

## Original Article

# Atorvastatin regulates pericardial patch healing via the microRNA140-ADAM10-ephrinB2 pathway

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**Abstract:** *Background:* Pericardial patches are frequently used in vascular surgery to close arteriotomies. The early healing of these patches is mediated by attraction of CD34 and ephrinB2-positive cells. Atorvastatin is a commonly used statin drug that promotes healing of cardiovascular injury. We hypothesized that atorvastatin attracts ephrinB2-positive cells by regulating the microRNA140-ADAM10-ephrinB2 pathway during patch healing in the arterial environment. *Methods:* Pericardial patches were used to close an infra-renal aortic arteriotomy in Wistar rats (male, 200-400 g). Atorvastatin was given to rats at a daily dose of 0 mg, 2.5 mg, 5 mg or 10 mg. Patches were harvested at 1 or 4 weeks and analyzed by histology, immunohistochemistry, immunofluorescence, western blot and qPCR. *Result:* Animals treated with atorvastatin showed a higher number of infiltrating cells and a thicker patch neointima than the control animals. Furthermore, ADAM10 protein expression decreased ( $P<0.01$ ) and ephrinB2 expression increased ( $P<0.01$ ) in time- and atorvastatin dose-dependent manner. Similarly, ADAM10 mRNA expression decreased ( $P<0.01$ ), while the expression of ephrinB2 mRNA and miR-140 mRNA expression increased ( $P<0.01$ ;  $P<0.01$ ) in a time- and dose-dependent manner. *Conclusion:* Atorvastatin regulates neointimal growth after pericardial patch angioplasty; atorvastatin is associated with infiltration of ephrinB2-positive cells, diminished ADAM10 expression, and increased ephrinB2 and miR-140 expression. These results suggest new mechanisms for regulating neointimal formation after vascular procedures. *Clinical relevance:* This study may help physicians to know more healing mechanism after pericardial patch angioplasty. Further, it may reveal some mechanism that how atorvastatin play roles in endothelium repair of the cardiovascular system.

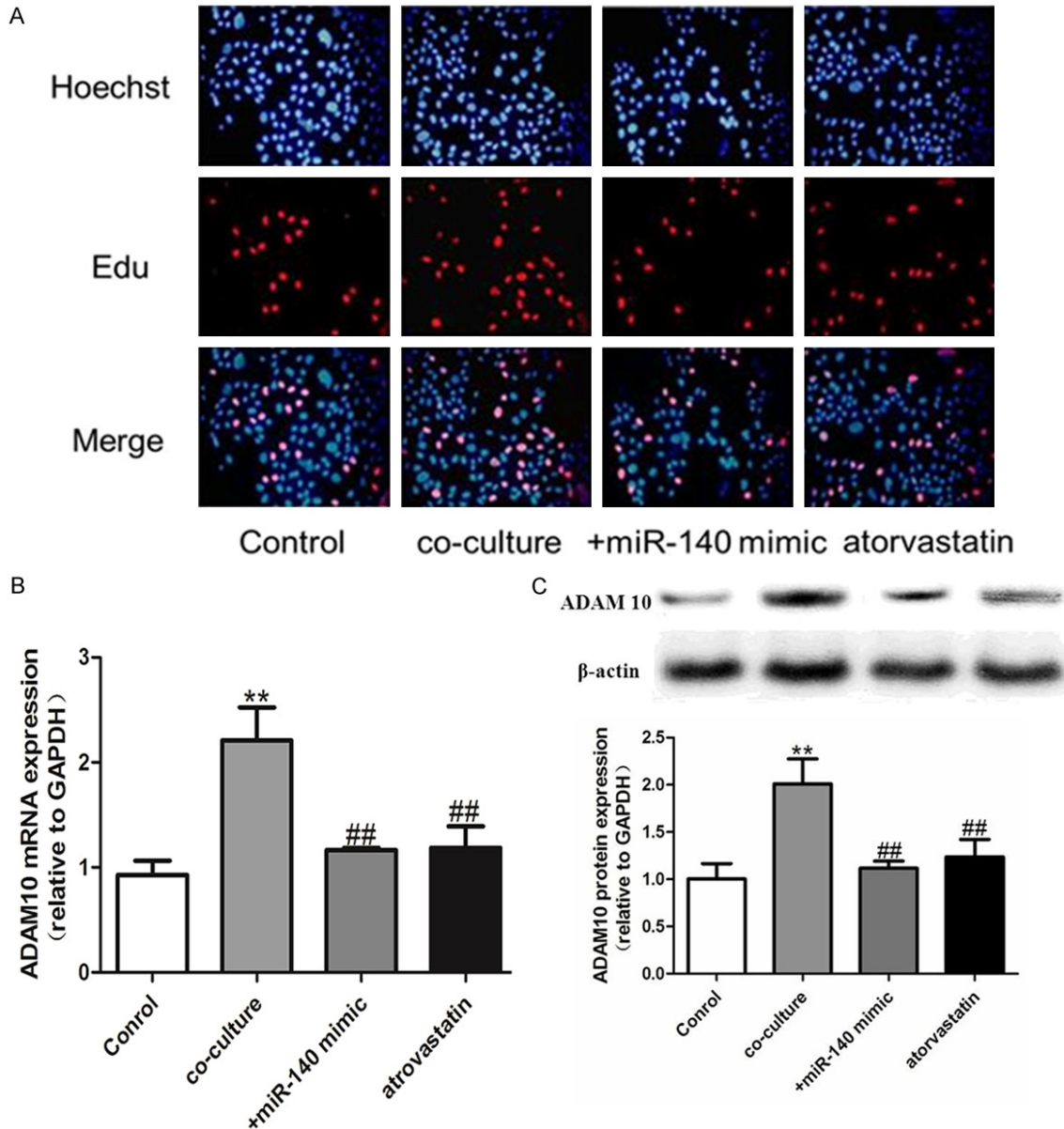
**Keywords:** Pericardial patch, atorvastatin, endothelial progenitor cells, microRNA-140, ADAM10, ephrinB2

## Introduction

Pericardial patches are commonly used in vascular surgery for closing arteriotomy. This operation may be used in carotid endarterectomy (CEA) or the femoral artery endarterectomy in lower limb extremity artery occlusion. Meta-analysis provides strong evidence that carotid patch angioplasty after CEA provides both peri-operative and long-term post-CEA benefits, supporting the standard use of patching during conventional CEA [1]. However, some patch related limitations may cause mid- or long-term

complications such as restenosis, calcification, pseudoaneurysm, or thrombosis after patch angioplasty [2]. Recent research in a rat model showed that CD34, vascular endothelial growth factor receptor 2, and ephrinB2-positive cells are recruited in the early stage of the patch healing process after patch angioplasty [3]. These cells are considered to be endothelial progenitor cells (EPCs), which can differentiate to endothelial cells. Sun et al identified the important roles that reendothelialization plays in preventing endothelial hyperplasia and vascular restenosis after patch angioplasty [4].

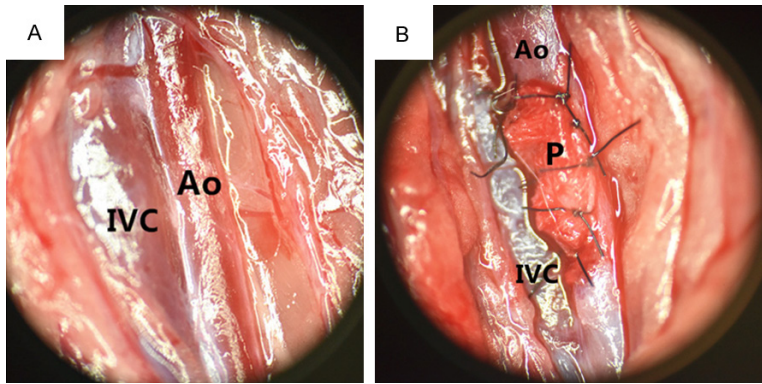
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**Figure 1.** (A) Shows EdU assay result of pericardial patch and EPCs have been co-cultured together. EPCs' proliferation slightly elevates both in co-culture, treated by miR-140 and treated by atorvastatin; (B) Shows rt-PCR data of ADAM10 mRNA expression in EPCs co-cultured with pericardial patch. After co-cultured, EPCs' ADAM10 mRNA expression significantly elevated. The ADAM10 mRNA level slightly elevated compare to control group, however the difference is not significant; (C) Shows western blot data (WB bands and densitometry) of ADAM10 protein in EPCs co-cultured with pericardial patch. After co-cultured, EPCs' ADAM10 protein significantly increased. The ADAM10 protein was slightly increased compare to control group, however the difference was not significant.

Statins are one of the primary compounds that inhibit 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase [5, 6], the major enzyme in the cholesterol synthesis pathway. Atorvastatin is frequently used as a lipid-lowering agent and exerts benefits on endothelial function by preventing endothelial senescence [7]. Atorvastatin can mobilize EPCs to localize to

the injury site of endothelium and then accelerate the endothelial repair process [8, 9]. Additionally atorvastatin can inhibit inflammation, stabilize atherosclerotic plaque, and improve endothelial cells function [10]. Furthermore, according to some reports, an atorvastatin-eluting coronary stent has a significantly lower restenosis rate than a normal bare



**Figure 2.** Animal model construction. A: Showed before the aorta arteriotomy and pericardial patch angioplasty, aorta and inferior vena cava have been exposed. Ao = aorta, IVC = inferior vena cava. B: Showed the surgical result of pericardial patch angioplasty, patch has been intermittent sutured to the aorta wall by 10-0 nylon sutures. After releasing the aorta clamps, blood flow restored and lumen of aorta refilled. Ao = aorta, P = pericardial patch, IVC = inferior vena cava.

**Table 1.** Primer pairs used in PCR analysis

Name	Forward primer (5'-3')	Reverse primer (5'-3')
ADAM10	TGCAGATCACTCCGTCTTTG	GAGTTGTGCGCTTTTTTCCTC
EphrinB2	GATGGTACATCCCCATCAG	ATTTGGTCCGATGCCAGTTG
GAPDH	GGGTGTGAACCACGAGAAAT	ACTGTGGTCATGAGCCCTTC

stent [4]. Because atorvastatin can mobilize EPCs to the stent delivery location through the circulatory blood, its restenosis-preventing mechanism is closely related to EPC differentiation and proliferation [11]. However, Miyauchi et al reported that atorvastatin has a reendo-vascularization function after stent implanta- tion [12] but has no effect on endothelial hyper- plasia to restenosis [13]. We wanted to explore whether atorvastatin affects the healing pro- cess in pericardial patch angioplasty.

MicroRNA (miR) can regulate cardiac function, including the conductance of electrical signals, heart muscle contraction, heart growth, and morphogenesis [14]. Our previous study found out that after treating EPCs with atorvastatin, miR-140 expression significantly increase (data not show). The bio-informative forecast showed that disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), a multi-functional sheddase, is one of the targets of miR-140. Our previous in vitro study also showed when pericardial patch and EPCs have been co-cultured, the proliferation level and ADAM10 expression elevate. However, after the miR-140 and atorvastatin treated, the patch

co-cultured EPCs' prolifera- tion level and ADAM10 ex- pression only showed slightly elevation without significant difference (Figure 1 showed). This previous ex vivo result may reveal some relevance in those molecules. However, the in vivo healing process of patch may be different. Furthermore, in *Xenopus laevis* embryogenesis, ephrinB2 protein levels in the absence of flotillin-1 expression are specific and partly the result of an increased susceptibility to cleavage by ADAM10 [15]. These findings mean ADAM10 may regulate ephrinB2 and play roles in vascular reendo-vascularization. Therefore, we hypothesize that atorvastatin can regulate ephrinB2 throug- h the miR-140-ADAM10 pathway in a rat model for assessing pericardial patch angioplasty healing.

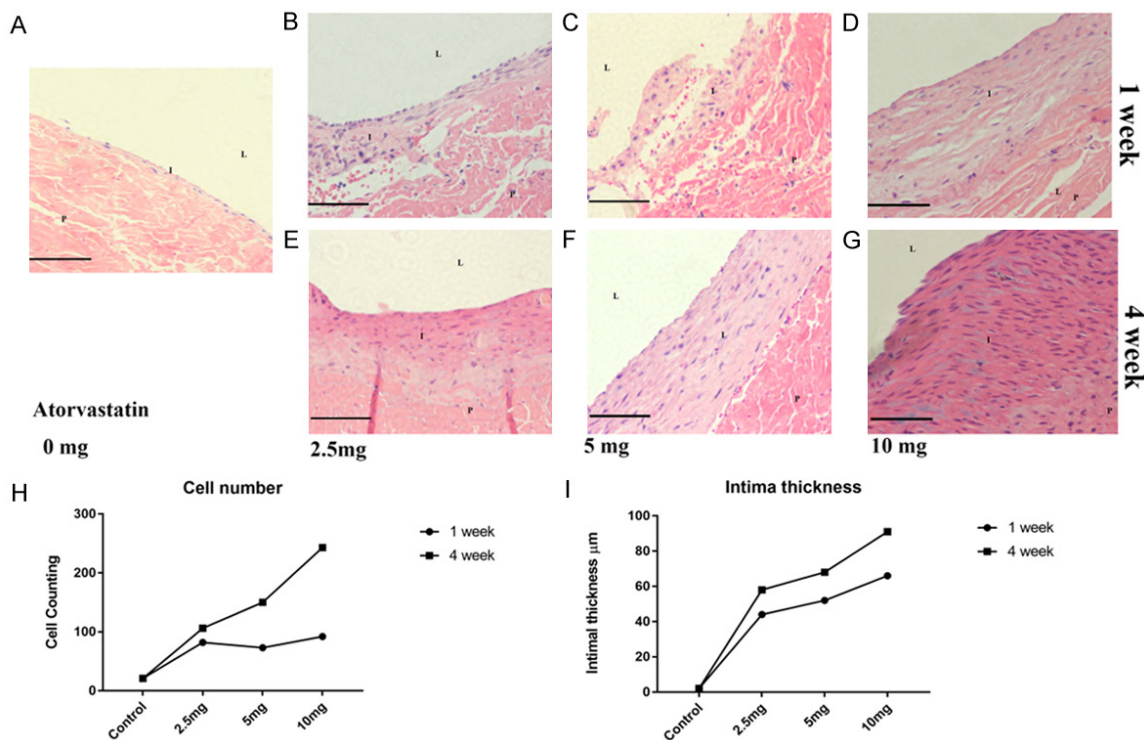
**Materials and methods**

*Animal model*

All animal experiments are approved by the Ethics Committee of Secondary Xiangya hospi- tal, Central South University (Ethics verify file # 2015-057). Male Wistar rats (8-10 weeks) were used to establish the model (n=42), and the average weight was 310±54 g. Rats were anesthetized by intra-abdominal injection of 10% chloral hydrate (0.3 ml/100 g). The sur- gery was performed under a dissecting micro- scope (Cossim). First, a middle longitudinal inci- sion was made, and then the inferior renal artery aorta was dissected. Following subcuta- neous heparin injection (100 U/100 g), the infrarenal aorta was clamped, and a longitudi- nal 3 mm length of arteriotomy was made in the anterior aorta wall. The pericardial patch (XenoSure, LeMaitre Vascular, Inc) was trimmed to a size of 3 mm × 1 mm and sutured to the aorta using 10-0 nylon suture (Figure 2). After the patch angioplasty, the microclamps were removed and aortic flow was restored. Then, 5-0 silk suture was used to close the abdomen incision. After the surgery, rats were intragastri-



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**Figure 3.** Increasing number of cells and neointimal thickness on the patch. In all the panels, P is stand for the patch, Ao is stand for the aorta, L is stand for the aortic lumen, I is stand for the intima on the patch. Animals treated with atorvastatin showed increased numbers of infiltrating cells at both 1 and 4 weeks; there were no cells in the patches prior to implantation (A). Patch implanted without atorvastatin treating (B-G). Similarly, there was increased neointimal thickening on the luminal surface of the patch in animals treated with atorvastatin. Scale bar is with 40 microns. (H) Graph of cell counting in the patch over time, mean of 2 higher fields per patch, n=2 representative pictures ( $P<0.01$ ); (I) Graph of neo-intima thickness over time, mean of 2 higher fields per patch, n=2 representative pictures ( $P<0.01$ ).

cally administrated of 2.5 mg, 5 mg and 10 mg atorvastatin (Lipitor, Pfizer Inc, USA) daily. Control rats were fed normal food without atorvastatin. The patches have been harvested at 7 days and 28 days after surgery.

### Histology

The tissue were fixed by transcardial perfusion of phosphate-buffered saline, followed by 10% formalin. Patches were harvested from rats, fixed overnight in 10% formalin, and then immersed in 70% alcohol for 24 hours. Tissue were embedded in paraffin and sectioned. Hematoxylin and eosin-stained section were used for the observation of morphologic characteristics of the patch after the angioplasty.

### Immunohistochemistry

The tissue sections were de-paraffinized using xylene and a graded series of alcohols. Then the sections were incubated in peroxidase

blocking solution and then with antibodies against CD34 (Abcam 81289) and proliferating cell nuclear antigen (PCNA; Abcam 29) overnight at 4°C. Immunohistochemistry detection was performed using DAB and NovaRED substrate (Vector Labs). Sections were counterstained with Mayer's hemotoxylin.

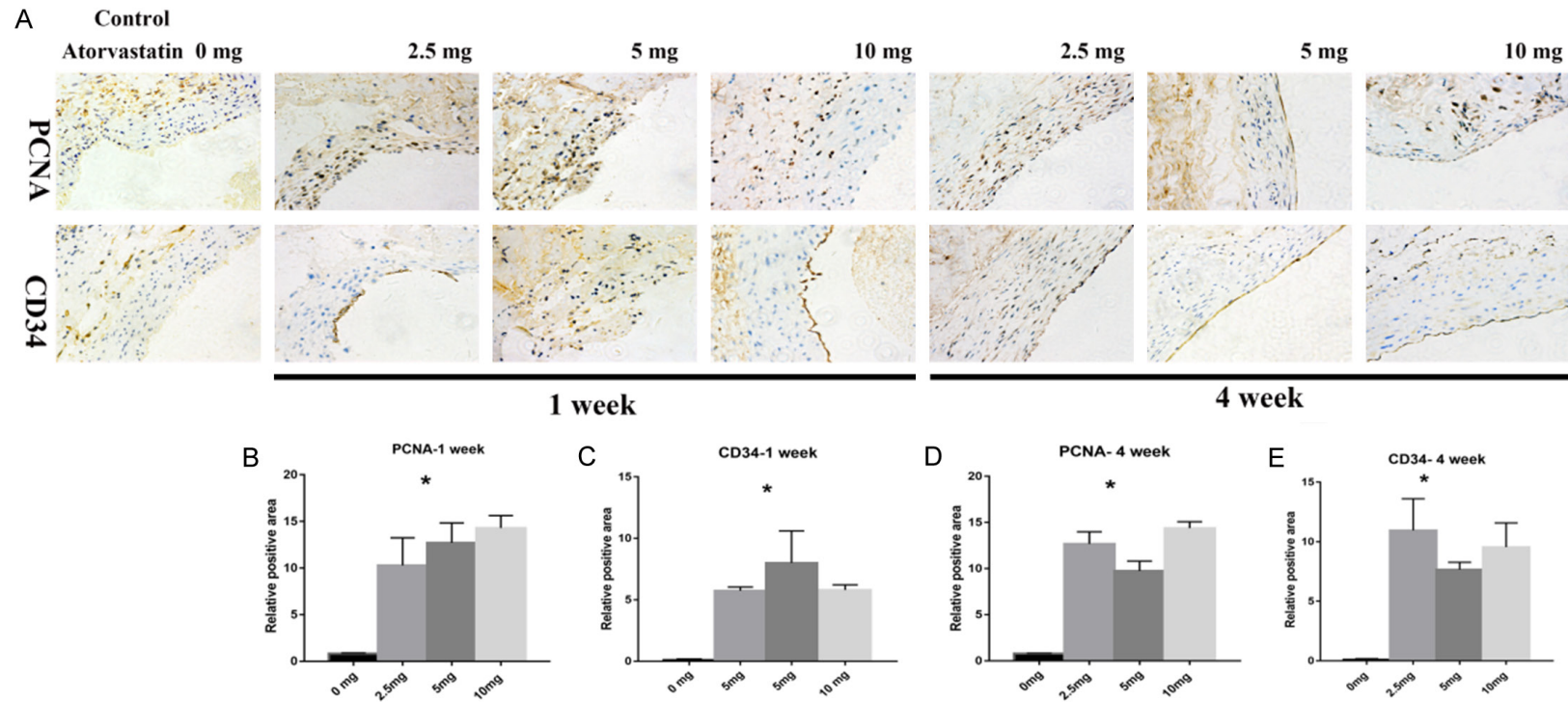
### Immunofluorescence

Tissue sections were de-paraffinized and stained using primary antibodies against ADAM10 (Abcam ab1997) and ephrinB2 (Abcam ab131526). Appropriated fluorophore-conjugated secondary antibodies were chosen for incubation. A antifade reagent with DAPI nuclear stain (Servicebio) was used prior to coverslip placement.

### PCR analysis

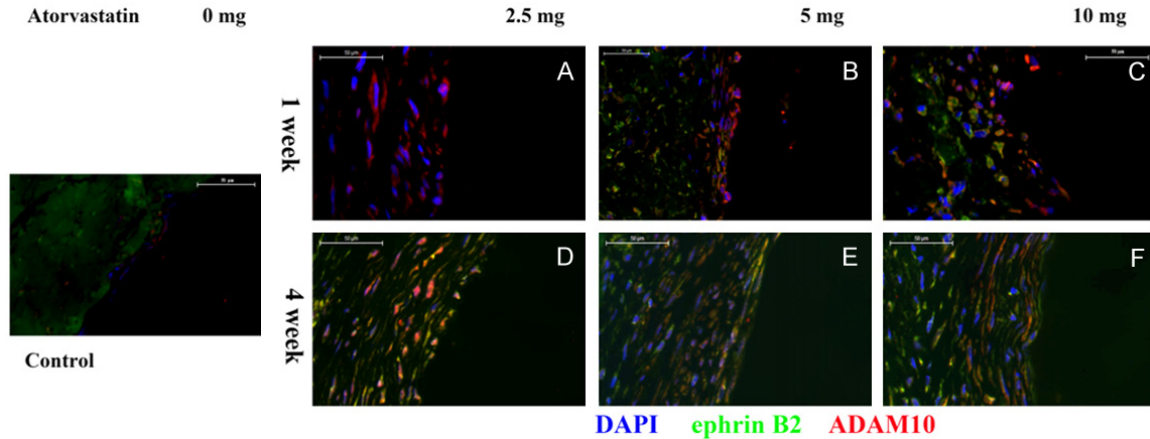
After the patch was harvested, it was isolated from surrounding vascular tissue. Total RNA

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**Figure 4.** (A) Immunohistochemistry result of different atorvastatin dose on 1 week and 4 weeks patch. (B) At 1 week, there was a trend towards increased cell proliferation (PCNA) with increasing doses of atorvastatin ( $P=0.0076$ ). (C) However, there were increased number of CD34-positive cells with increasing doses of atorvastatin ( $*P=0.0158$ ). (D) Similar in the 4 weeks, there was a trend towards increased cell proliferation (PCNA) with increasing dose of atorvastatin ( $*P=0.0040$ ). (E) And there were increased number of CD34-positive cells with increasing dose of atorvastatin ( $*P=0.0104$ ). Bar graph (B-E) is the calculation of the positive area of one representative figure.

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**Figure 5.** Immunofluorescence result of neo-intima on the patch in different time group and atorvastatin dose group. These figures are the cell infiltrate to the patch co-stained with DAPI (blue), ADAM10 (red) and ephrinB2 (green) together. Control panel is the patch not treated with the atorvastatin. At 1 week, ephrinB2 increasing as atorvastatin dose goes up, and ADAM10 showed tiny decreasing trend as atorvastatin dose goes up. Similar as 1 week, at 4 weeks, ephrinB2 signal showed increasing as atorvastatin dose goes up and ADAM10 signal showed obvious diminish trend as atorvastatin dose goes up. Scale bar is 50  $\mu$ m.

was isolated from the patches using TRIzol reagent (Invitrogen). RiboGreen RNA assay kit (Invitrogen) was used to measure the total RNA. Reverse transcription was performed using the SuperScript III first-strand Synthesis Supermix (Invitrogen) according to manufacturers' instructions. Real-time PCR was performed using SYBR Premix Ex Taq™ (Tli RNaseH Plus) and amplified for 40 cycles using ViiA7 Dx Real-time PCR detection system (Life Technologies). The samples were normalized by GAPDH amplification. The sequences for the primers to amplify ADAM10, ephrinB2 and GAPDH were synthesized and are showed in **Table 1**. The primer to amplify miR-140 was purchased from Bulge-Loop™ miRNA.

### Western blot analysis

After harvesting, the patches were snap-frozen in liquid nitrogen. When extracting the protein, the patches were crushed and mixed with buffer including protease inhibitors (cwbiotech) prior to sonication and centrifugation. Equal amounts of protein from each experiment group were loaded for SDS-PAGE, followed by western blot analysis. The membranes were probed with antibodies against ADAM10 (Abcam, ab1997) and ephrinB2 (Abcam, ab131536). Membrane signals were detected using the ECL detection reagent (Clarity™ Western ECL Substrate, Bio-Rad). Patches were analyzed individually, without combination of other tissues.

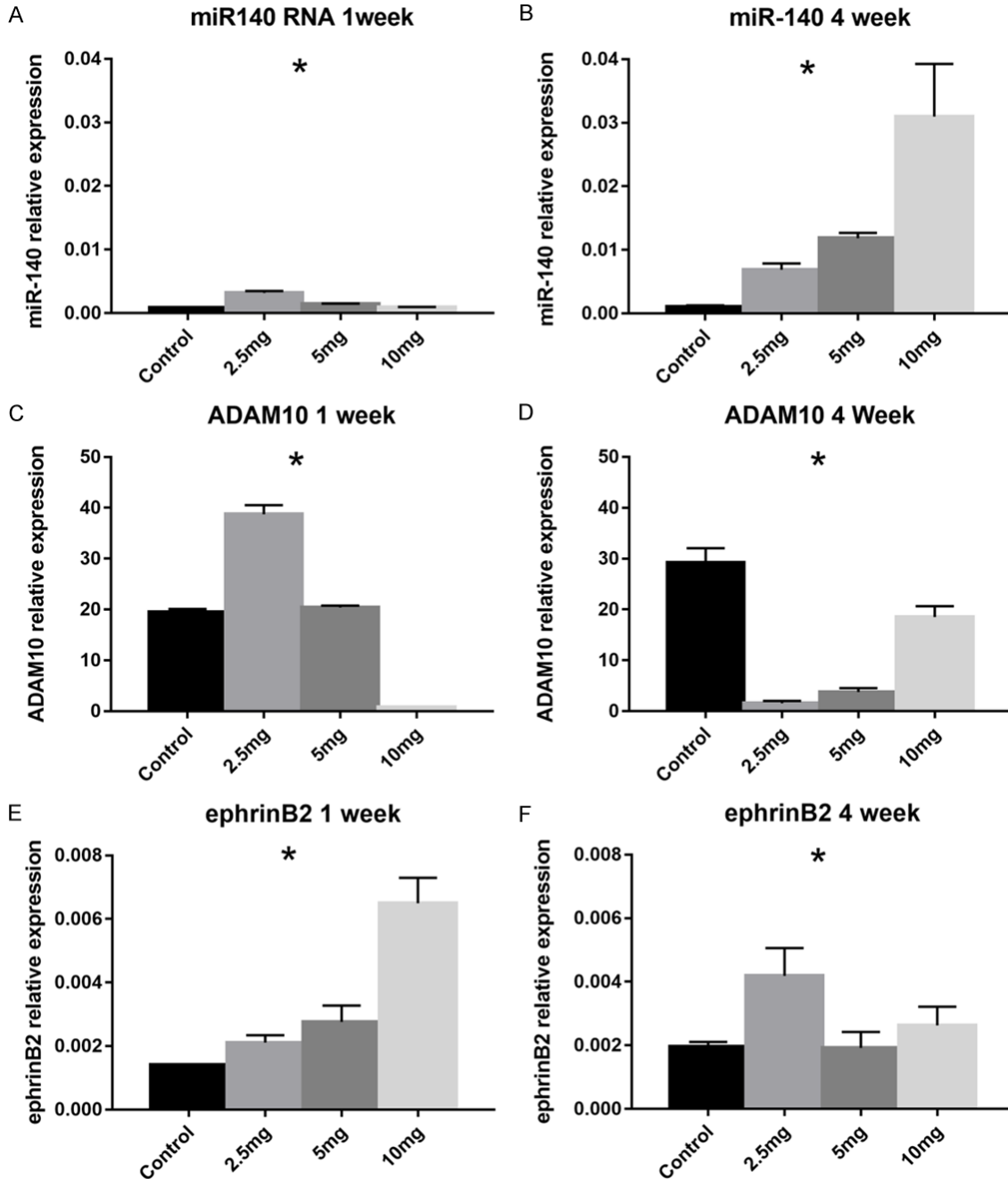
### Statistical procedures

Statistical software is GraphPad Prism 7.0 (Graphpad software, CA, USA). Statistic method of cell number and neo-intima thickness in different time and different atorvastatin dose; IHC positive area in different atorvastatin dose; RNA relative expression in different atorvastatin dose and WB densitometry in different atorvastatin dose is one-way ANOVA.

### Result

To determine whether atorvastatin attracts ephrinB2-positive cells during patch healing in the arterial environment, we placed patches into the infrarenal aorta (**Figure 2**). Animals treated with atorvastatin showed increased numbers of infiltrating cells at both 1 and 4 weeks (**Figure 3**); no cells existed in the patches prior to implantation, as previously described [3]. Similarly, an increased neointimal thickening on the luminal surface of the patch was observed in animals treated with atorvastatin (**Figure 3**).

Because atorvastatin increases the patch neo-intima and the number of infiltrating cells, we determined whether these cells were proliferating. At 1 week, a trend towards increased PCNA positive area with increasing doses of atorvastatin ( $P=0.0076$ ; **Figure 4**). In addition, an increased number of CD34-positive cells was found with increasing doses of atorvastatin

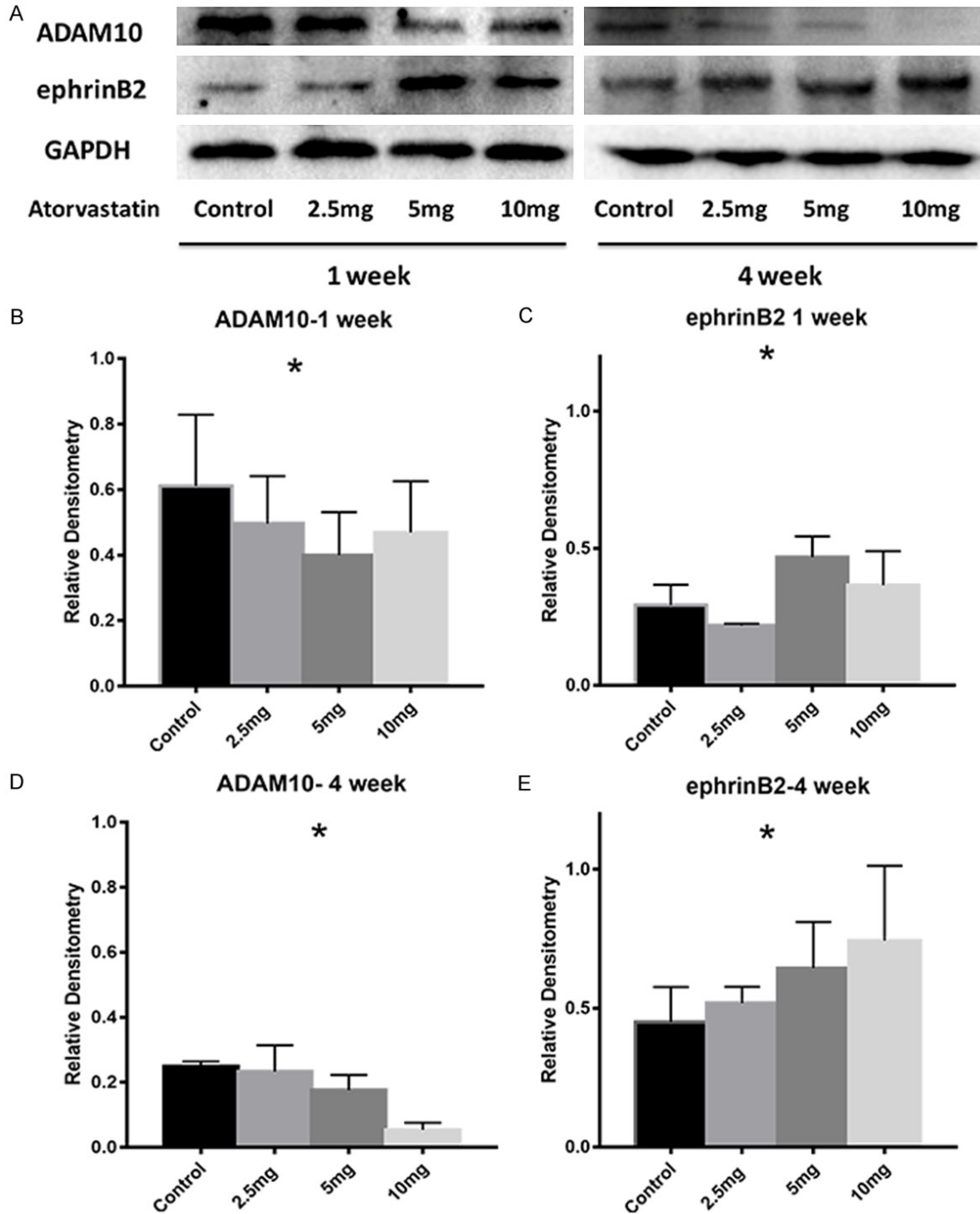


**Figure 6.** PCR result of miR-140, ADAM10 and ephrinB2 in 1 week and 4 weeks group. The real-time PCR result showed that ephrinB2 mRNA increased in a dose-dependent fashion (1 week), miR-140 mRNA increased in a dose-dependent fashion (4 weeks), and ADAM10 mRNA decreased in a dose-dependent fashion. Both group statistic method is one way ANOVA. And both \*P<0.01, N=2.

(P=0.0158; **Figure 4**). As expected, by 4 weeks, no differences among any of these markers (**Figure 4**). These results suggested that atorvastatin increases patch thickness by increasing early cell infiltration rather than by cell proliferation.

Because these results suggest that atorvastatin increases infiltration of CD34-positive progenitor cells, we determined whether infiltrating cells were ephrinB2-positive. Atorvastatin increased ephrinB2-positive cells in the patch in a time- and dose-dependent manner (**Figure**

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**Figure 7.** Western blot result of ADAM10 and ephrinB2 in 1 week and 4 weeks patch. The western blot bands of ADAM10, ephrinB2 and GAPDH are showed in (A). Densitometry bar (B-E) showed that ephrinB2 expression increased in both time- and dose-dependent fashion, whereas ADAM10 expression decreased in both time- and dose-dependent fashion. All the densitometry data statistic method is one way ANOVA, both \* $P < 0.01$ ,  $N = 2$ .

5); simultaneously, ADAM10 immunoreactivity decreased in a time- and dose-dependent manner (Figure 5). Similarly, ephrinB2 mRNA at 1 week and miR-140 mRNA at 4 weeks increased

in a dose-dependent manner, while ADAM10 mRNA at 1 week decreased in a dose-dependent manner (Figure 6). Western blot confirmed that ephrinB2 expression increased and



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ADAM10 expression decreased in time- and dose-dependent fashion (**Figure 7**).

### Discussion

We showed that atorvastatin-treated rats with pericardial patching after angioplasty had significantly higher cell numbers and thicker neointima than the control animals. In addition, the cell proliferation marker PCNA and endothelial progenitor cell biomarker CD34 increased at 1 and 4 weeks in rats that received patching plus atorvastatin treatment. Furthermore, on a molecular level, after atorvastatin treatment, the miR-140 and ephrinB2 expression was elevated, while ADAM10 expression decreased. On a protein level, ADAM10 decreased and ephrinB2 increased in atorvastatin-treated rats. These results suggest that atorvastatin treatment with arterial angioplasty pericardial patch improves the proliferative and differentiation abilities of cells in the neointima. In addition, the neointima formation or the adaptation to arterial circulation may be regulated by atorvastatin through molecules miR-140, ADAM10, and ephrinB2.

#### *Atorvastatin treatment thickens patch neointima*

In the short term, within one month, the pericardial patch adapts to the new environment of arterial circulation [3]. Neointima forming on the patch is very important for the healing process for preventing thrombosis on the patch after first stage of the implantation [16, 17]. Atorvastatin has protective effect against vascular smooth muscle cells proliferation and inflammation in cardiovascular remodeling [18]. In addition, it protects vascular and neural function by reducing inflammation [19]. We found out that rats treated with atorvastatin after angioplasty had thicker neointima and more cells on the patch than the control animals. In the early stage after angioplasty, thicker and regular neointima forming may provide satisfactory intimal function, preventing early stage thrombosis. Xu et al reported that intensive atorvastatin improves endothelial function by significantly reducing serum endothelin-1 and ADP-induced platelet clot strength, resulting in less major adverse cardiovascular events after percutaneous coronary intervention (PCI) [20]. Four weeks after angioplasty, the aorta is normally patent, indicating the neointima is

well formed. Atorvastatin should be protective in this pericardial patch healing stage.

#### *Cells on the patch have proliferative and differentiation abilities*

As shown by immunohistochemistry, the cells on the atorvastatin treated rats patches' neointima exhibited increased levels of PCNA, the EPCs marker CD34 and the endothelial cell marker CD31 (data not show). This findings suggest that EPCs are actively involved in the process of the endothelialization and proliferation. Some EPCs already differentiated into endothelial cells and well organized in the surface of the neointima. Previous studies have identified the roles of atorvastatin for mobilizing EPCs localized to endothelium injury site and aggregating endothelial repair [8, 9]. Chiang et al reported that atorvastatin improved neovascularization in a mouse hindlimb ischemia model; this effect may have been mediated by increased CXCR4 expression in EPCs [21]. This is one of pathways that atorvastatin may affect the repair function of EPCs in reendothelialization. However, we investigated another possible pathway of this effect.

#### *Atorvastatin affects EPC function on the patch via regulation of the miR-140-ADAM10-ephrinB2 pathway*

Our previous study found out that miR-140 expression significantly increased after treating EPCs with atorvastatin. The bio-informative forecast showed ADAM10, a multi-functional sheddase, is one of the target of miR-140. Moreover, ADAM10 can cleave the ephrin molecule in ephrin-Eph complex [15], indicating that ADAM10 may regulate ephrinB2 and play roles in vascular reendothelialization. Therefore, we hypothesized that atorvastatin can regulate ephrinB2 via the miR-140-ADAM10 pathway in a rat model for assessing pericardial patch angioplasty healing.

Although numerous studies have reported that miR-140 is related to tumor growth [22-24], miR-140 is also closely related to cardiovascular disease [14]. As shown in **Figure 6A** and **6B**, as the atorvastatin dose increased, miR-140 mRNA expression significantly increased in time- and dose dependent manner. ADAM10 is one of the direct target gene of miR-140. miR-140 may repress the ADAM10 expression, as

well as reciprocal [25]. The PCR and western blot identified that ADAM10 expression was downregulated as miR-140 expression increased. Furthermore, as ADAM10 was downregulated, ephrinB2 was upregulated, according to results of the immunofluorescence, PCR, and western blot. EphrinB2 has been identified in the endothelial cells in the pericardial patch. Ephrins ligands and Eph receptors belongs to the largest of 14 receptor tyrosine kinase sub-families [26]. EphrinB2 protein levels are partly the result of an increased susceptibility to cleavage by ADAM10 [15]. This regulatory function of ADAM10 and ephrin molecule explains how the downregulation of ADAM10 on a gene and protein level occurs concurrently with the upregulation of the ephrinB2 protein.

In summary, our findings supported our hypothesis that atorvastatin plays roles in pericardial patch healing after arterial angioplasty. First, in the patch neointima, miR-140 expression was upregulated in the EPCs, which exhibited good proliferative and differentiation abilities after atorvastatin treatment in time and dose-dependent manner. Second, as miR-140 increased, one of its target genes *ADAM10* was downregulated. As ADAM10 protein ephrin cleavage function decreased, ephrinB2 expression increased in the EPCs of the patch. Lastly, through the above pathway, EPCs more aggressively differentiated into endothelial cells on the patch. This resulted in thicker neointima on the patch after atorvastatin treatment in the rat arterial angioplasty model.

### Conclusion

Atorvastatin play roles in pericardial patch healing after arterial angioplasty. Atorvastatin may improve EPCs proliferative and differentiation abilities to arterial characteristic endothelial cells by upregulating miR-140 expression. As the consequence, ADAM10 expression decreased, causing ephrinB2 to increase. These results infer that atorvastatin improves angioplasty pericardial patch healing by regulating the microRNA140-ADAM10-ephrinB2 pathway in the rat model.

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### Disclosure of conflict of interest

None.

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