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Molecules and Cells

Pim Protein Kinase-3 Is Regulated by TNF-α and Promotes Endothelial Cell Sprouting

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Tumor necrosis factor-α (TNF-α) plays an important role in pathological angiogenesis associated with inflammatory response. Pim-3 kinase belonging to serine/threonine protein kinases is a potent suppressor of myc-induced apoptosis. We have recently demonstrated that Pim-3 plays an essential role in endothelial cell (EC) spreading and migration. In this study, we showed that TNF-α transiently increased Pim-3 mRNA expression, and this was mediated through Tumor necrosis factor-α receptor-1 (TNFR1) pathway in ECs. TNF-α could promote stabilization of Pim-3 mRNA in ECs. Small-interfering RNA (siRNA)-mediated gene knockdown of Pim-3 significantly impaired TNF-α-induced formation of EC membrane protrusions *in vitro*. Furthermore, Pim-3 silencing inhibited EC sprouting in subcutaneous Matrigel *in vivo*. eNOS mRNA abundance was lower in Pim-3 siRNA transfected ECs compared with the control ECs. These observations suggest that Pim-3 plays a role in TNF-α-induced angiogenesis.

INTRODUCTION

Angiogenesis is an essential component of inflammation. In recent years, large advances in angiogenesis research have occurred, especially in oncology (Kamba and McDonald, 2007; Trivella et al., 2007). Traditionally, mechanisms in inflammation angiogenesis were inferred from tumor angiogenesis. Recent research, however, has highlighted the similarities and dissimilarities between these processes. Endothelial cells participate in this process through the synthesis and secretion of pro-inflammatory cytokines, including VCAM, ICAM and interleukin (IL) (Benelli et al., 2006; Carmeliet, 2003). These molecules are also important components in the remodeling of vessels, mainly capillaries and venules, during inflammation (Ezaki et al., 2001; Thurston et al., 1998). One of the major inflammatory cytokines, tumor necrosis factor-α (TNF-α), is known to influence endothelial cell migration. (Bradley, 2008; Gao et al., 2002; Pan et al., 2002; Wu et al., 2005) Studies show that TNF-α can display either proangiogenic or antiangiogenic effects depending on experimental conditions. (Kociok et al., 2006; Mori et al., 2002).

Pim-3 belongs to a family of proto-oncogenes that encode serine/threonine protein kinases. Previously, studies have found that Pim-3 is aberrantly expressed in human colon cancer cells (Popivanova et al., 2007), hepatocellular carcinomas (Fujii et al., 2005; Wu et al., 2010) and pancreatic tumor cell lines. (Jian et al., 2009; Li et al., 2006; 2009) Pim-3 plays an important role in regulating the biology of tumor cells and inhibiting apoptosis. Pim-3 can promote tumor cell growth by regulating apoptosis and cellular metabolism through phosphorylating their substrates. (Amaravadi and Thompson, 2005; Muraski et al., 2007; Wood et al., 2009) Recently, we found that Pim-3 is concentrated at the cellular lamellipodia in ECs and plays an essential role in EC spreading and migration. (Zhang et al., 2009) The purpose of this study is to investigate the possible implication of Pim-3 in the TNF-induced EC sprouting. We report here that Pim-3 is a molecular target of TNF in ECs.

MATERIALS AND METHODS

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords according to the procedure described previously and cultured on plates coated with collagen I. (Zhang et al., 2009) Cells were maintained in M199 supplemented with 16% fetal bovine serum (FBS), 20 mM HEPES (pH 7.4), 1 ng/ml recombinant human acidic fibroblast growth factor (aFGF), and 90 μg/ml heparin and antibiotics at 37°C in 5% CO2. For all experiments, early passage (3-5) HUVECs were used. Medium 199, Earle, OPTI-MEM, Lipofectamine LTX reagent and FBS were purchased from Invitrogen (USA). Collagen I and aFGF were from Sigma (USA). Matrigel was obtained from BD company (USA) and the TNF-α was from Promega (USA).

RNA isolation and reverse transcription

Total cellular RNA was isolated using TRIZOL reagent (Invitrogen, USA). RNA concentrations were determined spectroscopically at 260 nm. cDNA was synthesized from 2 μg of total RNA by using the Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, USA) according to the manufacturer's protocol.

Semi-quantitative RT-PCR

Pim-3 mRNA expression was assessed using RT-polymerase chain reaction. A volume of 1 μl of cDNA from the RT reaction was used for the PCR reaction containing 0.2 μM of each for-

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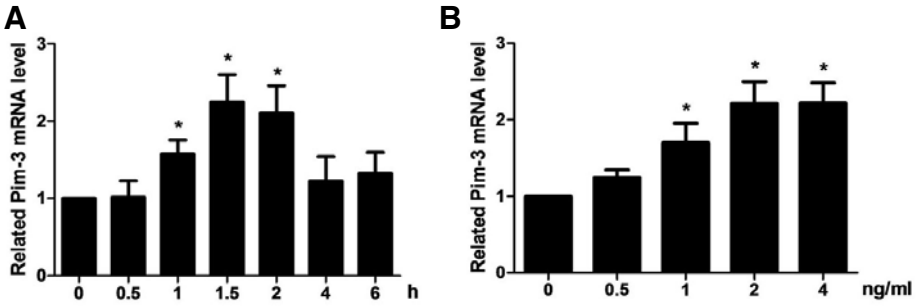

Fig. 1. Effects of TNF-α on Pim-3 mRNA expression in ECs. Confluent ECs were treated with different concentrations of TNF-α for indicated time. Pim-3 mRNA expression was analyzed using RT-PCR. The results represent the mean ± S.E.M. for triplicate experiments. *$P < 0.05$.

ward and reverse primer and 12.5 μl 2× Taq Plus PCR MasterMix (TIANGEN BIOTECH, CN). The sequences of the primers used for PCR are as follows: 5′-GCACACACAATGCAAG TCCT-3′ and 5′-AGAGGCAGACTGCTCAGAGG-3′ for Pim-3; 5′-ACCAAGTGCCACAAAGGAAC-3′ and 5′-CTGCAATTGAA GCACTGGAA-3′ for TNFR1; 5′-CTCAGGAGCATGGGGAT AAA-3′ and 5′-AGCCAGCCAGTCTGACATCT-3′ for TNFR2; 5′-GACGCTACGAGGAGTGGAAG-3′ and 5′-CCTGTATGCC AGCACAGCTA-3′ for eNOS. The reactions were performed for 23 to 25 cycles using the following conditions: 94°C 30 s, 56°C 30 s and 72°C 30 s. PCR reactions were analyzed on a 1.5% agarose gel and quantified using Image J software (NIH). The level of Pim-3 mRNA was normalized to beta-actin mRNA. Data were collected from three to four experiments.

mRNA stability assay

The half-life of Pim-1 and Pim-3 mRNA were determined by treating ECs with actinomycin D (10 μg/ml) to block transcription either alone or combined with TNF-α. During the following 5 h, the cells were harvested every hour, and total RNA was prepared. RT-PCR was performed as described.

siRNA transfection

siRNAs were purchased from Eurogentec (Jikai, Inc., China). The siRNA targeting human Pim-3 was synthesized with the sense sequence of GGCGUGCUUCUCUACGAUATT. TNFR1 and TNFR2 were knocked down by transient expression of siRNA directed against TNFR1 (5′-CAAAGGAACCUACUUG UACUU-3′) and TNFR2 (5′-GGGUGAUAAAUUGUUGAUA-3′). The annealed siRNA was transfected using Lipofectamine LTX according to the manufacturer's protocol. An RNA duplex targeting GFP was used as a control. The efficiency of target gene silencing was determined by RT-PCR analysis.

Membrane protrusion formation with an *in vitro* wound-healing assay

Confluent ECs were transfected with scramble or Pim-3 siRNA fragments for 24 h. Wounds were created on cell monolayers by using a sterile pipette tip. The debris was removed by washing the cells with serum-free M199 culture medium. The cells were photographed under an inverted microscope after further incubation in a 37°C incubator for 4 h in 2% serum M199 medium. A total of five areas were selected randomly on each culture plate.

Matrigel sprouting assay *in vivo*

Normal male mice (C57BL/6) 6-8 weeks of age and weighing ~20 g were used. The experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publications No.85-23, revised 1996) and was approved by the Animal Care and Use Committee of Hubei University of Medicine. *In vivo* angiogenesis measurements using Matrigel plugs were performed as previously described. (Bochkov et al., 2006; van den Berg et al., 2009) Confluent ECs were infected at 50 MOI with recombinant adenoviruses expressing green fluorescent protein (GFP) for 4 h. The siRNAs were then transfected into ECs for another 24 h before they were harvested for matrigel assay. Growth factor-reduced Matrigel (BD Biosciences) was thawed overnight at 4°C and mixed with 1×10^5 cells. Matrigel (0.4 ml) was injected into the ventral side of mouse. After three days of treatment, the mice were euthanized by CO_2 asphyxiation for plug exclusion. Matrigel plugs were isolated and fixed in 4% paraformaldehyde. Preparations were analyzed with a confocal microscope.

Western blot analysis

TNFR1 antibody was purchased from Santa Cruz Biotechnology (USA). Total protein from the cells was extracted using RIPA protein extraction reagent, supplemented with protease inhibitor cocktail (1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF; Calbiochem, USA). The protein was subjected to SDS-polyacrylamide electrophoresis and transferred to nitrocellulose membranes. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T). Then, the membrane was blotted overnight with TNFR1 antibody. The membranes were washed and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody. The membranes were washed and developed by chemiluminescence.

Statistic analysis

Data are expressed as the mean ± SEM. Differences between groups were analyzed by ANOVA followed by Student's *t*-test and were considered significant when $P < 0.05$.

RESULTS

TNF-α induces Pim-3 mRNA expression in ECs

We first investigated whether Pim-3 mRNA expression was regulated by TNF-α in ECs. Confluent ECs were incubated with TNF-α at different doses for the indicated times before total RNA was extracted with TRIZOL reagent. As shown in Fig. 1A, TNF-α treatment rapidly and transiently induced the expression of Pim-3 mRNA in ECs with the gene elevation within 90 min and peaking by 2 h. TNF-α significantly upregulated Pim-3 gene expression in a dose-dependent manner (Fig. 1B).

TNF-α acts *via* TNFR1 to stimulate Pim-3 mRNA expression in ECs

TNF-α acts on several different signaling pathways through two cell surface receptors, TNFR1 and TNFR2. To further investigate the contribution of TNFR1 and TNFR2 to TNF-α-induced Pim-3 expression, ECs were transfected with scramble, TNFR1

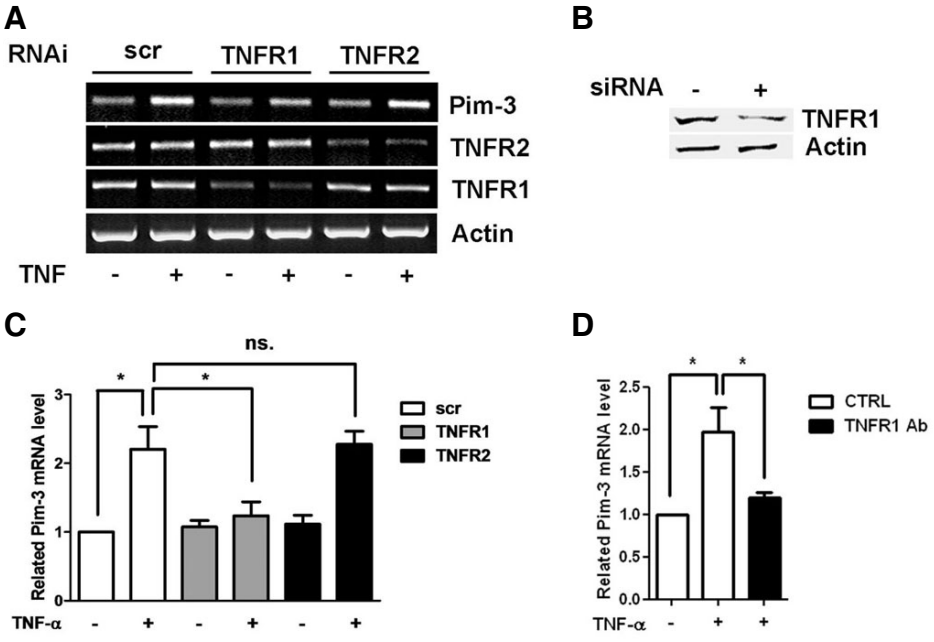

Fig. 2. TNFR1 silencing inhibited TNF-α-induced Pim-3 expression in ECs. TNFR1 and TNFR2 RNA interference in ECs were achieved by performing transfections with double-stranded RNA at 20 nM. (A) Silencing of TNFR1 and TNFR2 gene expression was confirmed using RT-PCR. (B) Silencing of TNFR1 protein expression was further confirmed by Western blot analysis. (C) Twenty-four hours after transfection, ECs were treated with TNF-α for 2 h. (D) ECs were preincubated for 30 min with a mAb blocking TNFR1 or a normal IgG (100 μg/ml each) before being treated with TNF-α for 2 h. RT-PCR analysis was performed to check Pim-3 mRNA expression. The results represent 3 independent experiments. *$P < 0.05$.

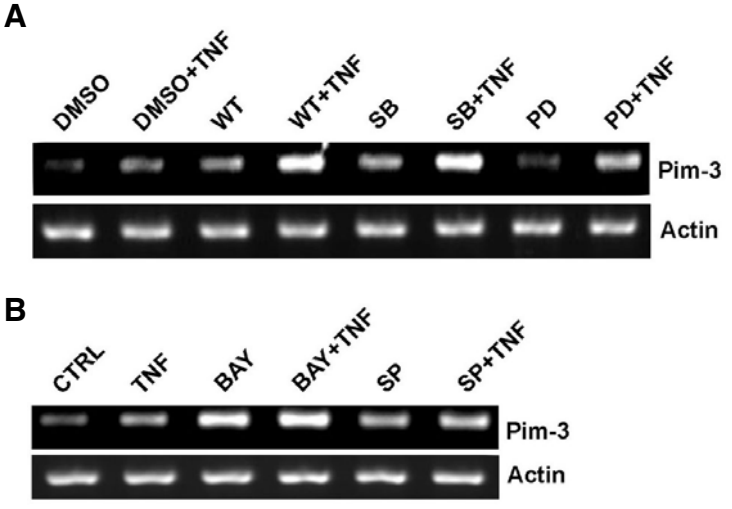

Fig. 3. Effects of signaling inhibitors on Pim-3 mRNA expression in ECs. ECs were pretreated for 30 minutes with or without 20 nM wortmannin (WT), 10 μM SB203580 (SB), 10 μM PD98059 (PD), BAY11-7802 (BAY), or SP60012570 (SP) before TNF-α treatment. Whole cell lysates were analyzed for Pim-3 mRNA expression by RT-PCR. The results represent 3 independent experiments.

or TNFR2 siRNA. As shown in Fig. 2A, TNFR1 and TNFR2 siRNA decreased TNFR1 and TNFR2 mRNA expression, respectively. TNFR1 silencing significantly inhibited TNF-α-induced Pim-3 expression in ECs whereas TNFR2 silencing had no effect on TNF-α-induced Pim-3 expression. Furthermore, neutralizing antibody against TNFR1 inhibited TNF-α-induced Pim-3 mRNA expression (Fig. 2D).

Wortmannin, SB203580, BAY11-7082 and SP600125 up-regulate Pim-3 mRNA expression in ECs

TNF-α has been shown to be a potent activator of p38 MAP kinase, ERK, PI3K/Akt, NF-κB and JNK kinase. To elucidate which signaling pathways contribute to TNF-α induced Pim-3 mRNA expression, we treated the ECs with several kinase inhibitors before TNF-α stimulation. The results showed that the PI3K inhibitor, wortmannin, the p38MAPK inhibitor, SB203580, the NF-κB inhibitor, BAY11-7082 and the JNK inhibitor, SP600125, all increased the Pim-3 mRNA expression in ECs. Moreover, these inhibitors and TNF-α had overlapping effects on Pim-3 mRNA levels.

TNF-α stabilizes endogenous Pim-3 mRNA

The expression of Pim kinases is regulated by transcriptional and post-transcriptional mechanisms, including mRNA stability and translation. Here, we examined the effect of TNF-α on endogenous Pim-3 mRNA stability in ECs. Actinomycin D, which blocks transcriptional activity, was used to determine the decay of Pim-3 mRNA in ECs. The mRNA half-life was about 1 h in control cells. In TNF-α-treated ECs, there was only a slight decrease during the first 1 h of actinomycin exposure. Pim-3 mRNA levels at each time point were more abundant than in the un-stimulated control. This result suggests an effect of TNF-α on Pim-3 post-transcriptional regulation (Fig. 4).

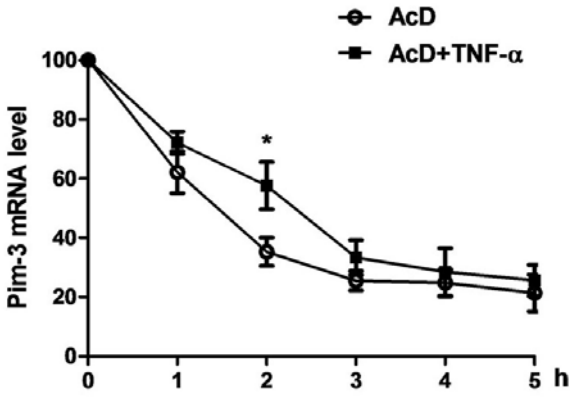

Fig. 4. Analysis of Pim-3 mRNA levels in TNF-α treated ECs. The ECs were preincubated with actinomycin D (10 μg/ml) and treated with TNF-α or vehicle alone for different time intervals (0-5 h). ECs were harvested at indicated time points. Fold change in Pim-3 mRNA expression was measured by RT-PCR as described. The mRNA level at zero time point was considered 100%. The results represent 3 independent experiments. *$P < 0.05$.

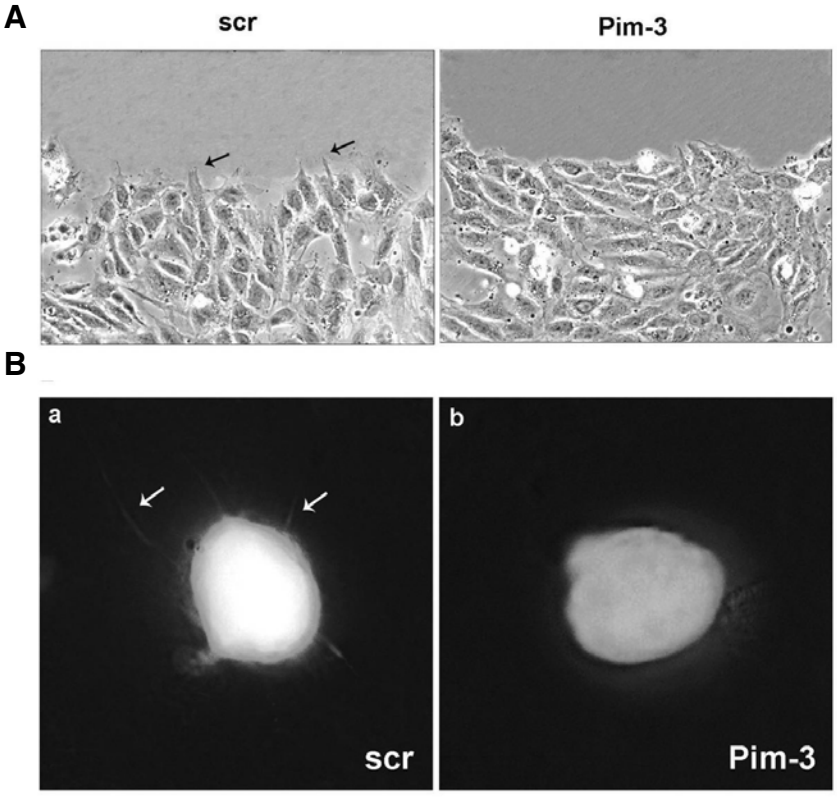

Fig. 5. The inhibitory effects of Pim-3 silencing on EC filopodia formation and sprouting. (A) Scramble and Pm-3 siRNA-transfected ECs were cultured in 2% FBS M199 culture medium for 4 h, and then treated with TNF-α (10 ng/ml) for another 4 h. Filopodia formation was analyzed in a wound-healing assay. (B) Ad-GFP infected and Pim-3 siRNA transfected ECs were harvested and mixed with matrigel, then injected into the ventral side of mouse. Matrigel plugs were isolated 3 days later. EC sprouting were observed with a confocal microscope. Representative results from three independent experiments are shown.

Effect of Pim-3 silencing on TNF-α-induced formation of membrane protrusions *in vitro* and endothelial cell sprouting *in vivo*

Exposure of ECs to TNF-α causes an increase in the formation of membrane protrusions and cell migration. Previously, we reported that Pim-3 plays an essential role in EC migration. We next examined the effect of Pim-3 silencing on the formation of membrane protrusions by using a well-established *in vitro* wound-healing assay. The results showed that scramble siRNA transfected ECs display more membrane protrusions than Pim-3 silenced ECs after TNF-α exposure (Fig. 5A). Furthermore, the function of Pim-3 in endothelial cell sprouting was also investigated *in vivo*. We used the mouse subcutaneous Matrigel plug assay to investigate Pim-3 angiogenic activity. Silencing of Pim-3 after siRNA transfection was confirmed by RT-PCR analysis (data not shown). The EC spheroids extended tube-like sprouts into the surrounding matrix (Fig. 5Ba). We found that Pim-3 silencing prevents the activation of EC sprouting more than in control cells (Fig. 5Bb). These data confirm that Pim-3 exerts pro-angiogenic characteristics *in vivo*.

Pim-3 contributes to eNOS mRNA abundance in ECs

Previous studies have shown that eNOS constitutes an important molecular signal that contributes to cell migration and actin polymerization. Cellular nitric oxide (NO) was also produced in a cell cycle-dependent manner. We observed the effect of Pim-3 silencing on eNOS expression in ECs. As seen in Fig. 6, a relatively low level of eNOS mRNA was detected in Pim-3 siRNA transfected cells. Thus, Pim-3 may regulate EC migration either directly or through eNOS signaling.

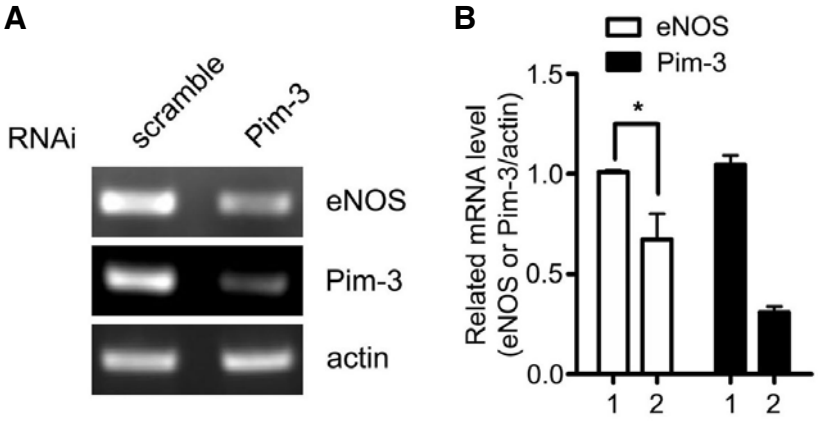

Fig. 6. Pim-3 silencing decreased eNOS mRNA expression in ECs. ECs were transfected with scrambled siRNA or Pim-3 siRNA. At 24 h post transfection, the mRNA levels of eNOS and Pim-3 were analyzed by semi-quantitative RT-PCR assay. *$P < 0.05$.

DISCUSSION

The results presented in this study demonstrate that Pim-3 kinase is a molecular target of TNF-α in ECs as TNF-α increased the mRNA stability of Pim-3 *via* TNFR1. Pim-3 silencing also inhibited TNF-α-induced membrane protrusion formation in ECs.

During wound healing, blood-derived monocytes accumulate and differentiate into tissue macrophages. These activated macrophages and monocytes synthesize and secrete several cytokines, including TNF-α, which activates local ECs (Li et al., 2005). The role of TNF-α in angiogenesis has been highly controversial. Numerous studies show that TNF-α can display either proangiogenic (Numasaki et al., 2004; Secchiero et al., 2004; Tanner, 2004; Zhu et al., 2006) or antiangiogenic effects (Frater-Schroder et al., 1987; Mori et al., 2002). TNF-α has been shown to inhibit activity and expression of VEGF receptors. Studies have also shown that TNF-α inhibits EC proliferation and migration *in vitro*, but TNF-α enhances angiogenesis *in vivo* (Frater-Schroder et al., 1987). On the other hand, TNF-α induces the release of metalloproteinases and vascular endothelial growth factor A, both potent angiogenic factors. TNF-α also increases the activation and ligation of $\alpha_5\beta_3$ and decreases the activation and ligation of $\alpha_5\beta_1$-integrins to facilitate EC migration (Gao et al., 2002). It has been reported that low concentration of TNF-α induced the high levels of tubule formation *in vitro*. We previously reported that Pim-3 kinase was concentrated in the lamellipodia in cultured ECs (Zhang et al., 2009). Additionally, Pim-3 plays an important role in regulating EC migration and proliferation. This study demonstrates that a low concentration of TNF-α induced Pim-3 mRNA expression in ECs. Reportedly, the structures of human Pim kinases demonstrate a conserved kinase domain and no regulatory domain (Amaravadi and Thompson, 2005). Pim-3 kinase activity depends on the level of Pim-3 gene expression. Thus, Pim-3 may partly mediate TNF-α-induced EC dysfunction.

There are two distinct types of receptors for TNF-α encoded by distinct genes. TNFR1 is expressed by all human tissues while TNFR2 expression is predominantly restricted to cells of hematopoietic origin and ECs. Contradictory results have been reported on the roles of TNFR1 and TNFR2 in angiogenesis. Pathological retinal neovascularization was significantly reduced in TNFR1- but not TNFR2-deficient mice (Kociok et al., 2006). On the other hand, Mori et al. (2002) reported that TNFR1 knockout mice exhibited an increment in vascular densities at wound sites. Another research group found that ischemia-initiated angiogenesis was enhanced in TNFR1 knockout mice but reduced in TNFR2 knockout mice (Luo et al., 2006). Activation of TNFR1 can cause inhibition of EC migration and EC apoptosis. These data demonstrate that TNFR1 and TNFR2 have opposite effects on EC survival and migration. We reported here that TNF-α acts *via* TNFR1 to stimulate Pim-3 mRNA expression in ECs. TNFR1 is the major signaling receptor for TNF-α in ECs. The present study expands our understanding of the mechanism of TNF-α induced vessel formation.

Other studies have demonstrated that the diverse TNF-α mediated biological responses are achieved through activating multiple signaling pathways. Our data show that pharmacological inhibitors of several signaling pathways increased the Pim-3 mRNA expression in ECs. Wortmannin is a PI3K and PI3K-like kinase inhibitor. We found that wortmannin up-regulates Pim-3 mRNA expression by inhibiting DNA-dependent serine/threonine protein kinase (DNA-PK) in ECs (our unpublished data). These observations suggest that other signaling pathways may be involved in TNF-α-induced Pim-3 expression in ECs.

The cellular biological activity of Pim kinases depends on protein quantity. The results from mRNA stability studies suggest that the level of Pim mRNA is also controlled post-transcriptionally as regulation of Pim mRNA stability partially determines Pim activity. The stability of the Pim-1 transcript is regulated in lymphocytes during the course of lymphocyte activation. In addition, IL2, IFNy and SLF can post-transcriptionally regulate the level of Pim-l mRNA (Wingett et al., 1991; Yip-Schneider et al., 1995). Our data indicate that the transient induction of Pim-3 gene expression by TNF-α is associated with regulation by mRNA stability. Previously, it was reported that the destabilizing (UAUU)n motif contributed to Pim-1 mRNA stability in germ cells and lymphocytes. Thus, whether the (UAUU)n motif also contributes to the increase in Pim-3 mRNA level observed after TNF-α exposure should be a subject of future study.

The initial step in directed cell movement is lamellipodial protrusion, an action driven by actin polymerization. Recently, both our research and that of other groups suggested that Pim-1 and Pim-3 play roles in controlling EC proliferation and subsequent vessel tube formation (Zhang et al., 2009; Zippo et al., 2004). Pim-1 and Pim-3 can regulate cytoskeletal organization (Bhattacharya et al., 2002). Reportedly, TNF-α can induce the formation of membrane ruffles, filopodia, and actin stress fibers in ECs. Increased formation of membrane protrusions was observed in TNF-α-treated ECs at the migration front *in vitro* (Gao et al., 2002). We report here that Pim-3 silencing inhibited TNF-α-induced formation of membrane protrusions *in vitro* and EC sprouting *in vivo*.

eNOS plays a predominant role in angiogenesis and vasculogenesis. EC movement requires a coordinated cycle of adhesion and detachment (Gavard et al., 2004), and NO participates in the cycle of lamellipodial extension and retraction (Fiedler, 2009). We showed that Pim-3 gene silencing led to the reduction in eNOS mRNA level in ECs. These data indicate that Pim-3 may regulate EC migration *via* eNOS/NO signaling pathway. The molecular details of how the Pim-3 silencing regulates eNOS mRNA level remain to be determined.

In summary, Pim mRNA expression is regulated by TNF-α in ECs and specific inhibition of the Pim-3-function suppresses TNF-α-induced EC sprouting. These data suggest that Pim-3 plays a role in TNF-α-induced angiogenesis.

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