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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. 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, yet autologous veins remain an important, convenient, and frequently used conduit for surgical revascularization. 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...
suggesting that localized inhibition of miR-21 may provide a novel model, we show that de-repression of several target genes is necessary veins (HSVs) were exposed to anti-miR-21 to investigate the translational application of these findings, human saphenous veins (SVs) were 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

### Methods

#### Vein graft models

Several established models of vein grafting were utilized in this study. We utilized two mouse models of vein grafting: interpositional grafting8 was used for miR-21 profiling, and the isogenic mouse vein graft model9 was used for all other experiments. Porcine SV-carotid interpositional grafting10 and ex vivo culture of human SV segments11 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 DataAssistTM 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 rate24 (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
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,
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
next performed immunohistochemistry for the fibroblast markers vimentin and FSP-1. 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. 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 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

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**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.
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

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**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.
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. 4,23,26 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. 35 In the setting of vascular injury, these studies are important since over-expression and gene knock-out 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.

**References**

Dysregulation of miRNA-21 in response to vein grafting


