

# 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.<sup>1</sup> Autologous veins are an important, convenient, and frequently used conduit for surgical revascularization.<sup>2</sup> A newly grafted vein undergoes remarkable changes in the mechanical environment, and these are attributed to higher flow velocities in the arterialized circulation.<sup>3</sup> 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.<sup>4</sup>

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.*<sup>5</sup> 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.<sup>6</sup> Recent research has shown that a specific

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  $\pm$  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 performed 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 grafted 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*<sup>12,13</sup>, the expression of miR-33 was assessed in grafted veins in order to demonstrate whether miR-33 is changed in the hyperproliferated 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*).

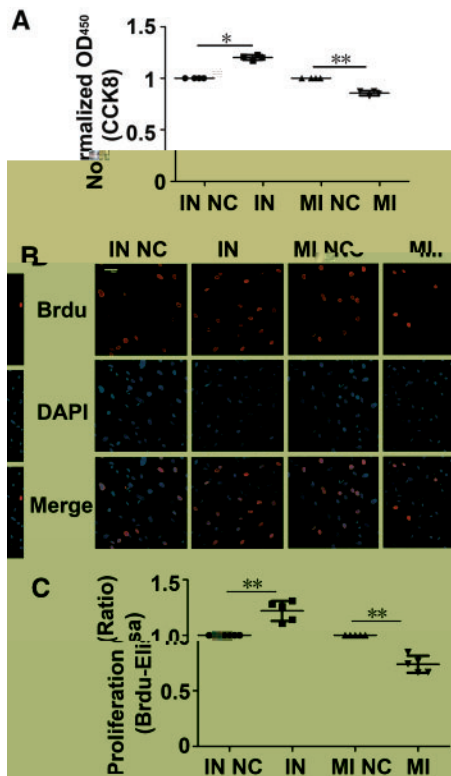
## 3.4 miR-33 targets on BMP3

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*).

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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



**Figure 2** miR-33 regulates venous SMC proliferation *in vitro*. (A) CCK-8 assay revealed that 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  $\pm$  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,<sup>5</sup> 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).

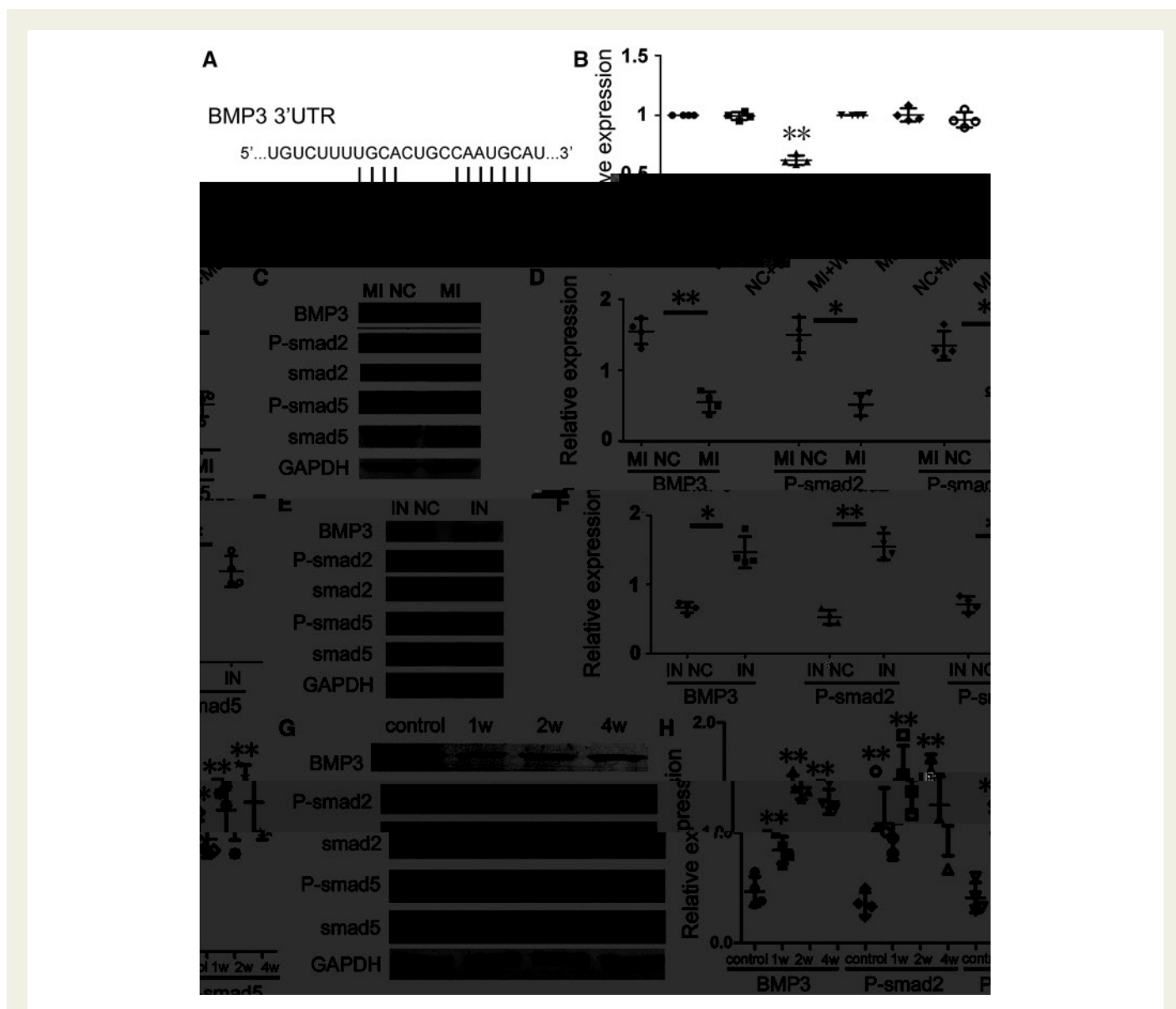
Western blot results showed that agomiR-33 reduced BMP3 expression and smad2 and smad5 phosphorylation (Figure 6C and D). agomiR-33 also markedly attenuated neointimal formation (Figure 6E and F) on Day 28 (4 weeks) and repress cell proliferation on Day 14 (2 weeks) (Figure 6G and H) in grafted vessels. 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 proliferation 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.<sup>3</sup> The increased cyclic stretch caused by the arterialized blood pressure has been shown to play important roles in neointimal thickening.<sup>3,4</sup> Using an *in vivo* animal model and an *in vitro* cyclic stretch application system, we investigated the potential molecular mechanisms of neointimal



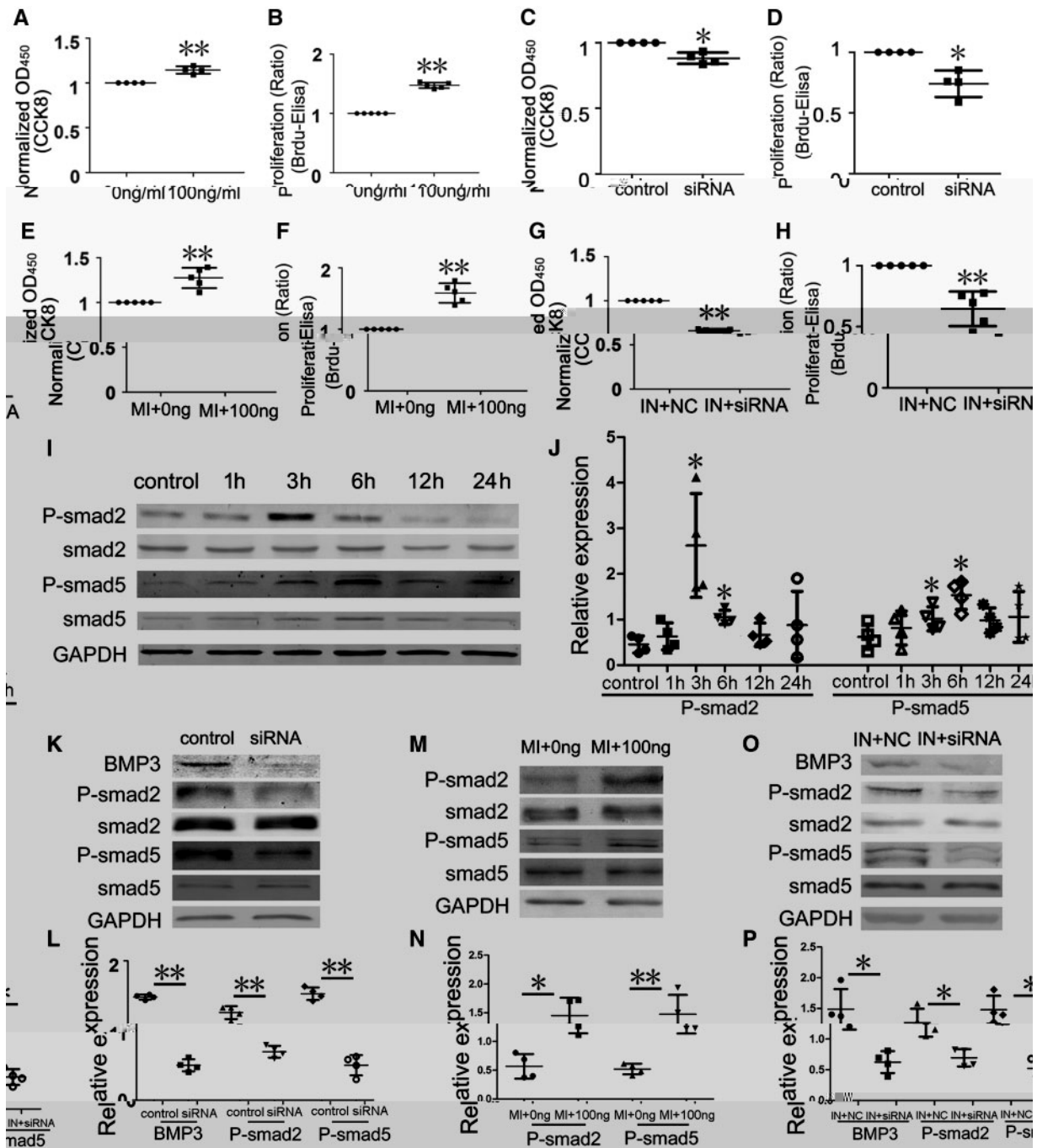
**Figure 3** miR-33 targets BMP3. (A) Sequence alignment between *miR-33* and its putative binding sites in the rat *BMP3* 3'-UTR. (B) Luciferase activity in wild-type (WT) and mutant (Mut) *BMP3* 3'-UTRs in negative control (NC) and *miR-33* mimics-treated HEK-293T cells. *miR-33* significantly reduced 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  $\pm$  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.

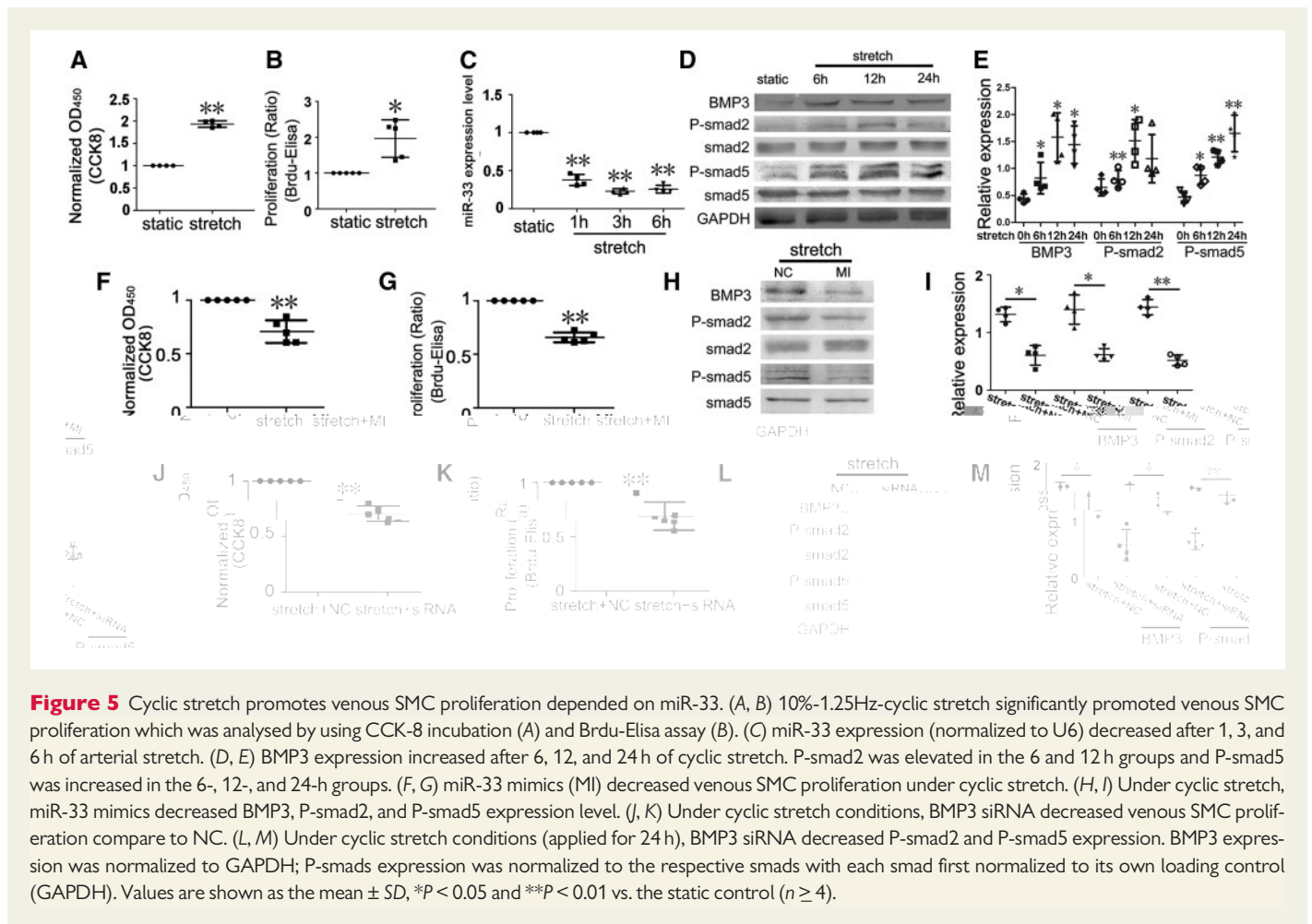
*miR-33* embedded within the intronic sequences of sterol regulatory element-binding proteins (SREBPs) genes. Two isoforms of *miR-33* exist in humans: *miR-33b*, which is present in intron 17 of the SREBP-1 gene on chromosome 17, and *miR-33a*, which is located in intron 16 of the SREBP-2 gene on chromosome 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*.<sup>9,23–26</sup>

*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.<sup>9,23,24</sup> Similar results have also been shown in non-human primates treated with anti-*miR-33* oligonucleotides.<sup>27,28</sup> Rotllan *et al.*<sup>29</sup> reported that *miR-33* therapeutic silencing inhibits the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice (*Ldlr*<sup>-/-</sup>) mice, whereas Marquart *et al.*<sup>30</sup> 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  $\pm$  SD, \* $P < 0.05$  and \*\* $P < 0.01$  vs. the control ( $n \geq 4$ ).



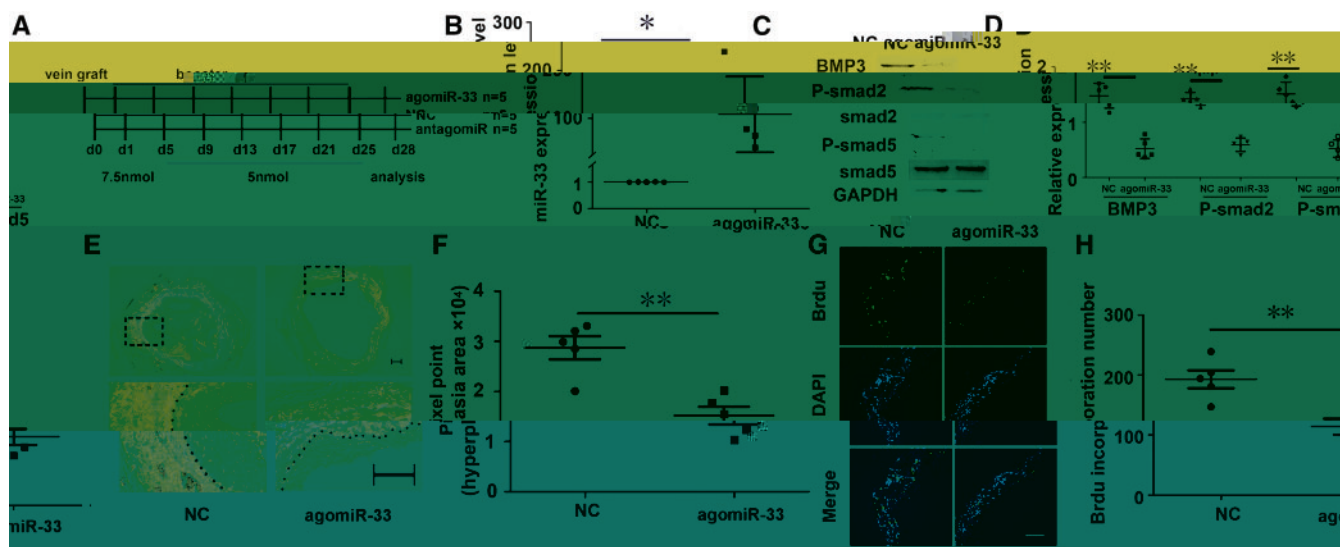
hepatic lipid accumulation in mice.<sup>31</sup> In addition to cholesterol efflux and lipid metabolism, miR-33 is also relevant to human liver regeneration<sup>12</sup> and haematopoietic stem cell self-renewal.<sup>14</sup> Cirera-Salinas *et al.*<sup>12</sup> elucidated that endogenous miR-33 antagonism 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*.<sup>24</sup> 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.<sup>12</sup> Here, we revealed a novel target of miR-33, BMP3, which is a member of the TGF- $\beta$  superfamily.<sup>4</sup> Although BMP3 was originally proved to be a trophic factor for bone growth,<sup>32</sup> subsequent study revealed that BMP3 is an anti-osteogenic<sup>33</sup> and stimulates mesenchymal stem cell proliferation via the TGF- $\beta$ /activin signalling pathway.<sup>22</sup> 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.<sup>34-36</sup>

Sorescu *et al.*<sup>35</sup> indicated that BMP4 is a mechanosensitive and inflammatory factor that plays a critical role in the early steps of atherosclerosis in lesion-prone areas. Chiu *et al.*<sup>34</sup> 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.<sup>34,36</sup> 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.<sup>12</sup> 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  $\mu$ m). (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  $\mu$ m). (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  $\pm$  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.

**Conflict of interest:** none declared.

## Funding

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