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MicroRNA-21 drives the switch to a synthetic phenotype in human saphenous vein smooth muscle cells

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Abstract

Aims: Cardiovascular disease is a leading cause of morbidity and mortality. Smooth muscle cells (SMC) comprising the vascular wall can switch phenotypes from contractile to synthetic, which can promote the development of aberrant remodelling and intimal hyperplasia (IH). MicroRNA-21 (miR-21) is a short, non-coding RNA that has been implicated in cardiovascular diseases including proliferative vascular disease and ischaemic heart disease. However, its involvement in the complex development of atherosclerosis has yet to be ascertained.

Methods: Smooth muscle cells (SMC) were isolated from human saphenous veins (SV). miR-21 was over-expressed and the impact of this on morphology, proliferation, gene and protein expression related to synthetic SMC phenotypes monitored.

Results: Over-expression of miR-21 increased the spread cell area and proliferative capacity of SV-SMC and expression of MMP-1, whilst reducing RECK protein, indicating a switch to the synthetic phenotype. Furthermore, PDGF-BB (a growth factor implicated in vasculoproliferative conditions) was able to induce miR-21 expression via the PI3K and ERK signalling pathways.

Conclusion: This study has revealed a mechanism whereby PDGF-BB induces expression of miR-21 in SV-SMC, subsequently driving conversion to a synthetic SMC phenotype, propagating the development of IH. Thus, these signaling pathways may be attractive therapeutic targets to minimise progression of the disease.

Keywords:

MicroRNA-21, PDGF, saphenous vein, smooth muscle cell, phenotype, remodelling

List of Abbreviations

BIRC3, baculoviral IAP repeat containing 3; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CVD, cardiovascular disease; EC, endothelial cell; ERK1/2, extracellular regulated kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IH, intimal hyperplasia; IL, interleukin; IL1R2, interleukin-1 receptor 2; IMA, internal mammary artery; ITGB2, integrin subunit beta 2; KDR, kinase insert domain receptor; MGM, minimal growth medium; miRs, microRNAs; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 matrix metalloproteinase; PCI, percutaneous coronary intervention; PDGF-BB, platelet-derived growth factor BB; PPARG, peroxisome proliferator-activator receptor gamma; RECK, reversion inducing cysteine rich protein with Kazal motifs; RQ, relative quantification; SMC, smooth muscle cell; SV, saphenous vein; VWF, von Willibrand factor.

Introduction

Despite continued advances in knowledge and therapeutic strategies, atherosclerosis remains the leading cause of cardiovascular disease (CVD) around the world [1]. Atherosclerosis is a chronic, progressive inflammatory disorder which involves interactions of different vascular cell types that are the underlying cause of angina, coronary artery disease (CAD) and stroke [2]. Atherosclerosis in the coronary arteries can block the blood supply to the heart tissue, causing myocardial infarction and necessitating vascular reconstruction.

The advent of percutaneous coronary intervention (PCI) has improved treatment options for patients, yet coronary artery bypass grafting (CABG) remains the mainstay for patients with multivessel disease and impaired ejection fraction [3]. The commonly used bypass conduits for CABG are the autologous internal mammary artery (IMA) and the saphenous vein (SV), with the SV graft remaining popular for multiple grafts due to its ease of harvesting and ready availability. However, during the first year post-CABG up to 15% of SV grafts fail with an expected 4-5% attrition rate each year thereafter [4]. Therefore, in 10 years SV graft failure rates are close to 50% and approximately 31% of all CABG surgeries in the United Kingdom necessitate re-intervention [5]. Conversely, 80-95 % of IMA grafts remain patent at 10 years [6].

The underlying pathological lesion of SV graft stenosis is the characteristic development of intimal hyperplasia (IH). IH can be detected approximately 4-6 weeks following SV grafting [7] and this can be further augmented by continued atherosclerotic lesion development in the vein graft within one year following vein implantation [8].

Smooth muscle cells (SMC) constitute the major cell type in the blood vessel wall, and their principal role is to maintain vascular tone and vessel homeostasis. In health, SMC exist in a contractile, differentiated state with a low turnover rate, but following injury and in response to circulating cytokines and growth factors, they switch to a synthetic phenotype that is characterised by increased proliferation, loss of contractility, and secretion of extracellular matrix degrading enzymes. This encourages migration of SMC into the intimal layer of the blood vessel, thus initiating a remodelling event as an adaptive response that ultimately can exacerbate the development of intimal hyperplasia and restenosis [9].

Platelet-derived growth factor BB (PDGF-BB) is a potent SMC mitogen and chemoattractant that induces cellular dedifferentiation into a synthetic phenotype, increasing proliferation and migration into the intima such as is evident in atherosclerosis and restenosis [10, 11]. In addition to being secreted from platelets, PDGF is secreted by endothelial cells (EC) and SMC [12]. PDGF-BB-induced SMC dedifferentiation is driven through the phosphoinositide-3-kinase/AKT and mitogen-activated protein kinase extracellular regulated kinase 1/2 (ERK1/2) intracellular transduction pathways [13]. As increased SMC migration and proliferation constitute the cornerstone of IH and SV graft failure, PDGF-BB is implicated in its progression [14].

MicroRNAs (miRs) are short, non-coding RNAs that negatively regulate gene expression. Emerging evidence reveals miR-21 to be one of the most dysregulated miRNAs in the cardiovascular system. miR-21 is expressed in most cardiovascular cell types including vascular SMC [15] and can mediate the pathological development of neointimal hyperplasia [15], atherosclerotic lesions [16], and cardiac hypertrophy and

fibrosis [17, 18]. Indeed, miR-21 expression is reportedly elevated in human atherosclerotic plaques [19] and, at least in murine models, is up-regulated and mediates neointimal formation following vein grafting [20].

Despite advances in modern therapies, vein graft failure is still a significant problem that requires identification of novel targets for development of new therapeutics to ameliorate adverse vascular remodelling. Thus, the aim of this study was to explore a relationship between miR-21 and PDGF in the cellular mechanisms underlying SV-SMC dysfunction that occurs in vein graft failure.

Experimental procedures

Cell culture

Human SV specimens were obtained from patients undergoing elective CABG surgery at the Leeds General Infirmary. Local ethical committee and informed, written patient consent was obtained and the study conformed to the Declaration of Helsinki. SV-SMC were cultured using an explant technique as previously described [21]. As we have previously demonstrated profound phenotypic changes in SV-SMC isolated from patients with Type 2 diabetes [22-24], all experiments were performed using cells cultured from patients without a diagnosis of diabetes, and used between passages 3 and 5.

miR-21 transfection

Subconfluent SV-SMC were transfected with 30 nM premiR-21 (hsa-miR21-5p; AM17100) or its associated negative control (AM17110; "premiR-negative") using Lipofectamine 2000 (all Life Technologies, Paisley, UK). Cells were harvested after 72 h and endpoints determined (cell morphology, F-actin staining, RNA isolation) or proliferation assays performed.

Cell morphology

Images of sub-confluent cells were captured under the light microscope at x100 magnification. For each patient population, 50 randomly selected cells were measured by tracing the cell boundaries and areas were calculated using ImageJ software (https://imagej.nih.gov/ij/) [25]. For cytoskeletal assessment, cells were cultured on glass coverslips in medium containing 0.4% foetal calf serum (minimal growth medium,

MGM) for 72h. After fixation in 4% paraformaldehyde, F-actin fibres were labelled using rhodamine phalloidin (1:40) as previously described [26]. Fluorescence images were captured using an LSM510 upright confocal microscope at x200 magnification.

Cell proliferation

SV-SMC proliferation was determined by in vitro counting of live cells in triplicate using trypan blue and a haemocytometer as we described previously [22].

Quantitative real-time RT-PCR

For quantification of miR-21 expression, cellular RNA was used to produce cDNA using TaqMan MicroRNA Reverse Transcription System. MiR-21 expression levels were measured using miR-21 TaqMan microRNA assay (Applied Biosystems) and real-time PCR performed according to manufacturer's protocols. Data were analysed using the comparative CT method and values normalised to U6 expression. For gene expression studies, cellular RNA was extracted and real-time RT-PCR was performed in triplicate using Applied Biosystems 7500 PCR system and intron-spanning human matrix metalloproteinase-1 (*MMP1*; Hs00233958_m1), interleukin 1-alpha (*IL1α*; Hs00174092_m1), reversion inducing cysteine rich protein with Kazal motifs (*RECK*; Hs01019179_m1) primers and TaqMan® probes. Data are expressed as percentage of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) endogenous control levels (Hs99999905_m1) using the formula $2^{-\Delta CT}$ x 100 or to a control sample using the formula $2^{-\Delta\Delta CT}$.

Atherosclerosis microarray

SV-SMC from four different patients were transfected with 30 nM premiR-21 or equivalent premiR negative control. Cellular RNA was extracted and reverse transcribed. Expression levels of 84 atherosclerosis-related genes were quantified using a focused SYBR green-based real-time PCR array (RT2 Profiler Human Atherosclerosis, SABiosciences / Qiagen PAHS-038ZA-12) and the Applied Biosystems 7500 Real-time PCR system. ΔCT vales for the target genes were calculated by subtracting the mean CT value (threshold cycle number) of the 5 housekeeping genes on the array from the CT value of the target genes. Data are expressed as relative quantification (RQ) values compared with the 5 housekeeping genes using the formula $RQ=2^{-\Delta CT}$.

Immunoblotting

Immunoblotting was performed essentially as per our previous protocol [27]. Whole cell homogenates were prepared and protein was separated using SDS-PAGE 10% polyacrylamide gels and transferred to PVDF membranes. Phosphorylation of AKT and ERK1/2 was determined by immunoblotting with phospho-specific monoclonal antibodies (#9106 mouse, #4060 rabbit respectively). Membranes were re-probed with AKT and ERK expression antibodies (#2920 and #4695 respectively, all Cell Signaling Technology). For RECK protein, a 7.5% SDS-PAGE gel was prepared and α-tubulin used as a loading control.

Statistical Analysis

All results are expressed as a mean ± SEM. Statistical analysis was performed using GraphPad Prism 7 by one-way ANOVA followed by Newman-Keuls multiple comparisons post-hoc test. Paired ratio *t*-tests were performed on log transforms of all

normalised data to test statistical significance where appropriate. $P < 0.05$ was considered statistically significant.

Results

Over-expressing miR-21 induces phenotypic and functional changes in SV-SMC

Cells over-expressing miR-21 exhibited an enlarged, rhomboid morphology which was visible under phase contrast microscopy (Fig. 1A), accounting for a 1.7-fold increased spread area relative to control premiR-negative transfected cells (Fig. 1B). This enlarged morphology was further emphasised by rhodamine phalloidin fluorescence microscopy, which revealed that the F-actin cytoskeleton appeared visually intact in these morphologically aberrant SMC (Fig. 1C). Cells over-expressing miR-21 also exhibited increased cell proliferation, resulting in a 1.8-fold increase in cell number relative to control premiR-negative transfected cells after 4 days (Fig. 1D).

Microarray identification of *MMP1* and *IL1A* as miR-21-regulated genes in SV-SMC

Using SMC over-expressing miR-21, we performed a focused microarray to explore expression of 84 potential target genes related to atherosclerosis and cardiovascular disease. The pattern of gene expression induced by miR-21 over-expression is presented in a volcano scatter plot (Fig. 2A). Of the 84 genes studied, 10 genes presented a fold change $\geq \pm$ 1.5. *MMP1*, *MMP3*, interleukin 1 receptor 2 (*IL1R2*), peroxisome proliferator-activator receptor gamma (*PPARG*) and integrin subunit beta 2 (*ITGB2*) displayed $\geq$ 1.5-fold increase in the premiR-21 transfected cells relative to control premiR-negative transfected SV-SMC. Conversely, interleukin-1α (*IL1A*), kinase insert domain receptor (*KDR*), Baculoviral IAP repeat containing 3 (*BIRC3*), von Willibrand

factor (*VWF*) and interleukin 5 (*IL5*) showed > 1.5-fold decrease in the premiR-21 transfected cells relative to premiR-negative controls (Table 1).

Genes identified by microarray were subsequently validated using specific TaqMan probes and RT-PCR using RNA from six different patients, which included the four original samples that were analysed by array. In this series of experiments, we confirmed a statistically significant increase in *MMP1* mRNA expression in the premiR-21 transfected cells versus premiR-negative controls (Fig. 2B). On the contrary, we were unable to validate the *IL1A* data where only a small but non-significant reduction was evident in the premiR-21 transfected cells relative to the control SV-SMC (Fig. 2C).

miR-21 over-expression downregulates RECK in SV-SMC

RECK is a recognised target of miR-21 that is known to be involved in cell proliferation [28]. In our study, miR-21 over-expression in SV-SMC for 72h led to ~50% reduction in *RECK* mRNA expression levels (Fig. 2D), yet there were no detectable changes in RECK protein levels at this time point (data not shown). Extended study revealed a ~40% reduction in RECK protein levels in response to premiR-21 transfection after 8 days (Fig. 2E,F).

PDGF-BB increases miR-21 expression via AKT and ERK1/2 pathways in SV-SMC:

To identify the potential source of miR-21 up-regulation in SV-SMC we treated cells with the potent chemoattractant and mitogen PDGF-BB which is known to be increased in proliferative vascular disorders [29]. Exposure of SV-SMC for 24 h to physiologically

relevant (1 ng/ml and 10 ng/ml; [30, 31]) concentrations of PDGF stimulated miR-21 expression; an effect that was maintained up to 72 h (Fig. 3A).

Binding of PDGF to its cognate receptor results in a signalling cascade that induces phosphorylation of both AKT and ERK1/2 [32]. To determine which of these pathways was involved in inducing miR-21 expression, we used specific pharmacological inhibitors. PDGF-BB treatment induced phosphorylation of both AKT and ERK1/2 as expected and pathway-specific phosphorylation events were inhibited by pre-treating with inhibitors of AKT (LY294002) and ERK1/2 (PD98059) pathways (Fig. 3B). Inhibiting either pathway independently was able to completely abrogate the induction of miR-21 expression by PDGF-BB, confirming the requirement for both pathways in miR-21 regulation in these cells (Fig. 3C).

PDGF-BB mimics the effect of miR-21 over-expression on MMP-1 and RECK expression

Having shown that PDGF-BB increased miR-21 expression (Fig. 3), and that miR-21 overexpression led to an increase in *MMP1* mRNA expression and inhibition of RECK expression (Fig. 2), we proceeded to seek evidence for a link between PDGF and these targets. PDGF-BB significantly induced *MMP1* mRNA at 24 h in a concentration-dependent manner, although this effect was transient and expression levels returned to baseline by 72 h (Fig. 3D). In parallel, *RECK* mRNA was significantly downregulated in a concentration-dependent manner, and this effect was maintained over the full 72 h period of study (Fig. 3E).

Discussion

Dysregulation of SMC differentiation is an event critical to the development of CVD. The presence of characteristically dedifferentiated SMC in IH is well recognised and thus investigations into the mechanisms contributing to this phenotypic reversion are of significant clinical importance. Two of the predominant mechanisms for SV graft failure are IH and accelerated atherosclerosis. In the present study, we utilised clinically relevant human SV-SMC and revealed signalling pathways whereby overexpression of miR-21 led to acquisition of a synthetic phenotype.

miR-21 as a driver of synthetic SMC phenotypes

The importance of miR-21 in the cardiovascular system is well recognised. It is aberrantly expressed in vascular SMC [15] and EC [33] following vascular injury, and is significantly up-regulated in human atherosclerotic plaques [19], injured rat carotid arteries [15] and following vascular grafting in mice [20]. However, its influence on human SV-SMC remodelling following bypass grafting is unknown. In this study we used an over-expression approach to determine the impact of miR-21 on SMC phenotype and function *in vitro*.

Synthetic SMC in remodelling vessels are known to adopt a rhomboid morphology and have a high turnover rate [34]. Both of these features were observed in miR-21 over-expressing SV-SMC, confirming that miR-21 was a driver of this dedifferentiation. Given the widespread reporting of miR-21 as a proto-oncogene that increases proliferation in a number of pathological models including vascular SMC [15, 35, 36], its impact specifically on SV-SMC is perhaps unsurprising. In direct contrast however,

miR-21 has been purported to drive a differentiated, contractile phenotype in human pulmonary artery SMC [37] and correlates with expression of smooth muscle differentiation genes [38]. Given this disparity, we perceived it was important to validate this hypothesis in clinically relevant human SV-SMC. Our present data highlight the species- and cell type-specific nature of miR-21's mechanism of action.

Identification of downstream miR-21 targets that contribute to vascular remodelling

As miR-21 over-expressing cells were dedifferentiated, and such SMC are common in IH, we proceeded to use a microarray-based approach to identify downstream targets of miR-21 in SV-SMC that may contribute to the development of CVD. *IL1A* is a pro-inflammatory cytokine that is elevated in atherosclerosis [39], however its regulation by miR-21 could not be corroborated in SV-SMC. In contrast, the array data for *MMP1* was validated using specific Taqman assays which identified *MMP1* as being regulated by miR-21 in these cells.

MMPs play an important role in degrading the ECM and can contribute to matrix destabilisation [40]. Furthermore, digestion of integrins by MMPs can facilitate SMC dedifferentiation [41]. Interestingly, in SV-SMC, there was a positive relationship between miR-21 and *MMP1* mRNA expression. Normally, miRs negatively regulate the expression of direct gene targets [42]. This suggests that the relationship between miR-21 and *MMP1* in SV-SMCs is complex and involves an intermediary step whereby the target of miR-21 firstly inhibits expression of a gene that normally acts as a repressor of *MMP1* expression.

Transcriptional regulation of MMPs is complicated, encompassing multiple intracellular signalling pathways and transcription factors [43]. RECK is a membrane-bound MMP inhibitor that has been reported to regulate the gelatinases MMP-2 and -9, and their activator membrane type 1-MMP (MT1-MMP) [44]. Previous studies have shown that miR-21 overexpression in various cancer cell lines reduces mRNA and protein levels of RECK [45]. Furthermore, in vascular SMC, knockdown of RECK led to increased MMP-2 and -9 expression, and an increase in cell proliferation [46]. In the present study, transfection with premiR-21 inhibited *RECK* mRNA expression after 3 days, and RECK protein levels after 8 days. Evidence of a direct effect of RECK on MMP-1 expression remains elusive, thus the relationship in SV-SMC is worthy of further investigation.

PDGF-BB is an important regulator of SMC function via miR-21

On the basis of the above evidence, elevated miR-21 expression can cause vascular SMC dysfunction leading to dedifferentiation, enhanced proliferation and degradation of the ECM. MiR-21 is known to be elevated in proliferative SMC present in IH and atherosclerotic plaques, thus identifying the factor(s) responsible for this elevation is of clinical relevance.

PDGF-BB is a potent SMC chemoattractant and mitogen, which can promote SMC dedifferentiation and proliferation [10, 11] and is elevated following vascular injury and angioplasty [29]. PDGF-BB is purported to regulate miR-21 in a number of cell types, including rat aortic SMC [47]. Our observations of a modest yet consistent up-regulation of miR-21 by PDGF-BB in SV-SMC that persists over time aligns with this. However, a recent study raised the possibility of a more complex relationship whereby miR-21 could

prevent the mitogenic effects of PDGF [36]. Further experiments examining any attenuation of PDGF-BB signalling through over-expressing miR-21 may answer this question.

Both the PI3K/AKT and ERK1/2 pathways are activated upon binding of PDGF-BB to its receptor, leading to SMC dedifferentiation [13, 32]. In our study, inhibition of either pathway was sufficient to completely ablate miR-21 expression by PDGF-BB, indicating that both pathways are required. Interestingly, miR-21 itself can stimulate the AKT pathway (via suppression of PTEN; [15, 35]) and the ERK pathway [48] potentially causing a positive feedback loop that reinforces the effect of PDGF-BB on miR-21 expression.

Treatment of SV-SMC with PDGF-BB was sufficient to increase expression of *MMP1* mRNA and reduce expression of RECK. Reports of a relationship between PDGF-BB and RECK in the literature are lacking, although one study demonstrated that loss of RECK in fibroblasts caused a disordered chemotactic response to PDGF [49]. To our knowledge, ours is the first report of PDGF-BB regulating RECK expression in human SMC via up-regulation of miR-21. The relationship between PDGF and MMP-1 is however, better recognised and previous studies have reported a positive relationship between PDGF and MMP-1 [50]. Our data concur with these findings, and further suggest a mechanism mediated via miR-21.

Conclusion

During pathological vascular remodelling, secretion of cytokines and growth factors from multiple vascular cell types are increased. Here, we have demonstrated how one such growth factor, PDGF-BB, controls SV-SMC phenotype, at least in part, through miR-21. This has downstream effects on RECK and MMP-1 expression and leads to SV-SMC dedifferentiation (see Fig. 4 for summary diagram). Targeted inhibition of PDGF-BB signalling may be a beneficial therapeutic strategy following coronary artery bypass grafting to limit post-graft development of neointimal hyperplasia.

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Conflict of interests

The authors declare no conflict of interest.

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Figure Legends

Figure 1: Overexpression of miR-21 alters SMC phenotype. SV-SMC were transfected with 30nM premiR-21 and cultured for 72h. (A) Representative images of transfected SV-SMC and (B) quantification of spread cell area (n=7, $^{**}P$<0.01). (C) Representative images of the F-actin cytoskeleton stained with rhodamine phalloidin. (D) After the 72h period, cells were placed in proliferation assays and direct cell counts taken after 4 days. Data is presented as a fold increase in cell number from day 0 (n=5, $^{**}P$<0.01).

Figure 2: miR-21 over-expression alters atherosclerosis-related gene expression (A) Volcano plot of the RT2 Profiler Human Atherosclerosis PCR Microarray data. The difference in expression level (log 2 scale) is indicated on the x-axis and the y-axis indicates statistical significance (-log 10 scale). Values located above the horizontal dotted line indicate $P < 0.05$. Only genes that had ≥ ±1.5-fold changes are labelled; genes upregulated by miR-21 over-expression are indicated in grey and those down-regulated are indicated in black (n=4). The array data was validated using TaqMan® intron-spanning primers for (B) MMP-1 and (C) IL-1A (both n=6). (D) Quantification of RECK mRNA in SV-SMC transfected with premiR-21 (or premiR-ve) for 72 h (n=8). (E) Quantification of RECK protein in transfected cells after 8 days (n=5, ***P<0.001). (F) Representative immunoblot with α-tubulin as a loading control. $^{***}P$<0.001, $^{**}P$<0.01, $^{*}P$<0.05, ns=not significant.

Figure 3: PDGF induces miR-21 expression via AKT and ERK1/2, and inhibits MMP-1 and RECK1. (A) SV-SMC were treated with increasing concentrations of

PDGF and miR-21 expression quantified after 24-72h (*n*=5, **P<0.01, *P<0.05, ns=not significant. (B) SV-SMC were treated with the PI3K inhibitor LY294002 (LY; 10μM) and ERK1/2 inhibitor PD98059 (PD; 30μM) for 1 h prior to PDGF stimulation (20 min). V refers to vehicle control. Representative Western blot demonstrating the efficacy of the inhibitors. (C) SV-SMC were treated with V, LY or PD for 1 h prior to the addition of PDGF. miR-21 expression was monitored 48 h later (*n*=5, *P<0.05). SV-SMC were treated with increasing concentrations of PDGF and the expression of (D) MMP-1 and (E) RECK quantified after 24-72h (*n*=5, ***P<0.001, **P<0.01, *P<0.05, ns=not significant).

Figure 4: PDGF - miR-21 axis in SV-SMC. PDGF-BB in the circulation signals through both AKT and ERK to up-regulate expression of miR-21. In turn, this inhibits MMP-1 and RECK1, and drives a phenotypic switch towards a synthetic SMC such as the type prevalent in atherosclerosis and neointimal hyperplasia.

FIGURE 1

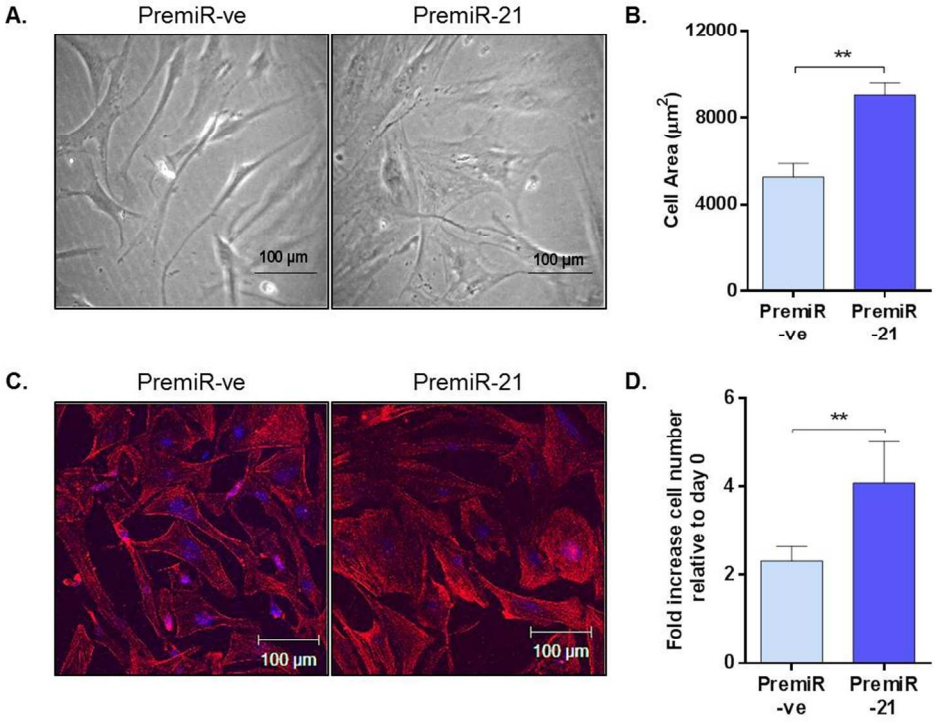

Figure 1: Overexpression of miR-21 alters SMC phenotype

184x148mm (150 x 150 DPI)

FIGURE 2

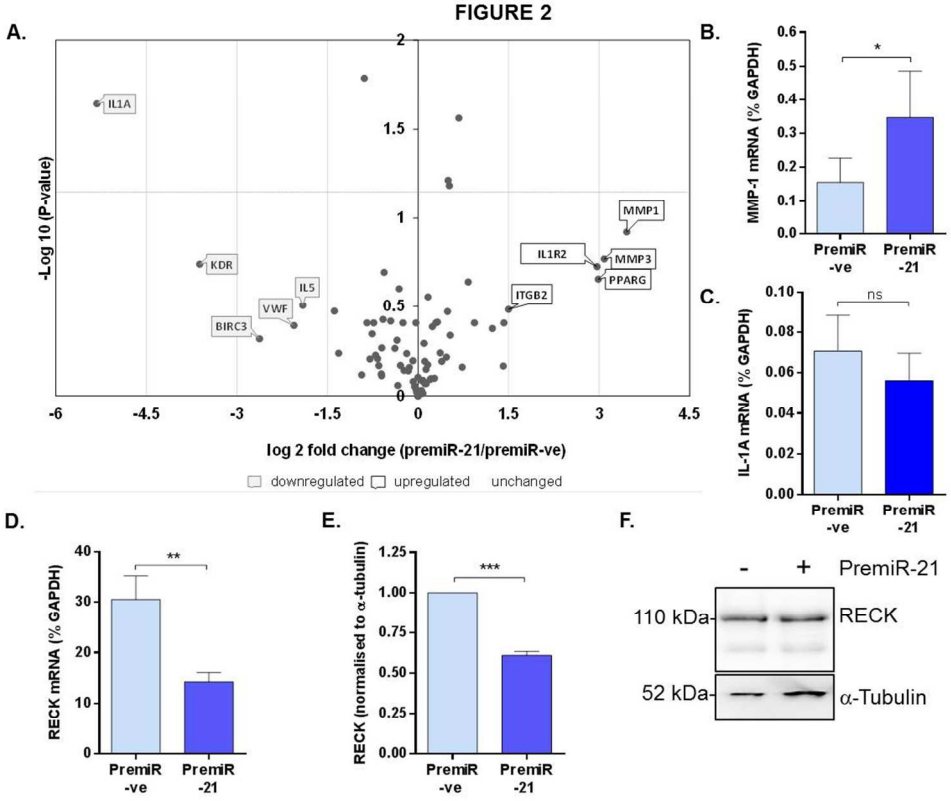

Figure 2: miR-21 over-expression alters atherosclerosis-related gene expression

202x167mm (150 x 150 DPI)

FIGURE 3

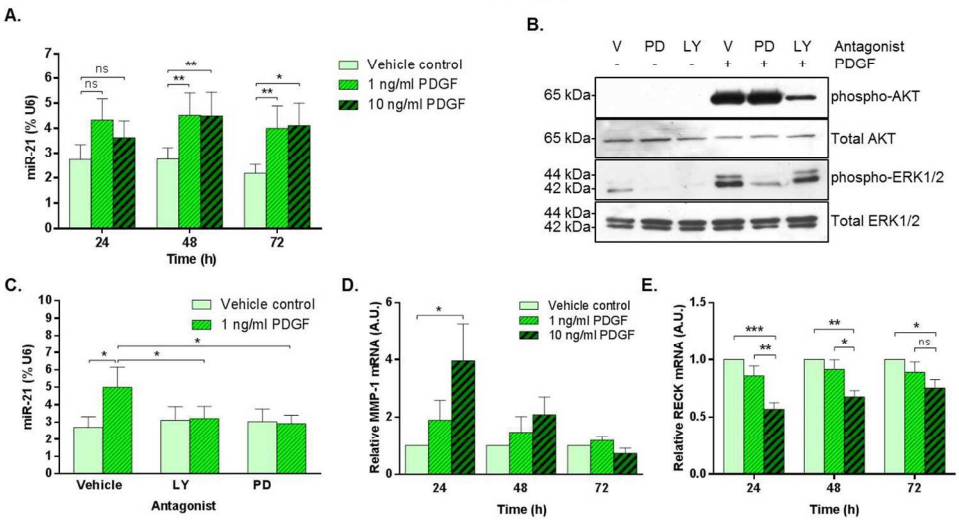

Figure 3: PDGF induces miR-21 expression via AKT and ERK1/2, and inhibits MMP-1 and RECK1

238x132mm (150 x 150 DPI)

FIGURE 4

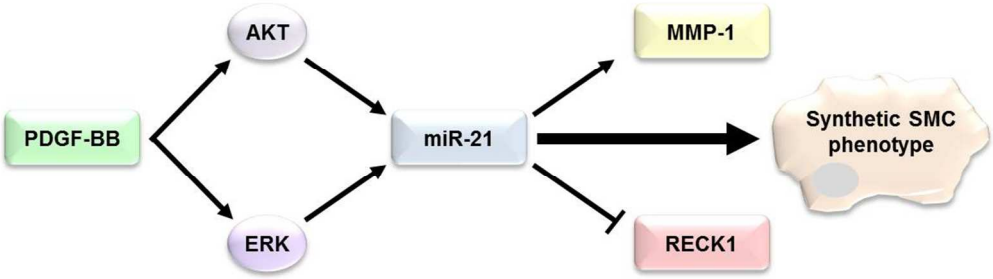

Figure 4: PDGF - miR-21 axis in SV-SMC

198x63mm (150 x 150 DPI)

Table 1: Genes modulated by miR-21 overexpression in the Human Atherosclerosis Microarray

Gene	Log 2 fold change (treated/control)	*P* value (ratio paired *t*-test)
Upregulated:		
MMP1	3.46	0.12
MMP3	3.08	0.17
PPARG	2.99	0.22
IL1R2	2.96	0.19
ITGB2	1.5	0.33
Downregulated:		
IL5	-1.91	0.34
VWF	-2.06	0.41
BIRC3	-2.62	0.49
KDR	-3.62	0.18
IL1A	-5.32	0.02

Cells over-expressed miR-21 or the negative control, and RNA was subsequently analysed using the Human Atherosclerosis Microarray. Table shows genes with a log 2 fold change greater or equal to 1.5 upregulation or downregulation, and the related *P* value.