High expression of ubiquitin-specific peptidase 39 is associated with the development of vascular remodeling

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Abstract. Vascular remodeling is the primary cause underlying the failure of angioplasty surgeries, including vascular stenting, transplant vasculopathy and vein grafts. Multiple restenosis-associated proteins and genes have been identified to account for this. In the present study, the functions of ubiquitin-specific peptidase 39 (USP39) were investigated in the context of two vascular remodeling models (a mouse common carotid artery ligation and a pig bilateral saphenous vein-carotid artery interposition graft). USP39 has previously been observed to be upregulated in ligated arteries, and this result was confirmed in the pig vein graft model. In addition, Transwell assay results demonstrated that vascular smooth muscle cell (VSMC) migration was suppressed by lentiviral vector-mediated downregulation of USP39 and enhanced by upregulation of USP39. Furthermore, knockdown of USP39 inhibited VSMC cell proliferation and the expression of cyclin D1 and cyclin-dependent kinase 4, as analyzed via cell counting, MTT assay and western blotting. These results suggest that USP39 may represent a novel therapeutic target for treating vascular injury and preventing vein-graft failure.

Introduction

Vascular remodeling is the primary reason for the failure of angioplasty surgeries, including vascular stenting (1), transplant vasculopathy (2) and vein grafts (3), and represents a major problem for vascular surgical procedures (4). Coronary artery bypass grafting is widely performed using autologous saphenous vein and internal mammary artery grafts, however,

~50% of patients who undergo the procedure require reoperation 10-15 years later to treat vascular restenosis (5).

Cell proliferation, migration and apoptosis are the main active processes in vascular remodeling (6), particularly in vascular smooth muscle cells (VSMCs) (7,8). Several animal models of vascular remodeling have been developed to explore the molecular mechanisms responsible for intimal hyperplasia, including wire-insertion, vein bypass grafting and common carotid artery (CCA) ligation models (9). In a murine CCA ligation model, leukocyte infiltration occurred 1 week after ligation (10), and smooth muscle cell (SMC)-rich neointima formation and an 80% luminal area reduction was observed 4 weeks after ligation (11).

Numerous restenosis-associated proteins and genes have been identified, including osteopontin (12), periostin (13), connexin 34 (14) and microRNA-21 (15), however, the association between ubiquitin-specific peptidase 39 (USP39) and restenosis remains to be investigated. USP39 is a 65-kDa SR protein-related member of the ubiquitin-specific protease family, which harbors a deubiquitinating enzyme (DUB) domain (16). Significantly increased USP39 expression has been observed in breast cancer tissues compared with paracancer tissues and normal tissues (17) and the embryonic zebrafish brain (18). *In vitro* removal of small ubiquitin-like modifiers (SUMOs), termed deSUMOlyation, strengthened the proliferation-enhancing effect of USP39 in prostate cancer cells (19). However, USP39 lacks three residues critical for protease activity and has been revealed to be inactive as a DUB (20). However, to the best of our knowledge, no previous study to date has assessed the involvement of USP39 in the context of intimal hyperplasia and vascular remodeling. In the present study, the expression and potential novel functions of USP39 in relation to vascular remodeling were investigated. USP39 protein expression levels were determined in ligated arteries in mice and in a pig vein graft model, and the involvement of USP39 in VSMC proliferation and migration was examined.

Materials and methods

Animals and cell culture. All animal procedures were approved by the Animal Care and Use Committee of Xiamen University [Xiamen, China; license no: SYXK (Min) 2008-0003, issued May 6, 2008]. C57BL/6J mice (male; 8 weeks old; 27-30 g; n=18) were obtained from the Xiamen University Class SPF Animal

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Laboratory Center (Xiamen, China). The mice were assigned randomly into two groups (control and surgery) and kept in a 12/12 h light/dark cycle, 25°C, with *ad libitum* access to food and water. Large White pigs weighing 35-45 kg (male; n=16) were obtained from the Prince of Wales Hospital Institute, Chinese University of Hong Kong (Hong Kong, China), and were kept in a 12/12 h light/dark cycle, 26°C, with *ad libitum* access to food and water prior to surgery. Pigs were assigned randomly into four groups, according to the time point at which vein grafts were to be harvested: Postoperative, and at 2, 4 and 12 weeks (12). Other steps were performed as described previously (21). C57BL/6 mouse VSMCs (Nanjing Mucyte Bio Tech Co., Ltd.; http://www.mucyte.com/index.php) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin in a 95% humidified, 5% CO_2 incubator at 37°C. Cells from passages 3-8 were used in all experiments. For VSMC stimulation, cells were cultured in 6-well plates in DMEM, grown to 70% confluence and washed with phosphate-buffered saline 12 h later. The medium was replaced with serum-free medium and the cells were stimulated with 0, 50, 100, 200, 300 or 400 ng/ml lipopolysaccharide (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for a further 12 h at 37°C.

Mouse CCA ligation. Mice underwent ligation of the left carotid artery near the distal bifurcation, as described previously (11). Mice were anesthetized with 10% chloral hydrate (400 mg/kg) by intraperitoneal injection. The left CCA was dissected free of connective tissue and completely ligated with 6-0 silk sutures just proximal to the common carotid bifurcation. In control mice, the suture was passed under the exposed carotid artery without ligation. At each time point (2 and 4 weeks after the procedure), mice were euthanized by CO_2 inhalation and the left CCAs were carefully excised and stored in liquid nitrogen.

Pig vein-carotid artery interposition grafting. The animals received humane care according to the Guide for the Care and Use of Laboratory Animals. Pigs underwent vein-carotid artery interposition grafting as follows. Anaesthesia was induced with ketamine (30 mg/kg) and atropine (0.6 mg/kg) administered intramuscularly. A section of the saphenous vein (~12 cm) from the right leg of each pig was dissected free from surrounding tissue. Two para-sternocleidomastoid muscle longitudinal neck incisions were made and the CCAs were carefully dissected from the internal jugular vein and vagus nerve within the carotid sheath. End-to-end anastomoses of the saphenous vein to CCA were then performed using continuous 7-0 polypropylene sutures. The pigs were sacrificed under general anaesthesia when the grafts were removed using an IV injection of ketamine (>150 mg/kg). Other steps were performed as described previously (12).

Morphometric analysis and immunohistochemistry. The arteries (mice) and veins (pigs) were dissected, embedded in paraffin, and serial 4 μm-sections were taken for morphometric analysis. Sections of carotid artery and vein grafts were stained with hematoxylin and eosin. Masson's trichrome

staining (cat. no. PT003; Shanghai Bogoo Biotechnology. Co., Ltd., Shanghai, China) was performed in mice carotid artery sections. The thickness of the neointima samples was examined by light microscopy (Olympus IX51; Olympus Corporation, Tokyo, Japan), and analyzed using dedicated image-analysis software (Image-Pro-Plus 6.0; Media Cybernetics, Rockville, MD, USA). Three serial sections of each vessel were analyzed to measure neointima thickness and USP39 protein expression, and the average was calculated as a standard for statistical analysis. Immunohistochemistry was performed using an immunohistochemistry kit (Elivision plus kit 9901; Fuzhou Maixin Biotechnology Co., Ltd., Fuzhou, China) and a DAB Color Developing Reagent kit (Fuzhou Maixin Biotechnology Co., Ltd.). USP39 antibody (1:200; cat. no. ab131244; Abcam, Cambridge, UK) was used as the primary antibody, with phosphate-buffered saline as the negative control. Goat anti-mouse immunoglobulin G (IgG) from the immunohistochemistry kit (cat. no. 9901; Elivision Plus kit; Fuzhou Maixin Biotechnology Co., Ltd., Fuzhou, China) was used as the secondary antibody. The primary antibody was incubated 4°C overnight and secondary antibody was incubated for 10 min at room temperature. Slides were observed under a light microscope (x400 magnification). Immunohistochemical positivity was determined by the ratio of USP39-positive cells to the total number of hematoxylin-positive nuclei in a defined field (four slides observed per condition, and three fields of view assessed per slide).

Western blotting analysis. Mouse VSMCs were pulverized in RIPA buffer (Sangon Biotech Co., Ltd., Shanghai, China) and sonicated to disrupt the integrity of cell membranes and extract total proteins. Whole cell lysates were then centrifuged at 4000 x *g* for 15 min at 4°C. Total protein was quantified using the Bradford method. Total protein (20 μg per lane) was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Other steps were performed as described previously (14). USP39 (cat. no. ab131244; 1:1,000; Abcam), cyclin D1 (cat. no. 60186-1-Ig; 1:1,000; ProteinTech Group, Inc., Chicago, IL, USA), and cyclin-dependent kinase 4 (CDK4; cat. no. 11026-1-AP; 1:1,000; ProteinTech Group, Inc.) antibodies were used as primary antibodies. β-tubulin antibody (cat. no. 10094-1-AP; ProteinTech Group, Inc.) was used to ensure equal loading quantities of protein samples.

Protein overexpression, virus packing and transfection of VSMCs. In order to overexpress USP39, plasmids were constructed in our laboratory using the pDEST_LTR_N_IRES_ puro vector, that added myc, hemagglutinin (HA) and FLAG tags to USP39. For USP39 knockdowns, packing plasmids (pHR and pVSVG; Shanghai Jikai Industry Co., Shanghai, China) were mixed with lentivirus vectors containing the following small interfering RNA (siRNA) sequences: siRNA usp39-70, 5'-GAA TAACATAAAGGCCAAT-3'; siRNA usp39-71, 5'-CGGGTA TTGTGGGACTGAA-3'; siRNA usp39-72, 5'-TTCCAGACA ACTATGAGAT-3'; siRNA usp39-73, 5'-TTTGGAAGAGGC GAGATAA-3' (Shanghai Jikai Industry Co.). The mixtures were then added to Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.). Lipofectamine 2000 (ProteinTech Group, Inc.)

was then mixed Opti-MEM for 5 min. The total transfection mixture was allowed to stand for ~20 min and was then added to 6-well plates containing 293T cells (Medical College of Xiamen University, Xiamen, China) at 70% confluence. The virus was harvested ~48 h later. Other steps were performed as described previously (14). For transfection, VSMCs at 70% confluence were cultured in 12-well plates and incubated with lentivirus for 24 h. The medium was replaced with DMEM 24 h later.

VSMC proliferation. VSMC proliferation was quantitated by cell counting using a hemocytometer (Bio-Rad TC20; Bio-Rad Laboratories, Inc., Hercules, MA, USA) and MTT assay. Transfected cells [siControl group (control vector), siRNA usp39-73 group, control group (empty vector) and myc-USP39 group] were cultured in 100 μl DMEM, seeded in a 96-well plate and incubated for 3 days. Cells were subsequently detached with trypsin and counted by a hemocytometer. For the MTT assay, cells of each group were incubated for 1, 3 and 5 days, then 5 h before the end of the incubation 20 μl MTT (5 mg/ml) was added to each well. Culture supernatants were then removed and resuspended in 400 μl isopropanol to dissolve the MTT formazan, and the absorbance was measured at 490 nm using an ELISA microplate reader (Bio-Rad Model 680; Bio-Rad Laboratories, Inc.). Each assay was performed in triplicate.

VSMC migration. Cell migration was assessed by Transwell assay, using the same groups as for the VSMC proliferation assays. Serum free DMEM medium (200 μl) containing 5,000 VSMCs was seeded into the upper chambers of 8 μm-pore size Transwell filters (cat. no. 14831; Corning Incorporated, Corning, NY, USA). In the lower chamber, 500 μl DMEM containing 10% fetal bovine serum was added, which served as a chemoattractant. Non-invading cells on the upper chambers were removed with a cotton-tipped swab following 12 h incubation. The lower sides of the filters were then fixed in 4% paraformaldehyde for 30 min and stained with methylrosanilinium chloride. The invading cells were viewed under an Olympus microscope (Olympus IX51; Olympus Corporation) and counted at x40 magnification (four fields of view for each group). The ability of the cells to invade was expressed as the mean number of cells in the entire field. The assay was performed three times.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistically significant differences were evaluated using Student's *t*-tests when 2 groups were compared or for multiple comparisons, analysis of variance followed by Student-Newman-Keuls tests. Data were analyzed using Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

USP39 is expressed in the ligated arterial wall. CCA ligation (Fig. 1A) resulted in vascular remodeling. The thickening of ligated arteries in mice was assessed 2 and 4 weeks following surgery (Fig. 1B). Morphometric analysis of hematoxylin and eosin (Fig. 2A) and Masson's trichrome-stained sections
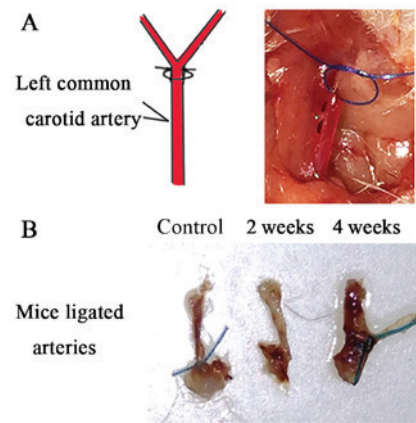

Figure 1. Murine model of vascular remodeling. (A) The left common carotid artery was completely ligated with 6-0 silk sutures just proximal to the common carotid bifurcation. (B) Thickening of ligated arteries in mice 2 and 4 weeks after surgery. The control was sacrificed immediately after the ligation was performed.

(Fig. 2B) was performed. Immunohistochemical analysis revealed positive staining for USP39, which was predominantly localized in the neointimal layer, and was also present in the nucleus (Fig. 2C). Analysis of mice carotid artery sections confirmed that the neointima thickness was increased in the ligated arteries 2 and 4 weeks after surgery, compared with the non-ligated control arteries (P=0.0125 and P=0.0046, respectively; Fig. 2D). Significantly increased USP39 protein expression was observed at 2 and 4 weeks in ligated carotid arteries compared with the non-ligated control arteries (P=0.0136 and P=0.0014, respectively; Fig. 2E) and USP39 was more abundant at 4 weeks in ligated carotid arteries compared with at 2 weeks (Fig. 2E).

USP39 expression is elevated during restenosis in pig vein grafts. The thickening of vein-grafts in the pig was assessed at 2 and 4 weeks after surgery (Fig. 3A). USP39 protein expression levels in the pig vein grafts at different time points following transplantation confirmed the results observed in the ligated mouse carotid arteries (Fig. 3B), and there were significantly more USP39-positive cells in the grafts at 2 and 4 weeks after surgery compared with the baseline levels prior to the operation (P=0.0028 and P=0.0023, respectively; Fig. 3C).

Knockdown of USP39 inhibits VSMC proliferation and the expression of cyclin D1 and CDK4. Wang *et al* (17) previously reported that RNA interference-mediated downregulation of USP39 markedly reduced the proliferation and colony-forming ability of MCF-7 cells, and induced G0/G1-phase arrest and apoptosis. The effect of overexpression (Fig. 4A) and knockdown (Fig. 4B) of USP39 on the USP39 protein was determined in the present study. USP39-73 siRNA was selected as the most effective siRNA at knocking down USP39 protein levels (Fig. 4B) and was, therefore, used for further experiments. Knockdown of USP39 in VSMCs inhibited cyclinD1 and CDK4 protein expression compared with transfection with control siRNA (Fig. 4C), which are essential proteins for G1/S phase transformation. Furthermore, USP39 knockdown in VSMCs significantly reduced proliferation compared with cells transfected with
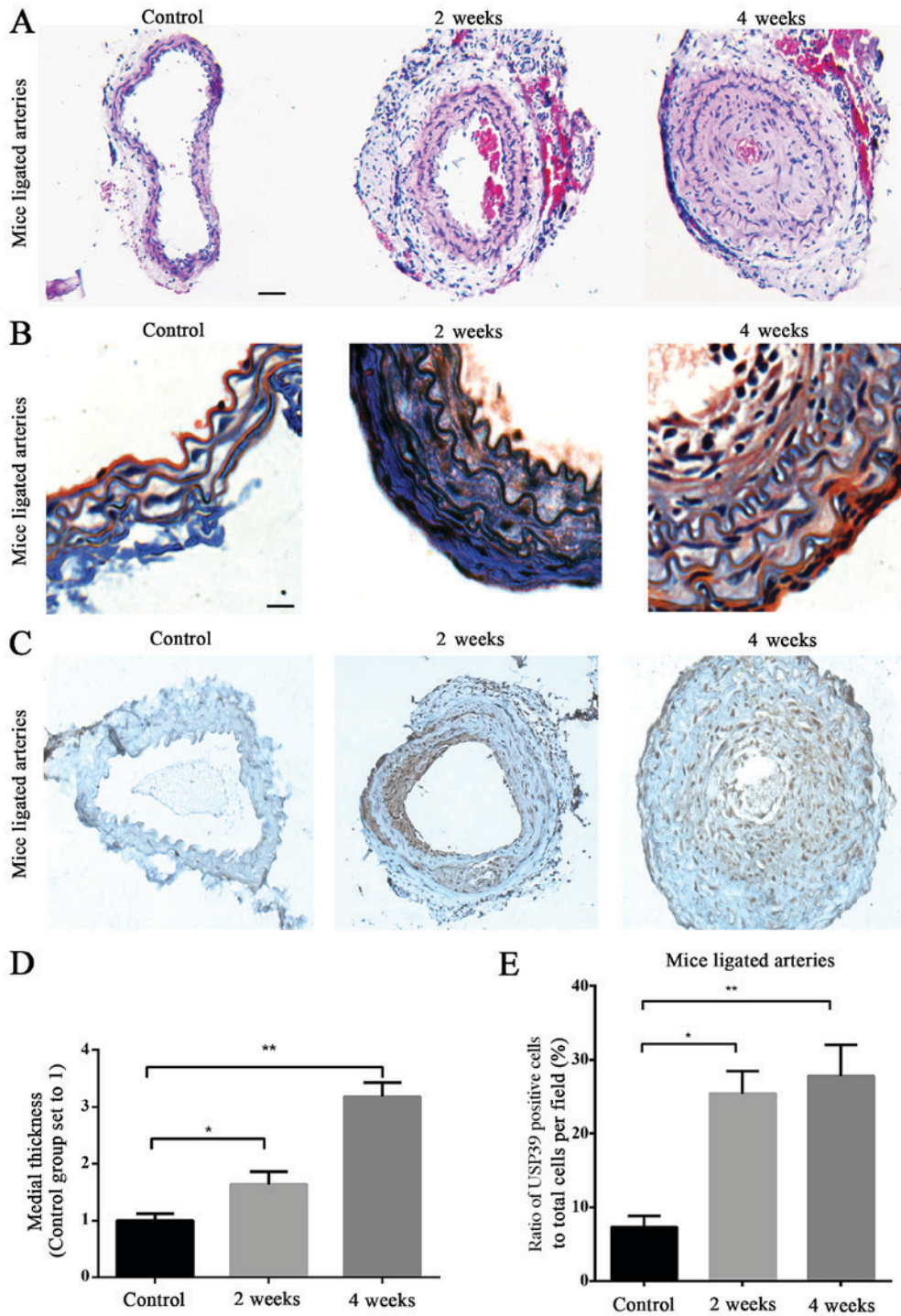
Figure 2. Sections of mouse carotid artery. Ligated mouse carotid arteries without ligation, and at 2 and 4 weeks after ligation, stained with (A) hematoxylin and eosin (scale bars: 25 μm, viewed at x400 magnification), and (B) Masson trichrome (scale bars: 50 μm. Original magnification, x400). (C) Immunohistochemical analysis revealed positive staining for USP39 at 2 and 4 weeks after ligation. (D) Neointima thickness 2 and 4 weeks after ligation. *P<0.05 and **P<0.01, comparison indicated by brackets; n=10, control group set as 1. (E) Percentage of USP39 positive cells. *P<0.05, **P<0.01 comparison indicated by brackets; n=10. USP39, ubiquitin-specific peptidase 39.

control siRNA (P=0.0238; Fig. 4D). The growth rate of VMSCs was demonstrated to be significantly suppressed by USP39 knockdown compared with cells transfected with control siRNA at 3 and 5 days (P=0.0478 and P=0.0002, respectively; Fig. 4E) via an MTT assay. However, USP39 overexpression had no significant effect on cell growth rate compared with cells transfected with empty vector (P>0.05; Fig. 4F).

USP39 is associated with VSMC migration. The migration of VSMC is a crucial event in the pathogenesis of vascular diseases and is characterized by intimal thickening (22). In the present study, VSMC migration was suppressed by knock-down of USP39 compared with transfection with control siRNA (P=0.0111; Fig. 5A) and migration was significantly increased in VSMCs overexpressing USP39 compared with control cells (P=0.0028; Fig. 5B and C).
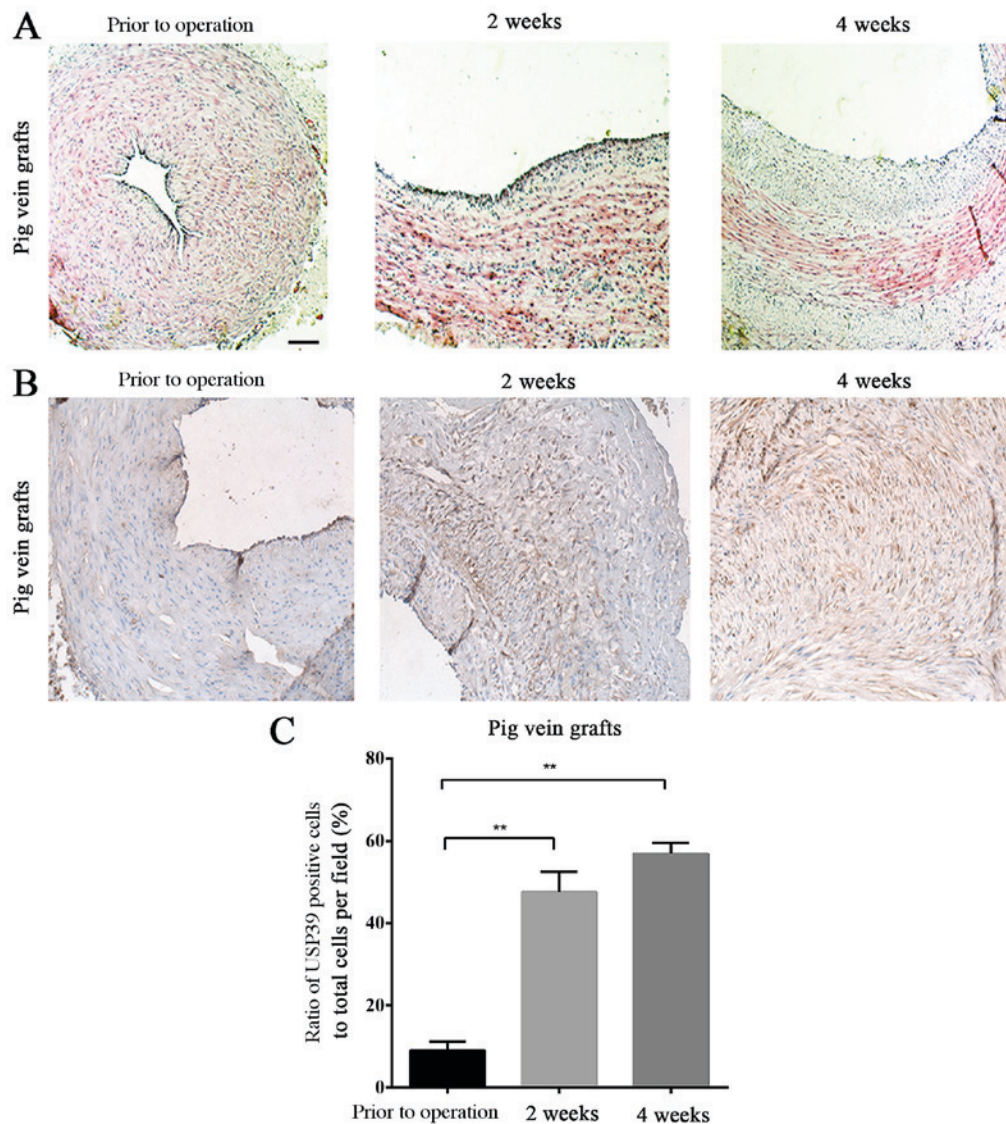
Figure 3. Sections of pig vein grafts at different time points. (A) Stained with hematoxylin and eosin (scale bars: 1 mm; x40 magnification). (B) USP39 protein levels in pig vein grafts at different time points visualized by immunohistochemistry (scale bars: 1 mm. Original magnification, x40). (C) Quantification of USP39 expression (ratio of USP39-positive cells to the total cells per field). **P<0.01 comparison indicated by brackets. USP39, ubiquitin-specific peptidase 39.

Discussion

The ubiquitin specific protease USP39 lacks three residues critical for protease activity, and thus lacks DUB activity despite the presence of a DUB domain (17,20). USP39 has previously been suggested to be involved in controlling cell growth in the pituitary gland, thus maintaining pituitary homeostasis in zebrafish (18). However, no exact mechanisms, specific substrates or other functions of USP39 have been identified.

To the best of our knowledge, the present study provides the first evidence for an association between USP39 expression and remodeling in ligated arteries and vein grafts, and USP39 may be involved in this pathological process in vein grafts and ligated arteries. The mouse carotid artery ligation and pig vein graft models closely resemble human venous bypass graft failure in terms of their morphological characteristics and development (23,24), and are thus useful for exploring the potential pathogenesis and novel therapies.

It has previously been reported that USP39 silencing affects spindle checkpoint function and cytokinesis by reducing Aurora B kinase levels (20). Furthermore, inhibition of USP39 induced G0/G1-phase arrest and apoptosis in breast cancer cells (17). Similarly, cyclin D1 and CDK4 protein expression levels, which maintain the cell cycle process, were decreased in USP39-knockout VSMCs compared with control cells, and VSMC proliferation was also significantly reduced by USP39-knockdown in the present study. Although it is not possible to conclude that USP39 has a direct effect on VSMC proliferation from these results alone, it appears to be associated with SMC proliferation, and may provide a novel target for the treatment of vascular restenosis. Vascular remodeling is the structural reorganization of a vessel and involves multiple cell activities, including SMC proliferation, migration and extracellular membrane restriction (7). The present study also demonstrated that VSMC migration was suppressed by downregulation of USP39 and enhanced by upregulation of USP39. The proliferation and migration of VSMCs is a key event in
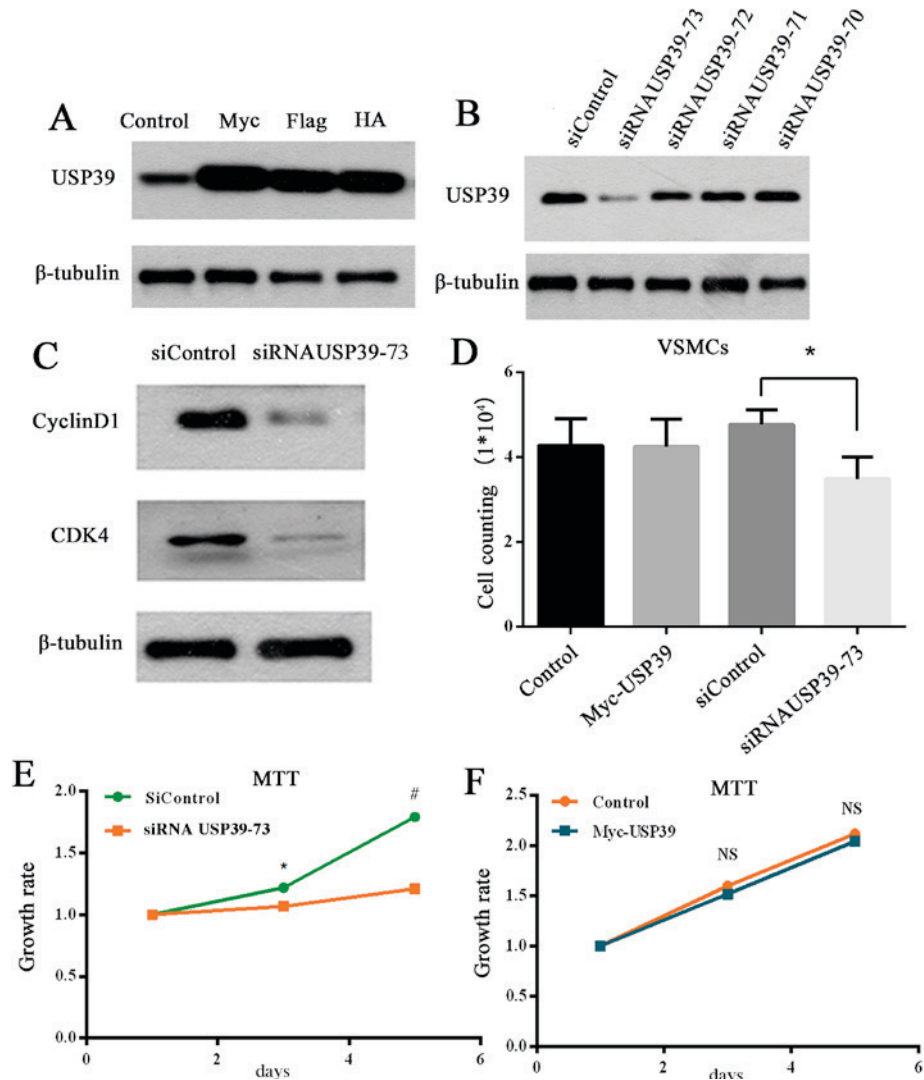
Figure 4. (A) Overexpression of myc-, Flag- and HA-tagged USP39 in VSMCs. (B) Knockdown of USP39 (siRNA USP39-70, 71, 72, and 73) in VSMCs. (C) Knockdown of USP39 in VSMCs decreased expression of cyclin D1 and CDK4. (D) USP39 knockdown inhibited proliferation in VSMCs. *$P<0.05$ comparison indicated by brackets. (E) Growth rate of siRNA USP39-73 group compared with siControl group VSMCs. *$P<0.05$ vs. siControl at 3 days, #$P<0.001$ vs. siControl at 5 days. (F) No significant difference in VSMC proliferation between the myc-USP39 group and the control VSMCs. HA, hemagglutinin; VSMC, vascular smooth muscle cell; USP39, ubiquitin-specific peptidase 39; siControl, control small interfering RNA; siRNA, small interfering RNA; CDK4, cyclin-dependent kinase 4; NS, no significance.
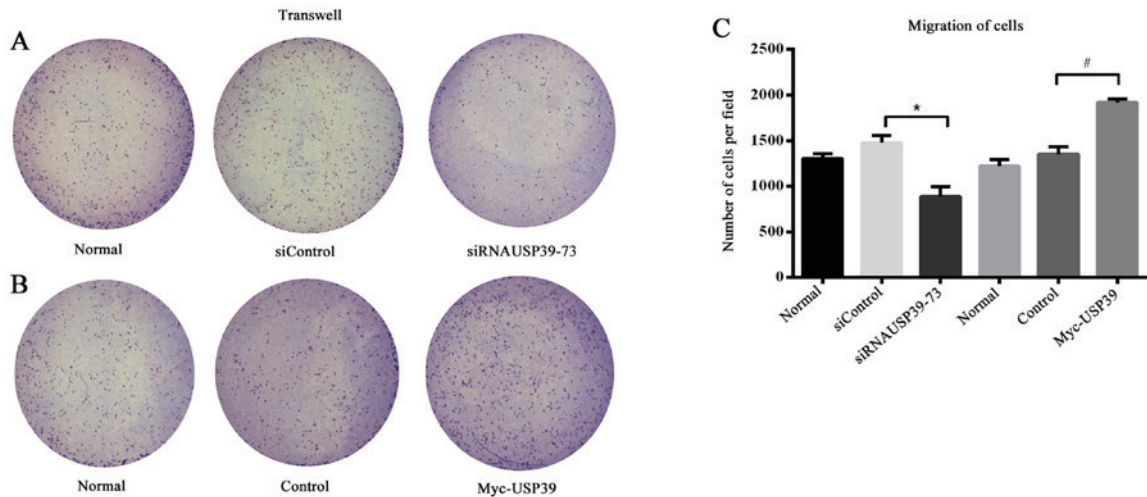

Figure 5. Migration of vascular smooth muscle cells following (A) USP39 knockdown by siRNA USP39-73 and (B) overexpression with Myc-USP39 (original magnification, x40), (C) The number of migrated cells per field was counted (four fields for each group). *$P<0.05$ and #$P<0.01$, comparison indicated by brackets. siControl, control small interfering RNA; siRNA, small interfering RNA; USP39, ubiquitin-specific peptidase 39.

the pathogenesis of vascular diseases and is characterized by intimal thickening, which is most commonly seen in atherosclerosis, vascular rejection, restenosis following vein grafting and coronary intervention (22,25,26). Multiple factors that affect the proliferation and migration of VSMCs. USP39 appears to be involved in proliferation and migration, but further studies are needed to assess potential underlying molecular mechanisms.

Once vascular injury occurs, the exposed subendothelial matrix rapidly attracts platelets and leukocytes, and infiltrated leukocytes may then release inflammatory cytokines several days following injury (12,24). Inflammation is believed to be associated with altered gene expression during the early stage of vascular remodeling (27-29). In the present study, USP39 expression was significantly increased in pig vein grafts and ligated mouse carotid arteries at 2 weeks after surgery.

The results of the present study indicate that USP39 may be involved in the early stages and development of vascular injury. However, inflammation and vascular remodeling are continuous processes, and specific mechanisms of USP39 throughout these processes require further investigation.

The present study provides the first evidence for an association between high USP39 protein levels and the development of vascular remodeling. USP39 regulates the cell cycle and affects proliferation and migration of VSMCs. USP39 may thus represent a novel therapeutic target for treating vascular injury and preventing vein-graft failure.

Acknowledgements

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