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Original Article

Polymer-based delivering of shRNA to rabbit aortic smooth muscle cells suppressed the expression of IGF-1R *in vitro* and *in vivo*

Xuebiao Li, Minjian Kong, Aiqiang Dong*, Jianfang Qian, and Daming Jiang

Department of Cardiothoracic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China
*Correspondence address. Tel/Fax: +86-571-87783643; E-mail: dr_dongaiqiang@sina.com

Restenosis is one of clinical limitations for vein graft in coronary bypass graft. It has been proved that signal pathway IGF-1 and its receptor (IGF-1R) activated by hemodynamic mechanical stretch are responsible for the vascular smooth muscle cells proliferation in vein graft neointima formation. Unfortunately, there is no routinely successful method to resolve this problem. Gene delivering to vein graft possesses great therapeutic potential to prevent neointima formation. Polymer is one kind of nanoparticles, which can activate the process of endocytosis of cells. In this study, we evaluated the transfection efficiency and therapeutic potential of polymer-based transfection of plasmids expressing GFP and shRNAs targeting IGF-1R (pGFPshIGF-1Rs) to smooth muscle cells and rabbit external jugular vein graft. Results showed that polymer-based transfection provided high efficiency of transgene expression in smooth muscle cells *in vitro*. *In vitro*, IGF-1R-specific shRNA transfected by polymer inhibited IGF-1R protein expression by 52 ± 3.6%, when compared with mock transfected cells. *In vivo* delivering efficiency of pGFPshIGF-1R plasmid into the rabbit external jugular vein graft was significantly high in the polymer-based transfection group, when compared with negative control group. *In vivo*, polymer-based transfection IGF-1R-specific shRNA efficiently inhibited the expression of IGF-1R protein by 77 ± 3.6%, 65.6 ± 4.9%, and 76.7 ± 4.3% at 24, 48, and 72 h, respectively, when compared with negative control group. Our findings indicated that polymer-based transfection may be a promising technique that allows the targeting of gene therapy for vein graft restenosis.

Keywords vein graft restenosis; gene therapy; polymers; igf-1r

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Introduction

Vein grafts used in coronary artery bypass graft often developed into stenosis due to the proliferation of vascular smooth muscle cells (VSMCs). A vital factor is the hemodynamic mechanical stress induced by blood pressure [1,2], which subsequently activates the IGF-1/insulin-like growth factor-1 receptor (IGF-1R) signaling pathway [3]. It has been demonstrated that IGF-1/insulin-like growth factor-1 receptor (IGF-1R) signaling pathway plays an essential role in VSMC proliferation, inducing neointima proliferation in vein grafts [4]. Proliferation of VSMCs can also be regulated by other growth factors including PDGF, bFGF, thrombin, and angiotensin II [5,6], but IGF-1 has a special role in this process because it regulates the cell cycle of VSMCs and functions both as an endocrine and an autocrine/paracrine growth factor [7,8]. Growth factors, such as bFGF and PDGF, activate IGF-1R transcription and up-regulate IGF-1R expression in VSMCs. Accordingly, the stimulation of an IGF-1/IGF-1R autocrine loop can be pivotal in mediating the growth factor-induced activation of VSMCs. In this regard, IGF-1R may be a new target for preventing vein graft stenosis.

Gene therapy for vein graft disease is effective; however, relatively low efficacy and safety remain to be problems in gene transfer technologies. Viral vectors can achieve efficient arterial gene transfer *in vivo* [9–11], but there is a potential of immunologic and cytotoxic complications [12]. Non-viral methods for *in vivo* gene transfer, such as hydrogel balloon-mediated gene delivery [13], cationic lipid [14], and electroporation-mediated gene transfection [15] have been widely used to introduce DNA into the arterial wall, but the efficiency is relatively low. Synthetic delivery systems such as non-lipid cationic polymers, e.g. polyethylenimine (PEI), polyamidoamine (PAMAM), and poly-L-lysine have been investigated as alternatives to viral vectors [16–18], because they are easier to produce, raise fewer safety issues, and pose fewer problems for repeated administration than viral gene transduction. However, the feasibility of polymer-based gene transfection to smooth muscle cells has never been systematically evaluated. In this study, plasmid DNA conjugated with polymer was applied to assess the efficiency of polymer-based gene transfection to smooth muscle cells *in vitro* and *in vivo*.

Materials and Methods

Cell culture

Under sterile conditions, aortic arteries were excised from the New Zealand rabbits (Zhejiang Academy of Medical Science, Hangzhou, China) as previously described [19]. The vessels were rinsed three times in phosphate buffered saline (PBS) containing antibiotics, then cut longitudinally and dissected into small pieces. Explants were placed in 100-mm culture plates. Five to six hours later, 5 ml of Dulbecco-modified eagle medium (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (Gibco) and antibiotics/antimycotic was carefully added to the culture plates in order not to disturb the adhered explants. Explants were cultured at 37°C in an incubator with 5% CO_2. Medium was removed and replaced every 3 days. Inverted light microscope was used to examine the morphology of culture growth every day. Cells started to grow from explants within 1 week. Two weeks later, cell cultures became ~75% confluent. Aortic smooth cells were used between passages 3 and 5.

Animals

The New Zealand rabbits were raised in clean environment and surgery procedure was performed according to Zhejiang University's institutional guidelines established for animal care and use.

Preparation of plasmid expressing GFP and shRNA targeting IGF-1R

Plasmids expressing GFP and shRNA targeting IGF-1R (pGFPshIGF-1R) were purchased from Genepharma (Shanghai, China) and the complementary sequences of ds-oligos encoding shRNAs and the negative control were as follows: IGF1R-shRNA1, 5′-GCGAGGTCTCCTTCTACTA CATTCAAGAGATGTAGTAGAAGGAGACCTCGCTT-3′; IGF1R-shRNA2, 5′-GCACAACTACTGCTCCAAAGATTC AAGAGATCTTTGGAGCAGTAGTTGTGCTT-3′; negative control shRNA (shNC), 5′-CTTCTCCGAACGTGTCACG TCAAGAGATTACGTGACACGTTCGGAGAATT-3′.

Polymer-based delivering of sh-RNA into smooth muscle cell *in vitro* and *in vivo*

In vitro transfection was performed according to manufacturer's instruction in a 6-well plate. Briefly, the transfection mixture contained 6 μl of MegaTran 1.0 (Origene, Rockville, USA) diluted in antibiotics and serum-free DMEM to a volume of 500 μl. A total of 2 μg of pGFPshIGF-1R was diluted in antibiotics and serum-free DMEM to 500 μl, added to the diluted polymer solution to a total volume of 1 ml, and mixed by pipetting. For the control, 500 μl of antibiotics and serum-free DMEM containing 6 μl of polymer were added instead of diluted pGFPshIGF-1R solution. Mixtures were incubated at room temperature for 15 min. Prior to transfection, the medium was removed and cells were gently washed twice with PBS. Transfection mixtures were added directly to the cells and the cells were subsequently incubated at 37 °C in a incubator with 5% CO_2 for 48 h. Lipo2000 (Invitrogen, California, USA) was used to deliver pGFPshIGF-1R into smooth muscle cells according to manufacturer's instruction.

To avoid possible nonspecific effects of the transfection reagents or the transfection methods, mock transfected cells were applied to provide a more severe control.

In vivo transfection was performed as previously described [20]. Briefly, animals were sedated with ketamine HCl (10 mg/kg IM) and anesthetized with pentobarbital (25–30 mg/kg IV). A segment of 1- to 2-cm external jugular vein graft was surgically exposed through the placement of vascular clamps on the distal external jugular vein and proximal common jugular vein, and the interternal jugular vein was ligated. The control group received 0.2 ml of PBS per rabbit, and the lipofection group received 0.2 ml of Lipo2000/DNA mixture. In polymer-based delivering group, a total of 0.2–0.3 ml of MegaTran 1.0/DNA mixture was instilled into the isolated external jugular veins segment through internal jugular vein. After 20 min of incubation, the vector-containing medium was withdrawn, and the blood flow through the common and external jugular vein was re-established.

Flow cytometry and fluorescence microscopy to analyze efficiency of transfection

Flow cytometry was performed as described previously [21]. Briefly, cultured smooth muscle cells in well plates were washed with PBS, and then trypsinized for 5 min to detach the cells from the well plates. The cell suspension was diluted with PBS to a volume of 500 μl. The expression of GFP was analyzed by using a FACSCalibur dual laser flow cytometer (Becton-Dickinson, Arlington, USA). For each cell sample, 1×10^5 events were collected. Transfection efficiency was expressed as the percentage of GFP-positive vital cells minus the mock transfected control cells. Cells were also visually examined for the presence of green fluorescence by using Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Reverse transcription polymerase chain reaction

Total RNA was extracted with TRIzol reagent (Invitrogen) according to manufacturer's instruction. The polymerase chain reaction (PCR) product (10 μl) was electrophoresed on 2% agarose, stained with ethidium bromide and analyzed by UV absorption using Quantity One Software (Quantity One 1-D; Bio-Rad, Hercules, USA).

Western blot analysis for IGF-1R expression *in vitro* and *in vivo*

Western blot assay was performed as described previously [21]. Briefly, 30 μg of total proteins from smooth muscle

cells and 60 μg of total tissue proteins from rabbit jugular vein graft were separated on 10% sodium dodecyl sulphate-polyacrylamide gels, then analyzed by using anti-IGF-1R and anti-β-actin (Bios, Beijing, China) antibodies.

Immunofluorescence staining

The smooth muscle cells were identified with the presence of α-smooth muscle actin by using monoclonal antibodies (Abcam, Cambridge, Britain). As described previously [22], a cover glass was placed in the culture dish before the cells were seeded. When 50% confluence was reached, the cover glass was fixed with 10% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed three times with PBST, and blocked with 5% BSA for 1 h. Then, mouse monoclonal anti-α-smooth muscle actin antibody (1 : 500 in PBST; Abcam) was applied after the cells were washed three times with PBST, then incubated at 4°C for12 h. The cells were incubated with goat anti-rabbit IgG/Alexa Fluor 555 at 37°C for 2 h, washed with PBST for three times (5 min per wash), further incubated with DAPI (Roche, Basel, Switzerland) at 37°C for 30 min, and then visualized by using an Olympus IX71 fluorescence microscope. For vein graft section staining, the same procedure was performed.

Statistical analysis

Data were expressed as mean ± SD from at least three parallel experiments. Student's two-tailed *t* test and one-way analysis of variance were used to determine the statistical significance of differences in two and multiple groups, respectively. $P < 0.05$ was considered statistically significant. SPSS 17.0 software (SPSS inc., Chicago, USA) was used to analyze data.

Results

IGF-1R-specific shRNA (pGFPshIGF-1R) efficiently inhibits IGF-1R expression

The growth of the cells was observed from vessel explants within 1 week (**Fig. 1**). Primary culture of rabbit aortic smooth muscle cells grew to 70% confluence in ~2.5 weeks. At confluence, cultured VSMCs had uniform morphology that was characterized by elongated cells in parallel rows and by the overlapping layers forming ridges, which is the characteristic of smooth muscle cell [22]. **Figure 1** showed that VSMCs morphology was retained after several passages and the cultured smooth muscle cells showed uniform immunostaining for α-smooth muscle actin. The purity of cultured smooth muscle cell reached almost 100%.

To assess the interference effect, reverse transcription (RT) PCR and western blot assays were performed to evaluate the interference effects of shIGF-1R on IGF-1R expression in smooth muscle cells. Results showed that IGF-1R mRNA level in smooth muscle cells 48 h after transfection with the pGFPshIGF-1R-1 and pGFPshIGF-1R-2 was 58.8 ± 4.7% and 50.7 ± 2.0%, respectively, when compared with shNC.

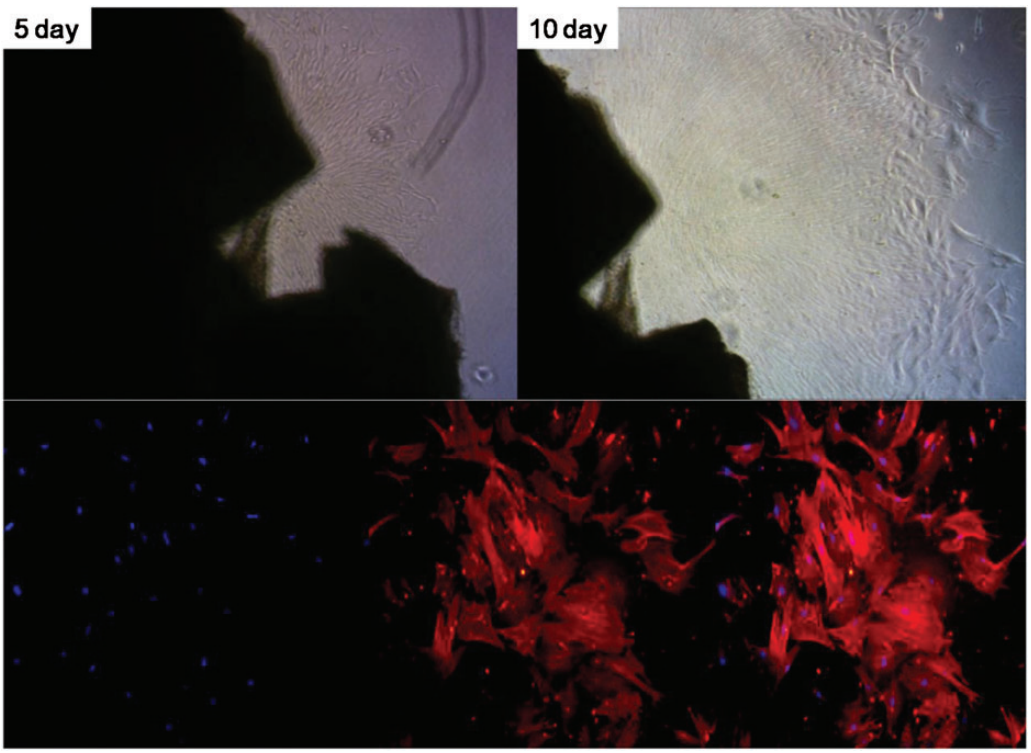

Figure 1. Growth of VSMCs cells from arterial explants VSMC cells started growing from explants within 1 week and grew to confluence within 4 weeks. Antibody staining of rabbit smooth muscle cells. Red: α-smooth muscle actin; blue: DAPI. Magnification, 100 ×.

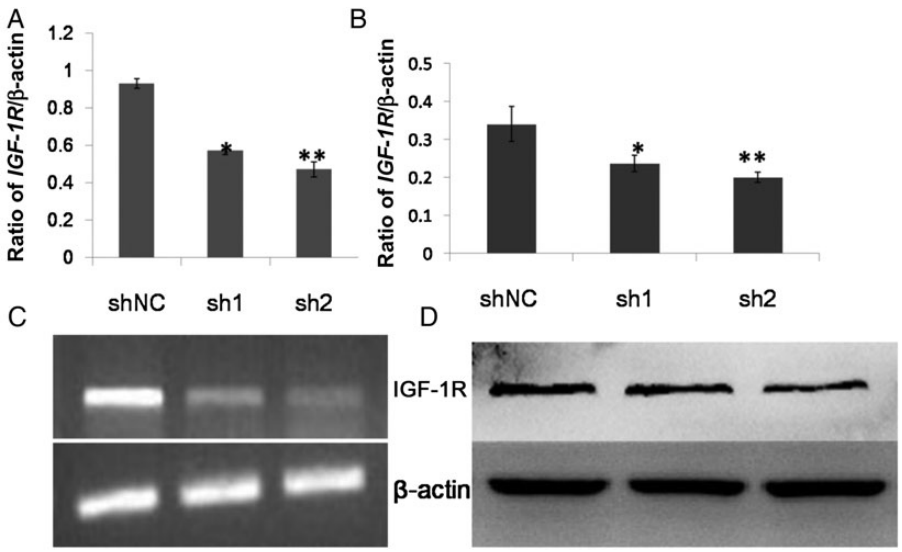

Figure 2. Expression change of IGF-1R in vascular smooth muscle cells (A,C) Downregulation of IGF-1R in vascular smooth muscle cells by plasmids expressing shRNA specific for IGF-1R *ex vivo*. (B,D) Silencing the IGF-1R expression in smooth muscle cells by plasmids expressing shRNA specific for IGF-1R *ex vivo*.

Compared with shIGF-1R-1 group, the shIGF-1R-2 group showed a more potent inhibition for IGF-1R mRNA expression (**Fig. 2A,C**, 49 ± 1.0% of shNC group). Moreover, compared with shNC group, shIGF-1R-2 inhibited IGF-1R expression by 45% ± 1.5% (**Fig. 2B,D**), while the interference effect of shIGF-1R-1 only reached 25.5 ± 0.7%, suggesting that the shIGF-1R-2 plasmid possessed a more potent inhibition for IGF-1R expression.

Polymer-based gene transfection has higher efficiency in cultured smooth muscle cells

In order to obtain higher transfection efficiency, we assessed transfection condition in 24-well plates by modulating the cell density per well and the ratio between shIGF-1R and polymer. When the cell confluence was >80%, the ratio of shIGF-1R between polymers was 1 : 3 (w/v) and the transfection efficiency reached the peak. Under fluorescence microscope, the polymer-based transfection group had higher fluorescence intensity than the lipofection group (**Fig. 3A,B**). Flow cytometry analysis indicated that the lipofection efficiency of pGFPshIGF-1R was 11.0 ± 4.1%, when compared with the control (mock transfected cells), while polymer-based transfection efficiency of shIGF-1R was 28.6 ± 1.2%, when compared with the control after 48 h (**Fig. 3D,E**).

Polymer-based transfection allows site-specific gene delivery *in vivo*

To evaluate whether polymer-based transfection can be a feasible strategy for target-specific gene therapy *in vivo*, local gene transfection was performed with MegaTran 1.0/DNA mixture to rabbit jugular vein graft. The DNA mixture without MegaTran 1.0 was used as control. High expression level of GFP was observed in the jugular vein graft after polymer-based transfection at 24, 48, and 72 h (**Fig. 4**). However, only scattered GFP expression was found in vein graft with lipofection, suggesting that polymer-based transfection could cause specific targeting and the uptake of plasmid DNA into specific target sites (data not shown).

Polymer-based transfection specifically silences IGF-1R protein expression *in vitro* and *in vivo*.

To evaluate the effect of IGF-1R gene silencing by delivering specific shRNA into smooth muscle cells using polymer, western blot assay was performed. Result showed that IGF-1R expression was down-regulated by 49 ± 2.0% in polymer group, when compared with control (**Fig. 5A,C**), however, in lipofection group, IGF-1R expression was down-regulated by 10.4 ± 0.5%, suggesting that polymer-based transfection was an effective way to deliver the target gene.

To further confirm whether the plasmid which expressed GFP and shRNA specific for IGF-1R is effective in the silencing of IGF-1R expression in vein graft, MegaTran 1.0/DNA mixture was locally applied into rabbit external jugular vein graft. Moreover, to determine the difference of GFP and shRNA expression in vein graft after transfection, fluorescence microscopy, and western blot assays were performed to analyze the efficiency of shRNAs delivered by polymer. The expression of GFP remained about 1 week in vein grafts. Western blot analysis showed that shRNAs delivered by both lipofection and polymer effectively inhibited IGF-1R expression *in vivo*. shRNAs delivered by polymer silenced IGF-1R expression more significantly than by lipofection (**$P < 0.05$). The difference within polymer group was not significant ($P > 0.05$). The efficiency of shRNAs delivered by polymer reached 77.0 ± 3.6%, 65.6 ± 4.9%, and 78.0 ± 4.3% at 24, 48, and 72 h, respectively, after pGFPshIGF-1R was instilled (**Fig. 5B,D**), while averagely

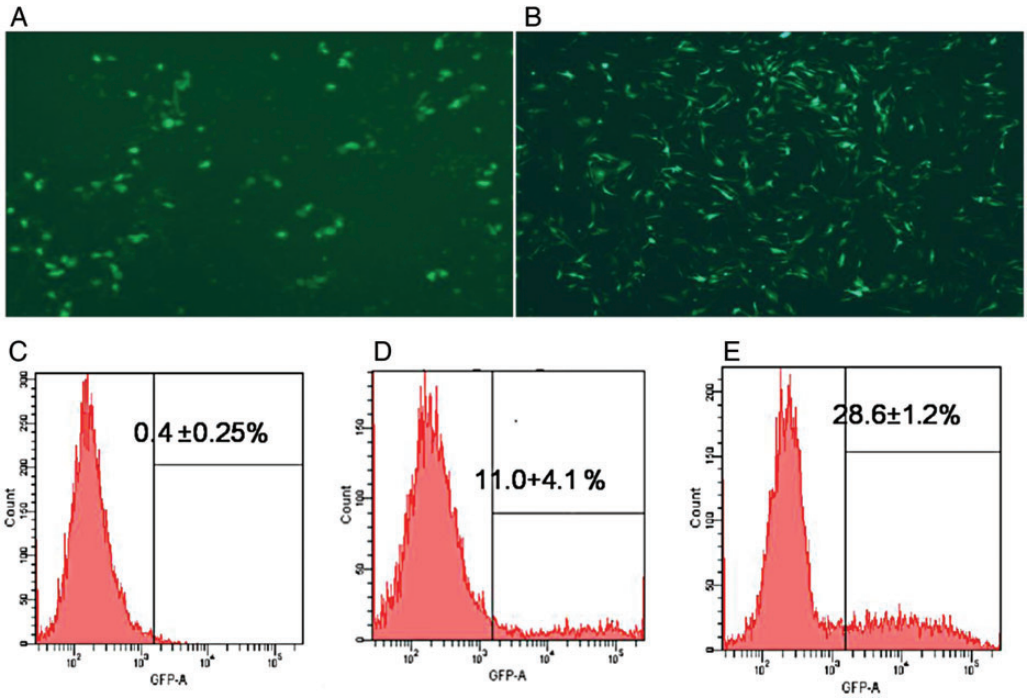

Figure 3. Transfection efficiency of pGFPshIGF-1R in aortic smooth muscle cells using the reporter gene of GFP (A,D) GFP-positive cell in lipofection group. (B,E) GFP-positive cell in polymer group. (C) Control, mock transfected cells. Magnification, 100 ×.

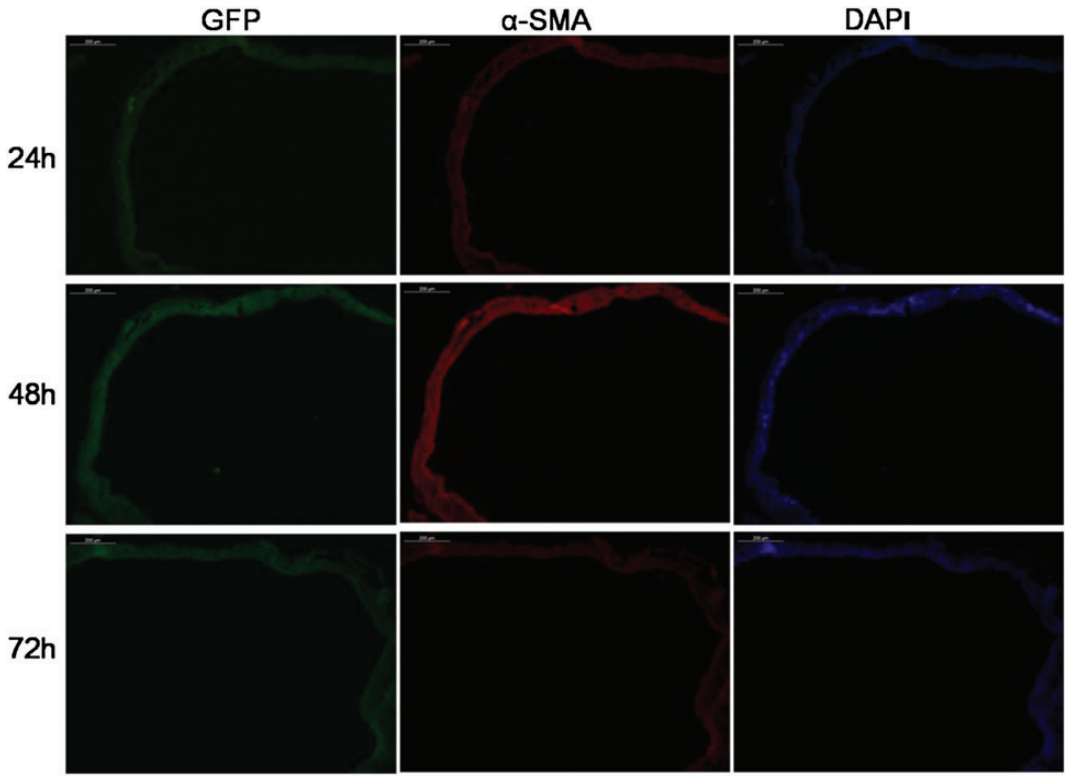

Figure 4. Polymer-based gene transfection *in vivo* Expression level of GFP was detected after polymer-based transfection at 24, 48, and 72 h. Magnification, 100 ×.

reached 52.8 ± 2.5% at 24, 48, and 72 h by lipofection. These results confirmed that the efficiency of gene transfection delivered by polymer was more effective than by lipo2000.

Discussion

Gene therapy can be effective for the treatment of restenosis after angioplasty. Although reports have suggested successful

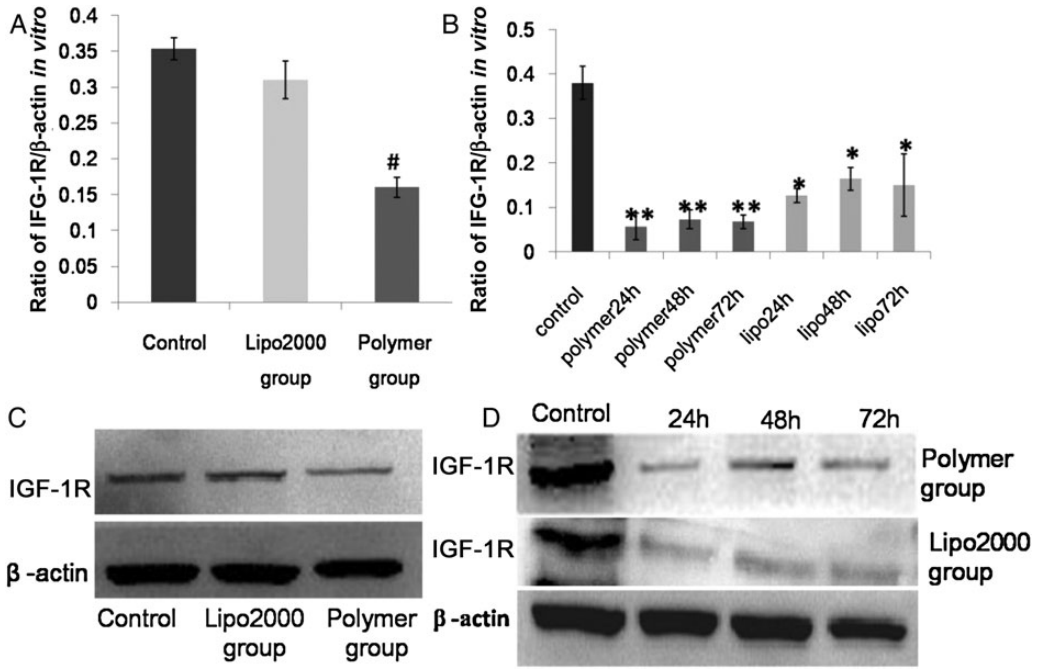

Figure 5. Down-regulation of IGF-1R in smooth muscle cells by plasmids expressing shRNA specific for IGF-1R delivered using polymer *in vitro* (A,C) and *in vivo* (B,D) *In vitro* IGF-1R gene expression was down-regulated by 49 ± 2.0%, when compared with the control, $^{\#}P < 0.03$). The *in vivo* silencing efficiency of shRNAs delivered by polymers reached 77.0 ± 3.6%, 65.6 ± 4.9%, and 78.0 ± 4.3% at 24, 48, and 72 h, respectively, $^{**}P < 0.01$. The statistical differences between 24, 48, and 72 h were not significant ($P > 0.05$).

gene therapy strategies for vascular disease in pre-clinical animal models with available non-viral gene transfer methods [23–26], efficacy and safety are major limitations of gene transfer techniques for humans [27]. The majority of the non-viral vectors used for gene therapy, such as PEI, PAMAM, poly-L-lysine, were developed for favorable transfection efficiency in gene transfer [16,28–30]. However, there are very few reports on introducing foreign DNA into smooth muscle cells using polymer. MegaTran 1.0 is an efficient and versatile agent for gene delivery, which can be used for *in vitro* transfection and possesses advantages including high efficiency, low toxicity, simple application, and extreme affordability [31–33].

Our results confirmed that polymer-based gene transfection can effectively deliver plasmid DNA into smooth muscle cells and vein graft in rabbit. In smooth muscle cells, the polymer-based transfection efficiency of pGFPshIGF-1R was 28.6 ± 1.2%, when compared with control (**Fig. 3**), in contrast, lipofection efficiency with Lipo2000 was only 11.0 ± 4.1% (**Fig. 3D**). We also found that the efficiency was affected by the ratio between shIGF-1R and polymer (data not shown). As other transfection methods, it is an endocytosis-dependent process [34], activating cellular uptake mechanisms rather than promoting uptake. This mechanism of the cellular uptake of transfected genes has been elucidated by transmission electron microscopy. Our results showed that polymer contributed to targeting delivery of therapeutic genes *in vivo*, indicating that the targeting of vessel with polymer increased the site specificity and thus the selectivity of the therapy, and contributed to the reduction of side effects and therapy cost. Furthermore, IGF-1R-specific shRNA of polymer into aortic smooth muscle cells silenced expression of IGF-1R with high suppression efficacy of 49 ± 2.0% (**Fig. 5A,C**), while IGF-1R-specific shRNA of lipofection with Lipo2000 silenced the expression of IGF-1R with a lower suppression efficacy of 10.4 ± 0.5% (**Fig. 5A,C**). This variability might be caused by different amounts of IGF-1R-specific shRNA delivered into aortic smooth muscle cells by polymer and lipofection. Furthermore, **Fig. 5(B,D)** showed that shRNA delivered by polymer down-regulated IGF-1R expression by 77.0 ± 3.6%, 65.6 ± 4.9%, and 78.0 ± 4.3% at 24, 48, and 72 h, respectively. Moreover, the inhibitory effect of shRNA became obvious when the time of pGFPshIGF-1R in the body was extended. And the silencing efficiency by lipofection reached only 52.8 ± 2.5% averagely at 24, 48, and 72 h.

Synthetic delivery systems have been investigated as an alternative to viral vectors because they are easier to produce, raise fewer safety issues, and pose fewer problems for repeated administration than viral gene transduction [35]. Depending on the type of transfected cell, delivery system, the size of the transfection system, or the ratio of DNA to reagent [36], the efficiency was acceptable though less efficient than viral vectors for gene transfection into smooth muscle cells.

In summary, we successfully applied commercially available polymer-mediated gene delivery in primarily cultured aortic smooth muscle cells *in vitro* and further achieved targeting transfection *in vivo*, which provided a novel method of targeting therapeutic gene delivery for gene therapy of vein graft disease. Based on this therapeutic potential, we hypothesized that the inhibition of IGF1R activity in vein grafts using polymer would be an effective approach to suppress graft neointimal hyperplasia in a rabbit model.

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References

1. Bassiouny HS, White S, Glagov S, Choi E, Giddens DP and Zarins CK. Anastomotic intimal hyperplasia: mechanical injury or flow induced. J Vasc Surg 1992, 15: 708–716.
2. Predel HG, Yang Z, von Segesser L, Turina M, Buhler FR and Luscher TF. Implications of pulsatile stretch on growth of saphenous vein and mammary artery smooth muscle. Lancet 1992, 340: 878–879.
3. Cheng JZ and Du J. Activation of the insulin-like growth factor-1 receptor mechanical stretch simulates proliferation of venous smooth muscle cells through. Arterioscler Thromb Vasc Biol 2007, 27: 1744–1751.
4. Du J and Delafontaine P. Inhibition of vascular smooth muscle cell growth through antisense transcription of a rat insulin-like growth factor I receptor cDNA. Circ Res 1995, 76: 963–972.
5. Shaw A and Xu Q. Biomechanical stress-induced signaling in smooth muscle cells: an update. Curr Vasc Pharmacol 2003, 1: 41–58.
6. Wilson E, Mai Q, Sudhir K, Weiss RH and Ives HE. Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. J Cell Biol 1993, 123: 741–747.
7. Du J, Meng XP and Delafontaine P. Transcriptional regulation of the insulin-like growth factor-I receptor gene: evidence for protein kinase C-dependent and -independent pathways. Endocrinology 1996, 137: 1378–1384.
8. Du J, Peng T, Scheidegger KJ and Delafontaine P. Angiotensin II activation of insulin-like growth factor 1 receptor transcription is mediated by a tyrosine kinase-dependent redox-sensitive mechanism. Arterioscler Thromb Vasc Biol 1999, 19: 2119–2126.
9. Arnold TE, Gnatenko D and Bahou WF. *In vivo* gene transfer into rat arterial walls with novel adeno-associated virus vectors. J Vasc Surg 1997, 25: 347–355.
10. Plautz G, Nabel EG and Nabel GJ. Introduction of vascular smooth muscle cells expressing recombinant genes *in vivo*. Circulation 1991, 83: 578–583.
11. Lee SW, Trapnell BC, Rade JJ, Virmani R and Dichek DA. *In vivo* adenoviral vector-mediated gene transfer into balloon-injured rat carotid arteries. Circ Res 1993, 73: 797–807.
12. Riessen R, Rahimizadeh H, Blessing E, Takeshita S, Barry JJ and Isner JM. Arterial gene transfer using pure DNA applied directly to a hydrogel-coated angioplasty balloon. Hum Gene Ther 1993, 4: 749–758.
13. Nabel EG, Plautz G and Nabel GJ. Site-specific gene expression *in vivo* by direct gene transfer into the arterial wall. Science 1990, 249: 1285–1288.
14. Channon KM, Qian HS, Youngblood SA, Olmez E, Shetty GA, Nepliouevа V, Blazing MA and George SE. Acute host-mediated endothelial injury after adenoviral gene transfer in normal rabbit arteries: impact on transgene expression and endothelial function. Circ Res 1998, 82: 1349–1351.
15. Matsumoto T, Komori K, Shoji T, Kuma S, Kume M, Yamaoka T, Mori E and Furuyama T. Successful and optimized *in vivo* gene transfer to rabbit carotid artery mediated by electronic pulse. Gene Ther 2001, 8: 1174–1179.
16. Li S and Huang L. Nonviral gene therapy: promises and challenges. Gene Ther 2007, 7: 31–34.
17. Luo D and Saltzman WM. Synthetic DNA delivery systems. Nat Biotechnol 2000, 18: 33–37.
18. Nishikawa M and Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. Gene Ther 2001, 12: 861–870.
19. Leik CE, Willey A, Graham MF and Walsh SW. Isolation and culture of arterial smooth muscle cells from human placenta. Hypertension 2004, 43: 837–840.
20. Ríos CD, Ooboshi H, Piegors D, Davidson BL and Heistad DD. Adenovirus-mediated gene transfer to normal and atherosclerotic arteries a novel approach. Arterioscler Thromb Vasc Biol 1995, 15: 2241–2245.
21. Chunmao W, Chao D, Minjian K, Aiqiang D, Jianfang Q and Daming J. Tumor-targeting magnetic lipoplex delivery of short hairpin RNA suppresses IGF-1R overexpression of lung adenocarcinoma A549 cells *in vitro* and *in vivo*. Biochem Biophys Res Commun 2011, 410: 537–542.
22. Teng B, Ansari HR, Oldenburg PJ, Schnermann J and Mustafa SJ. Isolation and characterization of coronary endothelial and smooth muscle cells from adenosine A 1 receptor-knockout mice. Am J Physiol Heart Circ Physiol 2006, 290: H1713–H1720.
23. Rekhter MD, Simari RD, Work CW, Nabel GJ, Nabel EG and Gordon D. Gene transfer into normal and atherosclerotic human blood vessels. Circ Res 1998, 82: 1243–1252.
24. von der Leyen HE, Gibbons GH, Morishita R, Lewis NP, Zhang L, Nakajima M, Kaneda Y, Cooke JP and Dzau VJ. Gene therapy inhibiting neointimal vas-cular lesion: *in vivo* transfer of endothelial cell nitric oxide syn-thase gene. Proc Natl Acad Sci USA 1995, 92: 1137–1141.
25. Matsumoto T, Komori K, Yonemitsu Y, Morishita R, Sueishi K, Kaneda Y and Sugimachi K. Hemagglutinating virus of Japan-liposome-mediated gene transfer of endothelial cell nitric oxide synthase inhibits intimal hyperplasia of canine vein grafts under conditions of poor runoff. J Vasc Surg 1998, 27: 135–144.
26. Yonemitsu Y, Kaneda Y, Tanaka S, Nakashima Y, Komori K, Sugimachi K and Sueishi K. Transfer of wild-type p53 gene effectively inhibits vascular smooth muscle cell proliferation *in vitro* and *in vivo*. Circ Res 1998, 82: 147–156.
27. Bai H, Morishita R, Kida I, Yamakawa T, Zhang W, Aoki M and Matsushita H, *et al*. Inhibition of intimal hyperplasia after vein grafting by *in vivo* transfer of human senescent cell-derived inhibitor-1 gene. Gene Ther 1998, 5: 761–769.
28. Narang AS, Cheng K, Henry J, Zhang C, Sabek O, Fraga D and Kotb M, *et al*. Vascular endothelial growth factor gene delivery for revascularization in transplanted human islets. Pharm Res 2004, 21: 15–25.
29. Mahato RI, Henry J, Narang AS, Sabek O, Fraga D, Kotb M and Gaber AO. Cationic lipid and polymer-based gene delivery to human pancreatic islets. Mol Ther 2003, 7: 89–100.
30. Benhamou PY, Moriscot C, Prevost P, Rolland E, Halimi S and Chroboczek J. Standardization of procedure for efficient *ex vivo* gene transfer into porcine pancreatic islets with cationic liposomes. Transplantation 1997, 63: 1798–1803.

31. Iliuk A, Liu XS, Xue L, Liu X and Tao WA. Chemical visualization of phosphoproteomes on membrane. Mol Cell Proteomics 2012, 11: 629–639.
32. Doyle JR, Fortin JP, Beinborn M and Kopin AS. Selected melanocortin 1 receptor single-nucleotide polymorphisms differentially alter multiple signaling pathways. J Pharmacol Exp Ther 2012, 342: 318–326.
33. Song B, Liu XS, Davis K and Liu X. Plk1 phosphorylation of Orc2 promotes DNA replication under conditions of stress. Mol Cell Biol 2011, 31: 4844–4856.
34. Schaffert D and Wagner E. Gene therapy progress and prospects: synthetic polymer-based systems. Gene Ther 2008, 15: 1131–1138.
35. Kiefer K, Clement J, Garidel P and Peschka-Süss P. Transfection efficiency and cytotoxicity of nonviral gene transfer reagents in human smooth muscle and endothelial cells. Pharm Res 2004, 21: 1009–1017.
36. Da Cruz MT, Simões S, Pires PP, Nir S and De Lima MC. Kinetic analysis of the initial steps involved in lipoplex-cell interactions: effect of various factors that influence transfection activity. Biochim Biophys Acta 2001, 1510: 136–151.