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Novel Role of Copper Transport Protein Antioxidant-1 in Neointimal Formation Following Vascular Injury

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Abstract

Objective—Vascular smooth muscle cell (VSMC) migration is critically important for neointimal formation following vascular injury and atherosclerosis lesion formation. Copper (Cu) chelator inhibits neointimal formation, and we previously demonstrated that Cu transport protein Antioxidant-1 (Atox1) is involved in Cu-induced cell growth. However, role of Atox1 in VSMC migration and neointimal formation after vascular injury is unknown.

Approach and Results—Here we show that Atox1 expression is upregulated in injured vessel, and it is colocalized with the Cu transporter ATP7A, one of downstream targets of Atox1, mainly in neointimal VSMCs at day 14 after wire injury. Atox1$^{-/-}$ mice show inhibition of neointimal formation and extracellular matrix expansion, which is associated with a decreased VSMCs accumulation within neointima and lysyl oxidase activity. Mechanistically, in cultured VSMC, Atox1 depletion with siRNA inhibits platelet-derived growth factor (PDGF)-induced Cu-dependent VSMC migration by preventing translocation of ATP7A and small G protein Rac1 to the leading edge as well as Cu- and Rac1-dependent lamellipodia formation. Furthermore, Atox1$^{-/-}$ mice show decreased perivascular macrophage infiltration in wire-injured vessels as well as thioglycollate-induced peritoneal macrophage recruitment.

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Disclosures None

Conclusions—Atox1 is involved in neointimal formation after vascular injury through promoting VSMC migration and inflammatory cell recruitment in injured vessels. Thus, Atox1 is a potential therapeutic target for VSMC migration and inflammation-related vascular diseases.

Introduction

Vascular smooth muscle cell (VSMC) migration is a critical event for the development of atherosclerosis and remodeling after vascular injury[1–3]. Platelet-derived growth factor (PDGF) is a key growth factor to promote neointimal formation and vascular remodeling primarily through the PDGF receptor β (PDGFR), by stimulating lamellipodia formation through Rac1 activation and translocation to the leading edge[1, 4]. Copper (Cu), an essential micronutrient, plays an important role in physiological repair processes including wound healing and angiogenesis as well as pathophysiologies including tumor growth, neurodegenerative disease, and atherosclerosis[4–11]. Cu levels are significantly increased in atherosclerotic lesions[12]. Implanting Cu cuff promotes neointima thickening in response to vascular injury[13], while Cu chelators inhibit neointimal formation[14]. Most recently, we found that PDGF-induced VSMC migration is inhibited by Cu chelators[15]. Thus, Cu or its regulators have been implicated in VSMC migration, vascular remodeling, and atherosclerosis. However, underlying mechanism is poorly understood.

Since Cu is essential catalytic cofactor in many biological responses but it is toxic, intracellular Cu concentration is tightly-regulated under physiological conditions[16]. Cu transport system is required for Cu uptake and its transfer to specific subcellular compartments and target proteins. Antioxidant-1 (Atox1) is cytosolic Cu chaperone that obtains Cu through Cu importer CTR1, and transports Cu to the Cu exporter ATP7A (Menkes ATPase)[17]. This in turn promotes ATP7A translocation from the trans-Golgi network (TGN) to the plasma membrane or to cytoplasmic vesicle[18], thereby delivering Cu to the secretory Cu enzymes such as extracellular superoxide dismutase (SOD) and lysyl oxidase (LOX)[15, 19, 20]. We previously demonstrated that Atox1 is involved in Cu-induced cell growth[21], and that Cu exporter ATP7A is involved in PDGF-induced VSMC migration by promoting ATP7A and Rac1 translocation to the leading edge as well as activating extracellular matrix (ECM) enzyme LOX, which is required for ECM maturation by regulating the cross-linking of collagen or elastin[15, 22]. However, whether Atox1 transmits PDGFR signal to the ATP7A, and Atox1 is involved in VSMC migration and vascular remodeling *in vivo* remain unknown.

In this study, we used wire injury model with Atox1 deficient mice to demonstrate that Atox1 plays an important role in neointimal formation and ECM expansion following vascular injury. Furthermore, Atox1$^{-/-}$ mice also show decreased LOX activity and reduced macrophage recruitment in injured vessels as well as thioglycollate-induced peritonitis model. In cultured VSMC, depletion of Atox1 with siRNA inhibits PDGF-induced VSMC migration by preventing Rac1- and ATP7A-translocation to leading edge and lamellipodia formation. These findings provide novel insight into Atox1 and Cu transporters as potential therapeutic targets for vascular remodeling and development of atherosclerosis.

Results

Atox1 is involved in neointimal formation and ECM expansion in response to vascular injury

To determine the role of Atox1 in vascular remodeling *in vivo*, we performed femoral artery wire injury in Atox1$^{-/-}$ and WT mice. The blood pressure was similar in the WT and Atox1$^{-/-}$ mice (98±3 vs. 103±8mmHg, *P*=NS). Twenty one days after wire injury, Atox1$^{-/-}$ mice show less neointimal formation compared to WT mice (Figure 1A). Quantitative

morphometric analysis revealed that Atox1$^{-/-}$ mice exhibited a marked decrease in intimal area, intimal to medial ratio (I/M) and reciprocal increase in lumen size without changes in medial area (Figure 1B). These morphometric measurements are consistent with marked pathological inward remodeling. Masson trichrome-staining showed less ECM expansion in Atox1$^{-/-}$ mice compared to WT mice (Figure 1A and B). These findings suggest that Atox1 is involved in neointimal formation and vascular remodeling following vascular injury.

Because VSMCs and macrophages contribute to the pathogenesis of neointimal formation, we also evaluated the VSMC and macrophage content in the injured vessels. Immunohistochemical analysis showed that SMC content within neointima after injury were markedly decreased in Atox1$^{-/-}$ mice (Figure 1C). The number of perivascular infiltrated Mac3-positive macrophage was significantly decreased in Atox1$^{-/-}$ mice at day 7 and 14 (Figure 1D). Furthermore, a reduction of inflammatory cell recruitment in Atox1$^{-/-}$ mice was also observed in a thioglycollate-elicited peritonitis model in which non-bacterial inflammation is involved[23] (Supplemental Figure IA). These results indicate that Atox1 is involved in neointimal formation after injury by increasing VSMC content within neointima as well as macrophage recruitment.

Atox1 expression is increased in neointima after vascular injury

We next examined the expression and localization of Atox1 and its key downstream targets Cu transporter ATP7A in wire-injured vessels. Immunofluorescence analysis showed that protein expression of Atox1 was significantly increased in the neointima in a time dependent manner (day 3 to 28), which was associated and colocalized with ATP7A expression (Figure 2A and 2B). Moreover, Atox1 expression was seen in αSMA-positive neointimal VSMCs (69 ± 8% of αSMA$^+$ cells; Figure 2C) and CD45-positive inflammatory cells (78 ± 7% of CD45$^+$ cells; Figure 2D) in wire-injured vessels. In addition, Atox1 expression was also observed in CD31-positive endothelial cells in neointima and adventitia (Supplemental Figure II) and Mac3-positive macrophages in neointima, media, and adventitia (Supplemental Figure III). Atox1 expression in VSMCs and macrophages were confirmed in cultured cells (Figure 2E).

Atox1 is involved in migration of VSMC, but not macrophage in vitro

We next examined the functional role of Atox1 in migration of VSMCs and macrophage, which contributes to the neointimal formation. Knockdown of Atox1 protein expression with siRNA which does not affect ATP7A expression in cultured VSMCs (Figure 3A) significantly inhibited PDGF-induced VSMC migration using modified Boyden chamber assays (Figure 3B) or wound scratch assay of confluent monolayer of VSMCs (Figure 3C), while Atox1 deficiency has no significant effect on PDGF-stimulated VSMC proliferation (Supplemental Figure IV). By contrast, lack of Atox1 in BM-derived macrophages did not affect migration in response to tumor necrosis factor α (TNFα), monocyte chemoattractant protein-1 (MCP-1), and lipopolysaccharide (LPS) (Supplemental Figure IB), although Atox1 was colocalized with ATP7A at the leading edge in stimulated macrophage (Supplemental Figure IC). Thus, Atox1 is involved in cell migration of VSMC, but not macrophage *in vitro*.

Atox1 associates with ATP7A in a Cu-dependent manner and is involved in ATP7A translocation to the leading edge in PDGF-stimulated VSMCs

Since we found that Atox1 colocalized with ATP7A, which was shown to obtain Cu from Atox1[17] and mediate VSMC migration[15], in the neointima in injured vessels, we next examined their association in cultured VSMCs. Co-immunoprecipitation analysis showed that PDGF stimulation rapidly promoted Atox1 binding to ATP7A, which was inhibited by Cu chelator, bathocuproine disulfonate (BCS) (Figure 4A and 4B). Immunofluorescence

analysis showed that PDGF stimulation promoted translocation of Atox1 and ATP7A to the plasma membrane at the leading edge where they colocalized in VSMCs (Figure 4C). Depletion of Atox1 with siRNA or Cu chelator BCS inhibited PDGF-induced ATP7A translocation (Figure 4D). These findings suggest that PDGF stimulation promotes Atox1 association with ATP7A in a Cu-dependent manner, and Atox1 is required for ATP7A translocation to the plasma membrane at the leading edge in VSMC.

Atox1 is involved in PDGF-stimulated lamellipodia formation

We previously demonstrated that ATP7A-dependent PDGF-induced VSMCs migration is mediated through Rac1-dependent lamellipodia formation[15]. To assess further the mechanism by which Atox1 is involved in VSMC migration, we examined whether Atox1 is involved in actin reorganization. Phalloidin staining showed that Atox1 siRNA significantly impaired PDGF-stimulated lamellipodia formation at plasma membrane in VSMCs (Figure 5A). By contrast, depletion of Atox1 had no effects on PDGF-stimulated Rac1 activity (Figure 5B).

Atox1 is involved in PDGF-induced Rac1 translocation as well as Rac1 association with ATP7A at the leading edge

Since Rac1 translocation to the plasma membrane is also important for lamellipodial formation and cell migration[24], we next examined the role of Atox1 in subcellular localization of Rac1 in PDGF-stimulated VSMCs. Knockdown of Atox1 with siRNA inhibited PDGF-stimulated Rac1 and ATP7A translocation to the leading edge (Figure 6A). Co-immunoprecipitation analysis showed that PDGF stimulation promotes Rac1 association with ATP7A, which is inhibited by Atox1 siRNA (Figure 6B) or Cu chelator BCS (data not shown). Taken together, these results suggest that Atox1 regulates PDGF-induced lamellipodia formation via regulating the interaction between ATP7A and Rac1 as well as their translocation to the leading edge, which contributes to PDGF-induced VSMC migration.

Atox1 is involved in PDGF-induced LOX activation

We previously demonstrated that ATP7A translocation to the plasma membrane is required for PDGF-induced activation of LOX, which is involved in VSMC migration and vascular remodeling[15, 22]. Therefore, we examined the role of Atox1 on PDGF-induced LOX activation, and found that Atox1 knockout reduced PDGF-induced LOX specific activity (Supplemental Figure VA). We confirmed that ATP7A mutant cells, which have a deletion of two conserved amino acids (Ala799 and Leu800) and lacks Cu transport function[20], also show significant decrease in LOX specific activity induced by PDGF (Supplemental Figure VB). Thus, both Atox1 and its downstream target ATP7A are required for PDGF-induced LOX activation.

Discussion

Previous studies show that Cu chelator TM (tetrathiomolybdate), which blocks Atox1 function[25], prevents tumor progression in clinical trials[5, 26, 27]. Cu transport protein Atox1 activates some secretory Cu enzymes/proteins by delivering Cu to the secretory pathway[19, 28–30], and regulates cell growth and survival[21, 31]. VSMC migration and proliferation are critical event for neointimal formation in response to injury[1–3]. However, the role of Atox1 in neointimal formation and vascular remodeling remains unknown. In the present study, we provide the first evidence that mice lacking Atox1 show suppressed neointima formation and ECM expansion after wire injury, which is accompanied by the decreased inflammatory cell recruitment and VSMCs accumulation within neointima (Figure 6C). We also found that Atox1 expression is increased in the neointima of femoral

arteries following vascular injury, which is associated with an increase in Atox1 positive infiltrated inflammatory cells and migrating αSMA$^+$ VSMCs. It has been shown that metallochaperone Atox1 is an essential regulator of Cu-exporting ATPases, ATP7A, by regulating their catalytic activity as well as by transferring Cu to activate secretory Cu enzymes and Cu excretion[17, 32, 33]. Most recently, we demonstrated that ATP7A is markedly increased in the neointima after vascular injury, and that ATP7A is involved in Cu-dependent PDGF-stimulated VSMC migration[15]. Our current study shows that Atox1 colocalizes with ATP7A in the neointimal VSMCs after wire injury *in vivo*. *In vitro* study also demonstrates that PDGF stimulation promotes Atox1 binding to ATP7A in cultured VSMCs, in a Cu-dependent manner. Given that Cu plays an important role in neointimal thickening after vascular injury and atherosclerosis, it is conceivable that association of Atox1 with ATP7A in the neointimal VSMCs may contribute the Cu-dependent neointima formation and vascular remodeling.

The small G protein family Rac1 plays a key role in lamellipodia formation by reorganizing of the actin cytoskeleton at the leading edge, which plays an important role in cell migration and vascular remodeling[34]. We recently reported that PDGF stimulation promotes association of Rac1 and ATP7A as well as their Cu-dependent translocation to the leading edge, and that ATP7A is required for Rac1 translocation and lamellipodial formation[15]. However, underlying mechanism of how Cu is involved in ATP7A-Rac1-mediated VSMC migration is unknown. In this study, we show that Cu chaperone Atox1 associates with ATP7A, which is enhanced after PDGF stimulation, in a Cu-dependent manner in VSMCs. Moreover, Atox1 knockdown with siRNA prevents PDGF-stimulated formation of ATP7A/Rac1 complex, thereby inhibiting ATP7A and Rac1 translocation to the leading edge, lamellipodial formation, and thus VSMC migration. By contrast, Atox1 is not involved in PDGF-induced VSMC proliferation, although Atox1 is shown to function as a Cu-dependent transcription factor to stimulate Cu-induced cell proliferation in mouse fibroblasts[35]. This suggests that Atox1-mediated cell proliferation may be either stimulant- or cell context-specific. In addition, although it is shown that reactive oxygen species (ROS) are involved in PDGF-induced VSMC migration[36], and that Atox1 regulates ROS levels by modulating SOD3 activity[37], SOD3 siRNA has no effect on PDGF-induced VSMC migration[15]. Thus, SOD3 seems not to be involved in Atox1-mediated PDGF-induced VSMC migration. Taken together, these findings suggest that PDGF activates Cu chaperone function of Atox1 for ATP7A, thereby promoting Cu-dependent Rac1 translocation to the leading edge and lamellipodia formation, which in turn stimulates VSMC migration.

Neointimal expansion in the injured vessel wall depends on not only increased cell migration but also expansion of the ECM, in which LOX is involved. Increased LOX expression is induced in rodent injured vessels, and LOX inhibition limits restenosis after balloon angioplasty[22, 38]. In the present study, we found that Atox1 deficient mice exhibit decreased ECM expansion assessed by Masson's trichrome staining, and Atox1 deficiency shows decrease in LOX specific activity induced by PDGF. We recently reported that knockdown of ATP7A inhibits PDGF-induced LOX activation[15]. Thus, it is likely that Atox1 may deliver catalytic cofactor Cu to the LOX to regulate its catalytic activity through interaction with ATP7A. Given that LOX is also involved in cell migration[15, 39], it is tempting to speculate that the Atox1-ATP7A-LOX pathway also regulates PDGF-induced VSMC migration. Taken together, Atox1 may promote VSMC migration as well as ECM accumulation, at least partially, through controlling LOX activation, thereby leading to neointimal formation in response to vascular injury.

Dysregulation of inflammatory response results in persistent vascular inflammation and adverse vascular remodeling, which contributes to the development of various vascular diseases[40]. Cu is a functional component of the innate immune system and Cu deficiency is

associated with altered inflammatory responses of inflammatory cells, endothelial cells or VSMCs[41, 42]. Importantly, we demonstrate that Atox1 is also expressed in infiltrating inflammatory cells, endothelial cells, as well as VSMCs. Functionally, Atox1$^{-/-}$ mice show impaired macrophage recruitment to the wire-injured vessels and thioglycollate-induced peritonitis. By contrast, Atox1 deficiency had no effect on inflammatory cell migration in response to various cytokines, although Atox1 is colocalized with ATP7A at the leading edge *in vitro*. Although migration of Atox1$^{-/-}$ macrophage is not affected *in vitro*, the Atox1/ATP7A pathway may be at least involved in Cu transport to the Cu-binding proteins or enzymes activated at the leading edge to promote macrophage infiltration to injured tissues *in vivo* and immune responses[43]. The discrepancy of results between *in vitro* and *in vivo* studies may be due to the possibility that microenvironment after injury may be required for Atox1-mediated inflammatory cell migration and invasion *in vivo*. Since the inflammatory cell adhesion and infiltration into the injured vessels is regulated by the activation of VSMCs and endothelial cells, Atox1 may be involved in this process. Additional studies are needed to clarify the precise underlying mechanism. Moreover, cell specific role of Atox1 in neointimal formation as well as mechanisms for upregulation of Atox1/ATP7A expression in response to injury; and role of Atox1/ATP7A in other vascular diseases/pathophysiologies, such as tissue ischemia should be investigated in future studies.

In conclusion, the present study provides the direct evidence that Atox1 plays an important role in vascular remodeling after injury via promoting Cu- and Rac1-depnedent VSMC migration, macrophage infiltration as well as Cu-dependent LOX activation (Figure 6C). Our findings provide insight into Atox1 as a potential therapeutic target for inflammation- and VSMC migration-related vascular diseases, such as atherosclerosis and postangioplasty restenosis.

Materials and Methods

Additional details are available in the online-only Data Supplement.

Animal

Atox1$^{-/-}$ mice (backcrossed eight times to C57Bl/6) were obtained from Mutant Mouse Regional Resource Centers. They were further backcrossed to C57Bl/6 mice more than ten times, as previously described[1]. Age matched C57Bl/6 mice used for control, wild type (WT) mice, were purchased from Jackson Laboratory. All mice were maintained at the University of Illinois at Chicago animal facilities. Mice at 12 to 16 weeks-old were used for experiments. Animal protocols were approved by the Animal Care and Use Committee of the University of Illinois at Chicago.

Vascular injury model

Transluminal arterial injury was performed in 12 to 16-week-old male mice. Briefly, mice were anesthetized using an intraperitoneal injection of ketamine (100mg/kg body weight) and xylazine (10 mg/kg) (Phoenix Scientific, Inc., St. Joseph, MO). A straight spring wire (0.25mm in diameter, Cook, Bloomington, IN) was inserted into the left femoral artery and placed there for 1 minute. Complete removal of endothelium was achieved by this procedure, as previously reported[2, 3].

Modified Boyden chamber assay

Migration assays using VSMCs and bone marrow (BM)-derived macrophages with the Modified Boyden Chamber method were conducted in duplicate 24-well transwell chambers as described previously[4, 56]. The upper insert (8-μm pores coated with 0.1% gelatin) containing VSMCs suspensions (5×10^4 cells) or BM-derived macrophages (2.5×10^5 cells)

were placed in the bottom 24-well chamber containing fresh serum free media with stimulant. The chamber was incubated at 37°C for the indicated time point. The membrane was fixed and stained using Diff-Quick. Cells were quantified under the microscope by blinded investigators.

Cell proliferation assay

VSMCs (3×10^5 cells) were seeded in 6-well plates, and cell number with and without PDGF in 0.1% bovine serum containing culture medium was determined by counting with a hemocytometer as described before[4, 5, 7].

Thymidine incorporation

VSMCs were seeded in 6-well plates at a density of 10^5 cells/well, treated with Atox1 siRNA or control siRNA for 48 hours, serum-starved for 24 hours with 0.1% bovine serum prior to stimulation, and PDGF-stimulated for 72 hours. During the last 6 hours of stimulation, cells were incubated with [^{3}H] thymidine (Amersham).

Thioglycollate-induced peritonitis

Mice were intraperitoneally injected with 1 ml of 4% thioglycollate broth (Sigma). Four days after the injection, mice were killed by CO_2 inhalation. Cells in peritoneal cavity were recovered by peritoneal lavage by injecting intraperitoneally with 3 changes of 3 ml of PBS containing 0.1% BSA, 0.5 mM EDTA and 10 U/ml of heparin as described previously[6]. Total cell and macrophage counts were determined by hemocytometer under microscopy.

Monoclonal Atox1 antibody production

Mouse monoclonal Atox1 antibody (Z3112) was developed by using baculovirus particles displaying the surface glycoprotein gp64-fusion protein as the immunizing agent. Briefly, the Atox1 cDNA was ligated into the gp64 gene to create a fusion protein that is expressed in the viral surface, as previously described[8]. Positive clones were selected using recombinant Atox1 protein or cells overexpressing Atox1 by adenovirus[7, 9].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

Copper (Cu), an essential nutrient, has been implicated in vascular remodeling and atherosclerosis with unknown mechanism. Since excess Cu is toxic, the bioavailability of intracellular Cu is tightly regulated by Cu transport proteins including Antioxidant-1 (Atox1), which are required for Cu uptake/efflux and Cu transfer to specific subcellular compartments and target proteins. The current study provides the compelling evidence that Atox1 plays an important role in vascular remodeling after injury via promoting Cu- and Rac1-depnedent vascular smooth muscle cell (VSMC) migration, macrophage infiltration as well as Cu-dependent lysyl oxidase activation. Mechanistically, in cultured VSMC, Atox1 depletion inhibits PDGF-induced Cu-dependent VSMC migration by preventing translocation of Cu transporter ATP7A and small G protein Rac1 to the leading edge as well as Cu- and Rac1-dependent lamellipodia formation. Our findings provide insight into Atox1 as a potential therapeutic target for inflammation- and VSMC migration-related vascular diseases, such as atherosclerosis and postangioplasty restenosis.

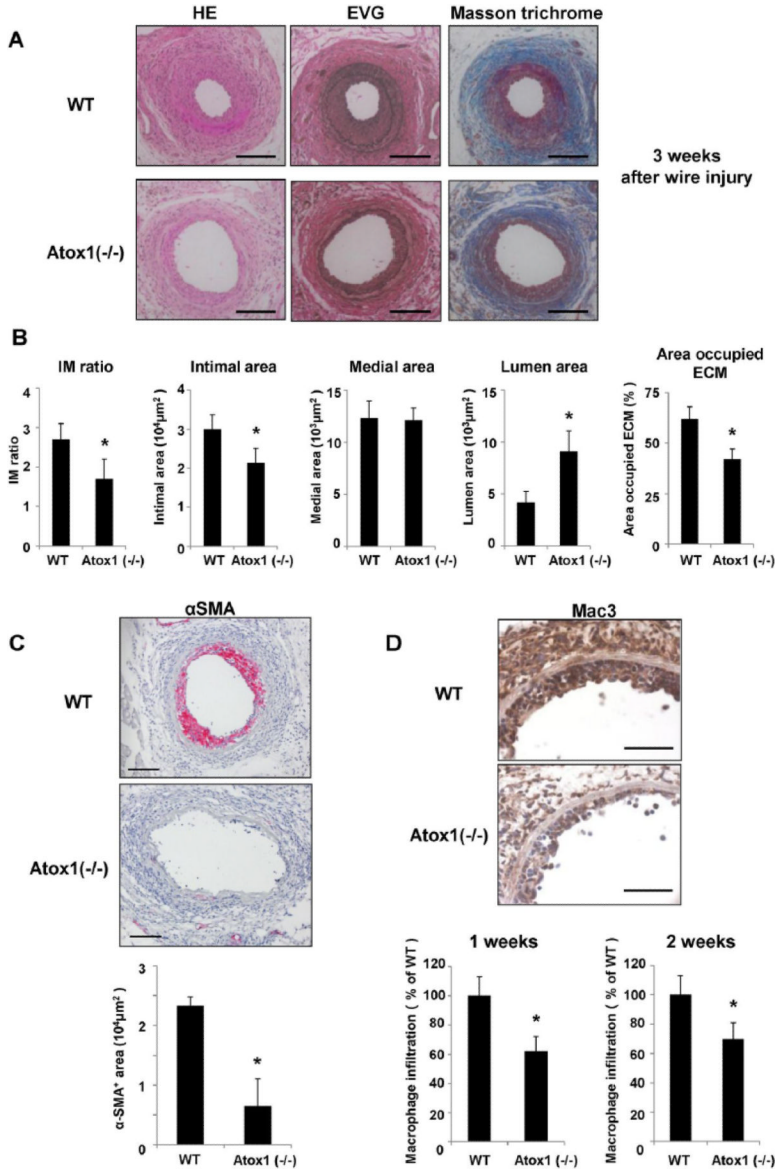

Figure 1. Atox1 is involved in neointimal formation and fibrosis in response to vascular injury

A: Hematoxylin & Eosin (HE), elastic van Gieson (EVG; delineates elastic laminae), and Masson trichrome staining of femoral arteries obtained from wild-type (WT) and Atox1 knockout (Atox1$^{-/-}$) mice, on day 21 after injury. **B:** Quantitative morphometric analysis of vessel remodeling in WT and Atox1$^{-/-}$ mice are expressed as mean ± S.E. of three sections from each of five vessels. Area occupied extracellular matrix (ECM) = Blue staining area/ Intimal area. **C and D:** Immunostaining of α-smooth muscle actin (αSMA) (**C**) and Mac3 (**D**) in wire-injured vessels in WT or Atox1$^{-/-}$ mice. Representative sections from injured vessels in WT and Atox1$^{-/-}$ mice on day 7 (**D**) or 14 (**C**) are shown. Bars represent 100μm. Graphs represent αSMA positive area or number of Mac3-positive cells. Values represent the means ± S.E. *$P < 0.05$ vs. WT.

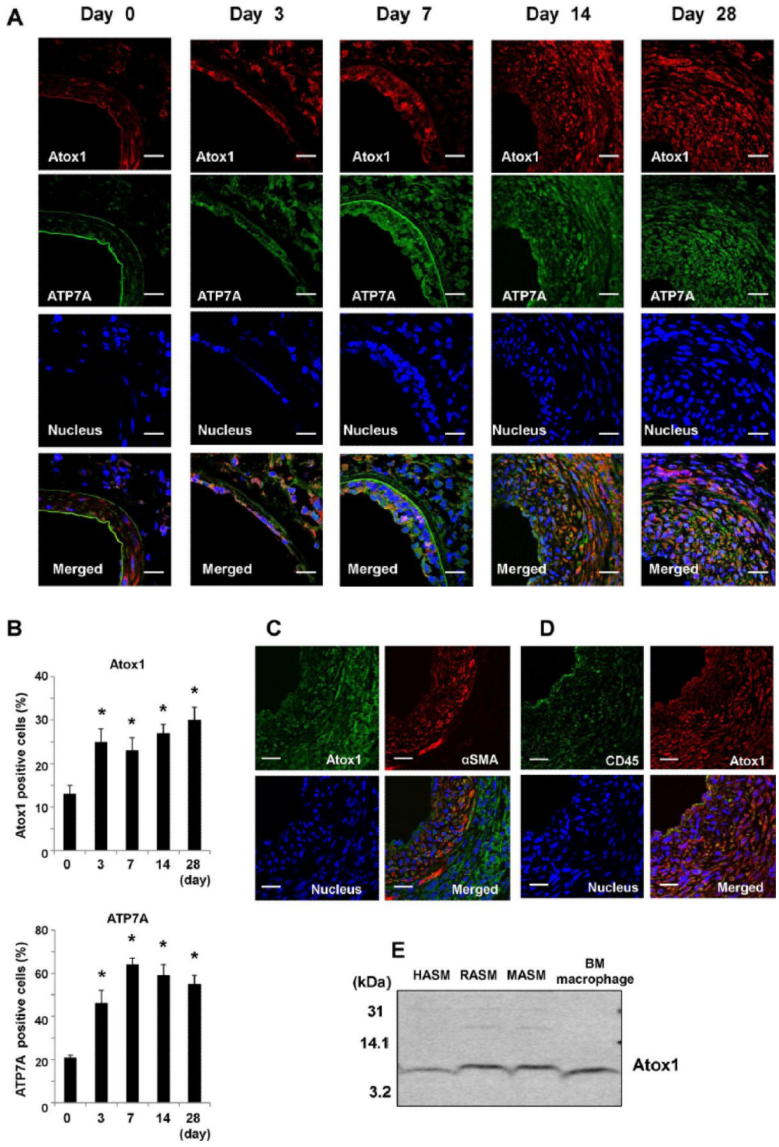

Figure 2. Atox1 expression is upregulated at vascular smooth muscle cells and macrophage in injured vessels and its expression colocalized with ATP7A-positive cells

A: Immunofluorescence analysis for uninjured (day 0) or injured (day 3, 7, 14, and 28) artery co-stained with anti-ATP7A (**green**) and Atox1 (**red**) antibodies, and Topro-3 (**blue**; nucleus marker). **B:** Time-course quantitative analysis of Atox1 or ATP7A-positive cells in wire-injured arteries. $*P < 0.05$ vs. uninjured artery. **C and D:** Representative staining for Atox1 (**green**) and α-smooth muscle actin (αSMA), SMC marker (**red**) (**C**), or Atox1 (**red**) and CD45, leukocytes marker (**green**) (**D**) on day 14 in wire-injured femoral arteries. Bars represent 20μm. **E**: Western blot analysis of Atox1 protein expression in human, rat, and mouse aortic smooth muscle cells (HASMs, RASMs, MASMs) and mouse bone marrow (BM)-derived macrophages.

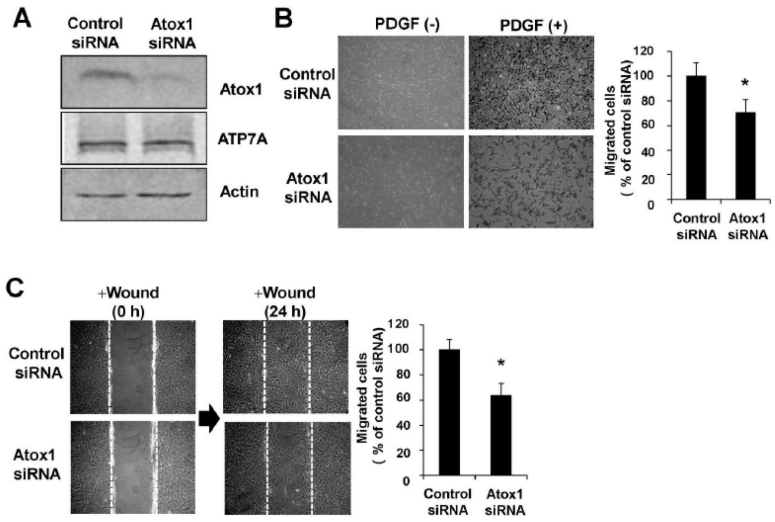

Figure 3. Atox1 is involved in PDGF-induced VSMC migration

Rat aortic smooth muscle cells (RASMs) were transiently transfected with Atox1 or control siRNAs for 48 hours. **A:** Western blot analysis of total cell lysates from RASMs transfected with Atox1 or control siRNA, using anti-Atox1, anti-ATP7A, and anti-actin antibodies. **B:** Cell migration was assessed by the modified Boyden chamber assay after stimulation with or without 50 ng/mL PDGF for 8 hours. **C:** Wound-scratch assay was performed in confluent monolayers of RASMs in the presence of PDGF (50 ng/mL). Images were captured immediately after rinsing at 0 hour and at 24 hours after the wounding in the cells. Values represent the means ± SD. *$P < 0.05$ vs. control siRNA-treated cells.

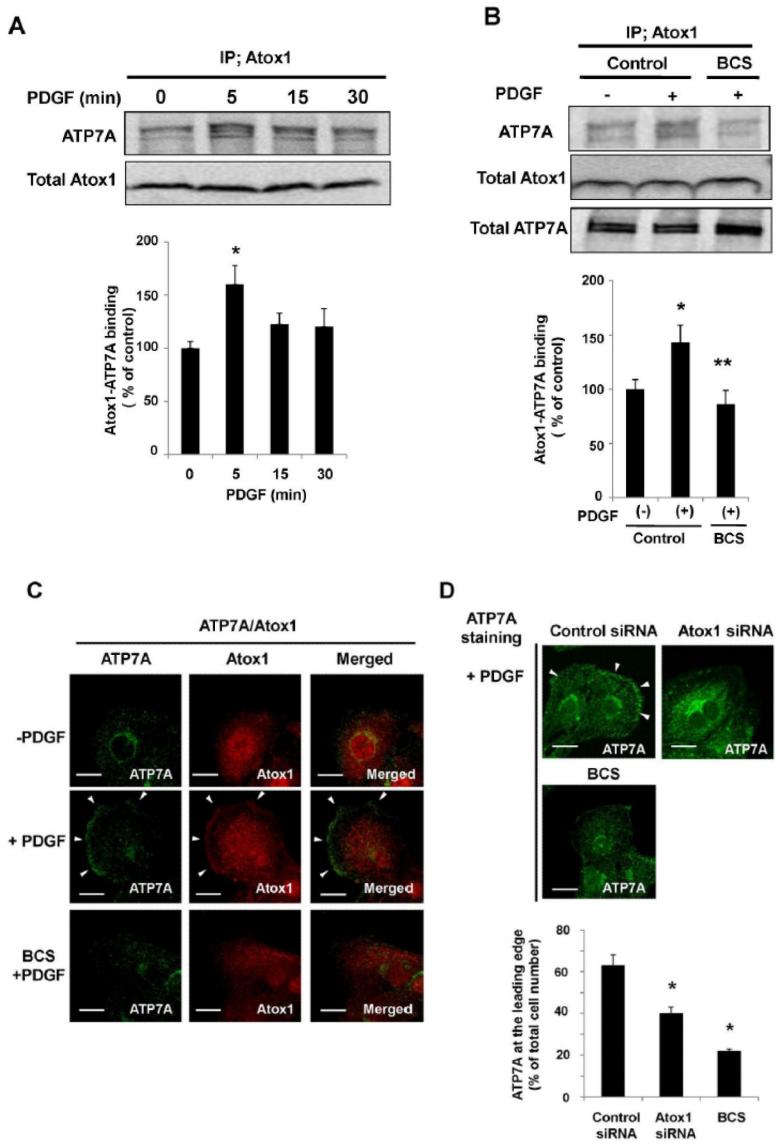

Figure 4. Atox1 associates with ATP7A after PDGF stimulation in a Cu-dependent manner and is involved in PDGF-stimulated ATP7A translocation to the leading edge in VSMC

Growth-arrested RASMs were stimulated with 50 ng/mL PDGF for indicated times (**A**) or 5 minutes (**B**). **A:** PDGF stimulation promoted Atox1 association with ATP7A in a time dependent manner. **B:** RASMs were treated with the copper chelator BCS (200μmol/L for 72 hours) with or without PDGF. PDGF stimulation promoted Atox1 association with ATP7A in a Cu-dependent manner. Lysates were immunoprecipitated (IP) with anti-Atox1 antibody, followed by immunoblotting (IB) with anti-ATP7A antibody. **C:** RASMs were treated with BCS with or without PDGF. Cells were stained with anti-ATP7A (**green**) and anti-Atox1 (**red**) antibodies. **D:** RASMs were treated with BCS or transfected with Atox1 or control siRNA for 48 hours with PDGF. Cells were stained with anti-ATP7A (**green**) antibody. White arrowheads points to the leading edge. All fluorescence images were taken at 5 different fields/well, and the cell images are representative of 3 different experiments. Bar represents 20μm. Values represent the means ± SD for 3 independent experiments. *$P <$ 0.05 vs. nonstimulated (**A**, **B**) or control siRNA-treated (**D**) cells. **$P < 0.05$ vs. control PDGF-stimulated cells.

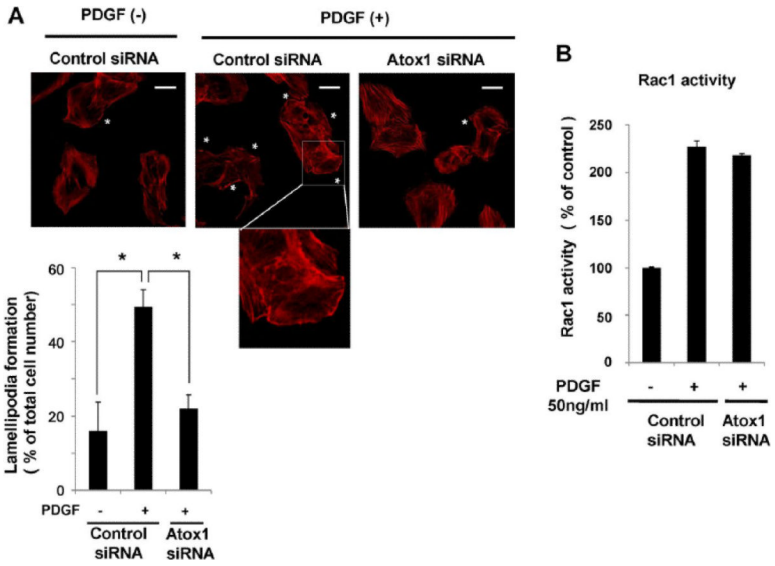

Figure 5. Atox1 is involved in PDGF-stimulated lamellipodia formation in VSMCs

A: RASMs transfected with control siRNA or Atox1 siRNA were stimulated with 50 ng/mL PDGF as described for Figure 4, and cells were stained for phalloidin to visualize lamellipodia formation. Cells with lamellipodia formation were expressed as percentage of total cell number (means ± SD, n=3). *$P < 0.05$. Bar represents 50μm. **B:** Effect of Atox1 siRNA on PDGF-induced Rac1 activation. Rac1 activities were determined by G-LISA assay.

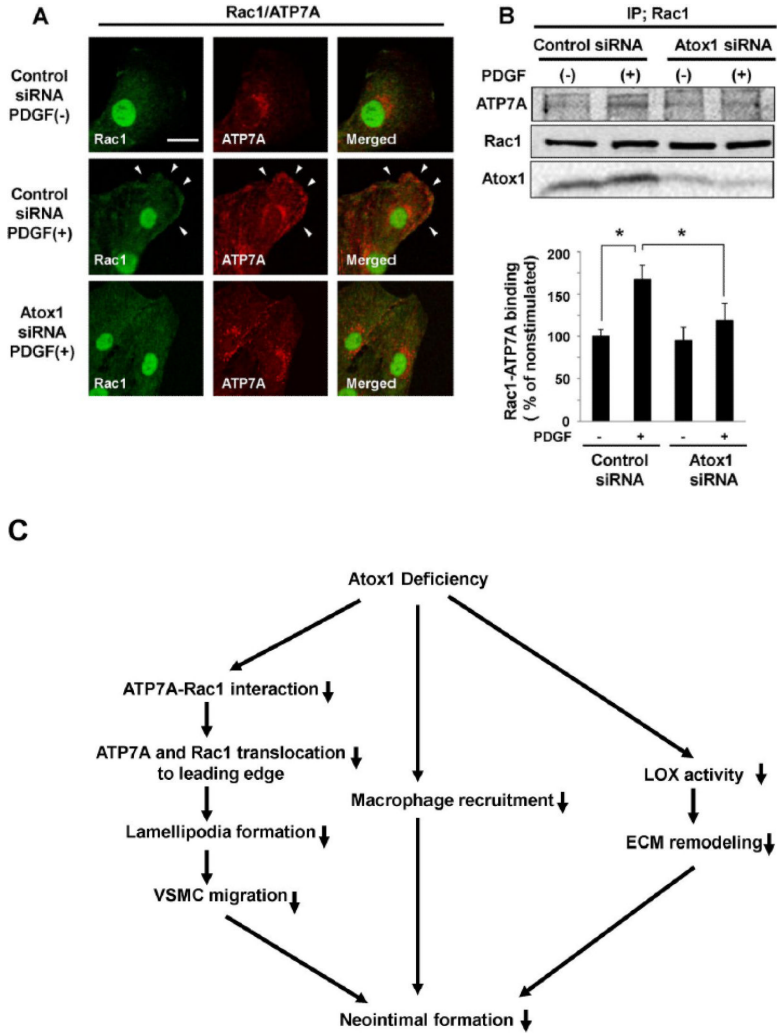

Figure 6. Atox1 is involved in PDGF-stimulated association of Rac1 and ATP7A and its translocation to the leading edge in VSMCs

RASMs transfected with Atox1 or control siRNA were stimulated with 50 ng/mL PDGF for 5 minutes. **A:** Cells were stained with anti-Rac1 (**green**) and anti-ATP7A (**red**) antibodies. White arrowheads points to the leading edge. All fluorescence images were taken at 5 different fields/well, and the cell images are representative of 3 different experiments. Bar represents 20μm. **B:** PDGF stimulation promoted ATP7A association with Rac1 in an Atox1-dependent manner. Lysates were immunoprecipitated (IP) with anti-Rac1 antibody, followed by immunoblotting (IB) with ATP7A antibody. Values represent the means ± SD for 3 independent experiments. $*P < 0.05$. **C:** Proposed model for role of Atox1 in vascular remodeling. Atox1 plays an important role in neointimal formation in response to vascular injury, which is at least due to the VSMC accumulation within neointima, macrophage recruitment, and extracellular matrix (ECM) remodeling. Mechanistically, Atox1 is involved in PDGF-induced VSMC migration by promoting Rac1- and ATP7A-translocation to leading edge and lamellipodia formation as well as activating ECM Cu enzyme LOX.