

Original Article

Hydrogen peroxide stress stimulates phosphorylation of FoxO1 in rat aortic endothelial cells

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Aim: To examine whether the phosphorylation of the O subfamily of forkhead transcription factors (FoxO) is involved in response to oxidative stress in rat aortic endothelial cells (RAECs).

Methods: RAECs were treated with H₂O₂ and phosphorylation status of proteins were evaluated by Western blot analysis. The subcellular localization of FoxO1 was determined by nuclear and cytosolic fractionation followed by Western blot analysis as well as immunocytochemistry. The transcriptional activity of FoxO1 in H₂O₂ stress was assessed by luciferase reporter assay. Expression of FoxO1 target gene was determined by real-time PCR analysis.

Results: H₂O₂ stress stimulated phosphorylation of FoxO1 at Thr24 and Ser256 in a concentration and time dependent manner in RAECs. Pretreatment of RAECs with PI-3K inhibitors abolished the activation of Akt and prevented the phosphorylation of FoxO1. Akt-mediated phosphorylation promoted nuclear exclusion of FoxO1. An IRS-driven luciferase activity transactivated by exogenous FoxO1 was modestly suppressed by hydrogen peroxide stress. The expression of Bim, a target gene of FoxO factors, was negatively regulated by Akt-mediated phosphorylation in response to hydrogen peroxide stimulation.

Conclusion: Our data demonstrate that phosphorylation of FoxO1 by PI-3K/Akt signaling is implicated in response to oxidative stress in vascular endothelial cells.

Keywords: oxidative stress; FoxO; endothelial cells; phosphorylation

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Introduction

Intracellular reactive oxygen species (ROS) have been linked to aging; neurodegenerative diseases such as Alzheimer disease and Parkinson disease; cancer; and vascular disease. In the vasculature, oxidative stress is associated with metabolic alterations (diabetes, obesity, and high cholesterol) and results in endothelial dysfunction. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis and its clinical complications (coronary disease, hypertension, and heart failure) and is thus considered a common risk factor for many cardiovascular diseases^[1,2].

One of the key determinants of ROS homeostasis and endothelial function is the O subfamily of forkhead transcription factors (FoxO). Consisting of the functionally related proteins FoxO1, FoxO3a, and FoxO4 (also known as FKHR, FKHL1, and AFX, respectively), FoxO factors regulate hormonal, nutrient, and stress responses and play a key role in endothelial homeostasis^[3]. A major regulator of FoxO activity

is protein kinase B (Akt), which directly phosphorylates and inactivates FoxO factors, triggering their translocation from the nucleus to the cytoplasm^[4]. In addition to phosphorylation, FoxOs are also regulated through other post-translational modifications including acetylation and ubiquitination^[5].

Several lines of evidence suggest a role for FoxO factors in ROS homeostasis. FoxO-deficient hematopoietic stem cells contain high concentrations of ROS and show reduced expression of genes involved in ROS detoxification^[3]. In particular, Foxo3a has been shown to directly activate transcription of three important antioxidant enzymes: MnSOD, catalase, and Prx3 and protects quiescent cells in vitro from oxidative stress^[6–8]. Interestingly, FoxO itself is also regulated by ROS. Treatment of cells with hydrogen peroxide, which increases cellular oxidative stress, results in acetylation or deacetylation of FoxO proteins^[9,10]. FoxO4 is monoubiquitinated under conditions of oxidative stress and results in nuclear translocation^[11]. FoxO proteins are also phosphorylated by other protein kinases, including JNK or Mst1 in response to oxidative stress and translocate to the nucleus^[12].

The role of FoxO factors in endothelial function is an active area of research. However, despite the well-established physi-

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ological relevance of ROS production to vascular function, the regulation of FoxO factors in endothelial oxidative stress has not been characterized. Here, we show that H₂O₂ activates PI-3K/Akt signaling and stimulates phosphorylation of FoxO1, which negatively regulates forkhead transcriptional activity in rat aortic endothelial cells (RAECs). Our results indicate that phosphorylation of FoxO by its major regulator Akt is implicated in response to oxidative stress in vascular endothelial cells.

Materials and methods

Cell isolation and culture

Male Sprague-Dawley rats were anesthetized and the thoracic aortas of rats were rapidly removed and collected in Dulbecco's Modified Eagle's Medium (Invitrogen, USA) and cleaned carefully of connective tissue and adherent fat. Isolated aorta was longitudinally cut open and cut into approximately 1 mm² sections and placed intimal side down into T25 flasks. DMEM containing 20% fetal bovine serum (HyClone, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, USA) was gently added to cover the tissues without disturbing the orientation of the explants and cultured at 37 °C in a humidified atmosphere of 5% CO₂. RAECs were allowed to grow out from the explants after 7–10 d, after which the tissues were removed. The cell culture purity (90%) was assessed by staining for factor VIII antigen, as previously described^[13]. Confluent cells were passaged by trypsinization with 0.25 trypsin-0.02% EDTA (Gibco, USA) and replated at a 1:3–1:4 dilution. Passages between 3 and 10 were used for all experiments. The study was approved by the Ethics Committee of nanjing medical University. Animal handling followed the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals.

Western blot analysis

80%–90% confluent RAECs were serum-starved overnight in DMEM medium before incubation with H₂O₂ at concentration and time as indicated. LY294002 was preincubated 1 h and wortmannin was preincubated 30 min before hydrogen peroxide stimulation. RIPA buffer containing protease inhibitors (Roche Diagnostics, Swiss) was added to RAECs to generate whole cell lysates. The samples were heated at 95 °C for 5 min and loaded on a 8% SDS-polyacrylamide gel. Protein extracts (50 µg) were separated by 8% SDS-PAGE gel and then transferred onto a methanol-activated PVDF membrane using a Bio-Rad transfer blotting system. Non-specific binding was blocked with 5% skim milk for 1 h at room temperature. Blots were incubated overnight with antibodies against phospho-FoxO1 (Thr24), phospho-FoxO1 (Ser256), phospho-Akt(Ser473), FoxO1, and Akt, respectively (Cell Signaling, USA). β-actin (Sigma, USA) was used as internal control. Membranes were incubated for 1 h at room temperature with a HRP-conjugated corresponding secondary antibodies after washing. Antigen detection was performed with an enhanced chemiluminescence detection system FluorChem (Alpha Inno-

tech, USA).

Cytosolic and Nuclear fractionation

After stimulation with or without H₂O₂ for 20 min, RAECs (5×10⁶ cells) were suspended in buffer A (10 mmol/L Hepes, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 0.4% NP-40, protease inhibitors), swollen for 10 min on ice, and centrifuged at 10000×g for 5 min. Supernatant was collected as a cytoplasmic extract. The pellet was washed, resuspended in buffer B (20 mmol/L Hepes, pH 7.9, 400 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT, 0.5 mmol/L PMSF, protease inhibitors), and vortexed for 20 min at 4 °C. The supernatant after centrifugation was used as nuclear extract. Proteins from cytosol and nuclear extract were subjected to immunoblotting.

Immunocytochemistry

RAECs were grown on glass slides and serum-starved overnight in DMEM medium. Cells were then treated with H₂O₂ (500 µmol/L, 20 min), fixed and washed twice with ice-cold PBS. Immunofluorescence staining using a primary antibody against phospho-FoxO1 (Thr24) and FITC-conjugated secondary antibody was performed. Samples were mounted with mounting medium and observed with a Nikon imaging system (Yokohama, Japan).

Plasmids, transfections and luciferase assay

Plasmid DNAs for FoxO1 and an IRS-driven luciferase (3×IRS-luc) reporter containing canonical insulin-responsive sequences were kindly provided from Dr Zhi-ping LIU (University of Texas Southwestern Medical Center at Dallas, USA). RAECs were transfected with reporter plasmid and plasmid encoding FoxO1 using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. At 24 h after transfection, cells were either untreated or stimulated with 200 µmol/L H₂O₂ for 12 h. Cell extracts were assayed for luciferase expression, using a luciferase assay kit (Promega, USA). Relative promoter activities were expressed as luminescence relative units normalized for cotransfected-Renilla luciferase expression in the cell extracts.

RNA and real-time PCR analysis

Total cellular RNA was extracted using Trizol reagent (Takara, Otsu, Japan) according to the manufacturer's instructions. The total RNA (2 µg) was reverse transcribed using PrimeScript[™] RT reagent Kit (Takara, Otsu, Japan). Real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, USA) using a Applied Biosystems 7500 Real-Time PCR System. The primer sequences used for Bim were: 5'-AAACGATTACCGAGAGGCGGAAGA-3' (sense), 5'-AATGCCTTCTCCATACCAGACGGA-3' (antisense). All primers were synthesized by Shanghai Invitrogen Corporation. The relative quantities of mRNA were determined using comparative cycle threshold methods and normalized against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA.

Statistical analysis

Statistical analysis was performed using functions from Microsoft Excel. Student's *t* test was used to assay the statistical significance.

Results

H₂O₂ stimulates phosphorylation of FoxO1 in RAECs via PI-3K/Akt signaling

Similar with human umbilical vein endothelial cells, FoxO1 and FoxO3a are the predominant FoxO factors in RAECs. The expression pattern of the forkhead factors FoxO1, FoxO3a, and FoxO4 in RAECs were characterized (data not shown). As reported previously with other types of endothelial cells, the available phosphospecific antibodies to FoxO3a yielded non-specific signals in Western blot analyses of RAECs, therefore we focused our study on FoxO1. Treatment of RAECs with H₂O₂ significantly enhanced phosphorylation of endogenous FoxO1 at Thr24 and Ser256 in a concentration-dependent manner (Figure 1A). Maximal phosphorylation of FoxO1 was observed at 20 min incubation with H₂O₂ (Figure 1B). In

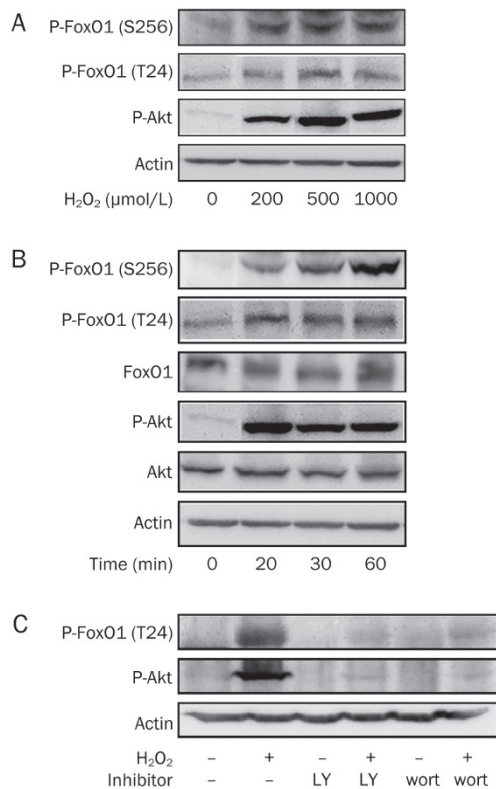


Figure 1. H₂O₂ stimulates phosphorylation of FoxO1 in rat aortic endothelial cells (RAECs) in a concentration- and time-dependent manner. RAECs were incubated with H₂O₂ for the indicated concentration for 20 min (A) or with 500 μmol/L H₂O₂ for the indicated time (B). Whole cell lysates were immunoblotted to detect endogenous expression of phosphorylated and total FoxO1 or Akt, respectively. (C) RAECs were treated with 500 μmol/L H₂O₂ for 20 min in the presence or absence of pretreatment with the PI-3K inhibitor LY294002 (10 μmol/L) or wortmannin (500 nmol/L) and phosphorylation of FoxO1 and Akt were determined by Western Blot analysis.

agreement with the known role of Akt in forkhead protein phosphorylation, exogenous hydrogen peroxide also resulted in a concentration- and time-dependent activation of Akt (Figure 1A&1B). Pretreatment of RAECs with LY294002 and wortmannin, two selective inhibitors of PI-3K, abolished the activation of Akt and prevented the phosphorylation of FoxO factors induced by H₂O₂, suggesting a PI-3K/Akt-dependent phosphorylation of FoxO1 was implicated in response to hydrogen peroxide stress in RAECs.

H₂O₂ treatment promotes nuclear exclusion of FoxO1 in RAECs

Akt-mediated phosphorylation of FoxO factors is thought to inhibit FoxOs function by promoting their export from the nucleus. To test whether H₂O₂-induced phosphorylation of FoxOs is accompanied by a change in FoxOs localization, nuclear and cytoplasmic fractions were prepared from H₂O₂-treated RAECs and subjected to immunoblot with phospho-specific FoxO antibodies. As shown in Figure 2A, a 20-min

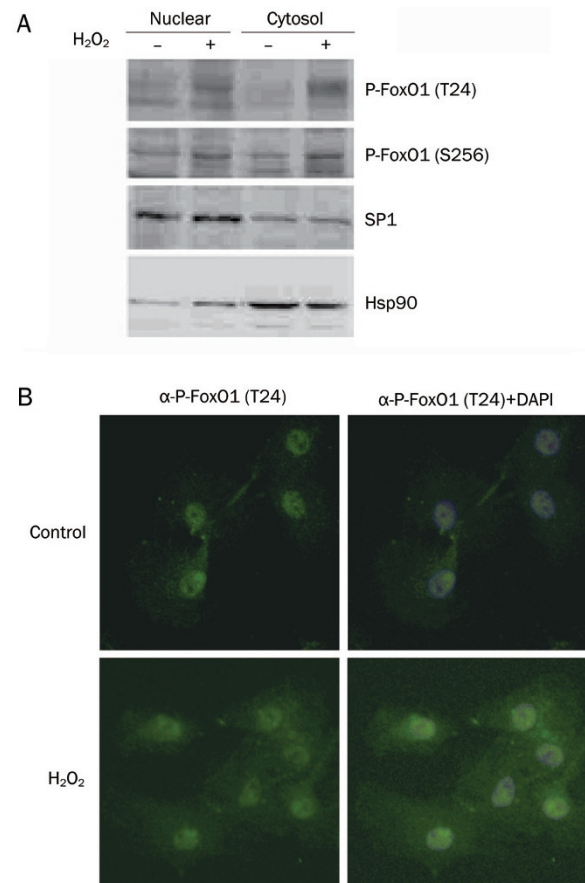


Figure 2. H₂O₂ promotes nuclear exclusion of FoxO1 in RAECs. (A) RAECs were serum-starved overnight and then either untreated or stimulated with 500 μmol/L H₂O₂ for 20 min. Cytoplasmic and nuclear fractions were prepared and subjected to Western blot with antibodies as indicated. Antibodies against SP1 and Hsp90 were used to validate the fractionation procedure. (B) Serum-starved RAECs were treated with vehicle or 500 μmol/L H₂O₂ for 20 min. Cells were immunostained with anti-phospho-FoxO1 antibody (left panels). Co-staining of the same cells with 4,6-diamidino-2-phenylindole (DAPI) is shown in the right panels. (×400)

treatment with 500 $\mu\text{mol/L}$ H_2O_2 induced a significant increase in the amount of phosphorylated FoxO1 in the cytoplasm. Immunostaining of RAECs with antibody against phospho-FoxO1 (Thr24) confirmed this result (Figure 2B). Certain amount of phosphorylated FoxO1 resided in the nucleus, suggesting that other modifications such as monoubiquitination or phosphorylation of other residues, which usually result in nuclear localization of forkhead factors, may also be present in these molecules simultaneously. We also tested the subcellular localization of total FoxO1 upon H_2O_2 treatment, but did not find detectable change with Western blot analysis and immunocytochemistry (data not shown), suggesting that phosphorylation of FoxO1 by Akt is minor fraction of total forkhead proteins, or other modifications may exist that affect the final distribution of FoxO1 in hydrogen peroxide stress.

Akt-mediated phosphorylation of FoxO1 negatively regulates forkhead transcriptional activity in hydrogen peroxide stress in RAECs

The transcriptional activity of exogenous FoxO1 in hydrogen peroxide stress was assessed by reporter assay using an IRS-driven luciferase construct. RAECs were transfected with luciferase reporter plasmid carrying IRS promoter and plasmid encoding FoxO1 as indicated. Twenty-four hours after transfection, cells were exposed to 200 $\mu\text{mol/L}$ H_2O_2 for 12 h, and were harvested for luciferase assay. Consistent with nuclear exclusion of FoxO1 shown above, IRS promoter activity transactivated by exogenous FoxO1 was modestly suppressed in response to hydrogen peroxide stress (Figure 3A).

To assess the effect of Akt-mediated phosphorylation on the transcriptional activity of endogenous FoxO factors in hydrogen peroxide stress, mRNA was extracted from RAECs after H_2O_2 stimulation. As known, one of the major roles of FoxO factors is the regulation of apoptosis. The pro-apoptotic gene Bim is suggested as one of target genes of FoxO transactivation and posttranslational modification of FoxOs, especially phosphorylation, is considered to play an important role in activating Bim. Therefore, we examined the effect of Akt-mediated phosphorylation of forkhead proteins on the transcription of Bim by real-time PCR. Consistent with previous reports, the level of Bim transcript was increased in 4 h of H_2O_2 treatment (Figure 3B). Pretreatment of RAECs with PI3K/Akt inhibitor LY294002 increased the induction of Bim in response to H_2O_2 stimulation, suggesting that Akt-mediated phosphorylation of FoxOs contributes, at least in part, to the negative regulation of forkhead transcriptional activity in oxidative stress.

Discussion

The function of FoxO proteins is controlled by different post-translational modifications that include phosphorylation, acetylation and ubiquitylation. The serine/threonine kinase Akt is one mediator of phosphorylation of FoxO factors and negatively regulates forkhead transcriptional activity. Previous studies have shown that various extracellular stimuli such as vascular endothelial growth factor (VEGF), angiopoietin-1, shear stress, and 11,12-epoxyeicosatrienoic acid activated

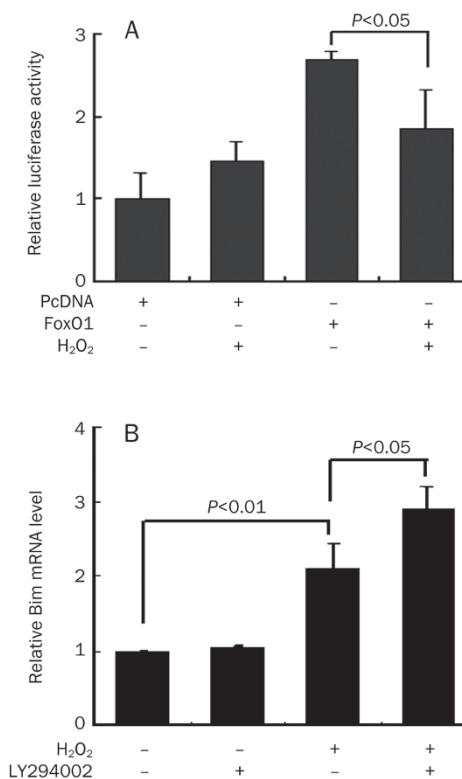


Figure 3. Akt-mediated phosphorylation of FoxO1 negatively regulates forkhead transcriptional activity. (A) RAECs were transfected with 3xIRS-luc reporter plasmid and plasmid encoding FoxO1 and pcDNA. At 24 h after transfection, cells were either untreated or stimulated with 200 $\mu\text{mol/L}$ H_2O_2 for 12 h. Cell extracts were assayed for luciferase expression and luciferase activity was normalized to Renilla activity for each well to control for transfection efficiency. Data shown are averages \pm standard deviations for three independent experiments. (B) RAECs were incubated with 200 $\mu\text{mol/L}$ H_2O_2 for 4 h in the presence or absence of pretreatment with the PI-3K inhibitor LY294002 (10 $\mu\text{mol/L}$). Total RNA was prepared for real-time PCR. The data are shown as means of fold-induction of each group over the control group. Data shown are averages \pm standard deviations for four independent experiments.

PI3K/Akt signaling and promoted the phosphorylation and transcriptional inactivation of FoxO factors in endothelial cells^[14-17]. Here, we demonstrated that ROS generation by exogenous sources such as H_2O_2 treatment also induced the phosphorylation and nuclear exclusion of FoxO1 through activation of Akt signaling in RAECs. As reported previously, Akt may be activated in EGF receptor-dependent manner upon ROS treatment^[18]. Hence, Our findings indicate that PI3K/Akt-mediated phosphorylation of FoxO factors contributes to the response to oxidative stress in endothelial cells.

Although little is known as to the regulation of FoxO factors in endothelial oxidative stress, several studies have focused on the regulation of FoxO factors in other cell types upon hydrogen peroxide stress. It has been reported that in PC12 cells FoxO3 was phosphorylated after exposure to hydrogen peroxide for 20 min and resulted in the redistribution of the protein to the cytosol^[19]. However, another study in human colon

carcinoma cell DLD1 demonstrated that H₂O₂ treatment induced the nuclear translocation and activation of FoxO4 by the activation of the small GTPase Ral, which results in a JNK-dependent phosphorylation of FoxO4 on threonine 447 and threonine 451^[12]. Our data indicated that although H₂O₂ induced a significant increase in the amount of phosphorylated FoxO1 in the cytoplasm, no detectable change in the distribution of total FoxO1 was observed upon H₂O₂ treatment. The distinct subcellular localization may result from a difference in cell types used or the specific FoxO factor studied. Since no conserved T447/451 phosphorylation sites are revealed by alignment of FoxO4 with FoxO1 and FoxO3a, whether FoxO1 and FoxO3a are potential substrates for JNK still needs to be determined. Therefore, our data extend understanding of the distribution of FoxO factors in response to oxidative stress in vascular endothelial cells.

In this study, we focused on the phosphorylation of FoxO factors. In fact, FoxOs are also regulated through other post-translational modifications such as acetylation and ubiquitination. Studies have shown that the acetylations of FoxO3 and FoxO4 are increased in response to hydrogen peroxide^[9, 10]. FOXO4 is also reported to be regulated by mono-ubiquitination after increased cellular oxidative stress, which increased nuclear localization of FOXO and hence increased transcriptional activity^[11]. Whether FoxO1 is acetylated or mono-ubiquitinated needs to be further investigated. Thus the regulation of FoxO in oxidative stress is complex, and different degrees of phosphorylation or other posttranslational modifications may confer distinct effects. Furthermore, the interaction of FoxO with other transcription factors such as nuclear factor- κ B (NF- κ B) would likely contribute to this complexity.

In summary, our data demonstrated that hydrogen peroxide stress activates PI-3K/Akt signaling and stimulates phosphorylation of FoxO1, which negatively regulates forkhead transcriptional activity in endothelial cells. Our results indicate that phosphorylation of FoxO by its major regulator Akt is implicated in response to oxidative stress in vascular endothelial cells.

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Author contribution

Hao LI designed research; Ye-yu WANG and Si-min CHEN performed research; Ye-yu WANG analyzed data; Hao LI wrote the paper.

References

- 1 Finkel T. Redox-dependent signal transduction. *FEBS Lett* 2000; 476: 52–4.
- 2 Maulik N. Redox signaling of angiogenesis. *Antioxid Redox Signal* 2002; 4: 805–15.

- 3 Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, et al. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell* 2007; 128: 309–23.
- 4 Huang H, Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci* 2007; 120 (Pt 15): 2479–87.
- 5 Greer EL, Brunet A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 2005; 24: 7410–25.
- 6 Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffey PJ, et al. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 2002; 419: 316–21.
- 7 Chiribau CB, Cheng L, Cucoranu IC, Yu YS, Clemens RE, Sorescu D. FOXO3a regulates peroxiredoxin III expression in human cardiac fibroblasts. *J Biol Chem* 2008; 283: 8211–7.
- 8 Alcendor RR, Gao S, Zhai P, Zablocki D, Holle E, Yu X, et al. Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* 2007; 100: 1512–21.
- 9 Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004; 303: 2011–5.
- 10 van der Horst A, Tertoolen LG, de Vries-Smits LM, Frye RA, Medema RH, Burgering BM. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). *J Biol Chem* 2004; 279: 28873–9.
- 11 Brenkman AB, de Keizer PL, van den Broek NJ, Jochemsen AG, Burgering BM. Mdm2 induces mono-ubiquitination of FOXO4. *PLoS One* 2008; 3: e2819.
- 12 Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL, et al. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J* 2004; 23: 4802–12.
- 13 Chen ZY, Feng GG, Nishiwaki K, Shimada Y, Fujiwara Y, Komatsu T, et al. Possible roles of neuropeptide Y Y3-receptor subtype in rat aortic endothelial cell proliferation under hypoxia, and its specific signal transduction. *Am J Physiol Heart Circ Physiol* 2007; 293: H959–67.
- 14 Abid MR, Guo S, Minami T, Spokes KC, Ueki K, Skurk C, et al. Vascular endothelial growth factor activates PI3K/Akt/forkhead signaling in endothelial cells. *Arterioscler Thromb Vasc Biol*. 2004; 24: 294–300.
- 15 Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, et al. Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J Biol Chem* 2000; 275: 9102–5.
- 16 Dimmeler S, Assmus B, Hermann C, Haendeler J, Zeiher AM. Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. *Circ Res* 1998; 83: 334–41.
- 17 Potente M, Fisslthaler B, Busse R, Fleming I. 11,12-Epoxyeicosatrienoic acid-induced inhibition of FOXO factors promotes endothelial proliferation by down-regulating p27Kip1. *J Biol Chem* 2003; 278: 29619–25.
- 18 Wang X, McCullough KD, Franke TF, Holbrook NJ. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. *J Biol Chem* 2000; 275: 14624–31.
- 19 Nemoto S, Finkel T. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science* 2002; 295(5564): 2450–2.