

## Original Article

# SUMO1 regulates endothelial function by modulating the overall signals in favor of angiogenesis and homeostatic responses

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**Abstract:** As a versatile regulatory mechanism, sumoylation has been found to be essential for ordered diverse cellular processes. However, the exact impact of sumoylation on endothelial function largely remained elusive. Here we investigated the role of small ubiquitin-like modifier 1 (SUMO1) mediated sumoylation in the regulation of endothelial function by examining its effect on angiogenesis and homeostatic responses. Adenoviral-mediated SUMO1 expression in porcine aortic endothelial cells (PAECs) dose-dependently promoted proliferation, migration and tube formation. In line with these results in PAECs, Matrigel plug assays in SUMO1 transgenic mice demonstrated a significant higher capacity for vascular neogenesis as compared with that of control littermates. Moreover, SUMO1 expression protected PAECs from serum starvation or H<sub>2</sub>O<sub>2</sub>-induced apoptosis. Mechanistic studies demonstrated that SUMO1 sumoylation modulates ERK1/2 activation and MMP13 expression as well as Jak2/STAT5 signaling to promote angiogenesis. SUMO1 sumoylation also suppressed NFκB and c-JUN transcriptional activity to provide protection for PAECs against oxidative stress-induced apoptosis. Given that sumoylation is a reversible process, dynamic regulation of the sumoylation function could be a novel strategy to modulate endothelial function in disease states.

**Keywords:** Sumoylation, endothelial cells, angiogenesis, SUMO1

## Introduction

The small ubiquitin-like modifiers (SUMO) belong to an evolutionarily conserved protein family found in all eukaryotes and are essential for viability of most eukaryotic cells including yeasts, nematodes, fruit flies, and vertebrate cells [1]. Post-translational attachment of SUMO defined as sumoylation involves a single SUMO-activating enzyme (Uba2-Aos1), an essential SUMO-conjugating enzyme (Ubc9), and a SUMO-E3 ligase such as the PIAS family and RanBP2 (also called Pc2) [2, 3]. SUMO covalently conjugates to target proteins using the same lysine residues by an isopeptide bond through their carboxyl termini as ubiquitin.

However, unlike ubiquitin which usually leads to protein degradation, SUMO addition to lysine residues is a remarkably versatile regulatory mechanism implicated in the regulation of signal transduction, gene transcription, genome integrity, mitochondrial fission and fusion, ion and protein transport, cell viability and apoptosis [3]. Importantly, sumoylation is a reversible process and, in some cases such as in the presence of stressful stimuli, dynamic cycles of sumoylation/desumoylation may be essential for the appropriate defensive cellular responses.

Given the importance of sumoylation in the regulation of normal function of many vital cellular

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proteins, it has been suggested to be implicated in the pathogenesis of human diseases such as cancer, diabetes, Huntington's disease, Parkinson's disease and Alzheimer's disease [4]. There is also evidence supporting its implication in the regulation of endothelial pathologies [5-8]. For example, sumoylation of ERK5 has been suggested to be implicated in diabetes-induced endothelial dysfunction [7]. While these discoveries are important and exciting, the exact impact of sumoylation on endothelial function, however, largely remained elusive. In the present report, we hypothesized that sumoylation dynamically regulates the signals in favor of endothelial angiogenesis and homeostatic responses. We have demonstrated direct evidence supporting that SUMO1 sumoylation enhances endothelial proliferation, migration and tube formation. Consistently, animals with transgenic SUMO1 expression showed significantly higher capacity for vascular neogenesis. Moreover, SUMO1 sumoylation protects endothelial cells (ECs) against oxidative stress-induced apoptosis. Our results suggest that dynamic regulation of the cellular sumoylation function could be a novel strategy to modulate endothelial function in disease states.

### Materials and methods

#### Reagents

Antibodies for  $\beta$ -actin, c-JUN, I $\kappa$ B $\alpha$ , Jak2, STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6 were purchased from Santa Cruz (Santa Cruz, CA, USA). The monoclonal His antibody was purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against MMP13 and VEGFR2 were purchased from Millipore (Billerica, MA). Rabbit anti-CD31 antibody was obtained from Abcam (Cambridge, MA, USA). Antibodies against ERK1/2, p-ERK1/2, p38, p-p38, AKT, p-AKT, and SUMO1 were purchased from Cell Signaling (Danvers, MA, USA).

#### Cell culture

PAECs were isolated and characterized from the main aortas of 6- to 7-month-old pigs as previously reported [9]. Third- to sixth-passage cells in monolayer culture were maintained in RPMI 1640 medium (Mediatech, Manassas, VA, USA) containing 4% FBS and antibiotics (10 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 20  $\mu$ g/

ml gentamicin, and 2  $\mu$ g/ml Fungizone), and were used 2 or 3 days after confluence.

#### Adenoviral production and transduction

Recombinant adenovirus carrying the SUMO1 coding region under the control of a CMV promoter (Ad-SUMO1) was generated from Vector Biolabs (Philadelphia, PA, USA). An empty vector with GFP only (Ad-GFP) was used as a negative control. Viral stocks used for the study ranged from  $10^9$  to  $10^{10}$  pfu/ml. PAECs 12 h after seeding were transduced with adenovirus at multiplicity of infections (MOI) of 0 to 150 for 24 to 48 h. The cells were then washed and supplemented with complete medium.

#### PAEC proliferation assay

PAECs 48 h after transduction were seeded in a 96-well plate ( $3 \times 10^3$  cells/well), and 12 h later the cells were pulsed with 0.5  $\mu$ Ci/well of  $^3$ H-thymidine (Amersham, Piscataway, NJ, USA) for 16 h. The cells were then harvested and counted in a 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (PerkinElmer, Boston, MA, USA) as reported [10]. PAEC proliferation was determined by  $^3$ H-thymidine incorporation and present as counts per minute (cpm).

#### Cell cycle analysis

PAECs at 50% confluence were transduced with adenovirus for 48 h. The transduced cells were subsequently synchronized by serum starvation in 0.1% bovine serum albumin (BSA) medium for 24 h, followed by serum stimulation for another 24 h. The harvested cells were then fixed with 70% ethanol at  $-20^\circ\text{C}$  overnight and stained with propidium iodide (PI) solution (50  $\mu$ g/ml PI, 0.1 mg/ml RNase A, 1% Nonidet P-40, in PBS) at  $37^\circ\text{C}$  for 30 min. The samples were analyzed using a BD FACSCalibur flow cytometer as reported [11].

#### Endothelial migration assay

The transduced PAECs ( $2.5 \times 10^5$  cells) were grown to confluence in a 12-well plate for 24 h. Two scratches in each well were created by scraping cell monolayer with a sterile 1,000  $\mu$ l pipette tip. The time course of PAEC migration was recorded for 36 h using a Nikon Eclipse microscope system (Nikon Instrument) equipped with the NIH ImageJ software. The

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width of wound sealing was measured using a Photoshop program. Data are present as the average migrated distance (Photoshop scale unit) of three independent experiments performed.

### *Analysis of tube formation*

Matrigel (BD Biosciences, San Jose, CA, USA; 60  $\mu$ L) was added into a 96-well plate and allowed solidification for 30 min at 37°C. PAECs ( $1 \times 10^4$  cells) 48 h after transduction were added into each well and incubated in 2% FBS EBM-2 endothelial cell basic medium at 37°C with 5% CO<sub>2</sub> for 6 h. Endothelial tubes were then examined under a light microscope (100X) by inspecting the overall branch points, and assessed by counting the number of branching points of the tubular network in randomly selected fields as previously reported [12].

### *Apoptosis assay*

Endothelial apoptosis assay was performed using a Cell Apoptosis DAPI Detection Kit (GenScript, Piscataway, NJ 08854, USA). In brief, PAECs 48 h after transduction were seeded into a 6-well plate ( $2 \times 10^5$  cells/well). The cells were then undergone either serum starvation for 3 days or H<sub>2</sub>O<sub>2</sub> treatment (0.2 mM) for 24 h. The cells were next fixed with 20% ethanol at RT for 10 min, and then stained with 49, 6-diamidino-2-phenylindole (DAPI) for 30 sec. Apoptotic cells were characterized by the irregular edges around the nucleus and nuclear pyknosis as previously described [13]. Images were taken under a computer-assisted Nikon Eclipse TE2000-S microscope. Apoptosis rate was assessed in 10 randomly selected fields under a microscope for each sample with 3 independent replications.

### *Generation of SUMO1 transgenic (SUMO1-Tg) mice*

The SUMO1 coding region with an N-terminal 6 x his-tag was cloned into a pHY-R vector using the *Hind* III and *Bam* HI cutting sites. A human  $\beta$ -actin promoter was used to drive the transgene expression. The expression cassette was released by *Xba* I digestion and then microinjected into NOD embryos (Joslin Diabetes Center, Boston, MA, USA). Pups resulted from foster mothers were genotyped by PCR followed by Southern blotting using the probes

from human  $\beta$ -actin promoter. Two founders, SUMO1-Tg1 and SUMO1-Tg2, were characterized with germline transmission after screening a total of 16 pups. All mice were housed in a SPF facility in microisolator cages supplied with autoclaved food and acidified water with a 12/12 h light/dark cycle. Experiments involving SUMO1-Tg model were carried out in SUMO1-Tg1 mice, while SUMO1-Tg2 mice were used for confirmation. All experiments involving mice were done according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Tongji Hospital.

### *Immunohistochemistry*

Tissues were fixed in 4% formaldehyde at 4°C overnight and then embedded in paraffin. Tissue sections (6  $\mu$ m) were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked with 3% H<sub>2</sub>O<sub>2</sub> and nonspecific proteins were blocked with 10% goat serum or rabbit serum for 30 min. The sections were then probed with a rabbit anti-CD31 (Abcam, Cambridge, MA, USA; 1:200) or anti-SUMO1 (Cell signaling, Danvers, MA, USA; 1:100) antibody at 4°C overnight, followed by incubation with an HRP conjugated goat anti-rabbit secondary antibody (1:100) at RT for 30 min. DAB substrate was applied for 5 min for color development as reported [14].

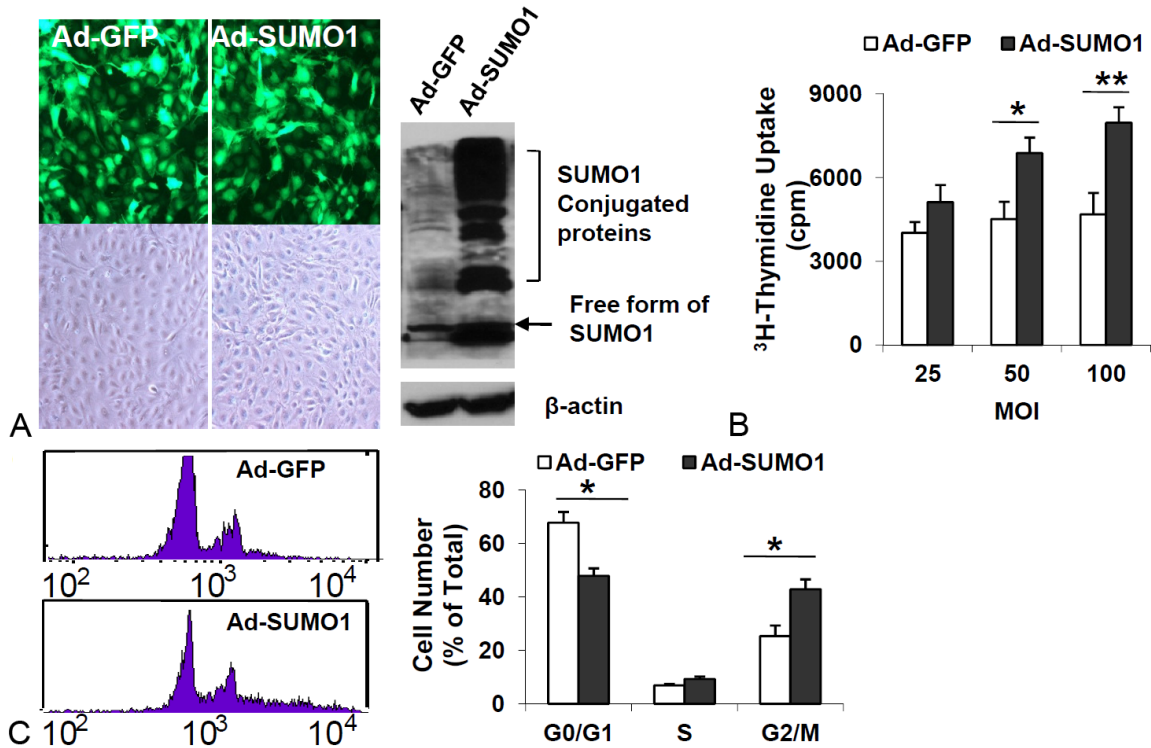
### *Matrigel plug assay*

Wild-type (WT) and SUMO1-Tg mice (females, 8 week-old) were injected subcutaneously on the back of both sides with 0.5 ml ice-cold 1:2 diluted Matrigel (BD Bioscience, CA, USA) containing 200 ng/ml VEGF (R&D, Minneapolis, MN) and 60 U/ml heparin (Sigma, St. Louis, MO, USA). One week later, the mice were sacrificed and gel plugs were harvested. Part of the plugs was subjected to immunohistochemical analysis of CD-31 as above. The rest part of plugs was weighed, chopped and immersed in 0.5 ml distilled water at 4°C overnight. The amount of hemoglobin in the plugs was then determined using Drabkin reagent (Sigma, St. Louis, MO, USA) as instructed.

### *Western blotting*

PAECs 48 h after transduction were harvested and lysed in RIPA buffer (50 mM Tris•HCl pH

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**Figure 1.** Ectopic SUMO1 expression dose-dependently promotes endothelial proliferation. A. Images for PAECs 24 h after Ad-SUMO1 or Ad-GFP transduction. Adenoviral transduced PAECs (100 MOI) were evidenced by GFP expression (left upper panel) as compared with total PAECs under a light microscope (left lower panel). Ad-SUMO1 transduction led to significantly higher levels of SUMO1 expression for both free and conjugated form (right). B. <sup>3</sup>H-thymidine incorporation assay demonstrated that SUMO1 dose-dependently promoted PAEC proliferation. The results are present as mean±SD (n = 3). C. Analysis of cell cycle progression. PAECs were first serum starved for synchronization followed by serum stimulation. The percentage of cells 24 h after stimulation in G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phase was analyzed by flow cytometry. \*, p < 0.05; \*\*, p < 0.01.

7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride (Amresco, Solon, Ohio, USA), protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and 10 mM N-ethylmaleimide (Sigma, St. Louis, MO, USA). Fifty micrograms of proteins were then used for Western blot analysis as previously reported [15].

### Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from Ad-SUMO1 or Ad-GFP transduced PAECs after H<sub>2</sub>O<sub>2</sub> treatment using a NE-PER® Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). EMSA was carried out with a LightShift® Chemiluminescent EMSA Kit (Thermo Scientific) as previously reported [16]. Biotin-labeled probes for NFκB and c-JUN are as follows: NFκB, 5'-biotin-AGT TGA GGG GAC TTT CCC AGG

C-3'/5'-biotin-GCC TGG GAA AGT CCC CTC AAC T-3'; c-JUN, 5'-biotin-CGG TTG ATG ACT CAG CCG GAA-3'/5'-biotin-TTC CGG CTG AGT CAT CAA GCG-3'.

### Statistical analysis

For pairwise comparison, the data were analyzed using a Student's *t* test. Comparison between multiple experimental groups was accomplished by one-way ANOVA using SPS 11.5 for windows. All data are present as mean±SD. In both cases, p < 0.05 was considered to be statistically significant.

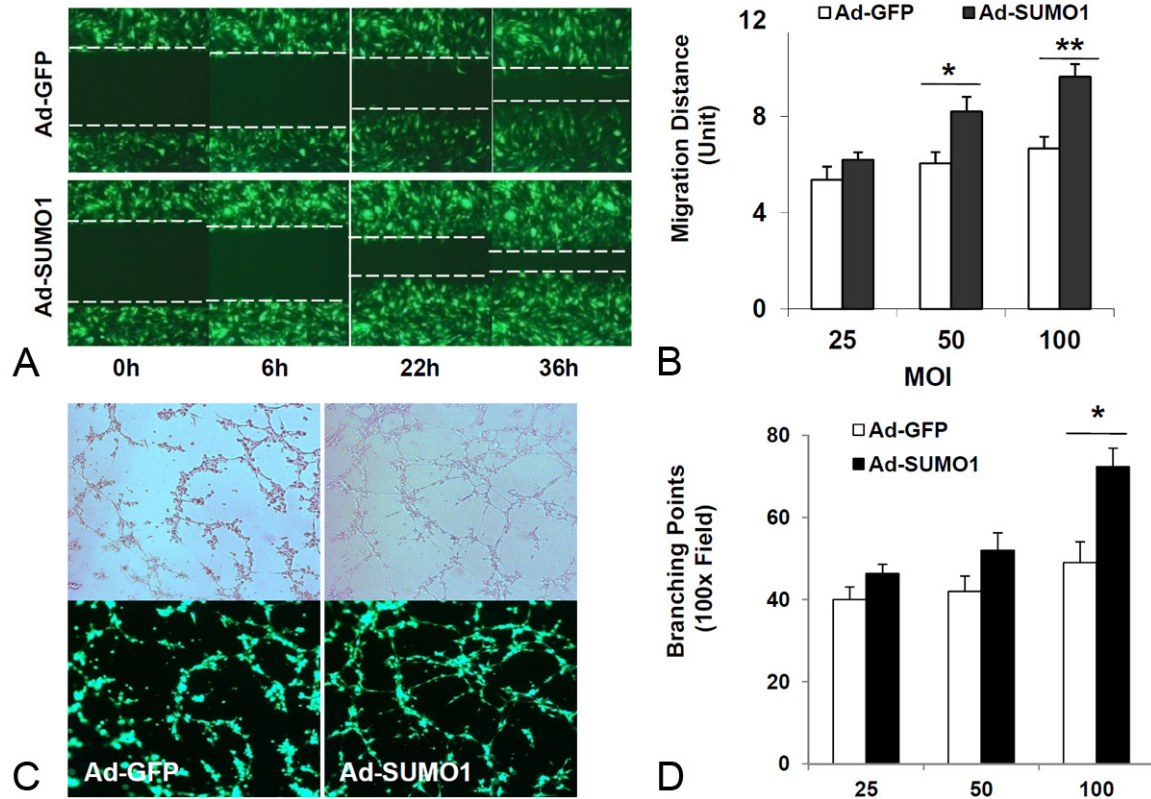
### Results

#### SUMO1 expression enhances porcine aortic endothelial cell (PAEC) proliferation

We first generated an adenoviral vector that expresses SUMO1 with a GFP reporter



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**Figure 2.** SUMO1 expression enhances endothelial migration and tube formation. A. Representative images for PAEC migration. B. A bar graphic figure showing the results of 3 independent experiments. Scratches were made in Ad-SUMO1 or Ad-GFP transduced PAEC monolayer. Time-course PAEC migration was monitored by automatic microscopy. C. A typical image showing the difference of tube formation between Ad-SUMO1 and Ad-GFP transduced PAECs. D. A bar graphic figure showing the mean tubular network branching points at different MOI (n = 3). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

(Ad-SUMO1) under the control of a CMV promoter (Vector Biolabs). An empty adenoviral vector with GFP only (Ad-GFP) was used as a control. Adenoviruses resulted from those viral vectors were then used to transduce PAECs, and their transducing efficiency was evaluated at different multiplicity of infections (MOIs). When PAECs transduced at 100 MOI, > 90% of cells were GFP positive 24 h after transduction (Figure 1A, left), and > 95% of cells were positive after another 24 culture (data not shown). Ectopic GFP expression culminated at day 3 of transduction and kept stable for at least one week. No obvious toxicity was observed for MOIs < 150, and therefore, MOI at 100 was then selected for the study. Western blot analysis demonstrated much higher levels of free and conjugated SUMO1 in Ad-SUMO1 transduced cells than that in Ad-GFP transduced cells (Figure 1A, right).

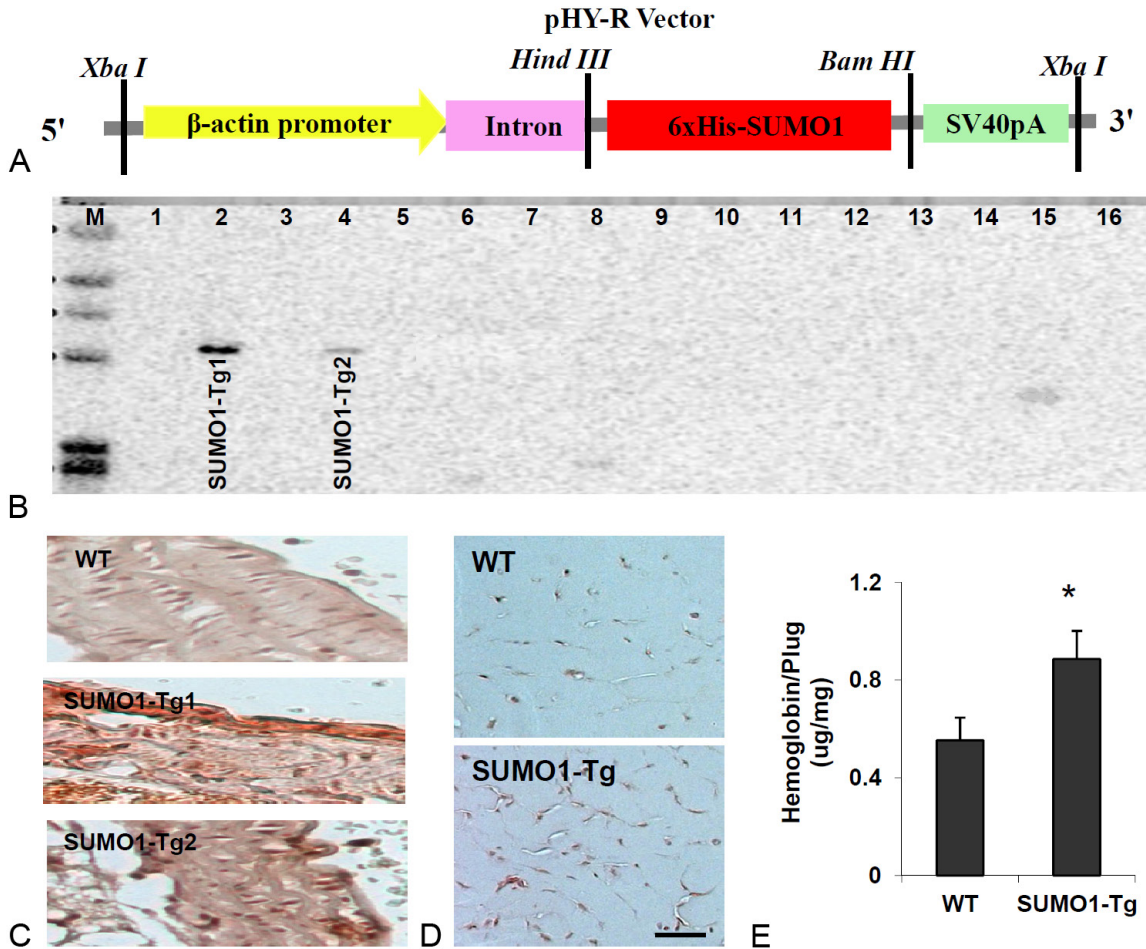
$^3\text{H}$ -thymidine incorporation assay was then employed to determine the role of SUMO1

expression in PAEC proliferation. SUMO1 showed a dose-dependent effect on PAEC proliferation. Significant higher  $^3\text{H}$ -thymidine uptake was detected in Ad-SUMO1 transduced PAECs at MOIs from 50 to 100 (Figure 1B). In line with this result, significantly higher percentage of Ad-GFP transduced PAECs was in the  $G_0/G_1$  phase ( $47.8 \pm 2.8\%$  vs.  $67.7 \pm 3.9\%$ ,  $p < 0.05$ ). In contrast, Ad-SUMO1 transduced PAECs were predominantly in the  $G_2/M$  phase ( $42.8 \pm 3.7\%$  vs.  $25.3 \pm 3.9\%$ ,  $p < 0.05$ ; Figure 1C). Together, those data suggest that SUMO1 expression enhances PAEC proliferation.

### SUMO1 expression promotes PAEC migration

We next checked the effect of SUMO1 expression on PAEC migration by *in vitro* scratch assay. The scratches were made in PAEC monolayer and time-course wound healing processes were monitored by automatic microscopy (Figure 2A). Similar as above, SUMO1 dose-dependently promoted PAEC migration.

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**Figure 3.** Generation of SUMO1-Tg mice and results for Matrigel plug assays. A. A diagram showing the strategy for generation of SUMO1-Tg model. B. Southern blot results for the identification of SUMO1-Tg pups. Genomic DNA from tails was first digested by *EcoR V* and then transferred onto a nitrocellulose membrane followed by hybridizing with a  $P^{32}$ -labeled probe using DNA fragment from the human  $\beta$ -actin promoter. C. Immunohistological staining for confirming transgenic SUMO1 expression in the aortas. D. Immunostaining results for Matrigel plug sections. The sections were stained with an antibody against CD31 (a marker for ECs) for indication of vascular neogenesis. E. A graphic figure showing the content of hemoglobin in the gel plugs derived from SUMO1-Tg and control mice ( $n = 8$ ). The assays were performed in SUMO1-Tg1 mice, while SUMO1-Tg2 mice were used for confirmation. \*,  $p < 0.05$ .

Significant differences for migration were noticed 22 h after the scratch. The migration rate 36 h after the scratch was significantly higher in Ad-SUMO1 transduced cells than that in control cells at both 50 MOI and 100 MOI (Figure 2B).

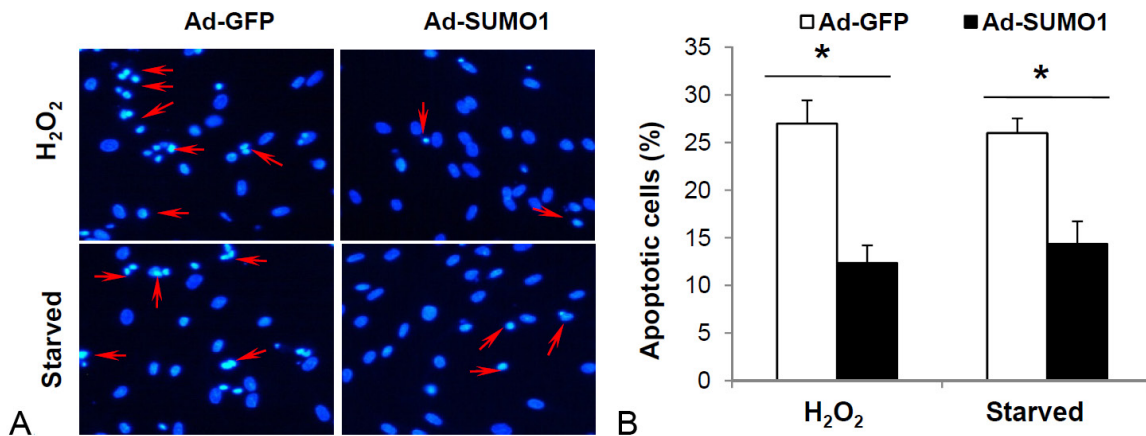
### *SUMO1 dose-dependently increases PAEC tube formation*

To assess the impact of SUMO1 on endothelial tube formation, PAECs 48 h after transduction were seeded into culture plates preconditioned with growth factor reduced Matrigel (BD Bioscience). Tube formation was then assessed 6 h later. Tubular network branching point, an

index for tube formation, was examined under a fluorescent microscope (100x) as described. Ad-SUMO1 transduced PAECs (100 MOI) showed significantly higher tubular network branching points than that of Ad-GFP transduced control cells ( $67.3 \pm 6.0$  vs.  $43.6 \pm 2.9$ ,  $p < 0.05$ ; Figure 2C & 2D). Collectively, our data indicate that SUMO1 dose-dependently promotes endothelial angiogenesis.

### *Transgenic SUMO1 expression enhances vascular neogenesis in mice*

To confirm the above observations in animals, we generated a SUMO1 transgenic (SUMO1-Tg) mouse model as described (Figure 3A). Two



**Figure 4.** Ectopic SUMO1 expression provides protection for PAECs against oxidative stress-induced apoptosis. A. Representative images for apoptotic PAECs after induction with H<sub>2</sub>O<sub>2</sub> or serum starvation. Typical apoptotic cells are indicated by arrows. B. A bar graph showing the protective effect of SUMO1 on PAECs against H<sub>2</sub>O<sub>2</sub> or serum starvation-induced apoptosis (n = 3). \*, p < 0.05.

founders (SUMO1-Tg1 and SUMO1-Tg2) were characterized with germline transmission by Southern blot analysis (Figure 3B). All SUMO1-Tg mice showed normal development without perceptible abnormalities in the vessels, heart, lung, kidney and other major organs. Immunohistochemical analysis further demonstrated transgenic SUMO1 expression in the aorta (Figure 3C).

Matrigel plug assay was then employed to examine the effect of SUMO1 on vascular neogenesis. The mice were anaesthetized and subcutaneously injected with 0.5 ml ice-cold Matrigel (BD Bioscience) on each side of the back and gel plugs were removed 1-week after injection. Gel plugs derived from SUMO1-Tg mice showed significantly higher number of CD31-positive cells (ECs) associated with more tubular like structures as compared with that from wild-type (WT) mice (Figure 3D). In line with this result, the content of hemoglobin in the gel plugs originated from SUMO1-Tg mice was significantly higher than that from WT mice (Figure 3E). Taken together, these results indicate that SUMO1-Tg mice have a higher capacity for vascular neogenesis than their control counterparts.

#### *SUMO1 expression protects PAECs against oxidative stress-induced apoptosis*

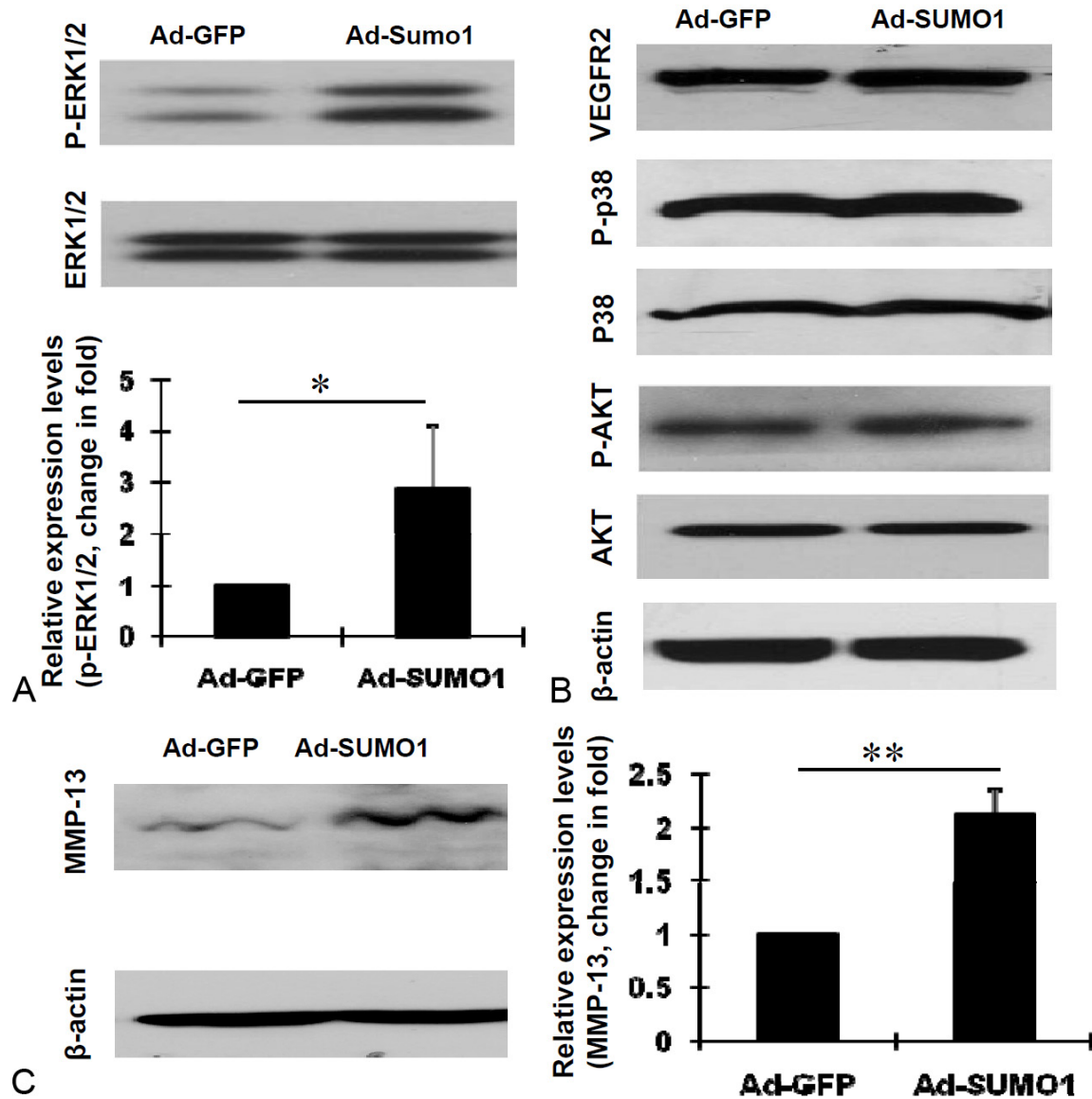
To demonstrate the role of SUMO1 in endothelial cell survival, Ad-SUMO1 transduced PAECs were induced for apoptosis either by H<sub>2</sub>O<sub>2</sub> (0.2

mM for 24 h) or by serum starvation (3 days). Apoptotic cells were identified by a DAPI detection kit as described (Figure 4A, indicated by arrows). As shown in Figure 4B, a 1.1-fold higher apoptosis was noted in Ad-GFP transduced cells as compared with that of Ad-SUMO1 transduced cells after H<sub>2</sub>O<sub>2</sub> treatment. Similarly, serum starvation resulted in an 80% higher apoptosis in Ad-GFP transduced cells as compared with that of Ad-SUMO1 transduced cells.

#### *SUMO1 modulates signals in favor of endothelial angiogenesis*

To dissect the underlying mechanisms by which SUMO1 regulates angiogenesis, we designed experiments to examine the overall impact of SUMO1 on signaling molecules pivotal for angiogenesis. We assumed that the phenotype resulted from ectopic SUMO1 expression could be relevant to SUMO1 modulating pro-angiogenic signals. We first examined the effect of SUMO1 on the expression and activation of VEGF-R2, ERK1/2, p38 and AKT, the four major signals essential for angiogenesis. SUMO1 expression did not result in a significant change for total ERK1/2, but a significantly higher levels of activated (phosphorylated) ERK1/2 (p-ERK1/2) was noted in Ad-SUMO1 transduced PAECs than that in control cells (Figure 5A). However, we failed to detect a significant difference for VEGF-R2 expression, and similarly, both total and activated p38 and AKT were the same (Figure 5B). In contrast, Western blot analysis characterized a 2.2-fold higher





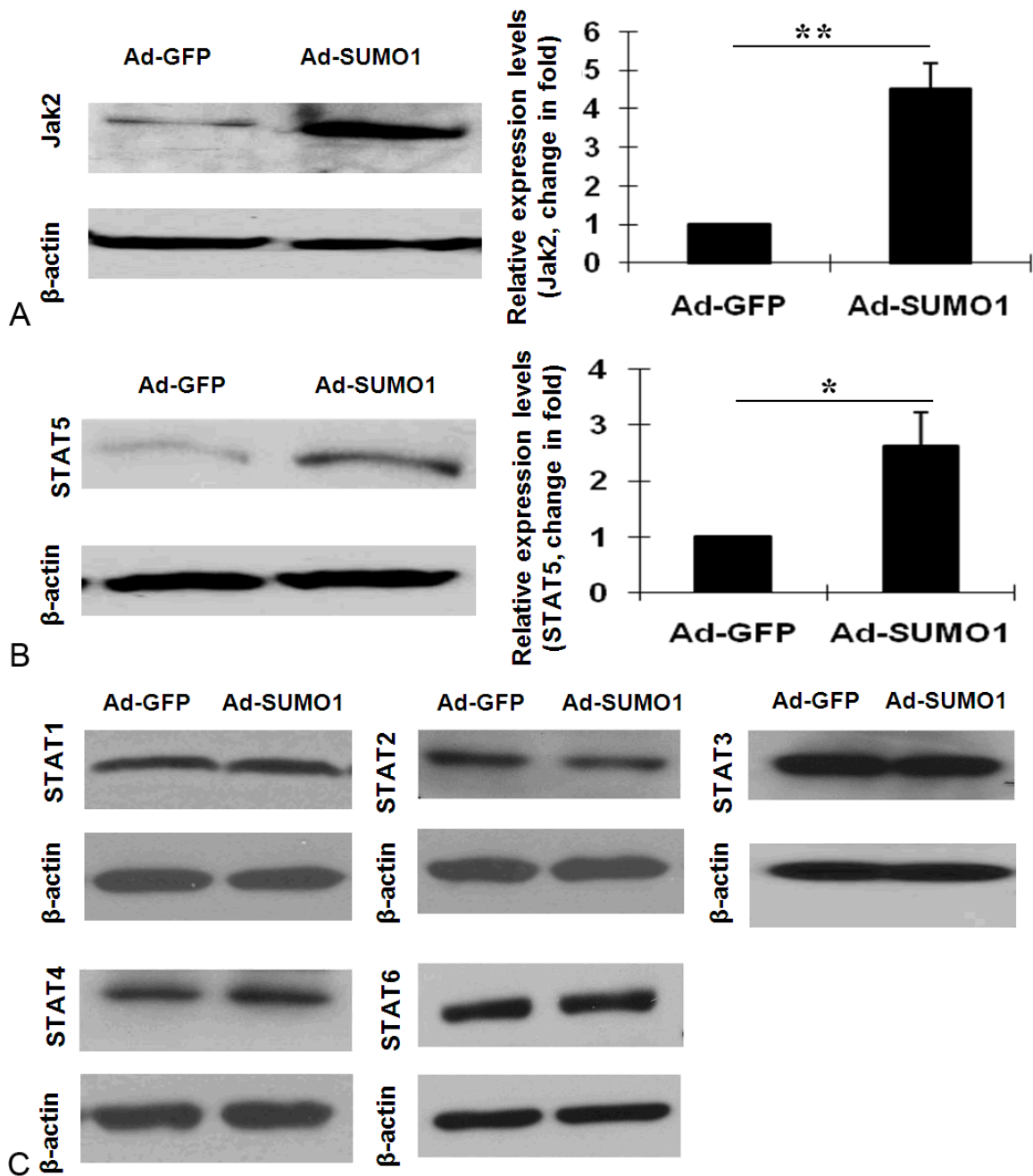
**Figure 5.** SUMO1 modulates signals to promote endothelial angiogenesis. PAECs 48 h after Ad-SUMO1 or Ad-GFP transduction (100 MOI) were harvested for Western blot analysis of VEGF-R2, ERK1/2, p38 and AKT expression and activation.  $\beta$ -actin was used for normalization. **A.** SUMO1 expression enhanced ERK1/2 activation as revealed by the detection of significantly higher levels of phosphorylated ERK1/2 (p-ERK1/2), but no discernable change for the total ERK1/2. **B.** No significant impact was observed for SUMO1 on VEGF-R2 expression, and similarly, both total and activated p38 and AKT were the same as that in control cells. **C.** A significant increase for MMP-13 expression was detected in Ad-SUMO1 transduced PAECs as compared with that of Ad-GFP transduced cells. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

MMP13 expression in Ad-SUMO1 transduced PAECs as compared with that in control cells (Figure 5C).

Next, we examined Jak2/STAT activity, a downstream pathway for the VEGF and FGF signaling [17-19]. Interestingly, significantly higher levels of Jak2 were observed in PAECs with ectopic SUMO1 expression (Figure 6A), which prompt-

ed us to examine the STAT family members. Surprisingly, enhanced Jak2 expression is only associated with increased STAT5 expression (Figure 6B) without a discernable impact on STAT1, 2, 3, 4 and 6 (Figure 6C). These results suggest that SUMO1 probably selectively modulates the Jak2/STAT5 axis in ECs. Collectively, our results indicate that SUMO1 may modulate signals in favor of angiogenesis.



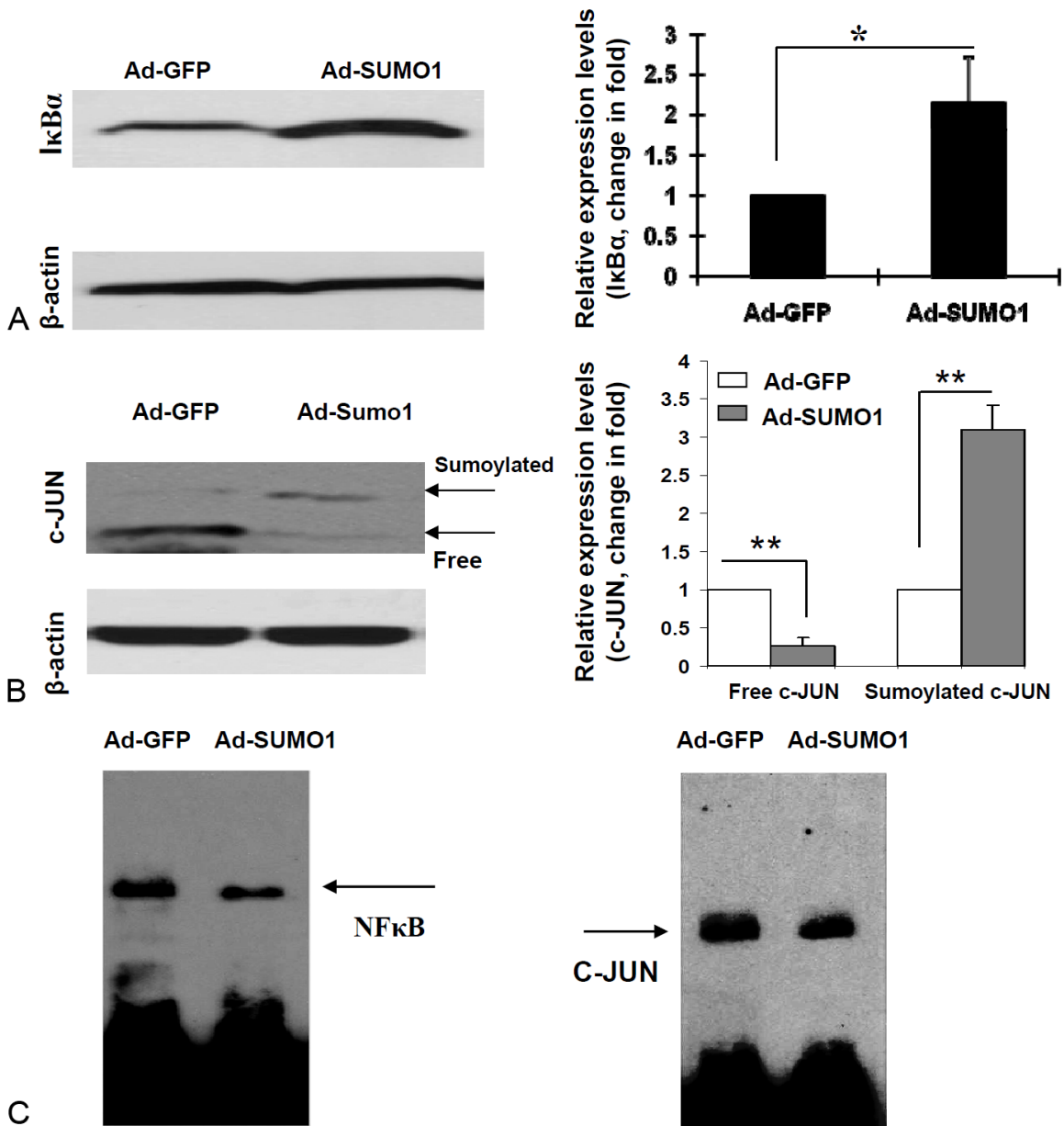


**Figure 6.** SUMO1 selectively modulates the Jak2/STAT5 axis. A. Ectopic SUMO1 expression led to a significant increase for Jak2 expression in PAECs. B. The enhanced Jak2 expression was associated with enhanced STAT5 signaling. C. No significant change was detected for STAT members 1, 2, 3, 4, and 6 between Ad-SUMO1 and Ad-GFP transduced PAECs.

*SUMO1 prevents endothelial apoptosis by suppressing NF $\kappa$ B and c-JUN transcriptional activity*

Given the important role of NF $\kappa$ B and c-JUN played in endothelial apoptosis [20, 21], we next examine the effect of SUMO1 expression on their transcriptional activity. We first ana-

lyzed the protein levels for I $\kappa$ B $\alpha$ , a repressor for NF $\kappa$ B nuclear translocation. In line with our previous reports [2], SUMO1 expression resulted in a significant increase for I $\kappa$ B $\alpha$  (**Figure 7A**). Interestingly, we detected a 2.2 fold increase for the reactive band with higher molecular weight for c-JUN in Ad-SUMO1 transduced cells (**Figure 7B**). Given the size for this reactive band



**Figure 7.** SUMO1 suppresses NFκB and c-JUN activity to prevent endothelial apoptosis. **A.** Ectopic SUMO1 expression increased IκBα protein levels. Much higher levels of IκBα after H<sub>2</sub>O<sub>2</sub> induction were detected in Ad-SUMO1 transduced PAECs as compared that of Ad-GFP transduced cells. **B.** Ectopic SUMO1 expression increased the sumoylated form of c-JUN along with a reduction for the free form of c-JUN. **C.** Ectopic SUMO1 expression significantly inhibited NFκB and c-JUN transcriptional activity as characterized by the reduced DNA binding activity.

is consistent with the addition of His-tagged SUMO1 to c-JUN, it could be the sumoylated form of c-JUN. In contrast, a 2.6 fold decrease for the free form of c-JUN was noted in Ad-SUMO1 transduced cells. We next examined their transcriptional activity by EMSA in PAECs after H<sub>2</sub>O<sub>2</sub> treatment. Consistent with the Western blotting results, PAECs with ectopic SUMO1 expression showed significantly lower DNA binding activity for both NFκB and c-JUN

than that of Ad-GFP-transduced control cells (**Figure 7C**). Taken together, our results suggest that SUMO1 modulates endothelial signals in favor of angiogenesis and homeostatic responses.

**Discussion**

Sumoylation carried out by the SUMO proteins has recently been demonstrated to be involved

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in diverse biological processes both in the physiological and pathological condition [3, 4, 22]. Particularly, Woo and colleagues reported that sumoylation mediated transcriptional repression of ERK5 implicates in shear stress or diabetes-induced endothelial dysfunction [7, 8]. It was also noted that Prox1, a master regulator for lymphatic development and lymphangiogenesis, is a target for SUMO1 sumoylation [6]. Together, these studies provided feasible evidence suggesting a role for sumoylation in the regulation of endothelial function. To further address this question, we now performed studies both in Ad-SUMO1 transduced PAECs and SUMO1-Tg mice, and demonstrated direct evidence indicating that SUMO1 sumoylation regulates endothelial function. Our data suggest that manipulation of the cellular dynamic sumoylation function could be a feasible strategy to modulate endothelial function in disease states.

Endothelial proliferation, migration and tube formation are essential features for angiogenesis. Using Ad-SUMO1 transduced PAECs we demonstrated that SUMO1 dose-dependently enhances endothelial proliferation, migration and tube formation (**Figures 1 & 2**). In consistent with these results, Matrigel plug assay in SUMO1-Tg mice revealed that transgenic SUMO1 expression enhances the capacity of mice for vascular neogenesis (**Figure 3**). Previous studies including ours suggested a possible role for sumoylation in regulating oxidative stress-induced apoptosis [16, 23, 24], and our studies in PAECs now provided additional supporting evidence as manifested by that PAECs with ectopic SUMO1 expression are resistant to serum starvation or H<sub>2</sub>O<sub>2</sub>-induced apoptosis (**Figure 4**).

Of interestingly note, the exact role for SUMO1 in embryo development has been somehow controversial, with one report indicating that a SUMO1 hypomorphic allele manifests an incompletely penetrant orofacial clefting phenotype [25], while the other one demonstrating that SUMO1 is dispensable in normal mouse development [26]. Similar as the later report, our studies in SUMO1-Tg mice failed to characterize a perceptible developmental abnormality for major organs and tissues. Also, these mice can breed normally and the resulting pups fit the expected Mendelian segregation ratio.

Similar as many biochemical pathways, signals relevant to angiogenesis are dynamically regulated in response to different stimuli, which is essential for the control of vascular neogenesis [27, 28]. Given the fact that sumoylation is a reversible process [3], we assumed that sumoylation could serve as a regulatory mechanism to finely tune endothelial function by modulating the signals in favor of angiogenesis and homeostatic responses. By keeping this in mind, we first examined several signals essential for endothelial angiogenesis which include VEGF-R2, ERK1/2, p38 and AKT. As expected, a significant increase for the activated ERK1/2 (p-ERK1/2) was noted along with ectopic SUMO1 expression, but the expression levels for total ERK1/2 remained the same (**Figure 5A**). Unexpectedly, no perceptible impact for SUMO1 expression on VEGF-R2, p38 and AKT activity was detected (**Figure 5B**). However, SUMO1 significantly increased MMP13 expression (**Figure 5C**). MMP13, also known as collagenase 3, is an interstitial collagenase that degrades interstitial collagens, collagen types IV, VII and X [29]. It can be secreted by a variety of cells including ECs and fibroblasts during the process of angiogenesis for digestion of ECM to facilitate endothelial migration and release sequestered angiogenic molecules [30]. As a result, animals deficient in MMP13 show impaired angiogenesis in wound healing process [31, 32]. By cooperating with other MMPs, MMP13 also reverses the inhibitory effect of connective tissue growth factor (CTGF) on VEGF by digesting the VEGF/CTGF complex [33]. Therefore, administration of chicken MMP13 can induce new blood vessel formation in chorioallantoic membrane onplant tissues [34]. As such, SUMO1-induced MMP13 expression could be an important contributing factor to the enhancement of endothelial migration.

We also characterized increased Jak2 expression (**Figure 6A**) along with enhanced STAT5 signaling (**Figure 6B**). Interestingly, it is likely that SUMO1 only selectively modulates the Jak2/STAT5 axis since other STAT members remained unchanged (**Figure 6C**). It has been well demonstrated that Jak2/STAT5 signaling transfers the pro-angiogenic signals derived from VEGF, FGF, Tie2, IL-20, erythropoietin and tissue factor/factor VIIa signaling [19, 35-38]. Therefore, Jak2/STAT5 may synergize with ERK1/2 and MMP13 to enhance endothelial angiogenesis.

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I $\kappa$ B $\alpha$  is a potent inhibitor for NF $\kappa$ B by preventing its nuclear translocation. Previous studies consistently demonstrated that sumoylation of I $\kappa$ B $\alpha$  prevents its phosphorylation and subsequent proteasome-dependent degradation, and therefore, sustains its inhibitory effect on NF $\kappa$ B activation [15, 39]. Similar as previous reports, SUMO1 expression in PAECs stabilized I $\kappa$ B $\alpha$  from signal-induced degradation (**Figure 7A**) along with suppressed NF $\kappa$ B transcriptional activity (**Figure 7C**, left). Altered NF $\kappa$ B activity had been suggested to play a pivotal role in hypoxia-induced endothelial apoptosis [20]. Its importance in endothelial function has recently been further underscored by studies in Tie2 promoter/enhancer-I $\kappa$ B $\alpha$ <sup>S32A/S36A</sup> transgenic mice, in which mice with suppressed endothelial NF $\kappa$ B activity show enhanced vascular density [40]. Thus, NF $\kappa$ B could be important in both endothelial angiogenic and homeostatic responses.

Similar as NF $\kappa$ B, the transcription factor c-JUN is induced by a variety of stimuli that perturb endothelial function. Particularly, c-JUN is essential in H<sub>2</sub>O<sub>2</sub>-induced endothelial apoptosis [21]. Furthermore, suppression of c-JUN activity has been found to inhibit endothelial proliferation, migration and tube formation associated with decreased blood vessel neogenesis [41]. Of note, SUMO1 expression in PAECs increased sumoylated form of c-JUN (**Figure 7B**) along with suppressed DNA binding activity (**Figure 7C**, right). Therefore, c-JUN could be another transcription factor essential for endothelial angiogenic and homeostatic responses.

In summary, we report for direct evidence supporting that SUMO1 sumoylation enhances endothelial angiogenesis and protects ECs against oxidative stress-induced apoptosis. The mechanism may involve a synergic action between signals from ERK1/2 and MMP13 as well as JAK2/STAT5 signaling along with suppressed NF $\kappa$ B and c-JUN transcriptional activity. Given the importance of endothelial function in vascular repair and homeostasis in response to different stimuli, manipulation of the cellular dynamic sumoylation function could be a novel therapeutic approach to modulate endothelial function in disease state.

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### Competing interests

The authors declare no competing financial interests. All authors have read and agreed the content within the manuscript.

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### References

- [1] Wilson VG and Rosas-Acosta G. Wrestling with SUMO in a new arena. *Sci STKE* 2005; 2005: pe32.
- [2] Guo D, Li M, Zhang Y, Yang P, Eckenrode S, Hopkins D, Zheng W, Purohit S, Podolsky RH, Muir A, Wang J, Dong Z, Brusko T, Atkinson M, Pozzilli P, Zeidler A, Raffel LJ, Jacob CO, Park Y, Serrano-Rios M, Larrad MT, Zhang Z, Garchon HJ, Bach JF, Rotter JI, She JX and Wang CY. A functional variant of SUMO4, a new I kappa B alpha modifier, is associated with type 1 diabetes. *Nat Genet* 2004; 36: 837-841.
- [3] Li M, Guo D, Isales CM, Eizirik DL, Atkinson M, She JX and Wang CY. SUMO wrestling with type 1 diabetes. *J Mol Med (Berl)* 2005; 83: 504-513.
- [4] Sarge KD and Park-Sarge OK. Sumoylation and human disease pathogenesis. *Trends Biochem Sci* 2009; 34: 200-205.
- [5] Gao C, Ho CC, Reineke E, Lam M, Cheng X, Stanya KJ, Liu Y, Chakraborty S, Shih HM and Kao HY. Histone deacetylase 7 promotes PML sumoylation and is essential for PML nuclear body formation. *Mol Cell Biol* 2008; 28: 5658-5667.
- [6] Pan MR, Chang TM, Chang HC, Su JL, Wang HW and Hung WC. Sumoylation of Prox1 controls its ability to induce VEGFR3 expression and lymphatic phenotypes in endothelial cells. *J Cell Sci* 2009; 122: 3358-3364.
- [7] Woo CH, Shishido T, McClain C, Lim JH, Li JD, Yang J, Yan C and Abe J. Extracellular signal-regulated kinase 5 SUMOylation antagonizes shear stress-induced antiinflammatory response and endothelial nitric oxide synthase expression in endothelial cells. *Circ Res* 2008; 102: 538-545.



## SUMO1 regulates endothelial function

- [8] Woo CH and Abe J. SUMO—a post-translational modification with therapeutic potential? *Curr Opin Pharmacol* 2010; 10: 146-155.
- [9] Su Y, Cao W, Han Z and Block ER. Cigarette smoke extract inhibits angiogenesis of pulmonary artery endothelial cells: the role of calpain. *Am J Physiol Lung Cell Mol Physiol* 2004; 287: L794-800.
- [10] Zhong J, Yang P, Muta K, Dong R, Marrero M, Gong F and Wang CY. Loss of Jak2 selectively suppresses DC-mediated innate immune response and protects mice from lethal dose of LPS-induced septic shock. *PLoS One* 2010; 5: e9593.
- [11] Han J, Zhong J, Wei W, Wang Y, Huang Y, Yang P, Purohit S, Dong Z, Wang MH, She JX, Gong F, Stern DM and Wang CY. Extracellular high-mobility group box 1 acts as an innate immune mediator to enhance autoimmune progression and diabetes onset in NOD mice. *Diabetes* 2008; 57: 2118-2127.
- [12] Rao X, Zhong J, Zhang S, Zhang Y, Yu Q, Yang P, Wang MH, Fulton DJ, Shi H, Dong Z, Wang D and Wang CY. Loss of methyl-CpG-binding domain protein 2 enhances endothelial angiogenesis and protects mice against hind-limb ischemic injury. *Circulation* 2011; 123: 2964-2974.
- [13] Zhang S, Zhong J, Yang P, Gong F and Wang CY. HMGB1, an innate alarmin, in the pathogenesis of type 1 diabetes. *Int J Clin Exp Pathol* 2009; 3: 24-38.
- [14] Zhang S, Lv JW, Yang P, Yu Q, Pang J, Wang Z, Guo H, Liu S, Hu J, Li J, Leng J, Huang Y, Ye Z and Wang CY. Loss of dicer exacerbates cyclophosphamide-induced bladder overactivity by enhancing purinergic signaling. *Am J Pathol* 2012; 181: 937-946.
- [15] Wang CY, Yang P, Li M and Gong F. Characterization of a negative feedback network between SUMO4 expression and NFkappaB transcriptional activity. *Biochem Biophys Res Commun* 2009; 381: 477-481.
- [16] Guo D, Han J, Adam BL, Colburn NH, Wang MH, Dong Z, Eizirik DL, She JX and Wang CY. Proteomic analysis of SUMO4 substrates in HEK293 cells under serum starvation-induced stress. *Biochem Biophys Res Commun* 2005; 337: 1308-1318.
- [17] Liu T, Castro S, Brasier AR, Jamaluddin M, Garofalo RP and Casola A. Reactive oxygen species mediate virus-induced STAT activation: role of tyrosine phosphatases. *J Biol Chem* 2004; 279: 2461-2469.
- [18] Ushio-Fukai M. VEGF signaling through NADPH oxidase-derived ROS. *Antioxid Redox Signal* 2007; 9: 731-739.
- [19] Yang X, Qiao D, Meyer K and Friedl A. Signal transducers and activators of transcription mediate fibroblast growth factor-induced vascular endothelial morphogenesis. *Cancer Res* 2009; 69: 1668-1677.
- [20] Matsushita H, Morishita R, Nata T, Aoki M, Nakagami H, Taniyama Y, Yamamoto K, Higaki J, Yasufumi K and Ogihara T. Hypoxia-induced endothelial apoptosis through nuclear factor-kappaB (NF-kappaB)-mediated bcl-2 suppression: in vivo evidence of the importance of NF-kappaB in endothelial cell regulation. *Circ Res* 2000; 86: 974-981.
- [21] Wang N, Verna L, Hardy S, Zhu Y, Ma KS, Birrer MJ and Stemerman MB. c-Jun triggers apoptosis in human vascular endothelial cells. *Circ Res* 1999; 85: 387-393.
- [22] Ulrich HD. The SUMO system: an overview. *Methods Mol Biol* 2009; 497: 3-16.
- [23] Manza LL, Codreanu SG, Stamer SL, Smith DL, Wells KS, Roberts RL and Liebler DC. Global shifts in protein sumoylation in response to electrophile and oxidative stress. *Chem Res Toxicol* 2004; 17: 1706-1715.
- [24] Yang W, Sheng H, Warner DS and Paschen W. Transient global cerebral ischemia induces a massive increase in protein sumoylation. *J Cereb Blood Flow Metab* 2008; 28: 269-279.
- [25] Alkuraya FS, Saadi I, Lund JJ, Turbe-Doan A, Morton CC and Maas RL. SUMO1 haploinsufficiency leads to cleft lip and palate. *Science* 2006; 313: 1751.
- [26] Zhang FP, Mikkonen L, Toppari J, Palvimo JJ, Thesleff I and Janne OA. Sumo-1 function is dispensable in normal mouse development. *Mol Cell Biol* 2008; 28: 5381-5390.
- [27] Hanahan D and Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; 86: 353-364.
- [28] Munoz-Chapuli R, Quesada AR and Angel Medina M. Angiogenesis and signal transduction in endothelial cells. *Cell Mol Life Sci* 2004; 61: 2224-2243.
- [29] Li JJ and Johnson AR. Selective MMP13 inhibitors. *Med Res Rev* 2011; 31: 863-894.
- [30] Nisato RE, Hosseini G, Sirrenberg C, Butler GS, Crabbe T, Docherty AJ, Wiesner M, Murphy G, Overall CM, Goodman SL and Pepper MS. Dissecting the role of matrix metalloproteinases (MMP) and integrin alpha(v)beta3 in angiogenesis in vitro: absence of hemopexin C domain bioactivity, but membrane-Type 1-MMP and alpha(v)beta3 are critical. *Cancer Res* 2005; 65: 9377-9387.
- [31] Hattori N, Mochizuki S, Kishi K, Nakajima T, Takaishi H, D'Armiento J and Okada Y. MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. *Am J Pathol* 2009; 175: 533-546.
- [32] Lederle W, Hartenstein B, Meides A, Kunzelmann H, Werb Z, Angel P and Mueller MM.

## SUMO1 regulates endothelial function

- MMP13 as a stromal mediator in controlling persistent angiogenesis in skin carcinoma. *Carcinogenesis* 2010; 31: 1175-1184.
- [33] Hashimoto G, Inoki I, Fujii Y, Aoki T, Ikeda E and Okada Y. Matrix metalloproteinases cleave connective tissue growth factor and reactivate angiogenic activity of vascular endothelial growth factor 165. *J Biol Chem* 2002; 277: 36288-36295.
- [34] Zijlstra A, Aimes RT, Zhu D, Regazzoni K, Kupriyanova T, Seandel M, Deryugina EI and Quigley JP. Collagenolysis-dependent angiogenesis mediated by matrix metalloproteinase-13 (collagenase-3). *J Biol Chem* 2004; 279: 27633-27645.
- [35] Dudley AC, Thomas D, Best J and Jenkins A. A VEGF/JAK2/STAT5 axis may partially mediate endothelial cell tolerance to hypoxia. *Biochem J* 2005; 390: 427-436.
- [36] Tritsaris K, Myren M, Ditlev SB, Hubschmann MV, van der Blom I, Hansen AJ, Olsen UB, Cao R, Zhang J, Jia T, Wahlberg E, Dissing S and Cao Y. IL-20 is an arteriogenic cytokine that remodels collateral networks and improves functions of ischemic hind limbs. *Proc Natl Acad Sci U S A* 2007; 104: 15364-15369.
- [37] Versteeg HH, Spek CA, Slofstra SH, Diks SH, Richel DJ and Peppelenbosch MP. FVIIa:TF induces cell survival via G12/G13-dependent Jak/STAT activation and BclXL production. *Circ Res* 2004; 94: 1032-1040.
- [38] Yasuda Y, Fujita Y, Masuda S, Musha T, Ueda K, Tanaka H, Fujita H, Matsuo T, Nagao M, Sasaki R and Nakamura Y. Erythropoietin is involved in growth and angiogenesis in malignant tumours of female reproductive organs. *Carcinogenesis* 2002; 23: 1797-1805.
- [39] Desterro JM, Rodriguez MS and Hay RT. SUMO-1 modification of I $\kappa$ B $\alpha$  inhibits NF- $\kappa$ B activation. *Mol Cell* 1998; 2: 233-239.
- [40] Kisseleva T, Song L, Vorontchikhina M, Feirt N, Kitajewski J and Schindler C. NF- $\kappa$ B regulation of endothelial cell function during LPS-induced toxemia and cancer. *J Clin Invest* 2006; 116: 2955-2963.
- [41] Zhang G, Dass CR, Sumithran E, Di Girolamo N, Sun LQ and Khachigian LM. Effect of deoxyribozymes targeting c-Jun on solid tumor growth and angiogenesis in rodents. *J Natl Cancer Inst* 2004; 96: 683-696.