administering a miR-145 inhibitor reduced atherosclerotic plaque progression through increasing levels of α-SMA, and attenuated inflammation through the nuclear factor kappa B (NFκB) pathway (43).

There are a number of potential explanations for these disparate results. It could be that miR-145 expression has no consistent effect on atherosclerotic development. This seems unlikely given the critical role of miR-145 in SMC physiology. A second explanation considers the

relative stability of the plaques. Stable plaques are associated with thick fibrous caps, caused by SMC proliferation, which minimise the likelihood of rupture. Conversely, vulnerable plaques have thin caps with lower SMC content. As miR-145 is intimately linked with the differentiation status of SMC it is entirely possible that SMC within different regions of the plaque may have differing levels of miR-145 expression. Thus, the lack of a consensus may be explained by the inevitable variability in the size of lesions between patients (or individual animals), and variation in the quantity and distribution of contractile, synthetic and foam cell-like SMC within these plaques.

Neointimal hyperplasia: Neointimal hyperplasia (NI) is characterised by accelerated SMC proliferation and migration resulting in increased wall thickness and decreased vessel lumen size. It is a normal physiological response to injury however when SMC proliferation persists this can lead to pathological changes and vessel occlusion. NI following coronary bypass surgery is one of the major causes of bypass failure requiring reintervention. The influence of SMC on NI development is much more consistent than in atherosclerosis, as it is driven purely by a proliferative SMC phenotype.

In a rodent model, miR-145 expression was markedly inhibited following carotid balloon injury in a time-dependent manner. This lifted the ordinarily inhibitory effect of miR-145 on KLF5 resulting in reduced expression of SMC markers including α-SMA and acquisition of the synthetic phenotype. Furthermore, adenoviral delivery of miR-145 was able to minimise NI development following injury demonstrating that miR-145 may be an attractive therapeutic target for NI (19). This is supported by studies in rabbits where transduction of the venous bypass with miR-145 via electroporation or nanoparticle delivery minimised NI development compared to control animals and was associated with increased expression of the SMC marker smooth muscle myosin heavy chain and impaired CD40 expression (44, 45). It is interesting to note that miR-145 knockout mice fail to develop NI at all following carotid artery ligation. Whilst in isolation, this observation may suggest that miR-145 drives pathological NI remodelling, a closer look at the study reveals a high mortality rate in these animals following

occlusion as their vessels are unable to respond to injury appropriately (10). Thus, there is a strong body of evidence that restoring miR-145 expression has a protective effect on NI development across multiple species. However, the factors that cause miR-145 downregulation in the first place have yet to be identified – is miR-145 downregulation a cause or a consequence of NI? Further research is needed to prove the relevance of these promising studies to human disease.

Hypertension: The importance of miR-145 in maintaining blood pressure was highlighted in murine knockout studies, where animals lacking miR-145 had pronounced hypotension due to a lack of vascular tone (10). One would predict that SMC in hypertension exhibit elevated miR-145 and contractile machinery, however the literature reveals that this is not the case.

Essential hypertension has been studied in spontaneously hypertensive rats (SHR). Here, miR-145 expression was consistently reduced in SHR, resulting in decreased SMC contractility and increased Akt phosphorylation, expression of complement C3 and fibroblast growth factor 10 (FGF10). This caused a downstream upregulation of KLF5 and activation of the renin-angiotensin system, and consequently hypertension (46-48). Interestingly, upregulation of the lncRNA taurine upregulated 1 (TUG1) acted as a sponge to inhibit miR-145 (48). Exercise restores miR-145 levels in SHR (47) and has very recently been demonstrated to inhibit TUG1 expression (49). This may provide a mechanistic link as to the benefit of lifestyle modification in ameliorating essential hypertension through restoration of miR-145 expression in SMC. Studies on human tissue in essential hypertension is not as clear-cut as examinations have only been carried out in patients who have hypertension and atherosclerosis together. In these patients, miR-145 expression was elevated in in the plaques of those patients who had hypertension compared to those who were normotensive (50). However, given the complicated SMC phenotypic landscape in atherosclerosis, it is impossible to determine the influence of miR-145 on hypertension alone from this study.

In PAH, hypoxia induces the proliferation of SMC which contributes to the hypertensive phenotype. miR-45 expression was increased in both human PAH patients and a rodent PAH

model, and, in contrast to its canonical role in suppressing proliferation, miR-145 contributed to enhanced SMC proliferation through inhibiting the cholesterol transporter ABCA1 (32, 33). Furthermore, therapeutic inhibition of miR-145 in murine and rodent models alleviated the symptoms of PAH (33, 51), confirming the detrimental impact of miR-145 in this pathology.

Diabetes: The prevalence of type 2 diabetes is growing globally at an alarming rate, and the leading cause of morbidity and mortality in these patients is cardiovascular diseases. T2DM is associated with dysfunction within the small blood vessels (microvasculature) leading to nephropathy and retinopathy, and within the large vessels (macrovasculature) contributing to myocardial infarction and stroke. Of note, tight glycaemic control in these patients reduces the microvascular complications, however the macrovascular complications persist for much longer and a beneficial impact is not seen for at least ten years (37). Furthermore, miR-145 can regulate the insulin signalling pathway by directly targeting insulin receptor substrate 1 (IRS1) and the insulin-like growth factor receptor 1 (IGF-1R) in SMC from some – but not all – vascular beds (13, 52).

Collaterals are small microvessels that develop in response to narrowed arteries and are part of the bodies' natural response to vascular disease. In a rodent model of metabolic syndrome, miR-145 was downregulated resulting in impaired collateral formation which could be restored through adenoviral miR-145 delivery (53). Diabetic retinopathy and neuropathy are caused by vascular leakage, and very recent studies have demonstrated that miR-145 can be protective in these scenarios through, for example, inhibiting the inflammatory mediator MyD88 and TGFβ-mediator ZEB2 (54-56).

miR-145 expression is increased in macrovascular venous SMC from patients with T2DM. Interestingly, this over-expression persists in culture across passages, when glucose levels have been normalised for at least 6 months (13). This demonstrates evidence of metabolic memory, where cells 'remember' the pathological environment long after it has been removed and may help to explain why macrovascular complications of T2DM persist for so long regardless of glycaemic control. As miR-145 expression increases in parallel with body mass

index in human saphenous veins and aortae (17), it is likely that elevated miR-145 levels are observed even in pre-diabetic patients, highlighting the importance of diagnosing the disorder early to minimise macrovascular complications.

In summary, miR-145 is implicated in the pathogenesis of multiple diseases where SMC have a key role (Figure 2). In some cases, such as neointimal hyperplasia and essential hypertension, the consensus demonstrates a protective role of miR-145 in the development of these diseases. In others miR-145 is clearly detrimental, such as PAH. Finally, in atherosclerosis where SMC may adopt multiple phenotypes (synthetic, contractile or foam cell-like), the contributory role of miR-145 is unclear (summarised in Table 1).

Plasma miR-145 as a biomarker for cardiovascular disease

Plasma miRs have proven useful as diagnostic or prognostic markers (57). Thus, if miR-145 could be used as a circulating biomarker for specific cardiovascular diseases it would have great benefits for identifying at-risk individuals or perhaps designing personalised therapeutics. However, the use of plasma miR-145 as a marker for cardiovascular diseases is contentious, with many conflicting data. For example, plasma miR-145 levels were significantly downregulated in the plasma of coronary artery disease patients compared with healthy controls (58, 59), and acute coronary syndrome patients compared to healthy controls (60) suggesting an overall atheroprotective role. In support of this, a polymorphism that causes a reduction in plasma miR-145 levels has been found to be more frequent in patients who have atherosclerosis, reinforcing the concept that miR-145 ordinarily protects against the disease (61). In contrast, very recently findings have associated higher plasma miR-145 levels with unfavourable plaque characteristics (62), suggesting an atherogenic role. The reasons underpinning these incongruencies are unclear, though Knoka *et al* (2020) suggest that a feedback loop may exist whereby low levels of miR-145 trigger miR-145 release into the circulation of their patient cohort, as a mechanism to maintain optimal miR-145 levels. Similarly, there is no consensus regarding plasma miR-145 levels in diabetes, with studies identifying either no difference between diabetes and control groups (63, 64), or reductions in

T2DM patients (65, 66). Due to the lack of consistency across studies, the utilisation of miR-145 as a circulating biomarker for cardiovascular disease remains unconvincing. Perhaps, evaluating plasma levels of a panel of miRs (including miR-145) would be more successful as a prognostic or diagnostic for different cardiovascular diseases (67).

Conclusion

miR-145 is a central regulator of SMC differentiation through a complex relationship with the SRF-myocardin pathway. By targeting multiple pathways controlling diverse SMC functions including proliferation, migration and contraction, miR-145 acts as a balancing factor ensuring that SMC respond correctly to any changes in the circulation. Accordingly, dysregulation of miR-145 has been implicated in numerous cardiovascular pathologies. The causes of dysregulated miR-145 are yet to be elucidated although they do include TGFβ (13) and mechanical stretch (27). The recent emergence of lncRNA controlling miR-145 through sponging (e.g. TUG1 (48), MEG3 (56) and MALAT1 (55)) open up exciting new avenues for research that may be useful in the treatment of cardiovascular disease. Whilst originally described as an SMC-enriched microRNA, miR-145 has recently been identified in a number of other relevant cardiovascular cell types including endothelial cells (60) and cardiomyocytes (68). Thus in the future, assessment of miR-145 and its functions in cells other than SMC would be beneficial to fully exploit it's therapeutic potential.

Perspectives

- miR-145 is a critical regulator of smooth muscle cell differentiation. Dysregulation of miR-145 contributes to the development of cardiovascular disease.
- Expression of miR-145 protects against neointimal hyperplasia and the microvascular complications of diabetes but is detrimental in pulmonary arterial hypertension.
- Therapeutic targeting of miR-145, for example through manipulation of long non-coding RNAs, may be a promising therapy for treating cardiovascular diseases.

Figure 1: miR-145 controls multiple SMC functions. Expression of miR-145 is controlled by transcription factors dependent on the extracellular signal. SMC differentiation and migration is regulated by bidirectional relationships between miR-145 and the SRF / RhoA pathways. miR-145 inhibits proliferation by targeting the 3'-UTR of different mitogenic pathways. Abbreviations: C/EBPβ, CCAAT enhancer binding protein beta; CD40, cluster of differentiation 40; FoxO1/3, forkhead box factors O1/O3; KLF4, Kruppel-like factor 4; MRTF, myocardin-related transcription factor; PI3K, phosphatidylinositol kinase; ROCK, Rho kinase; SMAD; SRF, serum response factor; TGFβ, transforming growth factor beta.

Figure 2: Dysregulation of miR-145 contributes to the development of cardiovascular disease. Under normal conditions, miR-145 inhibits a broad range of target messenger RNAs. Dysregulation of miR-145 can affect how these targets are expressed, contributing to the development of cardiovascular disease. Abbreviations: ABCA1, ATP binding cassette subfamily A member 1; α-SMA, alpha smooth muscle actin; C3, complement factor C3; CD40, cluster of differentiation 40; FGF10, fibroblast growth factor 10; IRS-1, insulin receptor substrate 1; KLF5, Kruppel-like factor 5; MALAT1, metastasis associated lung adenocarcinoma transcript 1; MEG3, maternally expressed 3; MyD88, myeloid differentiation primary response 88; NFκB, nuclear factor kappa B; NI, neointimal hyperplasia; PAH, pulmonary arterial hypertension; RAS, renin-angiotensin system; TUG1, taurine upregulated gene 1; ZEB2, Zinc finger E-box-binding homeobox 2.

Disease	miR-145 influence	Model	References
Atherosclerosis	Protective	Human	(39)
	Protective	Murine	(15, 40)
	Detrimental	Human	(41)
	Detrimental	Murine	(42, 43)
Neointimal hyperplasia	Protective	Murine	(10)
	Protective	Rodent	(19)
	Protective	Rabbit	(44, 45)
Essential hypertension	Protective	Murine	(46-48)
Pulmonary arterial hypertension	Detrimental	Human	(32, 33)
	Detrimental	Murine	(33)
	Detrimental	Rodent	(51)
Diabetes (microvascular)	Protective	Human	(54, 56)
	Protective	Murine	(55, 56)
	Protective	Rodent	(53)
Diabetes (macrovascular)	Detrimental	Human	(13, 17)

Table 1: Implication of miR-145 in protecting or driving vascular pathologies. Expression of miR-145 in SMC can either play protective or detrimental roles in the development of cardiovascular disease. Please see main text for further details.

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Author contributions

KRS had sole responsibility for drafting, reviewing and publishing the study.

Declaration of interests

The author declares no conflicts of interest

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