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miRNA-21 is dysregulated in response to vein grafting in multiple models and genetic ablation in mice attenuates neointima formation

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Aims The long-term failure of autologous saphenous vein bypass grafts due to neointimal thickening is a major clinical burden. Identifying novel strategies to prevent neointimal thickening is important. Thus, this study aimed to identify microRNAs (miRNAs) that are dysregulated during neointimal formation and determine their pathophysiological relevance following miRNA manipulation.

Methods and results We undertook a microarray approach to identify dysregulated miRNAs following engraftment in an interpositional porcine graft model. These profiling experiments identified a number of miRNAs which were dysregulated following engraftment. miR-21 levels were substantially elevated following engraftment and these results were confirmed by quantitative real-time PCR in mouse, pig, and human models of vein graft neointimal formation. Genetic ablation of miR-21 in mice or grafted veins dramatically reduced neointimal formation in a mouse model of vein grafting. Furthermore, pharmacological knockdown of miR-21 in human veins resulted in target gene de-repression and a significant reduction in neointimal formation.

Conclusion This is the first report demonstrating that miR-21 plays a pathological role in vein graft failure. Furthermore, we also provided evidence that knockdown of miR-21 has therapeutic potential for the prevention of pathological vein graft remodelling.

Keywords Vein graft failure • MicroRNA • Neointimal formation • Vascular remodelling

Introduction

Despite the increased utility of drug-eluting stents, coronary artery bypass grafting (CABG) remains the treatment of choice in patients with multi-vessel disease or diabetes due to increased freedom from recurrent angina, ischaemic events, and need for repeat intervention.[1–3]

A number of studies have demonstrated that patency rates of saphenous vein (SV) grafts are lower than those of arterial conduits such as the internal mammary artery,[4,5] yet autologous veins remain an important, convenient, and frequently used conduit for surgical revascularization.[6] A number of technical advances have been proposed over the past decade, such as off-pump CABG and no-touch SV harvesting; nevertheless, the rates of

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vein graft failure remain largely similar.[7,8] It is estimated that 20–50% of patients suffer from stenotic occlusion of the graft within 5 years.[9] The high failure rates highlight the need for novel therapeutic interventions in the setting of CABG. The principal processes responsible for vein graft failure are intimal hyperplasia [promoted by enhanced vascular smooth muscle cell (SMC) proliferation, migration, and extracellular matrix deposition] and superimposed atherosclerosis (reviewed in Wan *et al.*[10]). Although aggressive lipid-lowering therapy has delayed vein graft failure in patients, there is an urgent need for alternative therapies which can curtail the early pathological remodelling in the short term in order to improve long-term patency rates.

MicroRNAs belong to a recently discovered class of small, endogenous non-coding RNA molecules that negatively regulate gene expression by targeting specific messenger RNAs and induce their degradation or translational repression.[11] A number of reports have highlighted a role for miRNA in SMC proliferation and migration associated with vascular pathologies (reviewed in McDonald *et al.*[12]). MicroRNA profiling in normal and atherosclerotic human arteries demonstrated that miR-21 was up-regulated in the SMC layer of atherosclerotic vessels.[13,14] Furthermore, previous reports from two independent groups demonstrated that miR-145 levels were down-regulated following balloon injury and adenoviral-mediated over-expression of miR-145 reduced intimal lesion formation.[15,16] Torella *et al.*[17] demonstrated that adenoviral mediated over-expression of miR-133a reduced neointimal formation and antagomiR to miR-133a exacerbated neointimal formation following balloon injury. These studies highlight that miRNAs are dysregulated at the onset of vascular injury, and as a result, modulation of miRNA levels may represent a therapeutic target in a number of vascular pathologies. However, no studies to date have assessed the role of miRNAs in the context of neointima formation associated with vein graft failure. Accordingly, we sought to identify aberrantly expressed miRNAs in models of vein graft failure. We report using mouse, pig, and human models that a number of miRNAs were dysregulated during vein graft remodelling. We also highlight that genetic ablation of miR-21 substantially limited neointima formation in mouse vein grafts *in vivo*. To investigate the translational application of these findings, human saphenous veins (HSVs) were exposed to anti-miR-21 *ex vivo*. In this model, we show that de-repression of several target genes is achieved and a significant reduction in neointima size is observed, suggesting that localized inhibition of miR-21 may provide a novel therapeutic approach to prevent vein graft neointima formation.

Methods

Vein graft models

Several established models of vein grafting were utilized in this study. We utilized two mouse models of vein grafting; interpositional grafting[18] was used for miR-21 profiling, and the isogenic mouse vein graft model[19] was used for all other experiments. Porcine SV-carotid interpositional grafting[20] and *ex vivo* culture of human SV segments[21] were also used. In addition, we assessed failed grafts removed from patients at least 5 years post-CABG. miR-21 knockout mice were previously described.[22,23]

In vitro models

In vitro experiments were performed using isolated primary human SV-derived endothelial cells (EC) and SMCs.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). For the comparison of mean values, Bartlett's test for equal variances was performed—there was no evidence of heterogeneous variances between groups for any of the comparisons. Visual assessment was used to check for any lack of normality; as there was no evidence of this, one-way ANOVA followed by Tukey's multiple comparison test (for comparison of more than two groups) or Student's *t*-test (for comparison of two groups) was carried out. For all the quantitative real-time PCR (qRT-PCR) experiments, values are expressed as fold change. All statistical analyses were carried out using GraphPad Prism version 4 (GraphPad Software, USA), other than the microRNA array data. The microRNA array data were analysed in the DataAssist™ software (Life Technologies). Following manual normalization identification, miR-199a-3p was chosen as a reference gene to normalize the data, since its standard deviation across all samples was the lowest for all microRNAs on the chip. One-way ANOVA with Benjamini–Hochberg false discovery rate[24] (FDR) adjustment for multiple testing was carried out. We restricted our validation of miRNAs to those that were up-regulated >4-fold and which were significant at a 10% FDR in the TaqMan Low Density Array (TLDA) experiment.

For detailed descriptions of materials and methods, see Supplementary material online.

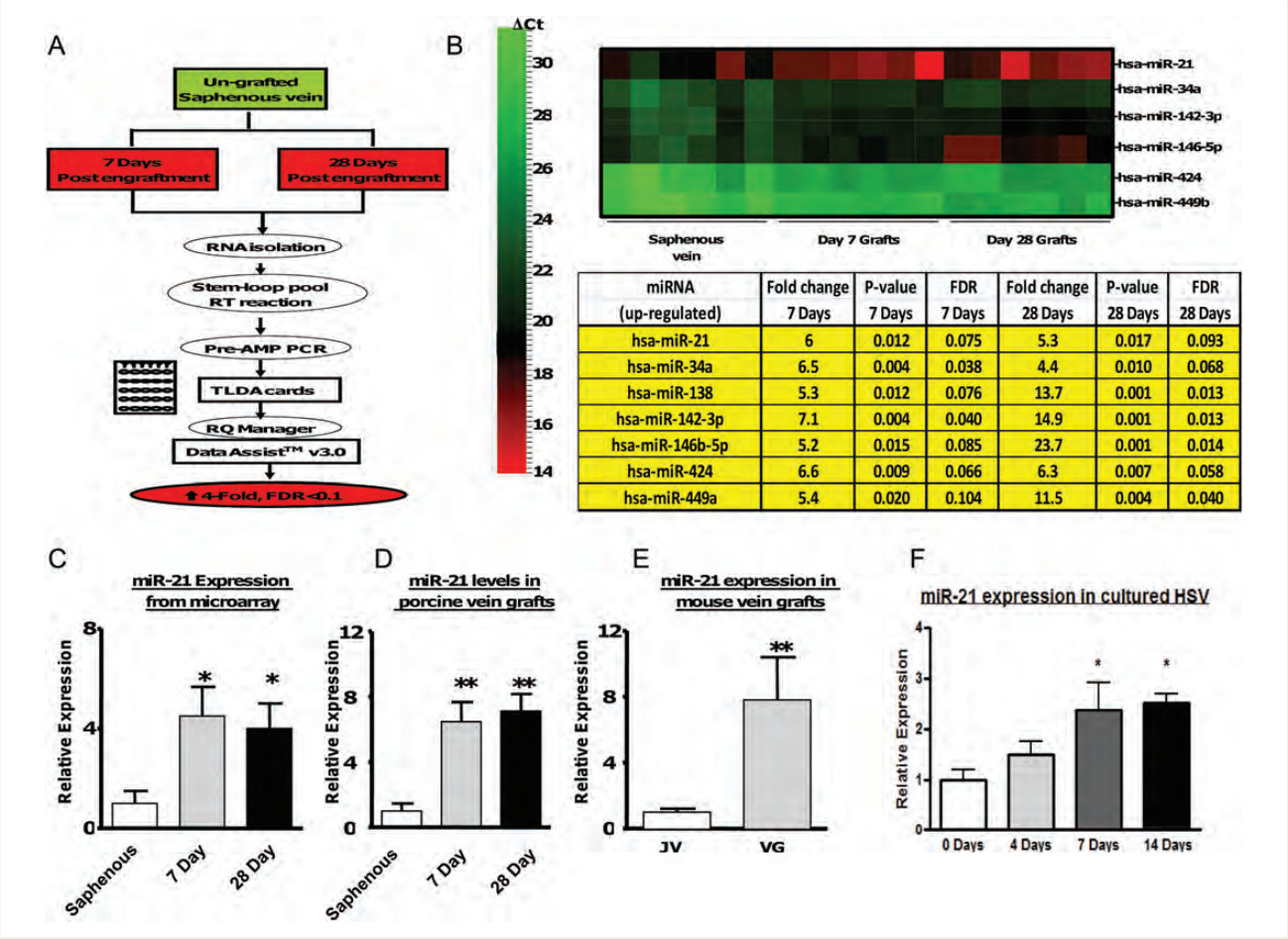
Results

Global microRNA profiling in ungrafted and grafted saphenous veins

In order to identify aberrantly expressed miRNA during neointima formation in the setting of vein graft failure, we compared miRNA expression patterns by microarray analysis in ungrafted and grafted porcine SVs isolated 7 and 28 days post-engraftment (*Figure 1A*). Global miRNA profiling analysis revealed that 21 out of 377 miRNAs were up-regulated following engraftment (see Supplementary material online, *Figure S1* and *Table S1*), 6 of which were up-regulated more than 4-fold (FDR < 0.1) at 7 and 28 days post-engraftment (*Figure 1B*). From this data set, we focused on miR-21 due to the substantial up-regulation observed in pig grafts compared with controls (*Figure 1C*) and since it has previously been implicated in SMC and fibroblast proliferation following acute vascular injury.[25,26]

miR-21 expression is elevated in multiple models of vein graft disease

In order to validate and further quantify the expression levels of miR-21 during the progression of vein graft neointimal formation, we profiled miR-21 levels in three well-characterized models of vein graft disease, using qRT-PCR analysis. These models were (i) *in vivo* porcine model of interposition grafting, (ii) the interposition mouse vein graft model (jugular vein into the right common femoral artery), and (iii) the *ex vivo* model of HSV neointimal formation using surplus vein tissue harvested at the time of CABG. In the porcine model, qRT-PCR confirmed that miR-21 levels were elevated at 7 days following grafting and remained elevated at 28 days (*Figure 1D*). We observed an ~7-fold increase in levels at



miRNA (up-regulated)	Fold change 7 Days	P-value 7 Days	FDR 7 Days	Fold change 28 Days	P-value 28 Days	FDR 28 Days
hsa-miR-21	6	0.012	0.075	5.3	0.017	0.093
hsa-miR-34a	6.5	0.004	0.038	4.4	0.010	0.068
hsa-miR-138	5.3	0.012	0.076	13.7	0.001	0.013
hsa-miR-142-3p	7.1	0.004	0.040	14.9	0.001	0.013
hsa-miR-146b-5p	5.2	0.015	0.085	23.7	0.001	0.014
hsa-miR-424	6.6	0.009	0.066	6.3	0.007	0.058
hsa-miR-449a	5.4	0.020	0.104	11.5	0.004	0.040

Figure 1 Global microRNA profiling and assessment of miR-21 in vein grafting. (*A*) A schematic diagram illustrating how the samples were prepared for global microRNA expression, using microfluidic cards. Ungrafted saphenous veins were compared with veins subjected to vein grafting for a period of 7 and 28 days. We evaluated only targets which were >4-fold up-regulated at both time points. (*B*) A heat map of the only microRNAs which were significantly up-regulated >4-fold; the table summarizes the fold change, *P*-values, and the false discovery rate (FDR) for each microRNA. (*C*) Relative quantification of the miR-21 array results normalized to miR-199a-3p. (*D*) Verification of TLDA array by real-time-PCR, demonstrating up-regulation of miR-21 in porcine veins following grafting for 7 and 28 days ($n = 6$/group). (*E*) Expression of miR-21 in control ungrafted jugular vein (JV) and vein graft (VG) from mice ($n = 5$/group). (*F*) Real-time PCR for miR-21 expression in HSV subjected to culture for a period of 0, 7, 14, or 28 days to allow neointimal lesions to develop ($n = 6$/group). Error bars denote SEM; $^*P < 0.05$, $^{**}P < 0.01$. Data were analysed by one-way ANOVA with a Tukey's post-test.

both time points compared with ungrafted SV ($P < 0.01$, $n = 6$/group), which is consistent with the changes observed in our TLDA analysis (*Figure 1C*). This elevation in miR-21 levels was paralleled in the mouse vein graft model, with miR-21 levels elevated in grafts at 28 days compared with ungrafted conduits (jugular veins) ($P < 0.001$, $n = 4–5$, *Figure 1E*). Finally, we assessed levels in HSV *ex vivo* organ cultures. miR-21 was significantly up-regulated 2.5-fold in vein segments cultured for 7 and 14 days ($P < 0.05$, $n = 6$, *Figure 1F*). Therefore, in three independent models representative of vein graft neointima formation, we observed that miR-21 levels were significantly elevated when analysed by qRT-PCR.

Localization of miR-21 in models of vein graft neointimal formation

In order to ascertain the localization of miR-21 in control vessels and veins post-grafting, we performed *in situ* hybridization for miR-21. miR-21 expression was low in porcine carotid arteries and undetectable in ungrafted SVs (*Figure 2A* and *B*). However, we observed positive staining for miR-21 in porcine vein grafts, in the adventitial, medial, and neointimal layers at both 7 and 28 days (*Figure 2C* and *D*). We performed immunohistochemistry with α-actin on serial sections since SMCs are integral to the formation of neointimal lesions. In vein grafts, we noted that miR-21 was located in all three layers of the vessel wall, in regions of the graft expressing α-actin (*Figure 2K* and *L*).

In a second mouse model of vein grafting, the isogenic graft model (vena cava into the right common carotid artery), miR-21 levels were undetectable in the ungrafted inferior vena cava and low in the carotid artery compared with the wide spread and high-level expression in grafted tissue (*Figure 3*). We performed immunohistochemistry for α-actin and mac-2 to determine whether the cells expressing miR-21 were SMCs or macrophages,

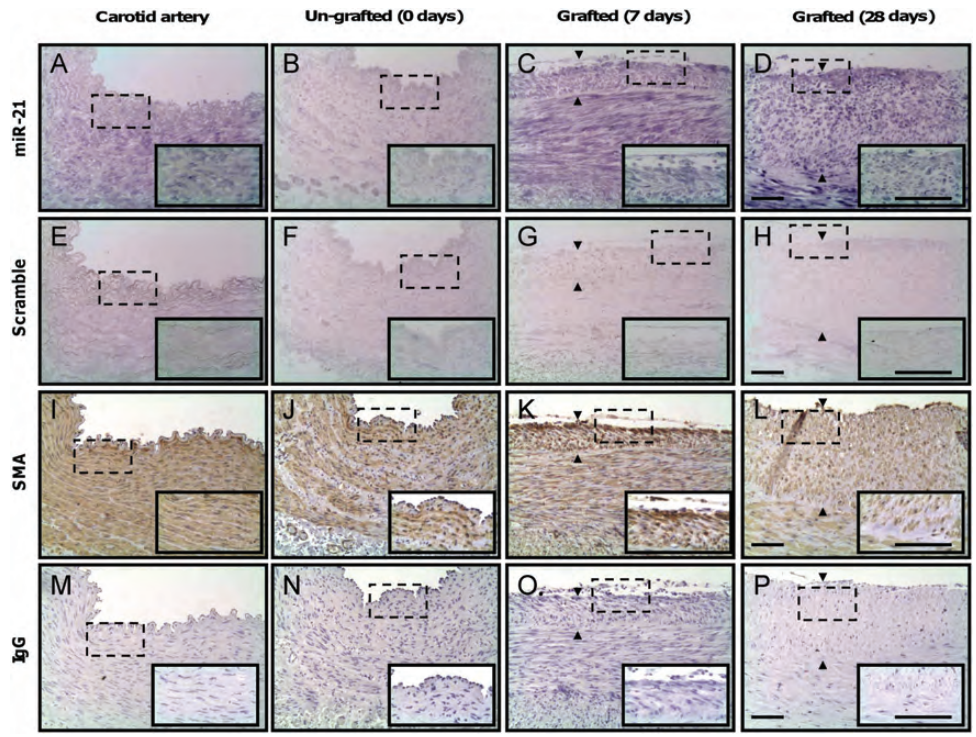


Figure 2 Localization of miR-21 at 7 and 28 days post-engraftment in a porcine model. (*A–D*) *In situ* hybridization (violet) of miR-21 in the carotid artery, ungrafted saphenous vein, and grafted saphenous vein at 7 and 28 days post-engraftment, respectively. (*E–H*) *In situ* hybridization on the same sections with a scrambled probe. (*I–L* and *M–P*) SMA and control IgG staining, respectively, in serial sections from the same vein grafts. Arrows indicate the neointimal layer; scale bar represents 100 μm.

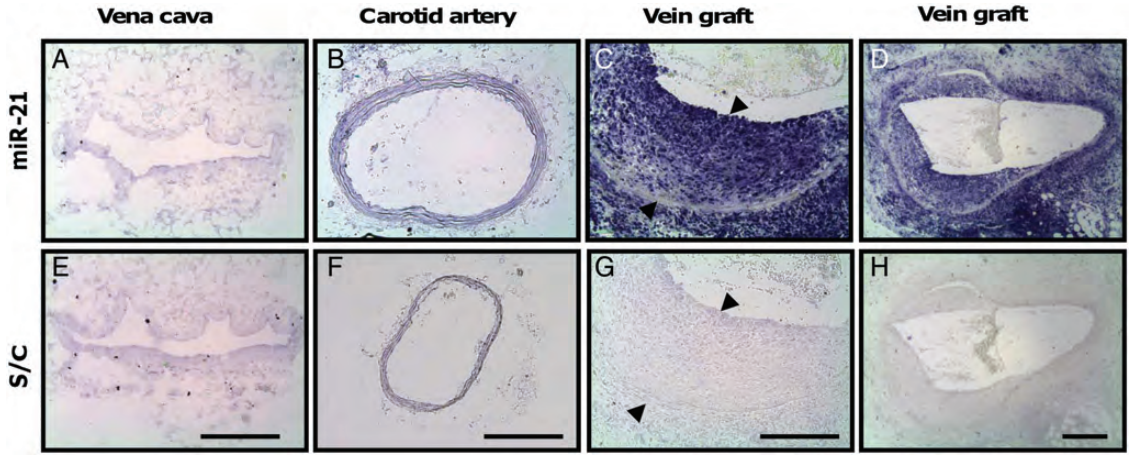


Figure 3 Localization of miR-21 in mouse vessels. *In situ* localization of miR-21 in the mouse inferior vena cava (*A*), carotid artery (*B*), and vein grafts harvested 28 days post-engraftment at high (*C*) and low magnification (*D*). (*E–H*) The corresponding control *in situ* hybridization with a scrambled probe. Filled arrows indicate neointimal thickness; scale bar represents 200 μm, applicable to all panels.

respectively. Comparison of the staining pattern for α-actin, mac-2, and proliferating cell nuclear antigen (PCNA) revealed that miR-21 is expressed in regions of the graft which stained positive for both SMC actin (α-actin) and macrophages (mac-2), although not all α-actin-positive cells expressed miR-21 (*Figure 4A–D*). These studies on sequential serial sections suggest that miR-21 is expressed in proliferating SMCs in the neointima and macrophages and actin-positive cells in the adventitial layer of the vein grafts. We

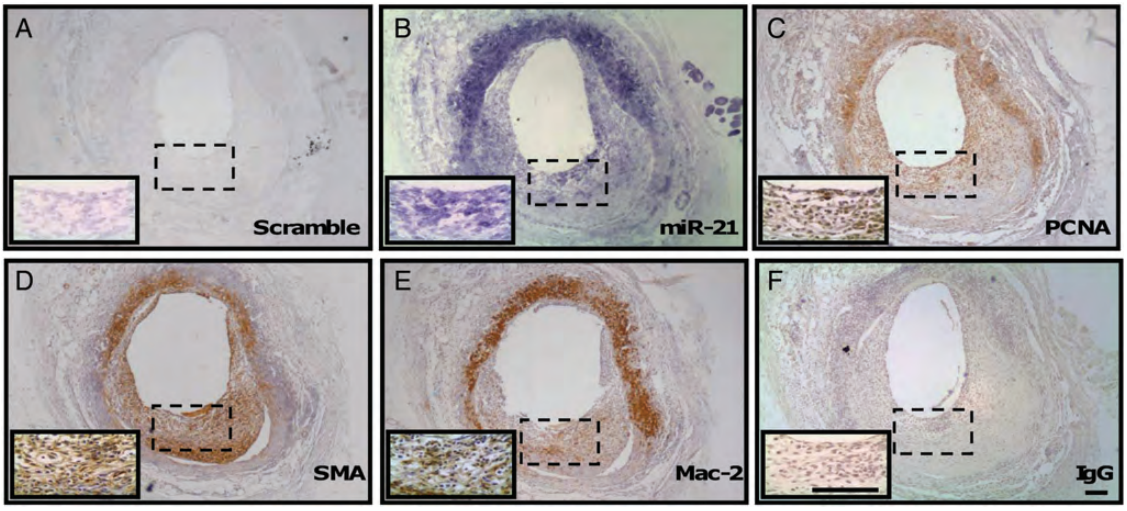


Figure 4 Immunolocalization of cells expressing miR-21 in murine vein grafts. (*A*) *In situ* hybridization with a scrambled LNA probe. (*B*) *In situ* hybridization for miR-21 in a mouse vein graft at 28 days. (*C–F*) Immunohistochemistry staining for proliferating cell nuclear antigens, smooth muscle cells (SMA), macrophages (mac-2), and control IgG, respectively. Scale bar represents 100 μm.

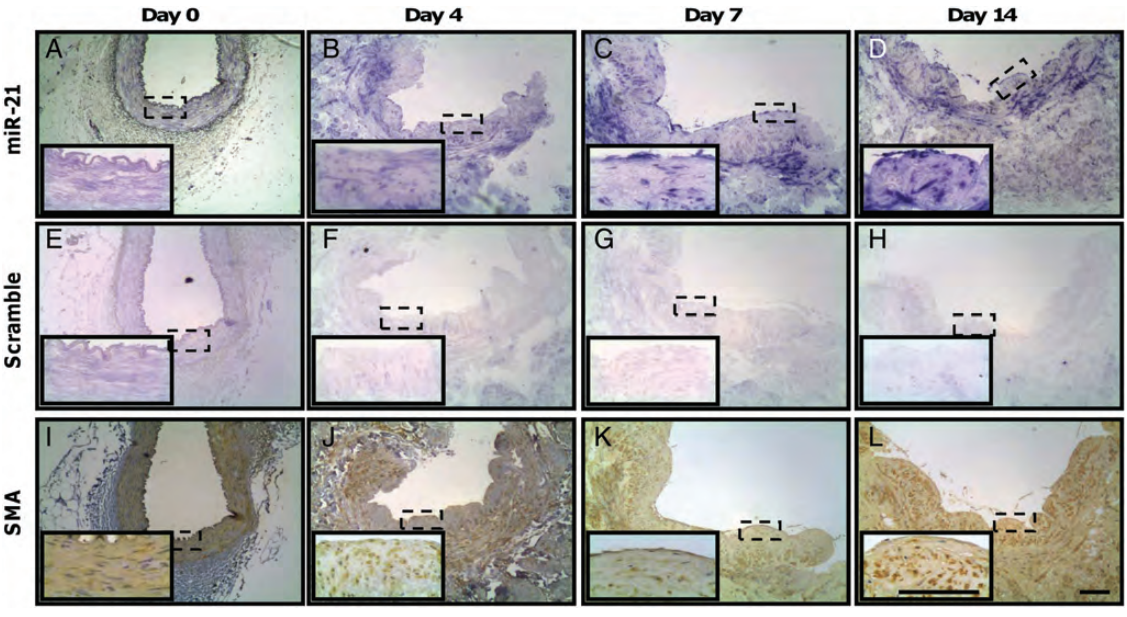


Figure 5 miR-21 expression in human vein subjected to culture. (*A–D*) *In situ* hybridization of miR-21 in human saphenous veins following 0, 4, 7, and 14 days of cell culture. (*E–H*) *In situ* hybridization on serial sections with a scrambled probe. (*I–L*) Serial sections stained with anti-α-actin antibodies (SMA) to determine smooth muscle cells. Scale bar represents 100 μm, applicable to all panels.

next performed immunohistochemistry for the fibroblast markers vimentin and FSP-1.[27] Cells staining positive for vimentin and FSP-1 were found extensively in the adventitia and a large proportion of cells in the neointimal layer, suggesting that myofibroblasts also contribute to miR-21 expression in the neointimal layer in this mouse model (see Supplementary material online, *Figure S2*). However, further detailed co-localization studies are needed to definitively demonstrate the cell types responsible for miR-21 expression.

In situ hybridization for miR-21 in surgically prepared HSV segments demonstrated that miR-21 was expressed in medial and neointimal SMCs (*Figure 5*). Immunohistochemistry in sequential

serial sections demonstrated that miR-21 expression was localized in areas which stained positive for α-actin (*Figure 5J–L*).

Thus, in concordance with the qRT-PCR analysis, we observed elevated levels of miR-21 in all three models of vein grafting, with the expression localized to multiple regions of the graft.

Effect of genetic loss of miR-21 on neointimal formation in vein grafts

In order to address whether the elevation of miR-21 plays an important role in the development of vein graft neointimal formation, we performed isogenic vein grafting in miR-21 knockout mice and wild-type controls. At 28 days post-engraftment, the neointimal area was dramatically reduced by 81% in miR-21 knockout mice compared with wild-type controls ($P < 0.001$, $n = 7–10$/group) (*Figure 6A* and *B*). Additionally, neointimal lesions in miR-21 knockout mice had significantly lower SMC content than controls (see Supplementary material online, *Figure S3*).

In this mouse model, it has previously been demonstrated that cells from both the donor vessel and recipient contribute to the progression of neointima formation.[28] To determine whether ablation of miR-21 in the donor vessel is sufficient to prevent neointima formation, donor veins from miR-21 knockout mice were engrafted into wild-type mice. Neointimal size was significantly reduced compared with wild-type grafts in wild-type mice (*Figure 6A*), suggesting that miR-21 expression in the engrafted vessel is critical to neointima formation.

Pharmacological knockdown of miR-21 expression in cultured HSV

To investigate the translational application of our findings and evaluate whether it is possible to achieve pharmacological knockdown of miR-21 in HSVs, the *ex vivo* culture model was utilized. Culturing HSV in the presence of anti-miR-21 for 7 or 14 days resulted in a >95% knockdown in miR-21 expression (*Figure 7A*). The expression of previously identified miR-21 target genes[25,29–31] was analysed by qRT-PCR. Compared with anti-miR-ctl-treated HSVs, anti-miR-21 treatment caused significant de-repression of STAT3, PTEN, and BMPR2 at 14 days (*Figure 7B*). However, PDCD-4 and TIMP3 expression were not significantly altered (*Figure 7B*). Neointima formation was significantly reduced in anti-miR-21-treated vessels (*Figure 7C* and *D*).

Owing to the limited availability of intact human SVs, we performed further experiments with anti-miR-control and anti-miR-21 in primary SV-derived EC and SMCs. In these experiments, we

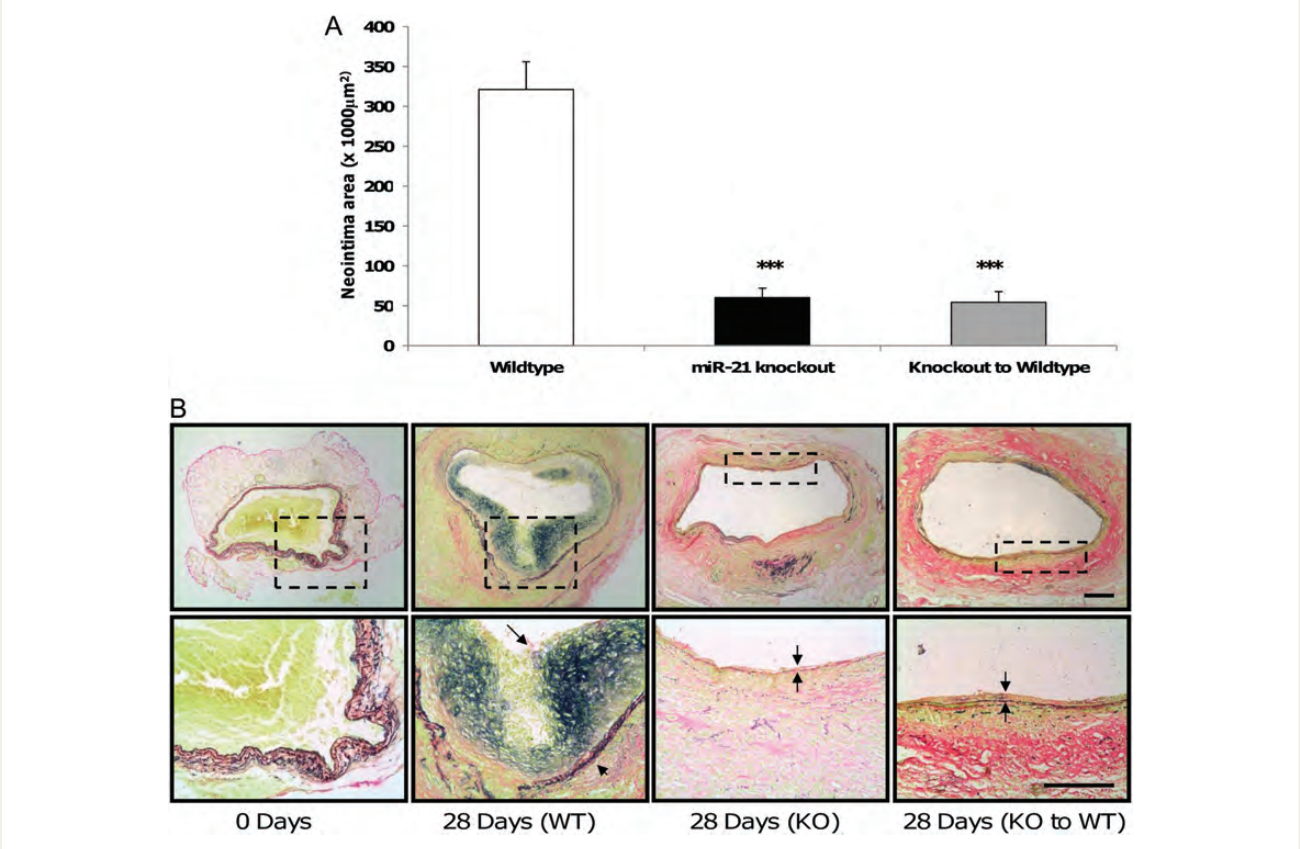


Figure 6 Effect of miR-21 ablation on neointimal formation in a mouse model of vein grafting. (*A*) The neointimal area 28 days post-grafting in wild-type mice, miR-21 knockouts, and miR-21 knockout veins engrafted into wild-type mice. (*B*) Vessel wall thickness in grafts from wild-type and knockout mice at 0 and 28 days; sections are stained with elastin van Gieson. Arrows indicate the neointimal layer. $n = 5–10$, ***$P < 0.001$ vs. wild-type. Scale bar represents 200 μm, applicable to all panels.

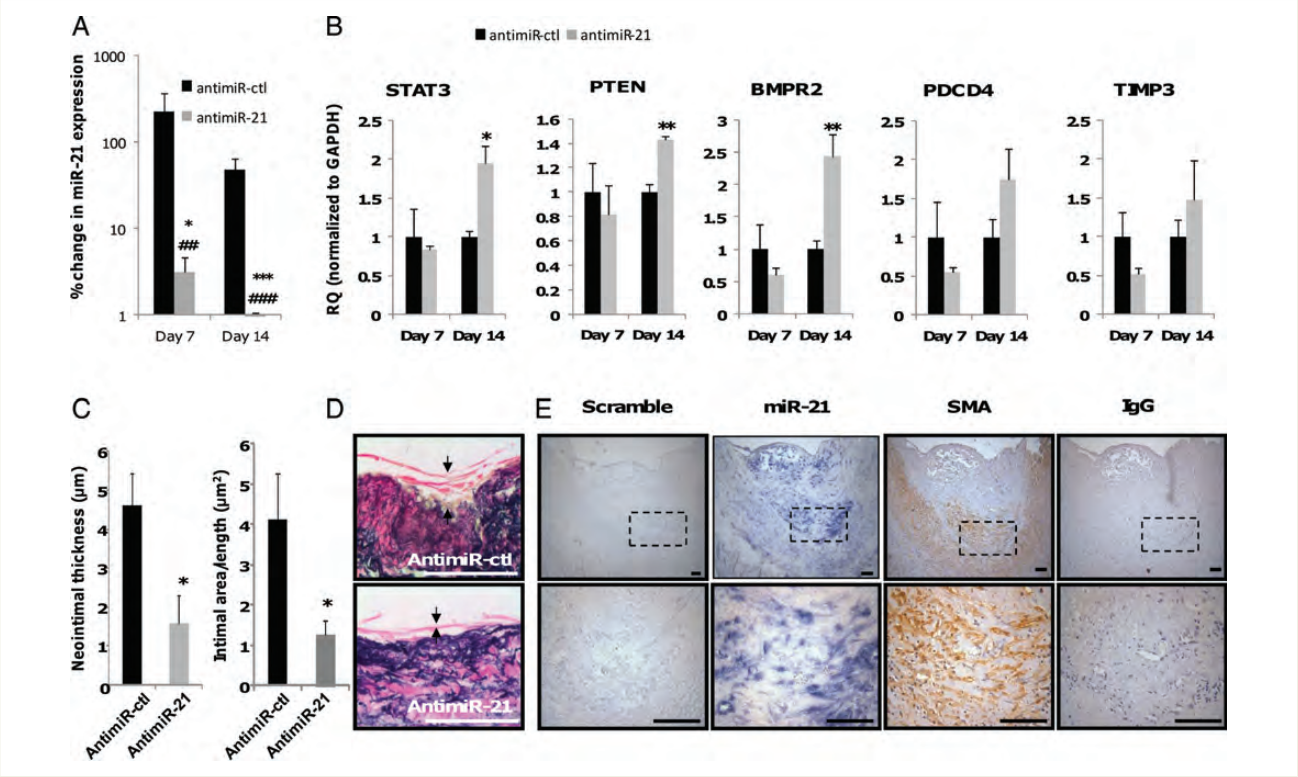


Figure 7 Effect of pharmacological knockdown of miR-21 in HSVs and miR-21 expression in failed human vein grafts. HSV segments were cultured for 7 and 14 days in the presence of 5000 nM anti-miR-ctl or anti-miR-21 ($n = 5$/group), then RNA was isolated from the vessels, and expression was measured by quantitative real-time PCR. (*A*) miR-21 expression. (*B*) Expression of putative miR-21 target genes. (*C*) Neointimal thickness. (*D*) Representative images of elastic van Gieson-stained sections from Day 14 samples. (*E*) *In situ* hybridization with a scrambled microRNA probe, miR-21 probe, and immunohistochemistry for smooth muscle cell actin in a failed human vein graft. Scale bar represents 100 μm, applicable to all panels. $^{*}P < 0.05$ vs. anti-miR-ctl; $^{**}P < 0.01$ vs. anti-miR-ctl, $^{***}P < 0.001$ vs. anti-miR-ctl, $^{\#\#}P < 0.01$ vs. Day 0, $^{\#\#\#}P < 0.001$ vs. Day 0.

aimed to identify any off-target effects of the anti-miR-control and anti-miR-21 treatments; hence, we transfected these cells with anti-miRs at a range of doses and analysed miR-21 expression compared with mock-transfected cells 72 h post-transfection. These data demonstrated that anti-miR-21, but not anti-miR-control transfection produced a substantial knockdown of miR-21 levels (see Supplementary material online, *Figure S4A* and *B*). We also noted that there were small but sometimes significant variations in miR-21 levels in anti-miR-control samples, but these are small and are unlikely to impact experimental outcome (see Supplementary material online, *Figure S4A* and *B*). Taken together, these results suggest that anti-miR-21 treatment is a viable strategy to knockdown miR-21 levels in venous SMC pre-engraftment.

miR-21 expression in failed human vein grafts

In order to demonstrate the importance of miR-21 in the clinical setting of vein graft failure, we performed *in situ* hybridization for miR-21 in failed human vein grafts. The staining pattern in these veins is very similar to that seen in the mouse and porcine vein grafts, with miR-21 expressed in regions of the graft which also stain positive for SMC-actin (see *Figure 7E* and Supplementary material online, *Figure S5*).

Discussion

This study is the first to document miRNA dysregulation in the context of vein graft neointima formation. It is also the first to demonstrate a functional role for miR-21 in neointimal formation following vein grafting. Our miRNA profiling identified 21 miRNAs which were significantly up-regulated in porcine vein grafts 7 and 28 days post-engraftment. We validated this sustained up-regulation of miR-21 expression in these porcine vein grafts and relevant murine and human models of vein graft neointimal formation by qRT-PCR. Our *in situ* hybridization studies demonstrated that miR-21 was abundantly expressed in the neointimal layer of the venous wall following engraftment in porcine and murine models and failed human grafts. Furthermore, our *ex vivo* HSV model confirmed this up-regulation of miR-21 levels in cells within the neointimal layer. These studies suggested that miR-21 might play a pathological role during neointimal formation in the setting of vein graft failure; hence, we performed vein grafting in miR-21-ablated mice. The absence of miR-21 in these mice substantially

attenuated neointimal formation and SMC accumulation in intimal lesions. Furthermore, engraftment of veins from miR-21 mice into wild-type mice resulted in the attenuation of neointima formation, suggesting that miR-21 expression in the engrafted vessel is critical to neointimal development. This has important implications for the development of miR-21 therapeutics, as it suggests that localized treatment to knockdown miR-21 expression in the vein may be sufficient for therapeutic efficacy.

In HSV samples treated with anti-miR-21, we analysed changes in gene expression of several previously proposed targets of miR-21. PTEN is an established target known to be expressed in fibroblasts, EC, and SMCs, where it regulates cell survival/apoptosis and is thought to have a key role in many cardiovascular diseases.[32] In agreement with previous studies,[14,26,33] we demonstrated an increase in PTEN expression in treated vessels. We also found significant de-repression of STAT3 and BMPR2, which have been shown to be direct targets of miR-21 in mesenchymal stem cells[31] and pulmonary vascular SMCs,[34] respectively. In wild-type mice, we found miR-21 expression localized to regions of the neointima and adventitia where PCNAs, SMCs, and fibroblast markers are also expressed. This suggests that the increase in miR-21 expression may contribute to the progression of neointima formation by promoting SMC and fibroblast proliferation and cell survival.

This is consistent with previous reports showing that knockdown of miR-21 levels in arterial SMCs and fibroblasts reduced rates of proliferation, migration, and neointimal formation in a rat balloon injury model, which was at least in part caused by de-repression of PTEN and BCL-2.[14,25,26] A subsequent study in a mouse model of abdominal aneurysm demonstrated that lentiviral-mediated over-expression of miR-21 reduced PTEN expression and increased SMC proliferation.[33] In the setting of vascular injury, these studies are important since over-expression and gene knockout studies demonstrate a role for PTEN in SMC proliferation and neointimal formation.[35,36] Moreover, previous studies suggested that PDCD-4 is down-regulated in the rat model of balloon injury, and over-expression with adenoviral vectors increased apoptosis and reduced SMC proliferation;[30] however, we did not find any significant change in PDCD-4 levels in our *ex vivo* HSV studies. There are also other reported targets of miR-21 which could play a role in vein graft failure. Recently, a study by Wang *et al.*[14] in atherosclerotic arteries demonstrated that miR-21 targeted tropomyosin-1, a protein implicated in the formation, stabilization, and regulation of cytoskeletal actin filaments. A change in tropomyosin may play a role in the reduced neointimal formation seen in the miR-21 knockout mouse vein graft study presented here, possibly via a reduction in SMC migration. Further detailed mechanistic studies are required to address the role of these targets in vein graft pathophysiology.

To investigate the translational application of our findings, we performed a pilot study to investigate the potential of knocking down miR-21 expression in intact human SVs. Although this *ex vivo* model has some limitations, such as the development of smaller neointimal lesions than those seen *in vivo* due to the lack of flow (shear stress) and infiltrating inflammatory cells, which contribute to neointimal formation *in vivo*, this model allows researchers to focus on the role of matrix remodelling, SMC proliferation, and migration. Using this model, we have demonstrated that it is possible to manipulate miRNA expression and observe changes in target gene expression in clinically relevant tissue samples.

Therapeutic potential

CABG provides an opportunity for the delivery of agents that can modulate the pathophysiology of vein graft neointima formation. In this study, we suggest that manipulation of miRNA may be one such therapeutic strategy. The manipulation of miR-21 levels in the venous wall at the time of engraftment through *ex vivo* treatment of the harvested vein could lead to localized manipulation of miRNA. This would negate the need for systemic delivery, which may result in the knockdown of miR-21 in other tissues, potentially creating off-target safety issues. We have demonstrated that anti-miRs can be used to knockdown miR-expression in HSV and that the level of inhibition achieved was sufficient to mediate target de-repression and reduce neointima formation. However, owing to the short exposure time available for *ex vivo* manipulation of the vein in the clinical setting (up to 30 min), it will be necessary to develop more efficient methods of inhibiting miR-21 in intact veins. A highly efficient delivery system is required to provide rapid and efficient uptake into the venous wall. Previous pre-clinical studies which have utilized decoy oligonucleotides directed against E2F showed efficient manipulation of the target and therapeutic efficacy when delivered to the vein under pressure,[37,38] although clinical trials failed to show efficacy.[39] It is well documented that viral vectors, specifically adenovirus, are efficient vein graft delivery vectors;[40] hence, the design of viral approaches for efficient delivery is a further option.

In summary, our study demonstrates that a number of miRNAs are modulated by the process of vein grafting and that miR-21 is significantly up-regulated in the neointimal layer of veins following engraftment. Further, genetic deletion of miR-21 substantially reduced neointimal formation and SMC accumulation within these lesions. This suggests that modulation of miR-21 level has therapeutic potential in the setting of vein graft failure.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: E.v.R. is co-founder and former employee of MiRagen Therapeutics. She is now Associate Professor at Hubrecht Institute, University Medical Center Utrecht.

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