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MicroRNA-33 protects against neointimal hyperplasia induced by arterial mechanical stretch in the grafted vein

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Aims Mechanical factors play significant roles in neointimal hyperplasia after vein grafting, but the mechanisms are not fully understood. Here, we investigated the roles of microRNA-33 (miR-33) in neointimal hyperplasia induced by arterial mechanical stretch after vein grafting.

Methods and results Grafted veins were generated by the 'cuff' technique. Neointimal hyperplasia and cell proliferation was significantly increased, and miR-33 expression was decreased after 1-, 2-, and 4-week grafts. In contrast, the expression of bone morphogenetic protein 3 (BMP3), which is a putative target of miR-33, and the phosphorylation of smad2 and smad5, which are potential downstream targets of BMP3, were increased in the grafted veins. miR-33 mimics/inhibitor and dual luciferase reporter assay confirmed the interaction of miR-33 and BMP3. miR-33 mimics attenuated, while miR-33 inhibitor accelerated, proliferation of venous smooth muscle cells (SMCs). Moreover, recombinant BMP3 increased SMC proliferation and P-smad2 and P-smad5 levels, whereas BMP3-directed siRNAs had the opposite effect. Then, venous SMCs were exposed to a 10%-1.25 Hz cyclic stretch (arterial stretch) by using the FX4000 cyclic stretch loading system *in vitro* to mimic arterial mechanical conditions. The arterial stretch increased venous SMC proliferation and repressed miR-33 expression, but enhanced BMP3 expression and smad2 and smad5 phosphorylation. Furthermore, perivascular multi-point injection *in vivo* demonstrated that agomiR-33 not only attenuates BMP3 expression and smad2 and smad5 phosphorylation, but also slows neointimal formation and cell proliferation in grafted veins. These effects of agomiR-33 on grafted veins could be reversed by local injection of BMP3 lentivirus.

Conclusion The miR-33-BMP3-smad signalling pathway protects against venous SMC proliferation in response to the arterial stretch. miR-33 is a target that attenuates neointimal hyperplasia in grafted vessels and may have potential clinical applications.

Keywords Vein graft • Neointimal hyperplasia • Mechanical stretch • MicroRNAs • Smooth muscle cells

1. Introduction

A coronary artery bypass graft is an economical clinical revascularization strategy for patients with 3-vessel or left main coronary artery disease.[1] Autologous veins are an important, convenient, and frequently used conduit for surgical revascularization.[2] A newly grafted vein undergoes remarkable changes in the mechanical environment, and these are attributed to higher flow velocities in the arterialized circulation.[3] It has been shown that mechanical cyclic stretch, which is defined as the repetitive deformation of cells caused by the vessel wall rhythmically distending and relaxing with blood pressure, plays important roles in neointima formation and the concomitant luminal obliteration of vein grafts, which are crucial pathological factors in bypass graft failure.[4]

Arterial cyclic stretch changes the functions of smooth muscle cells (SMCs) in grafted veins, including excessive proliferation and migration which mediates neointima formation during grafted vein remodelling. Kozai *et al.*[5] revealed that *in vitro* cyclic stretch promotes human saphenous vein SMC proliferation by inducing membrane accumulation of

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RhoA and phosphorylation of p42/p44 mitogen-activated protein kinase and Akt. Nuclear orphan receptor TR3 prevents cyclic stretch-induced proliferation in venous SMCs.[6] Recent research has shown that a specific microRNA (miRNA), miR-21, participates in neointimal formation, suggesting that miRNAs may represent a novel therapeutic target in vein graft failure.[7]

miRNAs are small, endogenous, non-coding RNA molecules that negatively regulate gene expression by targeting specific messenger RNAs and inducing their degradation or translational repression.[8,9] Although studies have revealed that miRNAs play crucial roles in SMC proliferation, which participates in the pathophysiological remodelling of vessel walls,[7,10,11] the molecular mechanisms by which mechanical stretch induces SMC proliferation in neointimal formation during vein grafting are still unclear.

miR-33, which is a novel miRNA, has been shown to regulate the proliferation of many different cells *in vivo* and *in vitro*. Recent studies have revealed that miR-33 is involved in human liver regeneration[12] and that miR-33a inhibition promotes the proliferation of the WM35 and WM451 cell lines.[13] miR-33 also controls haematopoietic stem cell self-renewal through p53, and targeting miR-33 may lead to the prevention and treatment of haematopoietic disorders.[14] miR-33 has also been proved to relate with cardiovascular diseases. miR-33 overexpression lead to decreases secretion of apolipoproteins and triacylglycerides (TAG) in primary hepatocytes.[15] miR-33 was suggested to be an early biomarkers for cholesterol levels in childhood,[16] and plays important role in regulation of high-density lipoprotein (HDL) metabolism and atherogenesis.[17] In addition, recent researches revealed that miR-33 is a mechanosensitive miRNA which promotes osteoblast differentiation by targeting Hmga2.[18] However, the effect of arterialized stretch on miR-33, and the roles of miR-33 in neointimal hyperplasia during vein grafts are still unknown.

Here, we examined whether miR-33 modulates SMC proliferation and analysed the potential roles of miR-33 in neointimal formation after vein grafting in response to arterial mechanical stretch and their underlying mechanisms. The present results may provide a novel therapeutic approach to prevent vein graft failure.

2. Methods

An expanded Methods section is available in the, see Supplementary ma terial online, *Supplementary Material*.

2.1 Vein graft procedure, micrON^{TM} miRNA agomiR, and antagomiR treatments, and morphometry

Animal experiments were performed conform to the recommendations in the 8th Edition of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH revised 2011), and also conform to the Animal Management Rules of China (Documentation 55, 2001, Ministry of Health, China). The animal study was approved by the Animal Research Committee of Shanghai Jiao Tong University.

Male Sprague-Dawley rats were anaesthetized by 2% isoflurane at 2 L/min oxygen flow using Isoflurane vaporizer (MATRX VIP 3000). Vein grafts were performed using the 'cuff' technique as described (see Supplementary material online, *Supplementary Material*).[3,19] miR-33 agomiR and antagomiR as well as their corresponding negative control (NC) (Shanghai GenePharma Co., Ltd) were delivered (see Supplementary material online, *Supplementary Material*) to grafted vein rats. Specimen retrieval was done following perfusion under terminal anaesthesia using pentobarbital (75 mg/kg) via intraperitoneal injection. Two hundred millilitres of 0.9% saline solution was perfused at a rate of 20 mL/min via the apex of heart. Image Pro-Plus 6.0 software was used to quantitative the area of neointimal hyperplasia in Elastin Van Gieson stained (see Supplementary material online, *Supplementary material*) sections, and pixel point was used to represent the intimal hyperplasia area.

2.2 Cyclic stretch application

Cells were plated on gelatin-coated flexible silicone bottom plates (Flexcell International, USA) at a density of 3×10^5 cells per well ($9.32\ cm^2$). Twenty-four hours after seeding, the cells were incubated with DMEM for 24 h to arrest growth and synchronize the cells. Venous SMCs were then subjected to cyclic stretch, which was produced by a computer-controlled vacuum (FX-4000T Strain Unit, Flexcell International, USA) as described previously.[20] The following mechanical parameters were applied:[3,21] (i) a stretch magnitude of 10% to mimic arterial mechanical conditions, (ii) a frequency of 1.25 Hz, and (iii) a duration of 1, 3, 6, 12, or 24 h, respectively. Venous SMCs cultured under the same conditions but with no mechanical stretch applied were used as the static control.

2.3 mRNA and protein expression

RT-PCR (qPCR) (see Supplementary material online, *Supplementary Material*) was used to detect miR-33 expression level in grafted veins and venous SMCs. Protein contents of miR-33 downstream molecules were determined by western blotting (see Supplementary material online, *Supplementary Material*).

2.4 Cell proliferation assay

Two methods were used to analyse cell proliferation, BrdU incorporation and CCK-8 incubation.

For the BrdU incorporation assay *in vitro*, Cell Proliferation ELISA, BrdU kit (Roche) was used and the experiments were performed according to the manufacturer's protocol (see Supplementary material online, *Supplementary Material*), ELISA plate reader (Bio-Rad 680) was used to detect cell proliferation level. For the BrdU immunostaining *in vitro*, venous SMCs were seeded onto 22 mm coverslips (50% confluent) and transfected with miR-33 mimics or inhibitor (see Supplementary material online, *Supplementary Material*), fluorescence microscope (Olympus IX71) was used to observe the results.

For the BrdU incorporation assay *in vivo*, BrdU (Sigma-Aldrich) dissolved in sterilized PBS was intraperitoneally injected (20 mg/100 g rat body weight). Twenty-four hours later, the grafted vein, contralateral jugular vein were all surgically removed. Then the vessel was plunged into freezing isopentane and frozen-sectioned to 6-µm sections. BrdU immunostaining was performed according to the protocol of *In Situ* Cell Proliferation Kit (Roche) (see Supplementary material online, *Supplementary Material*). A fluorescence microscope (Olympus IX71) was used to observe cell proliferation.

For the CCK-8 assay, venous SMCs were subjected to cyclic stretch, or treated with miR-33 mimics/inhibitor, recombinant BMP3, BMP3 specific siRNA. Two hours before the end of the experiment, the original liquid was removed and cells were washed with PBS. Then CCK-8 solution (Dojindo) diluted with DMEM (1:10) was added and incubated for 2 h. OD values (450 nm) were detected with an ELISA plate reader (Bio-Rad 680).

The OD values of venous SMCs treated with miR-33 mimics/inhibitor, recombinant BMP3, BMP3 specific siRNA, or cyclic stretch were normalized to that treated with the corresponding control.

2.5 Dual luciferase reporter assay

For luciferase reporter experiments, the whole 3'-untranslated region (3'-UTR, 147-bp, positioned at 2012–2158) was obtained by gene synthesis. The mutated segment (MUT) that prevented binding between miR-33 and the BMP3 3'-UTR (MUT 3'-UTR), was obtained by gene synthesis (see Supplementary material online, *Tables S1–S3*). The segment (3'-UTR or MUT 3'-UTR) was inserted downstream of the luciferase reporter gene (psiCHECK-2, Promega). To determine whether miR-33 specifically targeted BMP3 through its 3'-UTR, HEK-293T cells were transfected with the reporter plasmid or the mutated vectors with miR-33 mimics or NC. Twenty-four hours later, firefly and Renilla luciferase activities were measured consecutively using the dual luciferase reporter assay system (Promega).

2.6 Statistical analysis

Each experiment was performed at least in biological triplicate, and all values are expressed as mean ± *SD*. The Student *t*-test was used to compare two groups, and multiple comparisons among the groups were performed using one-way ANOVA and Student–Newman–Keuls (S–N–K) test as the post hoc comparisons. A value of $P < 0.05$ was regarded as statistically significant.

3. Results

3.1 Neointimal hyperplasia and cell proliferation after vein grafting

To address the progress of venous remodelling after graft surgery, Elastin-Van Gieson staining was used to detect the neointima in 1-, 2-, and 4-week grafted veins. Compared with the contralateral jugular vein (control), the neointimal hyperplasia after 1-, 2-, and 4-week graft surgery was significantly thickened (*Figure 1A*), and the area of neointimal hyperplasia in the venous lumen was markedly increased, especially in the 4-week group (*Figure 1B*).

Brdu *in situ* cell proliferation was then preformed to detect cell proliferation level in 1-, 2-, and 4-week grafted veins. Compared with the contralateral jugular vein (control), the proliferation rate of cells in grated vein was significantly increased after 1-, 2-, and 4-week surgery. The most obvious proliferation was showed in the 2-week group (*Figure 1C and D*).

These results suggested that in the grafted veins, cell proliferation rate is time dependent and the peak was happened around 2 weeks, while the neointimal hyperplasia was progressive aggravation. SMCs underwent excessive proliferation, which may mediate significant neointimal hyperplasia of jugular veins after exposure to arterial mechanical conditions, and cause luminal obliteration or even blockage of vein grafts.

3.2 miR-33 expression is decreased in grafted veins

Since miR-33 regulate the proliferation of many different cells *in vivo* and *in vitro*[12,13], the expression of miR-33 was assessed in grafted veins in order to demonstrate whether miR-33 is changed in the hyper-proliferated vein graft. Real time RT-PCR results revealed that the miR-33 expression was markedly decreased in the 1-, 2-, and 4-week grafted veins compared with the self-contralateral jugular vein (*Figure 1E*).

Because SMC proliferation plays crucial roles in neointimal formation in grafted veins, the effects of the change in miR-33 expression on venous SMC proliferation and the possible mechanisms were further elucidated.

3.3 miR-33 regulates venous SMC proliferation *in vitro*

BrdU incorporation and CCK-8 incubation were used to reveal the effect of miR-33 on proliferation of venous SMCs *in vitro*. Using miR-33 mimics or inhibitor transfection, the expression level of miR-33 in venous SMCs was significantly changed compared with the corresponding NC (see Supplementary material online, *Figure S1*). CCK-8 assay revealed that compared with the respective controls, proliferation of venous SMCs was significantly attenuated by rno-miR-33-specific mimics, but accelerated with a specific inhibitor *(Figure 2A*). BrdU incorporation confirmed the effect of miR-33 mimics/inhibitor on the proliferation of venous SMCs (*Figure 2B* and *C*).

3.4 miR-33 targets on BMP3

To identify possible miR-33 targets, three web-accessible databases, PicTar (http://www.pictar.org/), TargetScan (http://www.targetscan.org/), and miRanda (http://www.microrna.org) were searched. BMP3 was predicted to be a putative target of miR-33 by all of these three algorithms (*Figure 3A*). The sequence alignment between miR-33 and BMP3 3′-UTR is well conserved among different species (see Supplementary material online, *Figure S2*).

A dual luciferase reporter assay was used to validate the results of the algorithm prediction. *Figure 3B* showed that compare with the BMP3-3′-UTR (see Supplementary material online, *Table S3*) alone, there was no significant change in luciferase activity in the HEK-293T cells that were co-transfected with the NC and BMP3-3′-UTR. In contrast, co-transfecting miR-33 mimics with the BMP3-3′-UTR reduced luciferase activity. None transfection of the mutated BMP3-3′-UTR (see Supplementary material online, *Table S3*) alone or co-transfection with the NC or miR-33 mimics had significant effects on luciferase activity compared with the BMP3-3′-UTR alone.

Subsequently, interactions between miR-33 and BMP3 were studied by overexpression or knockdown of miR-33 in venous SMCs with miR-33-specific mimics or inhibitor treatments, respectively. Western results indicated that miR-33 mimics significantly reduced BMP3 expression (*Figure 3C and D*), whereas the inhibitor increased BMP3 levels compared with the respective NC (*Figure 3E and F*). BMP3 expression also had the opposite change in miR-33 expression in 1-, 2-, and 4-week grafted veins (*Figure 3G* and *H*).

These results demonstrated that BMP3 is a miR-33 target and is negatively regulated by miR-33.

3.5 BMP3 induces venous SMC proliferation *in vitro*

To demonstrate the effect of BMP3 on proliferation of venous SMCs, recombinant BMP3 and BMP3 specific siRNA were used. These experiments revealed that 100 (*Figure 4A*) and 200 ng/mL BMP3 significantly increased venous SMC proliferation, whereas10, 25, and 50 ng/mL had no significant effect detected by CCK-8 assay (see Supplementary material online, *Figure S3*). Using Brdu-Elisa assay, the effect of BMP3 on venous SMC proliferation was verified, and the result revealed that 100 ng/mL

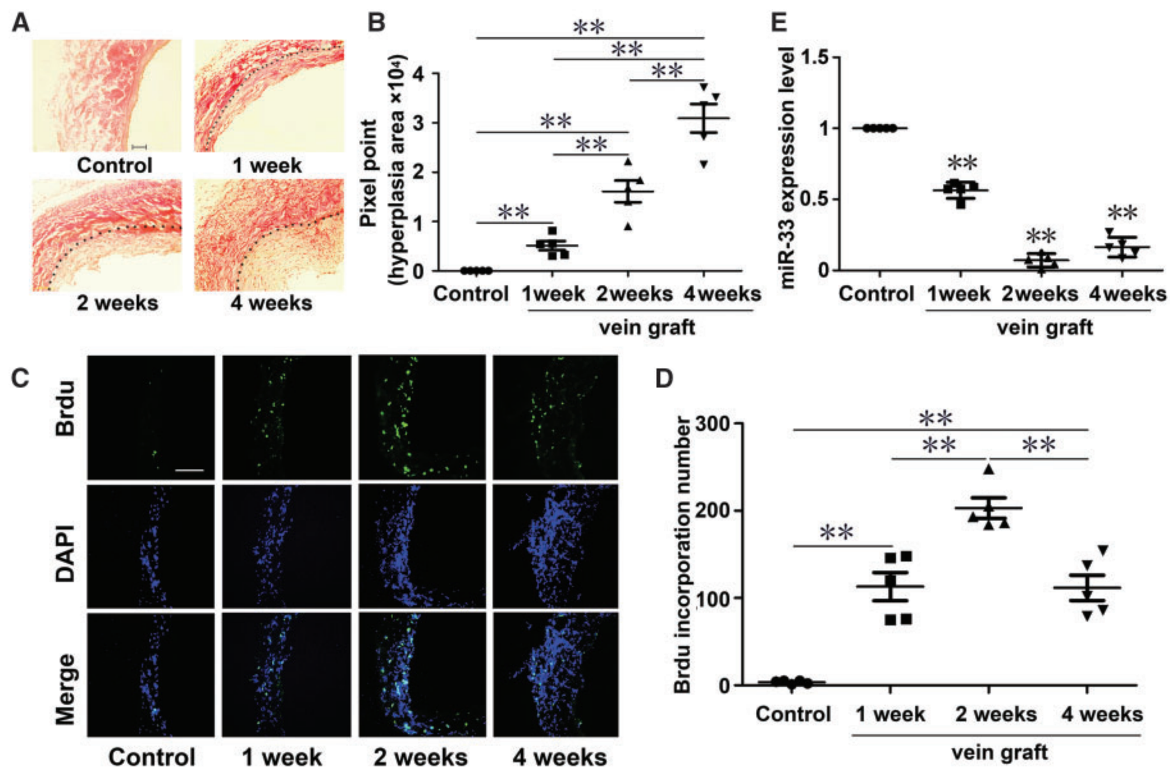

Figure 1 Neointimal hyperplasia, cell proliferation and miR-33 expression in grafted veins. (*A*) Elastin-Van Gieson staining revealed that the neointimal hyperplasia was significantly thickened after 1-, 2-, and 4-weeks graft surgery compared with the contralateral jugular vein (control). The extreme neointima was detected in the 4-week group (Bar = 20 µm). (*B*) The neointimal hyperplasia area, quantified with pixel point, was significantly increased after 1-, 2-, and 4-weeks graft surgery. (*C*) *In situ* BrdU proliferation assay revealed that cell proliferation was significantly increased in 1-, 2-, and 4-week grafted veins, especially in the 2-week group. Green is BrdU staining, and nuclei staining is shown in blue by 4, 6-diamidino-2-phenylindole [DAPI] (Bar = 100 µm). (*D*) The quantification of BrdU incorporated cells. (*E*) Real time RT-PCR results revealed that miR-33 expression (normalized to U6) was markedly decreased in grafted veins in the 1-, 2-, and 4-week groups compared with the self-contralateral jugular vein. Values were shown as the mean ± *SD*, $^{**}P < 0.01$ vs. control ($n = 5$).

BMP3 promoted venous SMC proliferation (*Figure 4B*). Moreover, BMP3 siRNAs decreased venous SMC proliferation which was detected by both CCK-8 and Brdu-Elisa assays (*Figure 4C* and *D*).

To determine whether miR-33 inhibits venous SMC proliferation via BMP3, two independent assays were performed. Recombinant BMP3 was added to cells transfected with miR-33 mimics, and exogenous BMP3 promoted venous SMC proliferation under miR-33-transfection (*Figure 4E* and *F*). Furthermore, miR-33 inhibitor and BMP3 specific siRNA were co-transfected. It revealed that compared with miR-33 inhibitor and siRNA NC group, the co-transfection of BMP3 siRNA reversed the up-regulated proliferation of miR-33 inhibitor in venous SMCs (*Figure 4G* and *H*).

Taken together, these results suggest that miR-33 targets on BMP3, which concentration-dependently induces venous SMC proliferation and may participate in neointimal formation in vein grafts.

3.6 BMP3 leads to the downstream phosphorylation of smad2 and smad5

BMP3 has been proved to promote proliferation in mesenchymal stem cells through the transforming growth factor β (TGF-β)/activin signalling pathway.[22] Hence, we analysed the phosphorylation of two important molecules, i.e. smad2 and smad5, in the TGF-β/activin signalling pathway.

Western blot results demonstrated that both smad2 and smad5 phosphorylation were significantly increased in 1-, 2-, and 4-week grafted veins (*Figure 3G* and *H*). miR-33 mimics significantly reduced P-smad2 and P-smad5 levels (*Figure 3C* and *D*), whereas miR-33 inhibitor increased P-smad2 and P-smad5 levels compared with the respective controls (*Figure 3E* and *F*). BMP3 recombinant proteins increased smad2 and smad5 phosphorylation following 3 and 6 h of treatment (*Figure 4I* and *J*); whereas BMP3 specific siRNAs significantly decreased mRNA and protein expression level of BMP3 (*Figure 4K* and *L*, see Supplementary material online, *Figure S4*), and repressed smad2 and smad5 phosphorylation (*Figure 4K* and *L*).

To confirm that miR-33-induced changes in smad2 and smad5 phosphorylation were dependent on BMP3, recombinant BMP3 was added to miR-33-transfected cells. It revealed that exogenous BMP3 promoted smad2 and smad5 phosphorylation in venous SMCs under miR-33-transfection (*Figure 4M* and *N*). miR-33 inhibitor and BMP3 specific siRNA co-transfection showed that compared with the miR-33 inhibitor and siRNA NC group, the phosphorylation of smad2 and smad5 were significantly decreased with co-transfection of BMP3 siRNA (*Figure 4O* and *P*).

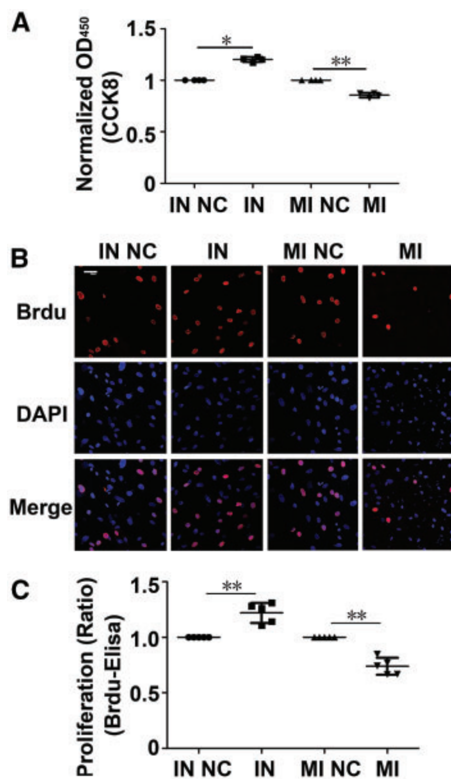

Figure 2 miR-33 regulates venous SMC proliferation *in vitro*. (*A*) CCK-8 assay revealed that rno-miR-33-specific mimics (MI) attenuated venous SMC proliferation compared with the respective negative control (NC), whereas specific inhibitor (IN) accelerated SMC proliferation. (*B*) BrdU incorporation confirmed that miR-33 mimics decreased venous SMC proliferation, whereas inhibitor increased venous SMC proliferation in compared with the respective control. Nuclei staining is shown in blue by DAPI; red is BrdU staining. (*C*) BrdU Elisa confirmed that miR-33 mimics decreased whereas inhibitor increased venous SMC proliferation in compared with the respective control. Values are shown as the mean ± *SD*, $*P < 0.05$ and $**P < 0.01$ vs. control ($n \geq 4$).

These results suggest that the effect of miR-33 on smad phosphorylation is dependent on BMP3 under static conditions.

3.7 Cyclic stretch regulates venous SMC proliferation *in vitro*

Because artery cyclic stretch induces proliferation of venous SMCs and plays important roles in neointimal formation in vein graft,[5] we detected whether miR-33 participates in venous SMC proliferation in response to mechanical cyclic stretch. *Figure 5A* and *B* showed that 10%-1.25Hz-cyclic stretch significantly promotes venous SMC proliferation. Compared with the static control, miR-33 expression was decreased after 1, 3, and 6 h of cyclic stretch (*Figure 5C*). In contrast, BMP3 expression was increased after 6, 12, and 24 h of cyclic stretch. Smad2 phosphorylation was elevated in 6- and 12-h groups, whereas smad5 phosphorylation was increased in 6-, 12-, and 24-h groups (*Figure 5D* and *E*).

miR-33 mimic transfection under cyclic stretch subjection was used to illustrate whether mechanical stretch modulates venous SMC proliferation and BMP3/smads signalling pathway via miR-33. It revealed that compared with the NC control miR-33 mimics decreased venous SMC proliferation (*Figure 5F* and *G*), and downregulated expression level of BMP3, P-smad2, and P-smad5 as well (*Figure 5H* and *I*).

To confirm whether the effect of cyclic stretch on venous SMC proliferation was dependent on BMP3, venous SMCs were transfected with BMP3 specific siRNA and then applied to 10%-1.25Hz-cyclic stretch. The results showed that BMP3 siRNA decreased venous SMC proliferation compared with NC under cyclic stretch condition (*Figure 5J* and *K*), and reduced P-smad2 and P-smad5 levels (*Figure 5L* and *M*). The effect of BMP3 specific siRNA was confirmed under both static and cyclic stretch conditions (see Supplementary material online, *Figure S5*).

These results demonstrate that arterial mechanical stretch (10%-cyclic-stretch) promotes venous SMC proliferation, which is consistent with previous *in vivo* studies, and suggests that the miR-33-BMP3-smad pathway participate in this process. To verify this pathway and detect the potential therapeutic role of miR-33 in neointimal formation, *in vivo* experiments were performed next.

3.8 agomiR-33 injection increases miR-33 expression, down-regulates the BMP3 pathway, and attenuates neointimal formation in grafted veins

Perivascular multi-point injection of agomiR-33 or antagomiR-33 was performed to detect the effect of miR-33 *in vivo*, and identify the potential therapeutic role of miR-33 in neointimal formation (*Figure 6A*). This experiment revealed that miR-33 expression was significantly up-regulated in grafted vessels by agomiR-33 injection compared with negative control injection on Day 28 (4 weeks) (*Figure 6B*).

Western blot results showed that agomiR-33 reduced BMP3 expression and smad2 and smad5 phosphorylation (*Figure 6C* and *D*). agomiR-33 also markedly attenuated neointimal formation (*Figure 6E* and *F*) on Day 28 (4 weeks) and repress cell proliferation on Day 14 (2 weeks) (*Figure 6G* and *H*) in grafted vessels. Compared with the negative control, multi-point injection of antagomiR-33 slightly, but not significantly, induced neointimal formation on Day 28 (4 weeks) and cell proliferaion in vein graft on Day 14 (2 weeks) (see Supplementary material online, *Figures S6* and *S7*).

BMP3 lentivirus was then transcutaneous injected around the grafted veins treated with agomiR-33 simultaneously. The results revealed that local injection of BMP3 lentivirus significantly increased the expression of BMP3, and increased neointimal hyperplasia areas, cell proliferation, and expression level of P-smad2 and P-smad5 (see Supplementary material online, *Figure S8*).

The results suggested that locally overexpression of miR-33 attenuates neointimal hyperplasia of grafted autologous vein. Therefore, miR-33 may be a potential therapeutic target in vein graft.

4. Discussion

Because blood pressure in the arterial system (80–120 mmHg) is much higher than that in the venous system (estimated to be 0–30 mmHg), the mechanical conditions are significantly different soon after a vein bypass graft.[3] The increased cyclic stretch caused by the arterialized blood pressure has been shown to play important roles in neointimal thickening.[3,4] Using an *in vivo* animal model and an *in vitro* cyclic stretch application system, we investigated the potential molecular mechanisms of neointimal

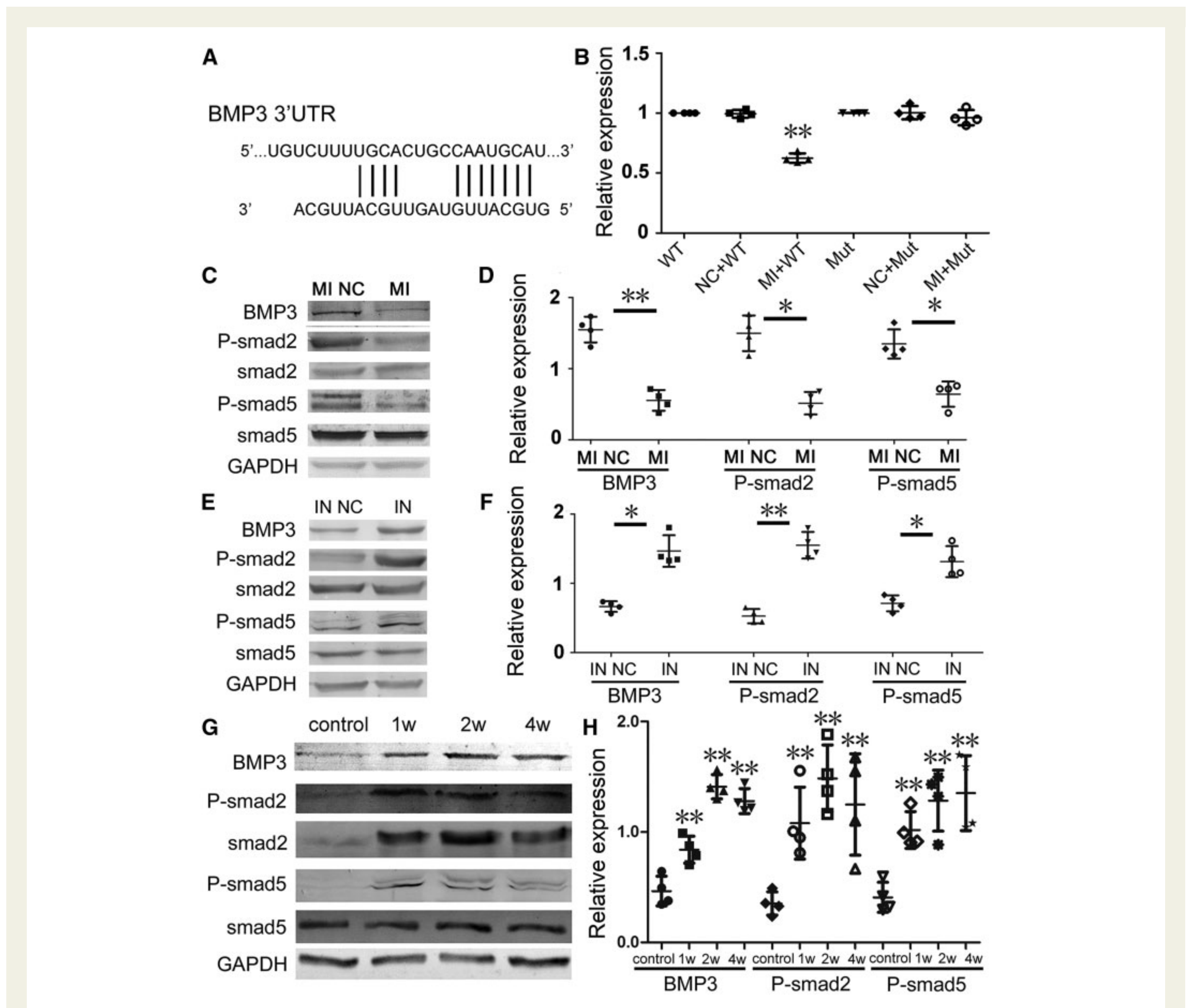

Figure 3 miR-33 targets BMP3. (*A*) Sequence alignment between rno-miR-33 and its putative binding sites in the rat BMP3 3'-UTR. (*B*) Luciferase activity in wild-type (WT) and mutant (Mut) BMP3 3′-UTRs in negative control (NC) and miR-33 mimics-treated HEK-293T cells. miR-33 significantly reduced luciferase levels of the BMP3-3'UTR luciferase reporter. (*C*, *D*) Western blot results indicated that in venous SMCs, miR-33 mimics (MI) greatly reduced BMP3 expression, as well as P-smad2 and P-smad5 compared with the NC. (*E*, *F*) In venous SMCs, miR-33 inhibitor (IN) increased BMP3, P-smad2, and P-smad5 expression compared with the NC. (*G*, *H*) BMP3 and smad2 expression and smad2 and smad5 phosphorylation revealed an opposite changes with miR-33 expression in 1-, 2-, and 4-week grafted veins. BMP3 expression was normalized to GAPDH; P-smads expression was normalized to the respective smads with each smad first normalized to its own loading control (GAPDH). P-smad2 in (*G*) was normalized to its own GAPDH because the expression of smad2 was also changed in the grafted vein compared to control. Values are shown as the mean ± *SD*, $^*P < 0.05$ and $^{**}P < 0.01$ ($n = 4$).

formation in response to arterialized mechanical stretch. Our study revealed that the miR-33-BMP3-smad signalling pathway modulates venous SMC proliferation, and that miR-33 may be a clinical target to prevent neointimal formation in vein grafts.

miR-33 embedded within the intronic sequences of sterol regulatory element-binding proteins (SREBPs) genes. Two isoforms of miR-33 exist in humans: miR-33b, which is present in intron 17 of the SREBP-1 gene on chromosome 17, and miR-33a, which is located in intron 16 of the SREBP-2 gene on chromosome 22. In rat, there is only one miR-33 isoform which is conserved with human miR-33a (located within intron 15 of the mouse SREBP-2 gene). Intron 17 in the mouse SREBP-1 gene lacks sequence homology to the human intronic sequences harbouring miR-33b.[9,23–26]

miR-33 has been proved to be involved in cholesterol efflux and lipid metabolism. Antagonism of miR-33 *in vivo* or genetic ablation of miR-33 results in a significant increase in circulating HDL cholesterol.[9,23,24] Similar results have also been shown in non-human primates treated with anti-miR-33 oligonucleotides.[27,28] Rotllan *et al.*[29] reported that miR-33 therapeutic silencing inhibits the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice (Ldlr-/-) mice, whereas *Marquart et al.*[30] showed that miR-33 had no effect on this process. Long-term miR-33 silencing increases circulating triglyceride levels and

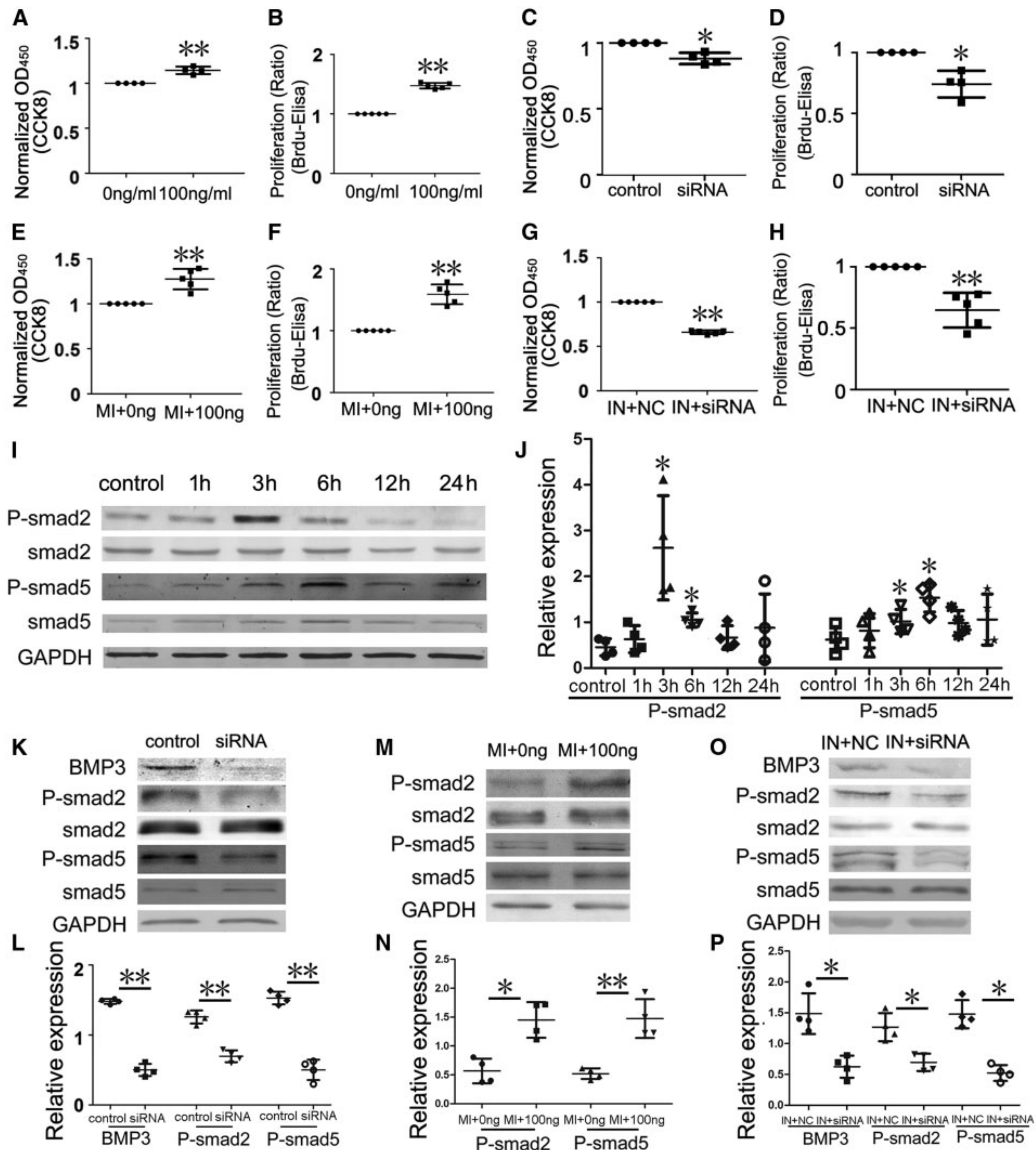

Figure 4 BMP3 promotes venous SMC proliferation and induces phosphorylation of smad2 and smad5. (*A*, *B*) Recombinant BMP3 (100 ng/mL) significantly increased venous SMC proliferation which was detected by both CCK-8 assay (A) and BrdU Elisa (*B*). (*C*, *D*) BMP3-specific siRNAs decreased venous SMC proliferation compared with the corresponding control, which was detected by CCK-8 assay (*C*) and Brdu-Elisa (*D*). (*E*, *F*) In venous SMC transfected with miR-33 mimics (MI), recombinant BMP3 (100 ng/mL) significantly increased cell proliferation. (*G*, *H*) In venous SMC transfected with miR-33 inhibitor (IN), BMP3 siRNA co-transfection repressed cell proliferation. (*I*, *J*) Western blot results indicated that recombinant BMP3 (100 ng/mL) increased P-smad2 and P-smad5 after treatment for 3 and 6 h. (*K*, *L*) BMP3-specific siRNAs significantly decreased the expression of BMP3 expression and P-smad2 and P-smad5. (*M*, *N*) Recombinant BMP3 promoted P-smad2 and P-smad5 expression under miR-33-transfection. (*O*, *P*) BMP3 siRNA, cotransfected with miR-33 inhibitor, repressed BMP3, P-smad2 and P-smad5 expression. BMP3 expression was normalized to GAPDH; P-smads expression was normalized to the respective smads with each smad first normalized to its own loading control (GAPDH). Values are shown as the mean ± *SD*, $*P < 0.05$ and $**P < 0.01$ vs. the control ($n \geq 4$).

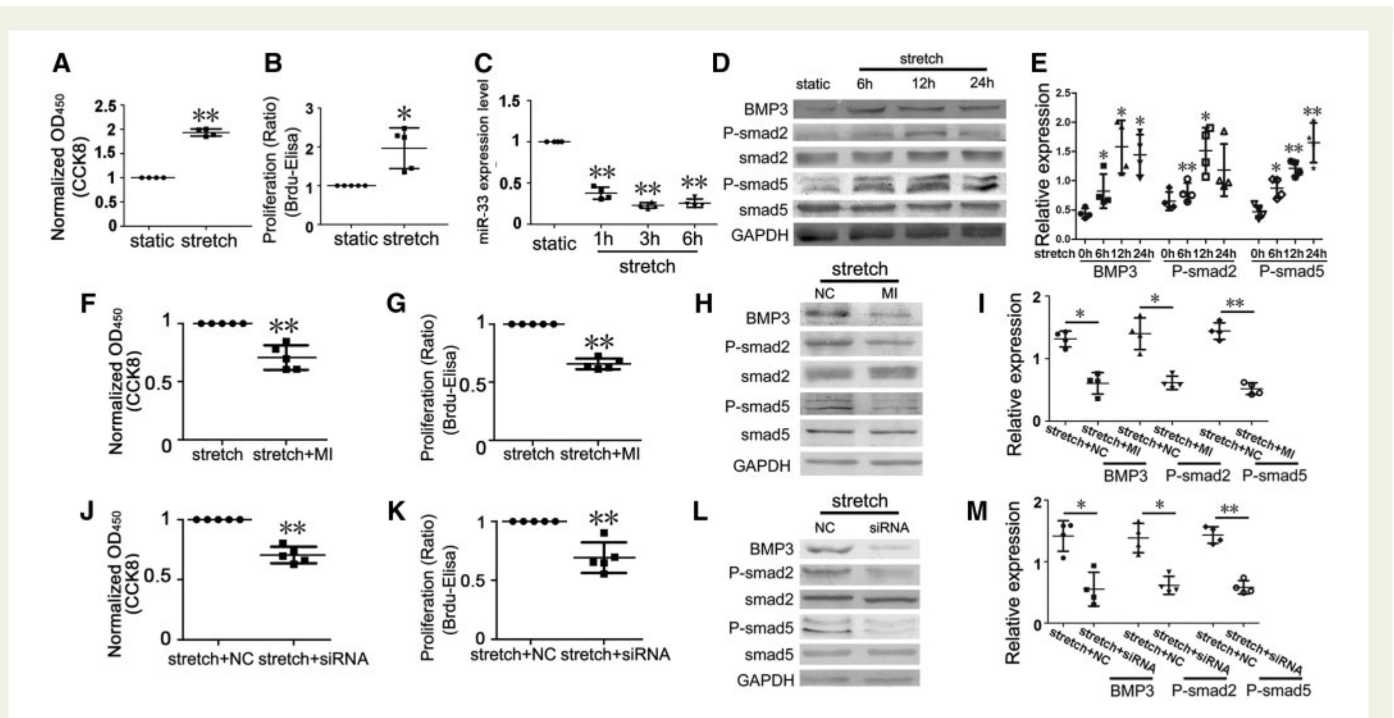

Figure 5 Cyclic stretch promotes venous SMC proliferation depended on miR-33. (*A*, *B*) 10%-1.25Hz-cyclic stretch significantly promoted venous SMC proliferation which was analysed by using CCK-8 incubation (*A*) and Brdu-Elisa assay (*B*). (*C*) miR-33 expression (normalized to U6) decreased after 1, 3, and 6 h of arterial stretch. (*D*, *E*) BMP3 expression increased after 6, 12, and 24 h of cyclic stretch. P-smad2 was elevated in the 6 and 12 h groups and P-smad5 was increased in the 6-, 12-, and 24-h groups. (*F*, *G*) miR-33 mimics (MI) decreased venous SMC proliferation under cyclic stretch. (*H*, *I*) Under cyclic stretch, miR-33 mimics decreased BMP3, P-smad2, and P-smad5 expression level. (*J*, *K*) Under cyclic stretch conditions, BMP3 siRNA decreased venous SMC proliferation compare to NC. (*L*, *M*) Under cyclic stretch conditions (applied for 24 h), BMP3 siRNA decreased P-smad2 and P-smad5 expression. BMP3 expression was normalized to GAPDH; P-smads expression was normalized to the respective smads with each smad first normalized to its own loading control (GAPDH). Values are shown as the mean ± *SD*, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the static control ($n \geq 4$).

hepatic lipid accumulation in mice.[31] In addition to cholesterol efflux and lipid metabolism, miR-33 is also relevant to human liver regeneration[12] and haematopoietic stem cell self-renewal.[14] Cirera-Salinas *et al.*[12] elucidated that endogenous miR-33 antagonism increases cell proliferation in Huh7 and A549 cells. Although these studies suggest that miR-33 is a key for multiple cell functions, the role of miR-33 in the vascular system is still unclear. Our present study revealed an anti-proliferative effect of miR-33 in venous SMCs. In addition, upregulation of miR-33 also decreases migration and increases apoptosis of venous SMCs, while knockdown of miR-33 reveals an opposite effect (see Supplementary material online, *Figure S9*). Therefore, miR-33 is an important molecule in venous SMCs.

miRNAs regulate cellular functions by targeting specific gene sequences. Previous studies have shown that miR-33 targets the adenosine triphosphate–binding cassette transporter A1 to regulate HDL *in vivo*.[24] Cyclin-dependent kinase 6 and cyclin D1 are also reported to be miR-33 targets and thereby participate in reducing cell proliferation and inhibiting cell cycle progression.[12] Here, we revealed a novel target of miR-33, BMP3, which is a member of the TGF-β superfamily.[4] Although BMP3 was originally proved to be a trophic factor for bone growth,[32] subsequent study revealed that BMP3 is an anti-osteogenic[33] and stimulates mesenchymal stem cell proliferation via the TGF-β/activin signalling pathway.[22] Our study revealed a pro-proliferation role for BMP3 in vein grafts *in vivo*, as well as under cyclic stretch *in vitro*, suggesting that BMP3 is a mechanosensitive molecule. The other BMP family members and the BMP receptor are also reported to be mechanosensitive and closely related to cardiovascular disease.[34–36]

Sorescu *et al.*[35] indicated that BMP4 is a mechanosensitive and inflammatory factor that plays a critical role in the early steps of atherogenesis in lesion-prone areas. Chiu *et al.*[34] demonstrated that BMP receptor -smad1/5 can be force-specifically activated by oscillatory shear stress in endothelial cells to cause cell cycle progression and to participate in atherosclerotic progression. Additionally, BMP ligand is essential for the activation of BMP receptor in vascular SMCs, although not necessarily in endothelial cells.[34,36] The present results revealed two downstream molecules of BMP3, i.e. P-smad2 and P-smad5, whose expression level were changed consistently with BMP3 both *in vivo* and *in vitro*. Interestingly, aside from its phosphorylation, smad2 expression was also elevated in grafted veins which suggested that there may be other molecules involved in smad2 regulation *in vivo*.

Additionally, in our present research, BMP3 recombinant protein and siRNA transfection revealed a minor effect on SMC proliferation than that of miR-33 mimics and inhibitor. Thus the influence of miR-33 in SMC proliferation may be exerted through other targets besides BMP3. For example, the known target of miR-33, CDK6 is proved to affect cell proliferation, cell cycle progression and relevant to human liver regeneration under miR-33 regulation.[12] CDK6 were also upregulated in jugular vein grafted for 1-, 2-, and 4-week groups (see Supplementary material online, *Figure S10*). Since our present research revealed that miR-33 plays crucial role in neointimal hyperplasia in vein graft, the multi-target effect of miR-33 and the potential signalling networks in mechanical stretch-induced proliferation of venous SMCs will be studied in the future.

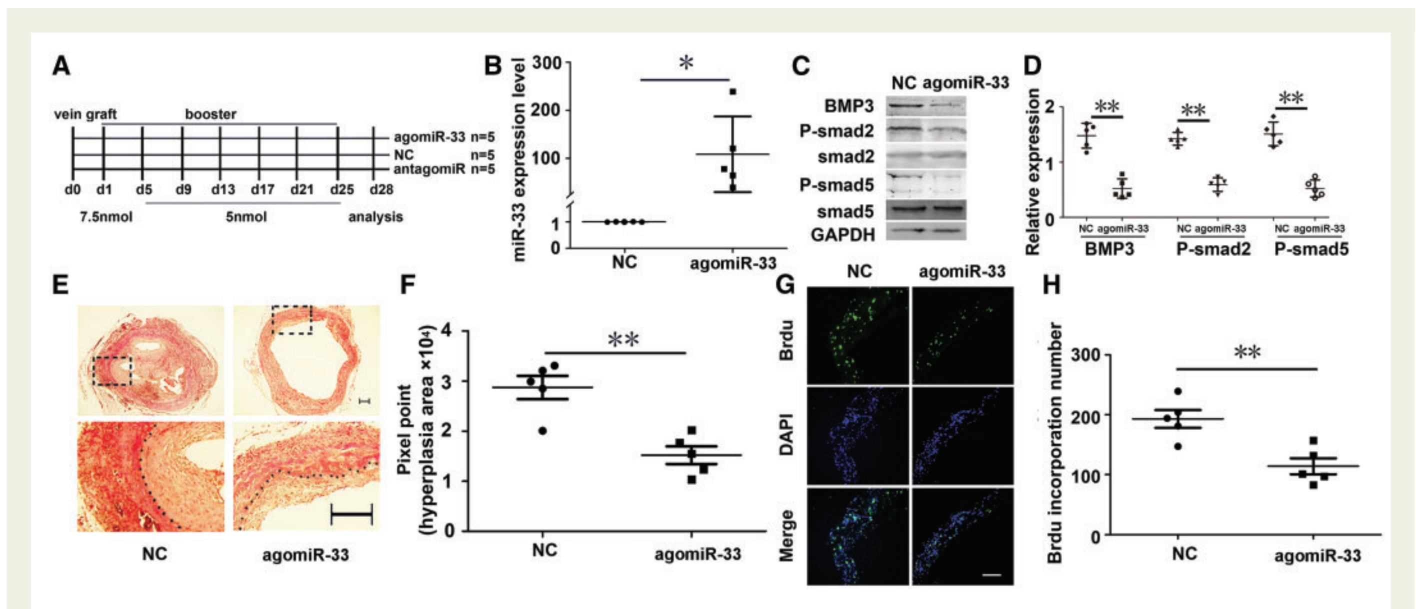

Figure 6 agomiR-33 attenuates neointimal formation in grafted vessels. (*A*) Perivascular multi-point injection of agomiR-33 or antagomiR-33 was performed in vein grafted rats. (*B*) agomiR-33 injection significantly up-regulated miR-33 expression (normalized to U6) in the grafted veins compared with the negative control injection on Day 28. (*C*, *D*) Western blot results showed that agomiR-33 reduced BMP3, P-smad2, and P-smad5 expression. (*E*) agomiR-33 significantly attenuated neointimal formation in grafted vessels on Day 28. (Bar = 30 µm). (*F*) The neointimal hyperplasia area was quantified with pixel point. (*G*) agomiR-33 markedly repressed cell proliferation in vein graft on Day 14. Green is BrdU staining, and nuclei staining is shown in blue by DAPI (Bar = 100 µm). (*H*) The quantification of BrdU incorporated cell. BMP3 expression was normalized to GAPDH; P-smads expression was normalized to the respective smads with each smad first normalized to its own loading control (GAPDH). Values are shown as the mean ± *SD*, $^*P < 0.05$ and $^{**}P < 0.01$ vs. the control ($n = 5$).

In conclusion, the miR-33-BMP3-smad signalling pathway is involved in mechanotransduction, which protects against venous SMC proliferation in response to arterial cyclic stretch. miR-33 is a target for attenuating neointimal hyperplasia in the grafted vessels. Therefore, miR-33 may be a potential therapeutic target in autologous vein grafted surgery, and locally overexpression of miR-33 may attenuates neointimal hyperplasia of grafted human saphenous vein.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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References

1. Cohen DJ, Osnabrugge RL, Magnuson EA, Wang K, Li H, Chinnakondepalli K, Pinto D, Abdallah MS, Vilain KA, Morice MC, Dawkins KD, Kappetein AP, Mohr FW, Serruys PW. Cost-effectiveness of percutaneous coronary intervention with drug-eluting stents versus bypass surgery for patients with 3-vessel or left main coronary artery disease: final results from the Synergy Between Percutaneous Coronary Intervention With TAXUS and Cardiac Surgery (SYNTAX) trial. *Circulation* 2014;**130**:1146–1157.
2. Goldman S, Zadina K, Moritz T, Ovitt T, Sethi G, Copeland JG, Thottapurathu L, Krasnicka B, Ellis N, Anderson RJ, Henderson W. Long-term patency of saphenous vein and left internal mammary artery grafts after coronary artery bypass surgery: results from a Department of Veterans Affairs Cooperative Study. *J Am Coll Cardiol* 2004;**44**:2149–2156.
3. Cheng J, Du J. Mechanical stretch simulates proliferation of venous smooth muscle cells through activation of the insulin-like growth factor-1 receptor. *Arterioscler Thromb Vasc Biol* 2007;**27**:1744–1751.
4. Deuse T, Hua X, Wang D, Maegdefessel L, Heeren J, Scheja L, Bolanos JP, Rakovic A, Spin JM, Stubbendorff M, Ikeno F, Langer F, Zeller T, Schulte-Uentrop L, Stoehr A, Itagaki R, Haddad F, Eschenhagen T, Blankenberg S, Kiefmann R, Reichenspurner H, Velden J, Klein C, Yeung A, Robbins RC, Tsao PS, Schrepfer S. Dichloroacetate prevents restenosis in preclinical animal models of vessel injury. *Nature* 2014;**509**:641–644.
5. Kozai T, Eto M, Yang Z, Shimokawa H, Luscher TF. Statins prevent pulsatile stretch-induced proliferation of human saphenous vein smooth muscle cells via inhibition of Rho/Rho-kinase pathway. *Cardiovasc Res* 2005;**68**:475–482.
6. de Waard V, Arkenbout EK, Vos M, Mocking AI, Niessen HW, Stooker W, de Mol BA, Quax PH, Bakker EN, VanBavel E, Pannekoek H, de Vries CJ. TR3 nuclear orphan receptor prevents cyclic stretch-induced proliferation of venous smooth muscle cells. *Am J Pathol* 2006;**168**:2027–2035.
7. McDonald RA, White KM, Wu J, Cooley BC, Robertson KE, Halliday CA, McClure JD, Francis S, Lu R, Kennedy S, George SJ, Wan S, van Rooij E, Baker AH. miRNA-21 is dysregulated in response to vein grafting in multiple models and genetic ablation in mice attenuates neointima formation. *Eur Heart J* 2013;**34**:1636–1643.
8. Ambros V. The functions of animal microRNAs. *Nature* 2004;**431**:350–355.
9. Najafi-Shoushtari SH, Kristo F, Li Y, Shioda T, Cohen DE, Gerszten RE, Naar AM. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. *Science* 2010;**328**:1566–1569.
10. Brock M, Samillan VJ, Trenkmann M, Schwarzwald C, Ulrich S, Gay RE, Gassmann M, Ostergaard L, Gay S, Speich R, Huber LC. AntagomiR directed against miR-20a restores functional BMPR2 signalling and prevents vascular remodelling in hypoxia-induced pulmonary hypertension. *Eur Heart J* 2014;**35**:3203–3211.
11. Torella D, Iaconetti C, Catalucci D, Ellison GM, Leone A, Waring CD, Bochicchio A, Vicinanza C, Aquila I, Curcio A, Condorelli G, Indolfi C. MicroRNA-133 controls vascular smooth muscle cell phenotypic switch *in vitro* and vascular remodeling *in vivo*. *Circ Res* 2011;**109**:880–893.
12. Cirera-Salinas D, Pauta M, Allen RM, Salerno AG, Ramirez CM, Chamorro-Jorganes A, Wanschel AC, Lasuncion MA, Morales-Ruiz M, Suarez Y, Baldan A, Esplugues E, Fernandez-Hernando C. Mir-33 regulates cell proliferation and cell cycle progression. *Cell Cycle* 2012;**11**:922–933.

13. Zhou J, Xu D, Xie H, Tang J, Liu R, Li J, Wang S, Chen X, Su J, Zhou X, Xia K, He Q, Chen J, Xiong W, Cao P, Cao K. miR-33a functions as a tumor suppressor in melanoma by targeting HIF-1α. *Cancer Biol Ther* 2015;**16**:846–55.
14. Herrera-Merchan A, Cerrato C, Luengo G, Dominguez O, Piris MA, Serrano M, Gonzalez S. miR-33-mediated downregulation of p53 controls hematopoietic stem cell self-renewal. *Cell Cycle* 2010;**9**:3277–3285.
15. Allen R1, Marquart TJ, Jesse JJ, Baldán A.Control of very low-density lipoprotein secretion by N-ethylmaleimide-sensitive factor and miR-33. *Circ Res* 2014;**115**:10–22.
16. Martino F, Carlomosti F, Avitabile D, Persico L, Picozza M, Barillà F, Arca M, Montali A, Martino E, Zanoni C, Parrotto S, Magenta A. circulating miR-33a and miR-33b are up-regulated in familial hypercholesterolaemia in paediatric age. *Clin Sci (Lond)* 2015;**129**:963–972.
17. Canfrán-Duque A, Ramírez CM, Goedeke L, Lin CS, Fernández-Hernando C. MicroRNAs and HDL life cycle. *Cardiovasc Res* 2014;**103**:414–422.
18. Wang H, Sun Z, Wang Y, Hu Z, Zhou H, Zhang L, Hong B, Zhang S, Cao X. miR-33-5p, a novel mechanosensitive microRNA promotes osteoblast differentiation by targeting Hmga2. *Sci Rep* 2016;**6**:23170.
19. Zou Y, Dietrich H, Hu Y, Metzler B, Wick G, Xu Q. Mouse model of venous bypass graft arteriosclerosis. *Am J Pathol* 1998;**153**:1301–1310.
20. Qi YX, Qu MJ, Yan ZQ, Zhao D, Jiang XH, Shen BR, Jiang ZL. Cyclic strain modulates migration and proliferation of vascular smooth muscle cells via Rho-GDIalpha, Rac1, and p38 pathway. *J Cell Biochem* 2010;**109**:906–914.
21. Yao QP, Zhang P, Qi YX, Chen SG, Shen BR, Han Y, Yan ZQ, Jiang ZL. The role of SIRT6 in the differentiation of vascular smooth muscle cells in response to cyclic strain. *Int J Biochem Cell Biol* 2014;**49**:98–104.
22. Stewart A, Guan H, Yang K. BMP-3 Promotes Mesenchymal Stem Cell Proliferation Through the TGF-b/Activin Signaling Pathway. *J Cell Physiol* 2010;**223**:658–666.
23. Marquart TJ, Allen RM, Ory DS, Baldan A. miR-33 links SREBP-2 induction to repression of sterol transporters. *Proc Natl Acad Sci U S A* 2010;**107**:12228–12232.
24. Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, Tamehiro N, Fisher EA, Moore KJ, Fernandez-Hernando C. MiR-33 contributes to the regulation of cholesterol homeostasis. *Science* 2010;**328**:1570–1573.
25. Horie T, Ono K, Horiguchi M, Nishi H, Nakamura T, Nagao K, Kinoshita M, Kuwabara Y, Marusawa H, Iwanaga Y, Hasegawa K, Yokode M, Kimura T, Kita T. MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL *in vivo*. *Proc Natl Acad Sci U S A* 2010;**107**:17321–17326.
26. Näär AM. Anti-atherosclerosis or no anti-atherosclerosis that is the miR-33 question. *Arterioscler Thromb Vasc Biol* 2013;**33**:447–448.
27. Rayner KJ, Sheedy FJ, Esau CC, Hussain FN, Temel RE, Parathath S, van Gils JM, Rayner AJ, Chang AN, Suarez Y, Fernandez-Hernando C, Fisher EA, Moore KJ. Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. *J Clin Invest* 2011;**121**:2921–2931.
28. Rottiers V, Obad S, Petri A, McGarrah R, Lindholm MW, Black JC, Sinha S, Goody RJ, Lawrence MS, deLemos AS, Hansen HF, Whittaker S, Henry S, Brookes R, Najafi-Shoushtari SH, Chung RT, Whetstine JR, Gerszten RE, Kauppinen S, Näär AM. Pharmacological inhibition of a microRNA family in nonhuman primates by a seed-targeting 8-mer antimiR. *Sci Transl Med* 2013;**5**:212ra162.
29. Rotllan N, Ramirez CM, Aryal B, Esau CC, Fernandez-Hernando C. Therapeutic silencing of microRNA-33 inhibits the progression of atherosclerosis in Ldlr-/- mice–brief report. *Arterioscler Thromb Vasc Biol* 2013;**33**:1973–1977.
30. Marquart TJ, Wu J, Lusis AJ, Baldan A. Anti-miR-33 therapy does not alter the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 2013;**33**:455–458.
31. Goedeke L, Salerno A, Ramirez CM, Guo L, Allen RM, Yin X, Langley SR, Esau C, Wanschel A, Fisher EA, Suarez Y, Baldan A, Mayr M, Fernandez-Hernando C. Long-term therapeutic silencing of miR-33 increases circulating triglyceride levels and hepatic lipid accumulation in mice. *EMBO Mol Med* 2014;**6**:1133–1141.
32. Sampath TK, Muthukumaran N, Reddi AH. Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. *Proc Natl Acad Sci U S A* 1987;**84**:7109–7113.
33. Bahamonde ME, Lyons KM. BMP-3: To be or not to be a BMP. *J Bone Joint Surg Am* 2001;**83**:S56–S62.
34. Zhou J, Lee PL, Tsai CS, Lee CI, Yang TL, Chuang HS, Lin WW, Lin TE, Lim SH, Wei SY, Chen YL, Chien S, Chiu JJ. Force-specific activation of Smad1/5 regulates vascular endothelial cell cycle progression in response to disturbed flow. *Proc Natl Acad Sci U S A* 2012;**109**:7770–7775.
35. Sorescu GP, Sykes M, Weiss D, Platt MO, Saha A, Hwang J, Boyd N, Boo YC, Vega JD, Taylor WR, Jo H. Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress stimulates an inflammatory response. *J Biol Chem* 2003;**278**:31128–31135.
36. Zhou J, Lee PL, Lee CI, Wei SY, Lim SH, Lin TE, Chien S, Chiu JJ. BMP receptor-integrin interaction mediates responses of vascular endothelial Smad1/5 and proliferation to disturbed flow. *J Thromb Haemost* 2013;**11**:741–755.