Prx1 promotes the proliferation and migration of vascular smooth muscle cells in a TLR4-dependent manner

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Abstract. The present study aimed to examine the effect of peroxiredocin 1 (Prx1) on vascular smooth muscle cell (VSMC) proliferation, invasion and migration abilities, and to examine the effect of Toll-like receptor 4 (TLR4) gene silencing on VSMC proliferation, invasion and migration induced by the overexpression of Prx1. The expression of Prx1 in rats with vein graft intimal hyperplasia (IH) was detected using western blot analysis. In addition, VSMCs were retrovirally transfected to establish stable cell lines overexpressing Prx1. An MTT assay was used to determine the effect of the overexpression of Prx1 on VSMC proliferation. A Transwell assay was used to detect the effect of the overexpression of Prx1 on the invasion of VSMCs and a wound-healing assay was used to determine the effect of the overexpression of Prx1 on the migration of VSMCs. The Prx1-overexpressed cells were then transfected with interference plasmids of TLR4, to detect the effect of silencing the TLR4 gene on the proliferation and migration of VSMCs using MTT and wound-healing assays. The results showed that the expression of Prx1 (1.067±0.03) in the IH group was significantly higher, compared with that in the control (0.677±0.05). The number of VSMCs able penetrated the membrane in Prx1-overexpressed group (150±17/visual field) was significantly higher, compared with that in the control (40±5/visual field). The VSMCs in the Prx1-overexpressed group possessed significantly higher migration ability, compared with those in the control group. The numbers of viable cells in the Prx1-overexpression groups were $5.625\pm0.1\times10^5$, $8.9\pm0.737\times10^5$ and $10.635\pm0.065\times10^5$, respectively, on days 1, 2 and 3 post-transfetion, which were significantly higher, compared with those in the control group ($3.0\pm0.025\times10^5$, $4.1\pm0.035\times10^5$ and $5.06\pm0.023\times10^5$. The overexpression of Prx1 promoted the proliferation, invasion and migration abilities of the VSMCs. Silencing of the TLR4 gene attenuated the Prx1-induced proliferation and migration of the VSMCs. Therefore, the promotion of VSMC proliferation and migration by Prx1 was dependent on TLR4, which may become a novel target in the diagnosis, treatment and prognosis of patients with IH.

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Introduction

The proliferation and migration of vascular smooth muscle cells (VSMCs), and the deposition of extracellular matrix (ECM) results in intimal hyperplasia (IH) (1-8). The proliferation and migration of VSMCs is stimulated and regulated by a series of factors in the injury response (9-16). Growth factors, metalloproteinases induced by matrix proteins, white blood cells, endothelial cells, fibroblasts and plasma are all involved in the proliferation and migration of VSMCs in response to injury (10,11,17). Also included are platelet-derived growth factor (PDGF), basic fibroblast growth factor, acidic fibroblast growth factor, insulin-like growth factor 1, tumor growth factor (TGF), epidermal growth factor, connective TGF, and vascular endothelial growth factor (VEGF) (18-22). Among these, PDGF may be vital in IH by stimulating the migration of VSMCs. There are a variety of factors, which affect VSMC proliferation and migration, however, the underlying mechanism remains to be fully elucidated, and there remains no effective therapeutic target for coronary artery bypass vein graft IH.

Peroxiredoxin-1 (Prx-1), as a member of the Prx family, is a class of antioxidant enzyme. Previous studies on Prx-1 have focussed predominantly on its roles in antioxidant activity, including ROS scavenging, which protect cells from damage caused by ROS (23,24). Previously, studies have shown that Prx1 is expressed at high levels in various types of tumor tissues, including esophageal squamous cell carcinoma (25), pancreatic cancer (26), astrocytoma (27) and prostate cancer (28), and it has been reported to be closely associated with tumor growth, proliferation, differentiation, invasion and migration (25-33). Although Prx1 has been shown to promote the proliferation of VSMCs (29), the effects of Prx1 on the invasion and migration of VSMCs and the underlying mechanisms remain to be fully elucidated.

The present study was designed to examine the effect of Prx1 on the proliferation, invasion and migration abilities of VSMCs, and to reveal the underlying mechanisms. The expression levels of Prx1 in the rat vein graft IH group were detected using western blot analysis. Retroviral transfection of the Prx1 plasmid was performed to construct stable VSMCs overexpressing Prx1. An MTT assay was used to detect the effect of overexpressed Prx1 on VSMC proliferation. The effect of overexpressed Prx1 on VSMC viability was measured using a Transwell assay. A wound-healing assay was used to detect the effect of overexpressed Prx1 on VSMC migration ability. The Prx1-overexpressing cells were then transfected with the TLR4 interference plasmid, and MTT and wound-healing assays were used to determine the effect of TLR4-silencing on the Prx1-induced proliferation and migration of the VSMCs.

Materials and methods

Antibodies and reagents. The rabbit-anti-rat Prx1 antibody (cat. no. ab208919) and goat anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody (cat. no. ab6721) were purchased from Abcam (Cambridge, MA, USA). The rabbit-anti-rat β-actin antibody (cat. no. sc-130656) was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The RevertAid™ First Strand cDNA Synthesis kit and DreamTaq™ Green PCR master mix were purchased from Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA), Lipofectamine™2000 and TRIzol® reagent were from Invitrogen; Thermo Fisher Scientific, Inc.). The gel DNA extraction kit, ribonuclease A and trypsin were from Takara Bio, Inc. (Otsu, Japan). The plasmid extraction and purification kit was from TianGen Biotech Co., Ltd. (Beijing, China). The GeneAmp PCR-System 9600 was from Applied Biosystems; Thermo Fisher Scientific, Inc.). TheUV-1700 spectrophotometer was from Shimadzu Corporation (Kyoto, Japan). Precooled Matrigel gum was from BD Biosciences (San Jose, CA, USA). Transwell chambers were purchased from EMD Millipore (Billerica, MA, USA).

Construction of the rat common carotid artery grafting model. Healthy, clean, male Sprague-Dawley rats (300-400 g; 10-12 weeks old; n=6) were purchased from the Laboratory Animal Center of Jilin University (Jilin, China). The rats were treated according to institutional guidelines of the Laboratory Animal Center of Jilin University, and the experimental protocol was approved by the local ethical committee. The rats were housed under controlled environmental conditions with ambient temperature of 25˚C, relative humidity of 65%, and a 12/12-h light-dark cycle. Food and water were provided *ad libitum*. Experiments were performed following 1 week of acclimation. All rats were anesthetized by intraperitoneal injection of chloral hydrate (30 mg/kg). The rats were fixed in the supine position and the cervical region were shaved and sterilized. A 3 cm long longitudinal incision was made along the median line of the anterior neck under an operating microscope to exposure the external jugular vein, followed by the injection of 1.5 mg/kg heparin saline into the common cardinal vein. The rats were then divided into two groups, the experimental group (n=3) and the control group (n=3). In the experimental group, a 0.5-0.8 cm long segment of the vessel was removed and the vessel was ligated using nylon 6 oligomer. The common carotid artery was exposed and a segment of the same length was cut. Immediately, a segment of the external jugular vein was anastomosed to the common carotid artery. No transplantation experiments were performed in the control group. Subsequently, the incisions in all six rats were closed. At 4 weeks post-transplantation, the rats were anesthetized by intraperitoneal injection of chloral hydrate (30 mg/kg). The graft was harvested, flushed with saline solution, cleaned of adipose tissue and then was fixed in 4% paraformaldhyde. Subsequently, the graft was stained with hematoxylin and eosin solution (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The arterial vasculature at same site and of the same length, were harvested from the normal rats (n=3), which had not received transplantation, and were cultured in the same environment as the experimental group. All animals survived 1 month following transplantation. The rats were sacrificed by an overdose of pentobarbital sodium (intraperitoneally, 100 mg/kg body weight; Sigma-Aldrich; Merck Millipore). The degree of IH was assessed and confirmed by morphometric analysis.

Cell culture and transfection experiments in vitro. The CRL-1476 rat VSMC line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2, and then seeded into six-well plates until the VSMCs were adherent and at logarithmic growth phase at 24 h. When 60-80% confluent, the cells were transfected with retrovirus for the overexpression of Prx1 and either the pGenesil-1 scramble short hairpin RNA or pGenesil-1-small interfering (si)RNA-TLR4 recombinant plasmid using Lipofectamine™2000 according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The mRNA expression level of Prx1 in the CRL-1476 cells was investigated using RT-qPCR analysis. Total RNA was extracted from the CRL-1476 cells using TRIzol reagent according to the manufacturer's protocol. The RNA preparations were quantified using agarose gel electrophoresis, following which the optical density (OD)260/OD280 ratio of the RNA was measured on a spectrophotometer and then stored at -20˚C.

cDNA was synthesized by using 1.25 μM oligo dT (Promega Corporation, Madison, WI, USA), 0.15 μg random primers (Invitrogen; Thermo Fisher Scientific, Inc.), and 200 U Super-Script III reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) from 2 μg total RNA in a total volume of 20 μl. The PCR mixture included 2.5 μl Taq buffer (10X), 5 μl $MgCl_2$ (25 mmol/l), 2 μl dNTP (2.5 mmol/l), 0.5 μl upstream primer (20 μmol/l), 0.5 μl downstream primer (20 μmol/l), 2 μl cDNA template, 0.3 μl Taq (5 U/μl), 0.6 μl TaqMan probe (10 μmol/l) and 11.6 μl double distilled H_2O. The sequences of the primers were as follows: Prx1, forward 5'-CCCGGATGC TTTTGTTCGAGA-3' and reverse 5'-CATGTGGCAGAA TAAGTAGCCAT-3'; TLR4, forward 5'-GCCTTGAATCCA GATGAAAC-3' and reverse 5'-CTGTGAGGTCGTTGAGGT TAG-3'; β-actin, forward 5'-CACGATGGAGGGGCCGGA CTCATC-3' and reverse 5'-TAAAGACCTCTATGCCAACAC AGT-3'. RT-qPCR analysis was performed at 95˚C for 5 min, followed by 40 cycles at 95˚C for 40 sec, 55˚C for 40 sec and

72˚C for 40 sec. Fluorescence was detected following each cycle and analyzed using the ABI PRISM 7000 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative mRNA level was calculated using the comparative threshold cycle (Cq) method ($2^{-\Delta\Delta Cq}$) (34) normalized by β-actin expression. The PCR products were analyzed using agarose gel and confirmed by melting curve analyses.

Western blot analysis. Protein was extracted from the cells and tissue samples from the vein grafts in each group for western blot analysis. Protein was isolated from cells using radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck Millipore) according to the manufacturer's instructions. Following centrifugation at 12,000 x g for 30 min at -4˚C, the supernatant was collected. The protein concentration was measured by the bicinchoninic acid protein assay (Sigma-Aldrich; Merck Millipore). Equal quantities of protein were separated on 12% SDS-PAGE gels, followed by transfer onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS) with Tween 20 for 1 h and probed with the rabbit anti-rat primary antibodies (1:800) overnight at 4˚C. Following three washes with Tris-buffered saline-Tween 20, the membranes were incubated with goat anti-rabbit IgG-HRP secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc.). The intensities of the bands were quantified using the NIH ImageJ software package (http://rsb.info.nih.gov/ij/). The expression level of β-actin served as an internal control for protein loading.

Transwell assay. VSMCs in logarithmic growth phase were collected and digested, and then resuspended with serum-free medium, adjusting the cell concentration to $1x10^5$/100 μl. Precooled Matrigel gum (BD Biosciences) was diluted with serum-free Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.), and added to evenly coat the bottom film of Transwell chambers (50 μl/well), followed by incubation for 30 min at room temperature. 100 μl cell suspension was added in each upper chamber of the Transwell chamber, 600 μl medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) was added in the lower chambers to avoid the generation of bubbles. The paved 24-well cell Transwell chamber (8 μm) cell culture plate was placed in a 37˚C incubator of saturated humidity and 5% CO_2, and cultured for 24 h. Following removal of the chamber, the medium was aspirated, washed twice using PBS, and a cotton swab was used to remove the cells on the upper chamber surface. Anhydrous methanol fixing was performed for 10 min, followed by 30 min staining with 0.05% crystal violet dye at room temperature. Following washing twice with PBS, the number of cells in three randomly selected visual fields per well were counted under a microscope, and the average calculated. Three replicate wells were used and the experiment was repeated three times.

Wound healing assay. The migratory ability of the cells was examined using a wound healing assay. Briefly, the VSMCs were seeded into six-well plates at a density of $1x10^5$ cells per well, and were cultured for 48 h to grow to full confluence in the plates. Subsequently, the cell layer were wounded using 200 μl pipette tips and washed with PBS to remove detached cells. The cells were then cultured with 5% CO_2 at 37˚C for 48 h. Photomicrographs of the initial wounds and final wounds were captured using an inverted microscope. The initial and final wound sizes were measured using AxioVision Rel.4.7 software (Zeiss GmbH, Jena, Germany).

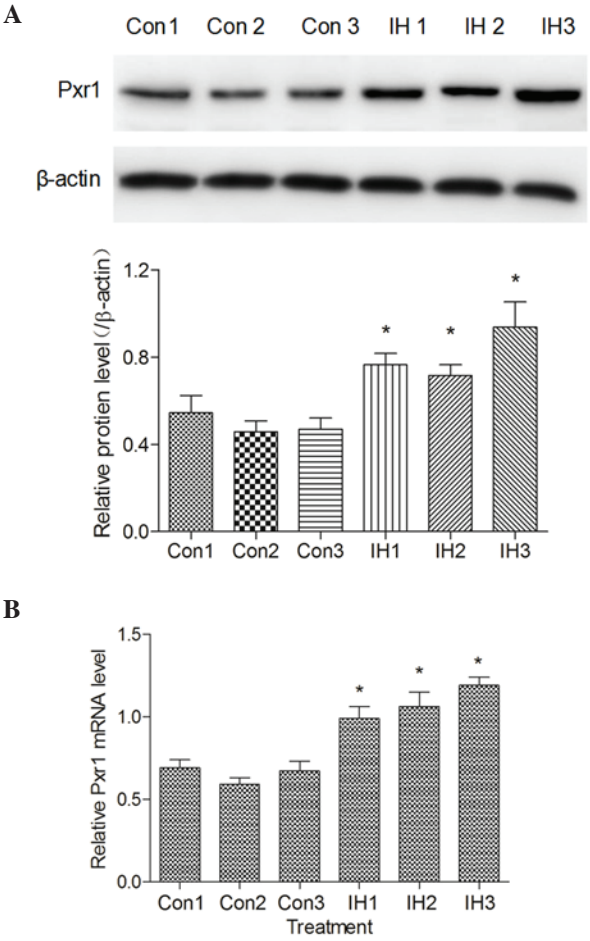

Figure 1. Analysis of the expression levels of Prx1 in rats with IH. (A) Western blot analysis was used to detect the expression of Prx1. (B) Relative mRNA expression levels of Prx1 in the IH and control groups were detected using reverse transcription-quantitative polymerase chain reaction analysis. Results are shown as the Prx1 mRNA copy number normalized to β-actin and are expressed as the mean ± standard deviation of three separate determinations. *P<0.05 vs. Con mean. Prx1 peroxiredocin 1; IH, intimal hyperplasia; Con, control.

MTT assay. Cell proliferation was determined using an MTT assay. The VSMCs were inoculated onto a 24-well plate at a density of $1x10^5$ cells per well 24 h post-transfection. The cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 for 24 h. Subsequently, 20 ml MTT dye was added (5 mg/ml), and incubation was continued for 4 h. Dimethyl sulfoxide (100 μl) was added and agitated for 5 min to mix thoroughly, and the absorbance was detected using ELISA at 595 nm.

Statistical analysis. Data are shown as the mean ± standard deviation of triplicate determinations, calculated using SPSS software (version 10.0; SPSS, Inc., Chicago, IL, USA).

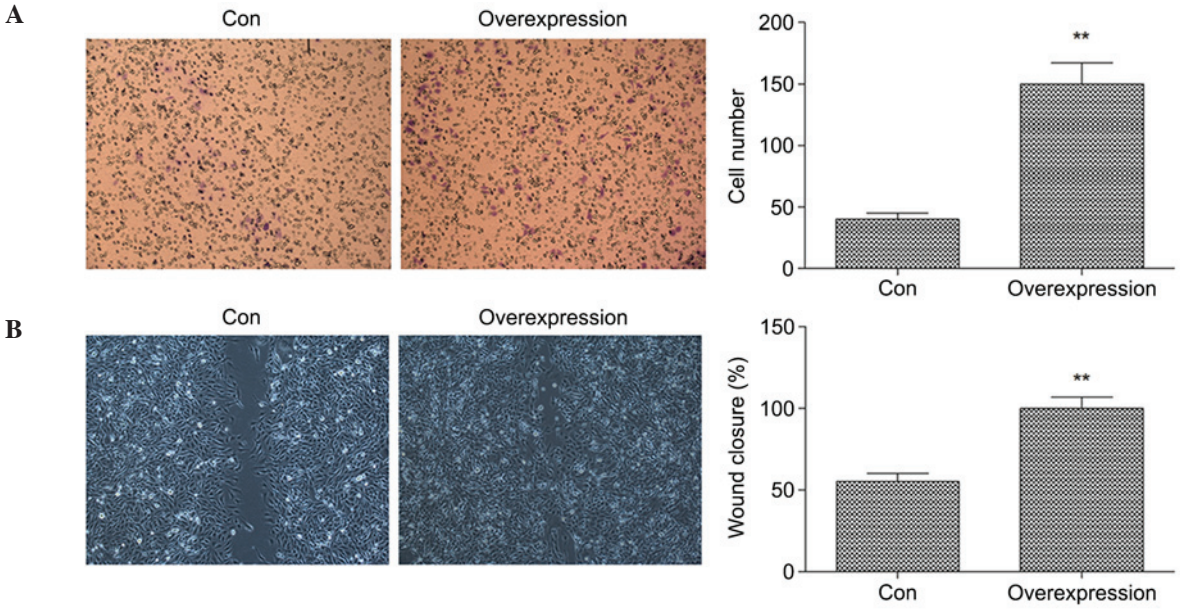

Figure 2. Overexpression of Prx1 enhances the invasion and metastatic abilities of vascular smooth muscle cells. (A) Representative photomicrographs of cell infiltration of the Transwell membrane in the control and Prx1 overexpression groups. (B) Representative photomicrographs of cell monolayers 48 h post-wounding. The bar graphs showed the results of quantification, with data shown as the mean ± standard deviation of three samples. Magnification, x200. **P<0.01 vs. Con. Prx1 peroxiredocin 1; Con, control.

Student's *t*-test was used for comparison between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Prx1 in grafted vessels of the IH rat model. The expression levels of Prx1 in the rat carotid artery bypass graft model were detected using western blot analysis. Additionally, the mRNA expression levels of Prx1 in each group were detected using RT-qPCR analysis. Each group was analyzed in duplicate and in three separate trials. The results showed that the protein (Fig. 1A) and mRNA (Fig. 1B) expression levels of Prx1 in the IH groups were significantly higher, compared with those in the control groups.

Overexpression of Prx1 promotes VSMC invasion and migration. A retroviral vector was transfected into VSMCs, and the transfection efficiency was detected using western blot and RT-qPCR analyses. The results showed that the expression of Prx1 in the retroviral cells was significantly higher, compared with that in the control group. Date analysis showed that the relative mRNA expression levels of Prx1 in the IH groups were increased by almost 50%, compared with those in the control groups (data not shown), which indicated that the VSMCs overexpressing Prx1 had been constructed successfully.

At 48 h post-VSMC transfection with the Prx1 gene, a Transwell assay was used to detect the effect of the overexpression of Prx1 on the invasion capability of the VSMCs. The results suggested that, compared with the control group, the number of cells able to pass through Transwell chamber was significantly increased from 40±5/visual field to 150±17/visual field (Fig. 2A), in the Prx1-overexpressed group, indicating that the overexpression of Prx1 promoted the invasive capability of the VSMCs. A wound healing assay was used to detect the effect of the overexpression of Prx1 on VSMC migration. The results showed that the VSMC migration ability in the Prx1-overexpressed group was significantly higher, compared with that in the control group, with the degree of wound healing increased from 55 to 100% (Fig. 2B), which suggested that the overexpression of Prx1 enhanced the invasion and migration capacities of the VSMCs.

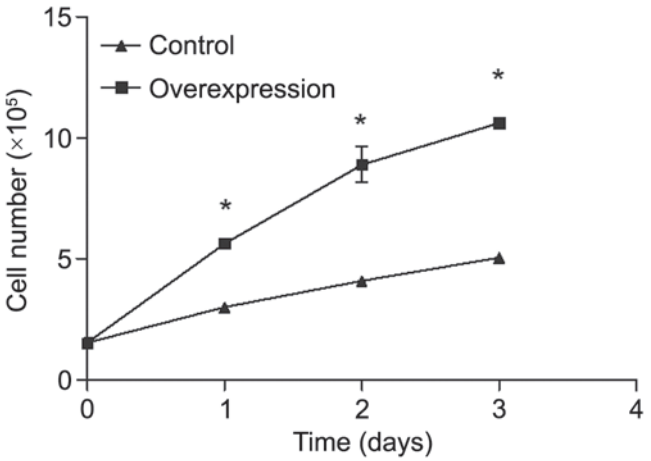

Figure 3. Overexpression of peroxiredocin 1 promotes vascular smooth muscle cell proliferation *in vitro*. Cell growth was determined using an MTT assay at 24 h. The initial cell number was $1x10^5$. Results are expressed as the mean ± standard deviation of triplicate determinations. *P<0.05, vs. control.

Overexpression of Prx1 promotes VSMC proliferation. Cell viability was determined using an MTT assay. The results showed that number of surviving cells in the Prx1-overexpressing group were $5.625±0.1x10^5$, $8.9±0.737x10^5$ and $10.635±0.065x10^5$ on days 1, 2 and 3, respectively, which were significantly

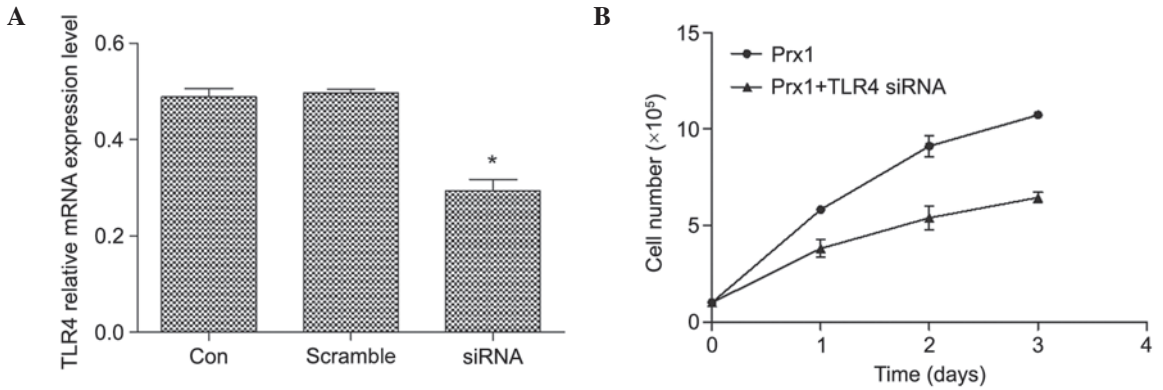

Figure 4. Gene silencing of TLR4 inhibits VSMC proliferation induced by the overexpression of Prx1 *in vitro*. (A) mRNA expression levels of TLR4 in the Prx1-transfected VSMCs either without siRNA transfection (Con), with non-targeting short hairpin RNA transfection (scramble) or co-transfected with retrovirus of Prx1 and TLR4 siRNA (siRNA). Results are shown as the TLR4 mRNA copy number normalized to β-actin and expressed as the mean ± standard deviations of three separate determinations. (B) Cell growth in the Prx1 overexpression group and in the group of cells co-transfected with retrovirus of Prx1 and TLR4 siRNA were determined using an MTT assay at 24 h. The initial cell number was $1x10^5$. *P<0.05 vs. Con. Prx1 peroxiredocin 1; TLR4, Toll-like receptor 4; siRNA, small interfering RNA.

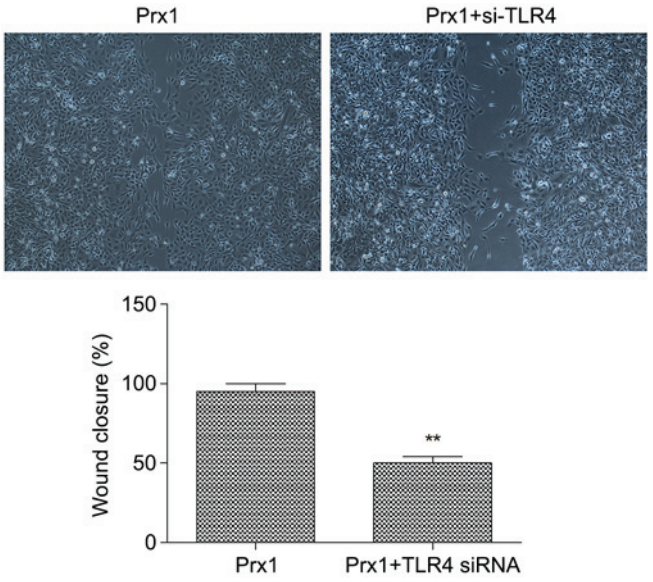

Figure 5. Gene silencing of TLR4 inhibits vascular smooth muscle cell metastatic ability induced by the overexpression of Prx1 *in vitro*. Representative photomicrographs of cell infiltration of the Transwell membrane in the Prx1 overexpression group and Prx1+TLR4 siRNA group (cells co-transfected with retrovirus of Prx1 and TLR4 siRNA). The bar graph shows the quantification of the results, with data experessed as the mean ± standard deviation of three samples. Magnification, x200. **P<0.01 vs. Prx1. Prx1 peroxiredocin 1, TLR4, Toll-like receptor 4; siRNA, small interfering RNA.

higher, compared with the number of surviving cells in the control group, which were $3.0\pm0.025x10^5$, $4.1\pm0.035x10^5$ and $5.06\pm0.023x10^5$ on days 1, 2 and 3, respectively (Fig. 3). These results demonstrated that the overexpression of Prx1 promoted cell proliferation of the VSMCs.

TLR4 silencing inhibits the Prx1-induced proliferation of VSMCs. To verify the transfection efficiency of TLR4 siRNA in the VSMCs, RT-qPCR analysis was performed, and the mRNA expression levels of TLR4 in the control group, scramble group and siRNA group were detected. The results showed that the mRNA expression of TLR4 was effectively inhibited in the TLR4 siRNA-transfected group, compared with those in the control and scramble groups (Fig. 4A). An MTT assay was then used to determine the proliferation of the Prx1-overexpressed VSMCs following TLR4 gene silencing. The results showed that the number of surviving cells in the TLR4 siRNA Prx1-overexpressed group on days 1-3 were $3.824\pm0.43x10^5$, $5.395\pm0.6x10^5$ and $6.456\pm0.28x10^5$, respectively, which were significantly lower, compared with the numbers in the Prx1-overexpressed group on these days (5.825 ± 0.12, 9.1 ± 0.5375 and $10.735\pm0.075x10^5$, respectively; Fig. 4B). This suggested that the silencing of TLR4 inhibited the cell proliferation induced by Prx1.

Silencing of TLR4 inhibits the Prx1-induced migration of VSMCs. The effect of TLR4 gene silencing on cell migration in the VSMC was determined using a wound healing assay. The results showed that, in the TLR4 interference Prx1-overexpressed group, the migration ability of the cells was significantly attenuated, compared with that in the Prx1-overexpressed group without TLR4 interference. The healing rate was decreased from 95 to 50% (Fig. 5) suggesting that silencing of TLR4 inhibited the cell migration induced by Prx1.

Discussion

Previous studies have demonstrated that Prx1 is expressed at high levels in esophageal squamous cell carcinoma (25), pancreatic cancer (26), astrocytomas (27) and prostate cancer (28), which is closely associated with tumor growth, cell proliferation, differentiation, invasion and migration. Gong *et al* (25) found that the downregulation Prx1 inhibits cell proliferation and invasion of esophageal squamous cell carcinoma, and promotes apoptosis. Taniuchi *et al* (26) revealed that Prx1 promotes pancreatic cancer invasiveness via the p38/mitogen-activated protein kinase pathway. Prx1 is important in invasion and malignancy in the development of astrocytoma, offering potential as a tumor marker and therapeutic target for astrocytoma (27). As a ligand of TLR4, Prx1 stimulates the inflammatory reaction, and

promotes the proliferation, metastasis and differentiation of endothelial cells in prostate cancer, whereas suppressing the expression of Prx1 can induce the expression of vascular endothelial growth factor and reduce angiogenesis (28,29). Prx1 has also been reported to promote the development of malignant lung cancer (30), and reducing the expression of Prx1 can induce the apoptosis of lung cancer cells (31). A study by Sun *et al* (32) showed that Prx1 is closely associated with tumor size, vascular invasion, edmondson grade, α-fetoprotein and lymph node metastasis in hepatocellular carcinoma. The overexpression of Prx1 also promotes the epithelial-mesenchymal transition and migration if non-small cell lung cancer cells induced by TGF-β1 (33). In the presence of TLR4, Prx1 promotes the expression of inflammatory cytokines, IL-6 and tumor necrosis factor-α, and induces the maturation of dendritic cells (29). Du *et al* (35) found that Prx1 is expressed at high levels in thyroid cancer, and the knockdown of Prx1 promotes apoptosis in thyroid cancer.

There are limited reports on the effect of Prx1 on VSMCs, and the effect of Prx1 on cell proliferation, invasion and migration in VSMCs remains to be fully elucidated. Jones *et al* (36) reported that denatured type I collagens prompt the expression of Prx1 and Prx2, and the overexpression of Prx1 promotes the growth of VSMCs. Denatured collagen protein can also promote the expression of tenascin-C, resulting in the proliferation of VSMCs, and the expression of Prx1 can activate the promoter, tenascin-1, with a 20-fold increase in activity. Jin *et al* (37) found that lipoma-containing lipoma preferred partner and the cytoskeleton-associated protein, palladin enhance the migration and proliferation of cells, which can be induced by angiotensin II, focal adhesion kinase and Prx1, further contributing to the migration of VSMCs (38). Investigations by Pi *et al (*38) showed that apocynin inhibited the generation of intracellular ROS, and then significantly inhibited the expression of TLR4 and pro-inflammatory cytokines, which inhibited the proliferation and migration of VSMCs. Knocking down TLR4 inhibited the formation, proliferation and migration of endometrial epithelial cells in the damaged carotid, although the inhibitory effect was not significant. These results showed that ROS offers potential for use as a therapeutic target in TLR4-associated cardiovascular inflammation and disease, as it is vital in the TLR4-mediated pro-inflammatory response and cell proliferation of the VSMCs.

In the present study, it was demonstrated that Prx1 was overexpressed in the vein grafts. The overexpression of Prx1 significantly promoted the proliferation, invasion and migration of the VSMCs. Compared, with the control group, the numbers of living cells in the Prx1-overexpressed group in the first 3 days increased 0.875-, 1.17- and 1.10-fold, respectively (Fig. 3). The results of the Transwell assay showed that the number of penetrating cells in the Prx1-overexpressed group increased from 40/visual field to 150/visual field (Fig. 2A), indicating the promoting effects of Prx1 on VSMC invasiveness. In the Prx1-overexpressed groups, the migration ability was significantly higher, compared with that in the control group ($P<0.05$; Fig. 2B). In further experiments, TLR4 siRNA vectors and recombinant plasmids of Prx1 were co-transfected into the VSMCs. The results demonstrated that Prx1 significantly inhibited the proliferation and migration of the VSMCs. Compared, with the Prx1-overexpressed group, the number of viable cells in the first 3 days decreased by 34, 41 and 40%, respectively, in the co-transfected groups (Fig. 4), indicating the significant inhibitory effect of TLR4 on Prx1-induced proliferation in VSMCs. The wound-healing assay demonstrated that the migration ability of the TLR4 siRNA and Prx1 co-transfected cells were attenuated significantly, compared with that in the Prx1-overexpressed group ($P<0.05$).

In conclusion, Prx1 was overexpressed in the vein graft IH group, and the overexpression of Prx1 significantly promoted the proliferation, invasion and migration ability of the VSMCs. Knocking down TLR4 significantly attenuated the Prx1-induced proliferation, invasion and migration of the VSMCs, which indicated that Prx1 promoted the proliferation and invasion of the VSMCs, which was TLR4-dependent. These results indicate the possibility of TLR4 as a novel target for the diagnosis, treatment and prognosis in IH.

Acknowledgements

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References

1. Muto A, Model L, Ziegler K, Eghbalieh SD and Dardik A: Mechanisms of vein graft adaptation to the arterial circulation: Insights into the neointimal algorithm and management strategies. Circ J 74: 1501-1512, 2010.
2. Dilley RJ, McGeachie JK and Tennant M: The role of cell proliferation and migration in the development of a neo-intimal layer in veins grafted into arteries, in rats. Cell Tissue Res 269: 281-287, 1992.
3. Guo H, Makarova N, Cheng Y, E S, Ji RR, Zhang C, Farrar P and Tigyi G: The early- and late stages in phenotypic modulation of vascular smooth muscle cells: Differential roles for lysophosphatidic acid. Biochim Biophys Acta 1781: 571-581, 2008.
4. Wolff RA, Malinowski RL, Heaton NS, Hullett DA and Hoch JR: Transforming growth factor-beta1 antisense treatment of rat vein grafts reduces the accumulation of collagen and increases the accumulation of h-caldesmon. J Vasc Surg 43: 1028-1036, 2006.
5. Jeremy JY, Nystrom ML, Barradas MA and Mikhailidis DP: Eicosanoids and septicaemia. Prostaglandins Leukot Essent Fatty Acids 50: 287-297, 1994.
6. Sasaki Y, Suehiro S, Becker AE, Kinoshita H and Ueda M: Role of endothelial cell denudation and smooth muscle cell dedifferentiation in neointimal formation of human vein grafts after coronary artery bypass grafting: Therapeutic implications. Heart 83: 69-75, 2000.
7. Yamashita A, Hanna AK, Hirata S, Dardik A and Sumpio BE: Antisense basic fibroblast growth factor alters the time course of mitogen-activated protein kinase in arterialized vein graft remodeling. J Vasc Surg 37: 866-873, 2003.
8. Jia G, Cheng G, Gangahar DM and Agrawal DK: Involvement of connexin 43 in angiotensin II-induced migration and proliferation of saphenous vein smooth muscle cells via the MAPK-AP-1 signaling pathway. J Mol Cell Cardiol 44: 882-890, 2008.
9. Libby P, Schwartz D, Brogi E, Tanaka H and Clinton SK: A cascade model for restenosis. A special case of atherosclerosis progression. Circulation 86 (6 Suppl): III47-III52, 1992.
10. Lyon CA, Johnson JL, Williams H, Sala-Newby GB and George SJ: Soluble N-cadherin overexpression reduces features of atherosclerotic plaque instability. Arterioscler Thromb Vasc Biol 29: 195-201, 2009.
11. Bhardwaj S, Roy H and Ylä-Herttuala S: Gene therapy to prevent occlusion of venous bypass grafts. Expert Rev Cardiovasc Ther 6: 641-652, 2008.

12. Ekstrand J, Razuvaev A, Folkersen L, Roy J and Hedin U: Tissue factor pathway inhibitor-2 is induced by fluid shear stress in vascular smooth muscle cells and affects cell proliferation and survival. J Vasc Surg 52: 167-175, 2010.
13. Chan J, Prado-Lourenco L, Khachigian LM, Bennett MR, Di Bartolo BA and Kavurma MM: TRAIL promotes VSMC proliferation and neointima formation in a FGF-2-, Sp1 phosphorylation-, and NFkappaB-dependent manner. Circ Res 106: 1061-1071, 2010.
14. Earley S and Brayden JE: Transient receptor potential channels and vascular function. Clin Sci 119: 19-36, 2010.
15. Jia G, Mitra AK, Gangahar DM and Agrawal DK: Regulation of cell cycle entry by PTEN in smooth muscle cell proliferation of human coronary artery bypass conduits. J Cell Mol Med 13: 547-554, 2009.
16. Mitra AK, Jia G, Gangahar DM and Agrawal DK: Temporal PTEN inactivation causes proliferation of saphenous vein smooth muscle cells ofhuman CABG conduits. J Cell Mol Med 13: 177-187, 2009.
17. Dong LH, Wen JK, Liu G, McNutt MA, Miao SB, Gao R, Zheng B, Zhang H and Han M: Blockade of the Ras-extracellular signal-regulated kinase 1/2 pathway is involved in smooth muscle 22 alpha-mediated suppression of vascular smooth muscle cell proliferation and neointima hyperplasia. Arterioscler Thromb Vasc Biol 30: 683-691, 2010.
18. Maile LA, Allen LB, Hanzaker CF, Gollahon KA, Dunbar P and Clemmons DR: Glucose regulation of thrombospondin and its role in the modulation of smooth muscle cell proliferation. Exp Diabetes Res 2010: pii: 617052, 2010.
19. Chiu JJ, Usami S and Chien S: Vascular endothelial responses to altered shear stress: Pathologic implications for atherosclerosis. Ann Med 41: 19-28, 2009.
20. Panchatcharam M, Miriyala S, Yang F, Leitges M, Chrzanowska-Wodnicka M, Quilliam LA, Anaya P, Morris AJ and Smyth SS: Enhanced proliferation and migration of vascular smooth muscle cells in response to vascular injury under hyperglycemic conditions is controlled by beta3 integrin signaling. Int J Biochem Cell Biol 42: 965-974, 2010.
21. Isenovic ER, Kedees MH, Haidara MA, Trpkovic A, Mikhailidis DP and Marche P: Involvement of ERK1/2 kinase in insulin-and thrombin-stimulated vascular smooth muscle cell proliferation. Angiology 61: 357-364, 2010.
22. Furgeson SB, Simpson PA, Park I, Vanputten V, Horita H, Kontos CD, Nemenoff RA and Weiser-Evans MC: Inactivation of the tumour suppressor, PTEN, in smooth muscle promotes a pro-inflammatory phenotype and enhances neointima formation. Cardiovasc Res 86: 274-282, 2010.
23. Egler RA, Fernandes E, Rothermund K, Sereika S, de Souza-Pinto N, Jaruga P, Dizdaroglu M and Prochownik EV: Regulation of reactive oxygen species, DNA damage, and c-Myc function by peroxiredoxin 1. Oncogene 24: 8038-8050, 2005.
24. Jamaluddin M, Wiktorowicz JE, Soman KV, Boldogh I, Forbus JD, Spratt H, Garofalo RP and Brasier AR: Role of peroxiredoxin 1 and peroxiredoxin 4 in protection of respiratory syncytial virus-induced cysteinyl oxidation of nuclear cytoskeletal proteins. J Virol 84: 9533-9545, 2010.
25. Gong F, Hou G, Liu H and Zhang M: Peroxiredoxin 1 promotes tumorigenesis through regulating the activity of mTOR/p70S6K pathway in esophageal squamous cell carcinoma. Med Oncol 32: 455, 2015.
26. Taniuchi K, Furihata M, Hanazaki K, Iwasaki S, Tanaka K, Shimizu T, Saito M and Saibara T: Peroxiredoxin 1 promotes pancreatic cancer cell invasion by modulating p38 MAPK activity. Pancreas 44: 331-340, 2015.
27. Zhou J, Liu Q, Wang J, Guo X and Song L: Expressions of peroxiredoxin 1, peroxiredoxin 6 and GFAP in human brain astrocytoma and their clinical significance. Nan Fang Yi Ke Da Xue Xue Bao 32: 1255-1259, 2012 (In Chinese).
28. Riddell JR, Bshara W, Moser MT, Spernyak JA, Foster BA and Gollnick SO: Peroxiredoxin 1 controls prostate cancer growth through Toll-like receptor 4-dependent regulation of tumor vasculature. Cancer Res 71: 1637-1646, 2011.
29. Riddell JR, Wang XY, Minderman H and Gollnick SO: Peroxiredoxin 1 stimulates secretion of proinflammatory cytokines by binding to TLR4. J Immunol 184: 1022-1030, 2010.
30. Jiang H, Wu L, Mishra M, Chawsheen HA and Wei Q: Expression of peroxiredoxin 1 and 4 promotes human lung cancer malignancy. Am J Cancer Res 4: 445-460, 2014.
31. Hwang KE, Park DS, Kim YS, Kim BR, Park SN, Lee MK, Park SH, Yoon KH, Jeong ET and Kim HR: Prx1 modulates the chemosensitivity of lung cancer to docetaxel through suppression of FOXO1-induced apoptosis. Int J Oncol 43: 72-78, 2013.
32. Sun QK, Zhu JY, Wang W, Lv Y, Zhou HC, Yu JH, Xu GL, Ma JL, Zhong W and Jia WD: Diagnostic and prognostic significance of peroxiredoxin 1 expression in human hepatocellular carcinoma. Med Oncol 31: 786, 2014.
33. Ha B, Kim EK, Kim JH, Lee HN, Lee KO, Lee SY and Jang HH: Human peroxiredoxin 1 modulates TGF-β1-induced epithelial-mesenchymal transition through its peroxidase activity. Biochem Biophys Res Commun 421: 33-37, 2012.
34. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
35. Du ZX, Yan Y, Zhang HY, Liu BQ, Gao YY, Niu XF, Guan Y, Meng X and Wang HQ: Suppression of MG132-mediated cell death by peroxiredoxin 1 through influence on ASK1 activation in human thyroid cancer cells. Endocr Relat Cancer 17: 553-560, 2010.
36. Jones FS, Meech R, Edelman DB, Oakey RJ and Jones PL: Prx1 controls vascular smooth muscle cell proliferation and tenascin-C expression and is upregulated with Prx2 in pulmonary vascular disease. Circ Res 89: 131-138, 2001.
37. Jin L, Kern MJ, Otey CA, Wamhoff BR and Somlyo AV: Angiotensin II, focal adhesion kinase, and PRX1 enhance smooth muscle expression of lipoma preferred partner and its newly identified binding partner palladin to promote cell migration. Circ Res 100: 817-825, 2007.
38. Pi Y, Zhang LL, Li BH, Guo L, Cao XJ, Gao CY and Li JC: Inhibition of reactive oxygen species generation attenuates TLR4-mediated proinflammatory and proliferative phenotype of vascular smooth muscle cells. Lab Invest 93: 880-887, 2013.