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ORIGINAL ARTICLE

# Antisense extracellular signal-regulated kinase-2 gene therapy inhibits platelet-derived growth factor-induced proliferation, migration and transforming growth factor-$\beta_1$ expression in vascular smooth muscle cells and attenuates transplant vasculopathy

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**Keywords**
extracellular signal-regulated kinase, gene therapy, migration, platelet-derived growth factor, proliferation, vascular smooth muscle cell.

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## Summary

Platelet-derived growth factor-BB (PDGF-BB) enables vascular smooth muscle cells (VSMCs) to proliferate, migrate and secrete connective tissue matrix, which are critical events in transplant vasculopathy. However, little is known about the intracellular pathways that mediate these biologic responses of VSMCs. Extracellular signal-regulated kinase (ERK) pathway plays a major role in cellular responses and vascular diseases. In this study, we observed that the inhibition of ERK2 activity by recombinant adenovirus encoding antisense ERK2 (Adanti-ERK2) significantly suppressed the proliferation, converting of cell cycle from $G_1$ phase to S phase and directed migration, and partially abrogated transforming growth factor-$\beta_1$ (TGF-$\beta_1$) expression in VSMCs stimulated with PDGF-BB. *Ex vivo* gene transfer of Adanti-ERK2 into rat aortic allograft attenuated chronic transplant vasculopathy by the inhibition of VSMC proliferation and migration. In conclusion, ERK2 is involved in PDGF-BB-induced VSMCs proliferation, migration and TGF-$\beta_1$ expression and may be a potential therapeutic target for transplant vasculopathy.

## Introduction

Chronic rejection (CR) remains a major limiting factor for long-term allograft survival [1]. It is characterized by a diffuse, concentric intimal thickening within the arteries of grafts, termed transplant vasculopathy [2]. The abnormal proliferation, migration and extracellular matrix (ECM) synthesis of vascular smooth muscle cells (VSMCs) are important pathologic processes in the development of transplant vasculopathy [3]. VSMCs are normally quiescent and proliferate at low indices but begin to proliferate and migrate after exposure to a variety of stimuli that include a number of growth factors [4]. Platelet-derived growth factor-BB (PDGF-BB), one of the most potent mitogen and chemoattractants for VSMCs, can induce VSMC proliferation and migration through activating multiple cellular signaling pathways [5]. It has also been demonstrated that the expression of PDGF-BB is prominent in chronically rejecting allografts and correlated to the intensity of intimal thickening [6]. Moreover, PDGF-BB induces transforming growth factor-$\beta_1$ (TGF-$\beta_1$) expression in VSMCs [7]. Overexpression of TGF-$\beta_1$, a principal profibrogenetic growth factor that stimulates cell hypertrophy and ECM production, plays a key role in chronic graft fibrosis that generally results from transplant vasculopathy [8]. However, the molecular mechanism of VSMC proliferation, migration and TGF-$\beta_1$ expression induced by PDGF-BB remains to be fully understood.

Extracellular signal-regulated kinase (ERK) is a subfamily of the mitogen-activated protein kinase (MAPK) signaling

pathways, which is involved in cellular responses including proliferation, differentiation, apoptosis and migration through mediating polypeptide growth factor signaling [9,10]. And transmission of signals is achieved by a sequential series of phosphorylation reactions wherein ERK is activated by phosphorylation. Activated ERK translocates to the nucleus and affects downstream regulatory molecules such as transcription factors, thereby causing changes in gene expression [11]. The important role of ERK in PDGF-induced VSMC responses is proposed [12,13].

So far no differential role was proven *in vitro* for the two main isoforms of the ERK pathway (ERK1 and ERK2), and they are activated by the same stimuli. However, *in vivo* ERK1 or ERK2 invalidation leads to different phenotypes, demonstrating different roles for ERK1 and ERK2 [14]. Indeed, ERK2 is essential for the transduction of signals, ERK1 could instead have an accessory role, possibly enabling a fine tuning of ERK2 activity [15]. Furthermore, ERK2 is well known both for its regulation of the entry into the cell cycle and for the progression of cells through the $G_1$ phase of the cell cycle [16]. Thus, in this study, by using gene transfer technique with adenoviral vector encoding antisense ERK2 (Adanti-ERK2), we examined the role of ERK2 in PDGF-BB-induced proliferation, migration and TGF-$\beta_1$ expression in VSMCs.

## Materials and methods

### Materials

Recombinant rat PDGF-BB, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit, dimethyl sulfoxide (DMSO), and propidium iodide (PI) were from Sigma (St Louis, MA, USA). Fetal bovine serum (FBS) and Dulbecco's minimal essential medium (DMEM) were from Gibco BRL (Carlsbad, CA, USA). Monoclonal antibody to α-smooth muscle actin (α-actin) was from Biodesign Company (Carmel, NY, USA). Phospho-specific ERK1/2 antibody was from Cell Signaling Technology (Beverly, MA, USA). The Boyden chamber was from BD Transduction Laboratory (San Jose, CA, USA). TGF-$\beta_1$ ELISA kit was from Promega (Madison, WI, USA). Adeno-X$^{TM}$ expression system kit was from BD Biosciences Clontech (Palo Alto, CA, USA). The plasmid of p3XFLAG-CMV7.1-ERK2 was generously provided by Dr Fred L. Robinson (University of Texas).

### Adenovirus construction

Adanti-ERK2 was constructed using the Adeno-X$^{TM}$ expression system, which is based on the procedure developed by Mizuguchi and Kay [17]. The wild-type rat ERK2 cDNA fragment, obtained by digesting the plasmid of p3XFLAG-CMV7.1-ERK2 with *Not*I and *Sal*I, was excised by *Dra*I digestion. The resulting 770-bp-length cDNA used for cloning was then separated by agarose gels and reversely ligated into the *Dra*I–*Not*I site of pShuttle vector to generate pShuttle-antiERK2. The expression cassette was excised from pShuttle-antiERK2 and inserted into replication-incompetent (E1/E3-deleted) Ad5 genome via PI–*Sce*I/I–*Ceu*I restriction sites. The recombinant adenoviral vector was packaged in human embryonic kidney (HEK) 293 cells (China Center for Type Culture Collection, Wuhan, China), purified by CsCl density gradient ultracentrifugation and stored at −80 °C. As vector control of Adanti-ERK2, recombinant adenovirus-containing bacterial β-galactosidase gene (Ad-*LacZ*) was also generated similarly. The titer of the virus was determined by limiting dilution in HEK 293 cells and expressed as plaque-forming units (PFU).

### Cell culture and adenovirus infection

Vascular smooth muscle cells were isolated from the thoracic aorta of adult male Sprague–Dawley rats (Experimental Animal Center of Tongji Medical College, Wuhan, China) and cultured by the modified explant method [18]. VSMCs were maintained at 37 °C, 5% $CO_2$ in DMEM containing 10% FBS, 100 U/ml penicillin and 100 g/ml streptomycin. At confluence, these cells formed a hill-and-valley pattern typical of cultured SMCs. The purity of VSMCs was identified by immunohistochemical staining with anti-α-actin monoclonal antibody. Experiments were performed with VSMCs at passages 3–6. When the cells were 60–70% confluent, the medium was changed to DMEM containing 2% FBS and viruses were added to the medium at a multiplicity of infection of 100. Following 18 h infection with adenoviruses, VSMCs were made quiescent in serum-free DMEM for 6 h and then stimulated with 10 ng/ml PDGF-BB as indicated [19]. As a control in all experiments, an identical group of cells was left uninfected but incubated 18 h in DMEM containing 2% FBS.

### Western blot

Cells were lysed in Triton lysis buffer and 50 μg total protein extracts from the cells were separated by 10% SDS-PAGE. Separated protein was transferred to nitrocellulose membranes and the membranes were blocked in tris-bruffer saline/0.05% tween 20 (TBST) plus 5% fat-free milk powder. The blots were incubated first with anti-phospho-ERK2 antibody at 4 °C overnight and then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Finally, immunoreactive proteins were visualized by enhanced chemiluminescense system. Anti-GAPDH antibody was used for normalization.

 

### Proliferation assay

Cells were trypsinized and plated in a 96-well plate at $1 \times 10^4$ per well. At the end of cell culture, 20 μl of MTT solution (5 mg/ml) was added to each well and cells were incubated for an additional 4 h. The supernatant was then discarded and the precipitate was suspended in 150 μl of DMSO. The absorbance values ($A$) were measured using a microplate reader at 570 nm. The absorbance of untreated cells was used as a reference and the proliferation rate of treated cells was calculated using the following formula modified from Yuan *et al.* [20]: $\text{Proliferation rate}\,(\%) = 100 \times \text{Absorbance}_{\text{treated cells}} / \text{Absorbance}_{\text{untreated cells}}$.

### Analysis of cell cycle

Cells ($1 \times 10^6$/ml) were harvested, rinsed with phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4 °C for 3 h. The fixed cells were centrifuged at 1620 *g* for 5 min and then resuspended in PBS containing 50 μg/ml RNAse at 37 °C for 1 h. Cells were stained with 50 μg/ml PI and incubated in dark for 30 min. The analysis of cell cycle distribution was performed by flow cytometry.

### Cell migration analysis

Cell migration was performed using the Boyden chamber method as described by Saito *et al.* [21]. Briefly, the lower chamber was filled with 600 μl of serum-free DMEM that contained with or without PDGF-BB. An 8 μm pores polycarbonate filter was placed between the upper and lower chamber. VSMCs transfected with Ad-*LacZ* or Adanti-ERK2 were harvested and resuspended in serum-free DMEM at a concentration of $10^5$ cells/ml and added to the upper chamber at 200 μl per well. After incubation at 37 °C for 6 h, cells on the upper surface of the filter were mechanically moved and the filter was fixed in 4% paraform for 20 min. VSMCs that migrated to the lower surface of the filter were stained with hematoxylin and eosin and enumerated under 200× magnifications. Five random microscopic fields were counted per filter and the number of migrated cells was demonstrated by mean value.

### Measurement of TGF-$\beta_1$ concentration

The concentration of TGF-$\beta_1$ in the culture supernatants was measured by specific ELISA assay in accordance with the manufacturer's protocol. The absorbance values ($A$) were measured in an ELISA microplate reader at 492 nm. The concentration of TGF-$\beta_1$ was obtained according to the standard curve constructed by plotting the mean absorbance for each standard against the concentration.

### *Ex vivo* gene transfer and aortic transplantation

Aortic transplantation was performed using Brown–Norway (BN, $RT1^n$) rats as donors and Lewis ($RT1^1$) rats as recipients. The rats were introduced from Charles River Laboratory (Boston, MA, USA) via Vital River Company (Beijing, China). The experimental procedure was approved by the Institutional Animal Care and Use Committee of China. The rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg)/xylazine (12 mg/kg) solution (Sigma). A segment of abdominal aorta (approximately 1.5 cm in length) from BN rat was excised and flushed with PBS (without $Ca^{2+}$/$Mg^{2+}$; pH 7.4). Ad-*LacZ* or Adanti-ERK2 ($5 \times 10^9$ PFU) in 200 μl of PBS was instilled into the aortic lumen and the two ends were clamped. Clamped aortic transplants were incubated in 4 °C University of Wisconsin solution for 45 min and then used for transplantation. The recipients were divided into three groups: (i) empty control group (no gene transfer, $n = 8$); (ii) vector control group (allografts transfected with Ad-*LacZ*, $n = 9$) and (iii) Adanti-ERK2 group (allografts transfected with Adanti-ERK2, $n = 6$). The aortic graft was transplanted orthotopically below the renal arteries and above the aortic bifurcation. There was no significant discrepancy in the size of the donor and recipient vessels. All anastomoses were performed using continuous 10-0 prolene suture in an end-to-end technique. The total operative time from the removal of the donor graft to the perfusion of the graft in the recipient was approximately 80 min. At day 60 after transplantation, the middle segments of aortic grafts were harvested for morphometric and immunohistochemical studies. The thickness of the intima was estimated by the percentage of intima value (intima/intima + media). VSMCs were semiquantitatively assessed by counting the number of α-actin-positive cells in 10 random fields per section under 400× magnifications.

### Statistical analysis

Data were expressed as mean ± SD. Differences in mean values were compared using Statistical Package for the Social Science (SPSS) 11.0 by one-way ANOVA and Student–Newman–Keuls test. Statistical significance was defined as $P < 0.05$.

## Results

### Adanti-ERK2 inhibits PDGF-BB-induced ERK2 phosphorylation in VSMCs

To elucidate whether Adanti-ERK2 affects PDGF-BB-induced ERK2 activity, ERK2 phosphorylation was

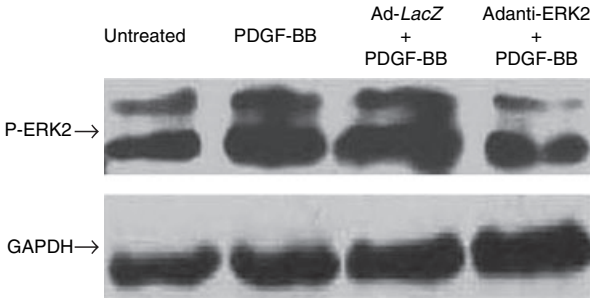


**Figure 1** Adenovirus encoding antisense extracellular signal-regulated kinase (Adanti-ERK2) inhibits platelet-derived growth factor (PDGF-BB)-induced ERK2 phosphorylation. Vascular smooth muscle cells were grown on six-well plates. Cells were transfected with Adanti-ERK2 or Ad-*LacZ* for 18 h before being treated with PDGF-BB for 20 min. Phosphorylated ERK2 protein in cell lysate was detected using Western blot analysis with an anti-phosphor-ERK1/2 antibody.

measured using Western blot with a phospho-specific ERK1/2 antibody. Untreated VSMCs exhibited low ERK2 activity, as evidenced by the faint signals produced by the phosphorylation of ERK2. Exposure of the cells to PDGF-BB for 20 min led to a remarkable upregulation of ERK2 phosphorylation, and this effect was remarkably inhibited by Adanti-ERK2 but not by Ad-*LacZ* (Fig. 1).

### Adanti-ERK2 suppresses PDGF-BB-induced VSMC proliferation

Compared with untreated cells, the proliferation rates were 169.7 ± 4.5% for VSMCs stimulated with PDGF-BB, 163.6 ± 9.1% for VSMCs cultured with Ad-*LacZ* and PDGF-BB and 112.1 ± 3.7% for VSMCs cultured with Adanti-ERK2 and PDGF-BB. These results indicate that Adanti-ERK2 infection inhibited PDGF-BB-induced VSMC proliferation, whereas Ad-*LacZ* infection did not significantly influence PDGF-BB-induced cell proliferation (Fig. 2).

### Adanti-ERK2 modulates PDGF-BB-mediated cell cycle progression

Compared with cells without treatment, cells stimulated with PDGF-BB showed an increased number of cells in S and $G_2$/M phase corresponding to a decreased proportion of $G_1$ phase. Infection of VSMCs with Adanti-ERK2 significantly attenuated PDGF-BB-induced increases in S entry of cell cycle, resulting in $G_1$ arrest, whereas Ad-*LacZ* did not inhibit PDGF-BB-induced progression of cell cycle. And the DNA profile of cells infected with Ad-*LacZ* did not obviously differ from that of VSMCs induced by PDGF-BB (Fig. 3).

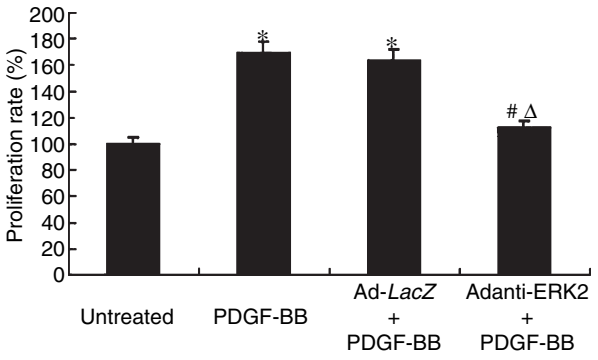


**Figure 2** Adenovirus encoding antisense extracellular signal-regulated kinase (Adanti-ERK2) suppresses platelet-derived growth factor (PDGF-BB)-induced vascular smooth muscle cells (VSMCs) proliferation. VSMCs were infected with Adanti-ERK2 or Ad-*LacZ* for 18 h, quiesced in serum-free medium for 24 h, treated with PDGF-BB for 18 h, and then proliferation rates were calculated using the absorbance values assessed by MTT. The proliferation rate of untreated cells was set as 100%. The data are representative of five independent experiments. $^{*}P < 0.01$ vs. untreated; $^{\#}P < 0.01$ vs. PDGF treatment; $^{\Delta}P < 0.01$ vs. Ad-*LacZ* and PDGF treatment.

### Adanti-ERK2 attenuates PDGF-BB-induced cell migration

As estimated by enumerating the number of cells that migrated to the lower surface of the filter, PDGF-BB stimulation significantly enhanced VSMC migration by 4.1-fold. Infection of VSMCs with Adanti-ERK2 suppressed PDGF-BB-induced VSMC migration by 68.1%; however, Ad-*LacZ* did not significantly alter PDGF-BB-induced cell migration (Fig. 4).

### Adanti-ERK2 partially inhibits PDGF-BB-induced TGF-$\beta_1$ expression in VSMCs

Compared with untreated cells, stimulation of VSMCs with PDGF-BB increased the protein level of TGF-$\beta_1$ in the culture supernatants by 3.8-fold. Infection of VSMCs with Adanti-ERK2 and Ad-*LacZ* inhibited PDGF-BB-induced increase in TGF-$\beta_1$ level by 33.2% and 6.3%, respectively. So Adanti-ERK2 could partially abrogate PDGF-BB-induced TGF-$\beta_1$ expression, whereas Ad-*LacZ* did not affect PDGF-BB-induced TGF-$\beta_1$ expression (Fig. 5).

### Adanti-ERK2 attenuates transplant vasculopathy after aortic transplantation

To further demonstrate the role of ERK2 in transplant vasculopathy, we tested the effect of Adanti-ERK2 on a rat aortic transplantation model. At day 60 after transplantation, typical vasculopathy lesions developed in empty control group and vector control group with con-

 

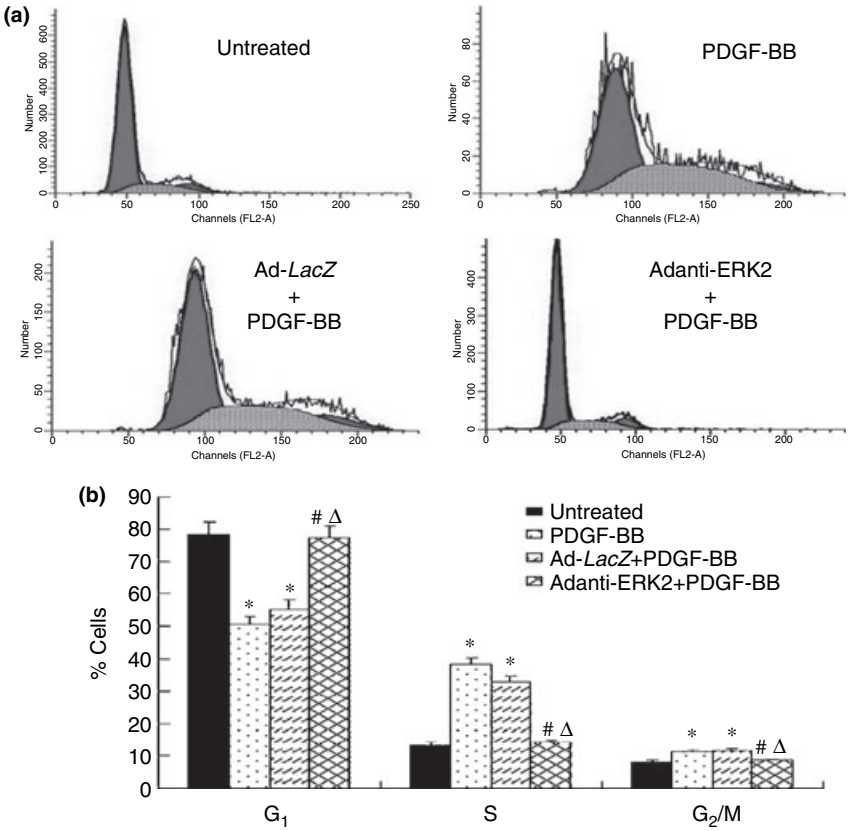


**Figure 3** Effect of adenovirus encoding antisense extracellular signal-regulated kinase (Adanti-ERK2) on cell cycle of vascular smooth muscle cells stimulated with platelet-derived growth factor (PDGF-BB). (a) Representative DNA histogram of propidium iodide fluorescence in cells was assessed by flow cytometry. (b) Percentage of cells in $G_1$ phase, S phase, and $G_2$/M phase of cell cycle. The results are representative of three independent experiments. $^*P < 0.01$ vs. untreated; $^{\#}P < 0.01$ vs. PDGF treatment; $^{\Delta}P < 0.01$ vs. Ad-*LacZ* and PDGF treatment.

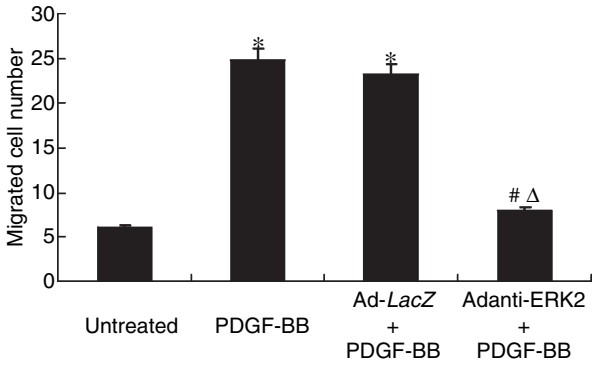


**Figure 4** Adenovirus encoding antisense extracellular signal-regulated kinase (Adanti-ERK2) inhibits platelet-derived growth factor (PDGF-BB)-induced migration of vascular smooth muscle cells. Migration of cells into the lower surface of the filter was quantified by microscopy. The results are representative of three independent experiments. $^*P < 0.01$ vs. untreated; $^{\#}P < 0.01$ vs. PDGF treatment; $^{\Delta}P<0.01$ vs. Ad-*LacZ* and PDGF treatment.

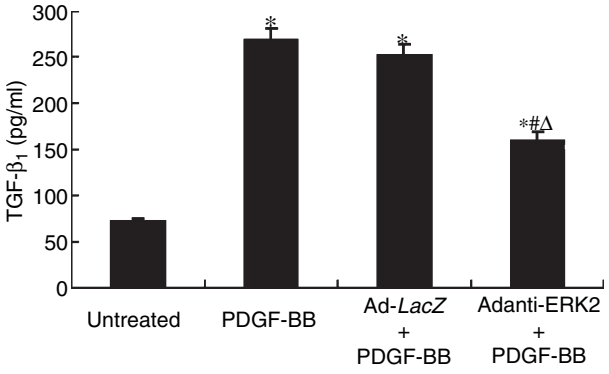


**Figure 5** Adenovirus encoding antisense extracellular signal-regulated kinase (Adanti-ERK2) partially inhibits platelet-derived growth factor (PDGF-BB)-induced transforming growth factor-$\beta_1$ expression. Vascular smooth muscle cells, infected with Adanti-ERK2 or Ad-*LacZ* for 18 h, were exposed to PDGF-BB for 24 h and then subjected to ELISA analysis. The data are representative of three independent experiments. $^*P < 0.01$ vs. untreated; $^{\#}P < 0.01$ vs. PDGF treatment; $^{\Delta}P<0.01$ vs. Ad-*LacZ* and PDGF treatment.

centric occlusive neointimal thickening, prominent loss of medial cellularity and the mean relative intimal thickening (intima/intima + media) were 84.1 ± 4.7% and 79.9 ± 10.4%, respectively. Moreover, the neointima contained mostly α-actin-positive VSMCs. The numbers of VSMCs in the intimal area were 71.3 ± 9.2 and 76.4 ± 11.3 cells per section, respectively. Compared with empty control group and vector control group, the grafted arteries in Adanti-ERK2 group showed slight inti-

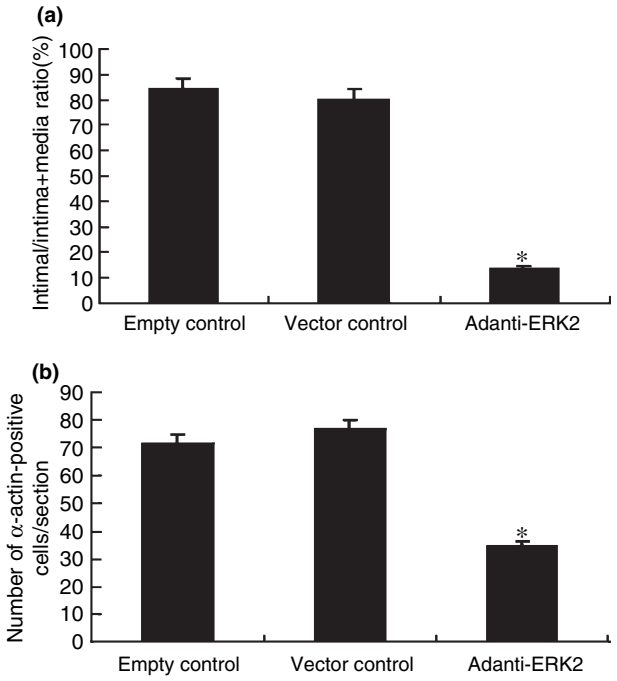


**Figure 6** *Ex vivo* gene transfer of Adenovirus encoding antisense extracellular signal-regulated kinase (Adanti-ERK2) to rat aortic allograft attenuated chronic transplant vasculopathy. At day 60 after transplantation, the aortic grafts were analyzed by histology and morphometry. (a) The thickness of the intima was estimated by the intima/intima + media ratio. Paraformaldehyde-fixed paraffin sections of the grafts were stained with hematoxylin and eosin to measure the intima and media using a computer-assisted morphometry system. (b) Quantitative analysis of α-actin-positive vascular smooth muscle cells in the neointima of grafts. Frozen sections of the grafts were fixed in acetone (–20 °C) for 15 min and stained with the two-layer indirect immunoperoxidase technique using α-actin antibody. The results represent three independent experiments of at least five animals per group. $*P < 0.01$ vs. empty control group and vector control group.

mal thickening (13.7 ± 5.2%) without pronounced neointimal SMC proliferation (34.8 ± 5.3 cells per section) (Fig. 6).

## Discussion

Although the availability of effective immunosuppressive agents may prevent or reverse most acute rejection episodes, the graft may still succumb to CR characterized by transplant vasculopathy. The events of transplant vasculopathy such as atherosclerosis and restenosis include migration of SMCs into the intima from the media and/or proliferation of resident or migratory SMCs and formation of a neointima, which results in intimal thickening, a decrease in caliber of the arterial lumena and graft fibrosis in late failing allografts [22]. Although the origin of the neointimal SMCs that form the basis of these lesions has been much debated [23], accumulating evidences indicate that the aforementioned serial events of VSMCs regardless of their origin are initiated by the release of cytokines and growth factors such as PDGF-BB, basic fibroblast growth factor and TGF-$\beta_1$ [24]. PDGF-BB has a substantial role in the stimulation of VSMC migration and proliferation in an autocrine or paracrine manner during the development of cardiac allograft vasculopathy [6]. Studies on PDGF and its receptor expression in CR have suggested a mitotic effect for PDGF on allograft vasculopathy [25,26]. Evidence suggests that inhibition of PDGF activity was able to decrease chronic allograft dysfunction [27]. PDGF-BB exerts its biologic function through activating multiple intracellular signaling transduction pathways that include SHP-2, Src, PLC-γ, Ras, protein kinase A and MAPK [28,29].

Ongoing studies have elucidated the signaling pathways involved in CR and utilized gene therapy to intervene in candidate pathways. And therapeutic genes may be easily delivered to the grafted tissue prior to transplantation [30]. Evidence supports the notion that ERK may be involved in vascular remodeling or diseases [31]. Our previous study had also suggested that MAPK family members increased expression and delivered extracellular signals to nucleus through Ras–MAPK pathway in CR response [32,33]. And antisense ERK1/2 gene therapy can attenuate graft arteriosclerosis and chronic allograft nephropathy [34,35]. However, most of these studies were carried out using specific inhibitors of the ERK pathway such as the mitogen-activated protein kinase (MEK) inhibitors (PD98059 or U0126) or antisense oligonucleotides. Notably, these approaches do not discriminate between ERK1 and ERK2. *In vivo* and *in vitro* evidences have shown that ERK2 is crucial for transduction of signals [14]. Activation of ERK2 is necessary for PDGF-BB stimulated migration in VSMCs [36]. Therefore, in this study, by using Adanti-ERK2 for specific inhibition endogenous ERK2, we examined the biologic role of ERK2 in PDGF-BB-induced responses of VSMCs.

In the present study, stimulation of VSMCs was carried out by PDGF-BB at a dose of 10 ng/ml in all experiments as PDGF-BB of 10 ng/ml was known to exert the maximal effects on VSMCs [37]. We observed transfection of VSMCs with Adanti-ERK2, through reducing the expression of activated ERK2, could significantly inhibit PDGF-BB-induced cell proliferation. VSMCs normally remain in the $G_0/G_1$ phase of the cell cycle transit through $G_1$ phase and enter into the S phase after stimulation with PDGF-BB, ultimately leading to DNA synthesis [38]. To further explore the mechanism mediating the inhibitory effect of Adanti-ERK2 in VSMC proliferation, a detailed analysis of the cell cycle phases by analysis of DNA content was

 

performed. We confirmed the infection of VSMCs with Adanti-ERK2, which significantly attenuated PDGF-BB-induced increase in S phase entry of cell cycle, resulting in $G_1$ arrest and subsequent growth inhibition. On the other hand, we demonstrated Adanti-ERK2 treatment suppressed PDGF-BB-induced migratory VSMCs. Thus, ERK2 has an important functional role in PDGF-BB-induced proliferation and migration in VSMCs.

Transforming growth factor-$\beta_1$ is a highly pleiotropic cytokine capable of inducing a variety of biologic effects that ultimately control the balance between cellular differentiation and proliferation [39]. It is involved in allograft fibrosis by contributing to the accumulation of ECM and prevention of ECM degradation [40]. Publications suggest that TGF-$\beta_1$ regulates the proliferation and migration of SMCs and ECM deposition in CR. Many effects of TGF-$\beta_1$ can be attributed to the activation of ERK [8]. PDGF-BB is well known to induce TGF-$\beta_1$ expression in VSMCs, which plays a pivotal role in vascular diseases [7]. However, whether ERK pathway is related to PDGF-induced TGF-$\beta_1$ expressions is poorly understood. In this study, we observed Adanti-ERK2 treatment could decrease but merely partially block the TGF-$\beta_1$ expression level of VSMCs stimulated with PDGF-BB. This indicates that several ERK-independent pathways are co-activated in a cell-specific manner in response to TGF-$\beta_1$ [41]. Thus, the expression of TGF-$\beta_1$ depends on the coordinated interplay of different signaling pathways. The biologic effect of ERK pathway is highly context and stimulus specific and is ultimately determined by extensive cross-talk with other kinase systems.

Previously, we reported that ERK pathway participated in intimal hyperplasia caused by transplant vasculopathy [34]. Here, using *ex vivo* gene transfer technique of Adanti-ERK2, we found that Adanti-ERK2 therapy effectively reduces the intimal thickening by suppressing SMC proliferation and migration. This treatment was inserted at the time of transplant, based on the concern that the process of transplant vasculopathy is initiated from the very beginning of transplant as intimal activation is triggered immediately by immunologic and nonimmunologic injuries [42]. Although this Adanti-ERK2 therapy aims at transfection of therapeutic gene into the graft itself, it is also capable of stabilizing the inflammation in the graft so as to lessen the innate immunity through downregulation of cytokines and growth factors, such as PDGF and TGF-β, which contributes in regulating the adaptive immunity. Furthermore, the virus-soaked transplant also could release some vectors, which then infect T cells, correlating with inhibition of immune response as ERK activation is an important event of T cell activation, differentiation and function [43]. However, Adanti-ERK2 gene therapy did not totally inhibit the onset of transplant vasculopathy in our study. One reason may be the limited duration of the therapeutic gene expression, failing to elicit a complete long-term inhibition after transplant. Alternatively, it is also possible that the blockade of ERK pathway *in vivo* is insufficient to prevent the activation of intimal thickening because of the multi-factorial and complex mechanism for transplant vasculopathy. Therefore, the vector improvement and therapeutic gene modification should be addressed in the future study.

In summary, our results suggest that antisense ERK2 gene therapy inhibits PDGF-BB-induced proliferation, migration and TGF-$\beta_1$ expression in VSMCs and attenuates transplant vasculopathy. Furthermore, knowledge about the elaborate molecular mechanisms of ERK pathway may contribute to promising novel and efficient therapeutic targets against CR.

## Authorship

X-LC: wrote the paper and performed research; Z-SC: supervised research and revised the paper critically; ZD: performed research; CD: performed research; HG: analyzed data; N-QG: designed research and revised the paper critically.

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